Synthesis of cyclic peptides as bioconjugation platforms

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Towards the synthesis of a fusion protein *via* a synthetic chemical ligation approach

Fusion proteins integrate two or more domains originating from different proteins into a single polypeptide chain. Such proteins are abundant in nature and scientists have copied nature's strategy to create synthetic fusion proteins that combine separate functions of the corresponding domains into a single entity.[1] Amongst the earliest examples are the polypeptide affinity tags fused to a protein of interest to simplify purification, or to enable its detection and monitoring, with minimal perturbation to its tertiary structure and biological activity. A prominent example is green fluorescent protein (GFP) that has been frequently used as a fusion

partner to act either as a tag to monitor the target's localization and fate or as an indicator, in which the GFP-fluorescence can be modulated post-translationally by its chemical environment or by protein-protein interactions.[2] Besides efforts to increase reaction rates in consecutive enzymatic reactions and to construct bifunctional enzymes, fusion proteins have also become an important class of therapeutic agents.[3–8] Fusion proteins have for instance found use in targeted drug delivery where the therapeutic protein can be linked to an antibody targeting a specific cell type or fused to a cell-penetrating peptide to efficiently transport the drug across the cell membrane.[9–11] In the context of biopharmaceuticals, fusion proteins, in which the biologically active protein is connected to Fc domains of antibodies or carrier proteins, have prolonged plasma half-lives and improved therapeutic efficacy compared to the unconjugated protein.[12,13] For instance, etanercept (tumor necrosis factor fused with Fc domain of IgG1) and aflibercept (extracellular domains of VEGF receptors 1 and 2 fused with Fc domain of IgG1) have found considerable success in the clinic, and both are listed in the ten top-selling biopharmaceuticals in 2017.[14]

Three main approaches exist for the construction of fusion peptides. The first one concerns domain insertion and entails insertion of the DNA-sequence encoding a domain of one protein into the gene of the second protein to establish the fusion. Tandem fusion is an approach in which the multiple domains are genetically connected end-to-end, usually via a linker peptide. Finally, post-translation conjugation comprises fusion of two proteins through (bio)chemical means after the proteins are individually expressed separating the fusion and expression step. Although the extra step in the latter approach inherently complicates the synthesis, this approach allows for modular pairing and assembly of the domains after expression in optimal hosts.[15] The conjugation of the domains can either be performed chemically through, for instance, lysine or thiol modifications, enzymatically by employing the frequently used sortase A or through split intein-mediated protein ligation (*Figure 1*).[16–18]

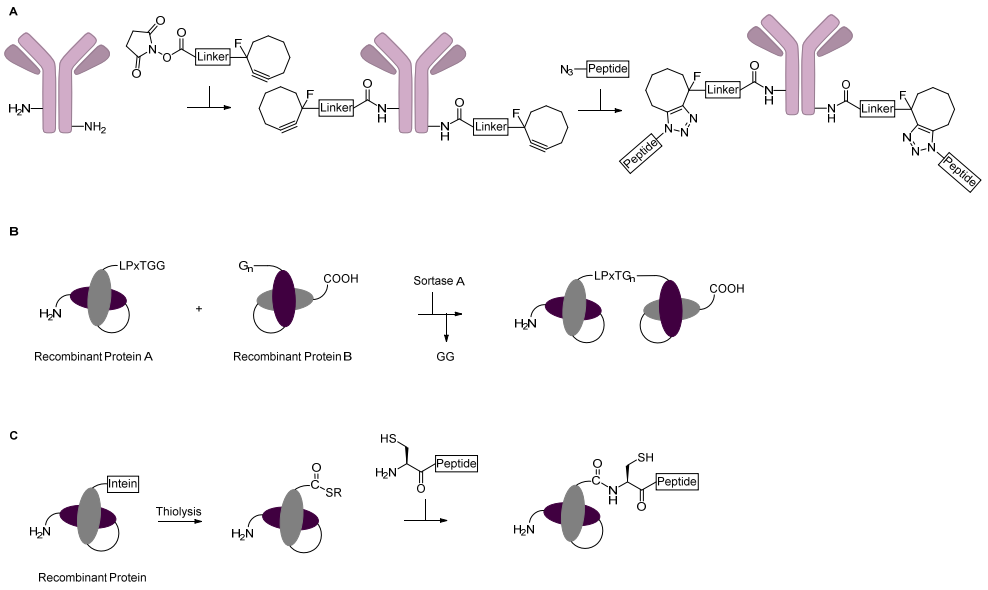


Figure 1. A. Chemical conjugation of multiple peptides to an antibody employing a copper-free conjugation strategy. *B.* Enzymatic fusion of two proteins by Sortase A. *C.* Split intein-mediated protein ligation with the generation of a C-terminal thioester.

The research described in this Chapter entails the synthesis of a fusion protein employing a chemical conjugation strategy with the emphasis on the late-stage derivatization of the individual proteins (*Figure 2*). For this purpose, both proteins will be genetically engineered with a thiol group for functionalization as this will allow for a variety of protein combinations to be assembled. Due to their unique characteristics, camelid single-domain antibodies were selected as a model system. Their good expression in microbial systems in combination with their beneficial biochemical properties (small size, good stability and solubility, high affinity and specificity for their antigen and strict monomeric behavior) makes VHH antibodies, also called nanobodies, well suited for the role of model proteins.[19] Nanobodies against PD-1 and CD4 are useful therapeutics, because $CD4^+$ T lymphocytes expressing PD-1 play a significant role in HIV persistence and T cell dysfunction and exhaustion in tumors. Hypothetically, a fusion protein of anti-PD-1 and anti-CD4 may accomplish a more selective targeting and restore antitumor response.[20–22] The respective nanobodies were genetically engineered to be equipped with a histidine tag for purification purposes and a C-terminal cysteine for conjugation purposes.

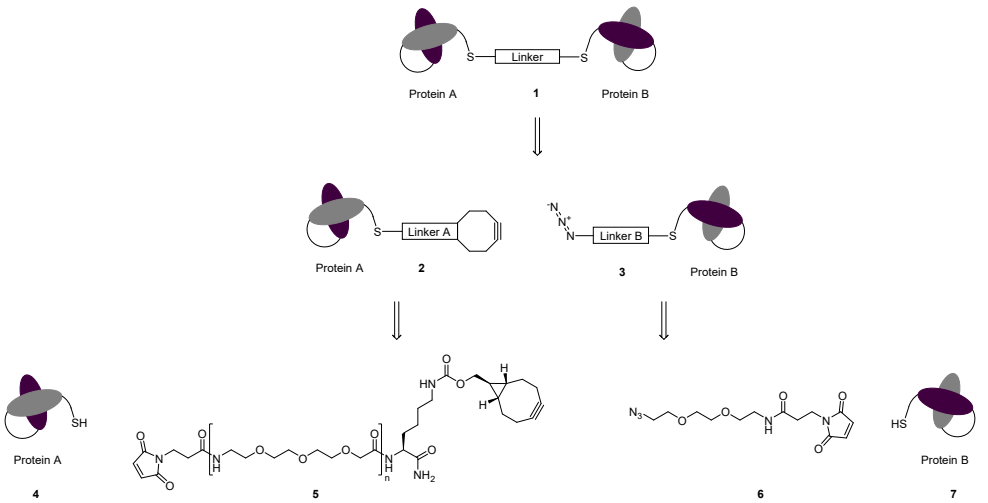


Figure 2. Design of a fusion protein with a late-stage conjugation strategy using a two-component linker system.

To construct the fusion protein, a two-component linker system was envisioned to facilitate late-stage fusion (*Figure 2*). First, bifunctional scaffold **5** equipped with a maleimide will be conjugated to the free thiol present on the anti-CD4 nanobody in a Michael addition reaction. Separately, the other VHH antibody (anti-PD-1) will be conjugated with bifunctional linker **6** in a similar fashion to construct derivatized protein **3**. With both nanobodies now bearing complementary conjugation partners (strained cyclooctyne and alkyl azide), the fusion will be achieved by a strain-promoted alkyne-azide cycloaddition (SPAAC) to furnish fusion protein **1**. The SPAAC was selected over the copper(I)-catalyzed Huisgen cycloaddition as the His-tag present on the VHH antibodies would hinder the reaction. The standard method with copper(II)sulfate and sodium ascorbate relies on the *in situ* reduction to the active copper(I) species, but bis(histidine)copper(II) complexes show negligible reduction to copper(I) by ascorbate in the presence of oxygen or argon.[23,24]

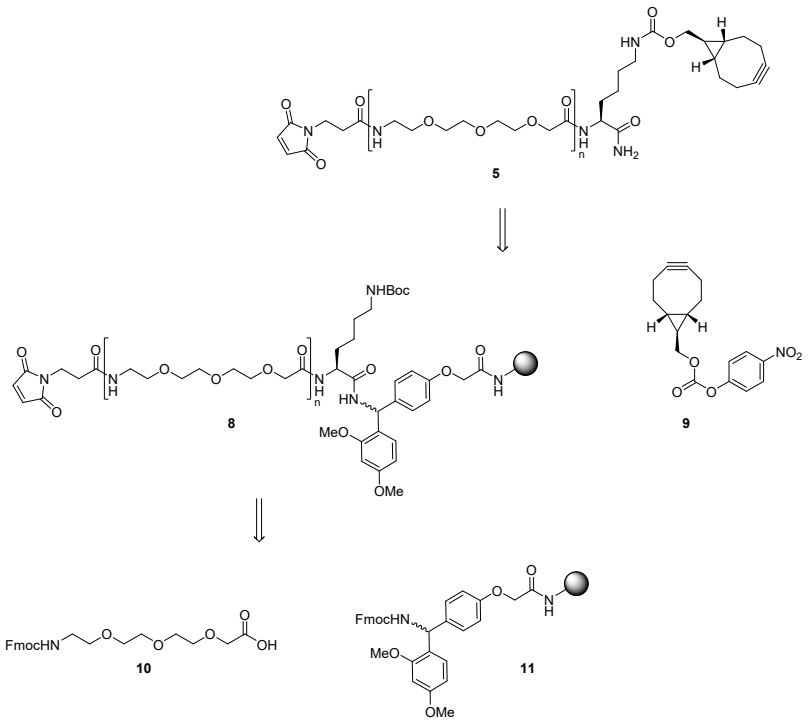


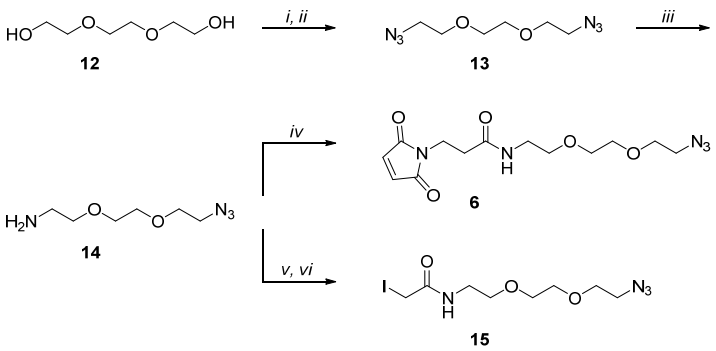
Figure 3. Retrosynthesis of bifunctional linker **5**.

Linkers play a crucial role in the fusion protein design providing an appropriate distance between the two domains in order to exhibit biological activity.[25] With that in mind, scaffold **5** was designed to allow for linker length variation by inserting ethylene glycol building block **10** multiple times (*Figure 3*). The synthesis of bifunctional linker **5** was envisioned using a solid-phase peptide chemistry approach. First, Rink amide resin **11** will be elongated with Fmoc-Lys(Boc)-OH for later functionalization. Then ethylene glycol building block **10** synthesized for use in an Fmoc-based solid-phase approach will be installed. The length of the linker can be varied at this stage based on the number of cycles with building block **10**. As an initial step, two ethylene glycol linkers will be installed. After installation of the maleimide functionality to furnish compound **8**, the linker will be cleaved from the resin liberating the ε-amine of the lysine. Reacting activated carbonate **9** with the free amine will finally give bifunctional linker **5**. The utility of linkers **5** and **6** is then demonstrated by the fusion of an anti-PD1 and anti-CD4 nanobody in the proposed manner (*Figure 2*). After conjugation of the linkers to either the anti-PD1 or anti-CD4

VHH antibody, the two differently functionalized proteins are fused through the strain-promoted alkyne-azide cycloaddition.

Results and discussion

First attention was focused on the synthesis of bifunctional linker **6** bearing an azide and maleimide moiety (*Scheme 1*). Starting from triethylene glycol **12**, tosylation of the diol with 4-toluenesulfonyl chloride and sodium hydroxide as the base furnished the corresponding ditosylate in 91% yield.



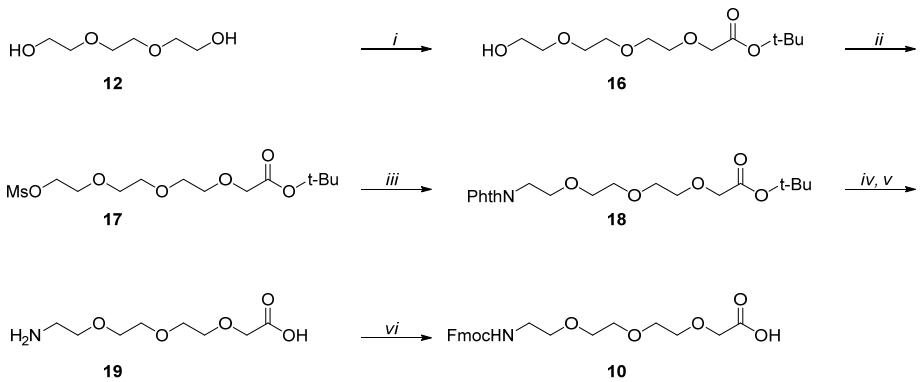
Scheme 1. Reagents and conditions: (*i*) TsCl, NaOH, DCM, 0 °C, 3 hrs, 91% (*ii*) NaN_3, TBAI, DMF, 80 °C, 17 hrs, 91% (*iii*) PPh_3, Et_2O, THF, 1.0 M aq. HCl, rt, 24 hrs, 71% (*iv*) (a) 3-maleimidopropionic acid NHS ester, DIPEA, THF, rt, 4 hrs, 10% (b) 3-maleimidopropionic acid, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, DIPEA, DCM, rt, 17 hrs, <5% (*v*) chloroacetyl chloride, Et_3N, DCM, 0 °C, 2 hrs, 68% (*vi*) NaI, acetone, rt, 48 hrs, 84%.

Overnight substitution of the ditosylate with sodium azide at elevated temperatures gave diazide **13** in 91% yield. At this stage in the synthesis desymmetrization of the molecule was achieved by employing a biphasic system. Diazide **13** was dissolved in a mixture of diethyl ether and 1.0 M aq. HCl solution with tetrahydrofuran added as a phase transfer reagent and a solution of triphenylphosphine in ether was added dropwise. Upon formation of the iminophosphorane, hydrolysis and protonation of the resultant amine in the acidic, aqueous layer prevented the reduction of the second azide. After vigorously stirring the mixture for 24 hours, amine **14** was isolated in a yield of 71%. Next the installation of the maleimide moiety was attempted but the synthesis thereof proved to be abortive. Neither 3-maleimidopropionic acid and peptide coupling reagents (diisopropylcarbodiimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were tried) nor the maleimide activated ester and a base gave satisfactory results. The yields were low and the isolation of maleimide derivative **6** was always accompanied with impurities. After a literature search it was found that this compound degrades in a matter of hours,

hence commercial products of this kind are sold as a two-component kit. It is hypothesized that the instability is in part caused by a [2+3] dipolar cycloaddition between the azide and maleimide. Usually this reaction occurs at elevated temperatures, but Constant and co-workers reported the formation of the dihydrotriazole at room temperature.[26–28]

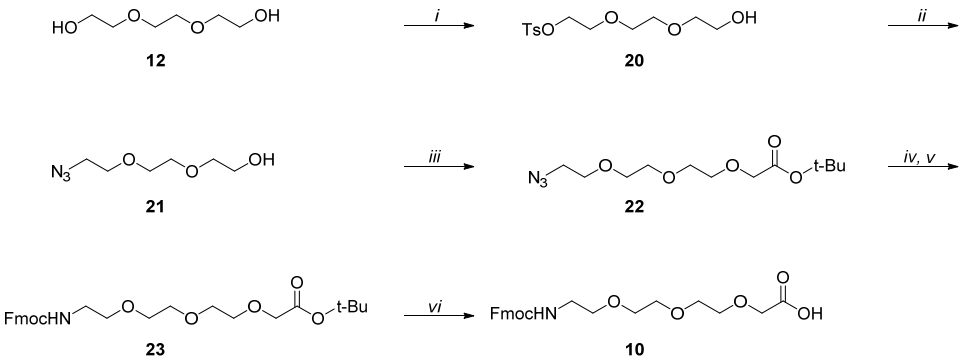
With the maleimide being incompatible in the designed conjugation strategy, an alternative linker was synthesized bearing the iodoacetamide capable of alkylating the sulfhydryl group. First, amine **14** and triethylamine were dissolved in DMF and chloroacetyl chloride was added dropwise at 0 °C to obtain the corresponding chloroacetamide. The chloroacetamide was converted into iodoacetamide **15** in a classic Finkelstein reaction with sodium iodide in acetone in a yield of 84%.[29]

With one component of the designed linker system in hand, attention was shifted to bifunctional linker **5** equipped with a strained cyclooctyne and maleimide moiety. To control the overall length of the linker, the core of **5** consists of several repeats of ethylene glycol building block **10**, which was synthesized first (*Scheme 2*). The functional groups of **10** resemble a standard amino acid building block featuring an Fmoc-protected N-terminus and an unprotected C-terminus. The synthesis started from the symmetrical triethylene glycol **12** leaving two options for desymmetrization; starting the synthesis from the C-terminus and installing the N-terminus later or *vice versa*. Both options were investigated.



Scheme 2. Reagents and conditions: (*i*) NaH, TBAI, *tert*-butylbromoacetate, THF, 0 °C to rt, 17 hrs, 46% (*ii*) MsCl, Et_3N, DCM, 0 °C, 3 hrs, 88% (*iii*) KPhth, DMF, 110 °C, 3 hrs, 97% (*iv*) TFA, DCM, 1 hr (*v*) hydrazine hydrate, MeOH, 90 min., 70% (*vi*) Fmoc NHS ester, *N*-methylmorpholine, H_2O, 1,4-dioxane, 0 °C to rt, 17 hrs, 11%.

Triethylene glycol **12** was alkylated using sodium hydride and *tert*-butylbromoacetate in THF with TBAI acting as a catalyst giving *tert*-butyl ester **16** in a 46% yield (*Scheme 2*). The remaining alcohol was then mesylated with methanesulfonyl chloride and triethylamine in DCM with a yield of 88%. Reaction of mesylate ester **17** with potassium phthalimide in DMF at an elevated temperature furnished phthalimide-protected amine **18** in a 97% yield. Deprotection of the phthalimide **18** with hydrazine in methanol would convert the *tert*-butyl ester into an acylhydrazide. Therefore, the *tert*-butyl ester was deprotected first using trifluoroacetic acid in DCM followed by treatment with hydrazine in methanol to furnish zwitterion **19** in a yield of 70% over two steps. Finally, Fmoc-protected amine **10** was obtained by reacting zwitterion **19** with Fmoc NHS ester and *N*-methylmorpholine in a mixture of THF and water in 11% yield. The reaction was accompanied by a number of byproducts presumably due to the formation of the asymmetric anhydride and subsequent side-reactions.



Scheme 3. Reagents and conditions: (*i*) TsCl, Et_3N, DCM, 0 °C to rt, 17 hrs, 98% (*ii*) NaN_3, DMF, 90 °C, 4 hrs, 82% (*iii*) KO^{t}Bu, *tert*-butyl bromoacetate, ^{t}BuOH, 30 °C to 50 °C, 6 hrs, 80% (*iv*) PPh_3, H_2O, THF, 0 °C to rt, 41 hrs, 89% (*v*) Fmoc NHS ester, *N*-methylmorpholine, DCM, 0 °C, 6 hrs, 96% (*vi*) 85% wt% aq. H_3PO_4, toluene, 4 hrs, 98%.

To first install the N-terminus, one alcohol was selectively tosylated by reaction with tosyl chloride and triethylamine in DCM giving tosylate **20** in a yield of 98% (*Scheme 3*). The azide group was employed as a masked amine instead of the phthalimido group to allow for milder deprotection conditions. Therefore, tosylate ester **20** was reacted with sodium azide in DMF at an elevated temperature to furnish azido **21** in 82% yield. The following installation of the C-terminus was achieved using different conditions than before as the quenching of the sodium hydride with water or methanol would lead to partial hydrolysis or transesterification of the *tert*-butyl

ester. Instead, a procedure by Heller and co-workers was followed where alcohol **21** was treated with potassium *tert*-butoxide as the base in *tert*-butanol at 30 °C followed by the addition of *tert*-butylbromoacetate and increasing the temperature to 50 °C.[30] After stirring for six hours, *tert*-butyl ester **22** was obtained in a yield of 80%.

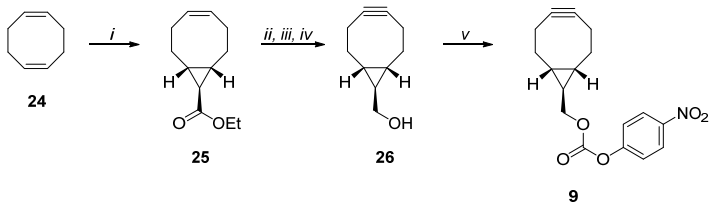
Hydrogenation of azide **22** with 10 mol% Pd/C and 1.0 equivalent of 1.0 M aq. HCl in ethanol for four hours furnished the corresponding amine in a yield of 79%. It should be noted that when 2.0 equivalents of 1.0 M aq. HCl was used premature cleavage of the *tert*-butyl group was observed. Moreover, when performing the reaction on a large scale partial cleavage was also observed with only 1.0 equivalent of 1.0 M aq. HCl prompting a change in the deprotection method.

As an alternative, Staudinger reduction was employed to deprotect the azide functionality. Azide **22** was dissolved in THF and triphenylphosphine was added. After 20 hours, water was added and the reaction was stirred an additional 21 hours. The mixture was then diluted with DCM and washed three times with sat. aq. $NaHCO_3$. However, this work-up method led to low yields as a significant portion of the amine remained in the aqueous layer. Therefore, a revised work-up was employed where the reaction mixture was diluted with toluene and the organic layer extracted three times with water. Evaporation of the combined aqueous layers afforded the free amine in 89% yield. The free amine was then taken up in DCM and Fmoc NHS ester was added portion wise at 0 °C followed by addition of *N*-methylmorpholine. The reaction was stirred for six hours at 0 °C affording Fmoc-protected amine **23** in a yield of 96%. The final step comprised deprotection of the *tert*-butyl group to give building block **10**. The common method for this deprotection is treatment with trifluoroacetic acid in DCM, but on a large-scale evaporation of trifluoroacetic acid is troublesome. Therefore, a different procedure was sought with a more convenient work-up. The group of Hamilton describes the use of zinc bromide in DCM as a mild reagent for the deprotection of *tert*-butyl esters.[31] The subsequent isolation of the free carboxylic acid would only require washing the organic layer with water. Employing this procedure, *tert*-butyl ester **23** was dissolved in DCM and treated with 5.0 equivalents of zinc bromide. After 24 hours, the deprotection was still incomplete and adding extra zinc bromide did not lead to full deprotection. In another report by Li and co-workers aqueous phosphoric acid is used for deprotecting *tert*-butyl esters where isolation of the free carboxylic acid was also achieved using only washing steps.[32] Therefore, *tert*-butyl ester **23** was dissolved in toluene and 5.0 equivalents of 85% aq. H_3PO_4 was added slowly. After

stirring for four hours, water and ethyl acetate were added and the layers were separated. The aqueous phase was extracted two times with ethyl acetate and the combined organic layers were evaporated to successfully give free carboxylic acid **10** in a 98% yield.

Comparing the two methods for desymmetrization, alkylation of triethylene glycol **12** with *tert*-butylbromoacetate was accompanied by the formation of byproducts hampering the isolation, especially in large scale reactions. On the other hand, tosylation of triethylene glycol **12** proceeded cleanly and tosylate ester **20** could be isolated by extraction, which was not possible for *tert*-butyl ester **16**. This allowed a larger excess of triethylene glycol to be used, thereby increasing the yield of the mono-substituted product. This made the installation of the N-terminus first the preferred strategy.

With building block **10** in hand, attention was focused on the synthesis of a strained cyclooctyne needed for bifunctional scaffold **5**. The bicyclo[6.1.0]non-4-yne group was chosen because of its relative ease of synthesis combined with its ability to add to alkyl azides with fast reaction rates (*Scheme 4*). An additional advantage is the symmetrical nature of the molecule which leads to the formation of a single regioisomer upon cycloaddition.[33]

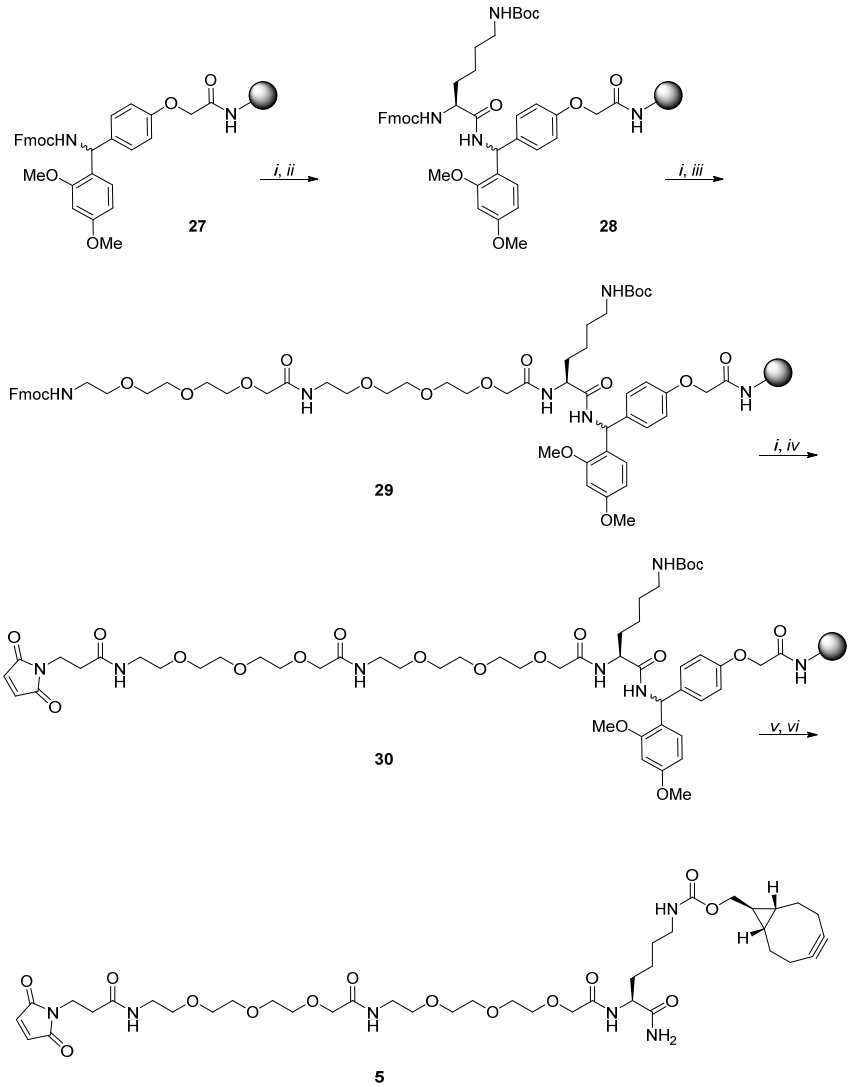


*Scheme 4.*Reagents and conditions: (*i*) ethyl diazoacetate, copper(II)acetylacetonate, EtOAc, reflux, 17 hrs, 44% (*ii*) $LiAlH_4$, Et_2O, rt, 15 min. (*iii*) Br_2, DCM, 0 °C, 5 min. (*iv*) KO^{t}Bu, THF, reflux, 2 hrs, 56% over 3 steps (*v*) *p*-nitrophenyl chloroformate, pyridine, DCM, rt, 15 min, 69%.

The first step was the cyclopropanation of 1,5-cyclooctadiene **24**. Instead of using rhodium acetate as the catalyst as was advocated in the first literature report on the synthesis of **25**, the less expensive copper acetylacetonate was employed.[33,34] Ethyl diazoacetate was slowly added to a solution of the copper catalyst and a large excess of 1,5-cyclooctadiene **24** in ethyl acetate after which the reaction was refluxed for 17 hours. Purification by silica gel column chromatography furnished *exo*-adduct **25** and the *endo*-adduct in a yield of 44% and 12% respectively. Continuing the synthesis, *exo*-adduct **25** was treated with lithium aluminum hydride in ether to give

the corresponding alcohol and bromination of the alkene followed by double elimination using potassium *tert*-butoxide in refluxing tetrahydrofuran afforded alkynol **26** in a yield of 56% over three steps. Finally, alcohol **26** was reacted with *p*-nitrophenyl chloroformate and pyridine in DCM to obtain activated carbonate ester **9** in a 69% yield.

Having both building blocks **9** and **10** now available, the synthesis of bifunctional scaffold **5** was undertaken next. To accommodate the option to vary the length of the linker, a solid-phase synthesis strategy was designed (*Scheme 5*). The choice was made for a C-terminal carboxamide over a carboxylic acid to simplify the synthesis and purification. To that end, the synthesis was started from TentaGel S RAM **27** and the amine was liberated by treatment with piperidine in DMF followed by six washing steps with DMF to remove any traces of piperidine.



Scheme 5. Reagents and conditions: (*i*) piperidine, DMF, rt, 20 min (*ii*) Fmoc-Lys(Boc)-OH, HCTU, DIPEA, DMF, rt, 1 hr (*iii*) **10**, HCTU, DIPEA, DMF, rt, 1 hr (*iv*) 3-maleimidopropionic acid NHS ester, DIPEA, DMF, rt, 4 hrs (*v*) TFA – H_2O – TIPS (190:5:5), rt, 3 hrs (*vi*) **9**, DIPEA, DMF, rt, 4 hrs, 25%.

Standard peptide condensation conditions were employed to attach Fmoc-Lys(Boc)-OH to the resin. The lysine building block was dissolved in DMF and pre-activated for five minutes with HCTU and DIPEA before the solution was added to the resin. The reaction was shaken for one hour after which the resin was washed with DMF and the Fmoc group was deprotected using piperidine in DMF. The resin was

washed with DMF (6 x 30 sec.) and two entities of building block **10** were then installed using the same procedure as for the lysine building block. After liberation of the amine, the resin was elongated with a maleimide moiety by treatment of 3-maleimidopropionic acid NHS ester and DIPEA in DMF for four hours. At this stage, the compound was liberated from the resin using a cleavage cocktail (190:5:5, TFA – TIPS – H_2O) for three hours while simultaneously removing the Boc-group exposing a functionalization handle for the strained cyclooctyne. The suspension was filtered and the resin was washed with additional cleavage cocktail and the filtrate was evaporated. The crude product was taken up in DMF and the strained cyclooctyne was installed by treatment with activated carbonate ester **9** and DIPEA for four hours. Purification by preparative reversed-phase HPLC was attempted, but the formation of a byproduct was observed during either evaporation of the aqueous mixture or lyophilization. Instead, bifunctional scaffold **5** was purified by size exclusion chromatography followed by silica gel column chromatography yielding **5** in 25% yield.

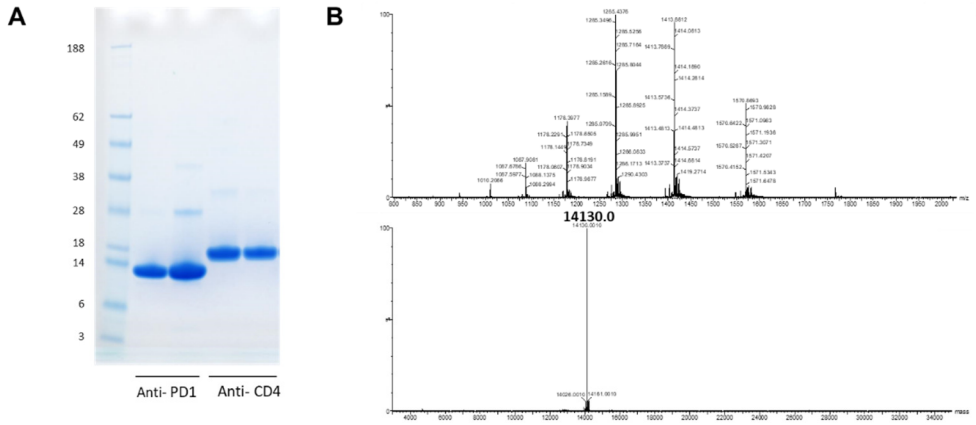


Figure 4.A. Visualization of the purified anti-PD1 and anti-CD4 VHH antibodies using SDS-PAGE. *B.* ESI-TOF spectrum of the anti-PD1 VHH antibody (upper panel), and deconvoluted mass spectrum (lower panel) with a calculated molecular weight of 14131.7 Da.

After having attained both bifunctional scaffold **5** and adapter **15** the feasibility of the protein conjugation chemistry was assessed. Towards that end the anti-PD1 (**4**) and anti-CD4 VHH antibodies equipped with a cysteine residue have been expressed and purified to near homogeneity (*Figure 4*). To test the conjugation approach, a homodimer of anti-PD1 antibodies was targeted. Nanobody **4** was first

reacted at its cysteine residue with an excess of scaffold **5** to provide protein **2** modified with the strained cyclooctyne and thus capable to react with alkyl azides under ambient and copper-free conditions. (*Figure 5*) The reaction was performed in aqueous buffer in the presence of TCEP to inhibit formation of unreactive disulfide bonded dimers from starting protein **4**. Progress of the reaction was monitored by MALDI-TOF spectrometry and complete conversion was observed after reacting the mixture at 0 °C for 30 minutes. Gel filtration of the solution afforded cyclooctyne-tagged protein **2**.

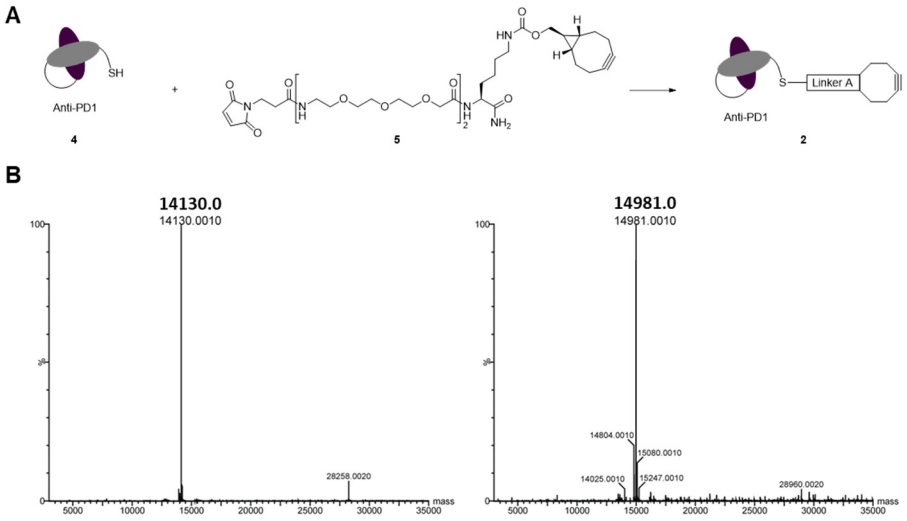


Figure 5.A. Reagents and conditions: compound **5** (30 equiv.), TCEP (1 mM), 0 °C, 30 min. *B.* Deconvoluted mass spectrum of anti-PD1 VHH antibody **4** (left panel) and deconvoluted mass spectrum of purified anti-PD1 nanobody conjugated to compound **5** (right panel) with a calculated molecular weight of 14982.6 Da.

Next, the anti-PD1 nanobody **3** equipped with an alkyl azide was prepared by a site-specific derivatization of the cysteine residue in protein **4** with iodoacetamide **15** (*Figure 6*). Compared to the previously performed thiol-maleimide conjugation, reaction of the iodoacetamide with the thiol proceeded at a slower rate. Optimization of the conjugation included increasing the equivalents of linker **15** and performing the reaction at room temperature. Under these conditions, complete conversion was achieved in two hours as observed by mass spectrometry. Lastly, purification by gel filtration furnished azido-tagged VHH antibody **3**.

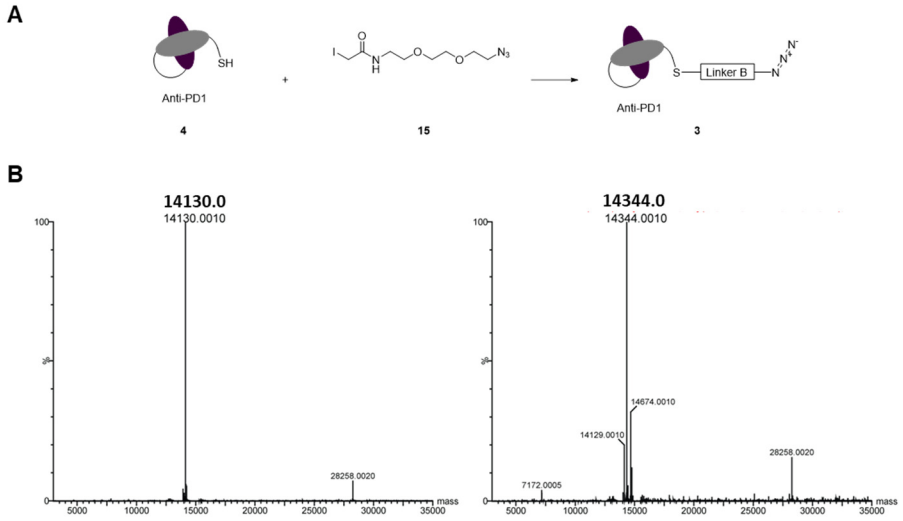


Figure 6.A. Reagents and conditions: compound **15** (50 equiv.), TCEP (1 mM), rt, 2 hrs. *B.* Deconvoluted mass spectrum of anti-PD1 VHH antibody **4** (left panel) and deconvoluted mass spectrum of purified anti-PD1 nanobody conjugated to compound **15** (right panel) with a calculated molecular weight of 14345.9 Da.

With both azido- and alkyne-tagged nanobodies **2** and **3** in hand, the preparation of a model fusion protein **1** by strain promoted cycloaddition reaction was attempted on a test scale following the reaction by mass spectrometry (*Figure 7*). Functionalized proteins **2** and **3** were mixed in aqueous buffer in a ratio of 1:2 (alkyne – azide). After 17 hours of incubation at room temperature showed the formation of expected fusion protein **1**. As a proof of principle, this result demonstrates the feasibility of fusing two VHH antibodies *via* the here-proposed synthetic chemical ligation. However, the reaction has not reached completion and to ensure synthesis of the bifunctional anti-CD4 and anti-PD1 fusion protein in the future further optimization is needed to gain access to suitable amounts of material that also need to be purified down the line. Performing the reaction at an elevated temperature and/or increasing the reaction time would be a reasonable starting point.

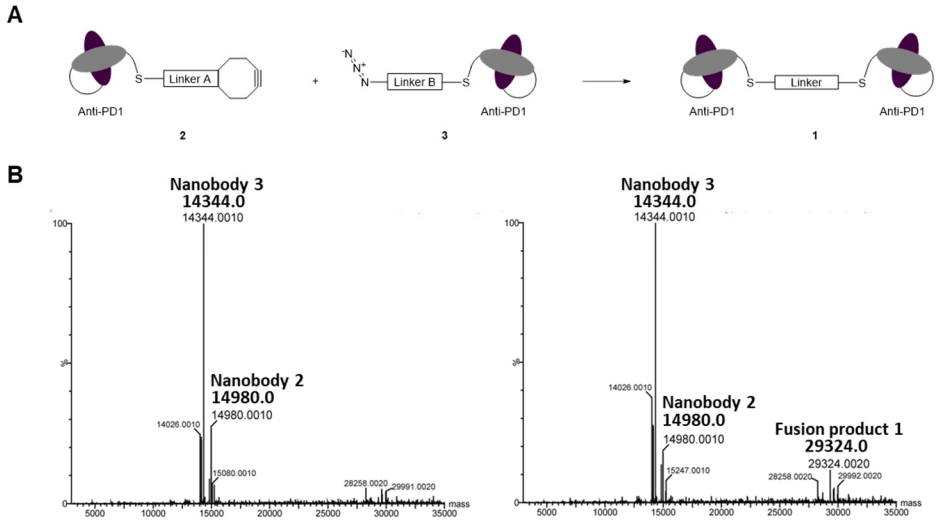


Figure 7.A. Reagents and conditions: nanobody **2** (1.0 equivalent), nanobody **3** (2.0 equivalent), rt, 17 hrs. *B.* Deconvoluted mass spectrum of reaction mixture at the start (left panel), and deconvoluted mass spectrum of the mixture after 17 hrs (right panel) showing formation of the fusion protein with a calculated molecular weight of 29328.5 Da.

Conclusion

This chapter demonstrates the feasibility of chemical conjugation of nanobodies *via* a new synthetic chemical ligation procedure. The designed approach focused on late-stage derivatization of the individual proteins necessitating a two-component linker system. Anti-CD4 and anti-PD1 VHH antibodies equipped with a C-terminal cysteine residue were expressed and purified to near homogeneity. Two linkers were envisioned equipped with a maleimide group to allow for ligation to the cysteine residue present on the nanobodies and either the strained cyclooctyne BCN or the complementary azido moiety. The linker equipped with the cyclooctyne and maleimide group was synthesized using a solid-phase approach. Separation between the two functionalities was achieved by an ethylene glycol building block which allowed for facile elongation during the on-resin synthesis. Purification by reversed-phase HPLC saw the formation of a single byproduct during either evaporation of the aqueous mixture or lyophilization. Instead, size exclusion chromatography followed by silica gel column chromatography proved successful in isolating the linker. As for the complementary linker, the combination of azide and maleimide functionalities proved to be incompatible and a switch was made to the iodoacetamide group. The conjugation of both linkers to anti-PD1 nanobodies

were readily achieved with the iodoacetamide-thiol ligation requiring additional equivalents of the linker and elevated reaction temperatures. A preliminary investigation was then conducted on the SPAAC ligation between the two anti-PD1 nanobodies, which demonstrated that fusion proteins can be formed in the proposed manner. Future research will demonstrate whether the procedure can be made more efficient in terms of scale at which it can be executed, yields and purities in terms of suppressing side reactions. Finally, the methodology can be utilized to produce homodimers, as shown here, but would be particularly attractive in the generation of heterodimers in a controlled fashion, yielding structurally homogeneous bioconjugates in which the nature, specifically also the size, of the spacer linking the two proteins can be controlled, simply through variation in the SPPS procedure as brought to bear in the construction of **5**, with respect to the number and nature of amino acids that are introduced at this stage.

General information

Materials, reactions and purification

Standard Fmoc-amino acids and resins for solid-phase peptide synthesis (SPPS), amino acids for solution-phase synthesis and peptide coupling reagents 2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU), *N*,*N*'-diisopropylcarbodiimide (DIC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), ethyl cyano(hydroxyimino)acetate (Oxyma Pure) and 1-hydroxybenzotriazole (HOBt) were purchased from Novabiochem or Sigma-Aldrich. The resin TentaGel S RAM (0.25 mmol/g) was bought from Rapp Polymere. 3-Maleimidopropionic acid NHS ester was available in-house. All other chemicals were purchased from Acros, Sigma Aldrich, VWR, Fluka, Merck and Fisher Scientific and used as received unless stated otherwise. Tetrahydrofuran (THF), *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), 1,4-dioxane and toluene were stored over molecular sieves before use. Commercially available ACS grade solvents were used for column chromatography without any further purification, except for toluene and ethyl acetate which were distilled prior to use. All reactions were carried out under a nitrogen atmosphere, unless indicated otherwise. Reaction progress and chromatography fractions were monitored by thin layer chromatography (TLC) on silica-gel-coated aluminium sheets with a F254 fluorescent indicator purchased from Merck (Silica gel 60 F_{254}). Visualization was achieved by UV absorption by fluorescence quenching, permanganate stain (4 g $KMnO_4$ and 2 g K_2CO_3 in 200 mL of H_2O), ninhydrin stain (0.6 g ninhydrin and 10 mL acetic acid in 200 mL ethanol). Silica gel column chromatography was performed using Screening Devices silica gel 60 (particle size of 40 – 63 μm, pore diameter of 60 Å) with the indicated eluent. Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Thermo Finnigan Surveyor HPLC system with a Phenomenex Gemini C_{18} column (4.6 mm x 50 mm, 3 μm particle size) with a flow rate of 1 mL/min and a solvent gradient of 10-90% solvent B over 8 min coupled to a LCQ Advantage Max (Thermo Finnigan) ion-trap spectrometer (ESI^+). Preparative RP-HPLC was performed with a GX-281 Liquid Handler and a 331 and 332-H2 primary and secondary solvent pump respectively with a Phenomenex Gemini C_{18} or C_4 column (250 x 10.0 mm, 3 μm particle size) with a flow rate of 5 mL/min and solvent gradients as described for each compound. HPLC solvent compositions: solvent A is 0.1% (v/v) TFA in H_2O; solvent B is MeCN. Preparative RP-HPLC was also performed on an Agilent 1200 HPLC system coupled to a 6130 Quadrupole Mass Spectrometer using a Nucleodur C_{18} Gravity column (250 x 10.0 mm, 5 μm particle size) with a flow rate of 5 mL/min and a gradient over 12 min. as described for each compound. HPLC solvent composition: solvent A is 0.2% (v/v) TFA in H_2O and solvent B is MeCN. All HPLC solvents were filtered with a Millipore filtration system equipped with a 0.22 μm nylon membrane filter prior to use.

Characterization

Nuclear magnetic resonance (^1H and ^{13}C APT NMR) spectra were recorded on a Brüker DPX-300, Brüker AV-400, Brüker DMX-400, Brüker AV-500 or Brüker DMX-600 in the given solvent. Chemical shifts are reported in parts per million (ppm) with the residual solvent or tetramethylsilane (0 ppm) as reference. High-resolution mass spectrometry (HRMS) analysis was performed with a Thermo Finnigan LTQ Orbitrap mass spectrometer equipped with an electronspray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10 ml/min, capillary temperature 250 °C) with resolution R = 60000 at m/z 400 (mass range m/z = 150 – 2000) and dioctyl phthalate (m/z = 391.28428) as a "lock mass". The high-resolution mass spectrometer was calibrated prior to measurements with a Thermo Finnigan calibration mixture. Nominal and exact m/z values are reported in daltons.

Solid-phase peptide synthesis

General methodology

Manual solid-phase peptide synthesis

Manual amino acid couplings were carried out using a fritted reaction syringe equipped with a plunger and syringe cap or a manual reaction vessel (SHG-20260-PI, 60 mL) purchased from Peptides International. The syringe was shaken using either a Heidolph Multi Reax vortexer set at 1000 rpm or a St. John Associates 180° Flask Shaker (model no. A5-6027). Fmoc deprotection was achieved by agitating the resin with 20% (v/v) piperidine in DMF (2 x 10 min.). After draining the reaction vessel, the resin was washed with DMF (6 x 30 sec.). The appropriately side-chain protected Fmoc-amino acid (5.0 equiv.) in DMF (5.0 mL) was pre-activated with HCTU (5.0 equiv.) and DIPEA (10 equiv.) for 5 min, then added

to resin and agitated for 60 min. After draining the reaction vessel, the resin was washed with DMF (4 x 30 sec.). The completion of all couplings was assessed by a Kaiser test and double coupling was performed as needed.

Automated solid-phase peptide synthesis

The automated peptide coupling was performed on a CEM Liberty Blue microwave peptide synthesizer or a Protein Technologies Tribute peptide synthesizer using standard Fmoc protected amino acids. For the Tribute peptide synthesizer, amino acids were presented as solids and 0.20 M HCTU in DMF was used as activator, 0.50 M DIPEA in DMF as the activator base, 20% (v/v) piperidine in DMF as the deprotection agent and a 90:10, DMF – Ac_2O mixture as the capping agent. Coupling of each amino acid occurred at room temperature for 1 hr followed by a capping step (2x 3 min.) betwixt two washing steps. Subsequently, Fmoc was deprotected using the deprotection agent (2x 3 min.) followed by two more washing steps. For the Liberty Blue microwave synthesizer, amino acids were presented as a solution (0.20 M in DMF) and 0.50 M DIC in DMF was used as activator, 1.0 M Oxyma Pure in DMF as additive and 20% (v/v) piperidine in DMF as the deprotection agent. Amino acid coupling in the microwave synthesizer occurred at 90 °C for 2 min. followed by Fmoc deprotection at 90 °C using the aforementioned deprotection agent (2x 90 sec.) and two washing steps.

Loading calculation

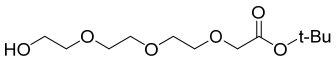
Resin was dried before loading calculation by washing with DCM (3x 30 sec.) and Et_2O (3x 30 sec.) followed by purging with N_2. A small amount of resin (5 – 10 mg) was weighed and DMF (0.80 mL) was added and the resin was swollen for 20 min. Piperidine (0.20 mL) was then added and shaken for 20 min. Following the deprotection, the suspension was filtered and diluted with 20% (v/v) piperidine in DMF to a total volume of 10 mL in a volumetric flask. The absorption of this solution was measured against a blank 20% (v/v) piperidine in DMF solution using a Shimadzu UV-1601 UV-VIS spectrometer with a Quartz cuvette (optical pathway = 1 cm). The loading was then calculated using the following equation:

$$\text{Loading}_{\text{resin}} = \frac{A_{301.0\,\text{nm}} * 10^6\ \text{mmol mol}^{-1}\ \text{mg g}^{-1} * V * D}{\varepsilon_{301.0\,\text{nm}} * m_{\text{resin}} * l}$$

where:

$\text{Loading}_{\text{resin}}$ = Fmoc substitution in mmol/g
$A_{301.0\,\text{nm}}$ = Absorption of sample at 301.0 nm
10^6 mmol mol^{-1} mg g^{-1} = Conversion factor of mmol to mol and mg^{-1} to g^{-1}
V = Total volume in L
D = Dilution factor
$\varepsilon_{301.0\,\text{nm}}$ = Molar absorption coefficient at 301.0 nm (8021 L mol^{-1} cm^{-1})
m_{resin} = sample weight of the resin in mg
l = optical path length of the cell in cm

***tert*-Butyl 2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)acetate**



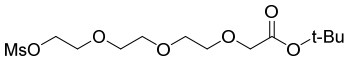
16

To a solution of triethylene glycol **12** (5.3 mL, 40 mmol, 2.0 equiv.) in THF (0.20 L, 0.10 M) at 0 °C was added sodium hydride (0.88 g, 22 mmol, 1.1 equiv.). After 5 min., Tetrabutylammonium iodide (0.74 g, 2.0 mmol, 10 mol%) and *tert*-butyl bromoacetate (3.0 mL, 20 mmol, 1.0 equiv.) were added and the reaction was stirred at room temperature. After 17 hrs, the reaction mixture was filtered. The filtrate was concentrated and purified by silica gel column chromatography (EA) obtaining ester **16** (2.4 g, 9.2 mmol, 46%) as a pale yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.03 (s, 2H, CH_2COO^{t}Bu), 3.76 – 3.65 (m, 10H, OCH_2, CH_2OH), 3.63 – 3.60 (m, 2H, OCH_2), 2.69 (br s, 1H, O*H*), 1.48 (s, 9H, COO^t*Bu*).

^{13}C NMR (101 MHz, $CDCl_3$) δ 169.7 (*C*OO^{t}Bu), 81.7 (*C*CH$_3$), 72.6 (OCH$_2$), 70.8 (OCH$_2$), 70.7 (OCH$_2$), 70.7 (OCH$_2$), 70.4 (OCH$_2$), 69.1 (*C*H$_2$COO^{t}Bu), 61.8 (*C*H$_2$OH), 28.2 (*C*H$_3$).

***tert*-Butyl 2-(2-(2-(2-((methylsulfonyl)oxy)ethoxy)ethoxy)ethoxy)acetate**



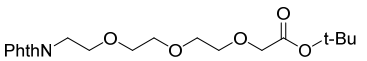
17

Ester **16** (2.0 g, 7.6 mmol, 1.0 equiv.) and Et_3N (2.1 mL, 15 mmol, 2.0 equiv.) were dissolved in DCM (77 mL, 0.10 M) and the solution was cooled to 0 °C. MsCl (0.67 mL, 8.7 mL, 1.2 equiv.) was slowly added and the reaction was stirred for 3 hrs at 0 °C gradually warming to room temperature. The solution was diluted with DCM and subsequently washed with 10% aq. $KHSO_4$ (3x), 10% aq. $NaHCO_3$ (3x) and brine (1x). The organic layer was dried (Na_2SO_4), filtered and concentrated *in vacuo*. After silica gel column chromatography (2:3, EA – Pentane to 4:1, EA – Pentane), mesylate **17** (2.3 g, 6.7 mmol, 88%) was furnished as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.42 – 4.35 (m, 2H, CH_2OMs), 4.01 (s, 2H, CH_2COO^{t}Bu), 3.81 – 3.74 (m, 2H, CH_2CH$_2$OMs), 3.72 – 3.64 (m, 8H, OCH_2), 3.08 (s, 3H, CH_3SO$_2$), 1.48 (s, 9H, COO^t*Bu*).

^{13}C NMR (101 MHz, $CDCl_3$) δ 169.7 (*C*OO^{t}Bu), 81.7 (*C*CH$_3$), 70.8 (OCH$_2$), 70.8 (OCH$_2$), 70.7 (OCH$_2$), 70.7 (OCH$_2$), 69.4 (OCH$_2$), 69.2 (*C*H$_2$OMs), 69.1 (*C*H$_2$COO^{t}Bu), 37.9 (*C*H$_3$SO$_2$), 28.2 (*C*H$_3$).

***tert*-Butyl 2-(2-(2-(2-(1,3-dioxoisoindolin-2-yl)ethoxy)ethoxy)ethoxy)acetate**



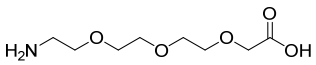
18

A solution of mesylate **17** (0.26 g, 0.75 mmol, 1.0 equiv.) and potassium phthalimide (0.18 g, 0.98 mmol, 1.3 equiv.) in DMF (3.0 mL, 0.25 M) was heated to 110 °C and stirred for 3 hrs. DMF was evaporated and the resulting residue was dissolved in DCM and H_2O. The layers were separated and the organic layer was dried ($MgSO_4$), filtered and concentrated *in vacuo*. The concentrate was purified by silica gel column chromatography (1:9, EA – Pentane to 1:1, EA – Pentane) giving phthalimidate **18** (0.29 g, 0.73 mmol, 97%) as a yellow-green oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 – 7.80 (m, 2H, C*H*-arom), 7.76 – 7.69 (m, 2H, C*H*-arom), 3.99 (s, 2H, CH_2COO^{t}Bu), 3.90 (t, J = 5.8 Hz, 2H, PhthNCH_2), 3.75 (t, J = 5.8 Hz, 2H, PhthNCH$_2$CH_2), 3.71 – 3.58 (m, 8H, OCH_2), 1.47 (s, 9H, COO^t*Bu*).

^{13}C NMR (101 MHz, $CDCl_3$) δ 169.7 (*C*OO^{t}Bu), 168.2 (*C*O-Phth), 133.9 (*C*H-arom), 132.1 (*C*q-arom), 123.2 (*C*H-arom), 81.4 (*C*CH$_3$), 70.7 (OCH$_2$), 70.6 (OCH$_2$), 70.6 (OCH$_2$), 70.0 (OCH$_2$), 69.0 (*C*H$_2$COO^{t}Bu), 67.9 (PhthNCH$_2$CH$_2$), 37.2 (PhthNCH$_2$), 28.1 (*C*H$_3$).

2-(2-(2-(2-Aminoethoxy)ethoxy)ethoxy)acetic acid



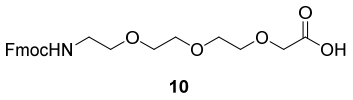
19

Phthalimidate **18** (0.20 g, 0.51 mmol, 1.0 equiv.) was dissolved in a mixture of DCM and TFA (1:1, TFA – DCM, 5.1 mL, 0.10 M) and the reaction was stirred at room temperature. After 1 hr, toluene was added and the solution was concentrated. The crude product was taken up in methanol (5.1 mL, 0.10 M) and hydrazine hydrate (0.25 mL, 5.1 mmol, 10 equiv.) was added. After stirring for 90 min. at room temperature, the solution was evaporated. Methanol was added, the resulting suspension filtered and the filtrate concentrated (15x) giving amine **19** (74 mg, 0.36 mmol, 70%) as a colorless oil.

^{1}H NMR (400 MHz, MeOD) δ 3.91 (s, 2H, CH_2COOH), 3.79 – 3.74 (m, 2H, H$_2$NCH$_2$CH_2), 3.74 – 3.61 (m, 8H, OCH_2), 3.16 – 3.08 (m, 2H, H$_2$NCH_2).

^{13}C NMR (101 MHz, MeOD) δ 178.0 (*C*OOH), 71.9 (OCH$_2$), 71.4 (OCH$_2$), 71.2 (OCH$_2$), 70.9 (OCH$_2$), 70.8 (OCH$_2$), 68.2 (*C*H$_2$COOH), 40.0 (NH$_2$*C*H$_2$).

1-(9*H*-Fluoren-9-yl)-3-oxo-2,7,10,13-tetraoxa-4-azapentadecan-15-oic acid



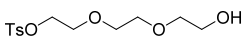
10

To a mixture of 1,4-dioxane and H_2O (2:1, 1,4-dioxane – H_2O, 21 mL, 0.13 M) were added amine **19** (0.55 g, 2.7 mmol, 1.0 equiv.) and *N*-methylmorpholine (0.88 mL, 8.0 mmol, 3.0 equiv.) and the solution was cooled to 0 °C. Fmoc *N*-hydroxysuccinimide ester (1.4 g, 4.0 mmol, 1.5 equiv.) was added portionwise and the reaction was stirred for 17 hours, gradually warming to room temperature. DCM and 1.0 M aq. HCl were added and the layers were separated. The organic phase was dried ($MgSO_4$), filtered and evaporated to dryness. Purification by silica gel column chromatography (3:97, MeOH – DCM to 1:9, MeOH – DCM) afforded title compound **10** (0.13 g, 0.29 mmol, 11%) as a pale yellow oil.

^{1}H NMR (400 MHz, MeOD) δ 7.76 (d, *J* = 7.5 Hz, 2H, C*H*-arom), 7.62 (d, *J* = 7.5 Hz, 2H, C*H*-arom), 7.37 (td, *J* = 7.5, 1.2 Hz, 2H, C*H*-arom), 7.29 (td, *J* = 7.4, 1.2 Hz, 2H, C*H*-arom), 4.33 (d, *J* = 6.9 Hz, 2H, CH_2-Fmoc), 4.17 (t, *J* = 6.9 Hz, 1H, C*H*-Fmoc), 3.97 (s, 2H, CH_2COOH), 3.64 – 3.55 (m, 8H, OCH_2), 3.51 (t, *J* = 5.4 Hz, 2H, FmocHNCH$_2$CH_2), 3.33 – 3.24 (m, 2H, FmocHNCH_2).

^{13}C NMR (101 MHz, MeOD) δ 176.6 (*C*OOH), 159.1 (NH*C*OO), 145.3 (*C*q-arom), 142.5 (*C*q-arom), 128.8 (*C*H-arom), 128.1 (*C*H-arom), 126.2 (*C*H-arom), 120.9 (*C*H-arom), 71.5 (FmocHNCH$_2$*C*H$_2$), 71.1 (O*C*H$_2$), 71.0 (O*C*H$_2$), 70.9 (O*C*H$_2$), 70.8 (*C*H$_2$COOH), 67.7 (*C*H$_2$-Fmoc), 48.4 (*C*H-Fmoc), 41.5 (FmocHN*C*H$_2$).

2-(2-(2-Hydroxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate



20

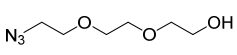
Triethylene glycol **12** (0.13 L, 1.0 mol, 10 equiv.) was dissolved in DCM (0.13 L, 0.80 M). The solution was cooled to 0 °C and Et_3N (21 mL, 0.15 mol, 1.5 equiv.) was added. Subsequently tosyl chloride (19 g, 0.10 mol, 1.0 equiv.) was added portionwise over 90 min. and the reaction was stirred for an additional 17 hrs gradually warming to room temperature. The mixture was washed with H_2O (3x) with each aqueous layer being back-extracted with DCM. The pooled organic layers were washed with 10% aq. citric acid (3x), dried ($MgSO_4$), filtered and concentrated *in vacuo* giving tosylate **20** (30 g, 98 mmol, 98%) as a pale yellow oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.82 – 7.77 (m, 2H, C*H*-arom), 7.37 – 7.33 (m, 2H, C*H*-arom), 4.18 – 4.15 (m, 2H CH_2OTs), 3.72 – 3.68 (m, 4H, CH_2OH, CH_2CH$_2$OTs), 3.60 (s, 4H, OCH_2), 3.58 – 3.55 (m, 2H, CH_2CH$_2$OH), 2.79 (br s, 1H, O*H*), 2.45 (s, 3H, CH_3).

^{13}C NMR (126 MHz, $CDCl_3$) δ 144.9 (*C*q-arom), 132.8 (*C*q-arom), 129.9 (*C*H-arom), 127.9 (*C*H-arom), 72.5 (CH$_2$CH$_2$OH), 70.7 (OCH$_2$), 70.2 (OCH$_2$), 69.2 (CH$_2$CH$_2$OTs), 68.6 (CH$_2$OTs), 61.6 (CH$_2$OH), 21.6 (CH$_3$).

HRMS (ESI-Orbitrap) calcd. for $C_{13}H_{21}O_6S$ $[M+H]^+$ 305.10534, found 305.10515.

2-(2-(2-Azidoethoxy)ethoxy)ethan-1-ol



21

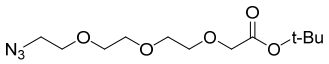
To a solution of tosylate **20** (0.12 kg, 0.40 mol, 1.0 equiv.) in DMF (0.18 L, 2.3 M) was added sodium azide (26 g, 0.40 mol, 1.0 equiv.) and the suspension was heated to 90 °C and stirred for 4 hrs. The mixture was filtered and the precipitate was washed with DMF. The filtrate was concentrated and the residue co-evaporated with toluene (2x). DCM was added, suspension was filtered and concentrated under reduced pressure (2x) affording azide **21** (58 g, 0.33 mol, 82%) as a yellow oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 3.74 (t, *J* = 4.3 Hz, 2H, CH_2OH), 3.71 – 3.67 (m, 6H, OCH_2), 3.64 – 3.60 (m, 2H, CH_2CH$_2$OH), 3.41 (t, *J* = 5.1 Hz, 2H, CH_2N$_3$), 2.75 (br s, 1H, O*H*).

^{13}C NMR (126 MHz, $CDCl_3$) δ 72.5 (CH$_2$CH$_2$OH), 70.6 (OCH$_2$), 70.4 (OCH$_2$), 70.0 (OCH$_2$), 61.7 (CH$_2$OH), 50.6 (CH$_2$N$_3$).

HRMS (ESI-Orbitrap) calcd. for $C_6H_{13}N_3O_3Na$ $[M+Na]^+$ 198.08491, found 198.08491.

***tert*-Butyl 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetate**

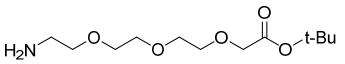


22

tert-Butanol (50 mL, 0.58 M) was warmed to 30 °C and azide **21** (5.0 g, 29 mmol, 1.0 equiv.) and potassium *tert*-butoxide (6.5 g, 58 mmol, 2.0 equiv.) were added. The reaction was stirred for 1hr after which *tert*-butyl bromoacetate (8.5 mL, 58 mmol, 2.0 equiv.) was slowly added. The suspension was further warmed to 50 °C and stirred for 5 hrs. The volatiles were removed under reduced pressure and the residue was taken up in DCM and H_2O. The layers were separated and the aqueous layer was back-extracted with DCM (3x). The pooled organic fractions were washed with brine, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by silica gel column chromatography (1:4, EA – Pentane to 3:2, EA – Pentane) furnished *tert*-butyl ester **22** (6.7 g, 23 mmol, 80%) as a yellow oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.03 (s, 2H, CH_2COO^tBu), 3.75 – 3.65 (m, 10H, OCH_2), 3.39 (t, *J* = 5.1 Hz, 2H, CH_2N_3), 1.48 (s, 9H, COO^tBu).
^{13}C NMR (126 MHz, $CDCl_3$) δ 169.7 (COO^tBu), 81.6 (CCH_3), 70.8 (OCH_2), 70.7 (OCH_2), 70.7 (OCH_2), 70.7 (OCH_2), 70.1 (OCH_2), 69.1 (CH_2COO^tBu), 50.7 (CH_2N_3), 28.1 (CH_3).
HRMS (ESI-Orbitrap) calcd. for $C_{12}H_{23}N_3O_5Na$ $[M+Na]^+$ 312.15299, found 312.15277.

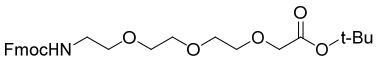
***tert*-Butyl 2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)acetate**



30

tert-Butyl ester **22** (0.50 g, 1.7 mmol, 1.0 equiv.) was dissolved in THF (13 mL, 0.13 M) and the solution was cooled to 0 °C. Triphenylphosphine (0.59 g, 2.3 mmol, 1.3 equiv.) was added portionwise and the reaction was stirred for 17 hrs slowly warming to room temperature. H_2O (81 μL, 4.5 mmol, 2.6 equiv.) was added and the reaction was stirred for an additional 24 hrs. Toluene and H_2O were added and the layers were separated. The organic layer was extracted with H_2O (2x) and the combined aqueous layers were concentrated under reduced pressure furnishing amine **30** (0.41 g, 1.5 mmol, 89%) as a yellow oil.
^{1}H NMR (400 MHz, $CDCl_3$) δ 4.03 (s, 2H, CH_2COO^tBu), 3.75 – 3.61 (m, 8H, OCH_2), 3.54 (t, *J* = 5.2 Hz, 2H, $CH_2CH_2NH_2$), 2.89 (t, *J* = 5.2 Hz, 2H, CH_2NH_2), 2.54 (s, 2H, NH_2), 1.48 (s, 9H, COO^tBu).
^{13}C NMR (101 MHz, $CDCl_3$) δ 169.7 (COO^tBu), 81.6 (CCH_3), 72.8 ($CH_2CH_2NH_2$), 70.6 (OCH_2), 70.5 (OCH_2), 70.5 (OCH_2), 70.2 (OCH_2), 69.0 (CH_2COO^tBu), 41.5 (CH_2NH_2), 28.1 (CH_3).
HRMS (ESI-Orbitrap) calcd. for $C_{12}H_{26}NO_5$ $[M+H]^+$ 264.18055, found 264.18045.

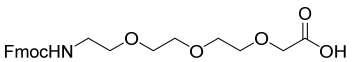
***tert*-Butyl 1-(9*H*-fluoren-9-yl)-3-oxo-2,7,10,13-tetraoxa-4-azapentadecan-15-oate**



23

At 0 °C, DCM (2.5 mL, 0.15 M) was added to amine **30** (0.10 g, 0.38 mmol, 1.0 equiv.) and Fmoc *N*-hydroxysuccinimide ester (0.14 g, 0.42 mmol, 1.1 equiv.) was added portionwise. The reaction was stirred at 0 °C for 1 hr before *N*-methylmorpholine (42 μL, 0.38 mmol, 1.0 equiv.) was added. After stirring for another hr, additional *N*-methylmorpholine (42 μL, 0.38 mmol, 1.0 equiv.) was added and the solution was allowed to stir for 4 more hrs at 0 °C. The solution was washed with H_2O and the aqueous layer was back-extracted with DCM. The pooled organic layers were dried ($MgSO_4$), filtered and the volatiles were removed under reduced pressure. The crude was purified by silica gel column chromatography (1:4, EA – Pentane to 1:1, EA – Pentane) obtaining Fmoc-protected amine **23** (0.18 g, 0.37 mmol, 96%) as a light yellow oil.
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (dt, J = 7.6, 0.9 Hz, 2H, C*H*-arom), 7.61 (d, J = 7.5 Hz, 2H, C*H*-arom), 7.40 (td, J = 7.5, 0.9 Hz, 2H, C*H*-arom), 7.31 (td, J = 7.5, 1.2 Hz, 2H, C*H*-arom), 5.43 (t, J = 5.7 Hz, 1H, N*H*Fmoc), 4.39 (d, J = 7.1 Hz, 2H, CH_2-Fmoc), 4.22 (t, J = 7.0 Hz, 1H, C*H*-Fmoc), 4.00 (s, 2H, CH_2COO^tBu), 3.74 – 3.61 (m, 8H, OCH_2), 3.58 (t, J = 5.0 Hz, 2H, $CH_2CH_2NHFmoc$), 3.40 (q, J = 5.3 Hz, 2H, $CH_2NHFmoc$), 1.46 (s, 9H, COO^tBu).
^{13}C NMR (126 MHz, $CDCl_3$) δ 169.8 (COO^tBu) , 156.7 (NHCOO), 144.1 (*C*q-arom), 141.4 (*C*q-arom), 127.8 (*C*H-arom), 127.2 (*C*H-arom), 125.2 (*C*H-arom), 120.1 (*C*H-arom), 81.7 (CCH_3), 70.8 (OCH_2), 70.7 (OCH_2), 70.7 (OCH_2), 70.4 (OCH_2), 70.2 ($CH_2CH_2NHFmoc$), 69.1 (CH_2COO^tBu) , 66.7 (CH_2-Fmoc), 47.4 (*C*H-Fmoc), 41.0 ($CH_2NHFmoc$), 28.2 (CH_3).
HRMS (ESI-Orbitrap) calcd. for $C_{27}H_{35}NO_7Na$ $[M+Na]^+$ 508.23057, found 508.23016.

1-(9*H*-Fluoren-9-yl)-3-oxo-2,7,10,13-tetraoxa-4-azapentadecan-15-oic acid

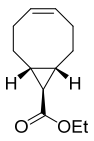


10

tert-Butyl ester **23** (39 g, 81 mmol, 1.0 equiv.) was dissolved in toluene (20 mL, 4.0 M) and 85% wt. aq. H_3PO_4 (28 mL, 0.40 mol, 5.0 equiv.) was slowly added. The reaction was stirred at room temperature for 4 hrs after which EA and H_2O were added. The layers were separated and the aqueous phase was extracted with EA (2x). The pooled organic fractions were dried ($MgSO_4$), filtered and concentrated under reduced pressure to furnish carboxylic acid **10** (34 g, 79 mmol, 98%) as a pale yellow oil.
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.6 Hz, 2H, C*H*-arom), 7.60 (d, *J* = 7.5 Hz, 2H, C*H*-arom), 7.38 (t, *J* = 7.5 Hz, 2H, C*H*-arom), 7.30 (td, *J* = 7.5, 1.2 Hz, 2H, C*H*-arom), 7.06 (br s, 1H, COO*H*), 5.58 (t, *J* = 5.9 Hz, 1H, Fmoc*H*N), 4.39 (d, *J* = 7.0 Hz, 2H, CH_2-Fmoc), 4.21 (t, *J* = 7.0 Hz, 1H, C*H*-Fmoc), 4.12 (s, 2H, CH_2COOH), 3.79 – 3.59 (m, 8H, OCH_2), 3.55 (t, *J* = 5.1 Hz, 2H, $CH_2CH_2NHFmoc$), 3.39 (q, *J* = 5.3 Hz, 2H, $CH_2NHFmoc$).

^{13}C NMR (126 MHz, $CDCl_3$) δ 172.7 (*C*OOH), 156.8 (NH*C*OO), 144.1 (Cq-arom), 141.4 (Cq-arom), 127.7 (*C*H-arom), 127.1 (*C*H-arom), 125.2 (*C*H-arom), 120.0 (*C*H-arom), 71.2 (OCH_2), 70.5 (OCH_2), 70.3 (OCH_2), 70.2 (OCH_2), 70.1 (OCH_2), 68.7 (CH_2COOH), 66.7 (CH_2-Fmoc), 47.3 (*C*H-Fmoc), 40.9 (CH_2NHFmoc).
HRMS (ESI-Orbitrap) calcd. for $C_{23}H_{28}NO_7$ $[M+H]^+$ 430.18603, found 430.18607.

(1*R*,8*S*,9*r*,*Z*)-Ethyl bicyclo[6.1.0]non-4-ene-9-carboxylate

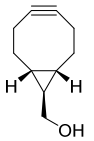


25

Cyclooctadiene **24** (0.20 L, 1.6 mol, 8.0 equiv.) and copper(II)acetylacetonate (1.1 g, 4.0 mmol, 2.0 mol%) were dissolved in EA (0.10 L, 2.0 M). A solution of ethyl diazoacetate (20 mL, 0.20 mol, 1.0 equiv.) in EA (0.10 L, 2.0 M) was added dropwise over 3 hrs and the reaction mixture was refluxed for 17 hrs. EA was removed under reduced pressure. Excess cyclooctadiene was removed by flushing the crude over silica (1:200, EA – Pentane) after which the product was eluted with EA. The solution was concentrated *in vacuo* and purified by silica gel column chromatography (toluene) to afford *exo*-product **25** (17 g, 88 mmol, 44%) and *endo*-product (4.6 g, 24 mmol, 12%) as light yellow oils.

Exo-product **25**
^{1}H NMR (400 MHz, $CDCl_3$) δ 5.66 – 5.56 (m, 2H, C*H*=C*H*), 4.09 (q, J = 7.1 Hz, 2H, CH_2-Ethyl), 2.32 – 2.24 (m, 2H, CH_2Cp), 2.22 – 2.13 (m, 2H, CH_2CH=CH), 2.11 – 2.00 (m, 2H, CH_2CH=CH), 1.61 – 1.52 (m, 2H, C*H*-bridgehead), 1.51 – 1.40 (m, 2H, CH_2Cp), 1.23 (t, J = 7.2 Hz, 3H, CH_3-ethyl), 1.17 (t, J = 4.6 Hz, 1H, C*H*).
^{13}C NMR (101 MHz, $CDCl_3$) δ 174.5 (*C*OOEt), 130.0 (*C*H=CH), 60.3 (OCH_2CH_3), 28.3 (CH_2CH=CH), 27.9 (*C*H), 27.8 (*C*H-Bridgehead), 26.7 (CH_2Cp), 14.4 (OCH_2CH_3).
Endo-product
^{1}H NMR (400 MHz, $CDCl_3$) δ 5.67 – 5.53 (m, 2H, C*H*=C*H*), 4.11 (q, *J* = 7.1 Hz, 2H, CH_2-Ethyl), 2.57 – 2.42 (m, 2H, CH_2Cp), 2.26 – 2.16 (m, 2H, CH_2CH=CH), 2.13 – 1.96 (m, 2H, CH_2CH=CH), 1.90 – 1.76 (m, 2H, CH_2Cp), 1.70 (t, *J* = 8.8 Hz, 1H, C*H*), 1.46 – 1.32 (m, 2H, C*H*-bridgehead), 1.26 (t, *J* = 7.1 Hz, 3H, CH_3-Ethyl).
^{13}C NMR (101 MHz, $CDCl_3$) δ 172.4 (*C*OOEt), 129.5 (*C*H=*C*H), 59.8 (OCH_2CH_3), 27.2 (CH_2CH=CH), 24.3 (*C*H-Bridgehead), 22.8 (CH_2Cp), 21.3 (*C*H), 14.5 (OCH_2CH_3).

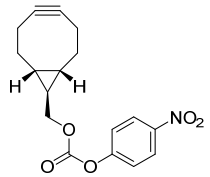
((1*R*,8*S*,9*r*)-bicyclo[6.1.0]non-4-yn-9-yl)methanol



26

$LiAlH_4$ (0.24 g, 6.3 mmol, 0.90 equiv.) was added to Et_2O (26 mL, 0.27 M) and the suspension was cooled to 0 °C. A solution of ethyl ester **25** (1.4 g, 7.0 mmol, 1.0 equiv) in Et_2O (26 mL, 0.27 M) was added dropwise over 30 min. The suspension was stirred for 15 min. at room temperature, then cooled down to 0 °C. H_2O was added slowly until the grey solid had turned white. The suspension was dried (Na_2SO_4), filtered and the residue washed with copious amounts of Et_2O. The filtrate was concentrated under reduced pressure. The crude was dissolved in DCM (55 mL, 0.13 M) and cooled to 0 °C. Bromine (0.40 mL, 7.7 mmol, 1.1 equiv.) was added dropwise until the yellow color persisted. The excess bromine was quenched with sat. aq. Na_2SO_3 and the mixture was extracted with DCM (2x). The pooled organic fractions were dried (Na_2SO_4), filtered and concentrated *in vacuo*. The crude dibromide was taken up in THF (65 mL, 0.11 M) and cooled to 0 °C. To this solution was added KO^{t}Bu (1.0 M in THF, 23 mL, 23 mmol, 3.3 equiv.) dropwise and the mixture was heated to reflux and stirred for 2 hrs. After the reaction was cooled, the mixture was quenched with sat. aq. NH_4Cl and subsequently extracted with DCM (3x). The combined organic layers were dried (Na_2SO_4), filtered and concentrated under reduced pressure. Purification by silica gel column chromatography (1:9, EA – Pentane to 2:3, EA – Pentane) afforded strained alkyne **26** (0.59 g, 3.9 mmol, 56%) as a white solid.
^{1}H NMR (300 MHz, $CDCl_3$) δ 3.55 (d, *J* = 6.3 Hz, 2H, CH_2OH), 2.47 – 2.37 (m, 2H, CH_2Cp), 2.36 – 2.22 (m, 2H, CH_2C≡C), 2.21 – 2.11 (m, 2H, CH_2C≡C), 2.09 (s, 1H, O*H*), 1.47 – 1.32 (m, 2H, CH_2Cp), 0.77 – 0.60 (m, 3H, C*H*-bridgehead, C*H*CH_2OH).
^{13}C NMR (75 MHz, $CDCl_3$) δ 98.9 (*C*≡*C*), 67.1 (*C*H_2OH), 33.5 (CH_2C≡C), 27.3 ($CHCH_2$OH), 22.6 (*C*H-Bridgehead), 21.5 (CH_2Cp).

((1*R*,8*S*,9*r*)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (4-nitrophenyl) carbonate



9

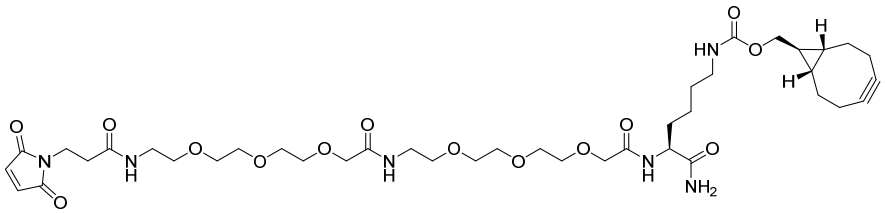
Alcohol **26** (2.7 g, 18 mmol, 1.0 equiv.) was dissolved in DCM (0.43 L, 43 mM) and pyridine (3.7 mL, 46 mmol, 2.5 equiv.) and *p*-nitrophenyl chloroformate (4.6 g, 23 mmol, 1.3 equiv.) were added. The solution was stirred at room temperature for 15 min after which the mixture was quenched with sat. aq. NH_4Cl. The mixture was subsequently extracted with DCM (3x) and the pooled organic layers were dried (Na_2SO_4), filtered and concentrated *in vacuo*. After silica gel column chromatography (1:9, EA – Pentane to 1:4, EA – Pentane), carbonate **9** (3.9 g, 13 mmol, 69%) was obtained as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 – 8.26 (m, 2H, C*H*-arom), 7.44 – 7.35 (m, 2H, C*H*-arom), 4.23 (d, *J* = 6.8 Hz, 2H, CH_2O), 2.51 – 2.41 (m, 2H, CH_2Cp), 2.38 – 2.25 (m, 2H, CH_2C≡C), 2.23 – 2.13 (m, 2H, CH_2C≡C), 1.51 – 1.35 (m, 2H, CH_2Cp), 0.93 – 0.78 (m, 3H, C*H*-bridgehead, C*H*CH_2O).

^{13}C NMR (101 MHz, $CDCl_3$) δ 155.7 (*C*=O), 152.7 (*C*q-arom), 145.4 (*C*q-arom), 125.4 (CH-arom), 121.9 (*C*H-arom), 98.7 (*C*≡*C*), 74.0 (CH$_2$O), 33.2 (*C*H$_2$C≡C), 23.3 (*C*H-Bridgehead), 23.0 (*C*HCH$_2$O), 21.4 (CH$_2$Cp).

HRMS (ESI-Orbitrap) calcd. for $C_{17}H_{18}NO_5$ [M+H]$^+$ 317.12130, found 317.19331.

((1*R*,8*S*,9*r*)-bicyclo[6.1.0]non-4-yn-9-yl)methyl ((*S*)-29-carbamoyl-1-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)-3,15,27-trioxo-7,10,13,19,22,25-hexaoxa-4,16,28-triazatritriacontan-33-yl)carbamate



5

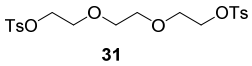
Using the procedure for manual peptide synthesis, Tentagel S RAM (0.25 mmol/g, 0.40 g, 0.10 mmol, 1.0 equiv.) was elongated with standard lysine building block and triethylene building block **10** to afford resin **28**. After Fmoc deprotection, a solution of 3-maleimido propionic acid NHS ester (68 mg, 0.30 mmol, 3.0 equiv.) and Hünig's base (52 μL, 0.30 mmol, 3.0 equiv.) in DMF (4.0 mL, 25 mM) was added to the resin and the resulting suspension was shaken for 4 hrs. The resin was washed with DMF (4x) and DCM (4x). A cleavage cocktail (190:5:5, TFA – H_2O – TIPS, 10 mL, 10 mM) was added and shaken for 3 hrs. The suspension was filtered and the filtrate was concentrated under a stream of N_2. The crude product was taken up in DMF (1.0 mL, 0.10 M) and Hünig's base (19 μL, 0.11 mmol, 1.1 equiv.) and BCN PNP carbonate **9** (35 mg, 0.11 mmol, 1.1 equiv.) were added. The reaction mixture was stirred for 4 hrs and subsequently evaporated at 20 °C. The crude product was purified by size exclusion chromatography (Sephadex LH-20, 1:1, MeOH – DCM) followed by silica gel column chromatography (1:99, MeOH – DCM to 7:93, MeOH – DCM) to afford linker **5** (21 mg, 25 μmol, 25%) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43 (d, *J* = 8.5 Hz, 1H, N*H*-Lys), 7.32 (t, *J* = 6.0 Hz, 1H, *N*HCH$_2$CH$_2$O), 7.05 (t, *J* = 5.3 Hz, 1H, *N*HCH$_2$CH$_2$O), 6.71 (s, 2H, C*H*-Maleimide), 6.67 (s, 1H, N*H*$_2$), 6.02 (s, 1H, N*H*$_2$), 5.04 (t, *J* = 5.7 Hz, 1H, N*H*-BCN), 4.49 (td, *J* = 8.4, 5.7 Hz, 1H, α-Lys), 4.08 – 4.03 (m, 4H, OC*H*$_2$C=O), 3.95 (d, *J* = 6.9 Hz, 2H, C*H*$_2$OC=O), 3.83 (t, *J* = 7.2 Hz, 2H, C*H*$_2$N(C=O)$_2$), 3.73 – 3.60 (m, 16H, OC*H*$_2$), 3.60 – 3.56 (m, 2H, NHCH$_2$C*H*$_2$O), 3.53 (t, *J* = 5.1 Hz, 2H, NHCH$_2$C*H*$_2$O), 3.49 – 3.42 (m, 2H, NHC*H*$_2$), 3.42 – 3.36 (m, 2H, NHC*H*$_2$), 3.20 – 3.13 (m, 2H, ε-Lys), 2.52 (t, *J* = 7.2 Hz, 2H, C*H*$_2$CH$_2$N(C=O)$_2$), 2.42 – 2.36 (m, 2H, C*H*$_2$Cp), 2.31 – 2.24 (m, 2H, C*H*$_2$C≡C), 2.17 – 2.10 (m, 2H, C*H*$_2$C≡C), 1.96 – 1.87 (m, 1H, β-Lys)), 1.75 – 1.65 (m, 1H, β-Lys)), 1.58 – 1.52 (m, 2H, δ-Lys)), 1.43 – 1.31 (m, 4H, γ-Lys, C*H*$_2$C≡C), 0.77 – 0.70 (m, 2H, C*H*-bridgehead), 0.69 – 0.55 (m, 1H, C*H*CH$_2$OC=O).

^{13}C NMR (126 MHz, $CDCl_3$) δ 174.0 (NH$_2$*C*=O), 170.7 (*C*=O), 170.5 (*C*=O), 170.4 (*C*=O), 170.2 (*C*=O), 157.0 (*C*=O-urethane), 134.3 (*C*H-maleimide), 98.9 (*C*≡*C*), 71.2 (OCH$_2$), 70.8 (OCH$_2$), 70.7 (OCH$_2$), 70.6 (OCH$_2$), 70.5 (OCH$_2$), 70.4 (OCH$_2$), 70.3 (OCH$_2$), 70.3 (OCH$_2$), 70.1 (OCH$_2$), 70.0 (OCH$_2$), 70.0 (OCH$_2$), 69.7 (OCH$_2$), 69.0 (*C*H$_2$OC=O), 52.2 (α-Lys), 40.6 (ε-Lys), 39.3 (CH$_2$NHC=O), 38.7 (CH$_2$NHC=O), 34.5 (*C*H$_2$N(C=O)$_2$), 34.5 (*C*H$_2$CH$_2$N(C=O)$_2$), 33.4 (*C*H$_2$Cp), 31.5 (β-Lys), 29.5 (δ-Lys), 23.8 (CHCH$_2$OC=O), 22.9 (*C*H-bridgehead), 22.8 (γ-Lys), 21.5 (*C*H$_2$C≡C).

HRMS (ESI-Orbitrap) calcd. for $C_{40}H_{63}N_6O_{14}$ [M+H]$^+$ 851.43968, found 851.43964.

Triethylene glycol ditosylate



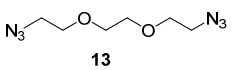
31

Triethylene glycol **12** (2.0 mL, 15 mmol, 1.0 equiv.) and *p*-toluenesulfonyl chloride (5.7 g, 30 mmol, 2.0 equiv.) were dissolved in DCM (75 mL, 0.20 M) and the solution was cooled to 0 °C. Powdered sodium hydroxide (4.8 g, 0.12 mol, 8.0 equiv.) was added portionwise and the mixture was stirred for 3 hrs at 0 °C. H_2O was added and the layers were separated. The aqueous phase was back-extracted with DCM (2x) and the combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by silica gel column chromatography (1:1, EA – Pentane) afforded ditosylate **31** (6.3 g, 14 mmol, 91%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 – 7.75 (m, 4H, C*H*-arom), 7.38 – 7.30 (m, 4H, C*H*-arom), 4.15 – 4.12 (m, 4H, CH_2OTs), 3.67 – 3.63 (m, 4H, CH_2CH_2OTs), 3.52 (s, 4H, OCH_2), 2.44 (s, 6H, PhCH_3).

^{13}C NMR (101 MHz, $CDCl_3$) δ 145.0 (*C*q-arom), 133.0 (*C*q-arom), 130.0 (*C*H-arom), 128.0 (*C*H-arom), 70.7 (OCH_2), 69.3 (CH_2OTs), 68.8 (CH_2CH_2OTs), 21.7 (PhCH_3).

Triethylene glycol diazide



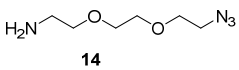
13

Ditosylate **31** (6.3 g, 14 mmol, 1.0 equiv.) and tetrabutylammonium iodide (0.25 g, 0.68 mmol, 5.0 mol%) were dissolved in DMF (20 mL, 0.70 M). Sodium azide (3.6 g, 55 mmol, 4.0 equiv.) was added and the mixture was heated to 80 °C and stirred for 17 hrs. The mixture was concentrated and further co-evaporation with *n*-heptane was performed to remove all traces of DMF. Et_2O was added, the resulting suspension was filtered and the filtrate concentrated (3x). Purification of the filtrate by silica gel column chromatography (2:3, EA – Pentane to 1:0, EA – Pentane) gave diazide **13** (2.5 g, 12 mmol, 91%) as a pale yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.72 – 3.66 (m, 8H, OCH_2), 3.40 (t, *J* = 5.0 Hz, 4H, CH_2N_3).

^{13}C NMR (101 MHz, $CDCl_3$) δ 70.8 (OCH_2), 70.2 ($CH_2CH_2N_3$), 50.7 (CH_2N_3).

2-(2-(2-azidoethoxy)ethoxy)ethan-1-amine



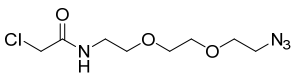
14

To diazide **13** (1.3 g, 6.3 mmol, 1.0 equiv.) was added a mixture of Et_2O, THF and 1.0 M aq. HCl (5:1:4, Et_2O – THF – 1.0 M aq. HCl, 19 mL, 0.32 M). A solution of PPh_3 (2.0 g, 9.3 mmol, 1.5 equiv.) in Et_2O (25 mL, 0.37 M) was added dropwise and the mixture was stirred vigorously for 24 hrs. To the mixture was added 4.0 M aq. HCl and the layers were separated. The aqueous layer was washed with Et_2O (2x) and subsequently brought to pH 14 by adding sodium hydroxide. The aqueous layer was then extracted with DCM (3x). The pooled organic fractions were dried ($MgSO_4$), filtered and concentrated *in vacuo* to furnish amine **14** (0.77 g, 4.4 mmol, 71%) as a yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.66 – 3.53 (m, 6H, OCH_2), 3.46 (t, *J* = 5.2 Hz, 2H, C$H_2CH_2NH_2$), 3.33 (t, *J* = 5.0 Hz, 2H, CH_2N_3), 2.80 (t, *J* = 5.2 Hz, 2H, CH_2NH_2), 1.60 (br s, 2H, NH_2).

^{13}C NMR (101 MHz, $CDCl_3$) δ 73.4 ($CH_2CH_2NH_2$), 70.6 (OCH_2), 70.3 (OCH_2), 70.0 (OCH_2), 50.6 (CH_2N_3), 41.7 (CH_2NH_2).

***N*-(2-(2-(2-azidoethoxy)ethoxy)ethyl)-2-chloroacetamide**



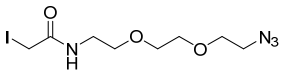
32

Amine **14** (35 mg, 0.20 mmol, 1.0 equiv.) and Et_3N (36 µL, 0.26 mmol, 1.3 equiv.) were dissolved in DCM (1.0 mL, 0.20 M) and the solution was cooled to 0 °C. Chloroacetyl chloride (21 µL, 0.26 mmol, 1.3 equiv.) was added dropwise and the reaction was stirred for 2 hrs at 0 °C. Additional DCM was added and the mixture was washed with sat. aq. $NaHCO_3$ (2x), H_2O and brine. The organic layer was dried ($MgSO_4$), filtered and the volatiles were removed under reduced pressure. Purification by silica gel column chromatography (1:49, MeOH – DCM) afforded chloroacetamide **32** (34 mg, 0.14 mmol, 68%) as a colorless oil.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.01 (br s, 1H, N*H*), 4.06 (s, 2H, CH_2Cl), 3.73 – 3.57 (m, 8H, OCH_2), 3.55 – 3.48 (m, 2H, CH_2NHClAc), 3.45 – 3.36 (m, 2H, CH_2N_3).

^{13}C NMR (75 MHz, $CDCl_3$) δ 166.1 (*C*=O), 70.7 (OCH_2), 70.5 (OCH_2), 70.2 (OCH_2), 69.6 (CH_2CH_2NHClAc), 50.8 (CH_2N_3), 42.7 (CH_2Cl), 39.7 (CH_2NHClAc).

_N_-(2-(2-(2-azidoethoxy)ethoxy)ethyl)-2-iodoacetamide



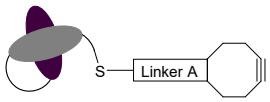
15

Chloroacetamide **32** (34 mg, 0.14 mmol, 1.0 equiv.) was dissolved in acetone (1.0 mL, 0.14 M) and sodium iodide (61 mg, 0.41 mmol, 3.0 equiv.) was added. The reaction was stirred for 48 hrs and subsequently concentrated *in vacuo* and purified by silica gel column chromatography (1:39, MeOH – DCM) to give iodoacetamide **15** (39 mg, 0.11 mmol, 84%) as a pale yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.57 (s, 1H, N*H*), 3.75 – 3.64 (m, 8H, OCH_2, CH_2I), 3.61 – 3.55 (m, 2H, CH_2CH_2N$_3$), 3.51 – 3.46 (m, 2H, CH_2NHIAc), 3.42 (t, *J* = 4.9 Hz, 2H, CH_2N$_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 167.3 (*C*=O), 70.6 (OCH_2), 70.4 (OCH_2), 70.3 (OCH_2), 69.5 (CH_2CH_2NHIAc), 50.8 (CH_2N_3), 40.2 (CH_2NHIAc), -0.5 (CH_2I).

Strained cyclooctyne-functionalized anti-PD1 nanobody

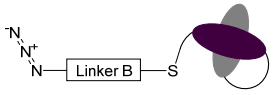


anti-PD1 **2**

A solution of anti-PD1 VHH antibody **4** (0.30 mg, 21 nmol, 1.0 equiv.) and tris(2-carboxyethyl)phosphine (57 µg, 0.20 µmol, 9.5 equiv.) in aqueous buffer (0.20 mL, 0.11 mM) was cooled to 0 °C and compound **5** (0.54 mg, 0.64 µmol, 30 equiv.) was added. The reaction was stirred for 30 min. at 0 °C after which conjugated nanobody **2** was purified by gel filtration.

MS (ESI-TOF) calcd. 14982.6 (MW), found 14981.0.

Azide-functionalized anti-PD1 nanobody

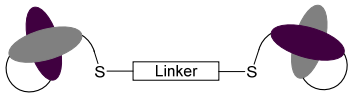


3 anti-PD1

To a solution of anti-PD1 VHH antibody **4** (0.30 mg, 21 nmol, 1.0 equiv.) and tris(2-carboxyethyl)phosphine (57 µg, 0.20 µmol, 9.5 equiv.) in aqueous buffer (0.20 mL, 0.11 mM) was added compound **15** (0.36 mg, 1.1 µmol, 50 equiv.) was added. The reaction was stirred for 2 hrs at room temperature after which conjugated nanobody **3** was purified by gel filtration.

MS (ESI-TOF) calcd. 14345.9 (MW), found 14344.0.

Anti-PD1 VHH antibody homodimer



anti-PD1 **1** anti-PD1

To a solution of cyclooctyne-functionalized nanobody **2** (10 µg, 0.67 nmol, 1.0 equiv.) in aqueous buffer (6.7 µL, 0.10 mM) was added azide-functionalized VHH antibody **3** (19 µg, 1.3 nmol, 2.0 equiv.). The reaction was stirred for 17 hrs at room temperature.

MS (ESI-TOF) calcd. 29328.5 (MW), found 29324.0.

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