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PAPER

Click-enabled heterotrifunctional template for sequential bioconjugations†

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A heterotrifunctional template was developed that utilizes thiol–maleimide and click chemistries (both copper-free and copper-mediated) to effect sequential biomolecule conjugations in a one-pot process. The breadth of compatible substrates was illustrated through highly efficient conjugations of protein, peptide, sugar, lipid, fluoroalkane, biotin and fluorophore molecules. This template should be useful for the creation of chemically-enhanced/enabled biotherapeutics, especially through the expression of discontinuous (and heterogeneous) epitopes.

Introduction

The ability to label biological molecules such as proteins/peptides, sugars and lipids with multiple functional groups is becoming a key focus for applications in biotherapeutics and chemical biology.[1–3] The modification of these biological systems relies on a 'toolbox' of suitably reactive reagents that can be efficiently conjugated to protein molecules. The increase in breadth of bioconjugation chemistries available for incorporation into these types of system augments this toolbox of multifunctional templates. For example Renard, Romieu and co-workers have developed a heterotrifunctional cross-linking template for the synthesis of bioconjugates which uses Copper-catalysed Azide–Alkyne Cycloaddition (CuAAC, Fig. 1 upper), oxime and thiol-based chemistries.[4,5]

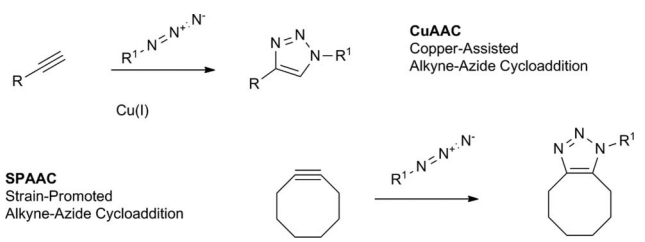

Fig. 1 CuAAC *vs.* SPAAC.

The synthesis of immunogens used to generate antibodies by the stimulation of an immune response has classically been performed using amide coupling/thiol-mediated chemistry to attach multiple copies of single peptides to proteins, such as Bovine Serum Albumin (BSA). The utilization of a multifunctional template to mimic a greater area of the target protein, a discontinuous epitope,[6] could greatly enhance the potency and selectivity of the antibodies produced.

Our initial goal to present discontinuous epitopes on protein surfaces, whilst keeping the two units physically linked, required a template which would satisfy specific requirements, including: the formation of stable, irreversible bonds between the coupled biomolecules and the template; the ability to derive a diverse array of constructs from simple azide monomers, ideally allowing a combinatorial one-pot methodology; incorporation of a thiol to enable attachment to proteins using reliable thiol–maleimide chemistry. In addition to this function, the ability to utilize this reactivity to build up 'tagging' reagents for proteins which had been labeled selectively with a suitably functionalized molecule was also of interest as a potential tool for chemical biology.[7–10]

To satisfy these stringent requirements for the linker, the work of Aucagne,[11] on sequential CuAAc reactions using silyl-protected alkynes, and also that of Kele,[12] on the incorporation of the functional groups required for both Strain-Promoted Azide–Alkyne Cycloaddition (SPAAC, Fig. 1 lower) and CuAAc onto Bovine Serum Albumin (BSA), were encouraging. These works suggest that by combining CuAAc and SPAAC into one template it is possible to incorporate orthogonality whilst maintaining the simplicity of the azide monomer set and avoiding the need for a protecting group strategy. This therefore indicates that sequential addition of diverse monomers to this template will allow for expedient synthesis of compound arrays.

CuAAC[13–16] and, more recently, SPAAC[17] have become popular methods for bioorthogonal labeling of biological systems. Both methods are based on the Huisgen cyclization,[18] the thermal reaction of an alkyne and an azide to give a 1,2,3-triazole as a mixture of regioisomers. The use of copper to catalyze this process allows the reaction to proceed at room temperature giving

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a single regioisomer (CuAAC). The introduction of ring strain to the alkyne-reacting partner lowers the required energy to affect cycloaddition so that reaction occurs at ambient temperature, giving a mixture of regioisomers (SPAAC).

The inherent trifunctionality of amino acids is such that the core of the template can be derived from many of the abundant amino acids, in particular lysine, cysteine, serine, aspartic and glutamic acid. Since thiol–maleimide chemistry was to be used for the attachment to proteins, the sulfur contained in cysteine immediately suggested itself for use as a core. In order to enable this chemistry, advantage was taken of the ability to deprotect thioacetates in the presence of molecules containing maleimides, using hydroxylamine, thus affecting a one-pot bioconjugation.[19] Due to the lack of suitable commercial starting points and concerns over transfer of the acyl group from sulfur to the β-amino functionality, as observed in native chemical ligation,[20] it was decided to use alternatives to cysteine. The ideal method for building the functionality onto the central core of the molecule is by way of amide coupling, and therefore the use of aspartic acid, glutamic acid and lysine offers more flexibility than serine. The size, reactivity and ease of synthesis of the fluoro-cyclooctyne acid, as described by Pigge,[21] showed promise as a reagent for incorporating the SPAAC functionality into the template. The presence of the carboxylic acid in the cyclooctyne enabled further refinement of the selection of amino acid core to lysine. An issue associated with this choice of cyclooctyne is the incorporation of a racemic centre into the template, resulting in two diastereomeric products. Additionally, the SPAAC step provides a mixture of regioisomers and therefore the final constructs are a mixture of four compounds. However, for our initial purposes (the colocation of two peptide epitopes on a single protein carrier) we decided to continue with this approach for our proof of concept studies. One could also argue that the four isomers formed by the template after the SPAAC and CuAAC step allow the two epitope fragments to sample a greater conformational space and therefore may be more likely to adopt the native protein conformation.

Results and discussion

The synthesis of the template was achieved by way of solution-phase chemistry using the readily available Fmoc-Lys(Boc)-OH (Scheme 1). The order of incorporation of the reactive functionalities onto the starting material was dictated by a need to avoid subjecting the cyclooctyne to strong acid, due to its instability in such conditions (unpublished observation). Initial amide formation to incorporate the terminal alkyne using 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate methanaminium (HATU) gave **2**[22] Fmoc deprotection of **2** using 4-methylpiperidine to give **3**[22] was followed by amide coupling with *S*-acetylthioacetic acid (SATA) using HATU, providing **4**. Subsequent Boc deprotection of **4** using HCl in 1,4-dioxane furnished the amine scaffold, **5**. **5** was coupled to 1-fluorocyclooct-2-yne carboxylic acid using HATU yielding the final template **6**.[23] This short and effective route enabled the preparation of the desired template in 5 steps and gave 6.3% overall yield as a 1 : 1 mixture of diastereomers.

The reactivity of the activated cyclooctyne towards azides in the initial SPAAC step was investigated and optimized using biotin-TEG-azide **15**[24] as a reporter model system (Scheme 2). Initial experiments showed that, at a reaction concentration of 1 mg ml^{-1} template and 1.1 equivalents of azide, conversion was clean but slow, reaching 89% conversion after 24 h, but with both starting materials remaining. By increasing the reaction concentration to 10 mg ml^{-1}, complete conversion was achieved within 6 h.[25] As observed by the HPLC traces in Scheme 2, significant conversion was achieved after one hour, suggesting either that the reaction would be complete within six hours, or that there was scope to lower the initial concentration of the reaction.

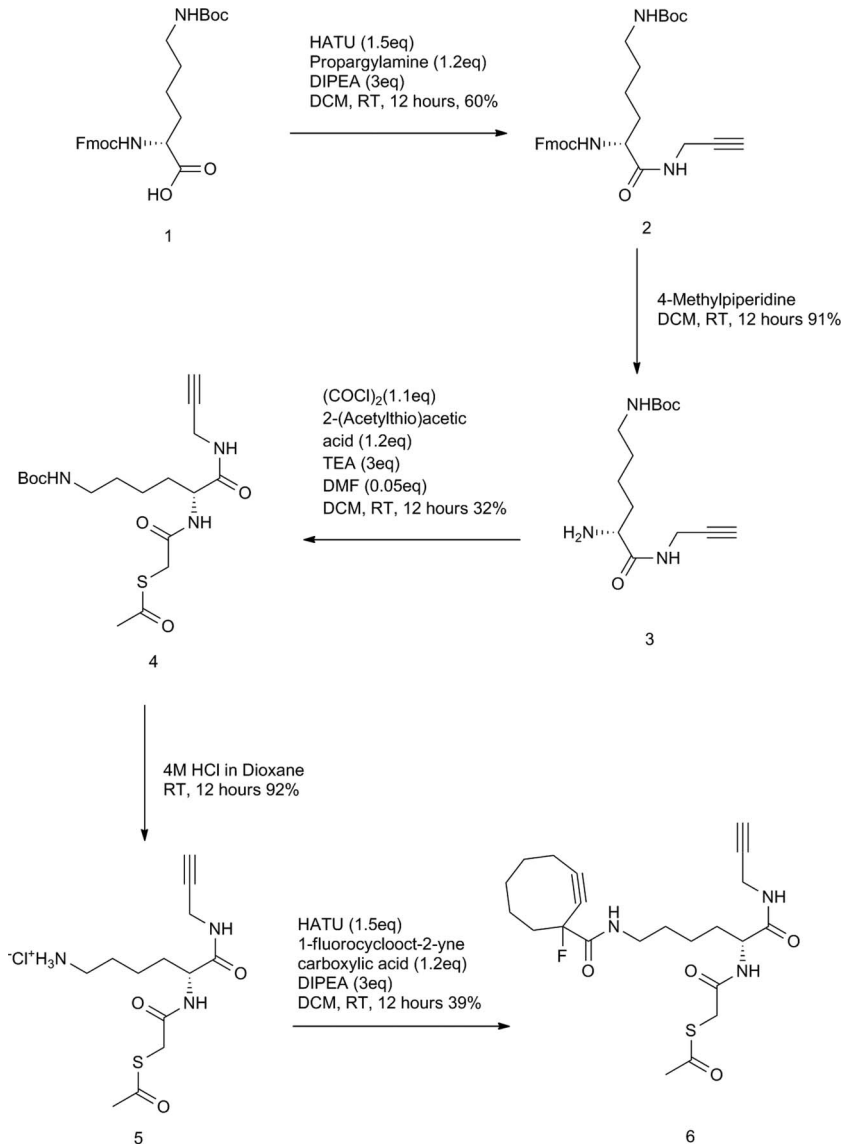
The subsequent CuAAC step (Scheme 3) was then explored using the fluorous tag **16**,[26] which is becoming increasingly common for enrichment of samples, employing classic 'click' conditions of $CuSO_4 \cdot 5H_2O$ and sodium ascorbate. The efficiency of the CuAAC between **7** and **16** was such that complete conversion of the intermediate template was achieved within 2 h and required no optimization. To exemplify the compatibility of the template with complex biomolecules, an array of click reactions with suitably functionalized azides was undertaken (Scheme 4).

The investigated monomer set included peptides with a variety of functional groups (aromatic, acidic and basic: the cyclic RGD peptide **10**[27] and two random peptides DAVE-N_3 **11** and FAKL-N_3 **12**), to benchmark the template's use for the display of discontinuous epitopes.

To illustrate the breadth of chemistry, representative sugar and lipid biomolecules **13** and **14**[28] were also conjugated. Other conjugated molecules included several tags common in chemical biology for enrichment, and the analysis of biomolecules from complex mixtures **15**[24] (biotin), **16**[26] (fluorous tag) and **17**[29] (fluorophore).

The results of this array show that various functional groups are tolerated by the template and the conditions required to produce the transformation. In some cases, such as **7a** and **7e**, a broadening of the LC-MS and a change in the peak shape was observed. This peak shape can be attributed to the four template components formed in the reaction, although there was not adequate separation to delineate the ratio of isomers created.

To demonstrate the potential of this system for the synthesis of immunogens, these molecules were incorporated onto commercial maleimide-activated BSA,[30] a standard starting material for the development of immunogens to be used for the generation of poly- and mono-clonal antibodies. Maleimide activation of BSA is achieved using 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, SMCC, and typically incorporates between 15 and 25 maleimides on each BSA molecule. The method of choice for analyzing protein conjugates is *via* mass spectrometry and, in particular, Matrix Assisted Laser Desorption Ionization (MALDI). Conversion of the parent BSA to the maleimide-activated species caused significant difficulties with the analysis of the system by MALDI-analysis. The suspected reason for this was the heterofunctional nature of the BSA activation giving different points of attachment, as well as the presence of numerous dimers, trimers and 'polymeric' species meaning that the sample contains many sub species rather than a single component (see gel Fig. 2a). This therefore makes analysis by mass spectroscopy unfeasible. The incorporation of the thiol-reactive template with the maleimide-activated species adds an extra layer of variation to the system and further deteriorates the mass spectroscopy data. For these reasons it was necessary to analyze the protein conjugates



Scheme 1 Synthesis of heterotrifunctional template.

which were synthesized with a qualitative method such as gel electrophoresis and subsequent imaging of the gels. Following gel electrophoresis, conjugates (Scheme 4, **9a–j**) were then analyzed using Western Blot (to assay for biotin incorporation, Fig. 2c), fluorescence scanning (to assay for dye incorporation, Fig. 2b) and Coomassie staining (to assay for molecular weight increase, Fig. 2a) to confirm addition of the templates to the protein (Table 1).

Conjugates **9b**, **9c** and **9d** all show appreciable movement to a higher molecular weight following Coomassie staining of the gel (Fig. 2a, line shows mid-point of the commercial maleimide-activated BSA band), displaying the successful reaction of the templates with BSA. Estimation of the construct 'loading' onto the BSA from these molecular weight shifts suggests that incorporation of up to five copies of the template onto the BSA was achieved. Confirmation of the attachment of **9c** and **9f** to the protein was achieved by scanning for the fluorescence on the gels at 488 nm (Fig. 2b), showing appendage of the desired templates to BSA. For the biotinylated samples **9f**, **9g**, **9i**, **9j**, **9h**, Western Blot analysis using an antibiotin antibody (Fig. 2c) demonstrates successful conjugation to the carrier protein. Unfortunately samples **9e** and **9a** do not display a movement to a higher molecular weight. Comparison with the line marking the mid-point of the maleimide activated BSA (Fig. 2a), shows a shift to a greater electrophoretic mobility for these conjugates.

Table 1 Description of the template composition added to BSA-maleimide. Assay method/treatment of gel: A = Coomassie stain, B = Coomassie stain and fluorescence scan, C = Coomassie stain, fluorescence scan and western blot, D = Coomassie stain and western blot (see supplemental)

	9a	**9b**	**9c**	**9d**	**9e**	**9f**	**9g**	**9h**	**9i**	**9j**
$\mathbf{R^1N_3}$	14	10	10	10	12	15	15	15	15	15
$\mathbf{R^2N_3}$	10	12	17	11	11	17	10	14	16	13
Assay	A	A	B	A	A	C	D	D	D	D

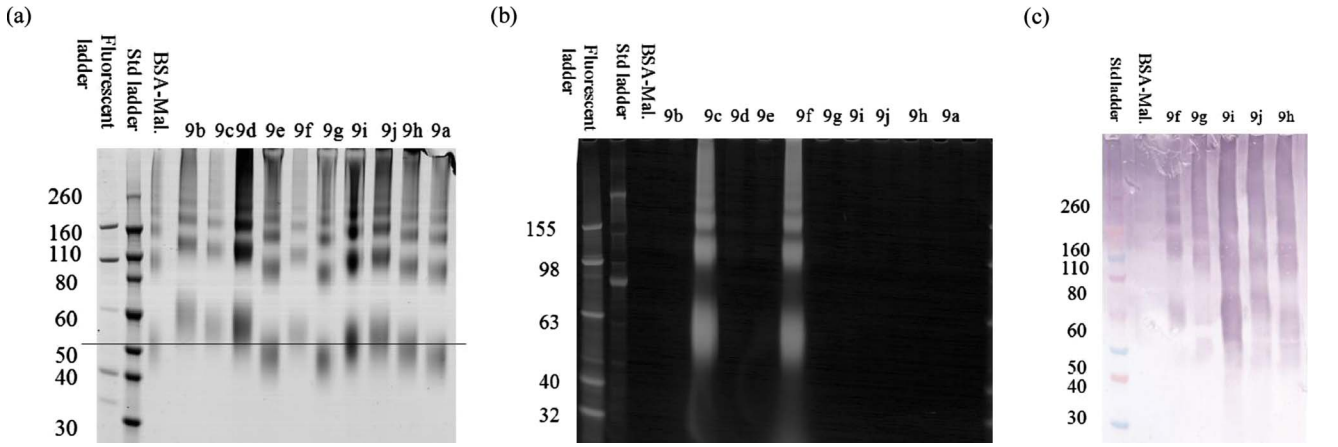
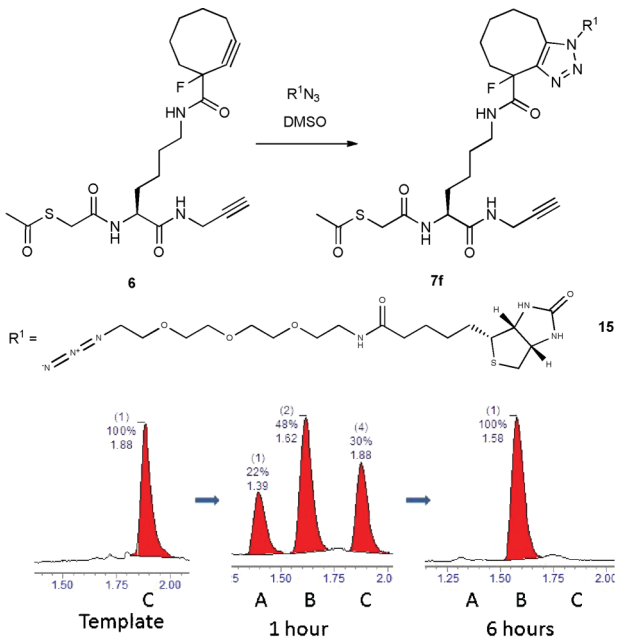


Fig. 2 Gel images of conjugates **9a–9j**: (a) Coomassie stain, (b) Fluorescence scan of gel of dye labeled samples, (c) Western blot analysis of biotinylated samples.



10mg/ml **6** treated with 1.1eq of Biotin-TEG-azide: A = Azide, B = **7f-j**, C = **6**, ELSD spectra.

Scheme 2 SPAAC reactivity at 10 mg mL^{-1}.

The functionality contained in these templates did not allow for visualization by alternative methods, and therefore no definitive evidence for conjugation can be taken from the gels of these samples (although through inference, it is highly likely these conjugations were also successful). Although it was possible to choose a simpler protein substrate to illustrate proof of concept for our template that would aid characterization, it was decided to pursue the more relevant BSA-maleimide system for our synthetic immunology programs.

The results of the array display the robustness and efficiency of both the SPAAC and CuAAC steps. Complete conversion was achieved for both steps, as assessed by LC-MS, irrespective of the azide-coupling partner utilized. The rate of both steps, combined with the avoidance of the need to purify at any point in the sequence, demonstrated that an array of trifunctional biomolecules could be synthesized in under two days. This approach appears to augment discontinuous epitope expressions for synthetic vaccine creation and antibody elicitation significantly and is under further investigation.

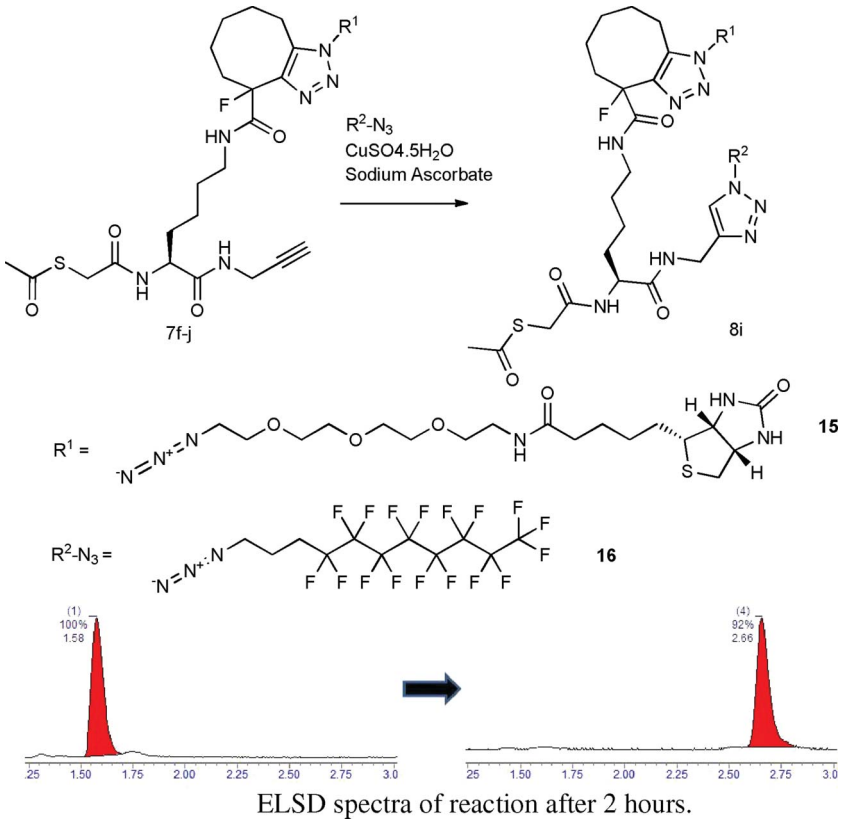
Conclusions

This newly-developed template is an effective method for combining various classes of molecule in a simple, robust and quick procedure which does not require purification. Moreover, the diversity available through this route offers great flexibility for the formation of multiple functions, including discontinuous epitope libraries, glycan arrays for tissue targetting and therapeutic delivery and the synthesis of multifunctional 'tags' for use in chemical biology. Further work in our group will explore templates that avoid diastereomeric mixtures and display multiple epitopes in rigidified architectures.

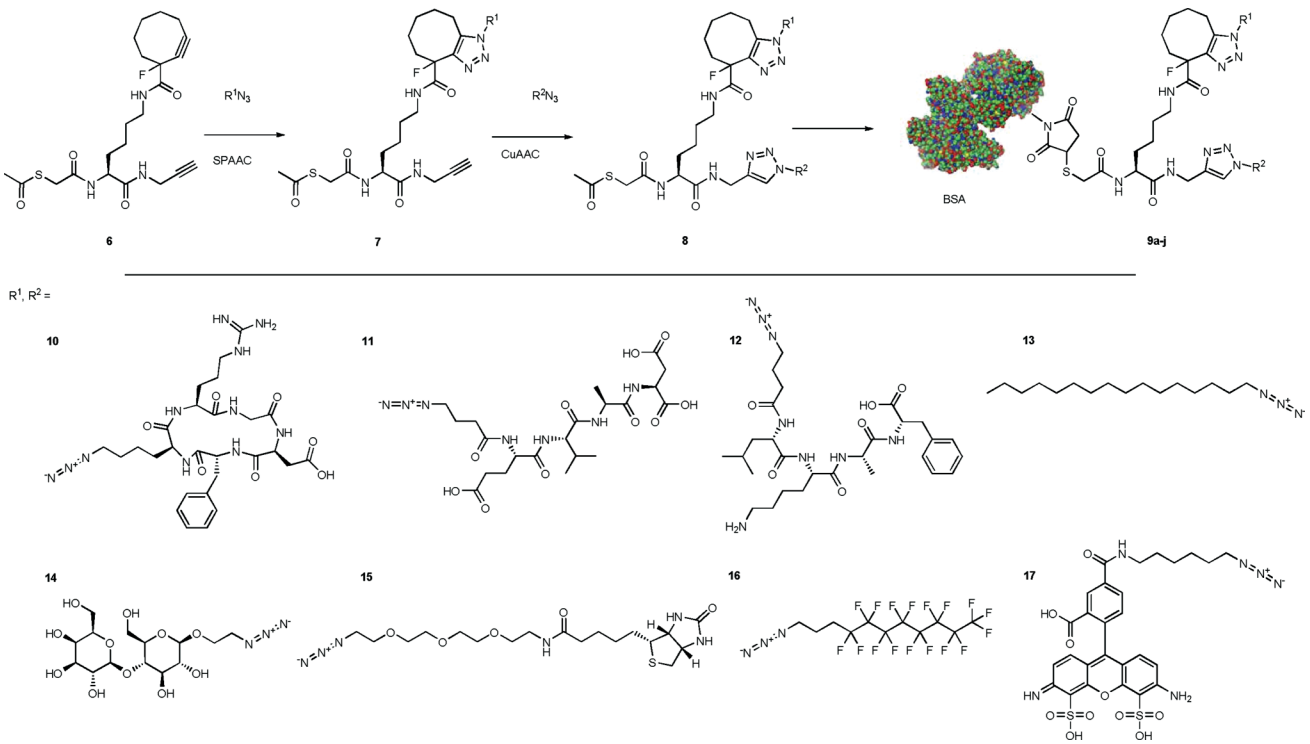
Experimental procedures

General methods

All reagents were purchased from Sigma-Aldrich, Fluka, VWR, Expedion, Berry and Associates, Fluorous Technologies, Invitrogen, Pierce, and Charnwood Molecular, and used without further purification, unless otherwise noted. Dry solvents were used as purchased from Sigma-Aldrich. Chromatographic purifications were performed on ISCO Companion Combiflash using Redisep cartridges (normal phase 69-2203-304 *etc.* and reverse phase 69-2203-410 *etc.* available from Teledyne ISCO) or using Merck silica gel 60 (0.040 mm–0.63 mm) 23-400 MESH, catalogue no. 1.09385 and the indicated solvent eluents. Analytical thin layer chromatography (TLC) was performed on Merck silica gel 60 F_{254} TLC glass plates. Infrared (IR) spectra were obtained using a Nicolet Avatar 360 FTIR. Proton, carbon and fluorine nuclear magnetic resonance spectra (^{1}H NMR, ^{13}C NMR and ^{19}F NMR) were recorded on a Varian Mercury 400BB spectrometer with solvent resonance as the internal standard. ^{1}H NMR data are reported as follows: chemical shift, multiplicity (s = singlet, BS = broad singlet, d = doublet, dd = doublet of doublets, dt = doublet of triplets, t = triplet, q = quartet, m = multiplet), coupling constants



Scheme 3 CuAAC reactivity – ELSD profile after 2 h.



Scheme 4 Synthesis of array of conjugates **9** from azide monomers **10–17**.

(Hz), and integration. LC-MS acquired on a Waters ZQ ESCI single quadrupole LC-MS using method: A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile; Column: Agilent Extend C18 phase 50 × 3 mm with 3 micron particle size; Gradient: 95–0% over 3.5 min. Accurate mass data was acquired on a Thermo LTQ Orbitrap ESI LC-MS or Bruker microTOF.

For details regarding the synthesis of azide monomers and known compounds synthesized by alternative methods, **2** and **3**, please see the supplemental information†.

(*R*)-*S*-2-(6-(*tert*-Butoxycarbonylamino)-1-oxo-1-(prop-2-ynylamino)hexan-2-ylamino)-2-oxoethyl ethanethioate (4)

SATA (592 mg/4.41 mmol) was dissolved in dichloromethane (10 ml) and to this solution the $(COCl)_2$ (339 µl/3.88 mmol) was added, followed by treatment with dimethylformamide (14 µl/0.176 mmol). The reaction was stirred at ambient temperature under N_2 for one hour. On stirring of the reaction effervescence was observed, caused by the liberation of CO_2. **3** (1 g/3.529 mmol) was dissolved in dichloromethane (5 ml) and treated with TEA (1.48 ml/10.6 mmol) and cooled to 0 °C. To this solution the acid chloride solution was added over one minute. The reaction was allowed to warm to ambient temperature and then stirred for one hour. The reaction was quenched by the addition of 2 M HCl (10 ml). The organics were extracted, dried over $MgSO_4$ and evaporated *in vacuo* to leave an oil. Acetonitrile was added to this oil causing precipitation and the resulting material was collected by filtration to give a white solid (450 mg, 32%) m.p. 135–137 °C. (The mother liquors contained a large amount of relatively pure product which was not isolated.)

IR: ν_{max}/cm^{-1} 3280 (C≡C–H), 2974 (amide), 2917 (amide), 1687 (C=O), 1629 (C=O), 1531 (C=O), 1249 (C–O), 792 (C≡C–H). **^{1}H NMR** (400 MHz, DMSO-d_6) δ (ppm) 1.14–1.28 (m, 2 H, HN-CH(CO)-CH_2C**H_2**CH_2CH_2NH), 1.28–1.40 (m, 11 H, NHCO-OC(C**H_3**)$_3$ + CH_2C**H_2**CH_2NHCO-O(CH_3)$_3$), 1.40–1.54 (m, 1 H, NH-CH(CO)C**H_2**CH_2CH_2), 1.54–1.68 (m, 1 H, NH-CH(CO)C**H_2**CH_2CH_2), 2.30–2.38 (m, 3 H, SCOC**H_3**), 2.86 (m, 2 H, CH_2C**H_2**NHCO-OC(CH_3)$_3$), 3.08 (t, J = 2.4 Hz, 1 H, CH2C≡C**H**), 3.65 (d, J = 1.2 Hz, 2 H, CH_3COSC**H_2**CONH), 3.81–3.86 (m, 2 H, NHC**H_2**C≡CH), 4.17 (td, J = 8.4, 5.2 Hz, 1 H, NHC**H**(CO)CH2), 6.60–6.79 (m, 1 H, CH_2CH_2N**H**CO-OC(CH_3)$_3$), 8.22 (d, J = 8.0 Hz, 1 H, CH_3COSCH$_2$CON**H**CH), 8.34 (t, J = 5.6 Hz, 1 H, HCCCH$_2$N**H**COCH). **^{13}C NMR** (100 MHz, DMSO-d6): δ (ppm) 22.5 (NH-CH(CO)CH_2**CH_2**CH_2), 27.9 (NH**CH_2**C≡CH), 28.2 (NHCO-OC(**CH_3**)$_3$), 29.1 (CH_2**CH_2**CH_2NHCO-O(**CH_3**)$_3$), 30.1 (SCO**CH_3**), 31.7 (NH-CH(CO)**CH_2**CH_2CH_2), 32.5 (CH_3COS**CH_2**CONH), 40.5 (CH_2**CH_2**NHCO-O(CH_3)$_3$), 52.6 (NH**C**H(CO)CH2), 72.9 (CH2C≡**C**H), 77.3 (O**C**(CH_3)$_3$), 80.9 (CH2**C**≡CH), 155.5 (**C**O), 166.7 (**C**O), 171.1 (**C**O), 194.5 (**C**O) **HRMS** (ESI) calc. For $C_{18}H_{29}N_3O_5SNa$ [M + Na]$^+$: 422.1726; found 422.1733, error = –3.1 ppm.

(*R*)-*S*-2-(6-Amino-1-oxo-1-(prop-2-ynylamino)hexan-2-ylamino)-2-oxoethyl ethanethioate·HCl (5)

Boc protected amine (**4**) (450 mg, 1.13 mmol) was treated with HCl in 1,4-dioxane (4 M, 10 ml, 40 mmol) and the reaction was stirred at ambient temperature under N_2 for one hour. The reaction was evaporated *in vacuo* to leave a yellow gum, which was washed with diethyl ether and then acetonitrile to give the product, HCl salt, as a white solid which was dried under vacuum (350 mg, 92%) m.p. 72–74 °C.

IR: ν_{max}/cm^{-1} 3264 (C≡C–H), 3043 (amide), 2929 (amide), 2855 (NH_2), 1634 (C=O), 1535 (C=O). **^{1}H NMR** (400 MHz, CD_3OD) δ (ppm) 1.34–1.55 (m, 2 H, HN-CH(CO)-CH_2C**H_2**CH_2CH_2NH$_3$Cl), 1.59–1.75 (m, 3 H, CH_2C**H_2**CH_2NH$_3$Cl + NH-CH(CO)C**H_2**CH_2CH_2), 1.81–1.93 (m, 1 H, NH-CH(CO)C**H_2**CH_2CH_2), 2.37 (s, 3 H, SCOC**H_3**), 2.58 (t, J = 2.45 Hz, 1 H, CH_2C≡C**H**), 2.92 (t, J = 7.6 Hz, 2 H, CH_2C**H_2**NH$_3$Cl), 3.63–3.74 (m, 2 H, CH_3COSC**H_2**CONH), 3.96 (t, J = 2.45 Hz, 2 H, NHC**H_2**C≡CH), 4.30–4.38 (m, 1 H, NHC**H**(CO)CH2). **^{13}C NMR** (101 MHz, $CDCl_3$) δ (ppm) 23.7 (HN-CH(CO)-CH_2**CH_2**CH_2CH_2NH$_3$Cl), 28.1 (CH_2C**H_2**CH_2NH$_3$Cl), 29.7 (CONH**C**H2CCH**)**, 30.2 (**C**H$_3$COSCH$_2$), 32.6 (NH-CH(CO)**C**H$_2$CH$_2$CH$_2$), 34.0 (CH$_3$-COS**C**H$_2$CONH), 40.7 (CH$_2$**C**H$_2$NH$_3$Cl), 54.6 (NH**C**H(CO)-CH2), 72.4 (CH2C≡**C**H), 80.5 (CH2**C**≡CH), 170.9 (SCH$_2$**C**ONH), 173.5 (CH**C**ONHCH$_2$CC, 197.1 (CH$_3$**C**OSCH$_2$). **HRMS** (ESI) calc. For $C_{13}H_{22}N_3O_3S$ [M + H]$^+$: 300.1382; found 300.1381, error = –1.70 ppm.

S-(2-(((2*R*)-6-(1-Fluorocyclooct-2-ynecarboxamido)-1-oxo-1-(prop-2-yn-1-ylamino)hexan-2-yl)amino-2-oxoethyl) ethanethioate (6)

To a stirred solution, under N_2, of amine (**5**) (189 mg, 0.56 mmol) in dichloromethane (5 ml) was added DIPEA (330 µl, 1.89 mmol). A solution of cyclooctyne (**19**) (204 mg, 1.2 mmol) in dichloromethane (4 ml) was then added to the amine solution followed by HATU (456 mg, 1.2 mmol). The solution was stirred overnight at ambient temperature. The solvent was evaporated *in vacuo* and the crude mixture suspended in ethyl acetate. The organic phase was washed with water (×2) and brine (×1), dried over ($MgSO_4$), filtered and concentrated under reduced pressure. The crude mixture was purified by flash column chromatography (silica gel, mesh, 20 to 100% ethyl acetate in heptane) to give the product as an off-white solid with a lower running impurity. The material was dissolved in ethyl acetate and washed with water (×3), dried over ($MgSO_4$), filtered and concentrated under reduced pressure to give the product as a white solid (94 mg, 39%) as a 1 : 1 mixture of diastereomers, m.p. 59–61 °C.

IR: ν_{max}/cm^{-1} 3272 (C≡CH), 3064 (C≡C in ring), 2929 (amide), 2871 (amide), 1662 (C=O), 1634 (C=O), 1531 (C=O), 1120 (C–O), 959 (C≡C–H). **^{1}H NMR** (400 MHz, $CDCl_3$ mixture of diastereomers) δ (ppm) 1.22–1.80 (m, 8 H), 1.81–2.16 (m, 5 H), 2.17–2.52 (m, 7 H), 3.15–3.44 (m, 2 H, CH_2C**H_2**NHCOCF), 3.53–3.69 (m, 2 H, CH_3COSC**H_2**CONH), 3.97–4.08 (m, 2 H, NHC**H_2**CCH), 4.31–4.44 (m, 1 H, NHC**H**(CO)CH2), 6.52 (m, 1 H, HC≡CCH$_2$N**H**COCH), 6.75 (m, 1 H, CH_3COSCH$_2$CON**H**CH), 6.88 (d, J = 7.6 Hz, 0.5 H, CH2N**H**COCF), 6.94 (d, J = 7.4 Hz, 0.5 H, CH2N**H**COCF). **^{13}C NMR** (101 MHz, $CDCl_3$ mixture of diastereomers) δ (ppm) 20.6, 22.1, 22.3, 25.7, 28.9, 28.9, 29.2, 30.3, 30.3, 30.9, 31.1, 33.2, 33.3, 33.9, 33.9, 38.6, 38.8, 46.3 (d, $^2J_{C-F}$ = 24.3 Hz), 46.4 (d, $^2J_{C-F}$ = 24.3 Hz), 53.2, 53.3, 71.5, 79.3, 79.3, 87.3 (d, $^2J_{C-F}$ = 31.7 Hz), 87.3 (d, $^2J_{C-F}$ = 31.7 Hz), 94.4 (d, J_{C-F} = 185.8 Hz), 94.5 (d, J_{C-F} = 185.8 Hz), 109.4 (d, $^3J_{C-F}$ = 10.3 Hz), 109.5

(d, $^{3}J_{C-F}$ = 10.3 Hz), 168.3, 168.4, 168.7 (d, $^{2}J_{C-F}$ = 19.3 Hz), 168.9 (d, $^{2}J_{C-F}$ = 19.3 Hz), 170.8, 170.9, 195.6. **^{19}F NMR** (376 MHz, $CDCl_3$ mixture of diastereomers) δ (ppm) −145.89—145.53 (m). **HRMS** (ESI) calc. For $C_{22}H_{30}FN_3O_4SNa$ $[M + Na]^+$: 474.1839; found 474.1840, error = −1.40 ppm.

SPAAC general method

A 10 mg ml^{-1} solution of **6** in DMSO was treated with 1.1 eq of an azide DMSO stock solution. The reaction was rolled for six hours at which point LC-MS analysis showed complete consumption of **6** and the formation of a single product, **7**. No purification was carried out before the CuAAC step was undertaken. For more information please see the supplemental information†.

CUAAC general method

The DMSO solution of compound **7**, from the SPAAC reaction, was treated with 2 eq of an azide DMSO stock solution, followed by the addition of 0.5 M sodium ascorbate (7 eq) and 0.25 M CuSO4.5H2O (5 eq). The reaction was rolled for two hours before LC-MS analysis of the reaction showed complete conversion from **7** to **8**. For further information please see the supplemental information†.

Conjugation to BSA-maleimide

BSA-maleimide (Pierce – 77115) 1 mg was reconstituted in 100 µl distilled water and then diluted to 1.5 ml with dPBS. Aliquots of 75 µl (74.5 nmol) were treated with 8.3 µl of PBS + 5 M $NH_2OH{\cdot}HCl$ +50 mM EDTA·2Na (pH had been adjusted to pH4). To an aliquot 10 µl of DMSO template solution **8** was added and then the reaction was incubated on a roller for four hours. These samples were then subjected to purification by size-exclusion chromatography, using a GE Healthcare illustra Nap 5 col. eluting with dPBS. The resulting solutions were then concentrated to approximately 95 µl using a Millipore Amicon ultra centrifugal filter device with a 3 KDa cut off (cat. No. UFC800324). Confirmation of conjugation to the protein was achieved by gel electrophoresis, using conditions described in the supplemental information.

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