DOI: 10.1002/anie.201200002

Molecular Scaffolds

Molecular Scaffolds Using Multiple Orthogonal Conjugations: Applications in Chemical Biology and Drug Discovery**

*David M. Beal and Lyn H. Jones**

bioconjugation · chemical biology · click chemistry · molecular scaffolds

Heteromultifunctional scaffolds that harness sequential "click" reactions will find significant utility in the areas of chemical biology and chemically enabled/enhanced biotherapeutics ("chemologics"). Here we review the existing synthetic technologies that illustrate the considerable potential of the field.

1. Introduction

Synthetic methodologies that efficiently derivatize biomolecules require urgent attention. Although recent breakthroughs in the area of "click" chemistry[1] have been made, the current toolbox of synthetic transformations that reliably and reproducibly enable chemistry at the interface with biology, particularly in a pharmaceutical setting, is still far from adequate. From our own work in the area of chemical biology we have consistently found there to be a paucity of useful transformations and in some ways this Minireview is a call to the synthetic community to further increase the size of this toolbox.

Advances in small-molecule synthetic organic chemistry will continue to be made, particularly when it is aligned to addressing the challenges of drug discovery, but more focus should be given to redirecting these chemistries to chemical biology needs. Equally, the existing toolbox can be significantly improved through the optimization of existing protocols tailored to bioconjugation.

Of specific interest to our group, and the focus of this Minireview, are reactions for the modification of biomolecules in a simple and reproducible manner, using scaffolds that have useful multiple functionalities. Although there are many scaffolds that contain a heterobifunctional cross-linker attached to, say, a biotin reporter, such as the commercially available Sulfo-SBED **1** (sulfosuccinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido)hexaneamido]ethyl-1,3′-dithiopropionate; Figure 1),[2] there are relatively few that contain three (or more) well-defined orthogonal synthetic handles that enable sequential biomolecule conjugations.

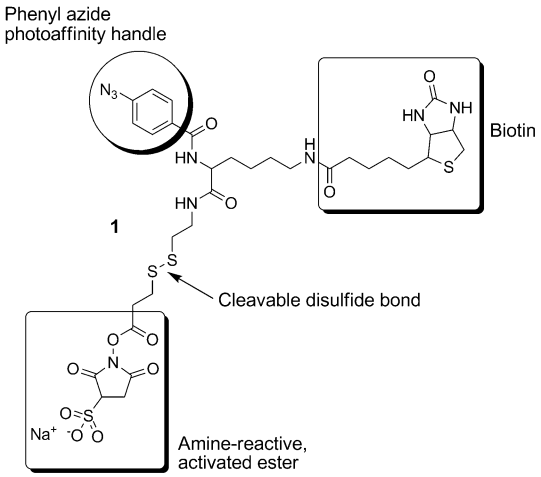


Figure 1. Bifunctional cross-linker Sulfo-SBED **1**.

Multifunctional scaffolds have a myriad of uses and can find significant utility in the biotherapeutic arena, particularly those scaffolds enabled or enhanced using chemical techniques, so-called "chemologics".[3] For example, in synthetic immunology, novel methods are required to mimic discontinuous epitopes to enable more sophisticated antigen design for the development of therapeutic vaccines or monoclonal antibodies (mAb).[4,5] In the area of oncology, in particular, antibody–drug conjugates (ADCs) can benefit through the facile bioconjugation of the mAb-targeting molecule to multiple cytotoxic agents, which may themselves be biomolecules, or complex, and possibly reactive, natural products.[6]

[*] Dr. L. H. Jones
Chemical Biology, BioTherapeutics Chemistry
WorldWide Medicinal Chemistry
Pfizer, 200 Cambridge Park Drive, Cambridge MA (USA)
E-mail: lyn.jones@pfizer.com
D. M. Beal
School of Biosciences, University of Kent
Canterbury, Kent, CT2 7NJ (UK)

[**] Both authors contributed equally to this Minireview.

Additionally, targeted delivery of oligonucleotide therapeutic cargos, including small interfering RNA, can utilize heterovalent expressions to gain cellular entry.[7]

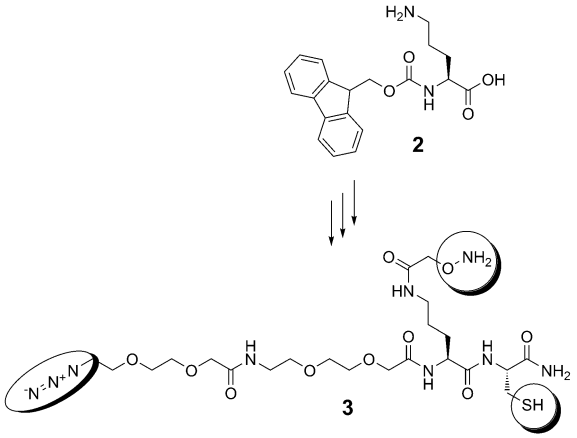
These same benefits can be leveraged in the chemical biology field, where, for example, activity-based probe molecules may combine protein reactivity with enrichment (e.g. biotin, fluorous reagents) and reporter functions (such as fluorescence and mass spectrometry tags).[8–11] The permutations and combinations for these functional molecules are almost endless, but they rely very heavily on the ability of synthetic chemistry to enable this "plug-and-play" strategy.

We hope this Minireview will not only highlight the current developments of heteromultifunctional scaffolds that can create complex biomolecular architectures, but also adequately demonstrate the potential value of this area that will trigger further investments in this exciting field. The sections are organized by the types of reactions used to build the desired complexity and functionality, and the chemical biology applications of the existing synthetic toolbox will be highlighted.

2. Oxime–Thiol–CuAAC

Recent work by Renard, Romieu, and co-workers illustrated a simple yet very effective approach to a multifunctional scaffold (**3**) derived from a lysine core, thus harnessing the intrinsic trifunctionality of the amino acid.[12,13] The lysine is decorated with three conjugation handles: a hydroxylamine for oxime ligation; a thiol for for reactions with maleimide or α-halo ketones; and an azide for copper-mediated azide–alkyne conjugation (CuACC).[14,15] The scaffold was synthesized in a convergent manner from Fmoc-Lys-OH **2** in 14 steps and an overall yield of less than 1% (Scheme 1). The order of reactivity at each position is key to the successful utilization of the scaffold. It is necessary to perform the CuAAC conjugation last to avoid copper/sodium ascorbate mediated formation of the disulfide from the thiol, as well as potential cleavage of the free aminoxy substituent by the copper.

The utility of this construct was then explored through the generation of a fluorescence resonance energy transfer (FRET) cassette for sensitive molecules such as the mycotoxin AFB2 (nucleophile-reactive lactone moiety). Reaction



Scheme 1. Oxime–thiol–CuAAC trifunctional scaffold **3**.

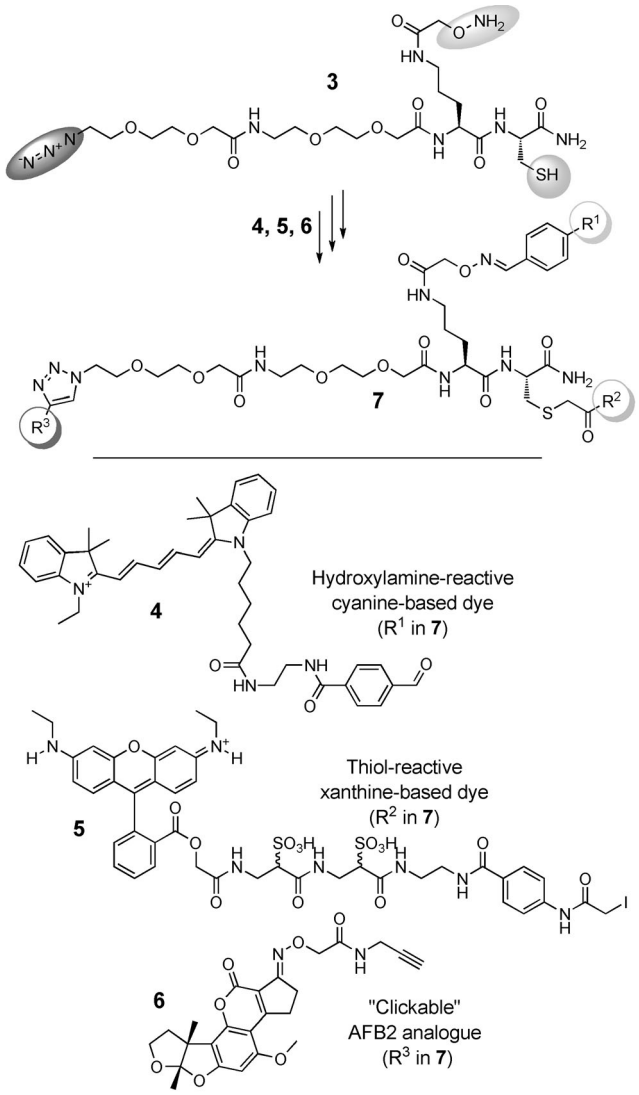
of the scaffold **3** (Scheme 2) with appropriately derivatized cyanine and xanthene dyes (**4** and **5**, respectively) provided the basic cassette (reverse-phase HPLC purification after each step was required). The sensitive nature of AFB2 had caused significant difficulties in previous attempts to create scaffolds of this type. The synthesis of the alkyne-functionalized AFB2 molecule **6**, through oxime ligation, enabled the CuAAC conjugation of the final component to the scaffold. This work successfully incorporates three sequential and orthogonal transformations to enable the synthesis of complex structures **7** without the need for protecting group manipulations. Additional scope was demonstrated using oligonucleotide coupling fragments and immobilization on solid surfaces with potential applications to create portable biosensors.

3. CuAAC–CuAAC–CuAAC

Owing to the chemoselectivity of CuAAC and the biocompatibility of the azide and alkyne coupling partners, it is becoming the bond-forming method of choice in biological systems. Aucagne and co-workers have presented a protecting group strategy to differentiate between alkynes and azides in the same molecules to provide a scaffold whereby three successive CuAAC reactions can be effect-

David Beal obtained his BSc in Chemistry from the University of Greenwich (UK) in 2002, whilst working for Pfizer Global Research and Development at Sandwich in Kent. He spent 14 years at Pfizer (1997–2011), during which time he was involved in several medicinal chemistry programs across different therapeutic areas in the Discovery Chemistry department, before moving to the Chemical Biology Group led by Dr. Lyn Jones. He is currently pursuing doctoral research into the nanoscale properties of amyloid fibrils under the supervision of Dr. Wei-Feng Xue at the University of Kent.

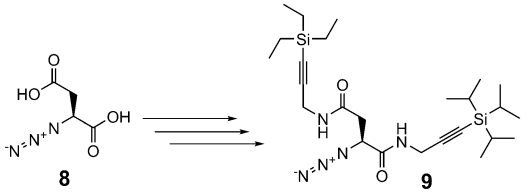
Lyn Jones received his undergraduate education at the University of Bath (UK) and then completed PhD studies with Prof. Alan Armstrong at the University of Nottingham in synthetic organic chemistry in 1998. He then conducted postdoctoral research with Prof. Kim Janda at The Scripps Research Institute in La Jolla (USA) in the area of chemical biology and biochemistry. In 2001 he joined Pfizer in Sandwich (UK) as a medicinal chemistry team leader. He recently transferred to Cambridge, Massachusetts (USA) to lead Pfizer's Chemical Biology and Orphan & Genetics Diseases Chemistry groups.



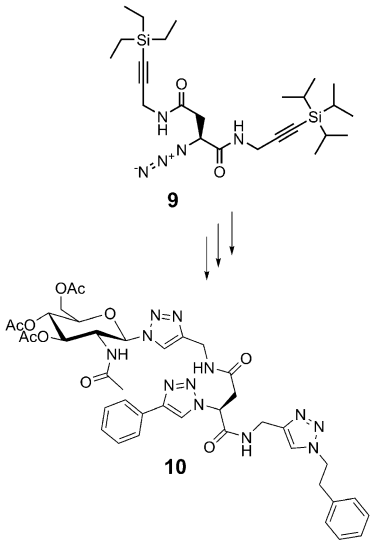
Scheme 2. Functionalization of the oxime–thiol–CuAAC scaffold **3** to create FRET–AFB2 conjugates **7**.

ed.[16] Starting from the commercially available azido aspartic acid **8** it was possible to generate the (differentially) silyl-protected scaffold **9** in five steps with an overall yield of 17% (Scheme 3).

The order in which the conjugations are implemented is fixed by the reactivity of the different silyl protecting groups of the alkyne functionalities. Initial CuAAC of the α-azide is followed by deprotection of the triethylsilyl (TES) alkyne using silver nitrate. Subjecting the resulting scaffold to CuAAC in the presence of another azide leads to the conjugation of the second component. Deprotection of the triisopropylsilyl (TIPS) alkyne is then effected using tetrabutylammonium fluoride (TBAF) to enable the third and final CuAAC conjugation. This method was used to conjugate three distinct components to the core scaffold to provide **10** in an impressive 72% overall yield (Scheme 4). Although only one of the components coupled here could be considered a biomolecule, the potential for this system to evolve into a trifunctional biomolecule-compatible scaffold is apparent.



Scheme 3. Synthesis of the CuAAC–CuAAC–CuAAC scaffold by Aucagne et al.



Scheme 4. Functionalization of the CuAAC–CuAAC–CuAAC scaffold by Aucagne et al.

A similar CuAAC–CuAAC–CuAAC strategy was utilized by Carell and co-workers recently, who applied alkyne-protecting groups to effect triple modification of DNA.[17] Standard phosphoramidite chemistry installed nucleotides containing a terminal alkyne and TIPS- and trimethylsilyl (TMS)-protected alkynes into a DNA chain (Figure 2). Highly efficient and sequential bioconjugations of a variety of azide-containing molecules (including a fluorescent dye, biotin, and a sugar) were demonstrated. The success of this approach is based upon the quantitative, selective removal of TMS using ammonia in the presence of the TIPS protecting group, which is then removed using TBAF prior to the final click coupling.

4. Oxime–Maleimide–4×CuAAC

Extending the lysine scaffold strategy, Boturyn and co-workers developed a linker that displays four alkyne moieties combined with oxime and maleimide coupling functions.[18] A decapeptide scaffold derived from six lysine, two proline, and two glycine residues, was created using standard solid-phase synthetic chemistry.

Use of the functionalized lysine derivatives **11** and **12** shown in Figure 3 ensures complete control over the synthesis

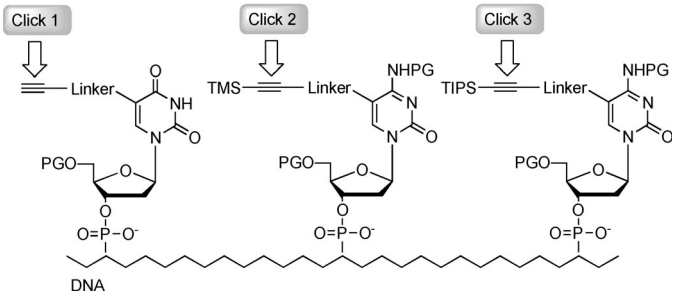


Figure 2. The scaffold designed by Carell et al. for CuAAC–CuAAC–CuAAC modifications. PG = protecting group.

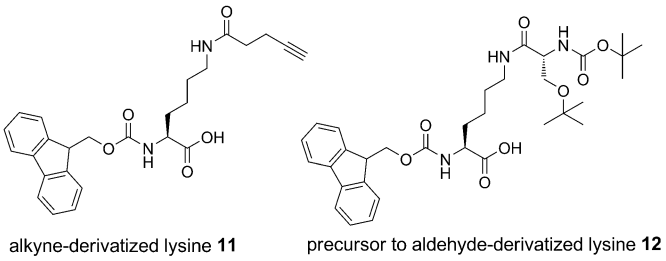


Figure 3. Functionalized lysine derivatives used to create scaffold **13**.

of the decapeptide linker. The maleimide was incorporated following solid-phase synthesis because of its incompatibility with the piperidine used in the Fmoc deprotection step on resin.

Owing to the instability of the aldehyde portion of the scaffold **13** (Figure 4), and the propensity of thiol coupling partners to oxidize in the presence of copper, the CuAAC conjugation was the last click reaction to be performed. The scope of this scaffold to link biomolecules was illustrated through sequential ligations with carbohydrate **14** (oxime), linear KLA peptide **15** (maleimide,) and cyclic RGD-peptide **16** (CuAAC) (Figure 5). Also, an oligonucleotide was installed using CuAAC onto a similar scaffold. A major advantage of this scaffold is that no intermediate purification is required en route to the final trifunctional conjugate. This work provides a proof-of-principle to create synthetic cancer vaccines that display multiple carbohydrate epitopes.

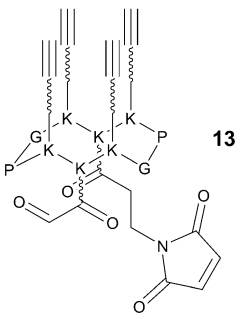


Figure 4. A clickable decapeptide scaffold.

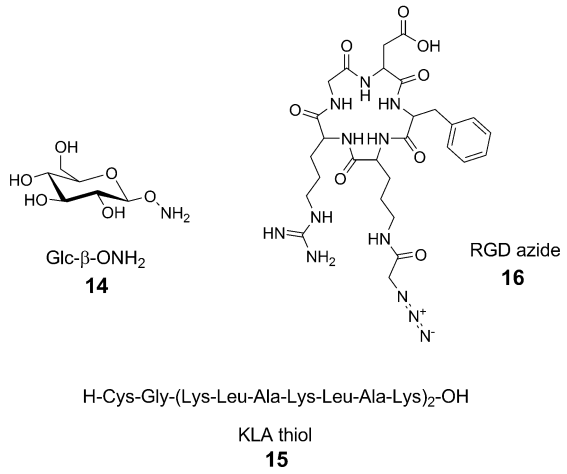
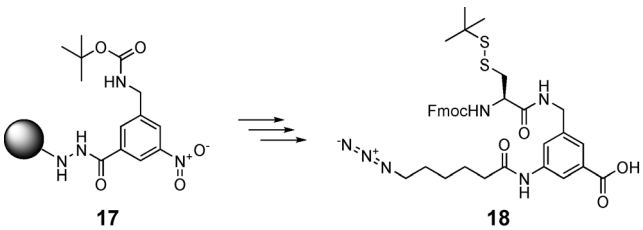


Figure 5. Biomolecules coupled to the decapeptide scaffold **13**.

5. Thiol–Amide–CuAAC

Goody, Waldmann, and co-workers have described several cores that have significant potential in creating biomolecular architectures.[19] This scaffold is based on a benzene ring core which is partially synthesized on resin from **17** (Scheme 5).

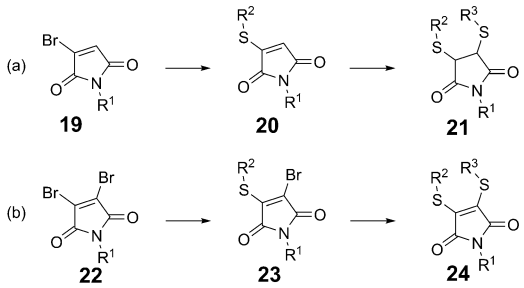
Functionalization of the carboxylic acid **18** with a fluorometric reporter is followed by deprotection of the cysteine to enable expressed protein ligation (EPL)[20] to an N-terminal thioester group on a protein (Ras in this case). The remaining azide then allows for facile coupling of the protein to a phosphane-functionalized glass surface using a Staudinger ligation. This work presents the possibility of creating protein microarrays for proteomic applications.



Scheme 5. Synthesis of a thiol–amide–CuAAC scaffold.

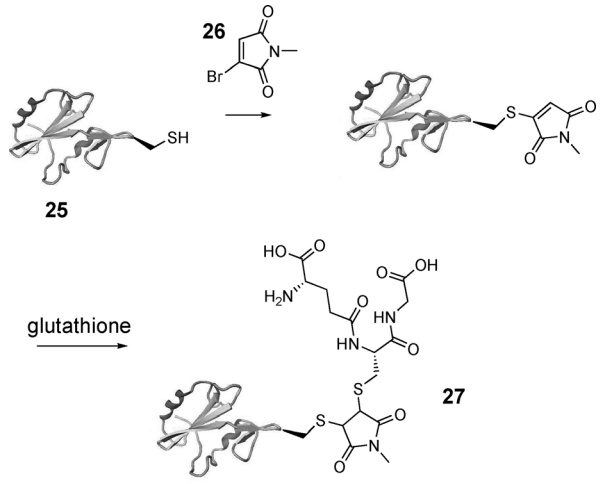
6. Functionalized Maleimides

Recently, Baker, Caddick, and co-workers developed functionalized maleimides as new reagents for bioconjugation chemistry, proving that significant diversity can be created from an extremely simple scaffold.[21,22] The reactivity of bromomaleimides **19** (Scheme 6a) is such that initial treatment with one equivalent of a thiol affects displacement of the bromide to yield **20**. A second thiol can then be added to the maleimide to give the saturated product **21**. In the case of the dibromomaleimide **22** (Scheme 6b) the unsaturated product **24** is formed by displacement of the second bromide **23**.



Scheme 6. Reactivity of a) bromomaleimides and b) dibromomaleimides.

The bioorthogonality of this process was benchmarked by the sequential addition of the Grb2 SH2 domain **25** (L111C) followed by glutathione to *N*-methyl bromomaleimide **26** to furnish **27** (Scheme 7).[22] Dibromomaleimide reacts in a similar manner with the same cysteine-containing protein, and the product was further reacted with glutathione or thioglucose to illustrate the scope of the scaffold.

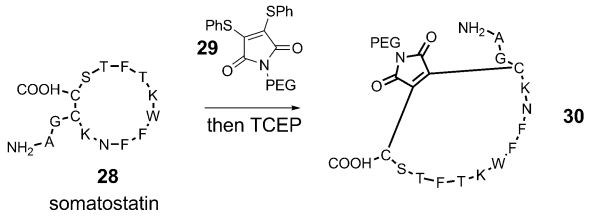


Scheme 7. Addition of Grb2 SH2 domain (L111C) protein and glutathione to *N*-methyl bromomaleimide.

Subsequent work on the bridging of disulfide-containing proteins, such as somatostatin **2**, using a bis-thiophenol-functionalized maleimide with a polyethylene glycol (PEG) group attached to the nitrogen atom (**29**) to yield **30** shows the potential for this reagent to be an extremely useful scaffold (Scheme 8).[22] Additionally, the reversibility of these reactions, through the addition of tris(2-carboxyethyl)phosphine (TCEP), points to potential applications of these motifs as novel prodrug scaffolds. Installation of a clickable handle on to the nitrogen atom of the maleimide would readily create a trifunctional scaffold for sequential bioconjugations.

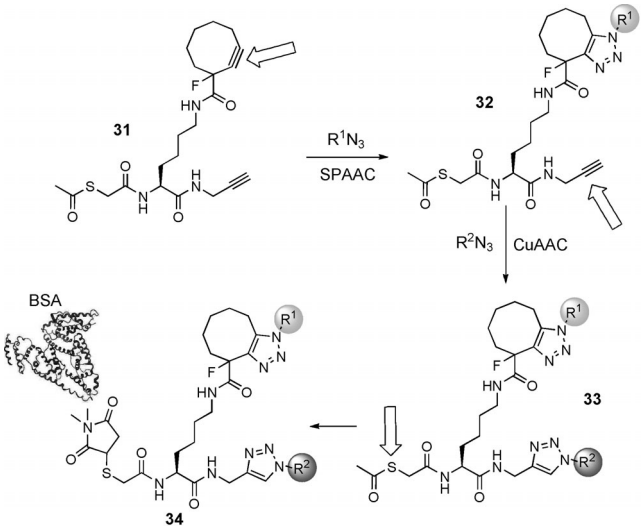
7. CuAAC–SPAAC–Thiol

Our group developed a scaffold that could sequentially append peptide azide monomers to create discontinuous



Scheme 8. Bridging of cyclic disulfide somatostatin with bis-thiophenol-functionalized maleimide.

epitope expressions on carrier proteins such as bovine serum albumin (BSA) for the development of synthetic vaccines and antibody elicitation in a one-pot process.[23] The scaffold **31** (Scheme 9) was prepared in just five steps from Fmoc-lysine in 6% overall yield and uses both copper-mediated and



Scheme 9. Addition of azide-containing monomers and the maleimide-BSA to the CuAAC–SPAAC–thiol scaffold.

strain-promoted azide–alkyne coupling (SPAAC) protocols.[24] The fluorinated cyclooctyne motif was used to enable the SPAAC-mediated conjugation with biomolecules.[25] The order of reaction with this scaffold is flexible but the SPAAC step to provide **32** must be carried out before the CuAAC (to give **33**) to prevent cross-reactivity. Acetate deprotection reveals the thiol for maleimide-mediated ligation to BSA (**34**). The scope of compatible substrates was illustrated through the highly efficient conjugation of peptide (**35**–**37**), sugar (**38**), lipid (**39**), biotin (**40**), and fluorophore (**41**) molecules (Figure 6). A perfluorinated aliphatic chain **42** was also conjugated, and these groups will find greater utility in chemical biology applications in the future.[26]

8. Summary and Outlook

The advent of click chemistry has significantly increased the varieties of architectures and functionalities that can be created in biorelevant systems. The ability to modify biomol-

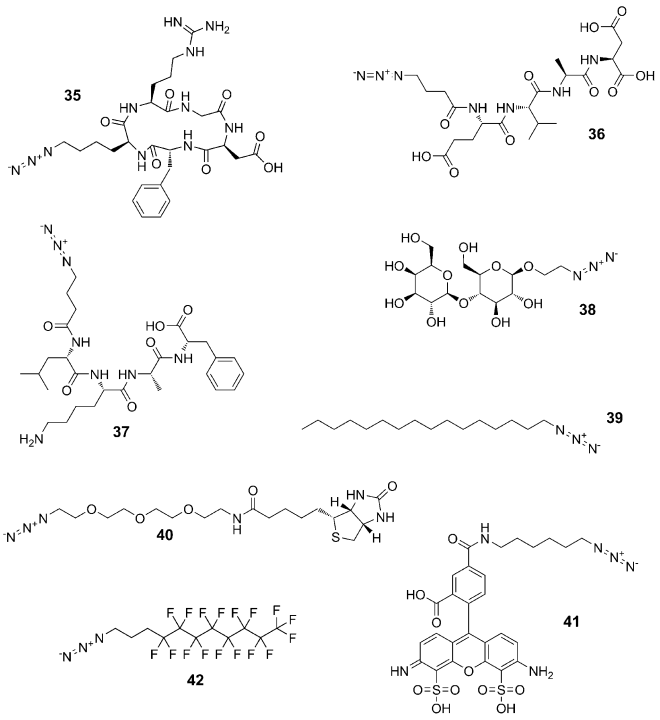


Figure 6. Biomolecules used in conjunction with the CuAAC–SPAAC–thiol scaffold **33** and attached at R^1 and R^2.

ecules and rapidly build additional function and complexity has advanced the areas of chemologics and chemical biology in particular. Specifically, multifunctional and biocompatible linkers are having considerable impact in the areas of synthetic immunology and immunogen design, activity-based proteomics, and technologies that enable therapeutic targeting and delivery. The goal of the synthetic community in these areas will be to create selective transformations that are close to quantitative yield without the need for protecting group manipulations or complex purification protocols—only then can highly complex biomolecular architectures (and in particular libraries and arrays of conjugates) be created reliably and reproducibly.

Each scaffold presented here has its advantages (and disadvantages). It is noticeable that some are simple to prepare, others less so. A common strategy is to harness the intrinsic trifunctionality of a simple amino acid core, although other simple scaffolds can be created using orthogonally functionalised aromatic systems, which can be particularly attractive for the creation of more rigidified architectures (to build discontinuous synthetic epitopes for example). Similarly, the cyclic decapeptide core possesses reduced flexibility and the ability to build multivalent constructs. Scaffolds that do not require protecting group manipulations will probably find greatest utility, and the remarkably simple and elegant application of functionalized maleimides will no doubt continue to have significant impact in chemical biology. Scaffolds that use the same "monomer" set to create complexity (as in the sequential addition of azide-containing biomolecules to a scaffold containing a terminal and strained alkyne) will likely be applied more in the future.

Another area that will significantly impact bioconjugation chemistry follows the pioneering work of Schultz that has expanded the genetic code by generating a unique codon-tRNA pair and the corresponding aminoacyl-tRNA synthetase.[27] Using this technique, site-selective incorporation of unnatural amino acids, bearing ketone, azide or alkyne functionality for example, into a target protein can be effected, so enabling selective biomolecular ligation.

Additionally, as the palette of click reactions is extended, the number of possible multifunctional architectures that can be created will increase exponentially, and the modification of biomolecules for practically any purpose will be realised. As a result, medicinal chemistry is entering a new era, since practically all chemical space is available for therapeutic applications.

Received: January 1, 2012
Published online: April 19, 2012

[1] H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem.* **2001**, *113*, 2056 – 2075; *Angew. Chem. Int. Ed.* **2001**, *40*, 2004 – 2021.
[2] http://www.piercenet.com/browse.cfm?fldID = 23A2097D-0154-4B13-9D95-3F08AAD8B2C9.
[3] L. H. Jones in *New Frontiers in Chemical Biology* (Ed.: M. E. Bunnage), RSC Publishing, Cambridge, **2011**, pp. 204 – 223.
[4] C. Chamorro, J. A. W. Krujitzer, M. Farsaraki, J. Balzarini, R. Liskamp, *ChemComm* **2009**, 821 – 823.
[5] J. D. Warren, X. Geng, S. J. Danishefsky, *Top. Curr. Chem.* **2007**, *267*, 109 – 141.
[6] P. R. Hamman, R. G. Dushin in *New Frontiers in Chemical Biology* (Ed.: M. E. Bunnage), RSC Publishing, Cambridge, **2011**, pp. 224 – 257.
[7] M. G. Stanton, S. L. Colletti, *J. Med. Chem.* **2010**, *53*, 7887 – 7901.
[8] G. C. Adam, E. J. Sorensen, B. F. Cravatt, *Mol. Cell. Proteomics* **2002**, *1*, 828 – 835.
[9] A. F. H. Berry, W. P. Heal, A. K. Taradfer, T. Tolmachova, R. A. Baron, M. C. Seabra, E. W. Tate, *ChemBioChem* **2010**, *11*, 771 – 773.
[10] M. Trester-Zedlitz, K. Kamada, S. K. Burley, D. Fenyö, B. T. Chait, T. W. Muir, *J. Am. Chem. Soc.* **2003**, *125*, 2416 – 2425.
[11] D. S. Wilbur, B. E. B. Sandberg, US pat, US/0023288 A1, **2001**.
[12] G. Clavé, H. Volland, M. Flaender, D. Gasparutto, A. Romieu, P.-Y. Renard, *Org. Biomol. Chem.* **2010**, *8*, 4329 – 4345.
[13] G. Clavé, H. Boutal, A. Hoang, F. Perraut, H. Volland, P.-Y. Renard, A. Romieu, *Org. Biomol. Chem.* **2008**, *6*, 3065 – 3078.
[14] C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057 – 3064.
[15] V. V. Rostovtsev, L. G. Green, C. V. V. Fokin, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 2708 – 2711; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596 – 2599.
[16] I. E. Valverde, A. F. Delmas, V. Aucagne, *Tetrahedron* **2009**, *65*, 7597 – 7602.
[17] P. M. E. Gramlich, S. Warncke, J. Gierlich, T. Carell, *Angew. Chem.* **2008**, *120*, 3491 – 3493; *Angew. Chem. Int. Ed.* **2008**, *47*, 3442 – 3444.
[18] M. Galibert, O. Renaudet, P. Dumy, D. Boturyn, *Angew. Chem.* **2011**, *123*, 1941 – 1944; *Angew. Chem. Int. Ed.* **2011**, *50*, 1901 – 1904.
[19] A. Watzke, M. Köhn, M. Gutierrez-Rodriguez, R. Wacker, H. Schröder, R. Breinbauer, J. Kuhlmann, K. Alexandrov, C. M. Niemeyer, R. S. Goody, H. Waldmann, *Angew. Chem.* **2006**, *118*, 1436 – 1440; *Angew. Chem. Int. Ed.* **2006**, *45*, 1408 – 1412.
[20] R. M. Hofmann, T. W. Muir, *Curr. Opin. Biotechnol.* **2002**, *13*, 297 – 303.

[21] M. E. B. Smith, F. F. Schumacher, C. P. Ryan, L. M. Tedaldi, D. Papaioannou, G. Waksman, S. Caddick, J. R. Baker, *J. Am. Chem. Soc.* **2010**, *132*, 1960 – 1965.
[22] F. F. Schumacher, M. Nobles, C. P. Ryan, M. E. B. Smith, A. Tinker, S. Caddick, J. R. Baker, *Bioconjugate Chem.* **2011**, *22*, 132 – 136.
[23] D. M. Beal, V. E. Albrow, G. Burslem, L. Hitchen, C. Fernandes, C. Lapthorn, L. R. Roberts, M. D. Selby, L. H. Jones, *Org. Biomol. Chem.* **2012**, *10*, 548 – 554.
[24] C. Jewett, C. R. Bertozzi, *Chem. Soc. Rev.* **2010**, *39*, 1272 – 1279.
[25] M. K. Schultz, S. G. Parameswarappa, F. G. Pigge, *Org. Lett.* **2010**, *12*, 2398 – 2401.
[26] M. Cametti, B. Crousse, P. Metrangolo, R. Milani, G. Resnati, *Chem. Soc. Rev.* **2012**, *41*, 31 – 42.
[27] X. Wu, P. G. Schultz, *J. Am. Chem. Soc.* **2009**, *131*, 12497 – 12515.