DOI: 10.1002/cbic.200700278

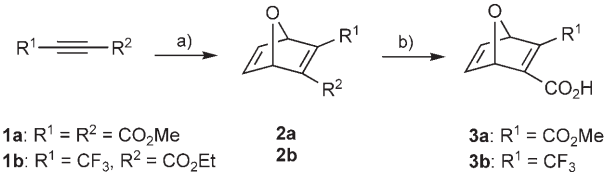
Metal-Free Triazole Formation as a Tool for Bioconjugation

Sander S. van Berkel, A. (Ton) J. Dirks, Marjoke F. Debets, Floris L. van Delft, Jeroen J. L. M. Cornelissen,* Roeland J. M. Nolte, and Floris P. J. T. Rutjes*[a]

The development of selective and site-specific bio-orthogonal conjugation methods is an important topic in chemical biology. A wide range of methods, such as the Staudinger ligation,[1] native chemical ligation,[2] genetic incorporation,[3] expressed-protein ligation,[4] Huisgen azide–alkyne cycloaddition,[5] and the Diels–Alder ligation[6] are currently employed in the selective modification of proteins and other biomolecules. In recent years, the Cu^{I}-catalyzed variant of the Huisgen 1,3-dipolar cycloaddition,[7,8] also referred to as "click reaction", has been increasingly applied in various fields of chemistry as a versatile and mild ligation method.[9] This method allows for the synthesis of complex materials, which include bioconjugates,[10] glycopeptides,[11] functionalized polymers,[12] virus particles,[13] and therapeutics.[14] However, due to the toxicity of the copper catalyst to both bacterial and mammalian cells applications that involve in vivo ligation are limited. In order to circumvent the use of copper ions, Bertozzi and co-workers[15] have devised a strain-promoted [3+2] cycloaddition reaction that involves azides and a strained cyclooctyne derivative. Recent reports by Ju et al. have also shown successful applications of copper-free 1,3-dipolar cycloaddition by using either elevated temperatures[16] or electron-deficient alkynes.[17]

We envisioned that the combination of ring strain and electron deficiency, as occurs in oxa-bridged bicyclic systems **2a** and **2b**, could also lead to an increased reactivity toward [3+2] cycloaddition reactions. Here, we report a spontaneous tandem [3+2] cycloaddition–retro-Diels–Alder ligation method that results in a stable 1,2,3-triazole linkage. This methodology can be applied to biomacromolecules that contain various functional groups under physiological conditions. The oxa-bridged bicyclic systems **2a** and **2b** were prepared by a Diels–Alder reaction of substituted propiolates with furan (Scheme 1).[19,20] Subsequent hydrolysis provided the desired carboxylic acid derivatives **3a** and **3b**, in excellent yield.

To compare the reactivity of Diels–Alder products **2a** and **b** with the corresponding alkynes, [3+2] cycloaddition reactions



Scheme 1. Synthesis of oxanorbornadienes. a) **2a**: 1 equiv furan, Et_2O, room temperature, 7 days (70%); **2b**: 1.25 equiv furan, neat, 40 °C, 4 days (71%); b) THF, 1 M NaOH, 0 °C→room temperature (30 min)[18] **3a** (80%), **3b** (83%).

were performed under ambient conditions by using benzyl azide, and monitored over time with ^{1}H NMR spectroscopy (Figure 1). The oxanorbornadienes **2a** and **2b** and their respective alkynes provided identical 1,4,5-substituted triazoles to the products.[21]

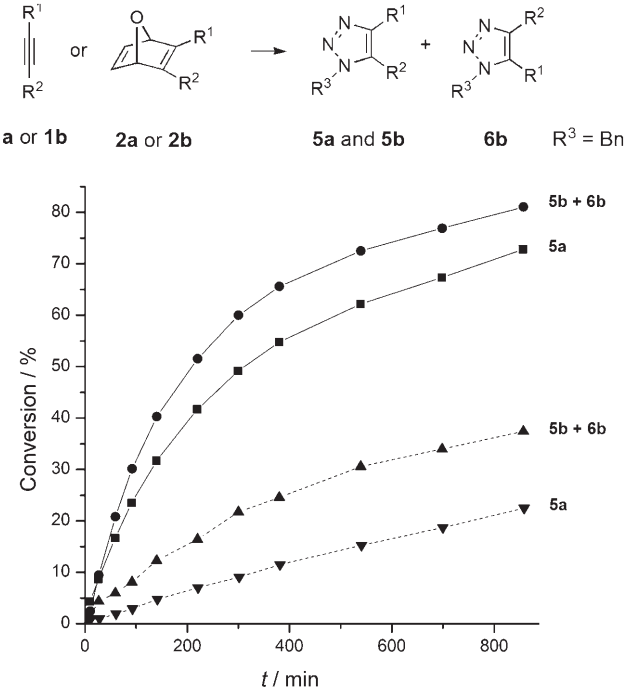


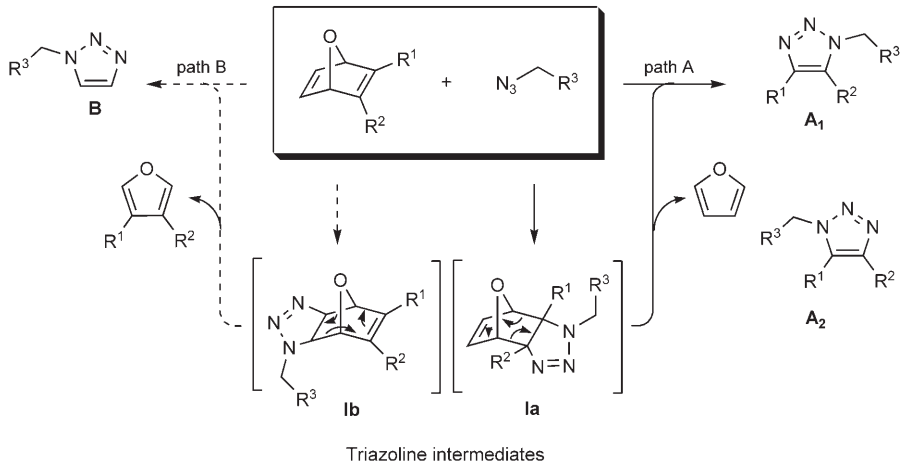
Figure 1. Rate of triazole formation. ^{1}H NMR spectroscopy experiments of alkynes **1a** (▼) and **1b** (▲), and the oxanorbornadienes **2a** (■) and **2b** (●) with benzyl azide (1:1) in [D_4]MeOH (100 mM) at 25 °C.

Interestingly, the reaction rates of oxanorbornadienes **2a** and **2b** were approximately fivefold higher than those of the corresponding alkynes. Additionally, the use of the trifluoromethyl substituent resulted in a rate increase (1.3- and 2.3-fold for the oxanorbornadiene system and alkyne, respectively) compared to the ester substituent. Triazole formation in the case of the oxa-bridged systems can be explained by the cycloaddition of the azide to the most electron deficient double bond (Scheme 2, path A), which initially resulted in the formation of the triazoline intermediate, **I a**. Subsequent loss of furan in a fast retro-Diels–Alder reaction led to the formation of the stable 1,4,5-tri-substituted 1,2,3-triazoles, $\mathbf{A_1}$ and $\mathbf{A_2}$.

[a] *S. S. van Berkel,[+] A. J. Dirks,[+] M. F. Debets, Dr. F. L. van Delft, Dr. J. J. L. M. Cornelissen, Prof. R. J. M. Nolte, Prof. F. P. J. T. Rutjes*
Institute for Molecules and Materials
Radboud University Nijmegen
Toernooiveld 1, 6525 ED Nijmegen (The Netherlands)
Fax: (+31) 24-365-3393
E-mail: j.cornelissen@science.ru.nl
f.rutjes@science.ru.nl

[+] *These authors contributed equally to this work.*

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.



Scheme 2. Reaction pathways for the formation of triazole compounds $\mathbf{A_1}$, $\mathbf{A_2}$, and **B** via triazoline intermediates **Ia** and **Ib**.

Azide cycloaddition onto the unsubstituted double bond (Scheme 2, path B), by intermediate **Ib**, resulted in the monosubstituted 1,2,3-triazole **B** and a 3,4-substituted furan derivative. Although both double bonds can react in the tandem [3+2] cycloaddition–retro-Diels–Alder reaction,[21b] we predominantly found the 1,4,5-substituted triazoles, $\mathbf{A_1}$ and $\mathbf{A_2}$.

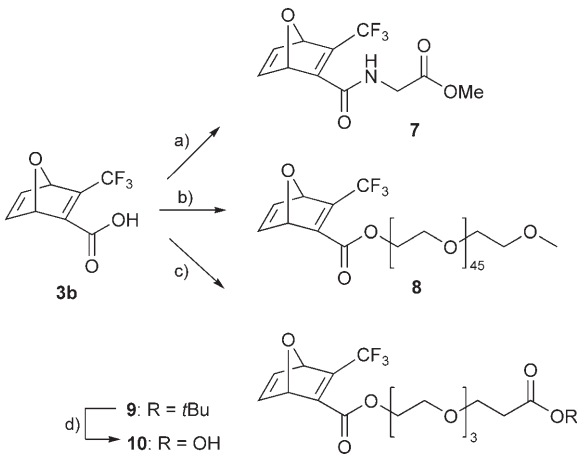
To explore the scope of this tandem [3+2] cycloaddition–retro-Diels–Alder reaction a diverse set of reaction conditions and reactants were tested and monitored with ^{1}H NMR spectroscopy.[22] From the NMR spectral data, the conversions, the ratios of the two regioisomers, and the rate constants of the reactions were determined (Table 1). As shown in Figure 1, the rate of **2a** (Table 1, entry 1) was somewhat lower than that of **2b** (Table 1, entry 2). When we repeated the reaction described in entry 2 we obtained identical results; this demonstrates the high reproducibility of this reaction. When we performed the reaction at an elevated temperature (37 °C; Table 1, entry 3) we obtained a higher reaction rate without an increase in the formation of undesired byproduct **B**. Use of deuterated chloroform as a solvent (entry 4) had a negative effect on the reaction rate, which is more generally observed for cycloadditions in apolar solvents. The outcome of the reaction between the water-soluble compound **3b** and benzyl azide (entry 5) was comparable to the results obtained with compound **2b** (entry 2); this enabled us to change the solvent to D_2O. Alteration of the solvent also required a change of azide. Both 3-azidopropylamine and azidoacetic acid are adequate water-soluble alternatives for benzyl azide. Treatment of oxanorbornadiene **3b** with unprotected azidopropylamine (entry 6) led to increased quantities of byproduct **B**, which can be explained by salt-bridge formation between the amine and the carboxylic acid; this renders [3+2] cycloaddition on the unsubstituted double bond more probable. The treatment of **3b** and unprotected azidoacetic acid in D_2O resulted in the selective formation of a single regioisomer (1,4-dicarboxylic acid triazole), possibly due to electronic repulsion between the carboxylic acid moieties of the oxanorbornadiene (**3b**) and azidoacetic acid. When the R^2 substituent was switched from an ester or a carboxylic acid to an amide (i.e., compound **7**; entry 8) a significant decrease in both the reaction rate and selectivity was observed. This effect can be attributed to an altered polarization of the double bond by the amide bond. The amide bond changes the electron density of the adjacent carbon atom; this is supported by the fact that more of the favored $\mathbf{A_2}$ isomer was formed.

The reaction rates obtained under these conditions were of the same order of magnitude as those reported for the Staudinger ligation,[1d] most of the strain-promoted cyclooctyne-based [3+2] cycloadditions,[15a,b] and the Diels–Alder ligation.[6] In addition, the rates were significantly lower than those of the recently developed fluorinated cyclooctyne cycloadditions by Bertozzi et al.[15c] Since cycloaddition reactions do not generally occur with common amino-acid residues, this tandem [3+2] cycloaddition–retro-Diels–Alder (tandem crDA) reaction is envisaged to be a potentially useful metal-free bioconjugation method. To explore the viability of this ligation method we investigated the chemical modification of a model peptide and protein.

Table 1. Products and kinetic data of reactions between oxanorbornadiene derivatives and azido compounds (at 25 °C and 100 mM) obtained by monitoring the reactions with ^{1}H NMR spectroscopy (400 MHz).

	R^1	R^2	R^3	Equiv N_3	Solvent	**A** [%]	$\mathbf{A_1}$:$\mathbf{A_2}$	**B** [%]	$t_{1/2}$ [min]	Rate $\times 10^4$ [$M^{-1}s^{-1}$]
1	CO_2Me	CO_2Me	Ph	0.93	CD_3OD	95	–	5	285	6.9 ± 0.05
2	CF_3	CO_2Et	Ph	0.99	CD_3OD	97	1:1.5	3	205	8.7 ± 0.09
3[a]	CF_3	CO_2Et	Ph	0.85	CD_3OD	97	1:1.4	3	90	23.8 ± 0.43
4	CF_3	CO_2Et	Ph	1.17	$CDCl_3$	94	1:1.6	6	350	3.7 ± 0.03
5	CF_3	CO_2H	Ph	0.93	CD_3OD	96	1:1.4	4	230	8.5 ± 0.15
6	CF_3	CO_2H	$EtNH_2$	1.39	D_2O	84	n.d.	16	180	7.0 ± 0.10
7	CF_3	CO_2H	CO_2H	1.09	D_2O	>99	–[b]	trace	140	10.6 ± 0.05
8	CF_3	CO-Gly-OMe	Ph	1.32	CD_3OD	84	1:2.4	16	590	1.9 ± 0.03

[a] Performed at 37 °C; [b] exclusively one regioisomer was observed; n.d. = not determined.



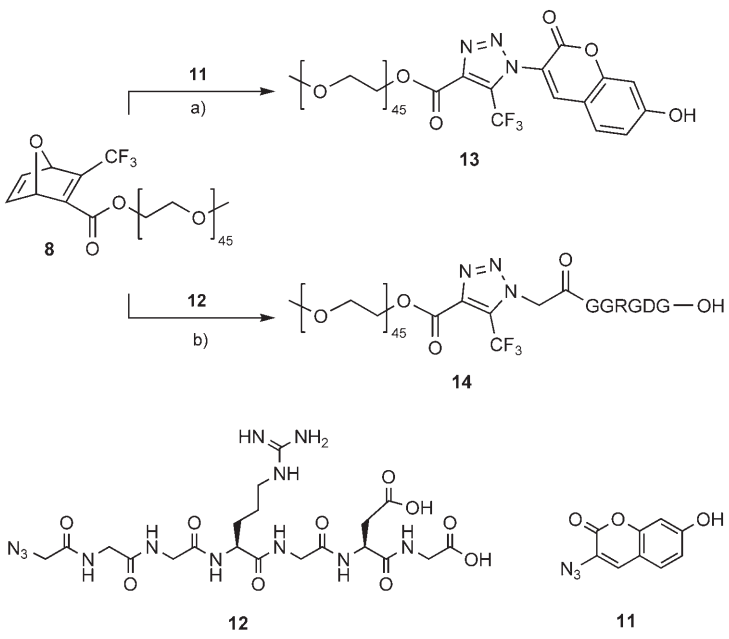
Scheme 3. Synthesis of oxanorbornadiene derivatives. a) H-Gly-OMe (1 equiv), DMAP (2 equiv), CH_2Cl_2, 0 °C then EDC·HCl (1.1 equiv), 0 °C→room temperature, 16 h; b) $mPEG_{45}$ (0.2 equiv), DMAP (0.5 equiv), CH_2Cl_2, 0 °C, then EDC·HCl (1.3 equiv), 0 °C→room temperature, 36 h; c) *tert*-butyl 12-hydroxy-4,7,10-trioxadodecanoate (1 equiv), DMAP (2 equiv), CH_2Cl_2, 0 °C, then EDC·HCl (1.1 equiv), 0 °C→room temperature, 18 h; d) CH_2Cl_2, 0 °C, then TFA, room temperature, 16 h. TFA: trifluoroacetic acid; EDC·HCl: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; DMAP: 4-(dimethylamino)-pyridine; $mPEG_{45}$: α-methoxypoly(ethylene glycol).

Oxanorbornadiene **3b** was functionalized with different groups—an amino acid, polyethylene glycol, and a linker molecule; this resulted in compounds **7**, **8**, and **10**, respectively (Scheme 3).

A well-established approach for improving pharmacological properties of biomolecules is conjugation with PEG (poly(ethylene glycol)), also termed PEGylation.[23] New synthetic strategies for covalent attachment of PEG to (poly)peptides are therefore of great interest. We investigated the applicability of the tandem-crDA reaction for this purpose. The suitability of **8** was initially examined by treating it with 3-azido-7-hydroxycoumarin (**11**; Scheme 4). Since 3-azidocoumarin derivatives are hardly fluorescent but become strongly fluorescent upon triazole formation,[24] they are highly suited as reaction indicators. The increase of fluorescence observed after the two components (**8** and **11**) were stirred in CH_2Cl_2 at room temperature for 15 h, indicated that coupling had taken place. By using ^{1}H NMR spectroscopy the degree of functionalization was subsequently determined to be 88%. The covalent attachment of coumarin to PEG (**13**) was confirmed with size-exclusion chromatography (SEC) by using both UV and refractive index (RI) detection.[22]

To further evaluate the concept of PEGylation with the tandem-crDA reaction, the azide-functionalized hexapeptide GGRGDG (**12**) was used. This oligopeptide was selected since the RGD segment is recognized by various cell receptors within the integrin family.[25] Hence, RGD conjugates can be used to stimulate cell adhesion and are of great interest for medical applications.[26] The GGRGDG sequence was synthesized on a Wang resin by using standard Fmoc protection chemistry; while still on the resin, azidoacetic acid was coupled to the N terminus of the peptide. After cleavage from the resin, the fully unprotected oligopeptide **12** was mixed with oxanorbornadiene functionalized PEG (**8**), and stirred in water for 36 h at 37 °C. This resulted in the formation of the desired PEGylated peptide, **14**, with an efficient conversion of 80%, which was determined by ^{1}H NMR spectroscopy. MALDI-ToF analysis of the mixture showed a distinct shift towards a higher molecular weight, with an expected mass difference that corresponded to the molecular weight of the oligopeptide (Figure 2). These results clearly demonstrate that the tandem-crDA reaction can be used as a method for PEGylation.

A different, though equally important application of bioconjugation chemistry is the labeling of proteins with fluorophores. To demonstrate the applicability of our ligation method in this field, a model protein, hen egg white lysozyme (HEWL), was functionalized with an oxanorbornadiene moiety by coupling derivative **10** to its lysine residues. Subsequently, the functionalized HEWL (**15**) was mixed with 3-azido-7-hydroxycoumarin (**11**) and gently shaken for 36 h at 25 °C (Scheme 5). As a control experiment native HEWL was also incubated with 3-azido-7-hydroxycoumarin (**11**) under the same conditions. The mixtures were run on a SDS-PAGE gel and analyzed with both UV illumination and Coomassie-blue staining. Upon exposure to UV light ($\lambda = 366$ nm) a distinct fluorescent band was observed for the product obtained from the reaction between functionalized HEWL **15** and coumarin **11**, which was not the case for the control experiment (Figure 3). Subsequent staining with Coomassie blue showed similar bands for both reaction products, as expected. These initial



Scheme 4. Tandem-crDA reaction of oxanorbornadiene functionalized PEG **8**, with 3-azido-7-hydroxycoumarin (**11**), and oligopeptide **12**. a) 1.2 mM PEG **8** (1 equiv), coumarin **11** (3.7 equiv), CH_2Cl_2, 15 h, 25 °C; b) 3.4 mM PEG **8** (1 equiv), oligopeptide **12** (2.7 equiv), H_2O, 36 h, 37 °C.

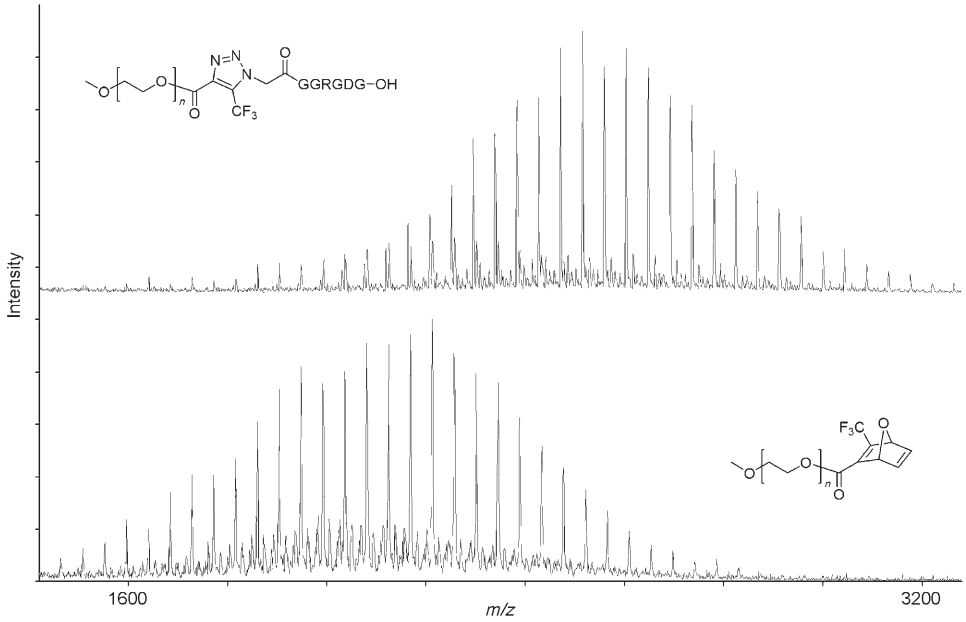
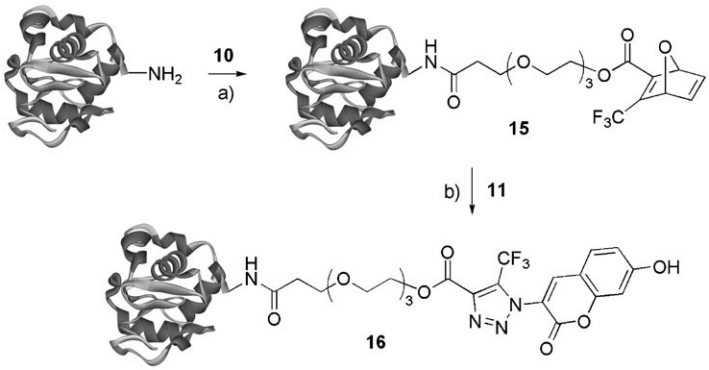


Figure 2. MALDI-ToF spectra of oxanorbornadiene functionalized PEG **8** before (lower panel) and after coupling with 2-azidoactyl–GGRGDG-OH (**12**; upper panel).



Scheme 5. Preparation of oxanorbornadiene functionalized HEWL **15** and subsequent treatment with 3-azido-7-hydroxycoumarin (**11**). a) 5.7 mg mL^{-1} HEWL (1 equiv), **10** (35 equiv), EDCI (48 equiv), sodium acetate buffer (100 mM, pH 5.5 with 9% THF), 14 h, 25 °C; b) 1.5 mg mL^{-1} HEWL (1 equiv), 3-azido-7-hydroxycoumarin (**11**; 48 equiv), sodium acetate buffer (20 mM, pH 5.5 with 9% THF), 36 h, 25 °C. EDCI: 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride.

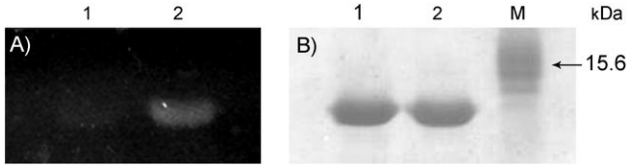


Figure 3. SDS-PAGE (15%) analysis of the HEWL–coumarin conjugate (**16**; lane 2) and a negative control experiment with native HEWL (lane 1). The SDS-PAGE gel was A) photographed under exposure to UV light (λ = 366 nm) and then B) stained with Coomassie blue; M: molecular weight marker in kDa.

results show that the tandem-crDA reaction is a sufficient method for the labeling of oxanorbornadiene functionalized HEWL **15**, and indicates that this method is a promising new tool for protein modification. A detailed exploration of the bio-orthogonality of this approach is currently under investigation.[27]

We have demonstrated that easily accessible trifluoromethyl-substituted oxanorbornadiene derivatives efficiently react with various azides in an elegant tandem [3+2] cycloaddition–retro-Diels–Alder (tandem crDA) reaction to form stable 1,2,3-triazole-linked compounds. Additionally, they possess an increased reactivity towards azides compared to their corresponding electron-deficient alkynes. These versatile compounds have a distinct potential for bioconjugation reactions as these reactions can be performed in aqueous media, at ambient temperature, and in the absence of a transition-metal catalyst, such as copper. The tandem-crDA reaction was found to be an effective method for the preparation of a PEGylated oligopeptide and initial experiments with HEWL as a model protein demonstrated that this reaction is a promising new bioconjugation method for the labeling and functionalization of proteins. A detailed evaluation of the scope of the new ligation method is currently under investigation in our laboratory.

Acknowledgements

These investigations were financially supported by the Netherlands Technology Foundation (STW) and the NRSC-Catalysis. The authors would like to thank Dr. J. W. Scheeren and R. Aben for their pioneering work and useful discussions on this research.

Keywords: click reaction · conjugation · cycloadditions · retro-Diels–Alder · triazoles

[1] a) E. Saxon, J. I. Armstrong, C. R. Bertozzi, *Org. Lett.* **2000**, *2*, 2141 – 2143; b) 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; c) M.-L. Tsao, F. Tian, P. G. Schultz, *ChemBioChem* **2005**, *6*, 2147 – 2149; d) F. L. Lin, H. M. Hoyt, H. van Halbeek, R. G. Bergman, C. R. Bertozzi, *J. Am. Chem. Soc.* **2005**, *127*, 2686 – 2695.

[2] P. E. Dawson, S. B. H. Kent, *Annu. Rev. Biochem.* **2000**, *69*, 923 – 960.

[3] A. Deiters, T. A. Cropp, M. Mukherji, J. W. Chin, J. C. Anderson, P. G. Schultz, *J. Am. Chem. Soc.* **2003**, *125*, 11782 – 11783.

[4] a) D. Schwarzer, P. A. Cole, *Curr. Opin. Chem. Biol.* **2005**, *9*, 561 – 569; b) N. Budisa, *ChemBioChem* **2004**, *5*, 1176 – 1179.

[5] R. Huisgen, G. Szeimies, L. Mobius, *Chem. Ber.* **1967**, *100*, 2494 – 2507.

[6] a) A. D. de Araújo, J. M. Palomo, J. Cramer, M. Köhn, H. Schröder, R. Wacker, C. Niemeyer, K. Alexandrov, H. Waldmann, *Angew. Chem.* **2006**, *118*, 302 – 307; *Angew. Chem. Int. Ed.* **2006**, *45*, 296 – 301; b) A. D. de Araújo, J. M. Palomo, J. Cramer, O. Seitz, K. Alexandrov, H. Waldmann, *Chem. Eur. J.* **2006**, *12*, 6095 – 6109.
[7] V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 2708 – 2721; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596 – 2599.
[8] C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057 – 3064.
[9] V. D. Bock, H. Hiemstra, J. H. van Maarseveen, *Eur. J. Org. Chem.* **2006**, 51 – 68.
[10] a) A. J. Dirks, S. S. van Berkel, N. S. Hatzakis, J. A. Opsteen, F. L. van Delft, J. J. L. M. Cornelissen, A. E. Rowan, J. C. M. van Hest, F. P. J. T. Rutjes, R. J. M. Nolte, *Chem. Commun.* **2005**, 4172 – 4174; b) A. J. Link, D. A. Tirell, *J. Am. Chem. Soc.* **2003**, *125*, 11164 – 11165.
[11] a) B. H. M. Kuijpers, S. Groothuys, A. R. Keereweer, P. J. L. M. Quaedflieg, R. H. Blaauw, F. L. van Delft, F. P. J. T. Rutjes, *Org. Lett.* **2004**, *6*, 3123 – 3126; b) A. Dondoni, P. P. Giovannini, A. Massi, *Org. Lett.* **2004**, *6*, 2929 – 2931.
[12] a) J. A. Opsteen, J. C. M. van Hest, *Chem. Commun.* **2005**, 57 – 59; b) V. Ladmiral, G. Mantovani, G. J. Clarkson, S. Cauet, J. L. Irwin, D. M. Haddleton, *J. Am. Chem. Soc.* **2006**, *128*, 4823 – 4830.
[13] a) S. S. Gupta, J. Kuzelka, P. Singh, W. G. Lewis, M. Manchester, M. G. Finn, *Bioconjugate Chem.* **2005**, *16*, 1572 – 1579; b) Q. Wang, T. R. Chan, R. Hilgraf, V. V. Fokin, K. B. Sharpless, M. G. Finn, *J. Am. Chem. Soc.* **2003**, *125*, 3192 – 3193.
[14] a) L. V. Lee, M. L. Mitchell, S.-J. Huang, V. V. Fokin, K. B. Sharpless, C.-H. Wong, *J. Am. Chem. Soc.* **2003**, *125*, 9588 – 9589; b) R. Manetsch, A. Krasinski, Z. Radic, J. Raushel, P. Taylor, K. B. Sharpless, H. C. Kolb, *J. Am. Chem. Soc.* **2004**, *126*, 12809 – 12818; c) G. G. Kochendoerfer, *Curr. Opin. Chem. Biol.* **2005**, *9*, 555 – 560.
[15] a) N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 15046 – 15047; erratum: b) N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2005**, *127*, 11196; c) N. J. Agard, J. M. Baskin, J. A. Prescher, A. Lo, C. R. Bertozzi, *ACS Chem. Biol.* **2006**, *1*, 644 – 648.
[16] a) S. J. Coats, J. S. Link, D. Gauthier, D. J. Hlasta, *Org. Lett.* **2005**, *7*, 1469 – 1472; b) T. S. Seo, Z. Li, H. Ruparel, J. Ju, *J. Org. Chem.* **2003**, *68*, 609 – 612.
[17] Z. Li, T. S. Seo, J. Ju, *Tetrahedron Lett.* **2004**, *45*, 3143 – 3146.
[18] S. Niwayama, *J. Org. Chem.* **2000**, *65*, 5834 – 5836.
[19] a) A. Nezis, J. Fayn, A. Cambon, *J. Fluorine Chem.* **1991**, *53*, 285 – 295; b) M. G. Barlow, N. N. E. Suliman, A. E. Tipping, *J. Fluorine Chem.* **1995**, *70*, 59 – 69.
[20] D. Cristina, M. De Amici, C. De Micheli, *Tetrahedron* **1981**, *37*, 1349 – 1357.
[21] a) D. N. Reinhoudt, C. G. Kouwenhoven, *Tetrahedron Lett.* **1974**, *15*, 2163 – 2166; b) L. Fišera, F. Považanec, P. Zálupský, J. Kováč, D. Pavlovič, *Coll. Czech. Chem. Commun.* **1983**, *48*, 3144 – 3153.
[22] For a detailed experimental procedure see the Supporting Information.
[23] a) J. M. Harris, R. B. Chess, *Nat. Rev. Drug Discovery* **2003**, *2*, 214 – 221; b) F. M. Veronese, J. M. Harris, *Adv. Drug Delivery Rev.* **2002**, *54*, 453 – 456; c) P. Caliceti, F. M. Veronese, *Adv. Drug Delivery Rev.* **2003**, *55*, 1261 – 1277.
[24] K. Sivakumar, F. Xie, B. M. Cash, S. Long, H. N. Barnhill, Q. Wang, *Org. Lett.* **2004**, *6*, 4603 – 4606.
[25] a) E. Ruoslahti, M. D. Pierschbacher, *Science* **1987**, *238*, 491 – 497; b) E. Ruoslahti, *Annu. Rev. Cell Dev. Biol.* **1996**, *12*, 697 – 715.
[26] a) U. Hersel, C. Dahmen, H. Kessler, *Biomaterials* **2003**, *24*, 4385 – 4415; b) J. Auernheimer, C. Dahmen, U. Hersel, A. Bausch, H. Kessler, *J. Am. Chem. Soc.* **2005**, *127*, 16107 – 16110; c) Y. Mei, K. L. Beers, H. C. M. Byrd, D. L. Vanderhart, N. R. Washburn, *J. Am. Chem. Soc.* **2004**, *126*, 3472 – 3476.
[27] Initial experiments have shown that Michael addition processes can, to a minor extent, interfere with the reported ligation method. In short, **2b** was incubated with a pool of all 20 naturally occurring amino acids (in excess) in phosphate buffer at room temperature for 72 h. For the basic amino-acid residues (i.e., Lys, Arg, His) traces of addition products with **2b** could be detected. Adducts of Cys could not be detected, but are likely to be formed.

Received: May 22, 2007
Published online on July 13, 2007

