Biomolecular Assemblies

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One-Pot Approach to Well-Defined Biomolecular Assemblies by Orthogonal Chemoselective Ligations**

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The use of highly efficient and chemoselective ligations is crucial to construct biomolecular assemblies. The major barriers to the chemical synthesis of these systems arise from their high molecular weight and/or from the incompatibility of the reaction conditions that are involved in the chemistry of carbohydrates, peptides, and nucleic acids. Numerous chemoselective reactions have proved to be particularly useful to tailor proteins,[1] and oligonucleotide[2] or carbohydrate derivatives.[3] We successfully exploited oxime bond formation to prepare diverse bioconjugates;[4] this reaction benefits from the high chemoselectivity and reactivity between the aminooxy and the carbonyl groups. Among other chemoselective reactions,[5] the copper(I)-catalyzed alkyne–azide cycloaddition reaction[6] is a powerful tool to prepare new peptidic derivatives by a triazole linkage.[7] The chemoselective formation of two successive triazole linkages ("click–click" chemistry) in one pot allows a direct access to complex structures.[8] The method utilizes copper(I)-catalyzed cycloaddition reactions combined with a silver(I)-catalyzed (trimethylsilyl)alkyne deprotection. During the preparation of this manuscript, dual labeling of model compounds was carried out by using similar click–click chemistry.[9] The feasibility of this approach was demonstrated by means of orthogonal systems encompassing cyclooctyne and terminal alkyne allowing respectively copper-free[10] and copper(I)-mediated click chemistry.

Herein we report a novel strategy for the synthesis of well-defined biomolecular assemblies using orthogonal oxime bond formation and copper(I)-mediated alkyne–azide cycloaddition reactions in a stepwise or in a one-pot approach. We believe that this strategy could be a very convenient method to access to highly sophisticated assemblies. To illustrate this strategy, we performed the regioselective ligation of biologically relevant RGD peptides onto a cyclopeptidic scaffold. Arginine–glycine–aspartic acid (RGD) peptides are used to specifically target tumor tissue through the $\alpha_v\beta_3$ integrin receptor.[11] We designed tetrameric RGD peptides[12] with desirable biological properties, showing the benefit of the multivalency presentation.[13] To study the contribution of each RGD motif, we synthesized an array of compounds containing from one to four copies of the RGD monomer (compounds **1**–**5**, Figure 1). Previously, we used a combinatory assembling strategy to explore all possible positions of the RGD motifs on the cyclodecapeptide scaffold.[14] To obtain molecules with similar shape, similar steric hindrance, and close molecular weights, we opted to substitute RGD ligands with nonsense RβAD peptide. Both were grafted to the cyclodecapeptide scaffold by stepwise oxime bonds. However, we were unable to isolate the different isomers that differ in the position of cyclic RGD pentapeptides onto the cyclodecapeptide scaffold. We then chose to exploit Huisgen dipolar cycloaddition to introduce RGD motifs onto the scaffold, nonsense RβAD peptides being introduced by orthogonal oxime bond formation. Using this strategy, we are able to synthesize well-defined biomolecular assemblies in which the position of RGD units can be distinguished within, for example, the related compounds **3** and **4** (Figure 1).

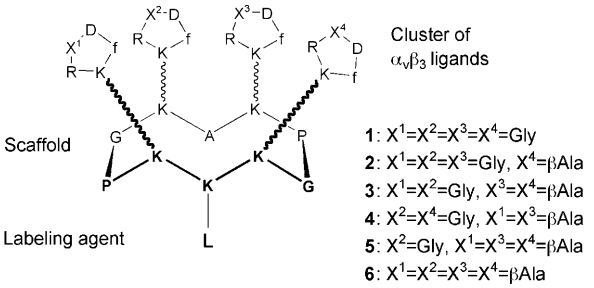

Figure 1. Structures of tetrameric RGD/RβAD-containing peptides **1**–**6**. L = labeling agent.

To incorporate chemoselective functions within the cyclodecapeptide chain, we synthesized building blocks **7** and **8**, which contain protected aminooxy and alkyne groups, respectively (Scheme 1). The introduction of building blocks during the solid-phase peptide synthesis (SPPS) considerably reduces the number of steps involved and the combination of protecting groups required for the synthesis of functionalized peptides. For this purpose, we developed 1-ethoxyethylidene (Eei) as a new protecting group for the aminooxy moiety.[15] We completed the peptide-chain elongation by the stepwise incorporation of Fmoc-protected amino acids, including building blocks **7** and **8**. Alkyne-containing amino acid **8** is entirely compatible with SPPS conditions, and it may be easily exploited to prepare alkyne-containing peptides ready for

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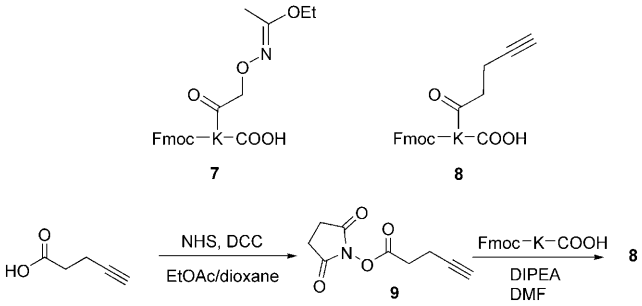
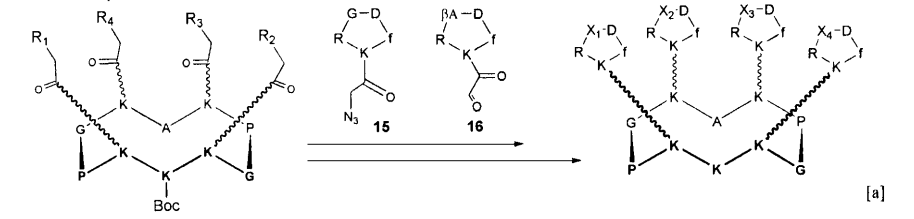
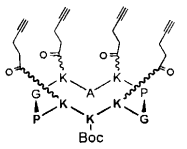
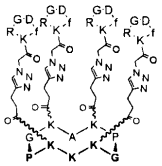
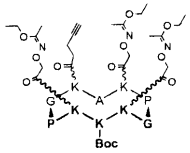
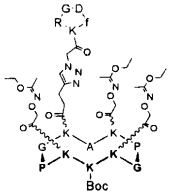
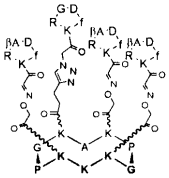
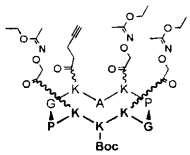
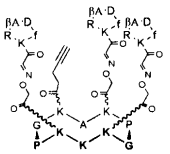
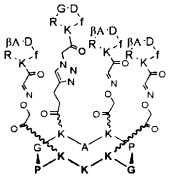
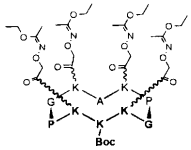
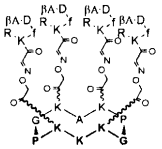


Scheme 1. Structures and syntheses of building blocks **7** and **8**. Compound **7** was prepared according to reference [15]. Fmoc = 9-fluorenylmethoxycarbonyl, NHS = *N*-hydroxysuccinimide, DCC = dicyclohexylcarbodiimide, DIPEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, K = lysine.

copper(I)-mediated click chemistry. Glycine at the *C*-terminal end was essential to ensure the subsequent head-to-tail cyclization from epimerization. Peptides **10**–**14** (Tables 1 and 2) were isolated in sufficient purity to carry out subsequent chemoselective assemblies.

Table 1: Stepwise chemoselective assemblies of **15** and **16**.[a]



Entry	Method[b]	Scaffold	Intermediate	Product	Yield[d]
1	A[c]	 **10**		 **1**	70%
2	A,B	 **11**	 **17**	 **5**	51%
3	B,A	 **11**	 **18**	 **5**	45%
4	B	 **12**		 **6**	72%

[a] R_n = CH_2−C≡CH or O−N=C(OEt)CH_3, X_n = Gly or βAla. [b] A: **15** (1.2 equiv per site), Cu^0 (1 equiv per site), *t*BuOH/PBS (7:3), room temperature, 18 h; B: **16** (1.5 equiv per site), TFA/H_2O (9:1), room temperature, 2 h. PBS = phosphate buffer saline. [c] TFA/H_2O (9:1), room temperature, 2 h. [d] Yield of isolated product.

Cyclopentapeptides **15** and **16** (Table 1), bearing the prerequisite functions (azide and aldehyde groups, respectively), were prepared through a combination of solid and solution-phase syntheses (see the Supporting Information). We then determined appropriate reaction conditions to carry out the copper(I)-catalyzed azido–alkyne cycloaddition. A variety of conditions have already been reported;[16] the copper(I) species can be introduced directly or generated in situ by reduction of copper(II) with reducing agents.

Although this reaction allows, for example, fourth-generation triazole dendrimers from polyacetylene cores to be obtained,[17] the conversion of tetraalkyne-containing calixarene failed.[18] Recently, the conversion of a tetraalkyne-containing calixarene was reported, but only at high temperatures.[19] The initial formation of a copper acetylide species on one of the alkyne chains probably led to formation of complexes with preferred neighboring alkyne units, thus preventing the binding of azide.[20] Taking into account this result, we examined the conversion of tetraalkyne-containing cyclodecapeptide **10** in the presence of azidopeptide **15** using copper nanosize powder (Table 1, entry 1). This catalyst was successfully used in the cycloaddition of azides with terminal alkynes.[21] The desired cycloaddition proceeded cleanly, with up to quantitative conversion, over 18 hours at room temperature. When standard $CuSO_4$/sodium ascorbate was used for the generation of the active copper(I) species, the reaction was markedly less efficient (see the Supporting Information, Table S2).

The optimized conditions for the cycloaddition reaction then allowed the development of synthetic methods for access to successive chemoselective ligations of peptides **15** and **16**. The cycloaddition was carried out prior to oxime ligation (Table 1, method A,B; entry 2). As expected, we observed quantitative conversion of peptide **11** in the presence of azidopeptide **15**. It is important to note that Eei function was stable during the copper(I)-catalyzed cycloaddition. The following oxime ligation was then performed with the conditions used to yield compound **6** (Table 1, entry 4). One-pot aminooxy deprotection of **17** and subsequent ligation of aldehyde derivative **16** were carried out in aqueous TFA (trifluoroacetic acid) solution. Under these conditions, removal of the *tert*-butoxycarbonyl (Boc) group, thus triggering *tert*-butoxy cation and isobutene formation, did not affect highly nucleophilic residues

such as aminooxy functions: compound **5** with one copy of RGD monomer was obtained without the formation of undesirable by-products. This result emphasizes the utility of this method for peptide ligation starting from Boc/*t*Bu protected peptides.

To ensure the versatility of our chemical strategy, a reverse method B,A was performed (Table 1, entry 3). Starting with peptide **11**, ligation of derivative **16** was effectively achieved. Subsequent cycloaddition of azidopeptide **15** furnished compound **5** quantitatively. As a result, we have shown that the oxime linkage is stable under copper(I)-catalyzed cycloaddition conditions, even in the presence of a slight excess of azidopeptide **15**. Once again, this two-step method was efficient in terms of chemoselectivity and reactivity. However, even if these methods permit quantitative peptide ligations, low overall yields were obtained from HPLC purifications.

To avoid a lengthy separation process and purification of intermediate peptides, we decided to perform both chemoselective ligations in a one-pot approach (Table 2). As copper(I)-catalyzed cycloaddition occurs under neutral conditions, it is necessary to adjust the pH to complete biomolecular assemblies, as oxime bond formation is very slow at pH 7 or higher.[22] During preliminary studies, it was found that aldehyde-containing peptide **16** is slightly unstable to the reaction conditions used for copper(I)-catalyzed cycloaddition. Therefore, peptides **13**, **15** (3.6 equiv), and **16** (1.2 equiv) were applied under mild acidic conditions using a solution containing 5% TFA and copper nanosize powder.

Rapid conversion of **13** was observed, affording principally aminooxy deprotection and the subsequent oxime ligation (Figure 2). Exclusive formation of the intermediate comprising one RβAD unit and three alkyne groups was obtained (See the Supporting Information, Figure S37). Neutralizing the pH resulted in complete disappearance of the intermediate and the exclusive formation of compound **19**. Starting with compounds **14** and **11** that have more aminooxy groups and then using higher concentration of aldehyde **16** (up to 3.6 equiv), one-pot reactions provided similar results.

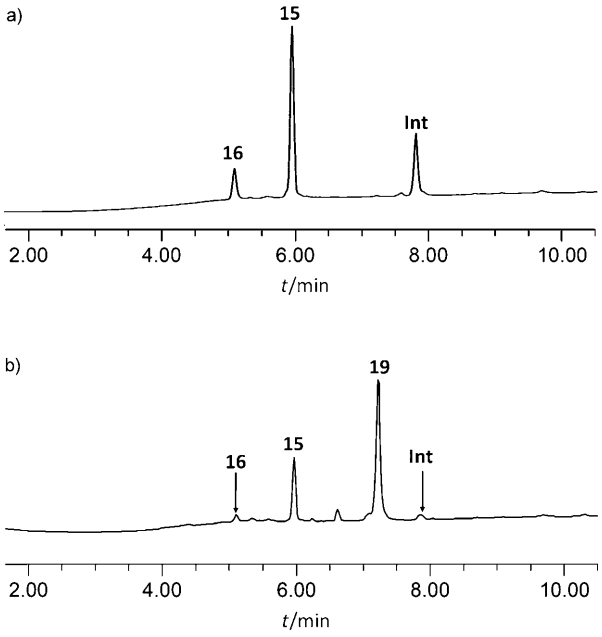
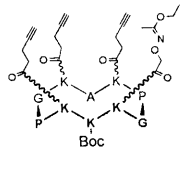
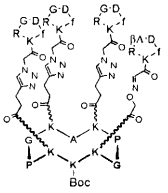
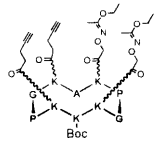
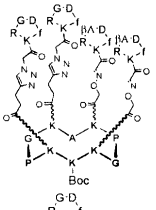
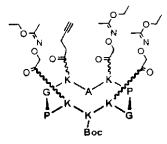
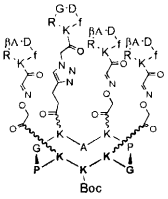


Figure 2. One-pot chemoselective assembly of peptides **13**, **15**, and **16**. HPLC traces are shown at a) 2 h and b) 18 h. Int = intermediate.

Table 2: One-pot chemoselective assemblies of **15** and **16**.[a]

Entry	Scaffold		Product		Yield[b]
1		**13**		**19**	68%
2		**14**		**20**	61%
3		**11**		**21**	63%

[a] **15** (1.2 equiv per site), **16** (1.2 equiv per site), Cu^0 (1 equiv per site), *t*BuOH/H_2O/TFA (50:45:5), room temperature, 2 h, then pH adjusted to 7 using DIPEA; 18 h. [b] Yield of isolated product.

In conclusion, we have expanded the scope of click–click chemistry by gaining access to sophisticated macromolecules. We have shown for the first time that well-defined biomolecular assemblies are possible by means of orthogonal oxime and copper-mediated click reactions in a stepwise or in a one-pot approach. The latter method is much desired, as it avoids purification of intermediates while increasing overall chemical yield. The simple reaction conditions suggest that this one-pot chemoselective ligation strategy could have wide potential for the chemical synthesis of complex systems. An extension of the method combining carbohydrates and peptides is in progress and will be reported in due course.

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