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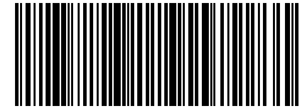

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Application of copper(I) catalyzed azide–alkyne [3+2] cycloaddition to the synthesis of template-assembled multivalent peptide conjugates†

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Here we describe the facile generation of tetravalent peptide conjugates *via* a copper(I) catalyzed azide–alkyne cycloaddition (CuAAC) using a cyclic peptide template as a versatile conjugation scaffold. This stable and rigid framework is a conformationally constrained cyclic β-sheet decorated with spatially defined alkyne moieties that serve as selectively addressable coupling sites. The proposed method allows for the effective coupling of unprotected peptide monomers in water at room temperature within comparatively short reaction times. The resulting conjugates display the ligands in an oriented manner, thus allowing for multivalent interactions with given target molecules, which may contribute to enhanced affinity and specificity. In addition, the selected scaffold offers an orthogonal coupling site for the incorporation of fluorescent labels or radioligands.

Introduction

A number of interactions taking place in nature occur in multivalent mode.[1] It is known that a multivalent display created through multiplication of biologically active monomers on an appropriate scaffold can significantly enhance the net affinity of a resulting construct towards its target in comparison to the interactions of individual monomers.[1,2] Linear or branched polymers, dendrimers, and peptides placed on various scaffold structures have already been used *e.g.* to enhance antigen immunogenicity, binding affinity, or selectivity.[3–6] Better understanding of the nature and mechanisms of multivalent interactions between the interplay partners is of particular research interest. Enhanced binding affinity towards desired targets, probably due to an improved steric orientation, make them potentially attracting objects in the design of antibodies, receptors, and inhibitors of proteases.[7,8]

Two different mechanistic models can be considered for the explanation of increased binding affinity of multimeric systems.[9] First, when a simultaneous binding to the corresponding receptors cannot take place, an increase in receptor–ligand binding can be explained by an apparent increase in local ligand concentration. Second, in cases of polyvalent binding, *e.g.* by interacting with receptors that are located on cell surfaces in several copies, co-operative interactions between multiple ligands and receptors can occur resulting in increased net affinity (avidity) of the receptor–ligand interaction.

In the context of multivalent peptide conjugation, the choice of a proper structural framework is of particular importance.[10] This scaffold should possess a three dimensional architecture providing free access to reactive moieties and making, therefore, possible the coupling of the ligands of choice without steric hindrance. Moreover, it is highly desirable for the scaffold to offer not only the possibility to oligomerize functional monomers but also to give an option for the introduction of other substituents, *e.g.* fluorescent dyes, lipophilic molecules for membrane association, chelators for incorporation of radionuclides, or additional orthogonal reactive moieties for the introduction of other bioactive ligands as shown schematically in Fig. 1.

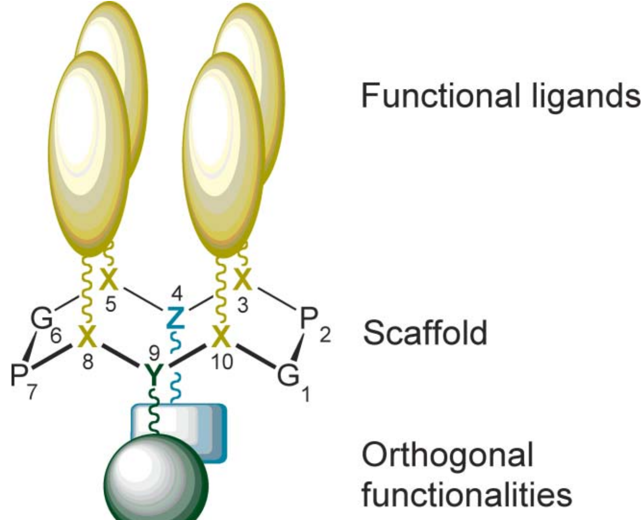

Fig. 1 Schematic representation of cyclic decapeptide scaffold decorated with different types of ligands. P, proline; G, glycine; X, chemo- and regioselectively addressable conjugation sites; Y, Z, sites for alternative (orthogonal) functionalization.

Cyclic peptides and their mimics are versatile molecules with particular properties. It was shown that cyclic decapeptides developed by Mutter and colleagues in the frame of their concept

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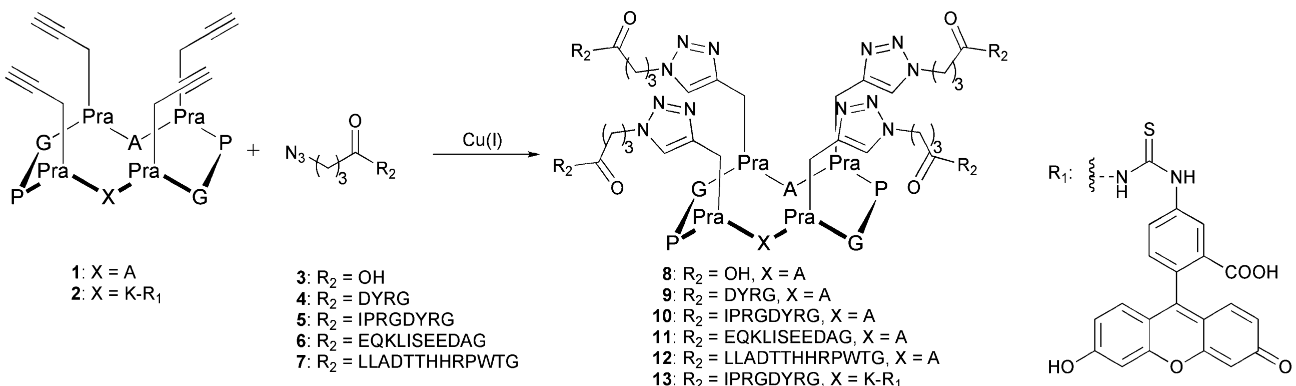
† Electronic supplementary information (ESI) available: RP-HPLC for peptides **1–13**, RP-HPLC traces of template **2** cyclization under activation with HBTU and PyBOP, ESI-MS for peptides **8–13**, GFC traces for conjugates **9–13** and corresponding monomers. See DOI: 10.1039/b908261a
‡ These authors contributed equally to this work.



Scheme 1 Synthetic strategy for the preparation of multivalent peptide conjugates on alkyne functionalized cyclic decapeptide scaffolds using copper(I) catalyzed azide–alkyne [3+2] cycloaddition.

of template assembled synthetic proteins (TASP)[11–21] possess due to the presence of two glycine-proline turns an architecture of an antiparallel β-sheet[21–23] that can also serve as scaffolds for coupling of a wide spectrum of ligands with diverse functionalities.[10,24–31]

Herein, we report the synthesis of tetravalent peptide conjugates by copper(I) catalyzed azide–alkyne [3+2] cycloaddition on a decameric peptide template bearing alkyne groups that were introduced using the commercially available non-natural amino acid building block Fmoc-L-propargylglycine (Pra). We show that unprotected peptide ligands can be effectively assembled on the framework alone or in combination with an orthogonally attached fluorescent reporter group (Scheme 1). Our presented strategy provides a short route towards efficient generation of multimeric compounds.

Results and discussion

General strategy

Cyclic decapeptides have been widely used in protein *de novo* design and drug discovery[32–42] due to their well-defined architecture furnishing a topological arrangement with sterically defined active groups, which provides a possibility for coupling of functional building blocks. A variety of synthetic strategies based on orthogonal protecting groups in combination with chemoselective ligations has been reported to date.[10,25,26,29–31,43,44] The concept of regioselectively addressable templates (RAFT) conventionally employs two main approaches aimed at the conjugation of unprotected peptides onto the functional scaffold. One involves the formation of amide bonds between the γ-amines of lysine side chains and linker molecules bearing reactive groups suitable for successive chemoselective ligations (Scheme 2A). The other exploits the particular reactivity of thiol functions of cysteine residues (Scheme 2B). Both approaches require the proper protection of the reactive moieties not only during assembly of linear precursors on the solid support but also in the course of head-to-tail cyclization. Lysine based templates also require additional steps for the introduction of selectively addressable moieties, usually aldehydes or aminooxy groups, for oxime bond formation[30] and recently reported alkynes and azides for the click coupling.[31] Cysteine based cyclic decapeptide scaffolds, though allowing for the direct chemoselective ligation *via* thio-ether bonds, are predisposed to the formation of undesired disulfides. Moreover, the regioselective conjugation is not possible with peptides or proteins containing unprotected cysteine residues.

Obviously very simple, our design was based on a construct derived from the well established *cyclo*-(Xaa-Ala-Xaa-Pro-Gly)$_2$ sequence. We reasoned that the incorporation of alkyne moieties in the desired positions (Xaa) of the decapeptide framework could be easily accomplished using Fmoc protected propargylglycine (Scheme 2C). This commercially available alkyne bearing building block could be easily incorporated in the growing peptide without side chain protection, therefore significantly reducing complexity of the whole procedure.

Synthesis of alkyne and azide building blocks

As alkyne components, two different decameric cyclopeptides were synthesized, **1** and **2**. Sharing the same topological features, they differ in the amino acid residue at position 9 (Fig. 1). While scaffold **1** contains no coupling sites other than the desired propargyl side chains, cyclopeptide **2** possesses an orthogonal reactivity in the form of a lysine γ-amino group that is labeled with FITC to demonstrate its utility for the coupling of additional ligands like fluorescent dyes, metal chelators, cell penetrating peptides, *etc.* prior to peptide conjugation *via* click reaction.

Solid phase synthesis of both templates, due to the influence of applied microwave irradiation, was fast and resulted in crude linear precursors of remarkable quality and in excellent yields. They were converted into the cyclic successors without further purification *via* active ester activation of the carboxy terminus. Our experiments corroborated the known fact[45] of *N*-terminal guanidination caused by uronium based reagent HBTU during head-to-tail cyclization. Therefore, phosphonium activator PyBOP was applied to the macrocyclization of template **2** (for further details refer to the supplemental material†).

Introduction of FITC as a model ligand for orthogonal coupling was carried out after macrocyclization of the corresponding precursor followed by cleavage of the Boc protecting group. Similar to previous synthetic steps, this transformation yielded a labeled alkyne functionalized RAFT scaffold in excellent yield (73% after four synthetic steps) and quality. Interestingly, no chromatographic purification was necessary during the whole course of the synthesis for template **2**.

Azide functionality was effectively introduced into model peptide ligands **4–7** during solid phase synthesis using

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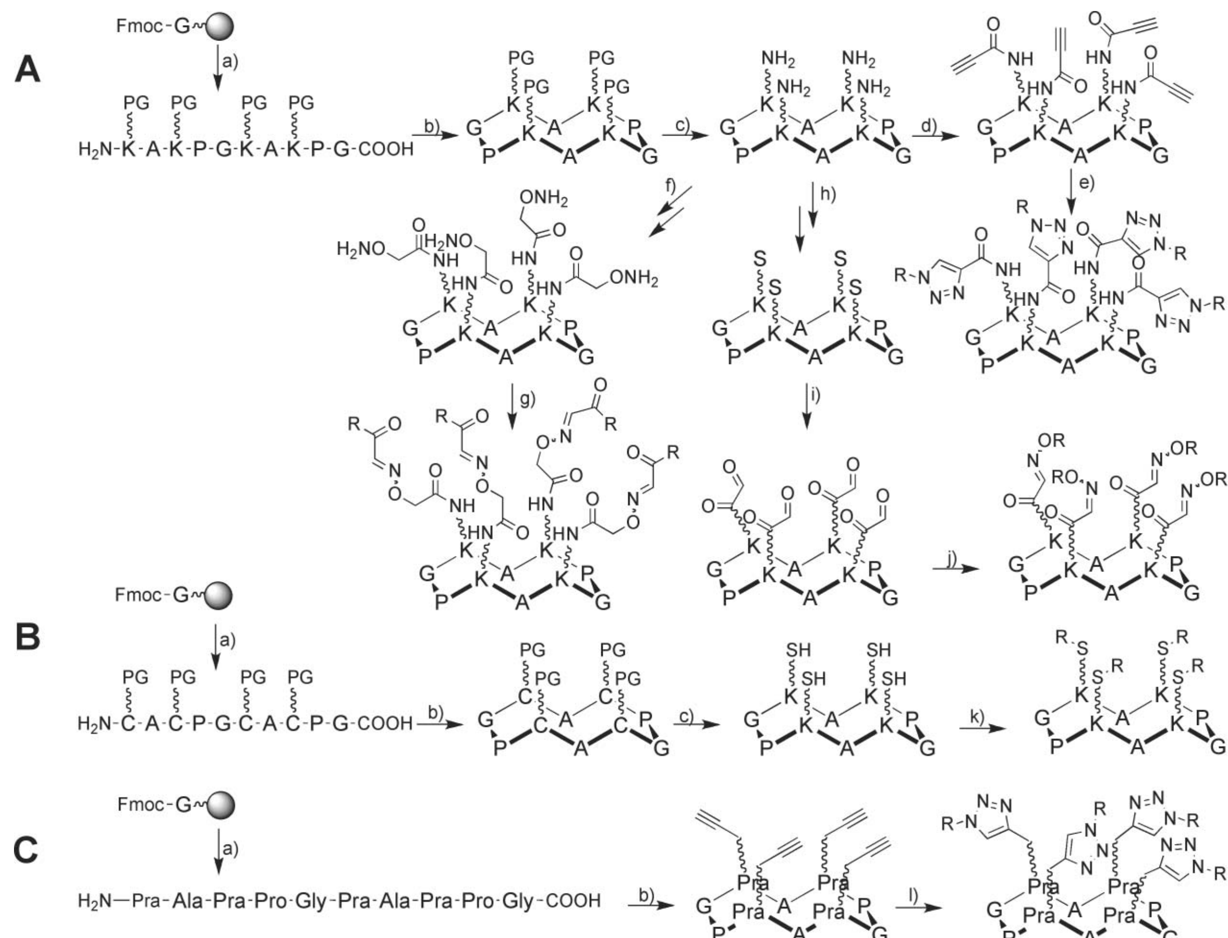

Scheme 2 Comparison of homo-tetramerization strategies using different RAFT scaffolds. A, selected approaches for the peptide conjugation using a lysine based template. B, general approach for the homo-multimerization through cysteine based templates. C, general scheme for the peptide conjugation *via* a progargylglycine based scaffold used in this study. PG, protecting group; R, unprotected bioactive ligand; (a) Fmoc SPPS and acidic cleavage from the support, (b) head-to-tail cyclization *via C*-terminal activation, (c) side chain deprotection, (d) propionic acid, DCC,[31] (e) R-N_3, $CuSO_4$, sodium ascorbate, t-BuOH–H_2O (1 : 1),[31] (f) BocNHOCH_2COOSu, DIEA, DMF, then Boc deprotection,[29] (g) CHO-CO-R, buffer pH = 4.6,[29] (h) Boc-Ser(t-Bu)OH, PyBOP, DIEA, DMF, then Boc deprotection,[30] (i) $NaIO_4$, H_2O,[30] (j) R-O-NH_2, 10% AcOH,[30] (k) R-Br, pH = 7.5,[43] (l) R-N_3, $CuSO_4$, Cu(0), sodium ascorbate, DIEA, H_2O.

4-azidobutanoic acid **3** as an *N*-terminal building block under standard coupling conditions generally applied in this work.

Click ligations

As a conjugation strategy, copper(I) catalyzed azide–alkyne cycloaddition [3+2] was chosen (Scheme 1). It is a highly efficient coupling method, orthogonal to most known ligation techniques, which gives an option to link together peptidic ligands as well as other functional substituents.[46–49]

To demonstrate the utility of the novel cyclic decapeptide scaffolds **1** and **2** towards the conjugation of unprotected peptide ligands and to investigate steric effects on ligation efficacy, azido-functionalized peptides of different length ranging from four to fourteen residues were used for coupling onto the presented scaffolds. Prior to the coupling of bulky peptidic building blocks, initial studies towards general applicability and optimization of reaction conditions were conducted using the small non-peptidic azido building block **3**. For this purpose, different click systems were tested such as iodo(trimethylphosphite)copper(I)/DIEA in MeOH–acetonitrile, $CuSO_4$/sodium ascorbate/DIEA in water, Cu-wire in water, $CuSO_4$/Cu wire/sodium ascorbate/DIEA in water. Combined addition of Cu(II) and Cu(0) in the presence of sodium ascorbate comprised the optimal system characterized with faster and cleaner conversions. We reasoned that as for bulky biological ligands reaction times could be prolonged, a continuous Cu(I) source in the form of a redox pair of Cu(II) and Cu(0) should be installed. The chromatographic traces of the chosen reaction system revealed the formation of synthesis intermediates already 5 min after catalyst addition. Complete conversion was reached within 50 min (Fig. 2). Conjugation of peptides **4–7** onto template **1** were accomplished in the same way. HPLC traces of the reaction components and conjugation products are shown in Fig. 3. Though HPLC monitoring proved that educts were completely consumed, the yields of the resulting conjugates decreased according to the length of the ligands, probably due to the tendency of particular peptides to aggregate in concentrated solutions (see Table 2). The irregular low yield of conjugate **11** could be explained by losses during preparative purification focused on obtaining a high quality product.

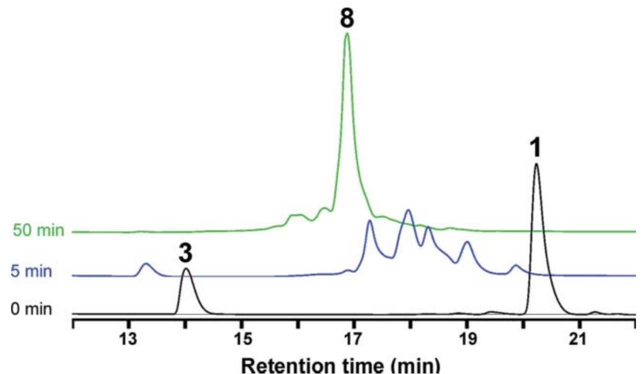

Fig. 2 HPLC monitoring of CuAAC reaction between alkyne **1** and azide **4** at 220 nm. Gradient: 9→45% acetonitrile in 0.1% aq. TFA over 30 minutes at flow rate 1 mL/min.

High resolution ESI-MS analysis proved the formation of tetrameric conjugates. Exemplary high resolution ESI-MS spectra for conjugate **12** are shown in Fig. 4; for the complete data set see the supplemental material.† Formation of the tetrapeptide conjugates **9–12** was further proved by analytical gel filtration. This analysis was of particular importance for the cases when the click conjugates and their corresponding monomeric peptide ligands showed similar elution behaviour under RP-HPLC conditions (Fig. 3B and C). Size exclusion chromatography made it possible to doubtlessly distinguish between the tetramers and monomers as shown exemplary for compounds **10** and **11** in Fig. 5. Click coupling onto additionally derivatized scaffold **2** differed from that for **1** carrying only alkyne moieties. Introduction of a bulky hydrophobic FITC substituent affected water solubility of template **2** significantly. Two modifications of the proposed click protocol were employed. Since it was not possible to dissolve the cyclic decapeptide **2** in water, a water–acetonitrile mixture was used. After two days of reaction the presence of click conjugation intermediates could still be detected in the HPLC trace (Fig. 6A), and the yield of the final product was significantly lower in comparison to the reactions carried out in pure water (13% and 30% after purification, respectively). Therefore, we increased the amount of tertiary amino base (DIEA) in the reaction system that made it possible to avoid the use of an organic co-solvent. This resulted in a remarkable acceleration of the reaction rate and yielded the target conjugation product within 5 h.

Nevertheless, taking into consideration the solubility problems caused by bulky substituents, it seems reasonable to introduce a solubilizing moiety like PEG into templates carrying hydrophobic ligands through one of the possible ligation sites (Fig. 1, *e.g.* position 4). This can be easily achieved *via* an incorporation of an additional orthogonal amino group or thiol function.

Experimental

General procedures

All chemicals and solvents purchased from Acros, Roth, Novabiochem, Aldrich or Sigma were of highest grade available. Fmoc-L-Pra-OH was obtained from Anaspec. All comprising a PrepStar

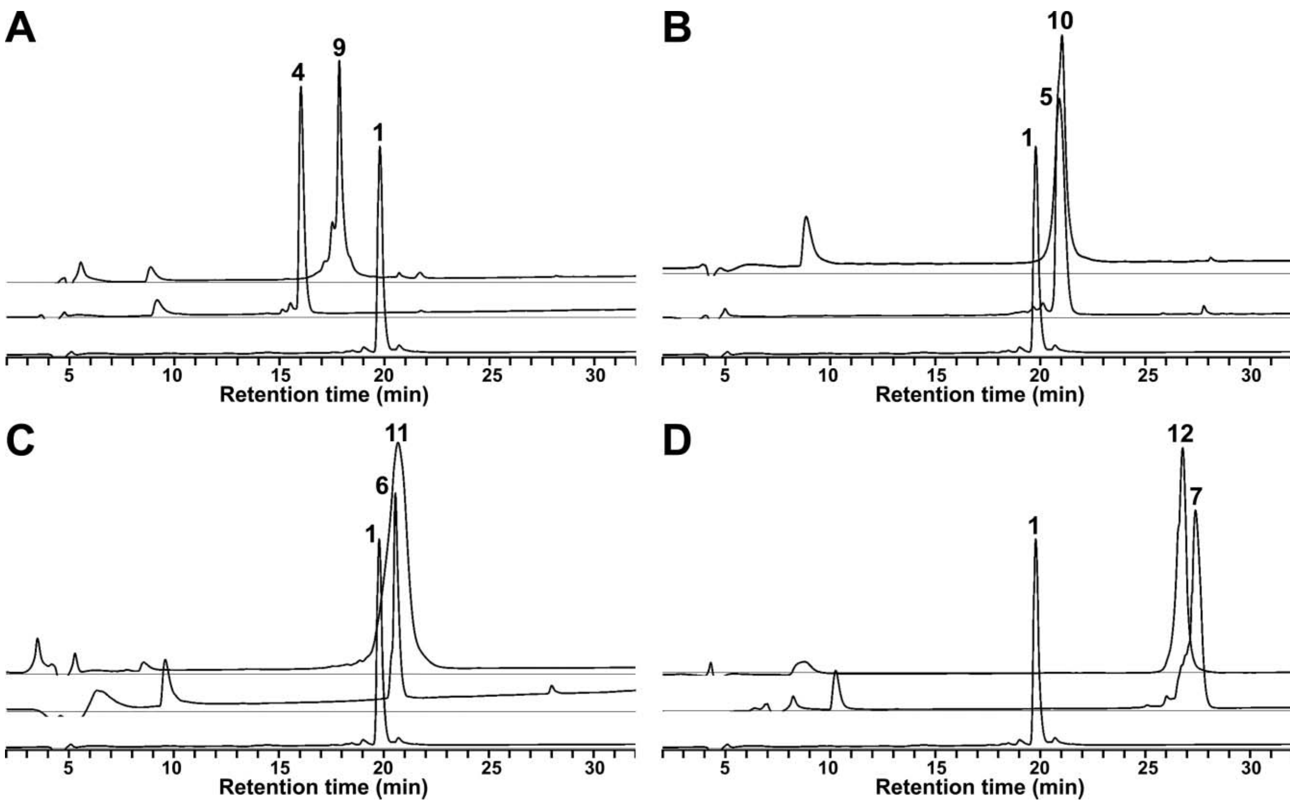

Fig. 3 HPLC traces of click reaction components and the resulting conjugates at 220 nm. Gradient: 9→45% acetonitrile in 0.1% aq. TFA over 30 minutes at flow rate 1 mL/min.

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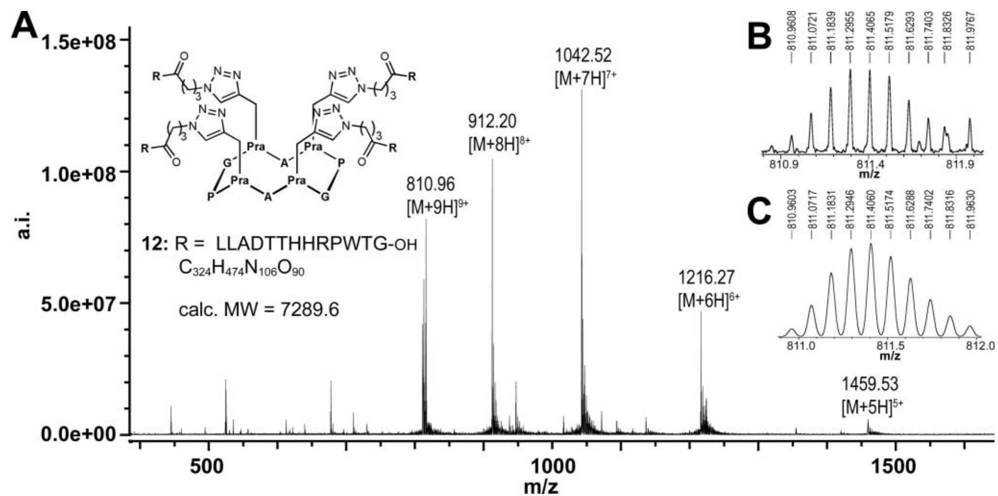

Fig. 4 Mass spectra of conjugate **12**. A, ESI-MS spectrum; B, high resolution spectrum recorded for $[M + 9H]^{9+}$; C, mass distribution calculated for $C_{324}H_{483}N_{106}O_{90}$ (9+).

Fig. 5 GFC traces of monomeric peptide ligands and corresponding tetra-conjugates at 220 nm. Eluent: 150 m aq. NaCl at flow rate 0.75 mL/min.

Fig. 6 HPLC monitoring of CuAAC reaction between alkyne **2** and azide **5** at 220 nm. Gradient: 9→72% acetonitrile in 0.1% aq. TFA over 30 minutes at flow rate 1 mL/min. A, click reaction in water–acetonitrile (2 : 1); B, click reaction in water with excess DIEA (2 eq. according to copper catalyst).

218 Solvent Delivery Module, a ProStar 410 HPLC AutoSampler and a ProStar 325 Dual Wavelength UV-Vis HPLC Detector using a Phenomenex Synergi 4u Hydro-RP 80 Å (250 × 4,6 mm, 4 μm, 8 nm) column for analytical runs and a YMC J'sphere ODS-H80, RP C-18 (250 × 20 mm, 4 μm, 8 nm) for semi-preparative ones. The solvent system consisted of eluent A (0.1% aq. TFA) and eluent B (90% aq. acetonitrile containing 0.1% TFA). ESI mass spectra

Table 1 Building blocks synthesized in the presented study

	Entry	Sequence	Calculated Mol. Wt.	Observed m/z	R_t [b]	Yield[c] [%]
Alkyne	**1**	G Pra A Pra P; P Pra A Pra G	830.4	853.6	20.29	36
	2	G Pra A Pra P; P Pra K Pra G; HN (4) FITC	1276.6	1299.6	22.71	73
Azide	**3**	N_3 … O, OH	129.1	n.d.[a]	13.54	78
	4	N_3 (3) O NH-DYRG-OH	620.3	621.3	16.04	33
	5	N_3 (3) O NH-IPRGDYRG-OH	1043.5	1044.6	20.92	17
	6	N_3 (3) O NH-EQKLISEEDAG-OH	1328.6	1329.7	20.54	5
	7	N_3 (3) O NH-LLADTTHHRPWTG-OH	1614.8	1616.0	27.41	23

[a] Characterized by 1H- and 13C-NMR.[32] [b] Compounds **1** and **3–7**: gradient: 9→45% acetonitrile in 0.1% TFA over 30 minutes at 1 mL/min; compound **2**: gradient: 9→72% acetonitrile in 0.1% TFA over 30 minutes at 1 mL/min. [c] Overall yield calculated from initial loading of the resin.

were recorded on a Bruker-Franzen Esquire LC and a Bruker Apex-Q IV FT-ICR mass spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were obtained with a Bruker DRX300 instrument (300 MHz). Peptides were synthesized on a manual Discover SPS Microwave Peptide Synthesizer (CEM) using the Fmoc strategy. Amino acids were employed in 4 eq. excess according to resin. HBTU (3.9 eq) and DIEA (8 eq.) were applied as coupling reagents. Coupling microwave conditions were 55 °C and 20 W within 10 min. Fmoc deprotection was performed by treatment with piperidine (20% in NMP) at 50 °C by 20 W microwave power and within 5 min. Cleavage from the Wang resin was done either at room temperature within 2 h or in the microwave at 40 °C, other amino acids were supplied by CEM; preloaded Fmoc-Gly-Wang-resin by Novabiochem; preloaded H-Gly-2Cl-Trt-resin by Iris Biotech. Both analytical and semi-preparative RP-HPLC were performed on a Varian modular system 20 W within 30 min using TFA/H_2O/anisole/TES (36:2:1:1, v/v/v/v) cleavage cocktail. Cleavage from 2-Cl-Trt resin was performed with acetic acid/DCM/MeOH (5:4:1, v/v/v) mixture within 2–3 h.

Synthesis of scaffold 1

The linear precursor peptide NH_2-Pra-Ala-Pra-Pro-Gly-Pra-Ala-Pra-Pro-Gly-OH was synthesized *via* microwave assisted Fmoc SPPS on Wang resin preloaded with Fmoc-Gly (loading capacity 0.61 mmol/g). Coupling efficacy was controlled by the Kaiser test. Both Fmoc-Ala-OH building blocks were double coupled as well as the *C*-terminal proline. After cleavage from the resin with 95% aq. TFA and lyophilization, cyclization was performed without further purification by treatment with HBTU (1 eq.) and DIEA (2 eq.) in diluted solution (1 mg peptide/1 mL DMF) overnight at ambient temperature. After solvent removal under reduced pressure, the crude cyclic decapeptide was purified by preparative HPLC to yield 55 mg of pure *cyclo*-(Pra-Ala-Pra-Pro-Gly)$_2$ **1** (Table 1).

Synthesis of scaffold 2

The linear precursor peptide NH_2-Pra-Lys(Boc)-Pra-Pro-Gly-Pra-Ala-Pra-Pro-Gly-OH was synthesized *via* microwave assisted Fmoc SPPS on a 2-Cl-Trt resin preloaded with Fmoc-Gly. Coupling efficacy was controlled by the Kaiser test. All amino acids were double coupled except for the Fmoc-Pra-OH building blocks. After cleavage from the resin with a cleavage cocktail containing 50% acetic acid, 40% DCM and 10% methanol followed by lyophilization, cyclization was performed directly with the crude linear peptide by treatment with PyBOP (1.2 eq.) and DIEA (3 eq.) in diluted solution (1 mg peptide/1 mL DMF) overnight

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Table 2 Peptide tetra-conjugates **8–13**

Entry	Educts: Alkyne	Educts: Azide	HPLC[a] R_t/min	GFC[b] R_t/min	Yield[c]/%
8	1	3	17.50	—	—
9	1	4	17.87	12.32	75
10	1	5	20.9	12.76	50
11	1	6	20.69	11.02	14
12	1	7	25.85	13.52	28
13	2	5	16.27	12.81	30

[a] Compounds **8–12**: gradient: 9→45% acetonitrile in 0.1% TFA over 30 minutes at 1 mL/min; compound **13**: gradient: 9→72% acetonitrile in 0.1% TFA over 30 minutes at 1 mL/min. [b] Eluent: 150 m aq. NaCl at flow rate 0.75 mL/min. [c] Pure yield after purification *via* HPLC; the loss of substance during routine monitoring of reaction progress and analytical purposes is not considered.

at ambient temperature. The solvent was removed under reduced pressure and the crude cyclic decapeptide *cyclo*-(Pra-Lys(Boc)-Pra-Pro-Gly-Pra-Ala-Pra-Pro-Gly) was treated with 95% aq. TFA to remove the Boc protecting group. Precipitation and washing with MTBE yielded crude *cyclo*-(Pra-K-Pra-P-G-Pra-A-Pra-P-G) in excellent quality that enabled its labelling with amino reactive FITC (2 eq.) in the presence of DIEA (2 eq.) in DMF without additional purification. After DMF removal under reduced pressure the excess of FITC was extracted with ether from a water–methanol (5:1) solution of labelled cyclic decapeptide **2**. This yielded FITC labelled alkyne functionalized RAFT scaffold **2** in excellent yield and quality (19 mg, Table 1).

Synthesis of *N*-terminal azido functionalized peptides 4–7

Peptide ligands were synthesized using microwave assisted Fmoc SPPS on Fmoc-Gly preloaded Wang resin using HBTU/DIEA activation in NMP. Each coupling cycle took 10 min, and the Fmoc deprotection was accomplished within 5 min. To introduce the *N*-terminal azido-group, 4 eq. of 4-azidobutanoic acid **3** synthesized from methyl 4-bromobutanoate following a reported procedure[50] were coupled onto the terminal amino group under the same coupling conditions. Cleavage from the solid support was conducted in the microwave oven within 30 min using 95% TFA at 38 °C. After microwave assisted cleavage from the solid support followed by MTBE precipitation, purification was done by preparative HPLC to yield pure azide functionalized peptide ligands **4–7** (see Table 1).

Copper(I) catalyzed azide–alkyne [3+2] cycloaddition (CuAAC) reaction

The click ligations of the alkyne scaffold **1** and the azido building blocks **3–7** were accomplished by treatment of an aqueous solution of **1** (2.4 μmol) and the corresponding azide (9.6 μmol) with $CuSO_4$ (28.8 μmol), sodium ascorbate (28.8 μmol), DIEA (28.8 μmol) and 15 mg of HNO_3-treated Cu-wire. Reaction progress was monitored by analytical HPLC (Fig. 2, 3 and 6) until conversion of both the azide and alkyne components was complete (50 min–4 h). The solvation of occasional precipitate was performed by addition of 0.3% ammonia. Conjugates **8–13** were isolated *via* semi-preparative RP-HPLC and characterized *via* RP-HPLC, GFC and ESI-MS as mentioned in Tables 2 and 3.

The click reaction between FITC labeled alkyne scaffold **2** and the azido building block **5** was conducted by treatment of the solution of **2** (1.56 μmol) and the azide (6.28 μmol) in water with $CuSO_4$ (18.72 μmol), sodium ascorbate (18.72 μmol) and DIEA (37.44 μmol). The reaction was continuously shaken at ambient temperature. Reaction progress was monitored by analytical RP-HPLC (Fig. 6B). The conjugation product was isolated by semi-preparative RP-HPLC yielding 2.6 mg of **13** as a yellow powder (Tables 2, 3). The formation of the desired conjugate was proven by GFC and ESI-MS.

Conclusions

We demonstrate the utility of the CuAAC reaction for the synthesis of tetravalent peptide conjugates on a cyclic peptide template. Unprotected biologically relevant azido peptides are efficiently converted into the corresponding cycloaddition products. The introduction of an azide moiety into the growing peptide chain by pre-activated 4-azidobutanoic acid is easy, efficient and needs no protecting groups or harsh reaction conditions. Alternatively,

Table 3 Summarized ESI-MS data for the synthesized peptidic tetra-conjugates **9–13**

	Entry	9	10	11	12	13
	Calc. MW	3311.4	5004.5	6144.9	7289.6	5450.6
	Formula	$C_{140}H_{194}N_{50}O_{46}$	$C_{216}H_{326}N_{78}O_{62}$	$C_{256}H_{402}N_{74}O_{102}$	$C_{324}H_{474}N_{106}O_{90}$	$C_{240}H_{344}N_{80}O_{67}S$
Measured	$[M + H]^+$	3312.45	5005.51	6145.92	7290.63	5451.6
	$[M + 2H]^{2+}$	—	—	—	—	—
	$[M + 3H]^{3+}$	1104.83	—	—	2430.88	—
	$[M + 4H]^{4+}$	828.87	1252.13	1537.74	—	—
	$[M + 5H]^{5+}$	663.30	1001.91	—	1459.53	—
	$[M + 6H]^{6+}$	—	835.0863[a]	—	1216.27	—
	$[M + 7H]^{7+}$	—	715.9316[a]	—	1042.52	—
	$[M + 8H]^{8+}$	—	626.5661[a]	—	912.2046[a]	—
	$[M + 9H]^{9+}$	—	—	—	810.9598[a]	—

[a] High resolution spectra (see Fig. 4 and supplementary information†).

azide bearing peptides and proteins are also readily accessible not only *via* Fmoc SPPS using the commercially available building blocks, *e.g.* Fmoc-L-azidohomoalanine, but through several selective protein modifications.[51,52] Azido group bearing proteins can be obtained by recombinant expression methods *via* growing *E. coli* strains deficient in methionine synthesis in the presence of azido homoalanine (AZH) resulting in the incorporation of AZH in place of methionine in proteins.[51] Moreover, a recently reported single-step azide introduction *via* an aqueous diazo transfer provides another possibility to functionalize native proteins with a clickable moiety.[52]

The alkyne functionalized cyclic peptide templates used in this study can be easily synthesized from commercially available building blocks and encompass two important features: (a) they have defined three dimensional architecture that provides free access to reactive moieties and allows for the coupling of bulky ligands without hindrance and (b) they enable conjugation of unprotected peptides in water using the simple, inexpensive and effective copper catalyst also providing an option for the oligomerization of native folded proteins. Click cycloaddition is fast and highly selective; the resulting conjugates are easily separable *via* HPLC or GFC.

It will be interesting to see whether larger peptides exceeding the length of 14 residues used in this study, miniproteins[53] or even proteins can be placed in fourfold copies onto the decapeptide scaffold and if steric constrains exist that might negatively influence conjugation efficiency. In this case, flexible linkers like polyethylene glycol[54] or oligoglycine could be introduced for enhancing conformational flexibility.

Peptide **5** of the model peptides used in this study (IP**RGD**YRG) contains an RGD motif and is known to bind GPIIb/IIIa receptor on the surface of platelets, thereby inhibiting platelet aggregation.[55] Systematic studies to compare biological activity of tetravalent peptide conjugates compared to their monomeric counterparts are currently underway aimed at obtaining a better understanding of the avidity effects imposed by the particular spatial orientation of peptide ligands induced by the conjugation onto a cyclic decapeptide scaffold.

Acknowledgements

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References

1 M. Mammen, S. K. Choi and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 1998, **37**, 2754–2794.
2 V. Martos, P. Castreno, J. Valero and J. de Mendoza, *Curr. Opin. Chem. Biol.*, 2008, **12**, 698–706.
3 R. J. Kok, A. J. Schraa, E. J. Bos, H. E. Moorlag, S. A. Ásgeirsdóttir, M. Everts, D. K. F. Meijer and G. Molema, *Bioconjugate Chem.*, 2002, **13**, 128–135.
4 G. Thumshirn, U. Hersel, S. L. Goodman and H. Kessler, *Chem.–Eur. J.*, 2003, **9**, 2717–2725.
5 S. E. Cwirla, P. Balasubramanian, D. J. Duffin, C. R. Wagstrom, C. M. Gates, S. C. Singer, A. M. Davis, R. L. Tansik, L. C. Mattheakis, C. M. Boytos, P. J. Schatz, D. P. Baccanari, N. C. Wrighton, R. W. Barrett and W. J. Dower, *Science*, 1997, **276**, 1696–1699.
6 O. Vadas, O. Hartley and K. Rose, *Biopolymers*, 2008, **90**, 496–502.
7 Z. Fan, Y. Lu, X. Wu and J. Mendelsohn, *J. Biol. Chem.*, 1994, **269**, 27595–27602.
8 Z. Hayouka, J. Rosenbluh, A. Levin, S. Loya, M. Lebendiker, D. Veprintsev, M. Kotler, A. Hizi, A. Loyter and A. Friedler, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 8316–8321.
9 H. J. Wester and H. Kessler, *J. Nucl. Med.*, 2005, **46**, 1940–1945.
10 Y. Singh, G. T. Dolphin, J. Razkin and P. Dumy, *ChemBioChem*, 2006, **7**, 1298–1314.
11 M. Mutter, R. Hersperger, K. Gubernator and K. Muller, *Proteins*, 1989, **5**, 13–21.
12 G. Tuchscherer, C. Servis, G. Corradin, U. Blum, J. Rivier and M. Mutter, *Protein Sci.*, 1992, **1**, 1377–1386.
13 R. Floegel and M. Mutter, *Biopolymers*, 1992, **32**, 1283–1310.
14 K. H. Altmann and M. Mutter, *Int. J. Biochem.*, 1990, **22**, 947–956.
15 G. Tuchscherer, V. Steiner, K. H. Altmann and M. Mutter, *Methods Mol. Biol.*, 1994, **36**, 261–285.
16 G. Tuchscherer and M. Mutter, *J. Biotechnol.*, 1995, **41**, 197–210.
17 G. Tuchscherer and M. Mutter, *J. Pept. Sci.*, 1995, **1**, 3–10.
18 M. Mutter and G. Tuchscherer, *Cell Mol. Life Sci.*, 1997, **53**, 851–863.
19 G. Tuchscherer, D. Grell, M. Mathieu and M. Mutter, *J. Pept. Res.*, 1999, **54**, 185–194.
20 D. Grell, J. S. Richardson and M. Mutter, *J. Pept. Sci.*, 2001, **7**, 146–151.
21 S. Peluso, T. Ruckle, C. Lehmann, M. Mutter, C. Peggion and M. Crisma, *ChemBioChem*, 2001, **2**, 432–437.
22 Z. H. Peng, *Biopolymers*, 1999, **49**, 565–574.
23 P. Dumy, I. M. Eggleston, G. Esposito, S. Nicula and M. Mutter, *Biopolymers*, 1996, **39**, 297–308.
24 A. S. Causton and J. C. Sherman, *J. Pept. Sci.*, 2002, **8**, 275–282.
25 H. K. Rau, N. DeJonge and W. Haehnel, *Angew. Chem., Int. Ed.*, 2000, **39**, 250–253.
26 R. Schnepf, P. Horth, E. Bill, K. Wieghardt, P. Hildebrandt and W. Haehnel, *J. Am. Chem. Soc.*, 2001, **123**, 2186–2195.
27 J. Hauert, J. Fernandez-Carneado, O. Michielin, S. Mathieu, D. Grell, M. Schapira, O. Spertini, M. Mutter, G. Tuchscherer and T. Kovacsovics, *ChemBioChem*, 2004, **5**, 856–864.
28 M. P. Glenn, L. K. Pattenden, R. C. Reid, D. P. Tyssen, J. D. Tyndall, C. J. Birch and D. P. Fairlie, *J. Med. Chem.*, 2002, **45**, 371–381.
29 D. Boturyn, J. L. Coll, E. Garanger, M. C. Favrot and P. Dumy, *J. Am. Chem. Soc.*, 2004, **126**, 5730–5739.
30 D. Boturyn, E. Defrancq, G. T. Dolphin, J. Garcia, P. Labbe, O. Renaudet and P. Dumy, *J. Pept. Sci.*, 2008, **14**, 224–240.
31 J. Wang, H. Li, G. Zou and L. X. Wang, *Org. Biomol. Chem.*, 2007, **5**, 1529–1540.
32 R. Hirschmann, K. C. Nicolau, S. Pietranico, E. M. Leahy, J. Salvino, B. Arison, M. A. Cichy, P. G. Spoors, W. C. Shakespeare, P. A. Sprengler, P. Hamley, A. B. Smith, T. Reisine, K. Raynor, L. Maechler, C. Donaldson, W. Vale, R. M. Freidinger, M. R. Cascieri and C. D. Strader, *J. Am. Chem. Soc.*, 1993, **115**, 12550–12568.
33 R. Boyce, G. Li, H. P. Nestler, T. Suenaga and W. C. Still, *J. Am. Chem. Soc.*, 1994, **116**, 7955–7956.
34 T. Sasaki and E. T. Kaiser, *J. Am. Chem. Soc.*, 1989, **111**, 380–381.
35 M. Lieberman and T. Sasaki, *J. Am. Chem. Soc.*, 1991, **113**, 1470–1471.
36 C. T. Choma, J. D. Lear, M. J. Nelson, P. L. Dutton, D. E. Robertson and W. F. DeGrado, *J. Am. Chem. Soc.*, 1994, **116**, 856–865.
37 M. R. Ghadiri, C. Soares and C. Choi, *J. Am. Chem. Soc.*, 1992, **114**, 825–831.
38 R. Hirschmann, P. A. Sprengeler, T. Kawasaki, J. W. Leahy, W. C. Shakespeare and A. B. Smith, *Tetrahedron*, 1993, **49**, 3665–3676.
39 M. Kahn, *Synlett*, 1993, 821–826.
40 J. Eichler, A. W. Lucka and R. A. Houghten, *Pept. Res.*, 1994, **7**, 300–307.
41 J. M. Ostresh, G. M. Husar, S. E. Blondelle, B. Dorner, P. A. Weber and R. A. Houghten, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 11138–11142.
42 M. Mutter and S. Villeumier, *Angew. Chem., Int. Ed. Engl.*, 1989, **28**, 535–554.
43 H. K. Rau and W. Haehnel, *J. Am. Chem. Soc.*, 1998, **120**, 468–476.
44 B. H. Monien, F. Drepper, M. Sommerhalter, W. Lubitz and W. Haehnel, *J. Mol. Biol.*, 2007, **371**, 739–753.
45 S. C. Story and J. V. Aldrich, *Int. J. Pept. Protein Res.*, 1994, **43**, 292–296.
46 C. W. Tornoe, C. Christensen and M. Meldal, *J. Org. Chem.*, 2002, **67**, 3057–3064.
47 V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 2596–2599.

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48 D. T. Rijkers, G. W. van Esse, R. Merkx, A. J. Brouwer, H. J. Jacobs, R. J. Pieters and R. M. Liskamp, *Chem. Commun.*, 2005, 4581–4583.
49 B. Jagadish, R. Sankaranarayanan, L. Xu, R. Richards, J. Vagner, V. J. Hruby, R. J. Gillies and E. A. Mash, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 3310–3313.
50 N. Khoukhi, M. Vauitier and R. Carrié, *Tetrahedron*, 1987, **43**, 1811–1822.
51 K. L. Kiick, E. Saxon, D. A. Tirrell and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 19–24.
52 S. F. van Dongen, R. L. Teeuwen, M. Nallani, S. S. van Berkel, J. J. Cornelissen, R. J. Nolte and J. C. van Hest, *Bioconjugate Chem.*, 2009, **20**, 20–23.
53 H. Kolmar, *FEBS J.*, 2008, **275**, 2684–2690.
54 A. Abuchowski, J. R. McCoy, N. C. Palczuk, T. van Es and F. F. Davis, *J. Biol. Chem.*, 1977, **252**, 3582–3586.
55 S. Reiss, M. Sieber, V. Oberle, A. Wentzel, P. Spangenberg, R. Claus, H. Kolmar and W. Losche, *Platelets*, 2006, **17**, 153–157.