A macrocyclic coumarin-containing tripeptide *via* CuAAC chemistry†

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A Cu-catalysed macrocyclisation was performed to obtain a macrocyclic coumarin-containing tripeptide for use in thrombin activity measurements.

Thrombosis is the basis of diseases such as cerebral and coronary infarction and pulmonary embolism and thus can be considered the major cause of death in the western world. A method for measuring the effect of newly developed thrombin inhibitors on the haemostatic system is the so-called thrombin generation test (TGT) devised by Hemker *et al.*[1] Upon selective hydrolysis of a fluorogenic peptide by developing thrombin, a fluorophore is released thereby providing a direct measure for the thrombin activity. Substrates used in the thrombin generation test are generally linear and flexible tripeptides bearing a *C*-terminal fluorophore (*e.g.* 7-amino-4-methylcoumarin, AMC).[2] An example of such a peptide is H-Gly-Pro-Arg-AMC (**1**, Fig. 1). This peptide is, however, hydrolysed extremely rapidly by thrombin (FIIa),[2] while a slow-reacting substrate is required for accurate and prolonged measurements. Moreover, the released AMC has a moderate solubility in aqueous solutions and thus interferes with the accurate assessment of enzyme activities as a result of fluorescent quenching.[3] In addition, AMC is able to act as a competitive inhibitor of thrombin at high concentrations.[4]

We envisaged a fluorophore-containing cyclic structure (*i.e.* **2**, Fig. 1) as a compelling strategy to (a) overcome the poor solubility of AMC in physiological media and (b) give rise to improved kinetic parameters. Constraining the linear peptide H-Gly-Pro-Arg-AMC in a bulky macrocyclic conformation is expected to hamper the entry into the thrombin active site, thus decreasing the affinity of the substrate for the binding pocket of thrombin resulting in favourable kinetic parameters (*i.e.* high K_M and low k_{cat}) suitable for the TGT. Moreover, precipitation and undesired competitive inhibition of AMC upon thrombin hydrolysis will not occur as a result of the triazole linkage of AMC to the tripeptide backbone.

The main difficulty in the synthesis of cyclic peptides is the macrocyclisation.[5] For penta- and especially tetrapeptides the cyclisation of all L-configured linear precursors is notoriously difficult. Various methods for macrocyclisation, such as lactonisation or lactamisation,[6] ring-closing metathesis[7] or cyclisation using auxiliaries[8] have been reported. With the introduction of the copper-catalysed azide–alkyne cycloaddition (CuAAC) reaction, a versatile and mild coupling method was established which rapidly found application in various fields of chemistry.[9] In addition, the CuAAC reaction had a significant impact on the synthesis of new peptidomimetics.[10] Studies have shown that a 1,2,3-triazole can be a suitable mimic for an amide bond in peptides,[11] moreover, the obtained 1,4-connected 1,2,3-triazole ring can serve as a β-turn mimic.[12] Recently, the CuAAC has also been applied as a tool for macrocyclisation both in solution[13] and on solid phase.[14] We therefore envisioned that the CuAAC could serve as an efficient cyclisation method for the synthesis of macrocycles **2a/b**.

Retrosynthetic analysis of *cyclo*-[-Gly-Pro-Arg-AMC-[triazole]-spacer-] analogues **2** leads to the linear peptides **3** (Scheme 1), containing the requisite alkyne and azide functionalities for the proposed CuAAC macrocyclisation. Optimal convergence of the route is obtained by splitting the linear peptide into an azidocoumarin-functionalised arginine residue **4** and an alkyne-containing Gly-Pro peptide **5**. The alkyne tail can be coupled to the Gly-Pro peptide unit either *via* an amide or carbamate type linkage (Scheme 2). The arginine residue is decorated with 7-amino-4-azidomethylcoumarin **13**, which in turn was thought to be derived from *N*-acetyl-3-aminophenol.

Synthesis of synthon **5a** commenced with coupling of 3-butyn-1-yl chloroformate to unprotected glycine (Scheme 2), which after acid–base extraction was obtained in excellent yield (95%). Next, coupling of L-proline methyl ester to **6a** was performed under standard peptide coupling conditions, to afford compound **8** in a reasonable yield of 64%. Finally, ester hydrolysis gave the desired synthon **5a** in good yield (70%). For the synthesis of synthon **5b** (Scheme 2), peptide coupling of glycine methyl ester with hexynoic acid was followed by hydrolysis of methyl ester **7**, resulting in compound **6b** in good yield. A second peptide coupling was performed to link proline *tert*-butyl ester to fragment **6b**, affording smooth formation of dipeptide **9** (97% yield). The

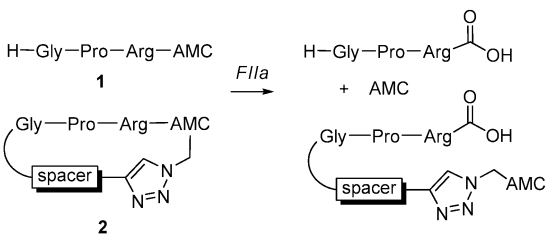

Fig. 1 Thrombin (FIIa) hydrolysis of linear (**1**) and cyclic (**2**) peptide.

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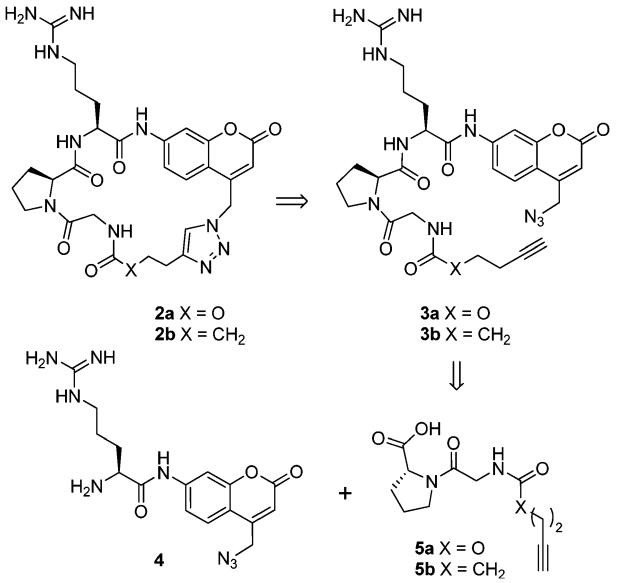
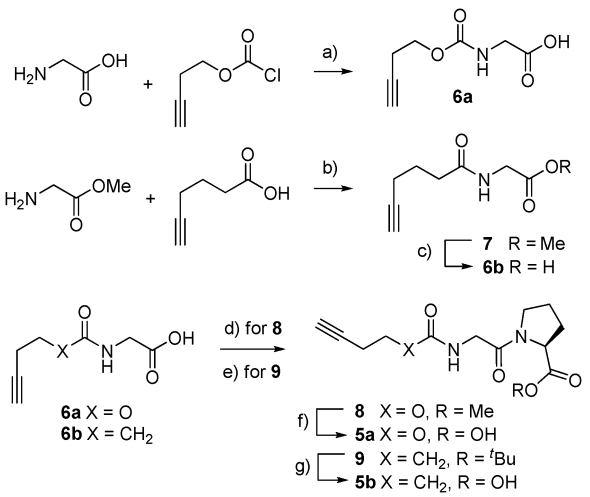
† Electronic supplementary information (ESI) available: Synthetic procedure for compounds **1–16**, click conditions, biological evaluation. See DOI: 10.1039/b906762k



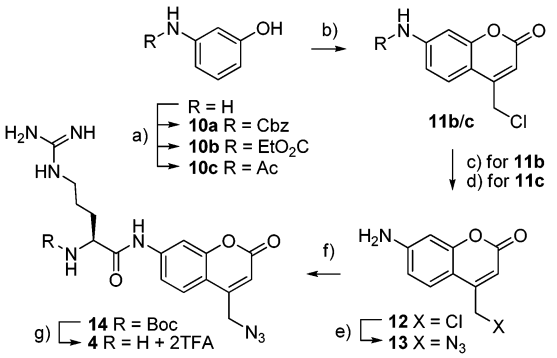
Scheme 1 Retrosynthesis of macrocycles **2**.



Scheme 2 *Reagents and conditions*: (a) NaOH (aq) pH 9.5–10.5, r.t., 72 h (95%); (b) hexynoic acid, EDC, DMAP, DMF, 0 °C to r.t., 18 h (97%); (c) NaOH (2M), dioxane, r.t., 16 h (70%); (d) H-Pro-OMe, DCC, NMM, EtOAc, 0 °C to r.t., 16 h (64%); (e) H-Pro-O^{t}Bu, EDC, HOBt, CH_2Cl_2, 0 °C to r.t., 18 h (97%); (f) NaOH (2M), dioxane, r.t. 18 h (77%); (g) TFA, CH_2Cl_2, r.t., 18 h (99%).

last step involved acid-catalysed hydrolysis of the *tert*-butyl ester to afford target compound **5b** in excellent yield (99%).

The fluorophore 7-amino-4-azidomethylcoumarin (AAMC, **13**), required for the synthesis of H-Arg-AAMC (**4**), is not commercially available so that a synthetic route was devised. Starting from 3-aminophenol both Lewis acid-catalysed and microwave-assisted Pechmann condensations were explored in order to obtain aminochloromethylcoumarin **12** (ACMC). A protective group was required since direct Pechmann condensation of 3-aminophenol under a variety of conditions turned out to be fruitless. Therefore, the amine functionality of 3-aminophenol was protected with different protecting groups in reasonable to good yields (Scheme 3). The *N*-protected aminophenols **10a–c** were subsequently subjected to the Pechmann condensation, typically applying a 60–70%

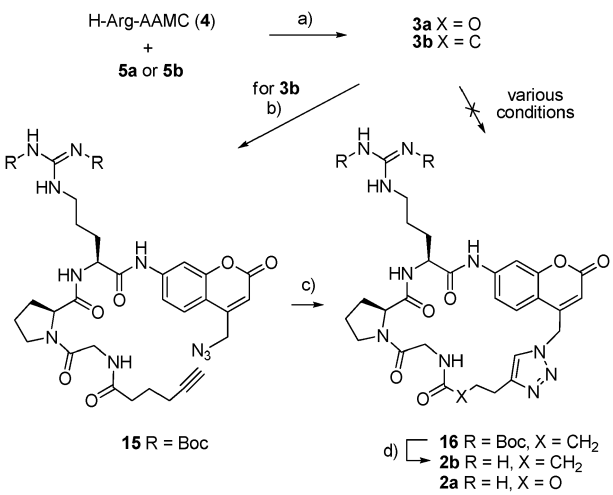


Scheme 3 *Reagents and conditions*: (a) Et_2O, Et_3N, r.t., 18 h, for **10a**: Cbz–Cl (51%), for **10b**: Ac_2O (99%) and for **10c**: EtO_2C–Cl (54%); (b) typically: 60–70% H_2SO_4, r.t. to 45 °C, 2–5 d (starting form **10a**: 0%, for **11b**: 20% and for **11c**: 70%); (c) various conditions (0%); (d) conc. HCl, 2-propanol, reflux, 16 h (72%); (e) NaN_3 (5 equiv.), MeCN–acetone, r.t., 48 h (95%); (f) Boc-Arg-OH, $POCl_3$, pyridine, −15 °C to r.t., 1 h (41%); (g) TFA, CH_2Cl_2, r.t. 16 h (86%).

solution of H_2SO_4. Cbz-protected aminophenol (**10a**) did not give the desired product under these conditions.

Conversely, *N*-ethoxycarbonyl-protected aminophenol **10b** produced coumarin **11b** in a satisfactory 70% yield, while acetyl-protected aminophenol **10c** gave only 20% of coumarin **11c**. Deprotection of **11b** applying various reaction conditions afforded mostly mixtures of unidentifiable compounds. Formation of ACMC **12** was then pursued by deprotection of coumarin **11c** with HCl in 2-propanol to produce **12** in good yield (72%). Finally, nucleophilic displacement of the chloride by an azide using NaN_3 in acetone–acetonitrile produced key intermediate **13** in excellent yield (95%).

Next, the fluorescent probe AAMC (**13**) was coupled to Boc-protected arginine using phosphoryl chloride in pyridine at −15 °C leading to Boc-Arg-AAMC (**14**). Purification of the product by counter-current chromatography provided Boc-Arg-AAMC (**14**) in a reasonable yield of 41%. Subsequent Boc-removal with TFA afforded synthon **4**.



Scheme 4 *Reagents and conditions*: (a) EDC, HOBt, DMAP, DMF, 0 °C, r.t., 18 h (**3a** (24%), **3b** (54%)); (b) Boc_2O (4 eq), THF, Et_3N, r.t., 16 h (50%); (c) CuBr/TMEDA, THF, 40 °C, 7 h (23%); (d) HCl in EtOAc, r.t., 16 h (99%).

Having key intermediates **4** and **5a/b** in hand, coupling of these fragments was required to obtain linear peptides **3a** and **3b** (Scheme 4). To this end, amide formation was performed utilizing EDC, HOBt and DMAP. Coupling of **5a** to **4** gave the linear carbamate **3a** in moderate yield (24%). Fortunately, coupling of **5b** with **4** proceeded significantly better so that linear amide **3b** was produced in a yield of 54% after purification by counter-current chromatography.

Copper-catalysed macrocyclisation of **3a** or **3b** was pursued applying modified literature procedures. Since we anticipated that elevated temperatures could well result in decomposition of compounds **3a** and **3b**, our attention was focused on intramolecular azide–alkyne cycloaddition at relatively low temperatures[15] and under high dilution (<0.001 M). Unfortunately, the different conditions applied on either **3a** or **3b** (see ESI†) failed to produce sufficient quantities of the desired macrocycle for adequate purification and identification. Since Cu strongly coordinates to the basic guanidine moiety, and thus hampers purification and possibly also cyclisation, compound **3b** was Boc-protected at the guanidine prior to macrocyclisation (Scheme 4). Addition of four equivalents of di-*tert*-butyl dicarbonate resulted in the formation of three products (*i.e.* mono-, di- and trisubstituted guanidine), of which the bis-Boc-protected compound **15** appeared to be the major product (50%).

Gratifyingly, subjecting **15** to Cu-catalysed ring-closing conditions (*i.e.* CuBr/TMEDA, THF, 40 °C, 7 h) resulted in the formation of the macrocyclic fluorophore-containing tripeptide **16** in an encouraging 23% yield.

Next, the kinetic parameters of both the linear and cyclic peptide were determined by constructing Michaelis–Menten and Lineweaver–Burk plots from which the binding constant (K_M) and the hydrolysis rate (k_{cat}) were determined (see ESI†). Comparing the binding affinity of the linear (**1**) and cyclic peptide (**2b**) showed a significant reduction (K_M = 746.9 μM and 3693.9 μM, respectively). Calculations of k_{cat} showed only a slight decrease for the cyclic peptide **2b** as compared to the linear peptide **1** (k_{cat} = 23.3 s^{-1} and 29.3 s^{-1}, respectively), but nevertheless remained high. Visual analysis of the turbidity of the samples after hydrolysis by thrombin, as a measure for the sedimentation of AMC, showed a clear difference between the cyclic and linear peptide. While the samples containing high concentrations of linear peptide **1** (>800 μM) clearly became turbid, no turbidity at these concentrations was observed for cyclic peptide **2b**, thus indicating an improved solubility of AMC as a result of its linkage to the hydrophilic peptide backbone.

In conclusion, we have successfully demonstrated that Cu-catalysed macrocyclisation of an azide- and alkyne-containing linear peptide resulted in a cyclic fluorogenic tripeptide. Biological evaluation of both the linear and cyclic peptide demonstrated that the latter had improved kinetic properties. The macrocyclic (bulky) shape of the cyclic peptide indeed resulted in a five-fold increase of the binding constant (K_M) as compared to the linear peptide, but the hydrolysis rate (k_{cat}) remained high. It clearly shows, however, the feasibility of this unprecedented cyclisation strategy to improve the kinetic properties of the fluorescent substrate. Further improvement of the kinetic parameters may be achieved by changing the Pro-Gly sequence and/or the alkyl spacer-length of the cyclic peptide. Moreover, other challenging strategies to construct the cyclic peptide involve coupling of the azide and alkyne fragments *via* CuAAC, and/or by invoking strain-promoted (Cu-free) 1,3-dipolar cycloaddition approaches, followed by an intramolecular lactamization reaction. These are all strategies currently under investigation in our laboratories.

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