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Polar Hinges as Functionalized Conformational Constraints in (Bi)cyclic Peptides

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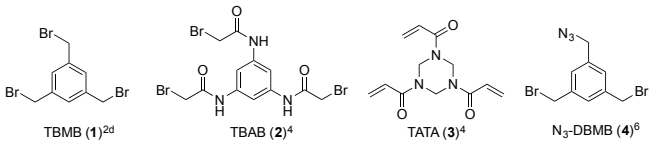
Abstract: Two polar hinges have been developed for cyclization of peptides leading to bicyclic peptides and cyclized peptides with improved solubility and biological activity. Increasingly, we note that a good aqueous solubility of peptides is an absolute prerequisite not only to be able to handle and purify our target peptides but it is also crucial for biological activity characteristics. Compared to earlier hinges, the structurally similar but chemically very different TATB and TBMT each containing three nitrogen atoms were accessible in one step from bromo-acetonitrile. TATB and TBMT are very suitable for the preparation of better soluble bicyclic peptides. Azide modified TATB and TBMT derivatives provide hinges for preparation of cyclized peptides for incorporation on scaffolds to afford protein mimics.

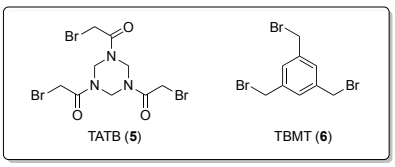
Introduction

Protein-protein interactions (PPI's) play essential roles on virtually all levels of cell function. As a result modulation or inhibition of PPI's is increasingly important in drug discovery. Despite the progress that has been made in the field of modulation of PPI's, the development of molecules that can interfere with protein-protein interactions remains difficult. Since contact surfaces involved in PPI's are normally large and flat, it is a serious challenge for the traditional "small-molecules" to modulate protein-protein interactions. Therefore, we wish to devise (cyclic) peptides and peptidomimetics as PPI inhibitors. These compounds are relatively large in size and thus are capable to make multiple contacts with the target surfaces, which is needed to obtain efficient PPI modulators. Moreover, by constructing *cyclic* macrocycles, instead of their linear analogues, conformationally constrained and more pre-organized structures will be obtained. This should lead to smaller losses of entropy upon binding and typically better binding affinities. In addition, cyclization of peptides and introduction of peptidomimetic moieties will improve the enzymatic stability and therefore biological availability of these macrocyclic compounds, which is crucial in applications of these biomolecular constructs.[1]

To further improve the rigidity, metabolic stability and binding specificity/affinity of cyclic peptidic PPI modulaters, *bicyclic* peptides were introduced.[2] Since it is difficult to rationally design macrobicyclic modulators of PPI's, a highly attractive approach involves the generation and screening of large libraries using the bicyclic phage display strategy developed by Heinis and Winter.[2d] In this approach, bicyclic-peptide libraries were generated by cyclizing tri-cysteine containing peptides displayed on a phage with a triple sulfhydryl-reactive hinge. By using iterative affinity selections, bicyclic peptides were found with binding affinities in the nanomolar or even picomolar range.[2d,3] In addition, these PPI inhibitors were tremendously selective towards the target protein.

The nature of the chemical hinge used in the bicyclization of tri-cysteine containing peptides was highly important for determining the actual conformation(s), and thus binding affinity of the generated bicyclic peptides. Recently, Chen *et al.* showed that when the original hinge, tris(-bromomethyl)benzene (TBMB, **1**), in a bicyclic peptide inhibitor of plasma kallikrein (PK15, K_i= 2 nM) was replaced by *N,N',N''*-(benzene-1,3,5-triyl)tris(2-bromoacetamide) (TBAB, **2**) or by 1,3,5-triacryloyl-1,3,5-triazinane (TATA, **3**) different conformations of the peptide backbone have been observed by NMR (Scheme 1).[4a] More importantly, by doing so the activity dropped by a factor of more than 1000 to around 4 µM, indicating that these relatively small hinges are capable of significantly influencing the conformation of bicyclic peptides. In a following-up study, it has been demonstrated that more hydrophilic hinge molecules in bicyclic peptides can promote non-covalent interactions and thus introduce additional conformational constraints, possibly leading to higher binding affinities.[5] Moreover, the more polar character may be beneficial for solubility. Therefore, polar hinges might have great advantages over the more hydrophobic counterparts in the construction of bicyclic PPI modulators.





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Scheme 1. Previously used hinges for bicyclization (**1**-**3**) and discontinuous epitope preparation (**4**), and the new hinges presented in this work (**5**, **6**).

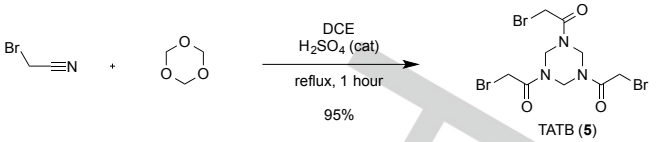
In the past we used a derivative of benzylic tribromide hinge **1** to tackle the complicated PPI's involved in discontinuous epitopes e.g. in antibody-antigen interactions.[6] By chemically creating cyclic peptides corresponding to the amino acid sequence of the epitopes using azido di(-bromomethyl)benzene **4** (N_3-DBMB) and attaching these to a molecular scaffold PPI modulators of the HIV-gp120 - CD4 interaction were obtained.[6b] Although cyclization of the peptides with the benzylic dibromide hinge **4** was very efficient and clean, solubility problems with the resulting cyclic peptides were encountered, probably caused by the used apolar hinge. These solubility problems might be remedied by the development and employment of more functionalized, polar hinges.

Here we describe the synthesis of two new small polar hinges, 1,1',1"-(1,3,5-triazinane-1,3,5-triyl)tris(2-bromoethanone) (TATB, **5**) and 2,4,6-tris(bromomethyl)-s-triazine (TBMT, **6**) (Schemes 1 and 2), which both contain three sulfhydryl-reactive groups, together with their application in peptide bicyclization as well as their use in the preparation of cyclic peptides necessary for the construction of discontinuous epitopes.[6,7]

Results and Discussion

Design and synthesis of the hinges

Two different small polar synthetic hinges, each containing three sulfhydryl-reactive groups, were designed and synthesized. Both hinges have a threefold rotational symmetry to ensure the formation of single isomers after peptide cyclization or azide displacement. The first hinge, TATB (**5**), is based on the same triazinane core as TATA (**3**).[4] It contains three electrophilic bromoacetyl groups, which are located in the 1-, 3-, and 5-positions of the triazinane. This makes each connection formed between peptide and hinge, after (bi)cyclization, one methylene unit shorter as compared to using the TATA hinge. The triazinane core adopts a chair-like conformation with the three amide groups pointing out to the same side of the ring to create a bowl-like shape.[4b] The TATB hinge (**5**) was efficiently and quickly synthesized by treatment of bromoacetonitrile with trioxane by acid catalysis in an excellent yield of 95% (Scheme 2). In addition, since only a filtration step was needed to yield pure TATB hinge **5**, this reaction could be performed on a multi-gram scale. By recrystallization from chloroform, suitable crystals for X-ray structural analysis were obtained, indeed showing a chair-like conformation with the three bromoacetyl groups pointing from the same side of TATB forming the bowl (**5**, Figure 1).



Scheme 2. Synthesis of the TATB (**5**) hinge.

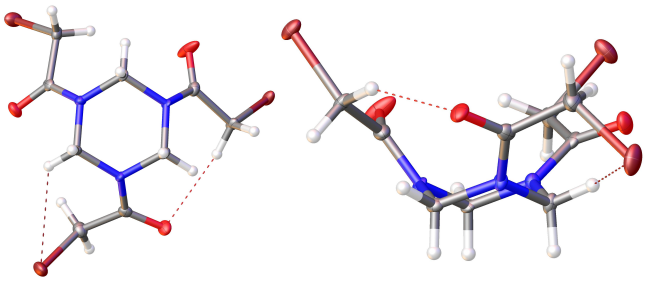
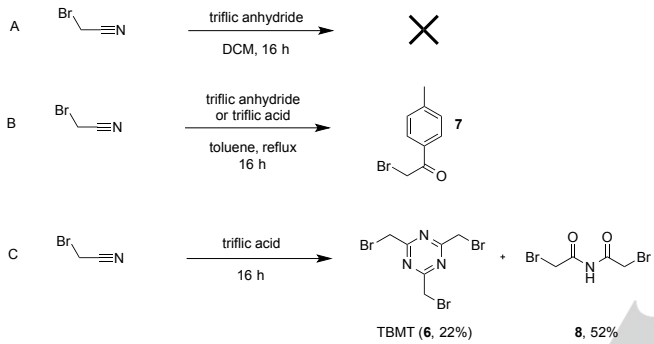


Figure 1. X-ray structure showing the bowl shape of the TATB (**5**) hinge. Displacement ellipsoids are drawn at 50% probability level.

The second designed hinge was the TBMT (**6**) molecule, which contains a sym-triazine core. This hinge has not been described previously and resembles the often-used TBMB hinge **1** (Scheme 1). The important difference is that the new core contains nitrogen atoms in the 1-, 3- and 5-positions of the aromatic ring. It was expected that this will significantly increase the polarity of the hinge, as was evident from the predicted cLogP values of 1.09 and 4.49 for TBMT (**6**) and TBMB (**1**), respectively.[8] In addition, the triazine core is susceptible to hydrogen bonding by hydrogen bond donating groups, which may lead to further conformational stabilization of the to be formed (bi)cyclic peptides. Synthesis of TBMT hinge **6** was attempted by following an in principle practical and easy protocol for obtaining 2,4,6-trisubstituted-s-triazines published by Herrera *et al.* in 2004.[9] This method, which involved treatment of bromoacetonitrile with triflic anhydride in DCM did not yield any product (Scheme 3a) in our hands. Next, a more recent procedure by the same authors was attempted for synthesis of 2,4-disubstituted-6-substituted 1,3,5-triazines, involving treatment of bromoacetonitrile with triflic anhydride or triflic acid in refluxing toluene.[10] Surprisingly, in both cases the only isolated product was 2-bromo-4'-methylacetophenone (**7**), resulting from electrophilic aromatic substitution of the solvent (Scheme 3b). Next, a much older protocol by Amer *et al.* was followed in which nitriles were treated with neat triflic acid.[11] This method required quenching of triflic acid, by slow addition of the reaction mixture to a stirred biphasic solution of DCM and aqueous sodium bicarbonate. Fortunately, these conditions led to s-triazine **6**, albeit in a low yield of 22% (Scheme 3c). X-ray crystallography confirmed the correct structure of TBMT hinge **6** (Figure 2). Since after work-up a significant amount of a white precipitate was formed, which was not TBMT (**6**), it was decided to characterise this solid in more detail to obtain more insights into the low yield of the TBMT–hinge. Although NMR analysis showed that the obtained solid closely resembles s-triazine **6**, it

was still difficult to derive a structure based on this information because of the limited number of NMR signals. Since MS and elemental analysis were also not conclusive, an X-ray crystal structure was obtained. This data showed that the obtained major product (52%) was 2-bromo-*N*-(2-bromoacetyl)acetamide (**8**, Scheme 3c, Figure 2). Formation of this product can be explained by assuming hydrolysis of the dimeric intermediate, which has to react to furnish a trimeric intermediate leading to TBMT. However, attempts to remove any residual water causing this hydrolysis from triflic acid by drying over molecular sieves or stirring with triflic anhydride, were unsuccessful in order to increase the yield of TBMT. Nevertheless, despite its low yield, TBMT can be obtained relatively straightforward in this manner.



Scheme 3. Attempted and successful synthesis of the TBMT (**6**) hinge.

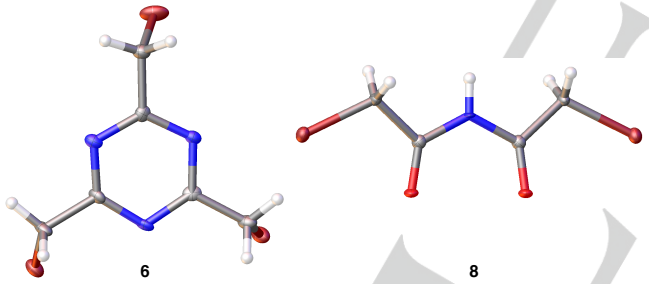
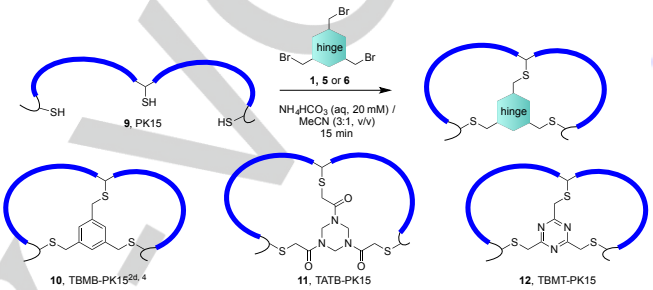


Figure 2. X-ray structure of the TBMT (**6**) hinge and hydrolysed dimer **8**. Displacement ellipsoids are drawn at 50% probability level.

Application of the hinges in peptide bicyclization

Bicyclization using the new hinges **5** and **6** was first tested on a 17-amino acid peptide containing three cysteine residues (**9**, peptide PK15: H-ACSDRFRNCPADEALCG-NH_2: cysteine residues are underlined, free amino terminus, C-terminal amide). The bicyclic version of this peptide TBMB-PK15 (**10**) was previously identified by screening random peptides cyclized with the TBMB hinge (**1**) using phage display. TBMB-PK15 (**10**) inhibits the serine protease plasma kallikrein with a K_i in the low nanomolar range.[2d,4] Both hinges **5** and **6** reacted fast, smoothly and clean with PK15 peptide (**9**). Even with only one equivalent of hinge at 1 mM concentration in an aqueous buffer (pH 7.9) containing acetonitrile (25%, v/v), complete conversion to the bicyclic peptides was observed within 15 minutes. Since these bicyclization reactions were found to be remarkably clean, it was decided to use the crude peptide in these reactions. In this way the reference bicyclic peptide TBMB-PK15 (**10**), TATB-PK15 (**11**) and TBMT-PK15 (**12**) were obtained by performing only one preparative HPLC purification (per bicyclic peptide) in an overall yield of 16%, 21% and 12%, respectively. This corresponded to an excellent average yield per step of 95% for bicyclic peptide **10**, 96% for bicyclic peptide **11** and 94% for bicyclic peptide **11** (Scheme 4).



Scheme 4. Bicyclization of PK15 peptide **9** (ACSDRFRNCPADEALCG) using hinges **1**, **5** and **6**, which yielded the corresponding bicyclic peptides **10**-**12**.

Influence of polar linkers on the biological activity of bicyclic peptides was studied in two examples: (1) bicycles capable of inhibition of human plasma kallikrein and (2) bicycles capable of inhibition of FXII.

Bicycles TATB-PK15 (**11**) and TBMT-PK15 (**12**) inhibited kallikrein with K_is of 540 ± 40 nM and 20 ± 9 nM, respectively, and thus with more than 100- and 10-fold higher K_is than TBMB-PK15 (**10**, 1.3 ± 0.4 nM) (Figure 3). This result was in line with previous findings showing that substitution of the original TBMB-hinge in phage-selected bicyclic peptides imposes different conformations onto the peptides' backbones and strongly affects their binding affinities. It is worth to note that even the hinge TBMT (**6**), which is structurally very similar to the hinge TBMB (**1**), is affecting significantly the binding affinity of the plasma kallikrein inhibitor.

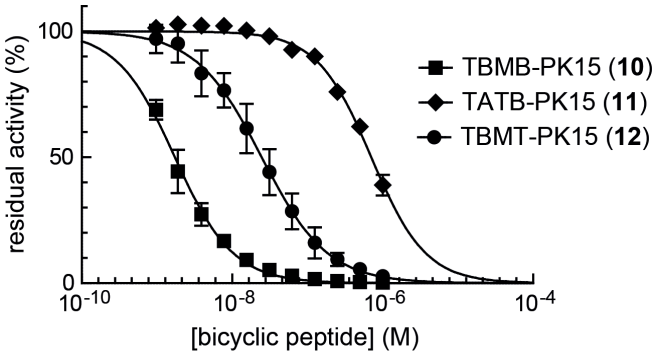
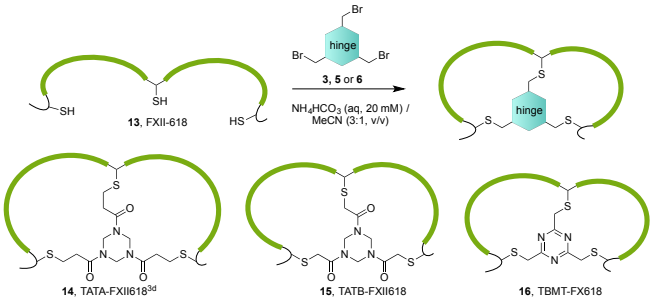


Figure 3. Inhibition of plasma kallikrein by TATB-PK15 (**11**) and TBMT-PK15 (**12**), compared to the original TBMB-PK15 (**10**) inhibitor. Residual protease activity was measured with a fluorogenic substrate in triplicate. Average values and standard deviations are indicated.

Another bicyclic peptide developed by phage display is the coagulation factor FXII (FXII) inhibitor **14** which was isolated from a random peptide library bicyclized with the previously reported polar hinge TATA (**3**)[3d] (K_i : 25 ± 6 nM). The peptide of this inhibitor could efficiently be cyclized with the new hinges **5** and **6** (Scheme 5).



Scheme 5. Bicyclization of FXII618 peptide **13** (RCFRLPCRQLRCR) using hinges **3**, **5** and **6**, which yielded the corresponding bicyclic peptides **14**-**16**.

The inhibitory activity of the bicyclic peptides **14** - **16** is shown in Figure 4. TBMT hinge containing bicyclic peptide **16** was the least active (K_i : 1220 ± 90 nM), indicative of a not allowed aromatic character of the hinge. Although the polarity of the TATB hinge (**5**) is similar to the TATA hinge (**3**) the resulting bicyclic peptide **15** was considerably less active (K_i : 340 ± 30 nM), showing the importance of the additional flexibility caused by an extra methylene unit in each of the arms of the TATA hinge.

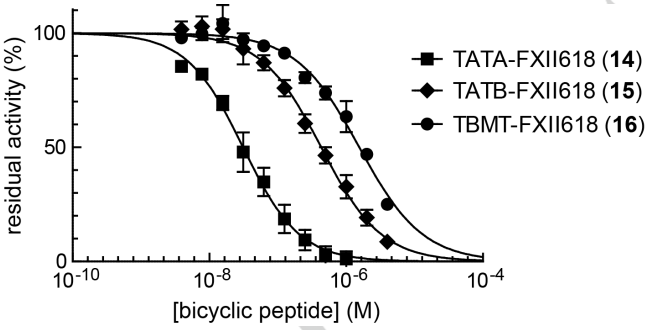
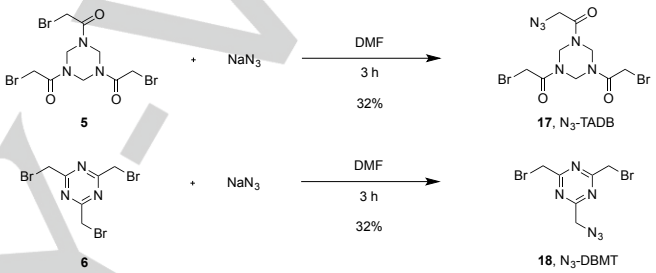


Figure 4. Inhibition of FXII by TATB-FXII618 (**15**) and TBMT-FXII618 (**16**), compared to the original TATA-FXII618 (**14**) inhibitor. Residual protease activity was measured with a fluorogenic substrate in triplicate. Average values and standard deviations are indicated.

The finding that cyclization of peptides with TBMB (**1**), TATB (**5**) and TBMT (**6**) yields macrocyclic structures with different backbone conformations makes them valuable reagents for the generation of large combinatorial libraries of bicyclic peptides. The hinges may be applied in parallel in separate reactions for the cyclization of phage-encoded peptide libraries.

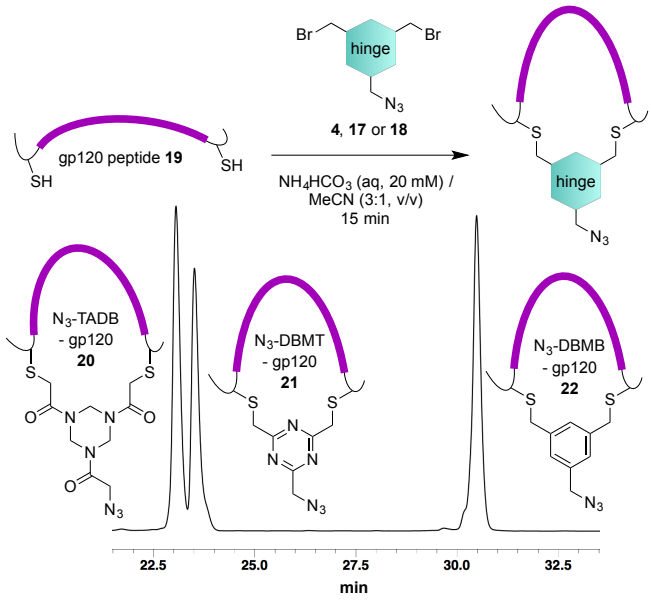
Application of the hinges in discontinuous epitope mimicry

To apply these novel hinges as hinges for the preparation of discontinuous epitope mimics, they first had to be outfitted with an azide handle. By simply performing a substitution reaction with sodium azide on hinges **5** and **6**, azido triazinanetris(2-bromoethanone) (**17**, N_3-TADB) and azido di(bromomethyl)-s-triazine (**18**, N_3-DBMT) were obtained in acceptable yields of 32%, each (Scheme 6), considering the expected sideproducts (di- and tri-substitution) in these reactions.



Scheme 6. Synthesis of azido hinges **17** and **18**.

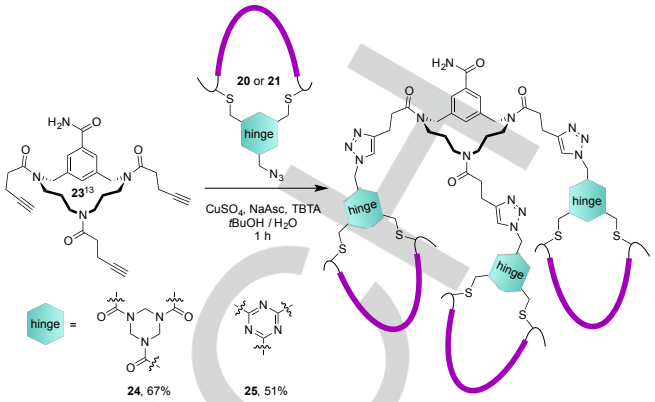
Next, these azide-containing hinges (**17** and **18**) were used in the cyclization of a C- and N-terminal cysteine containing peptide. It was decided to use a peptide sequence, corresponding to one of the three loops present in the HIV-gp120 discontinuous epitope interaction with CD4 (**19**, CLTRDGGKC, cysteine residues are underlined), which in the past gave considerable solubility problems when cyclized with the original aromatic dibromide hinge **4**, leading to N_3-DBMB-gp120 (**22**).[6b] Both hinges **17** and **18** reacted clean and fast with the crude linear peptide to form the cyclized peptides N_3-TADB-gp120 (**20**) and N_3-DBMT-gp120 (**21**) in good overall yields of 26% for cyclic peptide **20** and 28% for cyclic peptide **21** (Scheme 7).



Scheme 7. Cyclization of gp120 peptide **19** using azide containing hinges **17** and **18** as well as the earlier prepared hinge **4**. HPLC-trace (λ = 214 nm, 5 to 50% acetonitrile gradient in a water buffer in 48 minutes) of the cyclized peptides showing shorter retention times of **20** and **21** compared to **22** due to increased polarity of the hinges.

It was found that cyclic peptides **20** and **21** were much easier to handle than the original cyclic peptide **22**, which was cyclized with aromatic hinge **4**. Both **20** and **21** were soluble in an acetonitrile / water buffer (1:1, v/v) for purification, while cyclic peptide **22** needed a considerable amount of DMSO (10%, v/v) for dissolution which significantly reduced the overall yield to 18% after purification. In addition, when working with less water-soluble peptide sequences this issue may make the cyclic peptides almost insoluble in suitable preparative HPLC solvents, hence purification becomes a serious problem. From the HPLC retention times of the cyclic peptides (Scheme 7) it is evident that both N_3-DBMB-gp120 (**20**) and N_3-DBMT-gp120 (**21**) are significantly more polar than the original aromatic cyclic peptide N_3-DBMB-gp120 (**22**).

Next, it was investigated if the azide containing cyclic peptides **20** and **21** could be clicked to a molecular scaffold by using the chemoselective CuAAC reaction. The scaffold in this reaction, TriAzaCyclophane (TAC) scaffold **23**,[12] contained three alkyne substituents and was successfully used before.[7a] Both azido peptides (**20** and **21**) reacted fast and cleanly with TAC derived scaffold **23** to form the corresponding triple-loop containing protein mimics **24** and **25** in good yields after preparative HPLC (Scheme 8, 67% for **24** and 51% for **25**). This showed that both novel hinges (**5** and **6**) can be applied successfully in the preparation of protein mimics comprising discontinuous epitopes.



Scheme 8. Protein mimics by convergent introduction of peptide loops on alkynes containing TAC-scaffold.

Conclusions

In summary, we have developed two new small polar hinges, which both contain three sulfhydryl-reactive groups. The two hinges showed to react fast and cleanly with a tri-cysteine containing peptide to yield their corresponding bicyclic peptide. We found that their solubility characteristics have a large favourable impact on solubility of cyclized peptides and ensuing protein mimics. Although there are only small structural differences between the new hinges compared to the existing ones, we showed that by construction of a new generation of cyclized PK15 peptides, the impact of increasing the polarity by introduction of nitrogen atoms in the hinges is enormous. Interestingly, the new hinges impose different conformations onto peptides, which makes them attractive reagents for the synthesis of large combinatorial libraries of bicyclic peptides. Finally, increasing the solubility of cyclized peptides for epitope mimicry will facilitate the possibilities for development of protein mimics.

Experimental Section

General Procedures: All reagents and solvents were used as received. Fmoc-amino acids were obtained from Activotec (Cambridge, United Kingdom) and *N*,*N*,*N*′,*N*′-Tetramethyl-*O*-(6-chloro-1*H*-benzotriazol-1-yl)uranium hexafluorophosphate (HCTU) was obtained from Matrix Innovation (Quebec, Canada). Tentagel S RAM resin (particle size 90µm, capacity 0.25 mmol.g^{-1}) was obtained from IRIS Biotech (Marktredwitz, Germany). Methyl *tert*-butyl ether (MTBE), Hexane (HPLC grade) and TFA were obtained from Aldrich (Milwaukee, USA). DMF (Peptide grade) was obtained from VWR (Lutterworth, United Kingdom). Piperidine and D*i*PEA were obtained from AGTC Bioproducts (Hessle, United Kingdom), and 1,2-ethanedithiol (EDT) was obtained form Merck (Darmstadt, Germany). HPLC grade CH_2Cl_2 and acetonitrile were obtained from Fischer Scientific (Loughborough, United Kingdom). Solid phase peptide synthesis was performed on a PTI Tribute-UV peptide synthesizer. Lyophilizations were performed on a Christ Alpha 2-4 LD*plus* apparatus. Reactions were carried out at ambient temperature unless stated otherwise. Solvents were evaporated under reduced pressure at 40°C. Reactions in solution were monitored by TLC analysis and R_f-values

were determined on Merck pre-coated silica gel 60 F-254 (0.25 mm) glass plates. Spots were visualized by UV-light and/or by heating plates after dipping in a p-anisaldehyde or in a cerium molybdate solution (Hanessian's stain). Column chromatography was performed on Siliaflash P60 (40-63µm) from Silicycle (Canada) or on a Biotage Isolera One purification system using prepacked silica (ULTRA) Biotage SNAP cartridges. ^{1}H NMR data was acquired on a Bruker 400 MHz spectrometer in $CDCl_3$ or DMSO-d_6 as a solvent. Chemical shifts (δ) are reported in parts per million (ppm) relative to trimethylsilane (TMS, 0.00 ppm) or $CDCl_3$ (7.26 ppm). Splitting patterns are designated as a singlet (s). ^{13}C NMR data was acquired on a Bruker 400 MHz spectrometer at 101 MHz in $CDCl_3$ as solvent or DMSO-d_6 as a solvent. Chemical shifts (δ) are reported in ppm relative to the solvent residual signal, $CDCl_3$ (77.16 ppm) or DMSO-d_6 (39.52 ppm). Analytical high pressure liquid chromatography (HPLC) was carried out on a Shimadzu instrument comprising a communication module (CBM-20A), autosampler (SIL-20HT), pump modules (LC-20AT), UV/Vis detector (SPD-20A) and system controller (Labsolutions V5.54 SP), with a Phenomenex Gemini C18 column (110 Å, 5 µm, 250×4.60 mm). UV measurements were recorded at 214 and 254 nm, using a standard protocol: 100% buffer A (acetonitrile/H_2O 5:95 with 0.1% TFA) for 2 min followed by a linear gradient of buffer B (acetonitrile/H_2O 95:5 with 0.1% TFA) into buffer A (0-50%) over 30 min at a flow rate of 1.0 mL·min^{-1}. Purification of the peptidic compounds was performed on an Agilent Technologies 1260 infinity preparative system using both UV and ELSD detectors with a Phenomenex Gemini C18 column (110 Å, 10 µm, 250×20 mm). Auto-collection of fractions was used based on the UV measurements at 214 nm, using a standard protocol: 100% buffer A for 5 min followed by and linear gradient of buffer B into buffer A (0-50%) over 65 min at a flow rate of 12.5 mL·min^{-1} using the same buffers as described for analytical HPLC. Liquid chromatography mass spectrometry (LCMS) was carried out on a Thermo Scientific LCQ Fleet quadrupole mass spectrometer with a Dionex Ultimate 3000 LC using a Dr. Maisch Reprosil Gold 120 C18 column (110 Å, 3 µm, 150×4.0 mm), using a 0-100% linear gradient of buffer B into buffer A and the same flow rate and buffers as described for analytical HPLC. High-resolution electrospray ionization (ESI) mass spectra were measured on a Bruker micrOTOF-Q II in positive mode.

X-ray crystallographic analysis: Single crystal X-ray diffraction data were collected using a Nonius KappCCD with a Mo sealed tube source and equipped with an Oxford Cryosystems cryostream device, with the samples cooled to 100K. CCDC 1516966-1516968 contain the supplementary crystallographic data for this paper. The data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures.

General method for automated peptide synthesis: The peptides were synthesized on a PTI Tribute-UV peptide synthesizer. Tentagel S Ram resin (1.0 g, 0.25 mmol, 1.0 equiv or 400 mg, 0.1 mmol, 1.0 equiv) was allowed to swell (3 x 10 min). Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine in DMF using the RV_top_UV_Xtend protocol from the Tribute-UV peptide synthesizer followed by a DMF washing step (5 x 30 sec). The Fmoc-protected amino acids (with the 0.1 mmol scale 5 equiv was used and with the 0.25 mmol scale 4 equiv was used) were coupled using HCTU (with the 0.1 mmol scale 5 equiv was used and with the 0.25 mmol scale 4 equiv was used) and D*i*PEA (with the 0.1 mmol scale 10 equiv was used and with the 0.25 mmol scale 8 equiv was used) in DMF, as a coupling system, with 2 min pre-activation. The coupling time was 10 min when the peptide was synthesized on a 0.1 mmol scale and 20 min when the 0.25 mmol scale was conducted. After every coupling the resin was washed with DMF (6 x 30 sec). After the last amino acid coupling, the Fmoc group was cleaved using the normal deprotection conditions (described above) and the resulting free N-terminus was acetylated by treatment of the resin bound peptide with acetic anhydride (250 µL) and D*i*PEA (10 equiv for the 0.1 mmol scale and 8 equiv for the 0.25 mmol scale) in DMF using the standard coupling times (described above). After the last step the resin was washed with DMF (5 x 30 sec), DCM (5 x 30 sec), dried over a nitrogen flow for 10 min, followed by the cleavage of the resin-bound peptide. Cleavage and deprotection was achieved by treatment of the resin with TFA/H_2O/TIS/EDT (10 mL for the 0.25 mmol scale and 5 mL for the 0.1 mmol scale, 90:5:2.5:2.5, v/v/v/v) for 3 hours. Next, the peptide was precipitated by dropwise addition of the TFA mixture to a cold (4 °C) solution of MTBE/hexanes (1:1, 90 mL for the 0.25 mmol scale and 45 mL for the 0.1 mmol scale). After centrifugation (3500 rpm, 5 min) the supernatant was decanted and the pellet was re-suspended in MTBE/hexanes (1:1, v/v) and centrifuged again. Finally, the pellet was washed twice with MTBE/hexanes (1:1, v/v), each time collected by centrifugation, dissolved in *t*BuOH/H_2O (1:1, v/v) and lyophilized to yield the crude peptide.

Protease Inhibition assay: The inhibition of plasma kallikrein and coagulation factor FXII (FXII) was assessed by incubating the proteases with bicyclic peptide (2-fold dilutions) and measuring the residual protease activity using fluorogenic substrates. The inhibition assays were performed in buffer (150 µL) containing 10 mM TrisCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1mM $CaCl_2$, 0.1 % w/v bovine serum albumin (BSA) 0.01% v/v Triton-X100, and 5 % v/v DMSO. Final concentrations of human plasma kallikrein (Innovative Research) and human FXII (Innovative Research) were 0.25 nM and 10 nM, respectively. The fluorogenic substrates Z-Phe-Arg-AMC (plasma kallikrein) and Z-Gly-Gly-Arg-AMC (FXII) were used at a final concentration of 50 µM. Fluorescence intensity was measured with a Tecan Infinite M 200 Pro plate reader (excitation at 368 nm, emission at 468 nm). The inhibition constant (K_i) was calculated according to the Cheng and Prusoff equation $K_i = IC_{50}/(1+([S]_0/K_M))$, where IC_{50} is the functional strength of the inhibitor, $[S]_0$ is the total substrate concentration, and K_M is the Michaelis–Menten constant. K_M for plasma kallikrein and Z-Phe-Arg-AMC is 165 µM. K_M for FXII and Z-Gly-Gly-Arg-AMC is 180 µM.

General method for peptide bicyclization: To a solution of hinge molecule (**1**,**5** or **6**, 1 equiv) in acetonitrile (4.0 mM) was added an aqueous NH_4HCO_3 buffer solution (20 mM, pH 7.9) to give a concentration of 1 mM hinge in acetonitrile/buffer solution (1:3, v/v). This solution was added at once to the crude tri-cysteine containing peptide (PK15, 1 equiv, including the TFA salts for the molecular weight) and the resulting reaction mixture was stirred for 15 min before being lyophilized to yield the crude bicyclic peptide (**10** or **11**).

General method for peptide cyclization: To a solution of azido hinge (**17**, **18** or **4**, 1 equiv) in acetonitrile (4.0 mM) was added an aqueous NH_4HCO_3 buffer solution (20 mM, pH 7.9) to give a concentration of 1 mM azido hinge in acetonitrile/buffer solution (1:3, v/v). This solution was added at once to the crude di-cysteine containing peptide (1 equiv, including TFA salts in the molecular weight) and the resulting reaction mixture was stirred for 15 min before being lyophilized to yield the crude cyclic peptide (**20**, **21** or **22**).

General method for performing the CuAAC reaction: In an eppedorf tube triple-alkyne containing TAC scaffold[12] (**23**, 0.77 mg, 1.5 µmol, 1 equiv), azide containing cyclic peptide (**20** or **21**, 4.5 µmol, 3 equiv (1 equiv per arm), including the TFA salts for the molecular weight), $CuSO_4.5H_2O$ (0.34 mg, 1.35 µmol, 0.9 equiv), TBTA (0.36 mg, 0.68 µmol, 0.45 equiv) and sodium ascorbate (0.80 mg, 4.1 µmol, 2.7 equiv) were dissolved in *t*BuOH/H_2O (500 µL, 3:2, v/v) and shaken for 1 hour. The resulting mixture was diluted with 400 µL of HPLC buffer A and 100 µL of HPLC buffer B, and directly purified with preparative HPLC (0-50%

method). Next, the fractions corresponding to the product were pooled and lyophilized to yield the **24** or **25**.

Synthesis of hinge 5 (CCDC 1516998): To a stirred solution of 1,2-dichloroethane (DCE, 25 mL) in a 2-necked round bottomed flask was added bromoacetonitrile (8.71 mL 125 mmol, 1.5 equiv.) and a catalytic amount of concentrated H_2SO_4 (2 drops). After the reaction mixture was brought to reflux (100 °C), a solution of bromoacetonitrile (8.71 mL, 125 mmol, 1.5 equiv.) and 1,3,5-trioxane (7.5 g, 83 mmol, 1.0 equiv.) in DCE (25 mL) was added drop-wise over a period of 15 min and the resulting mixture was stirred for 1 hour while refluxing. The reaction mixture was allowed to cool to rt, upon which an off-white solid precipitated from the solution. The reaction mixture was filtered over a glass filter and the off-white precipitate was washed with cold MeOH until the solid was completely white. The filtrate was concentrated *in vacuo* and the resulting solid was suspended in MeOH and heated until reflux, cooled to rt, filtered and washed with cold MeOH until completely white. The combined solids were dried under high vacuum yielding hinge **5** as a white solid (35.32 g, 95%). R_f = 0.29 (EtOAc/Hexanes, 1:1, v/v); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.33 (s, 6H), 4.11 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.3, 57.9, 25.5; HRMS *m/z* calcd for $C_9H_{12}Br_3N_3NaO_3$ $[M+Na]^+$ 469.8321, found 469.8322.

Synthesis of hinges 6 (CCDC 1516997) and 8 (CCDC 1516996): To a stirred solution of triflic acid (40.0 mL, 448 mmol, 3.0 equiv) at 0 °C under N_2 atmosphere was added bromoacetonitrile (15.6 mL, 224 mmol, 1.0 equiv.). The resulting mixture was stirred overnight, during which a deep red colour appeared. Next, the reaction mixture was added drop-wise to a vigorously stirring solution of DCM (600 mL) and saturated $NaHCO_3$ (aq.) (600 mL). A white precipitate was formed which was filtered over a glass filter, washed with water and DCM, and dried under high vacuum to yield compound **8** as a white solid in a yield of 52% (g). R_f = 0.62 (EtOAc/Hexanes/HOAc, 50:50:1, v/v/v); ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.40 (s, 1H), 4.26 (s, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.0, 30.8; HRMS *m/z* calcd for $C_4H_5Br_2NNaO_2$ M+Na]$^+$ 279.8579, found 279.8579.

The aqueous phase of the filtrate was separated and back-extracted with DCM (200 mL). The combined organic layers were washed with brine (200 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The resulting deep red oil was purified by automated flash column chromatography using a linear gradient of EtOAc into Hexane (0-20%) to afford hinge **6** as a red / brown oil, which slowly crystallized over time into brown crystals (6.00 g, 22%). R_f = 0.72 (EtOAc/Hexanes, 1:1, v/v); ^{1}H NMR (400 MHz, DMSO) δ 4.68 (s, 6H); ^{13}C NMR (101 MHz, DMSO) δ 175.8, 32.3; HRMS *m/z* calcd for $C_6H_7Br_3N_3$ $[M+H]^+$ 357.8185, found 357.8181.

PK15 peptide 9[2d]: The peptide was synthesized according to the above described protocol on a 0.1 mmol scale. This gave 162.4 mg of crude peptide, which was used without any purification in the bicyclization step. t_R = 9.7 min (LCMS); ESI-MS *m/z* calcd for $C_{72}H_{117}N_{25}O_{25}S_3$ $[M+2H]^{2+}$ 913.89, found 914.42.

TBMB-PK15 peptide 10: According to the above described bicyclization method crude PK15 **9**[2d] (43.4 mg, 20 µmol, 1 equiv) was reacted with hinge **1** (7.14 mg, 20 µmol, 1 equiv). The resulting crude bicyclic peptide was dissolved in 2 mL of HPLC buffer B and 3 mL of HPLC buffer A, and was subjected to purification by preparative HPLC. Fractions containing the product were pooled and lyophilized to yield bicyclic peptide **10** as white fluffy solid (7.5 mg, 16% 95% average yield per step). t_R = 19.8 min; ESI-MS *m/z* calcd for $C_{81}H_{123}N_{25}O_{25}S_3$ $[M+2H]^{2+}$ 970.91, found 971.50.

TATB-PK15 peptide 11: According to the above described bicyclization method crude PK15 **9**[2d] (43.4 mg, 20 µmol, 1 equiv) was reacted with hinge **5** (9.46 mg, 20 µmol, 1 equiv). The resulting crude bicyclic peptide was dissolved in 2 mL of HPLC buffer B and 3 mL of HPLC buffer A, and was subjected to purification by preparative HPLC. Fractions containing the product were pooled and lyophilized to yield bicyclic peptide **11** as white fluffy solid (10.0 mg, 21% overall yield, 96% average yield per step). t_R = 18.4 min; ESI-MS *m/z* calcd for $C_{81}H_{126}N_{28}O_{28}S_3$ $[M+2H]^{2+}$ 1017.42, found 1017.67.

TBMT-PK15 peptide 12: According to the above described bicyclization method crude PK15 **9**[2d] (43.4 mg, 20 µmol, 1 equiv) was reacted with hinge **6** (7.20 mg, 20 µmol, 1 equiv). The resulting crude bicyclic peptide was dissolved in 2 mL of HPLC buffer B and 3 mL of HPLC buffer A, and was subjected to purification by preparative HPLC. Fractions containing the product were pooled and lyophilized to yield bicyclic peptide **12** as a white fluffy solid (5.40 mg, 12% overall yield, 94% average yield per step) t_R = 18.6 min; ESI-MS *m/z* calcd for $C_{78}H_{120}N_{28}O_{25}S_3$ $[M+2H]^{2+}$ 972.41, found 972.67.

FXII618 peptide 13[3b]: The linear peptide with sequence RCFRLPCRQLRCR was synthesized on a 0.03 mmol scale as described previously.[3b] This gave 30 mg of crude peptide, which was used without any purification in the bicyclization step. t_R = 10.38 min (analytical HPLC); ESI-MS m/z calcd for $C_{70}H_{124}N_{30}O_{14}S_3$ $[M+3H]^{2+}$ 569.70, found 569.50.

Bicyclic peptide 14[3b]**:** Crude FXII618 **13** (30 mg, 17 µmol, 1 equiv) was reacted with hinge **3** (6.5 mg, 26 µmol, 1.5 equiv). The resulting crude bicyclic peptide was subjected to purification by preparative HPLC. Fractions containing the product were pooled and lyophilized to yield bicyclic peptide **14** as white fluffy solid (9.2 mg, 27%). t_R = 10.2 min; ESI-MS *m/z* calcd for $C_{82}H_{139}N_{33}O_{17}S_3$ $[M+4H]^{2+}$ 489.51, found 489.75 $[M+3H]^{2+}$ 652.64, found 652.95.

Bicyclic peptide 15: Crude FXII618 **13** (30 mg, 17 µmol, 1 equiv) was reacted with hinge **5** (11.8 mg, 26 µmol, 1.5 equiv). The resulting crude bicyclic peptide was subjected to purification by preparative HPLC. Fractions containing the product were pooled and lyophilized to yield bicyclic peptide **15** as white fluffy solid (7.6 mg, 22%). t_R = 10.7 min; ESI-MS *m/z* calcd for $C_{79}H_{133}N_{33}O_{17}S_3$ $[M+4H]^{2+}$ 478.99, found 479.25; $[M+3H]^{2+}$ 638.23, found 638.75.

Bicyclic peptide 16: Crude FXII618 **13** (30 mg, 17 µmol, 1 equiv) was reacted with hinge **6** (9.5 mg, 26 µmol, 1.5 equiv). The resulting crude bicyclic peptide was subjected to purification by preparative HPLC. Fractions containing the product were pooled and lyophilized to yield bicyclic peptide **16** as white fluffy solid (6.5 mg, 20%). t_R = 10.6 min; ESI-MS *m/z* calcd for $C_{76}H_{127}N_{33}O_{14}S_3$ $[M+4H]^{2+}$ 456.48, found 456.75; $[M+3H]^{2+}$ 608.31, found 608.65.

Synthesis of azido hinge 17: Tribromo compound **5** (10.8 g, 24 mmol, 1.2 equiv) was dissolved in DMF (400 mL). Sodium azide (1.30 g, 20 mmol, 1.0 equiv) was added and the resulting mixture was stirred for 3 hours. The solvent was evaporated *in vacuo* and DCM (200 mL) was added. The salts were filtered off and the filtrate was concentrated *in vacuo*. The crude product was purified using column chromatography (40% (v/v) EtOAc in hexanes) to obtain pure product **17** as a white solid (2.65 g, 32%). R_f = 0.25 (EtOAc/Hexanes, 1:1, v/v); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.35 (s, 2H), 5.34 (s, 2H), 5.23 (S, 2H), 4.20 (s, 2H), 4.15 (s, 2H), 4.11 (s, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.0, 167.3, 167.2, 57.9, 57.7, 56.5, 25.5, 25.4; HRMS *m/z* calcd for $C_9H_{12}Br_2N_6NaO_3$ $[M+Na]^+$ 432.9230, found 432.9227.

Synthesis of azido hinge 18: Tribromo compound **6** (3.02 g, 8.4 mmol, 1.2 equiv) was dissolved in DMF (140 mL). Sodium azide (455 mg, 7 mmol, 1.0 equiv) was added and the resulting mixture was stirred for 3 hours. The solvent was evaporated *in vacuo* and DCM (50 mL) was added. The salts were filtered off and the filtrate was concentrated *in vacuo*. The crude product was purified by automated flash column chromatography using a linear gradient of EtOAc into Hexane (0-15%) to afford pure product **18** as a colourless oil (712 mg, 32%). R_f = 0.69 (EtOAc/Hexanes, 1:1, v/v); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.53 (s, 2H), 4.52 (s, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.9, 175.9, 54.2, 31.4; HRMS *m/z* calcd for $C_6H_6Br_2N_6Na$ $[M+Na]^+$ 342.8913, found 342.9810.

gp120 peptide 19[6b]: The peptide was synthesized according to the above described protocol on a 0.25 mmol scale. This gave 269.8 mg of crude peptide **19**, which was used without any purification in the cyclization step. t_R = 8.0 min (LCMS); ESI-MS *m/z* calcd for $C_{38}H_{69}N_{14}O_{13}S_2$ $[M+H]^+$ 993.46, found 993.54.

N_3-TADB - gp120 peptide 20: According to the above described cyclization method crude gp120 peptide **19**[6b] (48.8 mg, 40 µmol, 1 equiv) was reacted with azido **17** (16.5 mg, 40 µmol, 1 equiv). The resulting crude cyclic peptide was dissolved in 2 mL of HPLC buffer B and 3 mL of HPLC buffer A, and was subjected to purification by preparative HPLC. Fractions containing the product were pooled and lyophilized to yield cyclic peptide **20** as white fluffy solid (17.4 mg, 26% overall yield, 94% average yield per step). t_R = 17.9 min; ESI-MS *m/z* calcd for $C_{47}H_{79}N_{20}O_{16}S_2$ $[M+H]^+$ 1243.54, found 1243.52 .

N_3-DBMT – gp120 peptide 21: According to the above described cyclization method crude gp120 peptide **19**[6b] (48.8 mg, 40 µmol, 1 equiv) was reacted with azido hinge **18** (12.9 mg, 40 µmol, 1 equiv). The resulting crude cyclic peptide was dissolved in 2 mL of HPLC buffer B and 3 mL of HPLC buffer A, and was subjected to purification by preparative HPLC. Fractions containing the product were pooled and lyophilized to yield cyclic peptide **20** as white fluffy solid (17.3 mg, 28% overall yield, 94% average yield per step). t_R = 18.1 min; ESI-MS *m/z* calcd for $C_{44}H_{73}N_{20}O_{13}S_2$ $[M+H]^+$ 1153.51, found 1153.51.

N_3-DBMB - gp120 peptide 22[6b]: According to the above described cyclization method crude gp120 peptide **19**[6b] (48.8 mg, 40 µmol, 1 equiv) was reacted with the earlier described azido benzylic dibromide hinge[6] (**4**, 12.8 mg, 40 µmol, 1 equiv). The resulting crude cyclic peptide was dissolved in 500 µL DMSO, 2.5 mL of HPLC buffer B and 2 mL of HPLC buffer A, and was subjected to purification by preparative HPLC. Fractions containing the product were pooled and lyophilized to yield cyclic peptide **22** as white fluffy solid (10.2 mg, 18% overall yield, 93% average yield per step). t_R = 22.2 min; ESI-MS *m/z* calcd for $C_{47}H_{76}N_{17}O_{13}S_2$ $[M+H]^+$ 1150.52, found 1150.52.

Construct 24: By following the above described CuAAC method TAC scaffold **23**[7a] (0.77 mg, 1.5 µmol, 1 equiv) was reacted with cyclic peptide **20** (6.62 mg, 4.5 µmol, 3 equiv) to yield **24** (4.92 mg, 67%) as a fluffy white solid. t_R = 19.2 min; ESI-MS *m/z* calcd for $C_{171}H_{274}N_{64}O_{52}S_6$ $[M+4H]^{4+}$ 1061.98, found 1062.81; calcd for $C_{171}H_{273}N_{64}O_{52}S_6$ $[M+3H]^{3+}$ 1415.63, found 1416.13.

Construct 25: By following the above described CuAAC method TAC scaffold **23**[7a] (0.77 mg, 1.5 µmol, 1 equiv) was reacted with cyclic peptide **21** (6.22 mg, 4.5 µmol, 3 equiv) to yield **25** (3.54 mg, 51%) as a fluffy white solid. t_R = 19.3 min; ESI-MS *m/z* calcd for $C_{162}H_{256}N_{64}O_{43}S_6$ $[M+4H]^{4+}$ 994.45, found 995.34; calcd for $C_{162}H_{255}N_{64}O_{43}S_6$ $[M+3H]^{3+}$ 1325.60, found 1326.16.

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Keywords: keyword 1 • keyword 2 • keyword 3 • keyword 4 • keyword 5

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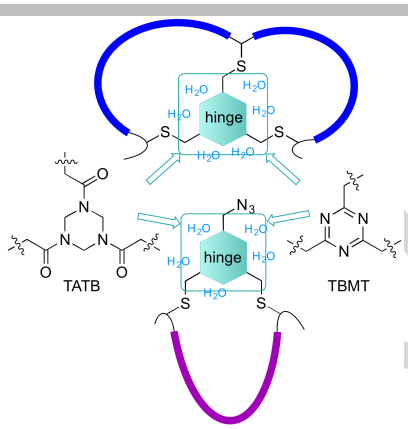
Entry for the Table of Contents (Please choose one layout)

Layout 1:

FULL PAPER

Two polar hinges have been developed for cyclization of peptides leading to bicyclic peptides and cyclized peptides with improved solubility and biological activity.



Helmus van de Langemheen, Valerijs Korotkovs, Joachim Bijl, Claire Wilson, Sangram S. Kale, Christian Heinis and Rob M. J. Liskamp**

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Polar Hinges as Functionalized Conformational Constraints in (Bi)cyclic Peptides

Layout 2:

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*Author(s), Corresponding Author(s)**

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