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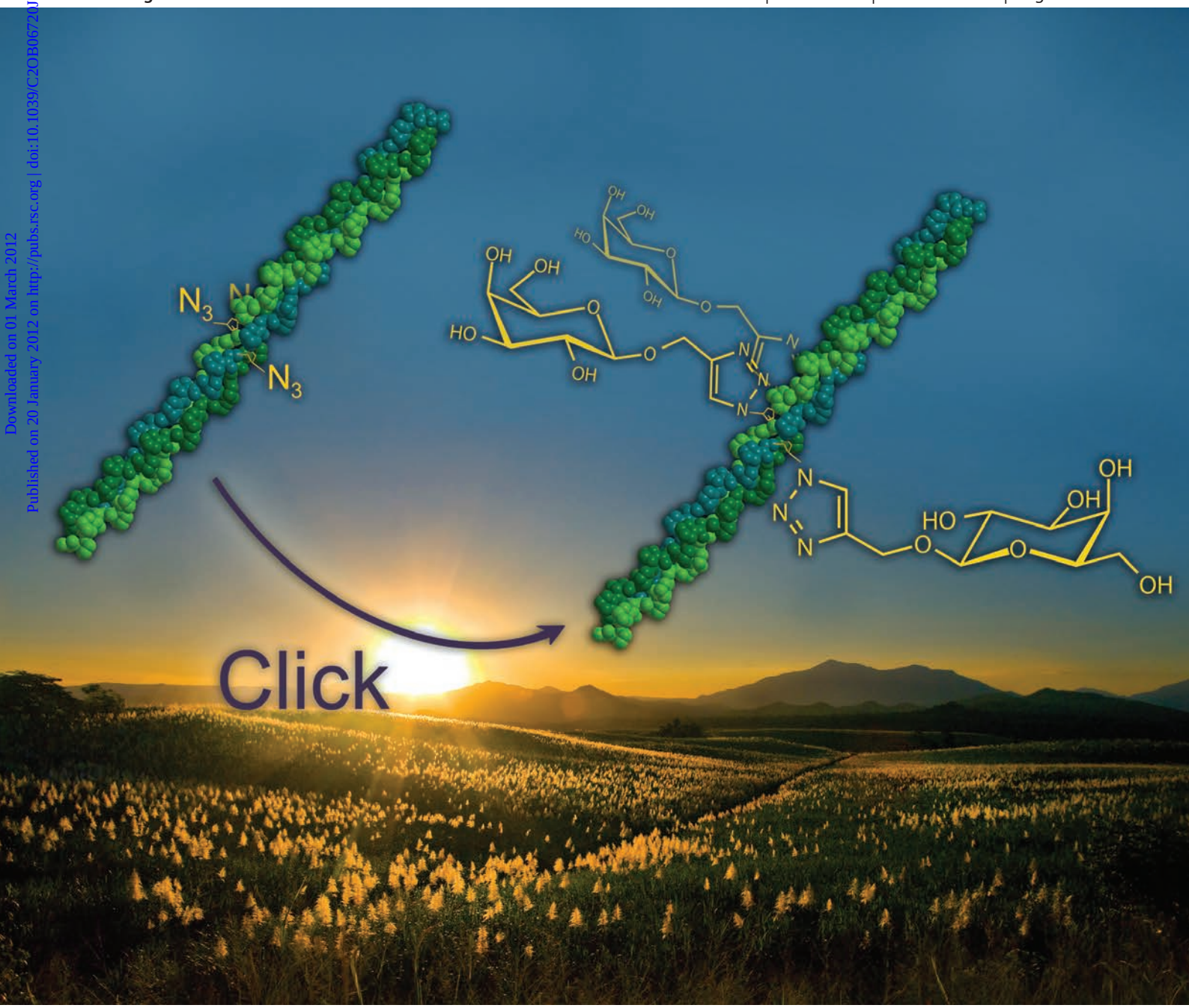

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PAPER

Conformational stability of collagen triple helices functionalized in the Yaa position by click chemistry†‡

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Click chemistry was used to introduce moieties as sterically demanding as monosaccharides into the Yaa position of collagen model peptides. The effect of different triazolyl derivatives as well as the configuration of the functionalized proline residue on the thermal stability of the collagen triple helices was examined.

Introduction

Collagen is the major constituent of the mammalian proteome. It provides structural stability in skin and bones and is involved in the modulation of several cellular activities.[1] In recent years, the development of collagen based functional materials has gained a lot of interest.[2] Such materials could be useful as scaffolds for cell growth, for wound healing and the development of artificial skin. The design of collagen model peptides (CMPs) that can be easily functionalized with a wide variety of different functional groups and still allow for the formation of stable collagen triple helices is therefore important.

Towards this goal, our group introduced CMPs containing azidoproline (Azp) moieties as sites for functionalization.[3,4] Previously we demonstrated that (4*R*)-configured Azp residues incorporated in the Yaa position of collagen allow for facile functionalization by click chemistry.[3] The resulting triazolyl functionalized CMPs form stable collagen triple helices, albeit with lower stability compared to the parent CMPs. Herein we demonstrate that (4*S*)-configured Azp residues incorporated into the Yaa position of CMPs can also be easily functionalized by click chemistry and allow for triple helix formation. Whereas a change from (4*S*)- to (4*R*)-configured proline derivatives has typically shown a significant impact on the conformational stability of the collagen triple helix,[5] the stability of the (4*S*)-configured triazolyl functionalized collagen triple helices is comparable not only to that of the parent (4*S*)Azp containing collagen triple helices but also to those of the diastereoisomeric (4*R*)-configured triazolyl functionalized collagen triple helices. Analysis of the conformational properties of simple triazolyl-functionalized proline derivatives shed light on the reasons for the comparable stabilities. The findings demonstrate that both (4*R*)Azp and (4*S*)Azp residues are versatile sites for functionalization of collagen triple helices in the Yaa position by click chemistry.

Within collagen, three single strands with polyproline II-like conformations coil around each other to form triple helices that further assemble to bundles and fibers.[1] The common repeat unit within the single strands is Xaa-Yaa-Gly with Pro as the most common amino acid in the Xaa position and (4*R*)hydroxyproline (Hyp) as the most abundant amino acid in the Yaa position.[1] In our previous work, we demonstrated that replacement of (4*R*)Hyp by (4*R*)Azp residues does not affect the stability of the collagen triple helix since (4*R*)Hyp and (4*R*)Azp have comparable conformational properties.[3,6] Due to stereoelectronic *gauche* effects exerted by the hydroxy and azido groups, both adopt C(4)-*exo* ring puckers preferentially and acylated derivatives have similar *trans* : *cis* amide bond ratios.[6] In contrast, the conformational properties of (4*S*)Azp (lower *trans* : *cis* amide bond ratio and (C4)-*endo* ring pucker, see below for details) differ significantly from those of (4*R*)Azp and (4*R*)Hyp.[6] As a result, replacing *all* of the (4*R*)Hyp by (4*S*)Azp residues within a 21mer led as expected to a CMP that does not form triple helices.[3]

Results and discussion

Design and synthesis of triazolyl functionalized CMPs

To explore whether CMPs containing (4*S*)Azp allow for derivatization by click chemistry with triazolyl moieties and formation of stable functionalized collagen triple helices, we started our investigations by synthesizing the CMPs **1S–4S** bearing a triazolyl moiety in the Yaa position of the middle repeat unit of a 21mer of Pro-Hyp-Gly units. Such so called host–guest CMPs

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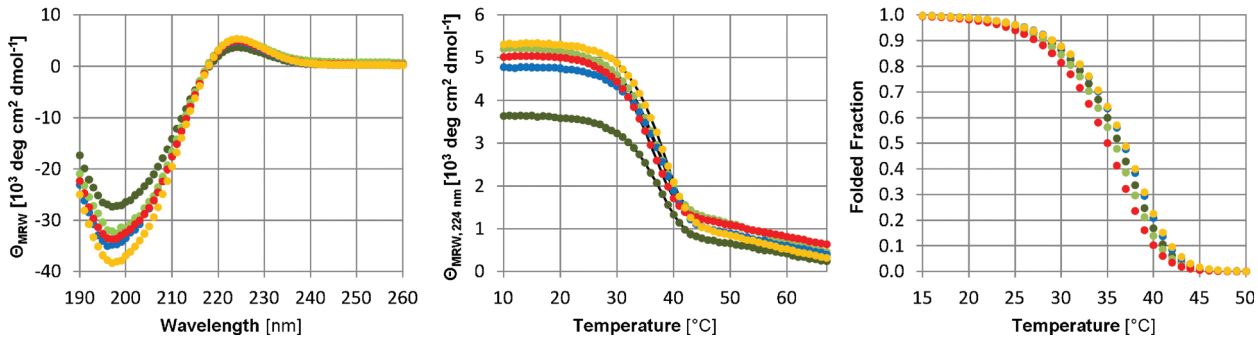
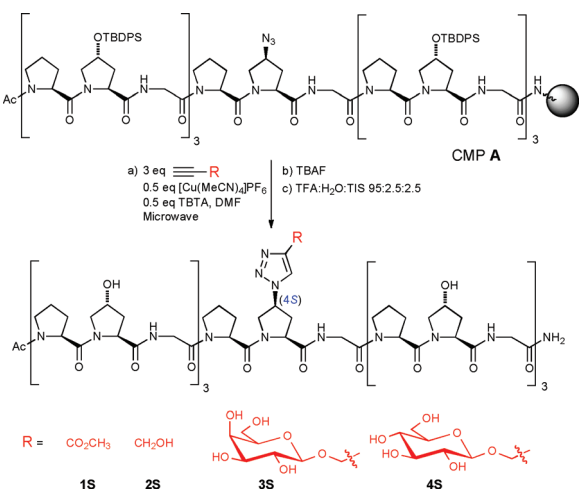


Fig. 1 a) CD spectra of 0.20 mM solutions of CMPs **1S** (●), **2S** (●), **3S** (●), **4S** (●), and **5S** (●) in 50 mM aqueous AcOH. b) Thermal denaturation curves; the black lines represent the fitted curves. c) Folded fraction of the collagen triple helices derived from the CMPs.

allow for investigating the effect of a single residue and thereby the detection of small effects on the conformational stability of collagen triple helices.[7] The triazolyl functionalized CMPs **1S–4S** were designed to bear simple ester and alcohol moieties but also the monosaccharides galactose and glucose. The same residues had been used in our previous studies with the respective (4*R*)-configured diastereoisomers and thus allow for a direct comparison of the impact of the absolute configuration at the γ-carbon of the triazolylproline (Tzp) residue on the conformational properties of CMPs.

The synthesis of CMPs **1S–4S** started with successive couplings of Fmoc-Pro-Hyp(TBDPS)-Gly-OH and Fmoc-Pro-(4*S*)Azp-Gly-OH on Rink Amide resin using 2-(6-Chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophos-phate (HCTU) as coupling reagent to yield the solid phase bound CMP **A**.[8] Derivatization of CMP **A** by click chemistry proved to be straightforward.[9] In the presence of substoichiometric amounts of Cu(I) and Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl) methyl] amine (TBTA)[10] under microwave irradiation the functionalization of the CMP with each of the four different alkynes proceeded cleanly.[11] Removal of the silyl protecting groups followed by cleavage from the resin and HPLC purification yielded the triazolyl functionalized CMPs **1S–4S** (Scheme 1). CMP **5S** bearing still the (4*S*)Azp residue was prepared by direct removal



Scheme 1 Synthesis of functionalized CMPs **1S–4S**.

Table 1 T_m values measured for the triple helices derived from CMPs **1S–5S**, **1R–5R** and **6** by thermal denaturation

Entry	CMP	T_m (°C)
1	**1S**	35
2	**1R**	36
3	**2S**	36
4	**2R**	35
5	**3S**	37
6	**3R**	37
7	**4S**	37
8	**4R**	37
9	**5S**	36
10	**5R**	43
11	**6**	43

of the silyl protecting groups of CMP **A** followed by cleavage from the resin. CMP **5S** was envisioned as a tool to probe the destabilizing effect of a single residue of (4*S*)Azp in each of the three strands of the collagen triple helix.

Thermal denaturation studies

The conformational properties of the CMPs were investigated using CD-spectroscopy and compared to those of the previously described diastereomeric CMPs **1R–5R** with (4*R*)- instead of (4*S*)-configuration at the triazolylproline (Tzp) and azidoproline residues, respectively. In addition, CMP **6** bearing the naturally most abundant (4*R*)Hyp residue was used for comparison.

Ac-(ProHypGly)$_3$-Pro**Yaa**Gly-(ProHypGly)$_3$-NH_2

5S: Yaa = (4*S*)Azp
1R: Yaa = (4*R*)Tzp(CO_2Me)
2R: Yaa = (4*R*)Tzp(CH_2OH)
3R: Yaa = (4*R*)Tzp(Gal)
4R: Yaa = (4*R*)Tzp(Glc)
5R: Yaa = (4*R*)Azp
6: Yaa = (4*R*)Hyp

The CD-spectroscopic analyses revealed that all of the (4*S*)-configured triazolyl functionalized CMPs **1S–4S** as well as **5S** form triple helices as indicated by the observed maximum at 225 nm that is typical of the collagen triple helix.[1e] Upon heating of the solutions of **1S–5S** sigmoidal curves with midpoints of thermal transition (T_m) in the range of 35–37 °C were observed (Fig. 1 and Table 1). These T_m values are lower

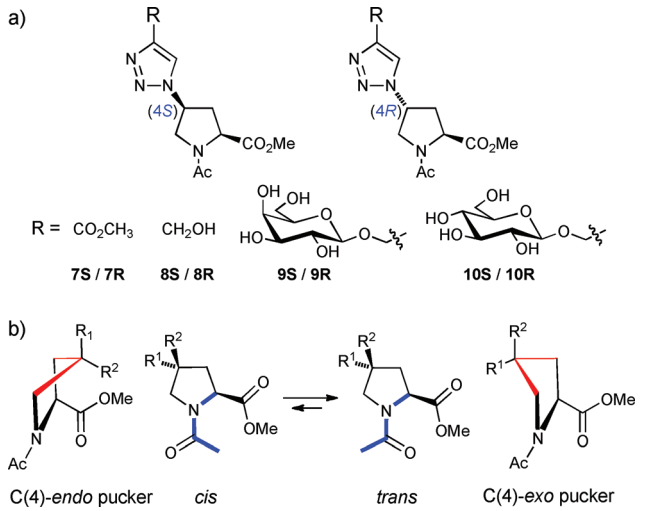


Fig. 2 a) Structures of proline derivatives **7S–10S** and **7R–10R**. b) *exo* and *endo* puckering as well as *cis* and *trans* amide isomers.

compared to those of CMPs **5R** and **6**, bearing (4*R*)Azp and (4*R*)Hyp residues, respectively, but are comparable to those of the (4*R*)-configured triazolyl functionalized CMPs **1R–4R**.[12] This finding shows that collagen triple helices derived from the (4*S*)-configured triazolyl functionalized CMPs have comparable relative thermal stabilities as those derived from the (4*R*)-configured analogues. This result is surprising since typically a large difference in the conformational stability is observed between collagen triple helices formed by CMPs that differ in the absolute configuration at the γ-carbon.[5] The results also demonstrate that the thermal stabilities of the collagen triple helices formed by the (4*S*)-configured triazolyl functionalized CMPs **1S–4S** are comparable to those formed by their unfunctionalized (4*S*)Azp containing parent CMP **5S**. Thus, in the case of the (4*S*)-configured proline derivatives, the larger steric demand of the triazolyl moieties compared to the azido group does not affect the thermal stability of the collagen triple helix to a significant extent.

The expected lower thermal stability of the triple helix derived from the (4*S*)Azp containing unfunctionalized CMP **5S** compared to those derived from CMPs **5R** and **6** can be easily explained based on our previous studies and work by others with (4*S*)-configured proline derivatives bearing electron withdrawing substituents in the γ-position.[3,5] All of these derivatives adopt preferentially an *endo*-ring pucker and have a *trans* : *cis* amide conformer ratio that is lower compared to those observed for the diastereomeric (4*R*)-configured derivatives.[5,6] The observed difference of 8 °C in the T_m value of the triple helix derived from **5S** compared to those derived from **6** and **5R** provides for a relative measure of the destabilizing effect of (4*S*)Azp residues in three symmetry related positions of the collagen triple helix. For monitoring such differences in the thermal stabilities of collagen triple helices, conformational analyses of acetylated methyl esters of proline derivatives (Ac-Xaa-OMe) have proven valuable as simple model compounds.[5] These studies showed that high *trans* : *cis* amide bond ratios observed in the model compounds and ring puckers matching those of the natural residues generally favor the formation of stable collagen triple helices. Thus, to gain a better understanding of how the triazolyl moieties affect the collagen triple helices derived from CMPs **1S–4S** and **1R–4R** we prepared and analyzed the conformations of the (4*S*)-configured triazolyl Ac-Xaa-OMe derivatives **7S–10S** and the respective (4*R*)-configured diastereoisomers **7R–10R** for comparison (Fig. 2).

Table 2 T_m *trans* : *cis* amide ratios of model compounds of the general structure Ac-Xaa-OMe determined in D_2O[a]

Entry	Ac-Xaa-OMe	*trans* : *cis*
1	**7S**	2.7 : 1
2	**8S**	2.7 : 1
3	**9S**	2.7 : 1
4	**10S**	2.7 : 1
5	**7R**	4.7 : 1
6	**8R**	4.5 : 1
7	**9R**	4.8 : 1
8	**10R**	4.7 : 1
9[b]	Ac-(4*S*)Azp-OMe	2.6 : 1
10[b]	Ac-(4*R*)Azp-OMe	6.1 : 1
11[b]	Ac-(4*R*)Hyp-OMe	6.1 : 1

[a] Determined by ^{1}H NMR spectroscopy at 25 °C, 80 mM concentration. [b] Data taken from ref. 6

Conformational analysis of Ac-Xaa-OMe derivatives

The ^{1}H NMR spectra of all of these model compounds show two sets of signals corresponding to the *cis* and *trans* isomers. In the case of the (4*S*)-configured proline derivatives, *trans* : *cis* isomer ratios of 2.7 : 1 were observed, regardless of the moiety attached to the triazole (Table 2, entries 1–4). Similarly also for the (4*R*)-configured diastereoisomers **7R–10R** comparable *trans* : *cis* isomer ratios of ~4.7 : 1 were observed demonstrating that the moieties at the triazole unit do not influence the ratio of rotational isomers around the amide bond to a significant extent. In comparison to the parent azido functionalized model compounds Ac-(4*S*)Azp-OMe and Ac-(4*R*)Azp-OMe (Table 2, entries 9 and 10),[6] the *trans* : *cis* conformer ratios of the triazoles are comparable in the case of the (4*S*)-configured derivatives and lower in the case of the (4*R*)-configured derivatives. Analysis of the vicinal ^{1}H,^{1}H coupling constants in D_2O revealed that the (4*S*)-configured derivatives **7S–10S** have a slight preference for a C(4)-*endo* ring pucker whereas the (4*R*)-configured derivatives **7R–10R** have a slight preference for C(4)-*exo* over C(4)-*endo* ring puckering. In comparison, the parent azido derivatives Ac-(4*S*)Azp-OMe and Ac-(4*R*)Azp-OMe have strong preferences for C(4)-*endo* and C(4)-*exo* ring puckering, respectively. These strong conformational preferences are due to the azido *gauche* effect that is comparable in its strength to that of hydroxy and fluorine groups.[6] The weaker preference for the analogous ring puckers in the case of the triazole moieties suggests that they exert a *gauche* effect that is, however, significantly weaker compared to that of the azido group and competes with steric effects.

These conformational studies allow for further insight into the observed thermal stabilities of the collagen triple helices derived from the triazolyl functionalized CMPs. The comparable thermal stabilities of triple helices derived from the CMPs **1S–5S** can be explained based on the similar *trans* : *cis* amide bond ratios and

the preference for C(4)-*endo* ring puckering of both (4*S*)-configured azidoproline and triazolyl-proline derivatives. This finding is remarkable since it demonstrates that the higher steric demand of the triazolyl moieties compared to the simple azido group within (4*S*)-configured proline derivatives in the Yaa position of collagen do not affect the conformational stability of the collagen triple helix.

Also the significant difference between the thermal stabilities of collagen triple helices derived from (4*R*)-configured azidoproline and triazolylproline derivatives (**5R** and **1R–4R**, respectively) can be explained based on the conformational properties of the model compounds. Whereas (4*R*)Azp derivatives have a strong preference for the C(4)-*exo* ring pucker and a high *trans* : *cis* amide bond ratio, not only the preference for the C(4)-*exo* ring pucker is lower but also the *trans* : *cis* amide bond ratio in the case of the triazolyl-functionalized derivatives.

The comparable thermal stabilities of the triazolyl-functionalized collagen triple helices regardless of the absolute configuration at the γ-carbon is difficult to explain solely based on the observed *trans* : *cis* amide bond ratios and the ring puckering preferences of the model compounds. Whereas the difference in the ring puckering and the *trans* : *cis* amide bond ratios of (4*S*)-*versus* (4*R*)-configured triazolylprolines is not as pronounced as those of the (4*S*)- and (4*R*)-configured azidoproline derivatives, they cannot entirely explain the essentially identical thermal stabilities. Thus, this finding demonstrates that not only matching ring puckering and high *trans* : *cis* amide bond ratios but also additional factors such as, steric effects or solvation need to be taken into account to explain the conformational properties of collagen triple helices. We are currently exploring the effect of steric constraints in more detail and will report these findings in due course.

Conclusions

In conclusion, we demonstrated that CMPs bearing (4*S*)Azp residues in the Yaa position can be easily functionalized with triazolyl moieties by click chemistry. Moieties as sterically demanding as monosaccharides installed in three symmetry related positions still allow for the formation of collagen triple helices that are thermally as stable as those derived from CMPs bearing an azido group in place of the triazole. The facile synthesis and functionalizability of the Azp containing CMPs and the stability of the resulting triple helices render them highly attractive for the development of functional collagen based biomaterials. Comparisons with the relative thermal stabilities of diastereomeric (4*R*)-configured triazolyl functionalized collagen triple helices and simple model compounds demonstrate that commonly used conformational properties such as *trans* : *cis* amide bond ratios and ring puckering can explain many but not all of the observed effects.

Experimental section

General aspects and materials

Materials and reagents were of the highest commercially available grade and used without further purification. For solid phase peptide synthesis Rink Amide-ChemMatrix resin from pcas BioMatrix (Saint-Jean-sur-Richelieu, Canada) was used. Reactions were monitored by thin layer chromatography using Merck silica gel 60 F254 plates. Compounds were visualized by UV light and ninhydrin. Flash chromatography was performed using Merck silica gel 60, particle size 40–63 μm. ^{1}H and ^{13}C NMR spectra were recorded on Bruker DPX 500 and DPX 400 spectrometers. Chemical shifts are reported in ppm using TMS as a reference. A Bruker Esquire 3000plus instrument was used for electrospray ionization (ESI) mass spectrometry measurements. Analytical HPLC was performed using a LiChrospher 100 RP-18e 5 μm (250 mm × 4 mm) column from Merck. Preparative HPLC was carried out on a LiChrospher RP-18e 5 μm (250 mm × 10 mm) column from Merck. For some peptides a Jupiter 4 μm Proteo 90 Å column (250 mm × 10.0 mm) was used. A Chirascan (Applied Photophysics Ltd, Leatherhead, UK) was used for CD measurements. The solutions were measured in a quartz cell with a pathlength of 1.0 mm (Hellma 110-QS). For automated peptide synthesis, a Syro I Peptide Synthesizer (MultiSynTech GmbH, Witten, Germany) was employed.

Synthesis of triazolyl functionalized CMPs 1S–4S

The peptides were synthesized on a Rink amide ChemMatrix resin (0.47 mmolg^{-1}) using a Syro I peptide synthesizer on a scale of 20-30 μmol. For the peptide couplings Fmoc-Pro-Hyp(TBDPS)-Gly-OH[8] (3 eq) was added to the resin swollen in DMF (0.4 mL) followed by HCTU (3 eq) and ^{i}Pr$_2$NEt (9 eq). The mixture was then agitated for 1 h. After Fmoc deprotection with a solution of 40% piperidine in DMF (0.4 mL) for 2 min and a solution of 20% piperidine in DMF (0.4 mL) for 10 min the coupling and deprotection steps were repeated twice followed by a coupling of Fmoc-Pro-(4*S*)Azp-Gly-OH. Three additional building blocks of Fmoc-Pro-Hyp(TBDPS)-Gly-OH were coupled as described above. The peptide was then acetylated by addition of Ac$_2$O and ^{i}Pr$_2$NEt (30 eq each) and agitation for 1 h. The alkyne (3 eq) was then coupled to the solid phase bound peptide by adding [Cu(MeCN)$_4$]PF$_6$ (0.5 eq) and TBTA (0.5 eq) to the resin swollen in DMF (0.4 mL) and allowing the mixture to stir under microwave irradiation at 60 °C for 2 h. The silyl protecting groups were removed by addition of a solution of TBAF in THF (1 M, 0.5 mL) and allowing the reaction mixture to agitate over night. The peptide was cleaved off the resin by addition of a solution of TFA/H$_2$O/TIS 95 : 2.5 : 2.5 (3 mL). After concentration the peptide was precipitated from Et$_2$O, lyophilized and purified by HPLC (for more details see supporting information†).

Synthesis of Ac-Xaa-OMe model compounds

Ac-(4*S*)Azp-OMe (0.15 mmol, 1.0 eq) was dissolved in ^{t}BuOH (100 μL) and H$_2$O (200 μL) was added. After the addition of the alkyne (3 eq) a freshly prepared aqueous solution of CuSO$_4$·5H$_2$O (0.4 M, 0.1 eq) and a solution of sodium ascorbate (1 M, 0.2 eq) were added. The suspension was stirred for 20 h at room temperature or 2 h at 60 °C under microwave irradiation. The reaction mixture was diluted with water (5 mL) and extracted with CH$_2$Cl$_2$ (4 × 3 mL). The combined organic layers

were concentrated under vacuum and the residue was purified by flash chromatography.

Abbreviations

Azp	4-Azidoproline,
Tzp	4-Triazolylproline
Hyp	4-Hydroxyproline
Pro	Proline
Gly	Glycine
CMP	Collagen model peptide
TBDPS	*tert*-Butyl diphenyl silyl
HCTU	2-(6-Chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate
TBTA	Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl] amine
T_m	Midpoint of thermal transition.

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11 For other examples of derivatisations of Azp residues or foldamers by click chemistry, see: (*a*) J. M. Holub, H. Jang and K. Kirshenbaum, *Org. Biomol. Chem.*, 2006, **4**, 1497; (*b*) H. N. Gopi, K. C. Tirupula, S. Baxter, S. Ajith and I. M. Chaiken, *ChemMedChem*, 2006, **1**, 54; (*c*) M. Kümin, L.-S. Sonntag and H. Wennemers, *J. Am. Chem. Soc.*, 2007, **129**, 466.

12 The same experimental conditions were used for the thermal denaturation studies as those used before (ref. 3). The program MicroMath Scientist 3.0 was used for deriving the T_m values from the thermal denaturation data. This fitting method is more accurate compared to that used for the determination of the T_m values reported in ref. 3. As a result, a few of the T_m values reported here differ slightly (maximum ±2 °C) from those reported in ref. 3. For more details, see the supporting information†.

