Oxidative α,ω-diyne coupling as an approach towards novel peptidic macrocycles

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Complete List of Authors:	Verlinden, Steven; Vrije Universiteit Brussel, Geudens, Niels; Ghent University, Martins, José; Ghent University, Tourwe, D; Vrije Universiteit Brussel, Ballet, S; Vrije Universiteit Brussel, Chemistry and Bio-engineering Sciences Verniest, Guido; VUB,

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AS. Verlinden,[a] N. Geudens,[b] J. C. Martins, [b] D. Tourwé,[a] S. Ballet[a] and G. Verniest[a]

The Glaser-Hay diyne coupling proved to be an efficient cyclisation approach towards diyne containing peptidic macrocycles. A variety of tetrapeptide-based macrocyclic 1,3-diynes were obtained from *O*-propargylated serine or tyrosine residues using $Cu(OAc)_2.H_2O$ and $NiCl_2$ under O_2-atmosphere. The effect of the linear 1,3-diyne on peptide conformations was studied by NMR and compared with a macrocycle bearing the saturated linker.

Introduction

Macrocyclisation represents a powerful method to develop bioactive peptides that possess improved metabolic stability, target selectivity and enhanced bioavailability.[1,2,3] Although many alternatives exist, the most common strategies used for peptide macrocylisations often rely on disulphide bridge formation, macrolactamisation, ring closing metathesis or azide alkyne click reactions.[4,5] Each of these methods has shown its value in the synthesis of cyclic peptides or mimetics thereof.[6] Nonetheless, there is still a high need for complementary methods which result not only in efficient macrocyclisation under mild conditions, but also allow further functionalisation of the introduced macrocyclic tether (e.g. the double bond in RCM reactions).[7] Moreover, cyclisation strategies that introduce additional conformational rigidity and allow to gain some conformational homogeneity can help to i) fix the peptide pharmacophore in a way that maximizes the peptide's interaction with its biological target, and ii) lower the intrinsic flexibility of the cyclic peptide.[8]

In search for new mild and functional group tolerant cyclisation strategies, oxidative alkyne-alkyne coupling reactions[9] were proposed as a method in peptide chemistry to form macrocycles. In addition, this method would introduce a 1,3-diyne unit as a reactive site for post-cyclisation modification of the peptidomimetic macrocycles.[10] Starting from one 'parent' compound, this could result in a variety of cyclic peptides, with potentially different conformations. Glaser-Hay type alkyne coupling reactions have been known for decades[11] and have been used to construct macrocyclic rings in non-peptidic applications.[12,13] However, only until very recently and simultaneously to our research, this macrocyclisation strategy had not been evaluated in cyclopeptidomimetic chemistry. Mallet and coworkers reported the synthesis of cyclic hexa- and octapeptides via alkyne-alkyne coupling reactions on solid phase.[14] In their study, propargylglycine (Pra) and *N*-propargylated amino acids were used to achieve cyclisation. Alternatively to direct alkyne-alkyne coupling, preformed diyne linkers have been incorporated in peptides to stabilize secondary structures.[15] Because of our interest in both alkyne functionalisation and new methodologies for peptide rigidification, intramolecular alkyne-alkyne coupling reactions on peptide substrates were evaluated.

Results and discussion

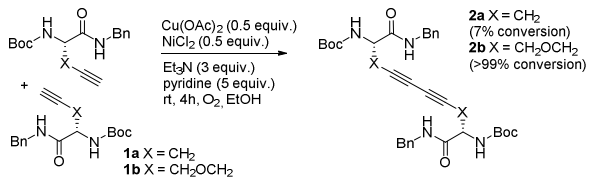
At first, optimal reaction conditions were explored for the oxidative 1,3-diyne formation starting from alkynylated glycine and serine derivatives. Over 50 different conditions were evaluated using variations in reaction temperature, Cu-catalyst, co-catalyst, base, solvent, substrate and oxidant. [11c,16] Due to the deprotonation of the amino acid carboxyl group and subsequent coordination to the Cu-salts, initial attempts with *N*-Boc- or *N*-Cbz-propargylglycine gave no satisfactory results under typical Glaser-Hay or Eglington reaction conditions (with variation of catalyst, base, oxidant). Indeed, changing the substrate to the corresponding amide derivatives **1** revealed that a clean conversion to the dimeric 1,3-diynes could be obtained. It was observed that dimerisation of *O*-propargylserine derivative **1b** with $Cu(OAc)_2.H_2O$ occurred much faster as compared to the propargylglycine analogue **1a**. This difference in reactivity was clearly shown upon use of a 1:1 mixture of substrates **1a** and **1b**. LC-MS-analysis of this

[a] *Research Group of Organic Chemistry, Department of Chemistry and Department of Bio-engineering Sciences, Faculty of Science and Bio-engineering Sciences, Vrije Universiteit Brussel, Pleinlaan (VUB) 2, B-1050 Brussels, Belgium; gvernies@vub.ac.be*

[b] *NMR and Structure Analysis Unit, Department of Organic and Macromolecular Chemistry, Ghent University, Krijgslaan 281-S4, 9000 Ghent, Belgium*

†Electronic Supplementary Information (ESI) available: Experimental data and spectroscopic data (^1H, ^{13}C NMR and ROESY spectra of **13a**, **14a** and **15a**) for all new compounds. See DOI: 10.1039/x0xx00000x

reaction mixture demonstrated that homodimer **2b** was formed as the major compound, a minor quantity of heterocoupled dimer and almost no homodimer **2a** was formed.[17] This reduced reactivity of **1a** can most probably be assigned to the increased sterical hindrance of the terminal alkyne moiety in **1a**.

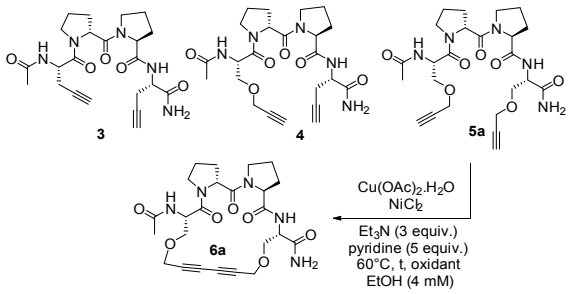


Scheme 1. Optimal conditions of the oxidative diyne coupling on protected propargylated Ser and Pra residues

In all cases, the use of $NiCl_2$ as a co-catalyst showed to improve yields and significantly reduce reaction times.[18] After screening several Cu- and Ni-catalysts, the combination of $Cu(OAc)_2.H_2O$ and $NiCl_2$ proved to be an optimal catalytic system for these type of substrates. In addition, the use of Et_3N as base (3 equiv.) and pyridine as ligand (5 equiv.) in EtOH under O_2-atmosphere provided the highest conversions towards dimer **2b**.

Subsequent to this model study, the optimal reaction conditions were used as a starting point for the envisaged intramolecular cyclisation of different tetrapeptides containing two alkynylated amino acid residues. At first, the linear tetrapeptide **3**, bearing two propargylglycine residues, was prepared via standard solid phase synthesis on Rink amide resin. A heterochiral D-Pro-L-Pro motif was initially chosen as central fragment, because this type II' β-turn inducer was envisaged to orient both triple bonds in close spatial proximity.[19] To prevent catalyst inactivation, the *N*-terminus was acetylated and in line with the intermolecular diyne formation (cf. Scheme 1), a C-terminal amide was introduced. Unfortunately, linear peptide **3** could not be cyclised. The expected increase in flexibility, decrease in steric hindrance on the terminal alkyne and decrease in ring strain following substitution of one propargylglycine residue with an *O*-propargylated serine residue (**4**, obtained from Boc-Ser-OH) was subsequently proposed to promote cyclisation. The cyclisation of **4** was however equally unsuccessful. Gratifyingly, using two *O*-propargylserine residues, a nice conversion of **5a** to macrocyclic peptide **6a** was obtained within 4h via treatment with catalytic amounts of $Cu(OAc)_2.H_2O$ and $NiCl_2$ in EtOH under O_2-atmosphere (Table 1, Entry 1). These cyclisation reactions proceeded slower or to a lower extent when performing the reaction open to the air or without the nickel co-catalyst (Table 1, Entry 2-3). A substrate concentration of 4 mM was found to be optimal in order to avoid the formation of oligomerisation products. It should be noted that the cyclisation needs to be performed in the absence of H_2O, as its presence proved to be detrimental for the cyclisation to occur, an observation also reported for related non-peptidic couplings (Table 1, Entry 4 vs. 5).[20]

Table 1. Oxidative diyne macrocyclization of **3**, **4** and **5a**

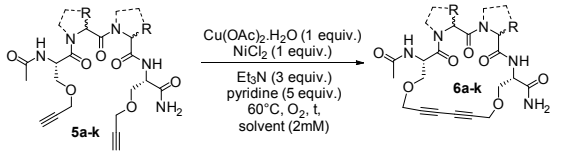


Entry	$Cu(OAc)_2.H_2O$ (equiv.)	$NiCl_2$ (equiv.)	Oxidant	t(h)	Conv. (%)
1	0.25	0.25	O_2	4	>99
2	0.25	0.25	Atm.	24	>99
3	0.25	/	Atm.	120	85
4	1	1	O_2	4	>99
5[a]	1	1	O_2	120	0

[a] excess H_2O was added

Although it was decided to focus on a solution phase cyclisation strategy, a slightly modified cyclisation protocol was performed successfully on solid supported substrates in catalytic conditions (see supporting information).[21]

Table 2. Scope of the oxidative diyne macrocyclization

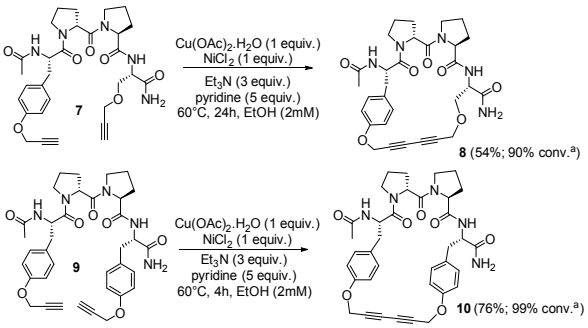


Entry	Linear peptide	Central segment	t(h)	Solvent	Cyclic peptide (% conv.)	Yield (%)
1	**5a**	D-Pro-Pro	4	EtOH	**6a** (>99)	68[a]
2	**5b**	D-Pro-Gly	24	EtOH	**6b** (>99)	39[b]
3	**5c**	D-Pro-Ala	4	EtOH	**6c** (90)	66[a]
4	**5d**	D-Pro-D-Ala	24	EtOH	**6d** (>99)	40[b]
5	**5e**	Aib-D-Ala	36	EtOH	**6e** (>99)	48[a]
6	**5f**	*N*Me-D-Ala-Pro	4	EtOH	**6f** (>99)	4[b]
7	**5g**	D-Ala-Ala	24	EtOH	**6g** (>99)	45[a]
8	**5h**	Ala-Ala	48	DMF	**6h** (80)	25[b]
9	**5i**	Asn-Gly	72	DMF	**6i** (50)	61[a]
10	**5j**	Glu(Bn)-Gly	24	DMF	**6j** (80)	5[b]
11	**5k**	Lys(Z)-Ala	4	DMF	**6k** (70)	17[b]

[a] Purification using silica gel chromatography (20% MeOH in CH_2Cl_2). [b] Isolated yield after preparative RP-HPLC.

To evaluate the scope of above described cyclisation approach, linear peptides **5b-d**, which are known to form II' β-turns, and linear peptide **5e**, which is prone to I' β-turn formation (Table 2, Entry 2-5),[22] were also prepared and subjected to the

optimised diyne coupling conditions. All tetrapeptides cyclised with high conversions (90 to 99%) within 4 to 36h. In an attempt to broaden the scope and to our delight, *N*-methylated amino acids (see **5f**, entry 6) could be used as well.[23] This result was indicative of a high amino acid variation potential in the central dipeptide motif. To extend the scope even further and investigate less hydrophobic sequences, central Ala-Ala, Asn-Gly, Glu(Bn)-Gly and Lys(Z)-Ala segments (**5g-k**, entry 7-11) were evaluated, and in these cases also, the corresponding macrocycles were obtained efficiently. The lower conversions of **5h**-**k** as compared to **5a**-**g** is most probably resulting from the use of DMF as a solvent because of the low solubility of **5h**-**k** in EtOH (Table 2).

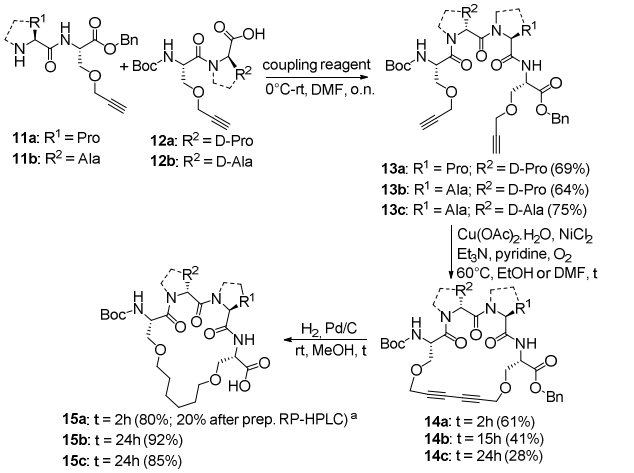


Scheme 2. Cyclisations involving *O*-propargyl-Tyr residues; [a]conversion as determined by HPLC.

It should be noted that a concentration of 2 mM is preferred for the intramolecular cyclisation, as higher concentrations resulted in the formation of oligomers. Due to solubility issues of peptides **5h-k** in EtOH, the reaction was performed in DMF. This modification was however accompanied by a decreased conversion. All obtained macrocyclic peptides **6a-k** were purified via preparative RP-HPLC (reverse phase) and isolated in high purity (>95%). Analytically pure samples of all obtained compounds **6a**-**k** could be obtained using reversed phase preparative HPLC but in general this resulted in very low to acceptable yields, mainly due to the use of unoptimized HPLC conditions. To demonstrate possible alternative purification methods, the mixtures of diynes, bases and catalysts were subjected to silica gel column chromatography using 20% MeOH in CH_2Cl_2 as eluent mixture. For compounds which have low solubility in CH_2Cl_2 such as **5g** and **5i**, the greenly colored copper salts eluted simultaneously with the diyne tethered peptide derivatives. To solve this inconvenience, the mixtures were first treated with metal chelators before silica gel chromatography. It was shown that the use of an excess of Na_3PO_4 indeed enabled the isolation of the peptide derivatives as white solids after silica gel chromatography in good yields up 68% (Table 2, for details see Supporting Information). In order to access larger ring systems and a larger structural diversity of peptide macrocycles, the synthesis of tetrapeptides **7** and **9** was also performed (Scheme 2). These tetrapeptides, containing a combination of *O*-propargyltyrosine and *O*-propargylserine (**7**) or two *O*-propargyltyrosines (**9**), gave rise to the corresponding macrocycles without side reactions under the above described reaction conditions in 90% and 99% conversion, respectively. The variation in both central dipeptide segment and alkynyl side chains clearly shows the extent of the cyclisation scope.

A solution phase synthesis of tetrapeptides **13a**-**c** was performed (Scheme 3) in order to access workable amounts of macrocycles in view of postcyclisation modifications. Whereas a linear synthesis towards **13a** resulted in poor yields because of a problematic D-Pro to Pro coupling reaction, the convergent approach depicted in Scheme 3 proved successful. Dipeptides **11a**-**b** were prepared starting via the coupling of Boc-Pro-OH or Boc-Ala-OH and propargylated H-Ser-OBn using EDC/HOAt or HATU, respectively. Dipeptides **12a**-**b** were synthesised via the coupling of H-D-Pro-OMe or H-D-Ala-OMe and Boc-Ser(*O*-propargyl)-OH with HATU or HOBt/EDC, respectively, followed by saponification of the methyl ester. Subsequently, the fragments **11a**-**b** and **12a**-**b** were coupled efficiently to give the linear peptides **13a**-**c** in good isolated yields (64-75%). Also in this case, these linear peptides were cleanly converted to the cyclic peptide **14a**-**c** in moderate yields using the above described alkyne-alkyne coupling conditions.

Finally, a first example of a postcyclisation modification of the 1,3-diyne linker was demonstrated via hydrogenation of the diyne under H_2 (1 atm) over Pd/C. Concomitantly with an *O*-debenzylation, the diyne was completely reduced to an aliphatic linker after 2h at room temperature.



Scheme 3. Solution phase synthesis of macrocycles **14a**-**c** and **15a**-**c** ([a] a high purity sample was obtained via RP-HPLC for NMR studies)

The conformational features of this new type of peptidic 1,3-diyne containing macrocycles was studied in more detail using NMR. More specifically, attention focused on the tetrapeptides **13a**-**14a**-**15a**, with the heterochiral diproline central fragment, as model systems.[24] These were selected as they were expected to allow a more straightforward analysis of the impact of cyclisation on backbone conformation, including the peptide bonds.

For all peptides, either linear or cyclised, three (**14a**) or four (**13a** and **15a**) different sets of resonances were found to be

present. Using ROESY spectra, exchange cross peaks connecting the different sets of resonances could be established, identifying these as different conformations in slow exchange on the NMR time scale.

As can be expected, the various conformers arise from *cis-trans* isomerisation of the two peptide amide bonds involving the Pro residues. This is confirmed by the mutually exclusive observation of specific sequential ROE correlations between H_α (i) and either H_α (i+1) or H_δ (i+1) for the *cis* and *trans* peptide bond connecting residues i and i+1, respectively.

By analyzing these characteristic ROE correlations for each conformer, the main isomer of **13a** is the *trans-trans* isomer with a population of 50%. Two minor isomers are found exchanging with the *trans-trans* major form and were identified as the *trans-cis* (30%) and the *cis-trans* (15%) isomers. The fourth and least populated conformer (5%) exchanges with both aforementioned minors but not the major conformer, which is consistent with a *cis-cis* conformer. Consequently, it seems that both peptide bonds in the linear peptide **13a** have a preference for the 'typical', naturally most abundant, *trans* orientation. When the peptide is in its cyclised form (**14a**), the population of the various *cis-trans* conformers is redistributed, the *trans-cis* isomer now being the main isomer (70%). Other isomers that are present in the NMR spectra are the *trans-trans* (28%) and the *cis-trans* isomer (2%). Since the major form is seen to exchange with each of these minor forms, it can be concluded that the constraint imposed by the rigid linker does not strongly affect the isomerisation barriers involved. The *cis-cis* isomer on the other hand appears to be absent in the NMR spectra.

When the diyne linker is reduced (**15a**), the *trans-trans* conformer increases to 40%, at the expense of the *trans-cis* isomer (50%) which remains the major form. There are two other minors present that must represent the *cis-trans* and *cis-cis* forms (7% and 3%), but cannot be identified with full confidence due to spectral overlap, even at 700 MHz. Nevertheless, it is clear that the introduction of conformational flexibility in the linker moves the relative populations closer to the ones originally found in the linear forms, most probably by alleviation of strain induced by the diyne linker. Since a 1,3-diyne as a linker appears to induce conformational bias and leads to a more conformationally constrained peptide as compared to the acyclic one, it might represent a handle to control the peptides' secondary structure.[25]

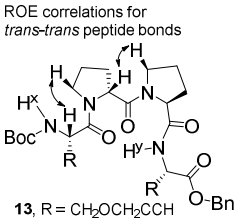
Table 3. The population of the different conformers of peptides **13a**, **14a** and **15a**[a]

	% conformers			
peptide	*trans-trans*	*trans-cis*	*cis-trans*	*cis-cis*
13a	50	30	15	5
14a	28	70	2	0
15a	40	50	7	3

[a] An absolute assignment of chemical shifts is only given for the main conformer (see SI file)

To shed some light on the control of the peptides' secondary structure, all amide proton temperature coefficients were determined in peptides **13a** to **15a** (see Table 4). Using -4ppb/K as a cut-off value for an 85% probability of hydrogen bond formation, only the amide protons of the most abundant minor (i.e. trans-trans) in peptides **14a** and **15a** seem to be involved in intramolecular hydrogen bonds. In this specific case, a reduction of the diyne linker seems to increase the conformer population implicated in turn structures (cf. trans-trans (**14a**) 28% to trans-trans (**15a**) 40%).

Table 4. The amide temperature coefficients of the main isomer and the main abundant minor



13, R = CH_2OCH_2CCH

peptide		**13**	**14**	**15**
	Proton	Temp. coeff. (ppb/K)		
trans-cis isomer	NH^x	-5.09	-3.90	-5.85
	NH^y	-2.95	-4.18	-6.13
trans-trans isomer	NH^x	-4.27	-2.07	-3.93
	NH^y	n.d.	-3.61	-1.98

From the point of view of the individual peptide bonds the following analysis can be made. For the Xxx–D-Pro dipeptide, the *trans* conformation is favoured for the peptide bond, and its overall population is promoted upon cyclisation as it appears in 98% of the overall conformer population against 80% in the linear form. Upon reduction of the linker this changes to 90%, probably indicating some relief of strain for the *cis* conformation. For the D-Pro–Pro peptide bond, the *trans* conformation becomes less favoured upon cyclisation.

Conclusions

In conclusion, oxidative alkyne-alkyne coupling reactions afford cyclic tetrapeptides in high conversion using $Cu(OAc)_2.H_2O$ and $NiCl_2$ as catalysts, on solid support as well as in solution. In view of future post-cyclization modifications and access workable amounts of material, a strong emphasis was however placed on the solution phase strategy. The method allows variation of the central segment of the tetrapeptide and the ring size of the macrocycles can be altered using different alkynylated amino acids. Cyclisation via 1,3-diyne coupling clearly results in conformational restriction of the peptide sequence as compared to the acyclic peptide and to the corresponding macrocycle bearing a fully saturated linker, as determined via NMR. As a first postcyclisation modification,

this 1,3-diyne motif was fully reduced, and resulted in a less constrained macrocycle. A full exploration of various transformations of this 1,3-diyne and an elaborate study on a potential conformational bias of peptide secondary structure formation are currently under investigation and will be reported in due course.

Experimental

General protocols Column chromatography purifications were conducted on silica gel 60 (40-63 µm; Grace Davisil) or with a Grace Reveleris X2 Flash Chromatography System on silica gel (prepacked 40µm; Grace Reveleris). TLC was carried out on glass plates precoated with silica gel 60F254 (Merck); the spots were visualized under UV light (λ=254 nm) and/or $KMnO_4$ (aq.) was used as revealing system. Preparative HPLC was conducted using a Gilson semi-preparative HPLC equipped with a Supelco Discovery Bio Wide Pore C18 column. Samples were analysed on an Agilent 1100 Series HPLC, equipped with a Supelco Discovery Bio Wide Pore C18 column (15cm x 2.1mm x 3µm). The solvent system consists of 0.1% TFA in water and 0.1% TFA in acetonitrile. The samples were then eluted through the column using a gradient ranging from 3% acetonitrile to 97% acetonitrile over 20 minutes (standard gradient) at a flow rate of 0.3mL/min. Melting points were acquired on a Buchi Melting Point B-540 and are uncorrected. IR absorption spectra were recorded on a Thermo Nicolet 700FT-IR spectrophotometer. NMR measurements were performed on either a Bruker Avance DRX spectrometer operating at ^{1}H and ^{13}C frequencies of 250.13 and 62.90 MHz, respectively, a Bruker Avance II spectrometer operating at ^{1}H and ^{13}C frequencies of 500.13 and 125.76 MHz, respectively; or a Bruker Avance II spectrometer operating at ^{1}H and ^{13}C frequencies of 700.13 and 176.05 MHz, respectively. The sample temperature was set to 298.2K. The deuterated solvent is mentioned in the analysis section and tetramethylsilane was used as an internal standard. Chemical shifts (δ) are given in parts per million (ppm), coupling constants (*J*) are given in Hertz (Hz). High resolution Mass Spectrometry was conducted on a Waters Micromass QTof in ES+ mode, using reserpine as the reference. Some peptide coupling reactions were performed using a BIOTAGE Initiator^{+} SP Microwave Synthesizer. Commercial Fmoc-protected amino acids, coupling reagents and Rink amide AM resin (100-200 mesh, 0.56 mmol/g) were purchased from NovaBioChem and Chem-Impex. All other used reagents and chemicals were purchased from Sigma Aldrich and used without further purification unless otherwise specified.

Solid Phase Peptide Synthesis protocols Linear peptides **5a**-**e**,**g**-**k**, **7** and **9** were synthesized manually by N^{α}-Fmoc methodology on Rink amide resin using (2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate) (HCTU)/NMM as coupling reagent. Fmoc-AA-OH (3 equiv.) and NMM in DMF (0.4 M) were added to the swollen solid support and the reaction mixture was shaken for 2 to 3h. Trityl, benzyl and benzyloxycarbonyl were used as Fmoc-amino side chain protecting group for Fmoc-Asn-OH, Fmoc-Glu-OH and Fmoc-Lys-OH, respectively. For the modified Fmoc-Ser(*O*-propargyl)-OH and Fmoc-Tyr(*O*-propargyl)-OH only 2 equivalents were used for the coupling. The resin was washed three times with DMF, three times with *i*-PrOH and three times with CH_2Cl_2. Completion of the reaction was tested by the Kaiser or chloranil test. In case of a positive color test, the coupling was repeated until a negative color test was obtained. Fmoc deprotections were carried out using 20% 4-methylpiperidine in DMF. Linear peptide **5f** was synthesised with a BIOTAGE Initiator^{+} SP Microwave Synthesizer. Coupling of the Fmoc amino acids occurred at 75 °C for 5min. Fmoc-*N*-Me-*D*-Ala-OH was coupled using DIC *(N,N'*-diisopropylcarbodiimide)/HOAt/DIPEA in DMF at 60°C (2x10min). After Fmoc-deprotection of the final coupled Fmoc-AA-OH, the free amine was acetylated by adding Ac_2O/CH_2Cl_2/DIPEA (0.5/8/1) to the resin and the resin was shaken for 1-2h. Final cleavage of the linear peptides from the resin was accomplished by treatment with TFA/triethylsilane (TES)/H_2O 95/2.5/2.5 for 1-2h. The crude peptides were isolated by filtration, lyophilised and purified via preparative RP-HPLC to obtain **5a**-**k**, **7** and **9** as white powders.

Cyclization of lineair peptide 5a: 5a (0.08 mmol), was dissolved in EtOH (20 ml). $Cu(OAc)_2.H_2O$ (0.02 mmol), $NiCl_2$ (0.02 mmol), triethylamine (0.24 mmol) and pyridine (0.40 mmol) were added and the reaction mixture was stirred for 4h at 60 °C under O_2-atmosphere. After cooling to room temperature, 5 equiv of Na3PO4 (as aq. sat. solution) was added to the mixture and stirred for 15 minutes. Afterwards the mixture was concentrated, loaded on a plug of silica gel (2cm, diameter 0.5cm) and eluted using 20% MeOH in CH_2Cl_2. After evaporation *in vacuo*, 5mL of hexane was added and the mixture was left without stirring to remove traces of pyridine. After decanting the hexane, the remaining solids were further evaporated at high vacuum (0,1 mbar) resulting in cyclic peptide **6a** as a white solid in 68% yield; HPLC (standard gradient): t_{ret} = 9.46 min.; HRMS Calcd for $[C_{24}H_{31}N_5O_7 + H^+]$: 502.2267. Found 502.2296. Alternatively, **5a** was isolated from the mixture via RP-HPLC in 9% yield as a white powder (see SI-file)

General procedure for the cyclization of peptides 5b-k, 7, 9: The linear peptides **5b**-**h**, **7** or **9**, were dissolved in EtOH (or DMF for linear peptides **5h**-**k**) towards a final concentration of 2 mM. $Cu(OAc)_2.H_2O$ (1 equiv.), $NiCl_2$ (1 equiv.), Et_3N (3 equiv.) and pyridine (5 equiv.) were added and the reaction mixture was stirred for 4-72h at 60 °C under O_2-atmosphere. The solvent was removed *in vacuo* and the cyclic peptides were afforded after preparative RP-HPLC purification as a white powder. For details regarding alternative isolation and purification is referred to the Supporting Information.

Solution Phase Peptide Synthesis The synthesis of **13a**-**15a** is described as representative example. For experimental details of all other new compounds is referred to the Supporting Information.

Benzyl *N*-*N*-(*tert*-butoxycarbonyl)-*O*-(prop-2-yn-1-yl)-L-seryl-D-prolyl-L-prolyl-*O*-(prop-2-yn-1-yl)-L-serinate 13a t*ert*-Butyl (*S*)-2-(((*S*)-1-(benzyloxy)-1-oxo-3-(prop-2-yn-1-yloxy)propan-2-yl)carbamoyl)pyrrolidine-1-carboxylate (0.65 mmol) was dissolved in a mixture of TFA (5 ml) and CH_2Cl_2 (5 ml). The reaction mixture was stirred for 1h closed from atmopsheric moisture with a $CaCl_2$-tube and concentrated *in vacuo* to obtain **11a** as a TFA salt .To a cooled solution of *N*-(*tert*-butoxycarbonyl)-*O*-(prop-2-yn-1-yl)-L-seryl-D-proline **12a** (0.59 mmol) in DMF (10 ml) was added HATU (0.71 mmol) and DIPEA (0.59 mmol) and the reaction mixture was stirred for 30min at 0 °C. The TFA salt (**11a**), dissolved in DMF (10 ml), was added to the reaction mixture, followed by the addition of

DIPEA (0.59 mmol). The reaction mixture was stirred for 15h at rt protected from atmospheric moisture with a $CaCl_2$-tube, diluted with brine (10 ml) and extracted with EtOAc (3x40 ml). The organic phase was washed with brine (3x40 ml), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The obtained residue was purified via column silica gel chromatography ($CHCl_3$/MeOH 99/1) to afford benzyl *N*-*N*-(*tert*-butoxycarbonyl)-*O*-(prop-2-yn-1-yl)-L-seryl-D-prolyl-L-prolyl-*O*-(prop-2-yn-1-yl)-L-serinate as a colourless liquid in 69% yield (0.27 g). IR (neat): 3284, 2113, 1744, 1652, 1633 cm^{-1}; ^1H NMR (700 MHz, CD_3CN): d 7.66 (d, *J* = 8.4 Hz, 1H)*, 7.37 (m, 5H), 5.82 (d, *J* = 8.7 Hz, 1H) 5.19 (d, *J* = 12.5 Hz, 1H)*, 5.11 (d, *J* = 12.5 Hz, 1H), 4.63 (m, 1H)*, 4.56 (m, 1H)*, 4.52 (m, 1H)*, 4.41 (m, 1H)*, 4.13 (m, 4H), 3.89 (m, 1H)*, 3.84 (m, 2H)*, 3.72 (m, 1H)*, 3.62 (m, 2H)*, 3.59 (m, 1H)*, 3.54(m, 1H,)*, 2.73 (m, 2H), 2.17 (m, 1H)*, 2.14 (m, 1H)*, 2.06 (m, 2H)*, 1.93 (m, 2H)*, 1.91 (m, 2H)*, 1.41 (m, 9H); ^{13}C NMR (176.05 MHz, CD_3CN): d 172.7, 172.0, 171.3, 169.4, 136.8, 129.2, 80.6, 80.4, 80.1, 76.3, 76.0, 70.3, 69.9, 67.7, 61.3, 59.1, 59.9, 53.2, 52.2, 48.4, 47.9, 30.1, 29.1, 28.5, 26.0, 24.8; HRMS Calcd for $[C_{34}H_{44}N_4O_9 + Na^+]$: 675.3000. Found 675.2977. (* Double signals due to rotamerism across the C-N bond of the amide. Only the resonance of the main isomer are given.)

(6*S*,17*S*,19*aS*,24*aR*)-6-((*tert*-Butoxycarbonyl)amino)-5,19,24-trioxoicosahydro-1H,5H-dipyrrolo[2,1-f:2',1'-i][1,14]dioxa[4,7,10]triazacycloicosine-3,8-diyne-17-benzyl ester 14a Benzyl *N*-*N*-(*tert*-butoxycarbonyl)-*O*-(prop-2-yn-1-yl)-L-seryl-D-prolyl-L-prolyl-*O*-(prop-2-yn-1-yl)-L-serinate **13a** (0.41 mmol), $Cu(OAc)_2.H_2O$ (0.41 mmol) and $NiCl_2$ (0.41 mmol) were added to EtOH (30 ml). Et_3N (1.22 mmol) and pyridine (2.03 mmol) were added and the reaction mixture was stirred at 60 °C under O_2-atmosphere for 2h. The reaction mixture was concentrated *in vacuo* and purified via column silica gel chromatography ($CHCl_3$/MeOH 99/1) to afford (6*S*,17*S*,19*aS*,24*aR*)-6-((*tert*-Butoxycarbonyl)amino)-5,19,24-trioxoicosahydro-1H,5H-dipyrrolo[2,1-f:2',1'-i][1,14]dioxa[4,7,10]triazacycloicosine-3,8-diyne-17-benzyl ester as a lightgreen solid in 61% yield (160 mg). mp: 79.9 – 83.2 °C; IR (neat): 3300, 1743, 1633 cm^{-1},^1H NMR (700 MHz, CD_3CN): d 7.38 (m, 6H)*, 5.53 (d, *J* = 8.1 Hz, 1H)*, 5.16 (d, *J* = 12.5 Hz, 2H), 4.86 (m, 1H)*, 4.62 (m, 1H)*, 4.51 (m, 1H)*, 4.31 (m, 1H)*, 4.21 (m, 2H), 4.15 (m, 2H), 4.00 (dd, J= 5.4 Hz and 9.4 Hz)*, 3.82 (dd, J = 2.8 Hz and 9.4 Hz)*, 3.76 (m, 1H)*, 3.73 (m, 1H)*, 3.68 (m, 1H)*, 3.61 (m, 1H)*, 3.42 (m, 2H)*, 2.22 (m, 1H)*, 2.13 (m, 1H)*, 2.09 (m, 1H)*, 1.97 (m, 1H)*, 1.91 (m, 1H)*, 1.84 (m, 1H)*, 1.76 (m, 2H), 1.40 (s, 9H)*; ^{13}C NMR (176.05 MHz, CD_3CN): d 173.4, 172.4, 170.2, 169.0, 136.8, 129.5, 80.0, 79.3, 78.7, 77.2, 76.8, 71.0, 70.1, 67.8, 60.8, 59.2, 59.1, 53.3, 52.9, 48.4, 47.8, 32.8, 30.2, 28.4, 25.6, 23.2; HRMS Calcd for $[C_{34}H_{42}N_4O_9 + Na^+]$: 673.2844. Found 673.2845. (* Double signals due to rotamerism across the C-N bond of the amide. Only the resonance of the main isomer are given.)

(6*S*,17*S*,19*aS*,24*aR*)-6-((*tert*-Butoxycarbonyl)amino)-5,19,24-trioxoicosahydro-1H,5H-dipyrrolo[2,1-f:2',1'-i][1,14]dioxa[4,7,10]triazacycloicosine-17-carboxylic acid 15a

To a mixture of (6*S*,17*S*,19*aS*,24*aR*)-6-((*tert*-Butoxycarbonyl)amino)-5,19,24-trioxoicosahydro-1H,5H-dipyrrolo[2,1-f:2',1'-i][1,14]dioxa[4,7,10]triazacycloicosine-3,8-diyne-17-benzyl ester (0.031 mmol) in MeOH (1 ml) was added palladium on carbon (10 wt-% Pd on C, 10 mg). The reaction mixture was stirred under H_2 (1 atm) at rt for 2h, filtered over Celite and concentrated *in vacuo* to afford 6*S*,17*S*,19*aS*,24*aR*)-6-((*tert*-butoxycarbonyl)amino)-5,19,24-trioxoicosahydro-1H,5H-dipyrrolo[2,1-f:2',1'-i][1,14]dioxa[4,7,10]triazacycloicosine-17-carboxylic acid as a white solid in 80% yield (14 mg). A sample of **15a** was purified for an extensive NMR study via preparative RP-HPLC powder in 20% yield (3.5 mg); IR (neat): 3311, 2933, 2871, 1636 cm^{-1}, ^1H NMR (700 MHz, CD_3CN):d 7.21 (d, J = 7.8 Hz, 1H)*, 5.72 (d, J = 8.0 Hz, 1H)*, 4.88 (m, 1H)*, 4.62 (m, 1H)*, 4.48 (m, 1H)*, 4.21 (m, 1H)*, 3.83 (dd, J = 4.9 Hz and 10.5 Hz, 1H)*, 3.74 (m, 1H)*, 3.68 (m, 1H)*, 3.67 (m, 1H)*, 3.61 (m, 1H)*, 3.53 (m, 3H), 3.48 (m, 1H)*, 3.43 (m, 1H)*, 3.39 (m, 1H)*, 2.34 (m, 1H)*, 2.17 (m, 1H)*, 2.04 (m, 1H)*, 1.93 (m, 1H)*, 1.86 (m, 1H)*, 1.84 (m, 1H)*, 1.83 (m, 2H)* ,1.54 (m, 1H), 1.49 (m, 2H), 1.46 (m, 1H) 1.41 (s, 9H)*; ^{13}C NMR (176.05 MHz, CD_3CN): d 173.9, 173.4, 172.3, 170.9, 80.8, 72.8, 72.1, 72.1, 71.7, 70.7, 61.4, 59.7, 55.2, 53.7, 49.1, 48.7, 33.9, 31.3, 31.0, 29.6, 29.2, 26.3, 24.2; HRMS Calcd for $[C_{27}H_{44}N_4O_9 + Na^+]$: 591.3000. Found 591.2994. (* Double signals due to rotamerism across the C-N bond of the amide. Only the resonance of the main isomer are given.)

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