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Turn conformations in a metallacyclictripeptide and a metallacyclictetrapeptide induced by tungsten–alkyne coordination

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Abstract

Tungsten–alkyne coordination was employed for the preparation of a metallacyclictripeptide and a metallacyclictetrapeptide. First, the dialkynyltripeptide **7** and the dialkynyltetrapeptide **9** were prepared using solution phase peptide synthesis. In both **7** and **9** the two alkynes were attached to the peptide at the N- and C-termini. Reaction of **7** with $W(CO)_3(dmtc)_2$ yielded the metallacyclictripeptide **10**, while reaction of **9** with $W(CO)_3(dmtc)_2$ yielded the metallacyclictetrapeptide **11**. The identities of **10** and **11** were established using ^1H NMR spectroscopy and positive ion electrospray mass spectrometry. The ^1H NMR spectra of **10** and **11** show that these species exist as a complex mixture of isomers that differ in how the ligands are arranged around the tungsten center. Interconversion between these isomers is slow at 23 °C, but the rate of interconversion increases as the temperature is raised. With **10**, all the alkyne hydrogen signals coalesce at 95 °C. However, with **11** the alkyne hydrogen signals do not fully coalesce, even at 105 °C. Related to this is the behavior of **10** and **11** during HPLC analysis; **10** elutes as a single peak, but **11** elutes as two overlapping peaks. The overlapping peaks and the high isomer interconversion temperature of **11** as compared to **10** are attributed to the presence of additional intramolecular hydrogen bonding interactions between the two ends of the peptide in **11**.

Keywords: Tungsten; Alkynes; Metallacyclicpeptide; Conformation; Turns

1. Introduction

Because linear peptides can adopt many different solution conformations, it often has been difficult to decipher the active conformations of bioactive peptides. One approach used to uncover the relationship between structure and activity in a peptide is the synthesis and evaluation of cyclicpeptides [1]. Judicious cyclization of the linear peptide will often severely limit the conformational freedom of the molecule. Thus, knowing the biological activity and the conformational limits of a cyclicpeptide can provide the structure–activity relationships that are difficult to obtain with the linear peptide [1].

In theory, there are innumerable ways in which peptides can be cyclized. To date, most of the cyclization methods employed have involved the formation of covalent links between two parts of the peptide [1]. An alternative to the use of covalent bonds is the use of metal–ligand interactions; cyclicpeptides generated using this chemistry are metallacyclicpeptides, since one of the atoms in the ring is a metal atom [2]. In a recently published work, we have demonstrated that tungsten-bis(alkyne) complexes [3] can be made from amino acid derivatives bearing alkyne groups [4]; also, we have described a novel method for generating cyclicpeptides that utilizes this tungsten–alkyne coordination chemistry [2].

The initial studies on metallacyclicpeptides that incorporate tungsten focused on exploration of the limits of the method. It was found that cyclization only occurred when the two alkyne groups could be parallel to each other and spaced approximately 6.5 Å apart. Thus, cyclization was

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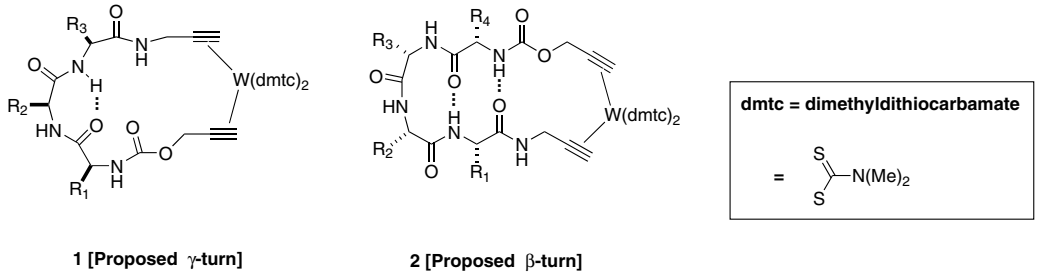
achieved with a single amino acid and a dipeptide with alkynes appended to their amine and carboxyl termini. The metallacyclicpeptides derived from these cyclizations were found to adopt a wide range of conformations associated with the location of the alkynes relative to the tungsten center [2].

In the present study, the initial research with these metallacyclicpeptides has been extended to the cyclizations of a tri- and tetrapeptide bearing alkyne ligands at their amine and carboxyl termini. It was thought that cyclization of the tripeptide might induce a γ-turn conformation [5–7] in the peptide, while cyclization of the tetrapeptide might generate a β-turn conformation [8,9] (see Scheme 1). This paper reports the synthesis and conformational behavior of these novel cyclicpeptides.

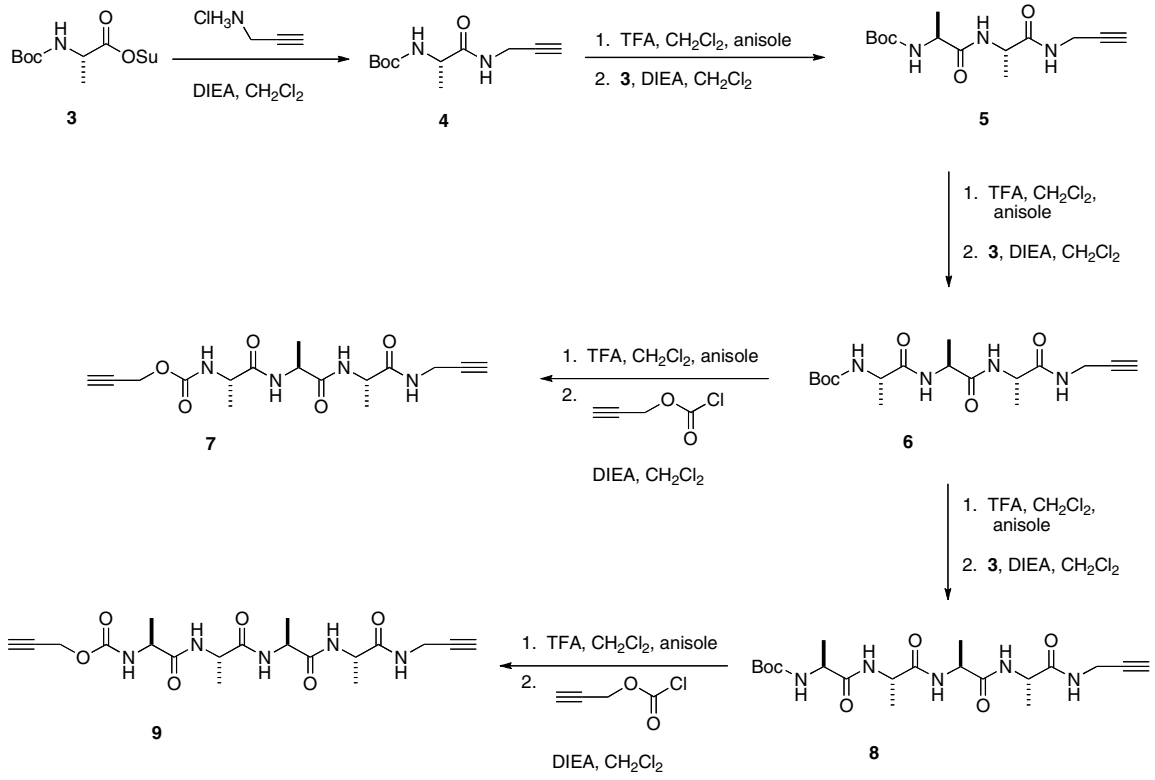
2. Results and discussion

2.1. Synthesis of dialkynyltripeptide 7 and dialkynyltetrapeptide 9

The dialkynylpeptides required for this study were **7** and **9**. They were prepared using solution phase peptide synthesis as outlined in Scheme 2. To start, Boc-Ala-OSu, **3**, was converted to **4** by treatment with propargylamine. Addition of the second, third and fourth amino acids was achieved in an iterative process in which the Boc protecting group was removed from the starting compound (either **4**, **5** or **6**) using trifluoroacetic acid, then the resulting amine salt was reacted under basic conditions with another molecule of **3**. Attachment of the second alkyne to **6** and **8** was



Scheme 1.



Scheme 2.

achieved by removal of the Boc group using trifluoroacetic acid, followed by treatment of the amine salt with propargyl chloroformate under basic conditions. Thus, **6** was converted to the dialkynyltripeptide **7**, while **8** was converted to the dialkynyltetrapeptide **9**. The peptide products here were purified to homogeneity by recrystallization; all were characterized by ^{1}H NMR and positive ion electrospray mass spectrometry (ESMS).

2.2. Cyclization of 7 by reaction with $W(CO)_3(dmtc)_2$

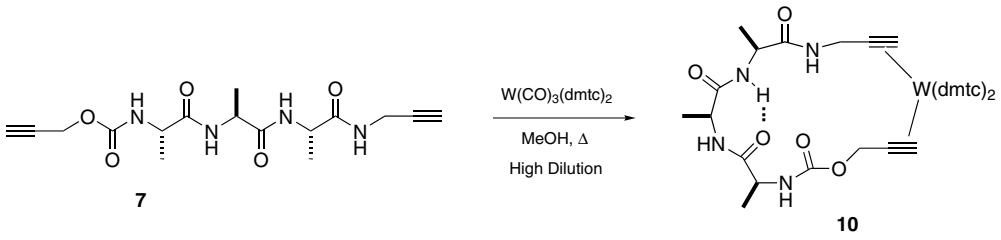
Dialkynyltrzipeptide **7** was reacted with $W(CO)_3(dmtc)_2$ [10] under high dilution conditions in an effort to form the desired metallacyclization product, **10** (Scheme 3). Addition of the $W(CO)_3(dmtc)_2$ to a refluxing, 1 mM solution of **7** in MeOH initially generated a light green solution, indicating formation of monoalkyne complexes [11]. On further heating, the reaction solution turned yellow, indicating formation of a bis-alkyne complex [12,13]. Workup of the reaction and analysis by TLC showed the presence of several yellow spots, with one of the spots being more intense than the others. This compound was isolated and purified to homogeneity by flash chromatography. Analysis of the amorphous, yellow solid showed it to be the desired metallacyclicpeptide, **10**. LC–MS of the purified product produced a chromatogram with one peak (Fig. 1); the UV–visible spectrum and ESMS of this peak were consistent with it being a tungsten bis(alkyne) complex [12,13]. The UV–visible spectrum showed an absorbance maximum at 270 nm and a shoulder at 320 nm. The ESMS [14,15] of the pure product produced a series of peaks centered around 797 *m*/*z*, which matched the isotope pattern peaks for the M+Na ion for **10**. The ^{1}H NMR spectrum also possessed all the resonances for the hydrogens expected in **10**, including the alkyne hydrogens at 11 ppm, the *N*-methyl hydrogens from the dithiocarbamates located in the 3.0–3.5 ppm region, and the alanine methyls located around 1.0–1.4 ppm.

2.3. Conformational analysis of metallacyclictripeptide *10*

As noted in previous work [2], metallacyclicpeptides like **10** can possess extensive conformational flexibility with regards to the orientation of the two alkynes and the two dmtc ligands to the tungsten. In the case of **10**, there are eight different possible orientations (**10A**–**H**, Scheme 4). Isomers **10A**–**D** possess one of the two possible orientations of the dmtc ligands; isomers **10E**–**H** possess the other. In addition, there are two possible orientations for the two alkyne groups in **10**; they can be either anti to each other (indicated by those isomers that have their alkyne C–H bonds pointing in opposite directions, **10A**, **10B**, **10E**, **10F**) or they can be syn to each other (indicated by those isomers that have their alkyne C–H bonds pointing in the same direction, **10C**, **10D**, **10G**, **10H**).

The number of isomers in **10** can be gleaned from inspection of the ^{1}H NMR signals for the alkyne hydrogens. These resonances appear around 11 ppm. Because they are far removed from other resonances in the molecule, and because they appear as singlets, simply counting the number of peaks in this region of the ^{1}H NMR provides an indication of the number of isomers present in solution. Given that each isomer shown in Scheme 5 should possess two different alkyne hydrogens, it was expected that each isomer would give rise to two singlets. Thus, if **10** adopts all eight possible orientations in solution, then 16 singlets should be observed around 11 ppm in the ^{1}H NMR spectrum.

Shown in Fig. 2 is the region around 11 ppm for the ^{1}H NMR spectrum of **10** in $CDCl_3$. As can be seen, nine singlets are visible in this region, indicating that at least five isomers are present in solution. The close proximity of the



Scheme 3.

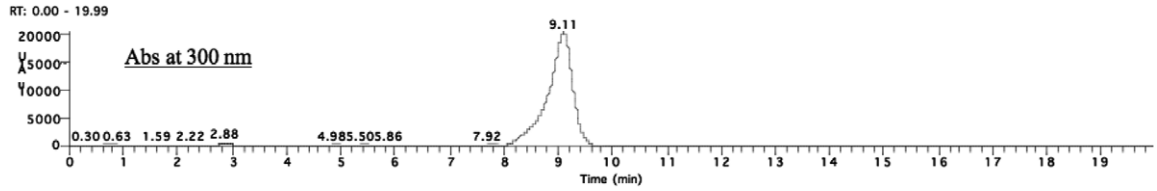
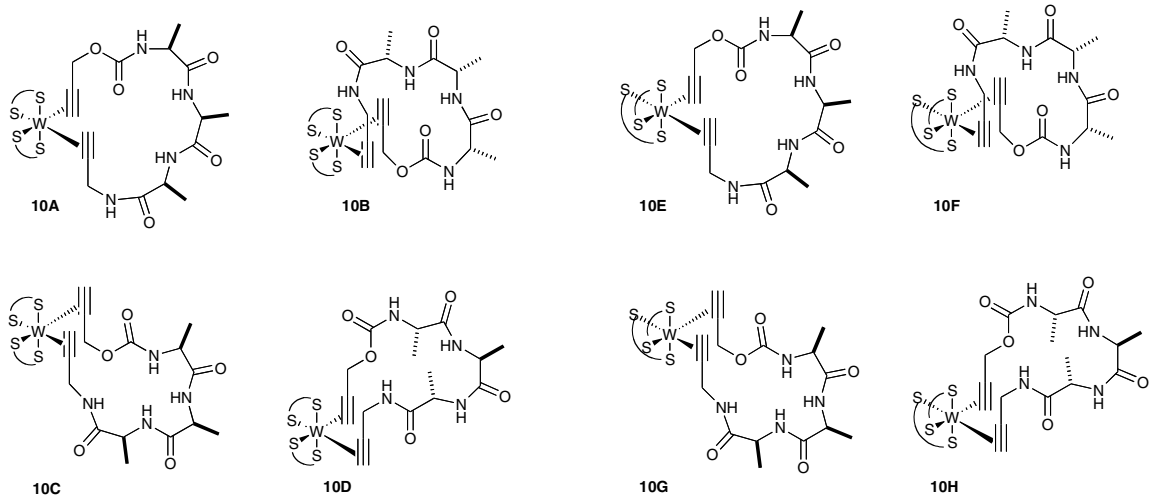
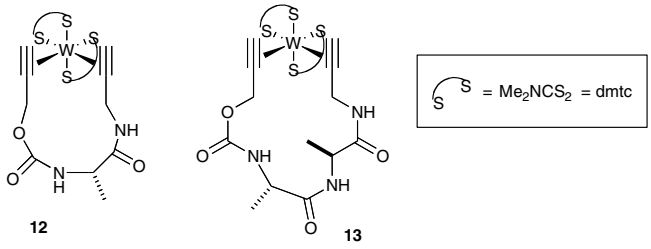


Fig. 1. HPLC trace of purified **10** showing a single peak at 9.11 min.



Scheme 4.



Scheme 5.

visible peaks makes it likely that some singlets are overlapping, meaning that it is possible that more than five isomers are present.

Are these various isomers in equilibrium, or are they frozen in the orientation in which they were formed? This question can be answered by heating the sample and recording its ^{1}H NMR spectrum at the various temperatures. If the alkyne hydrogen signals coalesce as the temperature is raised, this indicates that the isomers can equilibrate; if the signals do not coalesce, then this indicates that the isomers do not equilibrate. From prior work, it was found that the isomers of metallacyclicpeptide **12** do not equilibrate, but that the isomers of metallacyclicpeptide **13** do equilibrate (Scheme 5) [2].

Shown in Fig. 3 is the alkyne hydrogen region for **10** in d_6-DMSO at temperatures ranging from 25 to 95 °C. As the temperature is raised the various singlets gradually coalesce into one peak located at 11.1 ppm. Coalescence of the signals occurs at 95 °C. This data indicate that the isomers of **10** can and do equilibrate. To be sure that the coalescence of the peaks seen in this experiment was due to rapid equilibration of the various isomers and not from degradation of the complex, the sample was cooled back to 25 °C and the ^{1}H NMR spectrum recorded again. The spectra at 25 °C, before and after elevation of the temperature to

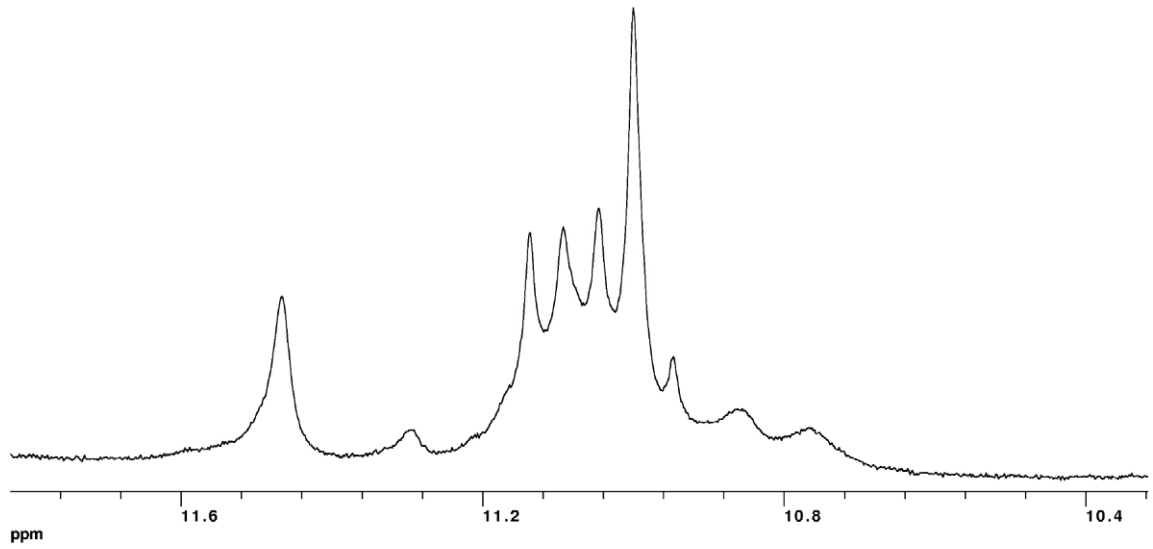


Fig. 2. Magnification of the alkyne hydrogen region in the ^{1}H NMR spectrum of **10** in $CDCl_3$.

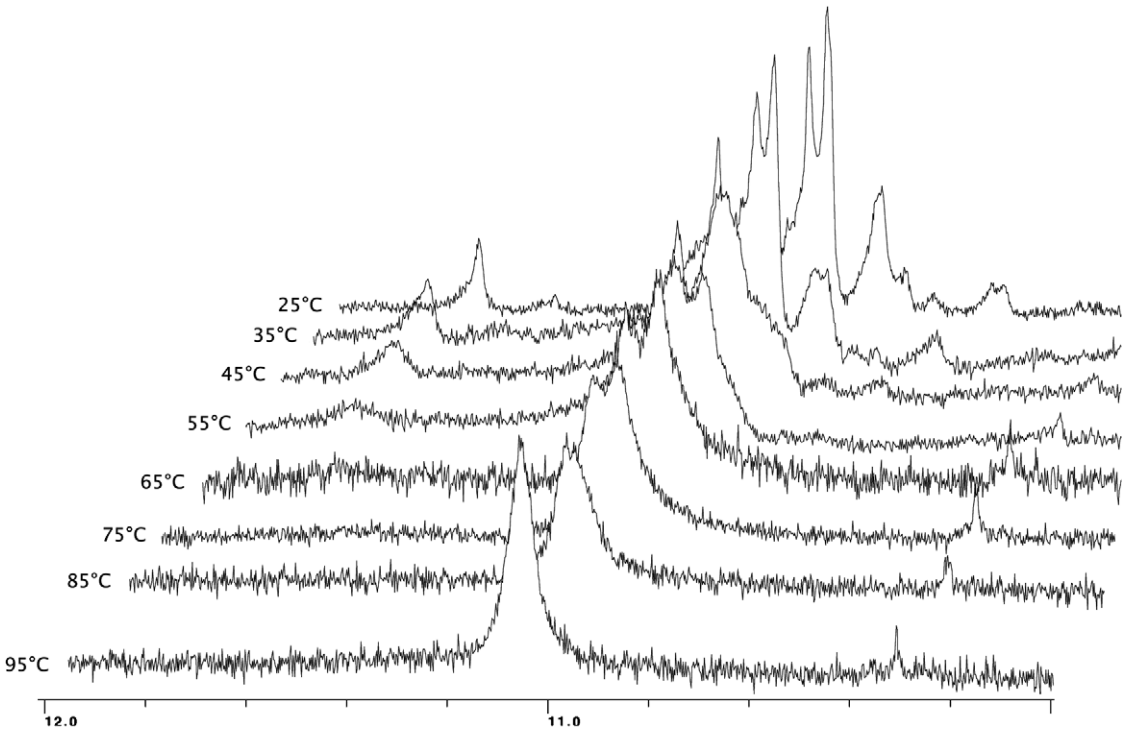


Fig. 3. Variable temperature ^{1}H NMR spectra of **10** from 25 °C to 95 °C. Only the terminal alkyne hydrogen region is shown. Convergence to a single peak is seen at 95 °C.

95 °C, were identical, indicating that the sample had not degraded during the course of the variable temperature experiment. Thus, the mixture of isomers obtained in the synthesis of **10** is the thermodynamic mixture of products.

To probe whether any of the amide NH protons in **10** are involved in an intramolecular hydrogen bond, a DMSO titration experiment was performed [16–21]. In this experiment, a solution of **10** in $CDCl_3$ was prepared and the ^{1}H NMR spectrum of the solution was recorded. Small increments of d_6-DMSO were then added to this solution, and the ^{1}H NMR spectrum of the new solution recorded. DMSO is a strong hydrogen bond acceptor, and its introduction to a $CDCl_3$ solution will typically cause a large change in chemical shift for amide NH protons exposed to the solvent; in contrast, amide NH protons involved in an intramolecular hydrogen bond tend to experience little or no effect from the addition of the DMSO [16–21]. Thus, the change in chemical shift of an amide NH proton upon addition of d_6-DMSO to a $CDCl_3$ solution of a peptide can be used to identify the presence of intramolecular hydrogen bonds.

Shown in Fig. 4 is the DMSO titration data for three NH proton signals that were readily visible in the ^{1}H NMR spectrum of **10**. The three signals initially located at 7.8, 6.2 and 6.1 ppm arise from one of the major conformers of **10**. The NH peak initially located at 7.8 ppm shows only a small change in chemical shift (0.6 ppm) over the course of the DMSO titration, indicating that it is likely involved in an intramolecular hydrogen bond. Amide NH protons that are involved in intramolecular hydrogen bonds also appear deshielded in the ^{1}H NMR spectrum, so the location of this proton at 7.8 ppm also indicates that this NH is part of an intramolecular hydrogen bond [21]. In contrast, the NH peaks initially located at 6.2 and 6.0 ppm would not be expected to participate in an intramolecular hydrogen bond based on their chemical shifts in $CDCl_3$; this conclusion is confirmed by their behavior in the

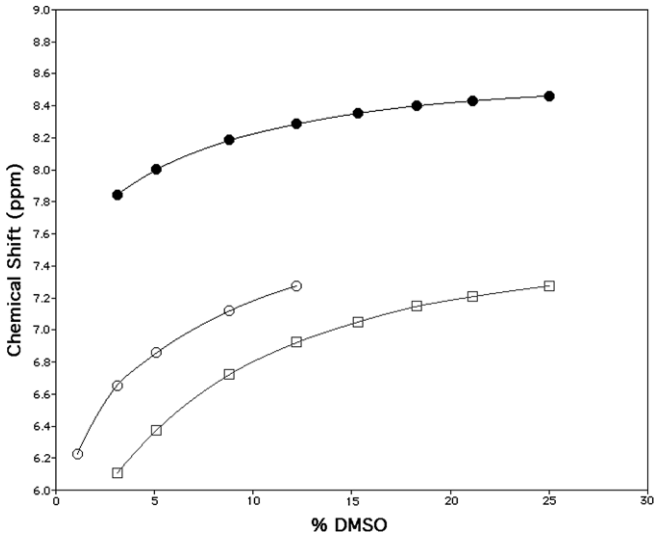


Fig. 4. DMSO titration data for three visible amide NH peaks in **10**. The peak that begins at 7.8 ppm (●) only changes its chemical shift by 0.6 ppm, while the two peaks that start at 6.2 ppm (○) and 6.1 ppm (□) change their chemical shifts over 1.0 ppm.

DMSO titration which showed a significant change in chemical shift (>1.0 ppm) over the course of the experiment. Although it is not possible to assign these particular resonances to specific amide NH protons in **10**, the DMSO titration data would be consistent with the peptide in one of the major conformers of **10** adopting a γ-turn conformation (see structure **1**). Unfortunately, because of signal overlap, it was not possible to determine whether other conformational isomers of **10** also possess an intramolecular hydrogen bond.

2.4. Cyclization of 9 by reaction with $W(CO)_3(dmtc)_2$

Dialkynyltetrapeptide **9** was reacted with $W(CO)_3(dmtc)_2$ [10] under high dilution conditions in an effort to form the desired metallacyclization product, **11** (Scheme 6). Addition of the $W(CO)_3(dmtc)_2$ to a refluxing, 1 mM solution of **9** in MeOH initially generated a light green solution, indicating formation of monoalkyne complexes [11]. On further heating the reaction solution turned yellow, indicating formation of a bis-alkyne complex [12,13]. Workup of the reaction and analysis by TLC showed the formation of several yellow spots. One of the major yellow spots was isolated and purified to homogeneity by flash chromatography. Analysis of the amorphous, yellow solid showed it to be the desired metallacyclicpeptide, **11**. Analysis by TLC showed it to be one spot. The ^{1}H NMR spectrum also possessed all the resonances for the hydrogens expected in **11**, including the alkyne hydrogens at 11 ppm, the *N*-methyl hydrogens from the dithiocarbamates located in the 3.0–3.5 ppm region, and the alanine methyls located in the 1.0–1.4 ppm region.

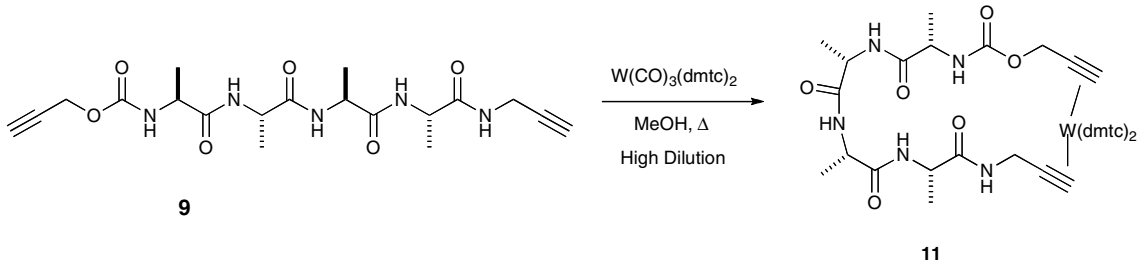
Metallacyclictetrapeptide **11** was also analyzed by LC–MS. The chromatogram is shown in Fig. 5. Instead of getting the expected single peak, two overlapping peaks located at 8.46 and 8.83 min were obtained. The UV–visible spectra of these two peaks, and the ESMS of the two peaks, shows that they both possess the expected molecular weight and absorbance properties for **11**. This means that the two overlapping peaks in this chromatogram arise from two isomeric forms of **11** that do not readily interconvert during the timeframe of the chromatographic analysis. The difference in the chromatograms of **10** and **11** suggest that the various conformational isomers of **10** readily interconvert, but that this is not the case with the various conformational isomers of **11**.

2.5. Conformational analysis of metallacyclictetrapeptide 11

Like **10**, metallacyclicpeptide **11** can potentially assume eight different orientations as illustrated in Scheme 7 (**11A**–**H**). Isomers **11A**–**D** possess one of the two possible orientations of the dmtc ligands; isomers **11E**–**H** possess the other. In addition, there are two possible orientations for the two alkyne groups in **11**; they can be either anti to each other (indicated by those isomers that have their alkyne C–H bonds pointing in opposite directions, **11A**, **11B**, **11E**, **11F**) or they can be *syn* to each other (indicated by those isomers that have their alkyne C–H bonds pointing in the same direction, **11C**, **11D**, **11G**, **11H**). The HPLC data for **11** (Fig. 5) suggest that at least two isomers are present and that they do not readily interconvert at 23 °C.

As with **10**, the number of isomers in **11** can be gleaned from inspection of the ^{1}H NMR signals for the alkyne hydrogens in the region around 11 ppm. Since each isomer shown in Scheme 7 should possess two different alkyne hydrogens, it is expected that each isomer should give rise to two singlets. Thus, if **11** adopts all eight possible orientations in solution, then 16 singlets should be observed.

Shown in Fig. 6 is the region around 11 ppm in the ^{1}H NMR spectrum of **11** in d_6-acetone; $CDCl_3$ could not be used because **11** is insoluble in this solvent. As can be seen, 10 singlets (which includes shoulders) are visible in this region, indicating that at least five isomers are present in



Scheme 6.

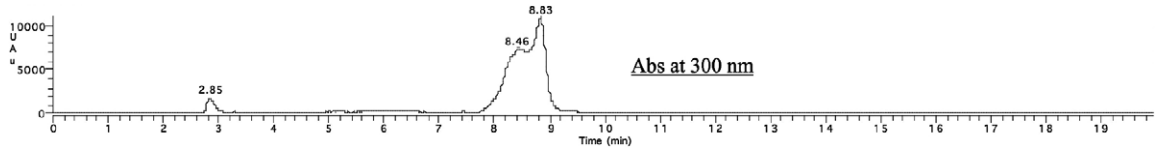
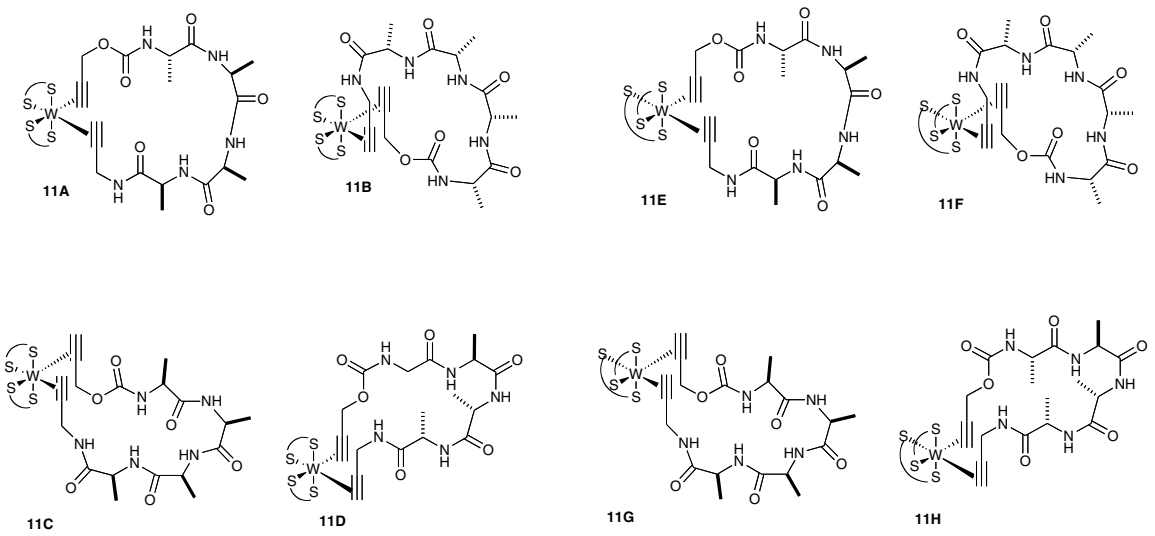


Fig. 5. HPLC trace of purified **11**. The chromatogram shows two overlapping peaks located at 8.46 and 8.83 min. Both peaks produced identical UV–visible spectra and positive ion ESMS.



Scheme 7.

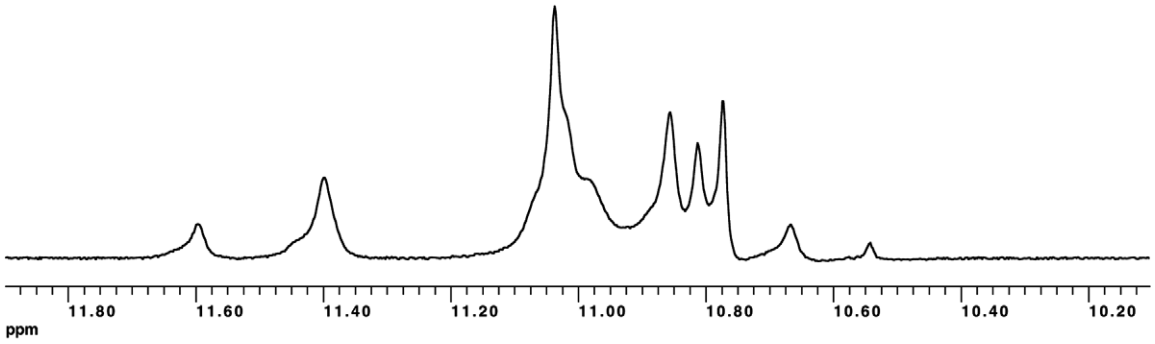


Fig. 6. Magnification of the alkyne hydrogen region in the ^{1}H NMR spectrum of **11** in d_6-acetone.

solution. The close proximity of the visible peaks, and the obvious shoulders for the main peak at 11.0 ppm makes it likely that some singlets are overlapping, meaning that it is not possible to count all the isomers present; what can be known is that, at a minimum, at least five of the eight possible isomers are present.

To determine if these isomers are in equilibrium, the ^{1}H NMR spectrum of **11** was recorded at varying, elevated temperatures. Unlike **10**, where all the alkyne resonances coalesced at 95 °C, the alkyne resonances in **11** never fully coalesce, even when the temperature of the complex is raised to 105 °C. Shown in Fig. 7 is the alkyne hydrogen region in the ^{1}H NMR spectra of **11** in d_6-DMSO at temperatures ranging from 25 to 105 °C. When **11** is heated to 95 °C or above, the alkyne hydrogen resonances appear as three singlets, and above 95 °C they do not appear to be converging on each other. The three singlets seen at 95 °C are likely to be four singlets, with two of the singlets overlapping each other. The presence of four singlets at 95 °C means that at this temperature **11** adopts two major orientations, and that these two orientations are not in rapid equilibrium with each other up to 105 °C. The behavior is mirrored in the HPLC behavior of **11** where two overlapping peaks are observed (Fig. 5).

An alternate explanation for the alkyne hydrogens coalescing to three singlets at 95 °C is that the sample decomposed upon heating. To test for this possibility, the sample used in this experiment was returned to 25 °C after it had been heated to 105 °C. The ^{1}H NMR spectrum of **11** at 25 °C was the same, before and after heating to 105 °C, indicating that the complex did not undergo decomposition during the variable temperature experiment. Thus, the coalescence to three singlets must arise from **11** adopting two distinctly different conformations – two conformations that do not readily interconvert.

2.6. Comparison of *10*, *11* and *12*

At first glance the difference in the temperature of coalescence between metallacyclictripeptide **10** and metallacyclictetrapeptide **11** seems counterintuitive; one would think that the larger ring and greater conformational freedom present in **11** would make it easier for the various isomers to interconvert, not harder. However, the reverse is true; it

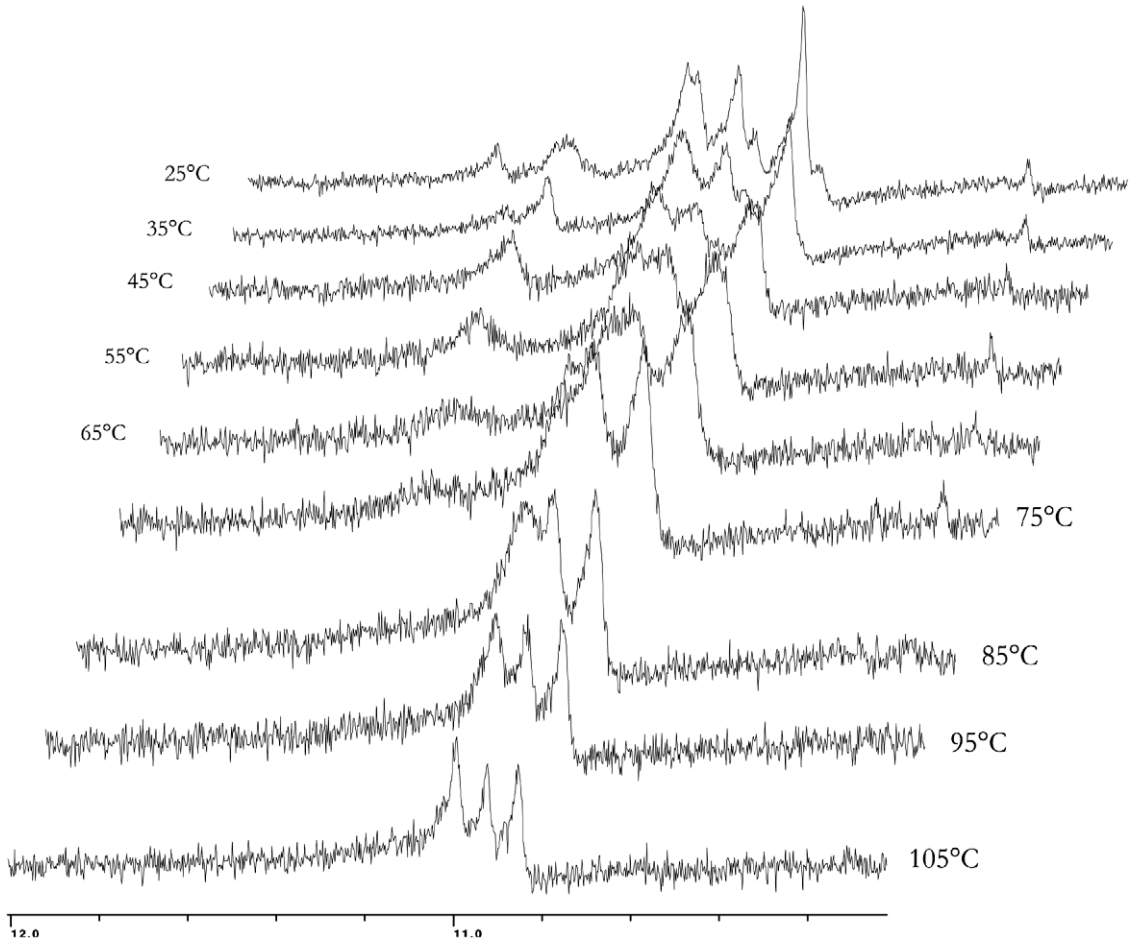


Fig. 7. Variable temperature ^{1}H NMR spectra of **11** in d_6-DMSO from 25 °C to 105 °C. Only the terminal alkyne hydrogen region is shown. Convergence to a single peak is not seen.

is harder to interconvert the isomers in **11** than in **10**. To see if this is a general trend, the behavior of **10** and **11** was compared to the behavior of the previously studied metallacyclicdipeptide **12** [2].

Of these three species, metallacyclicdipeptide **12** possesses the lowest temperature of coalescence at approximately 65 °C [2]. The next highest temperature is for metallacyclictripeptide **10** at 95 °C (Fig. 3). Lastly, metallacyclictetrapeptide **11** has the highest temperature, which is >105 °C (Fig. 7). Thus, the trend here is for the temperature of coalescence to increase as amino acid residues are added to the peptide chain. The data show that addition of amino acid residues to the peptide chain introduces a new energy barrier for interconversion between the various isomers in these metallacyclicpeptides.

What is the source of this energy barrier? One possible explanation is that there are intramolecular hydrogen bonds between the amide carbonyls and amide NH protons in metallacyclictripeptide **10** and metallacyclictetrapeptide **11** which are not present in metallacyclicdipeptide **12**. Dreiding models of the dialkynyldipeptide that is coordinated to tungsten in **12** show that it is nearly impossible to position the two alkynes parallel to each other and spaced 6.5 Å apart and to align an amide carbonyl and amide NH so that an intramolecular hydrogen bond can form. However, Dreiding models of the peptide components of **10** and **11** indicate that it is possible for these species to adopt conformations that possess intramolecular hydrogen bonds. With metallacyclictripeptide **10** the most likely conformation is a γ-turn, while for metallacyclictetrapeptide **11** the most likely conformation is a β-turn (structures **1** and **2** in Scheme 1). The β-turn conformation for the metallacyclictetrapeptide (**2**) would possess two intramolecular hydrogen bonds, while the γ-turn conformation for the metallacyclictripeptide (**1**) would only possess one intramolecular hydrogen bond. This difference in the number of intramolecular hydrogen bonds could be the source of the difference in temperature of coalescence between **10** and **11** because equilibration between the various isomers would require energy to overcome the barrier for alkyne rotation and for disruption of existing hydrogen bonds.

Does the peptide in **10** adopt a γ-turn conformation? Does the peptide in **11** adopt a β-turn conformation? Or do they adopt other turn structures? At present, it is not possible to answer these questions. The most direct way to get an answer would be to obtain an X-ray crystal structure of

10 and/or **11**. However, **10** and **11** (and **12** and **13**) are all amorphous solids that have not shown any propensity to crystallize. Alternatively, the solution structure of a peptide can often be solved by employing a variety of NMR experiments on reporter resonances in the peptide [22]. For example, one can use the Karplus equation to calculate dihedral angles based on coupling constants of the amide NH and their neighboring CH protons [23]. However, with **10** and **11** this approach is nearly impossible given the large number of solution conformations adopted by these complexes. If a complex adopts all eight of the possible conformations, then each NH and CH proton will produce eight different resonances. This large number of resonances is responsible for the complexity of the NMR spectra of **10** and **11**, which possess significant signal overlap, making it nearly impossible to extract the coupling constant values. It is the overlap of these multiple signals that makes conformational analysis of the peptide using ^{1}H NMR spectroscopy a difficult assignment.

Although at present the γ- and β-turn conformations in **10** and **11** cannot be confirmed, the evidence from the temperature of coalescence experiments and the DMSO titration experiments does suggest that these species assume turn conformations that feature intramolecular hydrogen bonds.

2.7. Summary

A metallacyclictripeptide **10** and a metallacyclictetrapeptide **11** that are tungsten-bis(alkyne) complexes have been prepared and purified. Both **10** and **11** assume a variety of different conformational isomers in solution. Temperature of coalescence experiments with **10** and **11** showed that interconversion between these isomers does occur, but that it is easier for the metallacyclictripeptide **10** to interconvert between its isomers that it is for metallacyclictetrapeptide **11**. A DMSO titration of **10** showed that this species possesses at least one NH proton (from one of the major conformational isomers) that is involved in an intramolecular hydrogen bond. Taken together the data indicate that **10** and **11** adopt turn conformations in solution, with the data being consistent with a γ-turn for **10** and a β-turn for **11**. Efforts to develop methods for probing the nature and conformation of these turns in **10** and **11** are underway.

3. Experimental

3.1. General procedures

Boc-Ala-OSu was purchased from ChemImpex International. Propargylchloroformate, propargylamine hydrochloride, anisole, and DMF (dimethylformamide) were purchased from Sigma–Aldrich. TFA, DIEA, and THF were purchased from Acros Organics. $CDCl_3$, d_6-acetone and d_6-DMSO were purchased from Cambridge Isotope Labs. Silica gel for flash chromatography was purchased from Silicycle. NMR spectra were obtained on a GE Omega 300 instrument. Electrospray mass spectra were obtained on a LCQ APCI/Electrospray LC MS–MS. Samples for mass spectral analysis were dissolved in MeOH (approximately 1 mg/mL) in borosilicate glass test tubes. Distance measurements using Dreiding models were done utilizing a ruler calibrated to convert distances in the models (in cm) to molecular distances (in Å).

3.2. Preparation of *4*

To a solution of 3.252 g (11.36 mmol) of Boc-Ala-OSu (**3**) and 1.300 g (14.20 mmol) of propargylamine hydrochloride in 70 mL CH_2Cl_2 was added 4.4 g (34 mmol) of DIEA. The resulting clear solution produced a white precipitate as it stirred at 22 °C for 13 h. The CH_2Cl_2 was evaporated and the residue that remained was partitioned between 100 mL EtOAc and 100 mL 1 M HCl. The layers were separated and the organic layer was washed 2×100 mL 1 M HCl, 3×100 mL saturated $NaHCO_3$, and 1×100 mL brine. The organic layer was dried with $MgSO_4$, filtered, and evaporated to give 1.893 g (74%) of pure **4** as a white solid: TLC, R_f 0.70 (EtOAc); ^{1}H NMR: (300 MHz, $CDCl_3$) δ 6.50 ppm (NH,1H, br s), 4.96 ppm (NH,1H, br s), 4.18 (Ala methine H, 1H, m), 4.05 ppm (H—C≡C—CH_2, 2H, m), 2.23 ppm (H—C≡C, 1H, t, $J = 2.7$ Hz), 1.46 ppm ($3 \times CH_3$, 9H, s), 1.37 ppm (Ala CH_3, 3H, d, $J = 3.4$ Hz); ^{13}C NMR: (75 MHz, $CDCl_3$) δ 172.30 ppm (amide C=O), 155.57 ppm (carbamate C=O), 80.41 ppm (O–*C*–$(CH_3)_3$), 79.35 ppm (CH_2—*C*≡C—H), 71.63 ppm (CH_2—C≡*C*—H), 50.27 ppm (Ala methine C), 29.21 ppm (*C*H_2—C≡C—H), 28.33 ppm ($3 \times CH_3$), 18.06 ppm (Ala CH_3); ESMS: M+Na ion calculated for $C_{11}H_{18}N_2O_3Na$ [24], 249 *m*/*z*; found 248.9 *m*/*z*.

3.3. Preparation of *5*

To a solution of 1.893 g (8.37 mmol) of **4** in 20 mL CH_2Cl_2 and 2 mL of anisole at 0 °C was added 7.5 mL of trifluoroacetic acid. The resulting solution stirred for 1 h at 0 °C, then the solvents were evaporated. The residue that remained was exposed to high vacuum for 2 h in order to remove the last traces of solvent. The crude amine salt was suspended in a solution of 10 mL tetrahydrofuran (THF) and 10 mL dimethylformamide (DMF). To this suspension was added 2.327 g (8.37 mmol) of **3** followed by 8.4 mL (67 mmol) of DIEA. Initially, a clear solution was obtained; within several minutes this solution produced a white precipitate. After stirring for 12.5 h at 22 °C, the solvents were evaporated. The residue that remained was resuspended in EtOAc. The suspension was chilled to 0 °C, and the undissolved white solid collected by vacuum filtration. The collected solid was then recrystallized from EtOAc (375 mL). A second crop of **5** was obtained by reducing the volume of the filtrate to 35 mL and then performing a second recrystallization. In this

way, 1.679 g (67%) of pure **5** was obtained as a white solid: ^{1}H NMR: (300 MHz, DMSO) δ 8.31 ppm (NH, 1H, br t), 7.85 ppm (NH, 1H, d, $J = 3.9$ Hz), 6.99 ppm (NH, 1H, d, $J = 3.7$ Hz), 4.23 ppm (Ala methine H, 1H, m), 3.94 ppm (Ala methine H, 1H, m), 3.85 ppm (H—C≡C—CH_2, 2H, m), 3.12 ppm (H—C≡C, 1H, s), 1.37 ppm ($3 \times CH_3$, 9H, s), 1.17 ppm (Ala $2 \times CH_3$, 6H, m); ^{13}C NMR: (75 MHz, DMSO) δ 173.75 ppm (amide C=O), 173.33 ppm (amide C=O), 156.63 ppm (carbamate C=O), 82.35 ppm (O–*C*–$(CH_3)_3$), 79.64 ppm (CH_2—*C*≡C–H), 74.50 ppm (CH_2—C≡*C*—H), 51.27 ppm (Ala methine C), 49.33 ppm (Ala methine C), 29.69 ppm ($3 \times CH_3$), 29.46 ppm (*C*H_2—C≡C—H), 19.80 ppm (Ala CH_3), 19.45 ppm (Ala CH_3); ESMS: M+Na ion calculated for $C_{14}H_{23}N_3O_4Na$ [24], 320 *m*/*z*; found 319.8 *m*/*z*.

3.4. Preparation of 6

To a solution of 1.679 g of **5** (5.65 mmol) in 20 mL of CH_2Cl_2 and 1.6 mL of anisole at 0 °C was added 7 mL of trifluoroacetic acid. The resulting solution stirred for 1 h at 0 °C, then the solvents were evaporated. The residue that remained was exposed to high vacuum for 2 h in order to remove the last traces of solvent. The crude amine salt was suspended in a solution of 10 mL tetrahydrofuran (THF) and 10 mL dimethylformamide (DMF). To this suspension was added 1.778 g (6.21 mmol) of **3** followed by 7.5 mL (45 mmol) of DIEA. Initially, a clear solution was obtained; within several minutes this solution produced a white precipitate. After stirring for 13.5 h at 22 °C, the solvents were evaporated. The residue that remained was resuspended in CH_2Cl_2 and vacuum filtered to give brittle off-white flakes. This solid was recrystallized from MeOH (170 mL) to yield 1.602 g (77%) of pure **6** as a white solid: ^{1}H NMR: (300 MHz, d_6-DMSO) δ 8.28 ppm (NH, 1H, br t, $J = 5.1$ Hz), 7.96 ppm (NH, 1H, d, $J = 3.7$ Hz), 7.87 ppm (NH, 1H, d, $J = 3.7$ Hz), 6.99 ppm (NH, 1H, d, $J = 3.7$ Hz), 4.23 ppm (Ala methine 2xH, 2H, m), 3.94 ppm (Ala methine H, 1H, m), 3.85 ppm (H—C≡C—CH_2, 2H, m), 3.12 ppm (H—C≡C, 1H, t, $J = 2.0$ Hz), 1.37 ppm ($3 \times CH_3$, 9H, s), 1.18 ppm (Ala $3 \times CH_3$, 9H, m); ^{13}C NMR: (75 MHz, d_6-DMSO) δ 173.98 ppm (amide C=O), 173.17 ppm (amide C=O), 156.60 ppm (carbamate C=O), 82.42 ppm (O–*C*–$(CH_3)_3$), 79.61 ppm (CH_2—*C*≡C—H), 74.47 ppm (CH_2—C≡*C*—H), 51.24 ppm (Ala methine C), 49.49 ppm (Ala methine C), 49.43 ppm (Ala methine C), 29.66 ppm ($3 \times CH_3$), 29.46 ppm (*C*H_2—C≡C—H), 19.64 ppm (Ala CH_3), 19.45 ppm (Ala CH_3); ESMS: M+Na ion calculated for $C_{17}H_{28}N_4O_5Na$ [24], 391 *m*/*z*; found, 390.5 *m*/*z*.

3.5. Preparation of 7

To a solution of 0.721 g of **6** (1.96 mmol) in 8 mL of CH_2Cl_2 and 0.8 mL anisole at 0 °C was added 3 mL trifluoroacetic acid. After stirring 1 h at 0 °C the solvents were evaporated. The residue that remained was exposed to high vacuum for 2 h in order to remove the last traces of solvent. The crude amine salt was suspended in a solution of 6 mL THF and 6 mL DMF. The addition of 0.191 mL (1.96 mmol) of propargyl chloroformate followed by 4.0 mL of DIEA (23.5 mmol) produced a clear solution with a yellow tint. A white precipitate formed after 45 min. The reaction was allowed to stir for 20 h, then the solvents were evaporated, first under aspirator vacuum, then under high vacuum. The resulting white slurry was resuspended in CH_2Cl_2 and vacuum filtered to yield a brittle off-white solid. Analysis of the solid by ESMS and ^{1}H NMR indicated that unreacted **6** was still present. Therefore, the crude product was re-reacted using conditions exactly as above, except for the reaction time with the trifluoroacetic acid, which was lengthened to 1 h and 40 min. Following the second suspension in CH_2Cl_2 and subsequent filtration, 0.364 g (53%) of pure **7** was obtained as a white solid: ^{1}HNMR (pure product): (300 MHz, d_6-DMSO) δ 8.30 ppm (NH, 1H, br t, $J = 5.4$ Hz), 8.03 ppm (NH, H, d, $J = 3.7$ Hz), 7.91 ppm (NH, 1H, d, $J = 3.7$ Hz), 7.58 ppm (NH, 1H, d, $J = 3.7$ Hz), 4.61 ppm (C≡C—CH_2—O, 2H, s), 4.23 ppm ($2 \times$ Ala methine H, 2H, m), 4.03 ppm (Ala methine H, 1H, m), 3.85 ppm (C≡C—CH_2—N, 2H, m), 3.51 ppm (H—C≡C, 1H, br t), 3.13 ppm (H—C≡C, 1H, t, $J = 2.4$ Hz), 1.37 ppm ($3 \times CH_3$, 9H, s), 1.19 ppm (Ala $3 \times CH_3$, 9H, m); ESMS: M+Na ion calculated for $C_{16}H_{22}N_4O_5Na$ [24], 373 *m*/*z*; found, 372.8 *m*/*z*.

3.6. Preparation of 8

To a solution of 0.874 g (2.37 mmol) of **6** in 10 mL of CH_2Cl_2 and 1 mL of anisole at 0 °C was added 4.0 mL of trifluoroacetic acid. After stirring 1 h at 0 °C the solvents were evaporated. The residue that remained was exposed to high vacuum for 12 h in order to remove the last traces of solvent. The crude amine salt was suspended in a solution of 7 mL THF and 7 mL DMF. The addition of 0.679 g (2.37 mmol) of **3** followed by 5.0 mL of DIEA (29 mmol) produced a clear solution with a yellow tint. A white precipitate formed quickly after addition of the DIEA. An additional 5 mL of DMF and 10 mL of THF were added to the solution to facilitate stirring. After 18 h the solvents were evaporated. The residue that remained was resuspended in 15 mL CH_2Cl_2 and the undissolved solid collected by vacuum filtration to yield 0.896 g (86%) of pure **8** as a white solid: ^{1}H NMR: (300 MHz, d_6-DMSO) δ 8.28 ppm (NH, 1H, br t), 8.07–7.82 ppm ($3 \times$ NH, 3H, m), 6.99 ppm (NH, 1H, d, $J = 3.2$ Hz), 4.23 ppm ($3 \times$ Ala methine H, 3H, m), 3.93 ppm (Ala methine H, 1H, m), 3.85 ppm (C≡C—CH_2—N, 2H, m), 3.62 ppm (1H, m), 3.12 ppm (H—C≡C, 1H, br t), 1.37 ppm ($3 \times CH_3$, 9H, s), 1.20 ppm (Ala $4 \times CH_3$, 12H, m).

3.7. Preparation of 9

To a solution of 0.884 g of **8** (2.01 mmol) in 8.5 mL of CH_2Cl_2 and 0.85 mL anisole at 0 °C was added 4.0 mL

trifluoroacetic acid. After stirring 1 h at 0 °C the solvents were evaporated. The residue that remained was exposed to high vacuum for 12 h in order to remove the last traces of solvent. The crude amine salt was suspended in a solution of 7 mL THF and 7 mL DMF. The addition of 0.196 mL (2.01 mmol) of propargyl chloroformate followed by 4.5 mL of DIEA (24 mmol) produced a clear solution with a yellow tint. After stirring for 20 h the solvents were evaporated. The residue that remained was resuspended in 15 mL CH_2Cl_2 and the undissolved solid was collected by vacuum filtration. The crude solid was crushed in a mortar and pestle, then rinsed with 15 mL CH_2Cl_2. Analysis by ^1H NMR showed that the crude product was a mixture of unreacted **8** and the product **9**. Therefore, the crude product was re-reacted using conditions exactly as above, except for the reaction time with the propargyl chloroformate, which was lengthened to 13.5 h. Following the suspension of the crude product in CH_2Cl_2 and subsequent filtration, 0.322 g (38%) of pure **9** was obtained as a white solid: ^1H NMR: (300 MHz, d_6-DMSO) δ 8.28 ppm (NH, 1H, br t, $J = 5.1$ Hz), 8.03 ppm ($2\times$ NH, 1H, d, $J = 3.9$ Hz), 7.93 ppm ($2\times$ NH, 2H, m), 7.57 ppm (NH, 1H, d, $J = 3.7$ Hz), 4.60 ppm (H—C≡C—CH_2—O, 2H, s), 4.23 ppm ($3\times$ Ala methine H, 3H, m), 4.03 ppm (Ala methine H, 1H, m), 3.85 ppm (H—C≡C—CH_2—N, 2H, m), 3.50 ppm (H—C≡C, 1H, s), 3.12 ppm (H—C≡C, 1H, br t), 1.20 ppm (Ala $4\times CH_3$, 12H, m); ESMS: M+Na ion calculated for $C_{19}H_{27}N_5O_6Na$ [24], 444 *m*/*z*; found 444.0 *m*/*z*.

3.8. Cyclization of 7; preparation of 10

Under N_2, a solution of 0.122 g (0.348 mmol) of **7** in 348 mL degassed MeOH in a three-necked round bottom flask fitted with a reflux condenser and an addition funnel was brought to reflux. Next, 0.177 g (0.348 mmol) of $W(CO)_3(dmtc)_2$ in 26 mL degassed CH_2Cl_2 was added dropwise to the MeOH solution over 30 min via the addition funnel. The solution initially turned an emerald green color during the dropwise addition phase of the experiment (indicating the formation of a monoalkyne complex). Continued heating of the solution for 2.5 h caused the color to change to a lemon yellow (indicating the formation of a bisalkyne complex). The solvents were evaporated, and the crude product was purified by flash chromatography (1:9 MeOH:EtOAc). Evaporation of the appropriate fractions yielded 75 mg (28%) of pure **10** as a light yellow amorphous solid: TLC, R_f 0.34 (9:1 EtOAc/MeOH); ^1H NMR: (300 MHz, $CDCl_3$) δ 11.60–10.63 ppm ($2\times$ C≡C—H, 2H, m), 8.00–6.46 ppm ($4\times$ NH, 4H, m), 6.20–3.66 ppm ($3\times$ Ala methine H and $2\times$ CH_2—C≡C—H, 7H, m), 3.37 ppm ($2\times$ dmtc CH_3 6H, m), 3.11 ppm ($2\times$ dmtc CH_3, 6H, m), 1.91–1.07 ppm ($3\times$ Ala CH_3, 9H, m); ESMS: M+Na theoretical isotope pattern for $C_{22}H_{34}N_6O_5S_4WNa$ [24]: 794 (0.1%), 795 (64.5%), 796 (54.0%), 797 (100.0%), 798 (34.2%), 799 (90.2%), 800 (26.2%), 801 (18.2%), 802 (4.6%), 803 (1.7%); Found: 794 (1.3%), 795 (69.3%), 796 (55.99%), 797 (100.0%), 798 (33.68%), 799 (90.34%), 800 (23.9%), 801 (15.89%), 802 (3.36%), 803 (1.31%).

3.9. Cyclization of 9; preparation of 11

Under N_2, a solution of 0.156 g (0.370 mmol) of **9** in 370 mL degassed MeOH in a three-necked round bottom flask fitted with a reflux condenser and an addition funnel was brought to reflux. Next, 0.188 g (0.370 mmol) of $W(CO)_3(dmtc)_2$ in 28 mL degassed CH_2Cl_2 was added dropwise to the MeOH solution over 20 min via the addition funnel. The solution initially turned an emerald green color during the dropwise addition phase of the experiment (indicating the formation of a monoalkyne complex). Continued heating of the solution for 2.0 h caused the color to change to a lemon yellow (indicating the formation of a bisalkyne complex). The solvents were evaporated, and the crude product was purified by flash chromatography (1:6.5 MeOH:EtOAc). Evaporation of the appropriate fractions yielded 72 mg (24%) of pure **11** as a light yellow amorphous solid: TLC, R_f 0.31 (7:1 EtOAc/MeOH); ^1H NMR (300 MHz, d_6-acetone): δ 11.65–10.68 ppm ($2\times$ C≡C—H, 2H, m), 8.14–6.83 ppm ($5\times$ NH, 5H, m), 6.24–3.96 ppm ($4\times$ Ala methine H and $2\times$ CH_2—C≡C—H, 8H, m), 3.43 ppm ($2\times$ dmtc CH_3 6H, m), 3.15 ppm ($2\times$ dmtc CH_3, 6H, m), 1.64–1.07 ppm ($4\times$ Ala CH_3, 12H, m); ESMS: M+H theoretical isotope pattern for $C_{25}H_{40}N_7O_6S_4W$ [24]: 842 (0.3%), 843 (0.1%), 844 (63.2%), 845 (55.2%), 846 (100.0%), 847 (37.2%), 848 (89.8%), 849 (29.0%), 850 (18.9%). Found: 842 (11.19%), 843 (31.92%), 844 (56.64%), 845 (68.09%), 846 (100.0%), 847 (53.94%), 848 (81.1%), 849 (25.62%), 850 (16.17%).

3.10. Variable temperature NMR

Samples were made by dissolving approximately 40–60 mg of either **10** or **11** in d_6-DMSO in an NMR tube. The spectrometer was set to the desired temperature and the sample was allowed to equilibrate at that temperature for a minimum of 5 min before the spectrum was collected. Spectra were taken at 10 °C intervals, starting at 25 °C. Once the spectrum at the maximum temperature had been recorded, the sample was cooled back to 25 °C, allowed to equilibrate to 25 °C, and the spectrum at 25 °C was retaken and compared to the first spectrum recorded at 25 °C.

3.11. DMSO titration of 10

Approximately 40–60 mg of **10** was dissolved in 750 μL of $CDCl_3$ in an NMR sample tube. A spectrum was obtained. The sample was then ejected, and a carefully measured quantity of d_6-DMSO was added via micropipette to the NMR tube. The tube was then inverted several times to ensure good homogeneity. The sample was then reinserted into the instrument and the spectrum recorded. This procedure was repeated for successive additions of d_6-DMSO until the percentage of DMSO reached 60.5%

(by volume). From concentrations of 0% to 18% DMSO the $CDCl_3$ was used as the lock signal; at concentrations above 18% DMSO the d_6-DMSO was used as the lock signal. The internal standard in these spectra was TMS.

3.12. LC–MS analysis of **10** and **11**

A Finnigan MAT LCQ APCI/Electrospray LC MS–MS system equipped with a Grace-Vydac C18 column (Model# 218TP54, 250 times 4.6 mm) was employed. The diode-array detector was set to obtain frequent (2 scans/s) full spectra from 200.0 to 768.0 nm. The solvents used in the mobile phase were 0.1% trifluoroacetic acid and CH_3CN. The gradient employed for these analyses ran from 80% of 0.1% trifluoroacetic acid to 50% of 0.1% trifluoroacetic acid over 20 min. This gradient program was used with the XCALIBUR software to obtain in-line UV and MS spectra of the compounds that eluted from the column.

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