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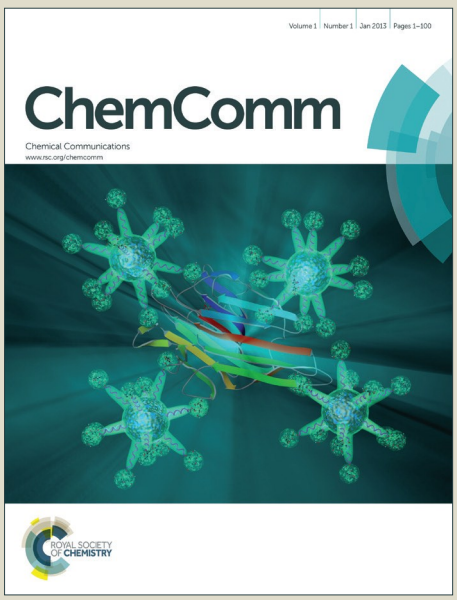





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# Stereoselective Synthesis and Structural Elucidation of Dicarba Peptides

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**A facile stereoselective synthesis of *cis* and *trans* unsaturated dicarba peptides has been established using preformed diaminosuberic acid derivatives as bridging units. In addition, characteristic spectral differences in the $^{13}$C-NMR spectra of the *cis*- and *trans*-isomers show that the chemical shift of carbons in the Δ4,5-diaminosuberic acid residue can be used to assign stereochemistry in unsaturated dicarba peptides formed from ring closing metathesis of linear peptide sequences.**

Cystine bridges are ubiquitous motifs crucial for the stabilisation of secondary and tertiary structure of peptides. They play an essential role in protein folding, allosteric control and maintenance of biological activity.[1] Consequently, the degradation of cystine bridges, commonly *via* reduction, polymerisation or enzymatic cleavage, can lead to deterioration or complete loss of protein activity.[2] The replacement of cystine bridges with synthetic isosteres provides an opportunity to explore their role in the structural, chemical and pharmacokinetic properties of the peptide.[3] In this context numerous synthetic isosteres including thioethers,[4] diselenides[5] and, more recently, the dicarba analogues[6] have been reported. In the latter case, olefin metathesis has gained increasing popularity as a highly diverse and straightforward method of installing unsaturated dicarba bridges.[6] The use of *S*-allylglycine (Agl) residues as the synthetic bridging precursor is of particular interest owing to the structural resemblance of the resultant diaminosuberic acid (DAS) derivative to native cystine.[7] However, most Ru-alkylidene olefin metathesis catalysts, such as **GII** and **HGII**, provide mixtures of both the *cis*- and *trans*- isomers in a ratio which is highly sequence dependent (Figure 1).[8] Subsequent reduction of the alkene, *cis* or *trans*, provides access to the saturated analogue. Interestingly, significant differences in biological activity can often be observed between the unsaturated and saturated isomers, with the saturated variant often shown to be less potent compared to the conformationally-restricted unsaturated derivatives.[9]

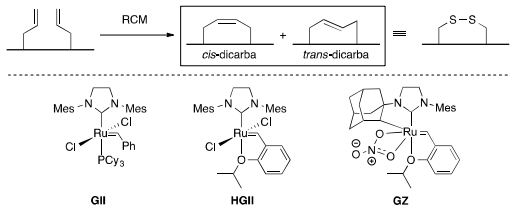


**Figure 1.** General scheme for RCM in peptide substrates and examples of olefin metathesis catalysts

Significantly, variation in activity can also be observed between *cis*- and *trans*-analogues, where the difference in potency arises from structural changes induced by the olefin geometry. The biological preference for one geometric isomer is crucial not only for the pharmaceutical potential of the peptidomimetic, but also for providing insight into the role of the disulfide bridge in influencing the native structure.[10] Therefore, there is a need to identify the structure of the more potent isomer and to achieve stereoselective synthesis of the target dicarba peptide.

Herein we exemplify a method for stereoselective synthesis of both *cis*- and *trans*-dicarba peptide isomers with dicarba oxytocin **1**. This approach utilises a preformed DAS bridging unit **2**, where the desired olefin geometry is both known and controlled. Orthogonal protection of the bridging unit allows simple insertion of the bridging motif into a desired peptide.

Oxytocin, a bioactive, cyclic nonapeptide, is a mammalian hormone responsible for uterine and mammary contraction.[11] Oxytocin was chosen as the peptide model for our stereoselective synthetic approach due to its commercial relevance and the availability of robust structural stereochemical assignment for the dicarba analogues from NMR studies.[9b,12] Studies commenced with the synthesis of the linear metathesis precursor **3** using standard Fmoc SPPS. [1,6]-Dicarba oxytocin **1** was then prepared *via* on-resin RCM of **3** using **GII** and delivered the

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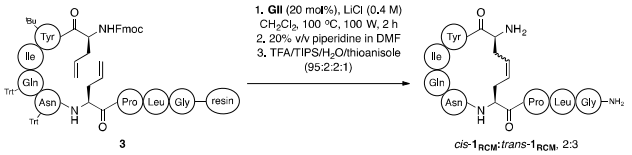
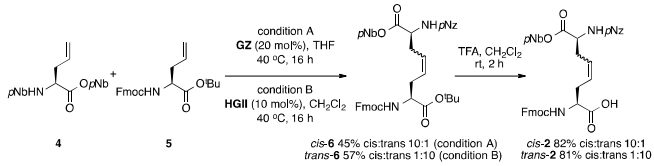



**Scheme 1.** Synthesis of dicarba oxytocin **1** using RCM

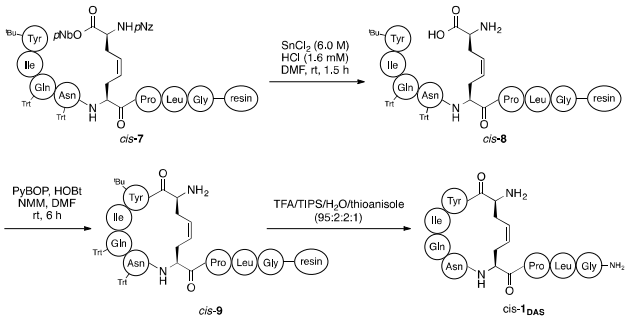
expected mixture of *cis*- and *trans*-dicarba oxytocin isomers **1** in a 2:3 ratio, respectively (Scheme 1). The *cis*- and *trans*-isomers were easily separated using RP-HPLC.

Previous work by Vederas and coworkers found that the *cis*-isomer of dicarba oxytocin **1** was an order of magnitude more potent than the *trans*- and saturated analogues in uterine muscle contraction.[9b] Thus, once the identity of a more potent analogue is known, strategies that facilitate stereoselective synthesis of the target dicarba isomer are desirable. Towards this end, new *cis*-selective metathesis catalysts provide the opportunity to access a single geometric isomer.[13] However, these catalysts are less reliable in peptide substrates and, to our knowledge, have only been reported for elongated bridge motifs.[13a] In addition, Grubbs and coworkers have established a method to selectively enrich either the *cis* or *trans* isomer in elongated olefin bridging systems using a RCM/ethenolysis strategy.[14] Rather than attempting to selectively form one geometric isomer post peptide construction, we explored the option of inserting a pre-formed DAS unit **2**. In this way the desired olefin stereochemistry could be installed prior to insertion into the peptide sequence. The DAS unit could then be readily incorporated into the peptide sequence, providing a controlled stereoselective synthesis of the target cyclic dicarba peptide.

Studies commenced with the selective synthesis of the *cis*-**2** and *trans*-**2** Δ4,5-DAS derivatives (Scheme 2). The DAS moiety required orthogonal protection compatible with Fmoc SPPS. *p*-Nitrobenzyl (*p*Nb)/*p*-nitrobenzyloxycarbonyl (*p*Nz) groups were chosen as they could be selectively removed using $SnCl_2$/HCl.[15] A generic cross-metathesis (CM) strategy was employed in the construction of the alkene bridge in both isomers, thus allowing not only rapid access to the DAS unit **2**, but also assisting the choice of catalyst to dictate the outcome of the olefin geometry (Scheme 2). Towards this end, readily available AgI derivatives **4** and **5** were subjected to CM firstly using the commercially available *cis*-selective **GZ** catalyst to afford predominantly *cis*-**6** in moderate 45% yield with a *cis*:*trans* ratio of 10:1.[16] Subsequent TFA mediated deprotection afforded primarily *cis*-**2** in excellent yield. Use of **HGII** under analogous CM reaction conditions gave predominantly the *trans*-**6** isomer (Scheme 2). A high degree of similarity was observed in the $^1$H- and $^{13}$C-NMR spectra of the *cis*-**2** and *trans*-**2** isomers but appreciable differences between the chemical shifts of the $C^{\beta}$ signals were observed (Table 2).




**Scheme 2.** Synthesis of orthogonally protected *E*- and *Z*-Δ4,5-DAS **2**




**Scheme 3.** Stereoselective synthesis of *cis*-$\mathbf{1}_{DAS}$

Stereoselective synthesis of the dicarba oxytocin isomers began with incorporation of *cis*-**2** into the linear octapeptide sequence at position 6 using standard Fmoc SPPS chemistry with HATU as the coupling reagent (Scheme 3). Subsequent removal of the *p*-nitrobenzyl ester and *p*-nitrobenzyloxycarbonyl protecting groups from resin-tethered *cis*-**7** using $SnCl_2$ (6 M) and HCl (1.6 mM) afforded *cis*-**8**. Lactamisation was performed using PyBOP and HOBt to provide *cis*-**9**. Finally, acid-mediated resin cleavage gave the *cis*-[1,6] dicarba oxytocin **1** as the major isomer. The *trans*-isomer of [1,6]-dicarba oxytocin analogue **1** was also prepared using analogous Fmoc SPPS conditions *via* insertion of *trans*-**2** (synthesis not shown). The diasterisomeric purity of the Δ4,5-DAS **2** isomers was preserved following their incorporation into the full peptide sequence.

The *cis*- and *trans*-isomers **1** obtained from the preformed DAS derivatives *cis*-**2** and *trans*-**2**, respectively, were compared with the *cis*- and *trans*-dicarba oxytocin derivatives obtained from RCM and were found to have identical retention times using reverse phase HPLC (Figure 2).

Numerous spectroscopic techniques have been employed previously to establish olefin geometry in a range of different dicarba peptide analogues. The simplest technique utilises $^1$H-NMR coupling constants, where $^3J_{\gamma\gamma'}$ of the *cis* analogue is consistently smaller than that of the *trans* (typical values are $J$ = 10 Hz and $J$ = 15 Hz, respectively).[8a,8c,17] However, this technique relies on resolved

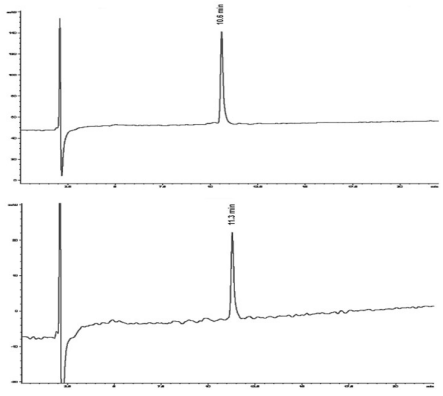
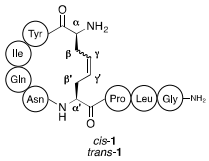



**Figure 2.** a) Co-injection of *cis*-$\mathbf{1}_{RCM}$ and *cis*-$\mathbf{1}_{DAS}$. b) Co-injection of *trans*-$\mathbf{1}_{RCM}$ and *trans*-$\mathbf{1}_{DAS}$.

**Table 1.** $^{13}$C-NMR assignments of *cis*-**1** and *trans*-**1**

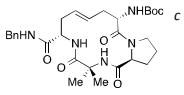
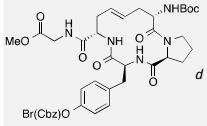



| Carbon environment | *cis*-**1**$^{a,b}$ | *trans*-**1**$^{a,c}$ |
|---|---|---|
| $C^{\alpha}$ | 53.4 | 53.0 |
| $C^{\alpha'}$ | 51.5 | 52.1 |
| $C^{\beta}$ | 30.8 | 34.6 |
| $C^{\beta'}$ | 29.7 | 34.2 |
| $C^{\gamma}$ | 125.9 | 126.2 |
| $C^{\gamma'}$ | 130.4 | 131.5 |

$^{a}$Spectra were run in $D_2O$, $^{b}$ $^{3}J_{\gamma\gamma'}$ 10.5 Hz, $^{c}$ $^{3}J_{\gamma\gamma'}$ 15.0 Hz

olefinic peaks in both isomers, which are often absent due to pseudo-symmetry of the DAS linker. Two-dimensional NMR experiments, particularly nOe, have also been applied to alkene geometry assignment.[10d,18] However, success here is highly dependent on structural context and assignment of olefin geometry is not always possible using this method.[8b,9a,10c,19] Thus, if the characteristic difference in $C^{\beta}$ chemical shifts of *cis*- and *trans*-analogues is a generic feature, it could be used as a rapid means for determining stereochemistry in other dicarba peptides.

**Table 2**. Dicarba peptide $C^{\beta}$ chemical shift and $^{3}J_{\gamma\gamma'}$ values

| Peptide | *Cis* isomer | | *Trans* isomer | | Ring size | Ref |
|---|---|---|---|---|---|---|
| | $C^{\beta}$ ppm | $^{3}J_{\gamma\gamma}$ Hz | $C^{\beta}$ ppm | $^{3}J_{\gamma\gamma}$ Hz | | |
| DAS **2**$^{a}$ | 30.2<br>30.1 | 10.4 | 35.5<br>35.3 | # | - | *e* |
| 2,8-Dicarba Vc1.1$^{b}$ | 31.9<br>31.3 | 10.7 | 36.7<br>36.7 | # | 23 | 10b |
| 3,16-Dicarba Vc1.1$^{b}$ | 30.9<br>31.9 | # | 36.2<br>36.6 | # | 44 | 10b |
| 2,8-Dicarba Iml$^{b}$ | 31.9<br>32.7 | # | -<br>- | 15.0 | 23 | 17a |
| 2,7-Dicarba vapreotide$^{b}$ | 30.7<br>30.7 | 10.3 | 36.0<br>36.0 | 15.0 | 20 | 20 |
| D-1,6-Dicarba oxytocin [D]-**1**$^{b}$ | 29.4<br>29.1 | 10.8 | 34.7<br>34.3 | 15.0 | 20 | *e* |
|  *c* | -<br>- | - | 34.9<br>34.4 | 15.0 | 14 | 7a |
|  *d* | -<br>- | - | 35.3<br>34.8 | 15.0 | 14 | 7b |

$^{a}$Spectrum was run in $CDCl_3$, $^{b}$Spectra were run in $D_2O$, $^{c}$Spectrum was run in $C_2Cl_2$, $^{d}$Spectrum was run in $(CD_3)_2SO$, $^{e}$Supplementary information. # = not determined.

The *cis*- and *trans*-dicarba oxytocin analogues **1** were therefore analysed by $^{13}$C-NMR spectroscopy. The key $^{13}$C-NMR resonances of the Δ4,5-DAS bridge on both the *cis*-**1** and *trans*-**1** analogues were assigned through the use of COSY and HSQC NMR experiments (Table 1). Although a high degree of similarity was observed between the two isomers at the $C^{\alpha}$ and $C^{\gamma}$ positions, significant differences were observed between the chemical shifts of the $C^{\beta}$ signal in the *cis*-**1** analogue (29.7 and 30.8 ppm) compared to the *trans*-**1** analogue (34.2 and 34.6 ppm) were observed. This trend was also observed in the original preformed DAS units **2** (Table 2).

Various other dicarba peptide examples were then investigated to determine whether this was a generic trend across all Δ4,5-DAS bridges in dicarba peptide systems. Chemical shifts of the $C^{\beta}$ available from the literature and our laboratory are shown in Table 2. In all cases, the alkene geometries have been assigned independently using $^{1}$H-NMR spectroscopy. Carbon NMR data for $C^{\beta}$ of each sample showed large and predictable differences between the two geometric isomers. The *cis*-isomer consistently had upfield $C^{\beta}$ chemical shifts relative to the trans-isomer, with values of 29.1-32.7 ppm and 34.4-36.7 ppm respectively. This difference in $C^{\beta}$ chemical shift was consistent across ring sizes ranging from 14 to 44 atoms. The stereochemically mutated oxytocin analogue [D]-**1** also followed the $^{13}$C-NMR trend showing that the inclusion of D-amino acids in the peptide sequence does not limit the application of this method. The assignments of the olefin stereochemistry are all in agreement with the $^{13}$C chemical shift method reported in this paper. To our knowledge, the only exception to this trend has been reported in alkene-bridged diastereoisomers of nisin DE-ring mimics.[21] The reported $^{13}$C NMR $C^{\beta}$ chemical shifts are consistent with the above described ranges for the *cis*- and *trans*-isomers but conversely the high-field resonances have been ascribed to the *trans*-isomers.[21] In these analogues, the reported $^{3}J_{\gamma\gamma}$ are in the range 4.0-6.8 Hz for DAS linkers assigned as *cis*, and 10.3-10.9 Hz for those assigned as *trans*. The lesser of these values fall well outside the range observed in other dicarba-bridged peptides, while the larger are more typical of *cis* (Table 2). Indeed, these values are atypical of olefins more generally in the absence of significant strain or strongly electron-withdrawing functionality; notably, the reported nicin analogues possess 14-membered rings and are not likely to be especially strained.[22] Accordingly, we suspect that the couplings reported as *cis* $^{3}J_{\gamma\gamma}$ are in fact $^{3}J_{\gamma\beta}$, and that the stereochemistry of each linker is systematically misassigned. If this is the case, the reported dicarba nisin analogues also conform to the above described $C^{\beta}$ chemical shift trend observed for the *cis*- and *trans*-stereoisomers.

In summary, we report a facile stereoselective synthesis of *cis*- and *trans*-dicarba oxytocin **1**. This was achieved by use of selective olefin metathesis catalysts to preform the DAS bridging unit which was subsequently incorporated into the peptide sequence. Furthermore, we report the use of $^{13}$C-NMR chemical shifts as an independent spectroscopic method for determining the Δ4,5-DAS bridge geometry in complex dicarba peptide systems. The latter is

particularly useful for assigning stereochemistry of dicarba peptides generated *via* RCM of linear sequences.

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