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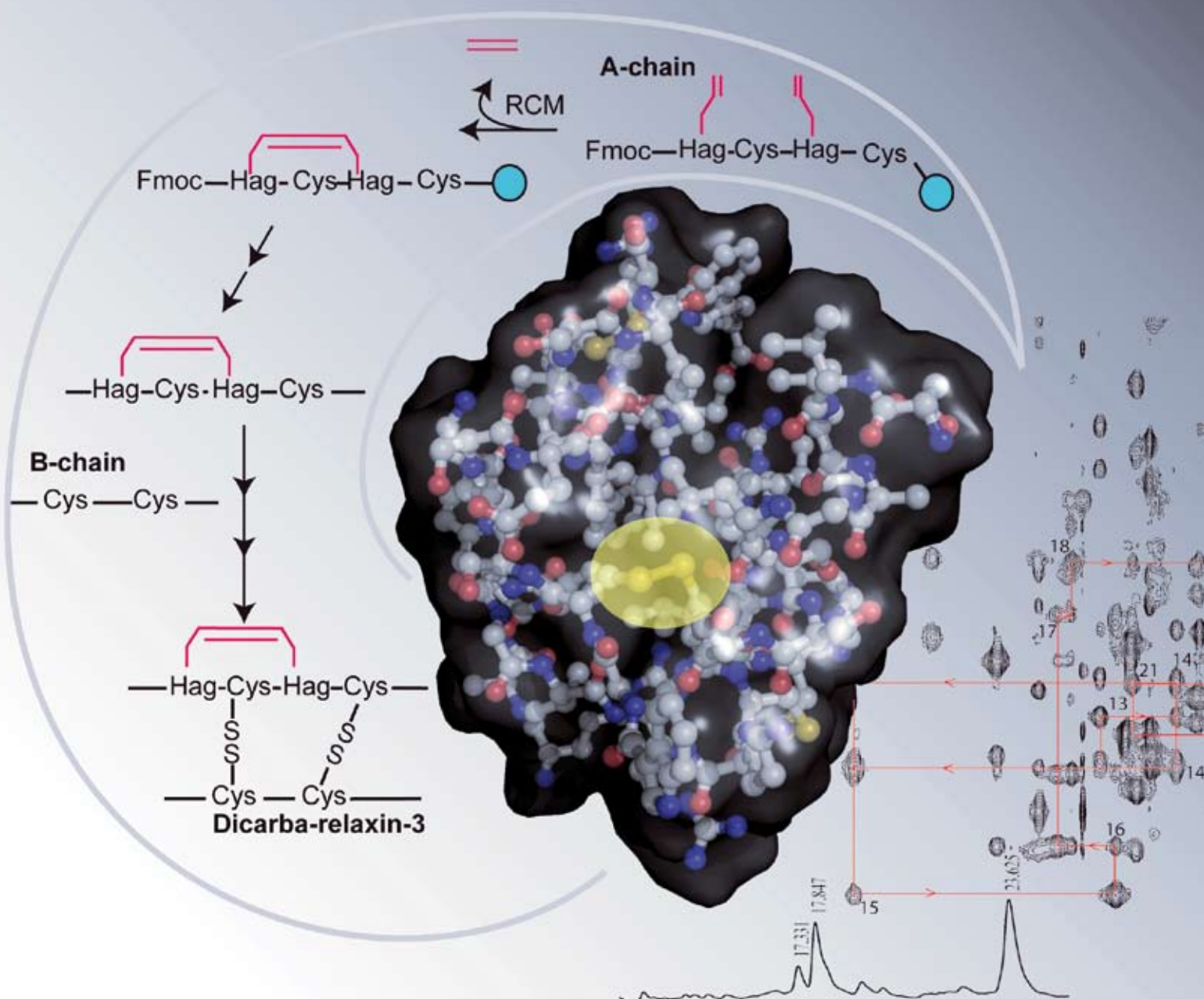
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Replacement of disulfide bonds with non-reducible isosteres can be a useful means of increasing the *in vivo* stability of a protein. We describe the replacement of the A-chain intramolecular disulfide bond of human relaxin-3 (H3 relaxin, INSL7), an insulin-like peptide that has potential applications in the treatment of stress and obesity, with the physiologically stable dicarba bond. Solid phase peptide synthesis was used to prepare an A-chain analogue in which the two cysteine residues that form the intramolecular bond were replaced with allylglycine. On-resin microwave-mediated ring closing metathesis was then employed to generate the dicarba bridge. Subsequent cleavage of the peptide from the solid support, purification of two isomers and their combination with the B-chain *via* two intermolecular disulfide bonds, then furnished two isomers of dicarba-H3 relaxin. These were characterized by CD spectroscopy, which suggested a structural similarity to the native peptide. Additional analysis by solution NMR spectroscopy also identified the likely *cis/trans* form of the analogs. Both peptides demonstrated binding affinities that were equivalent to native H3 relaxin on RXFP1 and RXFP3 expressing cells. However, although the cAMP activity of the analogs on RXFP3 expressing cells was similar to the native peptide, the potency on RXFP1 expressing cells was slightly lower. The data confirmed the use of a dicarba bond as a useful isosteric replacement of the disulfide bond.

## Introduction

Disulfide bonds are common structural motifs in many biologically active peptides and proteins. They play a crucial role in maintaining the three-dimensional structure of the biomolecule and are thereby often important for function and/or proteolytic stability.<sup>1,2</sup> However, intracellular components such as the enzyme disulfide reductase can disrupt such bridges and render the peptides inactive or susceptible to proteolytic attack, thereby limiting their therapeutic potential. Therefore substitution of the disulfide bonds of therapeutically important peptides with more stable bonds without significantly affecting the biological activity of the peptide is a major challenge for the synthetic chemist. Several strategies have been explored including replacing the disulfide bonds with more stable lactam,<sup>3</sup> diselenide<sup>4</sup> or, more recently, dicarba bonds.<sup>5</sup> In 2006, Zhang *et al.* synthesized a sunflower trypsin inhibitor analog in which the single disulfide bond was replaced by a diseleno bond.<sup>4</sup> The diseleno analog retained a high potency, showing only 30% reduced inhibitory activity compared to the native one. In another recent report, the dicarba analogs of the cyclic peptide enkephalin were shown to possess an even higher  $\mu$ -receptor agonist potency compared to the cystine-containing

parent peptide.<sup>5</sup> The chemically synthesized dicarba analog of the hormone oxytocin, where the cystine residues (disulfide bonds) were replaced by allyl glycine (dicarba bonds), also showed interesting bioactivity.<sup>6</sup> Biological testing on rat uterus strips shows that *cis* dicarba oxytocin has an EC<sub>50</sub> value of 38 ng/mL (EC<sub>50</sub> for native oxytocin is 2.7 ng/mL) whereas the saturated and *trans* olefins are less active.<sup>6</sup> Most importantly, increased metabolic stability has been observed by replacing the cysteine residue with dicarba isostere (*S,S*)-2,7-diaminosuberic acid ((*S,S*)-2,7-diamino octanedioic acid) in vasopressin,<sup>7</sup> natriuretic  $\beta$ -ANP,<sup>8</sup> oxytocin,<sup>9</sup> calcitocin<sup>10</sup> and bradykinin antagonists.<sup>11</sup> Therefore it is evident that replacement of the disulfide bond with more stable dicarba bond can lead to a development of more potent and stable bioactive peptides or proteins.

In the past, the replacement of the disulfide bond with a bis-methylene moiety (diaminosuberic acid) required a cumbersome multistep synthesis.<sup>12</sup> Recently, the use of ring-closing metathesis (RCM) was shown to be a more simple means of preparing a dicarba analogue of oxytocin.<sup>6</sup> The recent development of microwave technology and its application to the RCM reaction have significantly facilitated the preparation of dicarba analogs of small bioactive peptides.<sup>13</sup> However, to date, no successful example of the RCM reaction for the synthesis of dicarba analogues of complex peptides or proteins have been reported together with their structural details.

Human relaxin-3 (H3 relaxin), a recently discovered insulin-like peptide which is also known as insulin-like peptide 7 (INSL7), has been shown to have brain-specific functions and is found together with its G-protein-coupled receptor, RXFP3, in abundant

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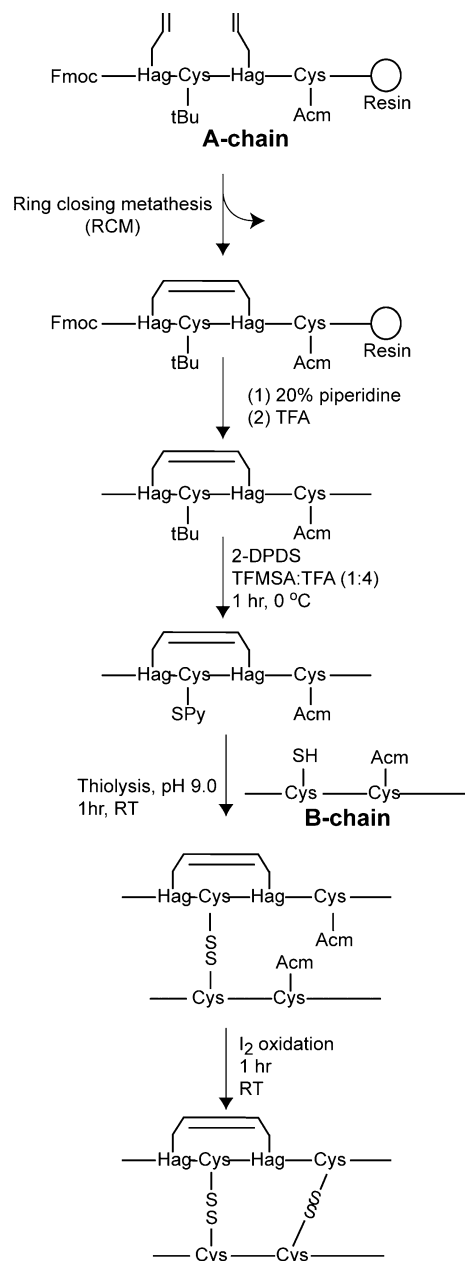
quantities in the brain.<sup>14</sup> Its involvement in neurological stress responses and effects on food intake makes it a potential drug for treatment of stress and obesity.<sup>15,16</sup> Because of its structural similarity to insulin, recently determined tertiary conformation and its potential application as a therapeutic, we undertook the synthesis of dicarba analogs of H3 relaxin. We report here, for the first time, the utility of efficient solid phase synthesis methods, together with a microwave-assisted RCM and regioselective disulfide bond formation to produce complex dicarba analogs of H3 relaxin that exhibit native structure and full biological activity.

## Results and discussion

The primary aim of this study was to replace the A-chain intramolecular disulfide bond of H3 relaxin with the non-reducible, isosteric dicarba bond and to determine if the native insulin-like structure and biological activity were retained. Microwave-assisted RCM methodology provides a highly efficient, on-resin route to carbocyclic peptides with quantitative conversions, fast reaction times (typically 1–2 h), high resin loadings and lower Grubbs' catalyst loading (5–10 mol%).<sup>13</sup> This approach was employed to prepare an intramolecular dicarba bridge within the resin-bound sidechain- and N-terminally-Fmoc-protected A-chain of H3 relaxin (Fig. 1). The Fmoc group was then removed with 20% piperidine in DMF and the resin treated with TFA in the presence of scavengers. The resulting mixture contained deprotected peptide chain of both the *cis* and *trans* isomers of the unsaturated dicarba bond together with unreacted starting peptide (Fig. 2B). On the basis of RP-HPLC peak integration, the two isomers (named DC1 and DC2 respectively) were in a ratio of approximately 20/80 and, collectively, represented about 50% of the total product with the remainder being starting bis-Hag A-chain. Despite several modifications to the protocol, no conditions were found which allowed an increase in the yield of cyclization. Parallel studies with the A-chain of human insulin and INSL3 showed that almost complete RCM-mediated cyclization could be achieved upon the introduction of a turn-inducing moiety between the Hag residues (unpublished data). The *cis/trans* identity of the two dicarba isomers of H3 relaxin A-chain was unknown but subject to attempted determination by NMR spectroscopy as described below.

The two-chain dicarba H3 relaxin (Fig. 2A) was generated by highly efficient regioselective disulfide bond formation between solid-phase peptide synthesis produced A- and B-chains, in which pairs of cysteines were selectively *S*-thiol-protected with masking groups that allowed individual removal and subsequent stepwise disulfide bond formation<sup>21,22</sup> (Fig. 1). The overall yield of the dicarba relaxin analogs following HPLC purification was about 5–6% relative to the starting B-chain peptide, which is satisfactory given the complexity of the analogues and the handling difficulties associated with the poor solubility of the A-chain. The purity of the two isomers (DC1 H3 relaxin and DC2 H3 relaxin) was confirmed by analytical HPLC (Fig. 2C). The identity of the peptides was confirmed by MALDI-TOF MS (calculated 5459.4 Da; found 5462.8 Da [DC1] and 5462.9 [DC2]) and they were quantitated by amino acid analysis.

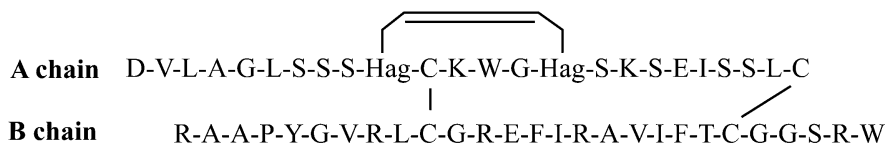
The key question regarding the dicarba H3 relaxin analogues was: Does the replacement of the disulfide bond affect the overall structure and the biological activity of H3 relaxin? In order



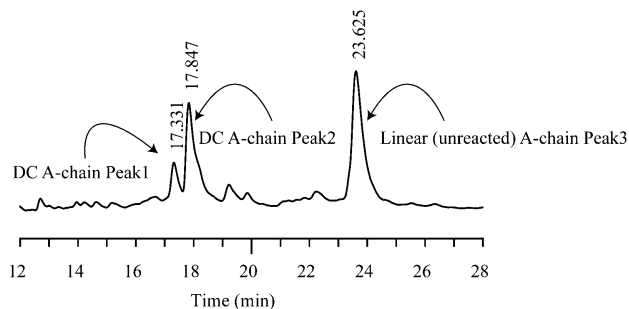
**Fig. 1** The synthesis scheme for the preparation of human dicarba relaxin-3.

to quickly assess whether any major conformational changes to the native H3 relaxin structure occurred as a result of the introduction of the dicarba bond, we analyzed the analogues by CD spectroscopy (Fig. 3). The CD spectra of native H3 relaxin and the two dicarba conformers showed a typical  $\alpha$ -helical pattern with double minima at 208 nm and 222 nm.<sup>26</sup> The mean residual weight ellipticity at 222 nm,  $[\theta]_{222}$ , can be used to calculate the helix content.<sup>27</sup> The  $[\theta]_{222}$  value for native H3 is  $-12958.7$ , which corresponds to an  $\alpha$ -helix content of 36.5%. The DC2 H3 relaxin analog showed no significant change in the  $[\theta]_{222}$  value,  $-13248.4$ , which corresponds to 37.2%  $\alpha$ -helix content. This result indicated that replacement of the disulfide bond with a dicarba bond in the A-chain could be achieved without disruption to the relaxin helical segments. However, a small decrease in the helicity ( $[\theta]_{222} = 11656$ ,

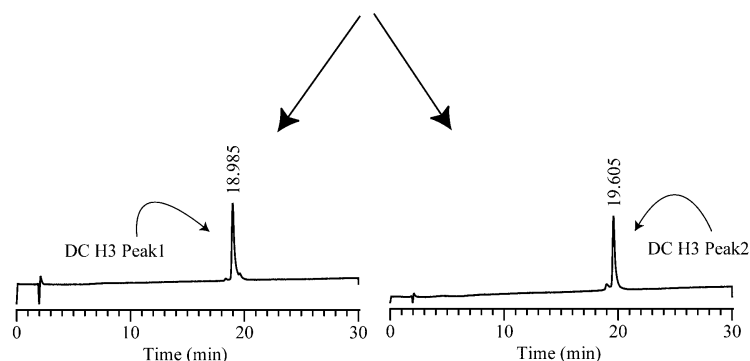
A)



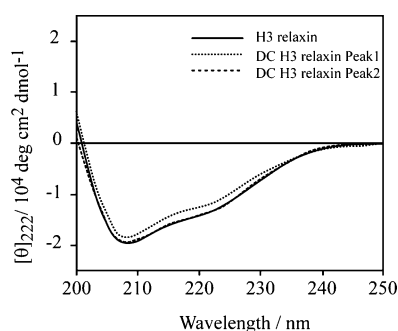
B)



C)



**Fig. 2** (A) Primary structure of human dicarba relaxin-3. (B) Analytical RP-HPLC of crude synthetic human dicarba relaxin-3 A-chain. Conditions: Eluent A: 0.1% aq. TFA; eluent B: 0.1% TFA in acetonitrile. Gradient = 20–50% B over 30 min. Column: Phenomenex C18 (pore size 300Å, particle size 5 µ, 4.6 × 250 mm). (C) Analytical RP-HPLC of purified isomers of human dicarba relaxin-3. Conditions as described above.



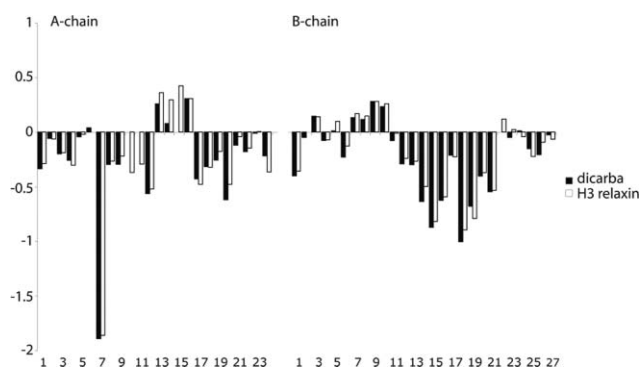
**Fig. 3** Circular dichroism spectra of H3 relaxin and DC1 and 2 H3 relaxin in 10 mM phosphate buffer with 120 mM NaCl (pH 7.4).

33% helicity) was observed for the DC1 H3 relaxin conformer, indicating that one of the two *cis/trans* conformers is less favorable for the H3 relaxin fold.

To gain further insight into how well the dicarba analogue folded and to confirm a H3 relaxin-like structure we subjected DC2 H3 relaxin, which was predicted on the basis of the CD spectral analyses to be the one structurally most similar to native H3 relaxin, to high-field solution NMR spectroscopy. Two dimensional proton TOCSY and NOESY datasets were recorded at 600 MHz and the data were of good quality in terms of signal dispersion, consistent with a structured peptide. However, similar to what has been seen

for several other relaxin peptides a large number of resonances were significantly broadened suggesting considerable internal structural flexibility. Nonetheless using sequential assignment strategies most resonances in the dicarba analogue could be identified. Among the notable exceptions were all resonances from the HagA10 residue and the olefinic proton from HagA15, as well as the HN protons of TrpA13, GlyA14, SerA16, IleB15, ArgB16 and CysB22. Consistent with the broadening in native H3 relaxin being most severe around the CysA10–CysA15 disulfide bond,<sup>28</sup> the broadening here appears worse around the dicarba bond between HagA10–HagA15. However, in the dicarba analogue broadening is also seen further away from the core including in the B-chain helix.

Secondary H $\alpha$  shifts, *i.e.* the difference between the observed chemical shifts and the chemical shifts expected for the particular amino acid in a ‘random-coil’ or unstructured peptide give a good indication about the presence of secondary structure. Fig. 4 shows a comparison of the secondary chemical shifts of H3 relaxin and its dicarba analogue. Strikingly the shifts are very similar, with the presence of the three helical segments and the short extended regions of the relaxin fold clearly visible as stretches of negative and positive numbers respectively. From here it is clear that like the CD spectrum the NMR data suggest that the replacement of the disulfide bond has not affected the overall structure but rather the dicarba analogue is in fact structurally very similar to the



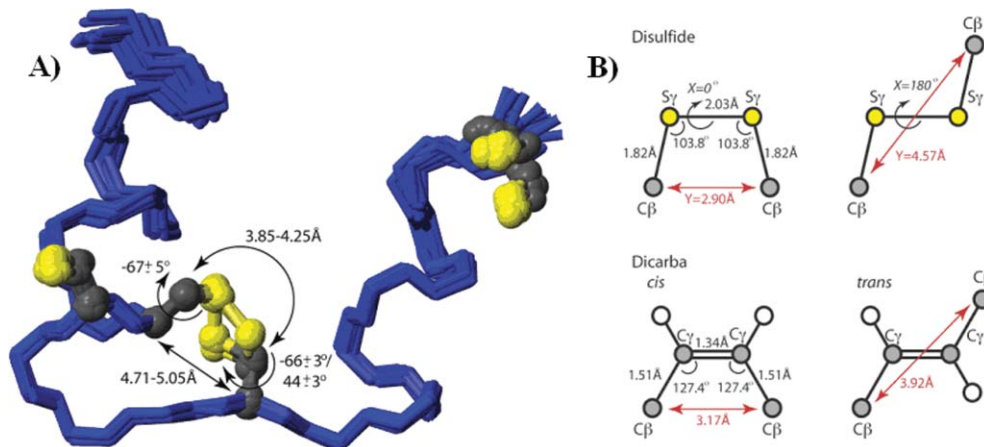
**Fig. 4** Secondary  $H\alpha$  shifts for H3 relaxin and DC2 H3 relaxin. Stretches of negative values are indicative of helical regions while stretches of positive numbers are consistent with extended regions. The DC2 H3 relaxin retains the secondary structure of the native peptide which includes three helical segments (A1–12, A17–24 and B11–21) and two short  $\beta$ -strands (A13–A16 and B7–10).

native peptide. A number of other features of the NMR data are also consistent with what has previously been seen for native H3 relaxin, e.g. the upfield shifts of SerA7  $H\alpha$  and the methyl groups of ValB18, which all interact with and show NOE cross peaks to the aromatic protons of PheB14. Other characteristic NOE contacts include the HagA15 HN to ValB7 HN, which confirms the pair of amide to carbonyl hydrogen bonds between these two residues. These data all support a structure without significant alterations from the native fold.

A key difference between the unsaturated dicarba bond and the disulfide bond is that the dicarba bond is conformationally 'locked' in either a *cis* or a *trans* conformation. Naturally we were interested in finding out which of the two conformations was most compatible with the H3 relaxin structure and thus would be the one causing minimal structural changes or strain. NMR spectroscopy would be expected to be the ideal technique for answering this question because of the distinct differences in coupling constant between the olefinic protons in a *cis* ( $\sim 10$  Hz)

and *trans* ( $\sim 16$  Hz) conformation, respectively. The nature of the double bonds also gives these protons a chemical shift of around 6 ppm, a region in which overlap is not expected to be a problem for a small protein. However, during our analysis it became clear that no resonance signals could be detected in the region expected for the olefinic protons. Furthermore, as noted above, during the course of the sequential assignment we could not identify a spin system resulting from HagA10, and although a spin system concluded to originate from the amide proton of HagA15 based on NOE contacts to residue 14 was present, it was broad and no cross-peaks to the olefinic protons could be seen in either the TOCSY or the NOESY. Based on these observations it was concluded that the region around the dicarba bond does not adopt a single stable conformation in solution but rather experiences structural rearrangement that result in chemical shift averaging and consequently causes broadening and loss of signals. Interestingly, these dynamic processes are also seen in native H3 relaxin,<sup>28</sup> as well as all other relaxins studied by NMR so far, as broadening of resonances in the vicinity of the intra-chain disulfide bonds are always observed. Consequently the NMR data could not be used to definitively confirm a *cis* or *trans* arrangement.

From a structural perspective it is interesting to consider what effect the dicarba bond may have on the fold by analyzing the available NMR structure of H3 relaxin. Fig. 5A shows the A-chain of H3 relaxin from the NMR structure and indicates the relevant observed distances and dihedral angles defining the geometry of the Cys10–Cys15 disulfide bond. The free rotation around the disulfide bond allows the distance between the  $\beta$ -carbons to vary between a theoretical minimum of 2.9 Å and a maximum of 4.6 Å at optimal bond lengths and angles (Fig. 5B), albeit not all conformations will be energetically favorable. In contrast in the dicarba analogue the lack of free rotation around the double bond results in a fixed distance between the  $\beta$ -carbons of 3.17 Å or 3.93 Å for a *cis* or a *trans* conformation respectively (Fig. 5B). Analysis of the 20 models representing the solution structure of H3 relaxin shows that this distance varies between 3.85 and 4.25 Å as a result of different side chain conformations. Thus a *trans* conformation



**Fig. 5** Structural consequences of introducing a dicarba bond in H3 relaxin. (A) Superimposition of the A-chain of the 20 structural models that have been calculated based on the NMR data and represent the solution structure of native H3 relaxin. The distances between the key  $C\beta$  atoms vary between 3.85 and 4.25 Å, which is primarily the result of the  $\chi_1$  angle of CysA15 adopting two different conformations ( $\sim 60^\circ$  or  $\sim 180^\circ$ ) within the ensemble. (B) Comparison of the geometry of a disulfide bond and a dicarba bond. The bond lengths and angles of the disulfide bond are taken from the CNS forcefield used for the NMR structure determination of H3 relaxin (topallhdg5.3.pro/parallhdg5.3.pro<sup>30</sup>) and the bond lengths and angles for the dicarba bond were generated by energy minimization using ChemDraw 3D Ultra, v.8.0.

**Table 1**

Ligand	RXFP1		RXFP3	
	Europium-H2 relaxin pIC50	cAMP pEC50	125I-H3/INSL5 pKi	cAMP pEC50
H3 relaxin	8.19 ± 0.17 (3)	9.36 ± 0.21 (4)	8.47 ± 0.09 (3)	9.08 ± 0.06 (3)
DC1 H3 relaxin	7.88 ± 0.11 (3)	8.13 ± 0.04 (3) <sup>a</sup>	8.90 ± 0.13 (3)	8.62 ± 0.08 (3) <sup>b</sup>
DC2 H3 relaxin	7.99 ± 0.015 (4)	8.30 ± 0.16 (4) <sup>a</sup>	8.70 ± 0.10 (3)	8.72 ± 0.002 (3) <sup>b</sup>

<sup>a</sup> p < 0.001 vs. H3 relaxin. <sup>b</sup> p < 0.001 vs. H3 relaxin.

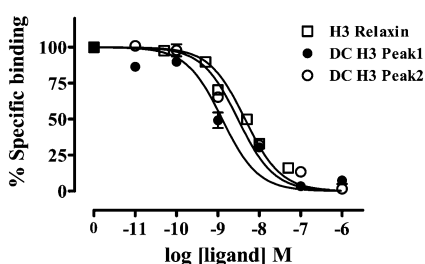
would be expected to fit very well with the native structure, while in contrast a *cis* arrangement would be expected to introduce strain and require changes at the very least to the projections of the side chains involved in the bond. Therefore it seems likely that the DC2 H3 relaxin variant, with the most native-like structure, represents the *trans* conformation. It should also be noted that the disulfide bond is associated with the core of the molecule and thus there is a possibility that the introduction of the dicarba bond forces changes to the packing of the core due to its extra rigidity and the additional two hydrogen atoms. However these effects are more difficult to predict.

## Binding and cAMP assays

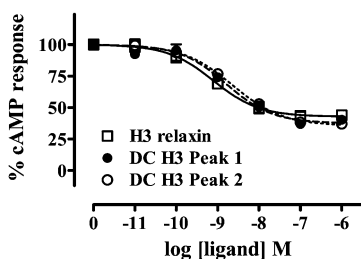
To test whether the replacement of the disulfide bond had resulted in impaired biological activity, the two dicarba H3 relaxin isomers were tested for their ability to bind to and activate the relaxin-3 receptor RXFP3. Strikingly, both of the isomers demonstrated high affinity binding to RXFP3 expressing cells (Fig. 6A, Table 1). Additionally, both were able to inhibit forskolin stimulated cAMP production with a similar potency to native H3 relaxin (Fig. 6B,

Table 1). Hence replacement of the A-chain disulfide bond with a stable dicarba bond had no effect on H3 relaxin activity on RXFP3. To further investigate any functional changes the peptides were then tested for their ability to bind to and activate the relaxin receptor RXFP1, for which H3 relaxin is also a high affinity agonist.<sup>29</sup> As with RXFP3, both of the isomers demonstrated high affinity binding to RXFP1 (Fig. 7A, Table 1). However, the potency was slightly lower compared to the native H3 relaxin (Fig. 7B, Table 1). That this effect is only seen on the RXFP1 receptor is perhaps not so surprising given that the RXFP1 receptor has been shown to be highly sensitive to structural changes in its ligands, H2 and H3 relaxin. In contrast, the RXFP3 receptor is less sensitive to such changes, as an A-chain truncated version of H3 relaxin, which lacks ordered structure, retains full activity on RXFP3.<sup>24</sup> These observations suggest that although the dicarba moiety does not significantly disrupt the fold as shown by the circular dichroism spectra and NMR spectroscopy data, it may still cause a destabilization of the structure, as suggested from the severe resonance broadening in the NMR data, which could explain this slight drop in potency on RXFP1.

A) <sup>125</sup>I-H3/INSL5 binding to RXFP3

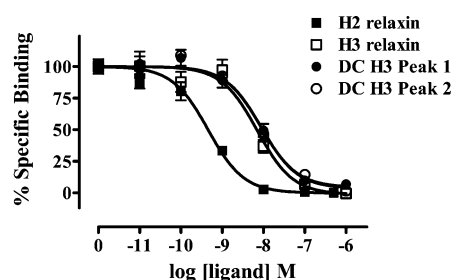


B) cAMP inhibition in RXFP3 expressing cells

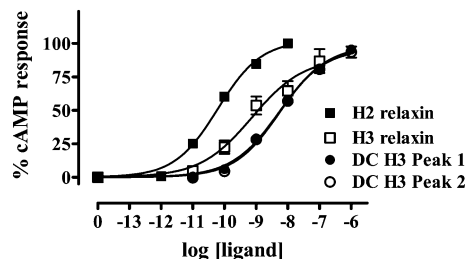


**Fig. 6** RXFP3 activity of the synthetic peptides. (A) Competition binding in RXFP3 cell membranes using <sup>125</sup>I-H3/INSL5. (B) Ability of the peptides to inhibit cAMP activity induced by 5 μM forskolin in RXFP3 expressing cells. Data are the mean ± SEM of pooled data from three independent experiments performed in triplicate.

A) Eu-H2 relaxin binding to RXFP1



B) cAMP stimulation in RXFP1 expressing cells



**Fig. 7** RXFP1 activity of the synthetic peptides. (A) Competition binding in RXFP1 expressing cells using europium-labelled H2 relaxin. (B) Ability of the peptides to stimulate cAMP in RXFP1 expressing cells. Data are the mean ± SEM of pooled data from three independent experiments performed in triplicate.



## Conclusions

A combination of solid phase peptide synthesis, RCM and regioselective disulfide bond formation were employed to successfully assemble two isomeric A-chain intramolecular dicarba bridge-containing analogs of H3 relaxin. CD and NMR spectroscopy analyses confirmed that both analogues possessed native H3 relaxin-like structure. The analogues also demonstrated H3 relaxin receptor binding and activation activity. The results showed that replacement of the intramolecular disulfide bond of this hormone with the isosteric dicarba bond is a viable means of obtaining analogues with potentially improved pharmacological properties. The acquisition of analogues with all three disulfide bonds—one intramolecular and two intermolecular—substituted by dicarba bonds is currently underway.

## Materials and methods

Fmoc-amino acid derivatives of the L-configuration for the peptide synthesis were purchased from Auspep Pty. Ltd. (Melbourne, Australia) or GL Biochem (Shanghai, China). RP-HPLC columns were obtained from Phenomenex (Torrance, California, USA). Solvents and chemicals were all of peptide synthesis or analytical grade. (2S)-Amino-4-pentenoic acid (L-allylglycine, Hag) was supplied by Peptech and Fmoc-protected according to a procedure described by Paquet.<sup>17</sup>

### Solid phase peptide synthesis

H3 relaxin consists of a twenty four residue A-chain and a twenty seven residue B-chain that are linked by three disulfide bonds (Fig. 2A). Both regioselectively S-protected A- and B-chains were separately synthesized by the continuous flow Fmoc solid-phase method<sup>18</sup> using an automatic PerSeptive Biosystems Pioneer peptide synthesizer (Framingham, MA, USA) as previously reported<sup>19</sup> or using microwave-assisted synthesis on the Liberty system (CEM corporation, North Carolina, USA). The solid support used for both A- and B-chains was Fmoc-PAL-PEG-PS. All amino acid side chains were protected by trifluoroacetic acid (TFA)-labile protecting groups except for Cys11 (But) and Cys24 (Acm) in the A-chain and Cys22 (Acm) in the B-chain. The acylation (coupling) reaction was carried out for between 30 min to 1 h. Deprotection of the Fmoc group was carried out with 20% piperidine/DMF. The two cysteines involved in the intramolecular disulfide bond in the A-chain were replaced with the Hag residue.

**Microwave-accelerated ring closing metathesis.** Tricyclohexylphosphine[1,3-bis(2,4,6-trimethylphenyl)-4,5-dihydro-imidazol-2-ylidene](benzylidene)ruthenium(II) dichloride (2nd generation Grubbs' catalyst) was supplied by Aldrich and stored under nitrogen. DCM and a 0.4 M solution of LiCl in DMF were degassed with high purity argon prior to use. Microwave reactions were carried out on a CEM Discover™ System fitted with the Benchmate™ option. The instrument produces a continuous focussed beam of microwave irradiation at a maximum power delivery selected by the user, which reaches and maintains a selected temperature. Reactions were performed in 10 mL high pressure quartz microwave vessels fitted with self-sealing Teflon septa as a pressure relief device. The vessels employed magnetic stirrer beads and the temperature of each reaction was monitored

continuously with a non-contact infrared sensor located below the microwave cavity floor. Reaction times were measured from the time the microwave reached its maximum temperature until the reaction period had elapsed (cooling periods not inclusive).

**Unsaturated dicarba relaxin-3 A-chain.** A microwave reactor vessel was loaded with resin-bound peptide (0.5 g, 0.1 mmol), DCM (10 mL), 0.4 M LiCl in DMF (200  $\mu$ L) and 2nd generation Grubbs' catalyst (16.97 mg, 20  $\mu$ mol, 20 mol%) in an inert environment. The system was sealed and the reaction mixture irradiated with 40 W of microwave energy and stirred at 100 °C for 1 h. The reaction mixture was cooled to room temperature, filtered through a fritted syringe and the resin washed with DCM (7 mL, 3  $\times$  1 min) and MeOH (7 mL, 3  $\times$  1 min) then left to dry *in vacuo* for 1 h. Post-metathesis, a small aliquot of resin-bound peptide was subjected to Fmoc-deprotection in the presence of 20% piperidine in DMF (1  $\times$  1 min, 2  $\times$  10 min) and washed with DMF (5  $\times$  1 min), DCM (3  $\times$  1 min) and MeOH (3  $\times$  1 min). After acid-mediated cleavage, RP-HPLC and mass spectral analysis supported formation of the desired cyclic peptide as two isomers in a 1 : 4 ratio and a cyclic product to linear starting material ratio of approximately 1 : 1. The resin-bound peptide was again subjected to identical metathesis conditions for an additional 1 h, however, RP-HPLC and mass spectral analysis (see below) showed that there was no improvement in the conversion from linear to cyclic.

### Cleavage and purification

The A-chain, after the on-resin RCM reaction, was treated with 20% piperidine/DMF to remove the N-terminal Fmoc group. Final cleavage of both the A- and B-chains from their solid supports and simultaneous side chain deprotection was achieved by a 2 h treatment with TFA (94%) in the presence of scavengers: anisole (3%), 3,6-dioxa-1,8-octanedithiol (DODT, 2%)<sup>20</sup> and triisopropylsilane (TIPS, 1%). The crude A- and B-chains were then purified by preparative reverse-phase (RP) HPLC as previously described.<sup>21</sup>

### Regioselective disulfide bond formation to produce H3 relaxin analogues

For each of the two A-chain peptide isomers, the *t*Bu group of Cys11 was converted to S-pyridinyl group by reaction of the peptide with 2-dipyridyldisulfide (2-DPDS) in the presence of trifluoromethanesulfonic acid (TFMSA).<sup>21,22</sup> The resulting Cys-S-pyridinyl A-chains were then each combined separately with H3 B-chain which had a single free thiol group on Cys 21.<sup>21,22</sup> This led to thiolysis of the pyridinyl group of the A-chain forming the first intermolecular disulfide bond. The resulting AB heteromeric peptide containing a Cys(Acm) in each of the two chains was then treated with iodine in acetic acid to form the 2nd and final intermolecular disulfide bond.<sup>21,22</sup> The two separate isomeric analogues of H3 relaxin were then purified by preparative RP-HPLC. The purity of the peptides was analyzed by MALDI-TOF MS and analytical RP-HPLC (Fig. 2C).

### CD Spectroscopy

CD spectra were recorded by using JASCO J-185 spectrometer (Tokyo, Japan) at room temperature with a 1 mm path length cell.



The peptides were dissolved in phosphate buffer (10 mM) with NaCl (120 mM, pH 7.4).

### NMR Spectrometry

All NMR data were recorded on a Bruker Avance spectrometer operating at 600 MHz on a sample containing 0.5 mg dicarba H3 relaxin dissolved in 0.5 ml of 90% H<sub>2</sub>O/10% D<sub>2</sub>O. Recorded data sets included two-dimensional homonuclear TOCSY and NOESY (with a mixing time of 200 ms). All data were recorded with 4k data points in the F2 and 512 increments in the F1 dimension, which was subsequently zero-filled to 1k data points prior to transformation. All data were collected and processed using Topspin (Bruker) and referenced according to the water resonances at 4.79 ppm at 298 K.

**Binding and cAMP activity assays.** The peptides were tested for their ability to stimulate cAMP activity in HEK-293T cells stably expressing RXFP1<sup>23</sup> and inhibit forskolin stimulated (5  $\mu$ M) cAMP activity in SK-N-MC (human caucasian neuroblastoma cells) cells transiently expressing RXFP3,<sup>24</sup> as previously described. Peptides were measured in triplicate within each assay and each experiment was repeated at least three times. Competition binding using [<sup>125</sup>I]-H3 relaxin B chain/INSL5 A-chain chimeric peptide was performed on cell membranes prepared from CHO-K1 cells stably expressing RXFP3.<sup>24</sup> H2 relaxin was labeled with europium as previously described<sup>25</sup> and used in whole cell competition binding assays with HEK-293T cells stably expressing RXFP1.<sup>23</sup> All data is presented as the mean  $\pm$  SEM of the percentage of the total specific binding of triplicate wells, repeated in at least three separate experiments and the data were fitted using one-site binding curves in GraphPad Prism 4 (GraphPad Software, USA). Statistical differences in pIC<sub>50</sub> or pEC<sub>50</sub> were analysed using one-way ANOVA coupled to Newman-Keuls multiple comparison test for multiple group comparison.

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