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Ring closing metathesis of unprotected peptides†

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An efficient and expedient route to the synthesis of dicarba peptides from protecting group-free sequences is reported using Ru-alkylidene catalysed olefin metathesis. A range of cyclic peptides was prepared from linear peptides containing two *Z*-crotyl glycine residues. Free amine groups were masked as salts with Brønsted acids preventing *in situ* catalyst decomposition. Excellent RCM conversion was obtained in both DMF and methanol.

The feasibility of progressing bioactive peptides and proteins through to therapeutic agents is highly dependent on the cost and ease of bulk manufacture. Although chemical synthesis can be a highly effective method for the generation of small peptides, *via* solution and solid phase approaches,[1–4] chemical processing becomes more challenging with longer sequences.[5,6] Recombinant DNA technology provides an economical means for peptide and protein manufacture,[6,7] and peptide therapeutics such as insulin[8] and glucagon[5] are industrially manufactured in this way. Many bioactive peptides entering into clinical trials possess chemical modifications that improve *in vivo* stability and other physiochemical properties.[9–13] Aligning the chemistry used to install these structural modifications with recombinant technology has therefore become increasingly important.

Replacement of peptide disulfide bridges with stable isosteres can be used to improve bioavailability and protease stability.[14–18] Towards this end, Ru-alkylidene catalysed ring closing metathesis (RCM) of sequence incorporated olefinic amino acid residues can be used to generate dicarba peptide analogues.[14,19] Significantly, previous work by Tirrell[20–22] and Schultz[23] has described genetic incorporation of non-proteinogenic allyl and crotyl glycine residues, *inter alia*, into peptide sequences, highlighting the potential to recombinantly generate olefin metathesis primed peptide precursors. However, the metathesis chemistry required to deal with the resultant highly polar, unprotected and functionally diverse substrates remains highly challenging. Towards this end, Davis and co-workers report cross metathesis on allyl sulfide[24] and allyl selenide[25,26] functionalised peptides to achieve site selective protein modification in aqueous media. Pre-coordination of the chalcogen to the catalyst's Ru-centre leads to rapid initiation and metathesis prior to catalyst decomposition.[27] Additionally, under analogous reaction conditions, Schultz and co-workers have performed RCM on a large fluorescent protein encoded with crotyl serine residues.[23] Despite the success of this approach, the method relies on large stoichiometric equivalents of both catalyst and cross partner.[23–26] Additionally, although Schultz and coworkers were able to demonstrate the successful expression of crotyl serine, allyl cysteine was shown to be prone to oxidation during recombinant expression necessitating its post translational incorporation.[23]

Our group has a long-standing interest in the generation of dicarba isosteres of cystine-containing cyclic peptides.[19,28–30] RCM of sequence installed allyl glycine residues generates a biologically inert four carbon linkage and close structural mimetic of the native peptide. Work to date has largely centred on the development of solid phase chemistry where sidechain functionality and the *N*-terminus are protected during metathesis. In this manuscript, the development of methodology to align RCM with recombinantly expressed peptides is described. The metathesis is therefore performed in solution on sequences devoid of protecting groups.

Solution phase RCM of five linear, unprotected peptides was investigated to generate dicarba peptides **1–5** (Fig. 1). These sequences were chosen due to their cognate peptide's biological activity and pharmaceutical relevance. Oxytocin is a mammalian hormone responsible for the prevention of post-partum haemorrhage following birth.[31–33] α-Conotoxins Vc1.1 and Pu1.2, as well as fragments of these peptides, have been found to be promising drug leads for the treatment of chronic pain.[34] Octreotate is a potent inhibitor of growth hormone, and is involved in both the imaging and treatment of cancer.[35] Lastly, the insulin fragment **5** was chosen due to the hormone's importance in the treatment of diabetes and our long standing interest in the mechanism of action of this peptide.[36] Several factors needed to be considered to

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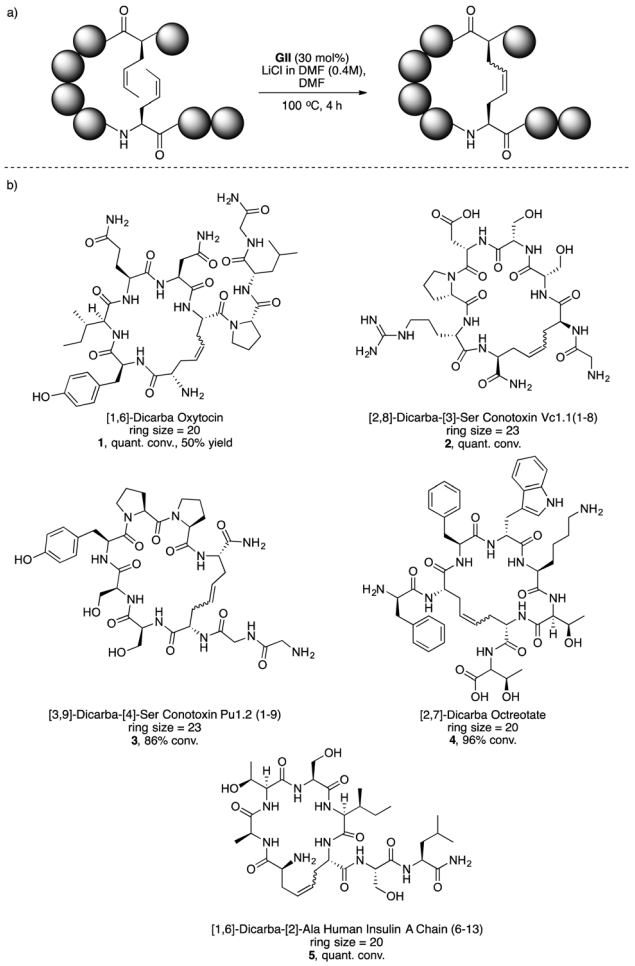


Fig. 1 (a) Generic scheme for RCM of protecting group-free peptides, and (b) unprotected dicarba peptides (**1–5**) synthesised using optimised RCM conditions.

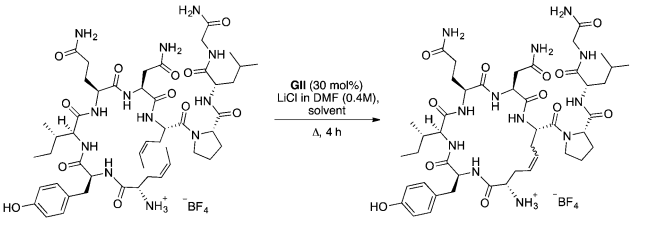
ensure optimum catalytic turnover. Towards this end, Ru-alkylidene catalysts are susceptible to deactivation and decomposition by functional groups that act as strong donor ligands.[37] Recent work has shown that Brønsted acids can be used to conveniently and reversibly mask problematic amine functionality rather than opting for covalent protection strategies.[38] Hence deactivation of peptidic amine functionality in the linear precursors, located in residue sidechains (*e.g.* lysine) and *N*-termini, was efficiently achieved by conversion to an ammonium derivative *via* ion exchange chromatography prior to RCM. Secondly, avoiding the use of terminal olefinic residues promotes catalytic cycling through robust Ru-alkylidene species; methylidene species are fragile and vectors for catalyst decomposition.[39] *Z*-Crotyl glycine, generated through partial hydrogenation of butynyl glycine with Lindlar's catalyst, was therefore chosen as the metathesis-active residue. Finally, the reaction solvent needed to be selected to ensure solubilisation of the unprotected, polar peptide and olefin metathesis catalyst, and deliver high catalyst activity. Initially DMF was chosen: although this solvent is widely considered to deactivate Ru-alkylidene catalysts, many successful examples of olefin metathesis using DMF have been reported.[40–43] After an extensive investigation of reaction temperature, time, catalyst loading and ammonium counter ion (ESI,† Table SI1), the optimised RCM conditions were applied to the above described unprotected peptide sequences using 30 mol% **GII** (Fig. 1). Gratifyingly, high conversions to the cyclic dicarba products **1–5** were observed for all of the peptides (Fig. 1). Product purification by RP-HPLC was conducted for dicarba oxytocin **1**, providing *cis*- and *trans*-isomers in a 50% yield, in a ratio of 55 : 45 *cis* : *trans*, and >97% purity. Significantly, this result is comparable with that obtained by Vederas and co-workers in their original RCM study of dicarba oxytocin where the precursor peptide was solid supported and fully protected.[44] In conclusion, the generic methodology described herein provides a powerful way to construct cyclic dicarba peptides with varying sequence and ring size. To our knowledge, it is the first example where the cystine-bioisosteric diaminosuberic acid bridging motif has been installed *via* solution phase RCM of an unprotected linear peptide sequence.

In view of DMF being considered an undesired solvent choice for industrial medicinal chemistry,[45–48] other solvents were explored using the oxytocin sequence **6** as the substrate for evaluation. Gratifyingly, green replacements for DMF (Table 1, entry 1) were identified; methanol gave an excellent 84% conversion and ethanol a good conversion of 66% (Table 1, entries 2 and 3). Aqueous solvent mixtures gave no conversion (ESI,† Table SI2). Similarly, the green solvent propylene carbonate and *N*-butylpyrrolidinone were not effective, nor were the dipolar aprotic solvents dimethylsulfoxide and acetonitrile (ESI,† Table SI2).

Prior to establishing the aforementioned optimised metathesis conditions several regio- and stereoisomeric oxytocin sequences were synthesised and evaluated. Interestingly, sequence replacement of the two *Z*-configured crotyl residues (Table 2, entry 1) with *E*-crotyl residues to give isomeric peptide **7** led to only 72% RCM conversion to **1** under identical reaction conditions (Table 2, entry 2). Furthermore, sequences containing crotyl and allyl glycine residues (**8** and **9**, entries 3 and 4) led to inseparable mixtures of **1** and homologous peptides. The metathesis sensitivity to sequence configuration was highly unexpected and merited further investigation.

Allyl glycine is commonly employed to form Δ4,5-diaminosuberic acid bridges using on-resin olefin metathesis chemistry.[14–16,18] It is not normally problematic, however terminally located allyl

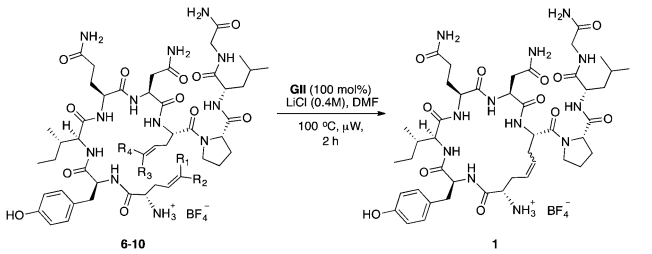
Table 1 RCM of linear oxytocin **6** under varying solvent conditions



6 1

Entry	Solvent	Temp. (°C)	Conv. to **1** (%)
1	DMF	100	Quant.
2	MeOH	Reflux	84
3	EtOH	Reflux	66

Table 2 RCM of linear oxytocin analogues **6**–**10**

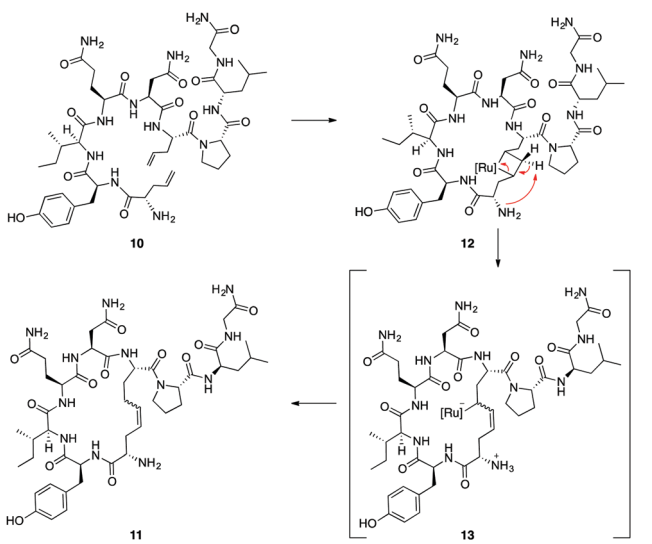


Entry	Compound	R_1	R_2	R_3	R_4	Conv. (%)
1	**6**	CH_3	H	CH_3	H	100
2	**7**	H	CH_3	H	CH_3	72
3	**8**	CH_3	H	H	H	*a*
4	**9**	H	CH_3	H	H	*a*
5	**10**	H	H	H	H	22

a Complex reaction mixture (including cyclic peptides **1** and **11**).

glycine residues, as in **10**, are traditionally *N*-acylated during the metathesis step. This led us to postulate that complications might be arising from *in situ* generation and errant reaction of an *N*-terminal allyl glycine substituted sequence. Significantly, reaction of the diallyl derivative **10** (entry 5) gave the desired cyclic product **1** but the major product (62%) was homoalkene **11** (Scheme 1).

The deleterious effect of nitrogen bases on metathesis reactions utilising Ru-alkylidene catalysts (*e.g.* **HGII**) has been extensively studied by Fogg and coworkers.[49] Under an analogous reaction mechanism, amine-assisted deprotonation of the 1,3-metallocyclobutane intermediate **12** would be facilitated by a 6-membered transition state (Scheme 1). The resultant anionic σ-alkyl complex **13** can then collapse with irreversible elimination of observed homologated peptide **11**. Such a mechanism is also consistent with the observed differences in reaction profile between *E*- and *Z*-Crt substituted peptides **6** and **7** where *Z*-Crt **6** provided clean quantitative conversion to the cyclic product **1**, while the *E*-Crt **7** gave a lower overall conversion to **1** as well as formation of unknown by-products.[50] Hence, despite our attempts to dampen the nucleophilicity of the amine as the ammonium tetrafluoroboronate salt, under some reaction conditions an equilibrium with the free amine presumably arises to facilitate proton abstraction and by-product **11** formation. It is important to note that the by-product **11** only arises during metathesis of *N*-terminal allyl glycine substituted sequences and this can be eliminated through the use of crotyl glycine residues.



Scheme 1 Proposed mechanism leading to homoalkene **11**.

In conclusion, an expedient route to the synthesis of cyclic dicarba peptides from protecting group-free, linear sequences is described. After temporary masking of primary amines as ammonium salts, crotyl glycine-containing linear sequences undergo efficient ring closing metathesis to afford dicarba cyclic peptides. This approach was exemplified by RCM of five structurally diverse peptide sequences **1**–**5**. The strategy described herein heralds an opportunity to access dicarba-stabilised cyclic peptides from feedstock derived from large scale recombinant processes.

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Conflicts of interest

There are no conflicts to declare.

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