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Synthesis and bio-assay of RCM-derived Bowman–Birk inhibitor analogues †

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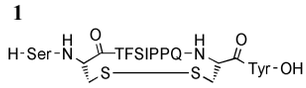
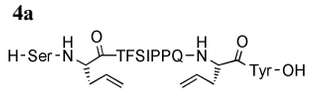
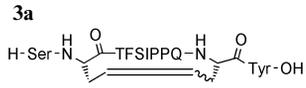
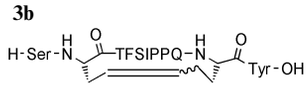
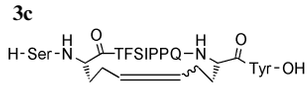
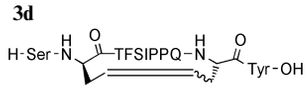
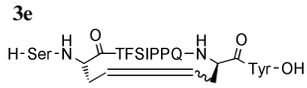
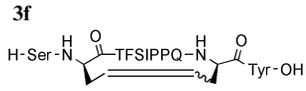
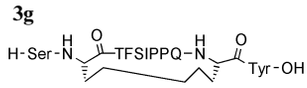
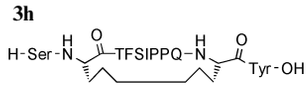
Bowman–Birk inhibitor analogues containing 2, 3 and 4-carbon analogues of the natural disulfide were synthesised *via* solid phase microwave-assisted RCM and found to have K_i values against chymotrypsin in the low to sub-micromolar range, the best replacement for the disulfide arising from the linkage by RCM of two L-homoallylglycine residues.

Cyclic peptides and their derivatives are of great importance as potential drug candidates as they often exhibit enhanced stability and activity relative to their linear counterparts.[1] In recent years, there has been an explosion of interest in the use of ring closing metathesis (RCM) to synthesise conformationally constrained cyclic peptides.[1b,2] Our interest in cyclic peptides is concentrated around the Bowman–Birk inhibitors[3] (BBI), naturally-occurring serine protease inhibitors with nine-residue disulfide-constrained loops of peptide that are responsible for inhibitory activity. It is well known that synthetic nine-residue-cyclic peptides based on the BBI loop sequence retain much of the loop structure[4] (a β-hairpin with type VIb turn) and activity[5] of the parent BBI, whereas the linear analogues are relatively inactive.[5c] One example of a synthetic mini-inhibitor is the cyclic disulfide **1** (Table 1), identified within our group from a solid phase combinatorial peptide library and found to have an equilibrium dissociation constant (K_i) against chymotrypsin of 19 nM.[5b] We wondered whether the disulfide could be replaced with an all-carbon link *via* RCM of a linear dienic peptide in which the flanking cysteine residues are replaced with olefin-containing residues such as L-allylglycine.[6]

In initial studies, we assembled resin-bound dienic peptide **2a**[7] from Fmoc-Tyr(tBu)-Wang polystyrene resin using standard Fmoc methods and found that the desired RCM proceeded with Grubbs' second generation ruthenium benzylidene catalyst in dichloromethane. The reaction could be carried out at reflux or, more efficiently, at 120 °C under microwave irradiation conditions to give cyclic peptide **3a**[8] upon deprotection and cleavage (Fig. 1).

RCM has recently been reported under microwave irradiation conditions for small molecules,[9] but to our knowledge this is the first report of microwave-assisted RCM of a peptide, and also the first such report for a resin bound substrate.[10] Encouraged by this success, we then turned our attention to the synthesis of a small array of inhibitor analogues whereby the length and stereochemistry of the all-carbon link was varied. This was accomplished simply by replacing one or both of the L-allylglycine residues in the linear diene precursor with either D-allylglycine or L-homoallylglycine. In particular when D-allylglycine was incorporated we found the use of our microwave-assisted conditions to be essential to drive the sluggish metathesis reactions towards the cyclised products.[11] In two examples, the olefin in the cyclic product was hydrogenated by diimide reduction[12] on solid phase[2f] to furnish the saturated analogue. With the array in hand, we conducted competitive

Table 1 Inhibition assay results for peptide array against chymotrypsin

Entry	Peptide	K_i/μM
1	**1** 	0.016[a]
2	**4a** 	11
3	**3a** 	0.49
4	**3b** 	0.68
5	**3c** 	0.12
6	**3d** 	2.4
7	**3e** 	30
8	**3f** 	2.2
9	**3g** 	2.9
10	**3h** 	1.0

[a] Literature value 0.019 μM.[5b]

† Both single letter and three letter amino acid codes are used in this communication.

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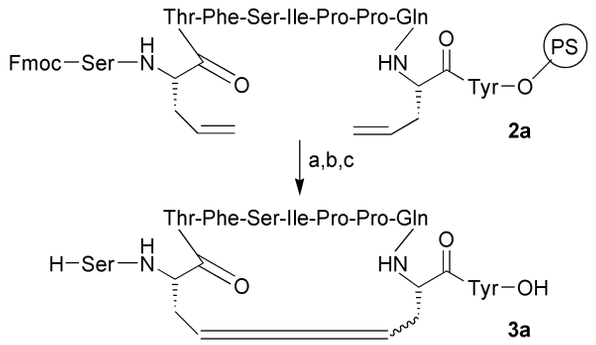

Fig. 1 Synthesis of cyclic peptide **3a** *via* RCM. *Reagents and conditions*: (a) $Cl_2(PCy_3)$(Imes)Ru=CHPh 6 mol%, CH_2Cl_2, 120 °C, microwave, 10 min, 4 iterations; (b) 20% piperidine; (c) 95% TFA. [Imes = 1,3-bis-(2,4,6-trimethylphenyl)-4,5-dihydroimidazol-2-ylidene].

inhibition assays[13] against chymotrypsin to determine K_i values for the inhibitors (Table 1).

For potent activity it was necessary to cyclise the peptides, with the linear analogue **4a** showing only weak inhibition (entry 2). The best RCM-derived inhibitors were cyclic olefins with the same linker stereochemistry as that found in the natural BBI sequences (entries 3–5). Of these, the 31-membered macrocycle **3c** was the most potent, with 7.5-fold less activity than the parent 29-membered macrocyclic disulfide (entry 1). Since RCM-derived cyclic peptides **3a**–**f** are assumed to be a mixture of olefin *E* and *Z* isomers,[8] the true potency of these compounds in an isomerically pure form may be significantly greater than that quoted in Table 1, if the other isomer is less active. It appears from the assay results that of those tested, the six-carbon olefinic link represents the best isosteric replacement for the disulfide-containing link of the parent. Altering the stereochemistry of the all-carbon link was found to greatly reduce inhibitory activity (entries 6–8), presumably due to destabilisation of the active inhibitory conformation. Hydrogenation of the double bond also led to a marked reduction in inhibition (entries 9–10), perhaps due to bond lengthening and/or extra flexibility producing a less optimal conformation.

Despite the wide application of RCM to cyclise peptides, there are few reports to date of the biological activity of RCM-derived cyclic peptidomimetic inhibitors. Therefore the inherent usefulness of the RCM reaction for this purpose and in particular for the replacement of a disulfide bridge remains largely unanswered. Of the reports that exist,[15] the results are in agreement with this study in that RCM was found to significantly improve biological activity compared to the linear counterpart[15b] although some of this improvement was lost upon hydrogenation to the saturated cyclic compounds,[15a,15c] which had markedly lower activity than the cyclic olefins in each case. However it must be emphasised that these reports are for different biological systems than that presented in this communication.

In summary, we have developed novel microwave-assisted solid phase RCM methodology for the synthesis of 29–31 membered macrocyclic peptidic inhibitors of varying stereochemistry. The use of microwave irradiation to promote the RCM reaction is novel for both a peptide substrate and a resin bound substrate. The RCM-derived inhibitors were found to have K_i values in the low to sub-micromolar range in competitive assays against chymotrypsin. To our knowledge this is the first account of inhibition data for a sizeable array of RCM-derived cyclic peptidic inhibitors.

Notes and references

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6 A known strategy for simpler peptides, see ref. 2*h* above.

7 Linear peptide precursors for the RCM reaction such as **2a** were fully side-chain protected using the following standard protecting groups: Ser(tBu), Thr(tBu) and Gln(Trt).

8 Compound **3a** was isolated by reversed phase HPLC as an inseparable mixture of *E* and *Z* alkene isomers in approximately equal ratio, as revealed by ^{1}H NMR spectroscopy. Representative procedure for microwave-assisted RCM is as follows: resin-bound Fmoc-protected peptide **1a** (60 mg, *ca*. 24 mmol) was suspended in CH_2Cl_2 (2.0 mL) in a silanated glass vial. To this was added a solution of tricyclohexylphosphine-[1,3-bis(2,4,6-trimethylphenyl)-4,5-dihydroimidazol-2-ylidene]-[benzylidine]-ruthenium (IV) dichloride (1.27 mg, 1.5 μmol, 6.25 mol%) in CH_2Cl_2 (125 μL) and the vial was sealed. The reaction mixture was heated to 120 °C and held at that temperature for 10 min by microwave irradiation (variable power, up to 300 W). The vial was then cooled and opened, and the process of adding ruthenium catalyst and heating was repeated a further 3 times as before. The resin was then transferred to a fritted vessel and washed with CH_2Cl_2 (5 × 2 mL) followed by a literature washing procedure using tris-(hydroxymethyl)-phosphine[2g] to assist in the removal of any residual ruthenium compounds. The resin was finally washed with CH_2Cl_2 (3 × 2 mL) and dried *in vacuo*. A portion of this resin product (29 mg) was Fmoc-deprotected and cleaved using standard conditions. HPLC purification yielded the cyclic olefinic peptide as a white powder (1.6 mg, 11% based on assumed resin loading of 0.40 mmol g^{-1}). HPLC purity > 95%; FAB-MS *m*/*z* 1205 ([M + H]$^+$, 100%). All other RCM-derived peptides were synthesised similarly, with characterisation by FAB-MS and HPLC purity > 90%, and with olefins assumed to be a mixture of *E* and *Z* isomers (ratios not determined).

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11 RCM conversions could be judged by analytical reversed phase HPLC of the crude product, following Fmoc-deprotection and cleavage. In each case, the cyclic olefinic peptides eluted after the acyclic diene precursors. Under reflux conditions [$Cl_2(PCy_3)$-(Imes)Ru=CHPh 25 mol%, CH_2Cl_2, 18 h] little or no conversion to give peptides **3e** and **3f** was observed, however 25% conversion was seen when microwave irradiation conditions[8] were employed. The effect of microwave irradiation on the more facile RCM of **1a** to give peptide **2a** (Fig. 1) was less pronounced, with 50% conversion under the reflux conditions (above) and 60% conversion for the microwave-assisted reaction.[8]

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13 Competitive inhibition assay conditions: assays were performed at 25 °C in aqueous tris-(hydroxymethyl)-aminomethane buffer (0.144 M, adjusted to pH 7.8 with HCl). Total in-assay enzyme (bovine pancreatic α-chymotrypsin EC 3.4.21.1) concentration was 0.33 nM,

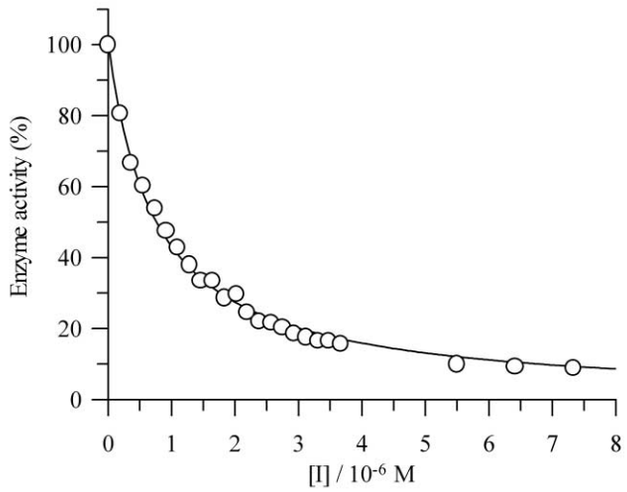

Fig. 2 Inhibition curve for RCM-derived peptide **3a** against bovine α-chymotrypsin. The plot shows the enzyme activity as measured by the relative rate of hydrolysis of Succinyl-Ala-Ala-Pro-Phe-AMC (Y-axis) versus inhibitor concentration [I]. Enzyme concentration was 0.33 nM, and the Michaelis constant for Succinyl-Ala-Ala-Pro-Phe-AMC with the enzyme was taken as 21 μM.[14]

total substrate (Succinyl-Ala-Ala-Pro-Phe-AMC) concentration was 10 μM and inhibitor concentration was varied. The enzyme and inhibitor were incubated for 10 min prior to the addition of substrate. Initial rate data was monitored by fluorescence (360 nm excitation, 460 nm emission) and processed using the non-linear regression features of the GraFit PC software (Leatherbarrow, R. J.; GraFit v. 5.0.6, Erithacus Software Ltd., Surrey, UK, 2001) to produce inhibiton curves (*e.g.* Fig. 2) and K_i values. K_i values quoted in Table 1 are the mean of two closely agreeing assays, except in the case of entries 1 and 7 when a single assay was performed. In the case of peptide **3a**, hydrolysis assays were also performed to demonstrate the stability of the peptide under the assay conditions. Thus, peptide **3a** was incubated with chymotrypsin (I : E ratio of 550 : 1) at 25 °C and pH 7.8. Analytical HPLC of an aliquot after 30 min showed no detectable hydrolysis of **3a** had occurred.

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