Chemical and biomimetic total syntheses of natural and engineered MCoTI cyclotides

Panumart Thongyoo, Núria Roqué-Rosell, Robin J. Leatherbarrow* and Edward W. Tate*

Received 30th January 2008, Accepted 15th February 2008
First published as an Advance Article on the web 6th March 2008
DOI: 10.1039/b801667d

The naturally-occurring cyclic cystine-knot microprotein trypsin inhibitors MCoTI-I and MCoTI-II have been synthesised using both thia-zip native chemical ligation and a biomimetic strategy featuring chemoenzymatic cyclisation by an immobilised protease. Engineered analogues have been produced containing a range of substitutions at the P_1 position that redirect specificity towards alternative protease targets whilst retaining excellent to moderate affinity. Furthermore, we report an MCoTI analogue that is a selective low-μM inhibitor of foot-and-mouth-disease virus (FMDV) 3C protease, the first reported peptide-based inhibitor of this important viral enzyme.

Introduction

Cyclotides[1,2] are a unique class of head-to-tail cyclic cysteine-rich microproteins up to 37 amino acids in length that exhibit a wide range of biological properties, ranging from anti-HIV[3–5] to insecticidal activity.[6,7] In contrast to many smaller naturally occurring cyclic peptides they are true gene products, and possess a highly stable cystine-knot† topology, whereby two disulfide bridges form a ring through which a third is threaded.[8–10] This combination of intricate structure, diverse biological activity and unusual biogenesis has inspired a growing research effort directed towards understanding the synthesis and function of cyclotides both in the cell and *in vitro*.[7,11–13] In addition, their high resistance to degradation *in vivo* has attracted recent and widespread interest in the potential of cyclotides as scaffolds to present structured protein domains for therapeutic applications.[14–17] To date, cyclotides have been isolated from a variety of plant species, including Cucurbitaceae (genus *Momordica*),[18,19] Rubiaceae[20] and Violaceae,[21,22] where they are thought to act as antifeedants.[7] Recent work, most notably by the Craik group, has provided the three-dimensional structures of a wide range of cyclotides by NMR.[23] Syntheses of several small to medium size cyclotides have been reported[24–28]

The cyclotides MCoTI-I and MCoTI-II were first isolated from the dormant seeds of *Momordica cochinchinensis*, and have been shown to be very potent (sub-nM) inhibitors of trypsin.[18] Whilst the MCoTI trypsin inhibitor cyclotides do not share significant sequence homology with other cyclotides beyond the presence of the three cystine bridges, solution NMR has shown that they do indeed adopt a backbone-cyclic cystine-knot topology (Fig. 1). The binding mode of these cyclotides is likely to be similar to that of related cystine-knot trypsin inhibitors such as EETI;[17,29,30] however, their rigidifying cyclic backbone contributes both enhanced stability and increased potency in comparison to these simpler structures.

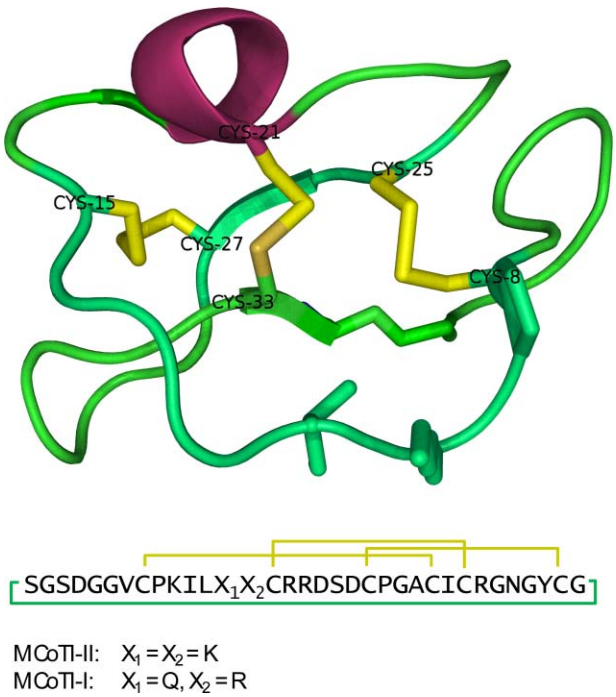


Fig. 1 Solution NMR structure of MCoTI-II and sequences of the known naturally-occurring MCoTI cyclotides, MCoTI-I and -II.[18,36] Key residues in the active loop (Pro9, Lys10, Ile11) and the cystine bridges are highlighted. Red: alpha helix; green: backbone; yellow: cystine bridges. Structure rendered with PyMOL.[37]

As part of our ongoing studies on the potential of cyclotides as stable scaffolds for drugs or as tools for chemical genetics[31] we wished to explore whether the structure of the MCoTI cyclotides may be re-engineered to exhibit novel inhibitory activity. Furthermore, previous studies suggest that cyclotides from *Rubiaceae* and *Violaceae* form *via* protease-mediated ring closure at a conserved motif,[7,12,32,33] and we hypothesised that the total synthesis of MCoTI cyclotides might be achieved *via* a biomimetic protease/ligase pathway. Here we report in full our recent studies in these two areas,[34,35] and describe the activity and selectivity of natural and engineered MCoTI cyclotides for a range of proteases.

Department of Chemistry, Imperial College London, Exhibition Road, London, SW7 2AZ. E-mail: e.tate@imperial.ac.uk, r.leatherbarrow@imperial.ac.uk; Fax: (+44) (0)2075941139

† Note that the term *cysteine*-knot is also used to describe this structural motif.

We also discuss the potential implications for future work aimed at realising the full potential of cyclotides as tools in medicinal and chemical biology.

Results and discussion

Chemical synthesis of engineered MCoTI cyclotides

Total synthesis of an engineered cyclotide presents three key challenges:

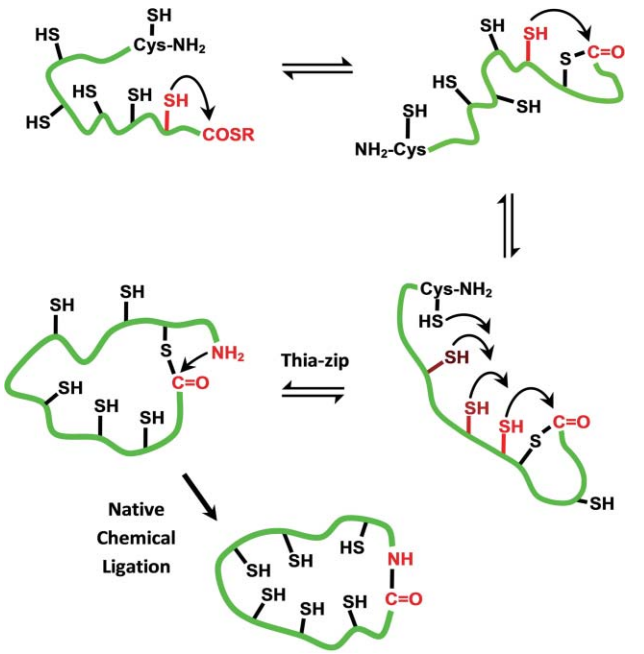
1. Synthesis of the backbone, which in the case of MCoTI contains a sensitive Asp-Gly sequence.
2. Head-to-tail backbone cyclisation.
3. Oxidative protein folding to form the three disulfide bonds in the correct cystine-knot geometry.

In practise, the strategy adopted for backbone synthesis lays the foundation for correct cyclisation and folding, and this may be approached using a range of methodologies. Boc-,[38] microwave-enhanced[24] and Fmoc-[14,30,34,35] based solid-phase peptide synthesis (SPPS) have all been used for cyclotide backbone construction, whilst recent work has shown that bacterial expression can also be an effective approach for smaller scale production of the cyclotide backbone.[25,28]

Backbone cyclisation may then be achieved by exposing a suitably side chain protected backbone peptide bearing an amino N-terminus and a carboxy C-terminus to standard peptide coupling conditions, but this approach suffers from multiple drawbacks including poor peptide solubility and the need for high dilution. Thia-zip native chemical ligation (TZ-NCL), a technique first pioneered by the Tam group,[39,40] has emerged as the most effective method for backbone cyclisation of cysteine-rich cyclic peptides, and is particularly applicable to the synthesis of cyclotides.

In this approach, an unprotected backbone peptide bearing an N-terminal cysteine and a C-terminal thioester is simply dissolved in a suitable buffer triggering a spontaneous cascade cyclisation reaction, resulting in eventual formation of a macrocyclic thioester that undergoes native chemical ligation to the target head-to-tail cyclic peptide (Scheme 1). The presence of multiple internal cysteine residues greatly accelerates the cyclisation by providing a series of thiolactone intermediates of inferior ring size that are readily populated under equilibrium conditions, and are eventually converted into the target compound by irreversible macrolactamisation of the largest ring *via* NCL. Similarly, in the bacterial expression of cyclotides constructs bearing a C-terminal intein permit a similar mechanism to operate.[28] Despite the potentially problematic synthesis of a C-terminal thioester, the overall sequence of SPPS followed by TZ-NCL has proven to be significantly more efficient and reliable than the analogous peptide bond formation at high dilution, and may be performed at relatively high concentration without detectable formation of oligomeric by-products.

Subsequent formation of the three disulfide bonds in the correct configuration is highly dependent on the inherent propensity of the cyclotide to adopt a cystine-knot.[8] In some cases, differential protection of cysteine residues with acetamidomethyl (Acm) groups is required to direct sequential formation of one or two disulfide bonds, followed by forced oxidation to the native state.[39] Unfortunately, this approach may reduce the efficiency of TZ-NCL due to reduced availability of internal cysteine residues, and disulfide bond exchange tends to reduce the purity of the final

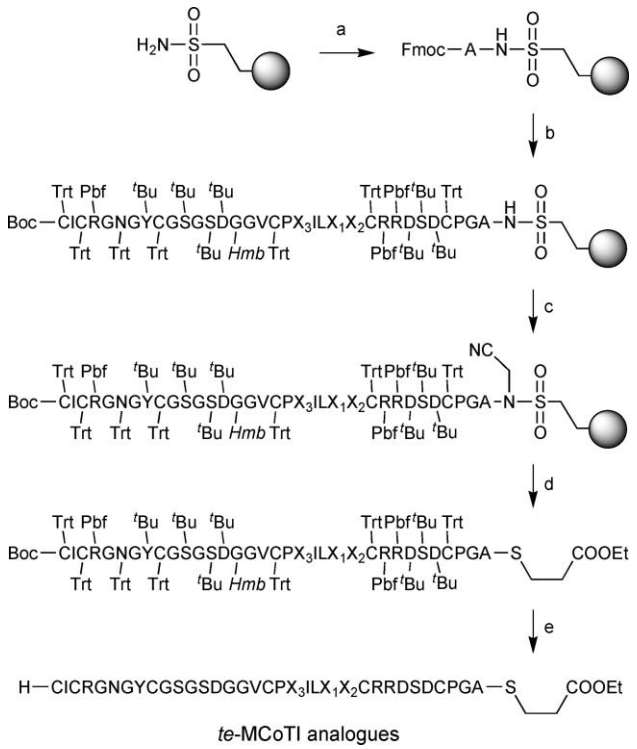


Scheme 1 Schematic representation of thia-zip native chemical ligation (TZ-NCL). Ring expansion occurs *via* an inter-converting series of macrothiolactones, the largest of which can be trapped by an N-to-C rearrangement (native chemical ligation, NCL) to yield the target macrolactam.

product. However, a recent systematic study on the mechanism of oxidative folding of plant-derived MCoTI-II by Craik *et al.* demonstrated that it has a strong tendency to adopt the native cystine-knot conformation,[9] so we did not anticipate significant problems with this step.

We therefore chose to adopt Fmoc-based SPPS synthesis of the backbone thioester followed by TZ-NCL for the total synthesis of MCoTI cyclotides and analogues. The Ala22–Cys23 amide bond was selected as the optimal position for backbone cyclisation, since among the possible Xaa-Cys disconnections it presents the lowest hindrance to NCL. SPPS was carried out on a sulfamylbutyryl 'safety-catch' linker[41] after loading of the C-terminal alanine residue under optimised conditions, as shown in Scheme 2. Activation and displacement of the resultant C-terminal sulfonamide was best achieved with iodoacetonitrile and ethyl 3-mercaptopropionate, resulting in excellent overall yield of linear thioester with respect to a sequence of 60+ synthetic steps. Introduction of the sensitive Asp-Gly motif as a dipeptide bearing a hydroxy-4-methoxybenzyl (Hmb)[42] protecting group on the glycine backbone nitrogen to suppress aspartimide formation was found to be essential, and omission of this group resulted in only trace quantities of linear product from SPPS.

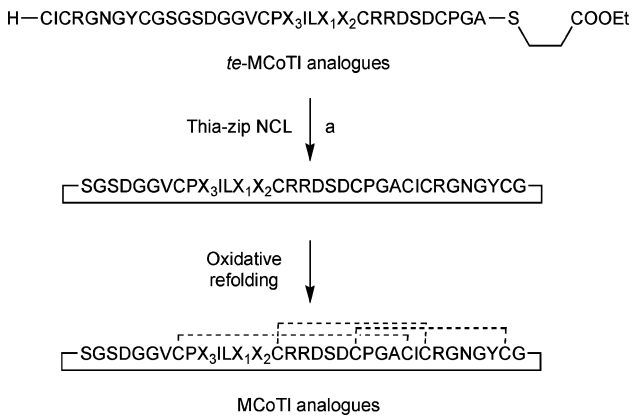
In addition to the naturally-occurring MCoTI-I and MCoTI-II cyclotides, a focussed series of MCoTI-II analogues bearing alternative residues in place of Lys10 was designed to enable a systematic investigation of the influence of this position on protease inhibition. Lys10 is recognised as the key determinant of binding to trypsin by analogy to well-characterised homologous cystine-knot trypsin inhibitors such as EETI,[29,43] and occupies the P_1 position in the substrate binding site. Engineering changes at this residue would therefore be expected to alter inhibitory



Scheme 2 Synthetic route for SPPS synthesis of MCoTI cyclotide backbone thioester (*te*-MCoTI) analogues, starting from sulfamylbutyryl AM resin. MCoTI-I: $X_1 = Q$, $X_2 = R$, $X_3 = K$; MCoTI-II: $X_1 = X_2 = X_3 = K$; MCoTI-II analogues: $X_1 = X_2 = K$, $X_3 = R, Q, F, V, A$ or AKQ. Protecting groups: Boc: *tert*-butyoxycarbonyl; Trt: trityl; Fmoc: 9-fluorenylmethoxycarbonyl; Pbf: 2,2,4,6,7-pentamethyl dihydrobenzo furan-5-sulfonyl; *Hmb*: hydroxy-4-methoxybenzyl (on backbone nitrogen). See Table 3 for overall yields of linear thioesters. Conditions: (a) Fmoc-Ala-OH, DIPEA, PyBOP, −20 °C, 8 h; (b) Fmoc–^{t}Bu SPPS; (c) NMP, DIEPA, iodoacetonitrile (excess), 24 h, r.t.; (d) sodium thiophenolate (cat.), ethyl 3-mercaptopropionate, DMF, 16 h, r.t.; (e) TFA, H_2O, EDT, TIPS (94 : 2.5 : 2.5 : 1).

activity against trypsin, and potentially enable the development of inhibitors of alternative proteases whose activity depends on the presence of a residue other than Lys at P_1, by analogy with previous work on peptide-based protease inhibitors.[44–46] Accordingly, open chain thioester MCoTI-II analogues (*te*-MCoTI) bearing positively charged (Arg), hydrophobic (Val, Ala), aromatic (Phe) or amide (Gln) side chains were synthesised (Scheme 2b), along with one analogue with an expanded active loop (Ala-Lys-Gln) to investigate the versatility of the synthetic strategy with a longer backbone.

Backbone cyclisation of the linear thioesters *via* TZ-NCL was achieved under standard NCL conditions (100 mM ammonium carbonate buffer, pH 7.5), and in the presence of a reducing agent such as tricarboxyethyl phosphine (TCEP) the reduced cyclic backbone with six free cysteine thiols was isolated. Mild oxidative refolding by exposure to air in the presence of a suitable disulfide bond-exchange mediator (glutathione, reduced form) resulted in smooth conversion to a new product within minutes, as determined by reverse-phase HPLC (RP-HPLC). Analysis of this material by MALDI-TOF showed the formation of 3 disulfide bonds, and the native cyclotide fold of synthetic MCoTI-II was confirmed by NMR following the protocols of Craik *et al.*,[23] and in inhibition studies (see below). Optimal results were obtained by combining cyclisation and refolding in a one-pot reaction, whereby the linear thioester is treated with glutathione in the same buffer, producing the target cyclotide in excellent overall yield (Scheme 3). The extended MCoTI-II variant MCoTI-II[AKQ] was formed in comparable yield and purity, suggesting that the MCoTI-II cyclotide fold tolerates the presence of additional residues in this loop.



Scheme 3 One-pot TZ-NCL-refolding of linear precursor thioester (*te*-MCoTI) analogues; X_1, X_2, X_3: see Scheme 2. Solid bond: backbone cyclisation; dashed bonds: disulfide bridges. See Table 4 for isolated yield of target cyclotide after purification by RP-HPLC. Conditions: (a) glutathione (1 mM, reduced form), aqueous ammonium carbonate (100 mM, pH 7.4), r.t., 24 h. The intermediate head-to-tail cyclic peptide undergoes spontaneous refolding under these conditions to yield the target cyclotide in a one-pot reaction.

Protease inhibition by engineered cyclotides

With a variety of natural and engineered MCoTI cyclotides in hand, we next analysed their activity against a panel of proteases (Table 1). As previously determined for the natural products, synthetic MCoTI-I and MCoTI-II are extremely potent trypsin inhibitors, with K_i values in the low-pM range.[29] MCoTI-II[R], which contains a conservative substitution that is tolerated in trypsin substrates, also maintains the potency of the parent natural product. These cyclotides also proved to be highly selective for trypsin: all other analogues tested had 10^3- to 10^4-fold lower activity in the assay, emphasising the importance of a positively-charged residue at P_1. Activity was effectively switched to chymotrypsin when Lys10 was changed to match the preferred P_1 residue (Phe), with only MCoTI-II[F] showing low-nM activity against this enzyme, although other uncharged residues (Q, V, A) were also tolerated to some degree. The MCoTI cyclotides appear to be selective inhibitors for trypsin family proteases, exhibiting micromolar or higher K_i against the serine proteases thrombin and subtilisin. Remarkably however, MCoTI-II[Q] and MCoTI-II[AKQ] show significant activity against 3C protease from foot-and-mouth-disease virus (FMDV $3C^{pro}$),[47] a recently identified *cysteine* protease essential for replication in this virulent pathogen. Inhibition is specific to these cyclotides, which contain elements of the preferred P_4-P_1 residues (PAKQ) of FMDV $3C^{pro}$,[48] with other cyclotide analogues showing no detectible activity. Crystallography studies have shown that FMDV $3C^{pro}$, like other picornaviral cysteine proteases, exhibits structural homology to mammalian

Table 1 Inhibition activity (K_i) of naturally-occurring and engineered MCoTI cyclotides against trypsin, chymotrypsin, thrombin and FMDV 3C protease. Note that the units for activity against trypsin and chymotrypsin are pM and nM respectively, and are μM for all other enzymes

	K_i Values				
Cyclotide Lys10 = [X]	Trypsin/pM	Chymo-trypsin/nM	Thrombin/μM	Subtilisin/μM	FMDV $3C^{pro}$/μM
MCoTI-I	29 ± 2	$>10^4$	>100	>100	>100
MCoTI-II	75 ± 5	$>10^4$	>100	>100	>100
MCoTI-II[R]	85 ± 7	$>10^4$	>100	>100	>100
MCoTI-II[Q]	5×10^6	2×10^3	>100	>100	41 ± 25
MCoTI-II[F]	0.4×10^6	9.8 ± 0.7	>100	49 ± 4	>100
MCoTI-II[V]	$>10^9$	$>10^4$	>100	>100	>100
MCoTI-II[A]	$>10^9$	$>10^4$	>100	>100	>100
MCoTI-II [AKQ]	$>10^9$	$>10^4$	>100	>100	56 ± 35

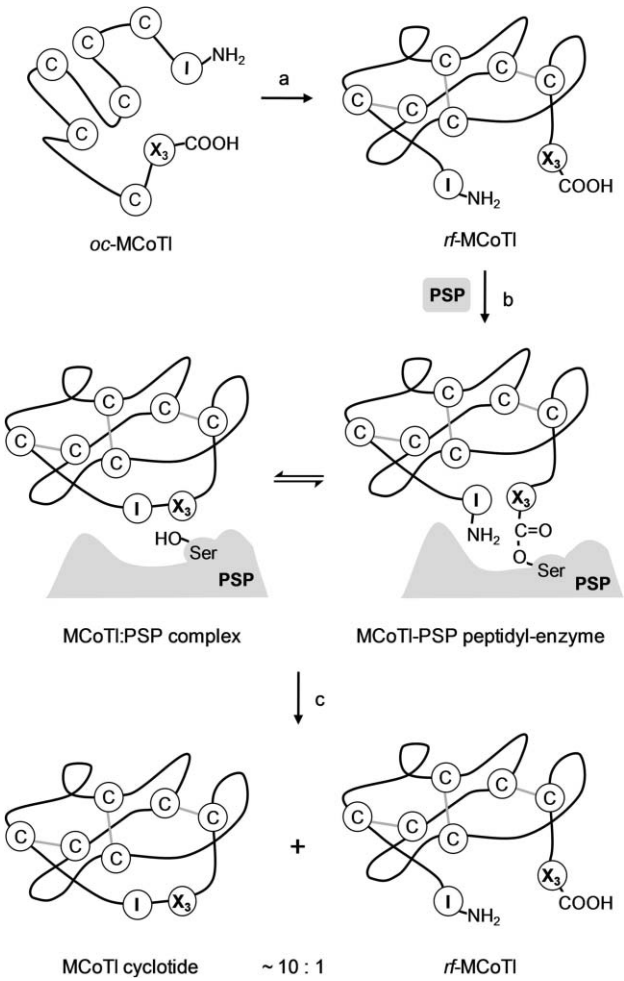
serine proteases including a chymotrypsin-like fold, which may favour the inhibitory activity of MCoTI cyclotides against this target.[47,48] MCoTI-II[Q] and MCoTI-II[AKQ] represent the first known protein-based inhibitors of this important viral protease.

Chemoenzymatic synthesis of engineered MCoTI cyclotides

Having established the inhibition activity and selectivity profile of the MCoTI cyclotides, we next sought to design a simplified synthesis of the head-to-tail cyclic cystine-knot scaffold by utilising a biomimetic (chemoenzymatic) approach. It is known that related cyclic peptidic trypsin inhibitors such as BPTI[49,50] and SFTI-I[51] bind to the enzyme active site in an inter-convertible mixture of non-covalent enzyme–peptide complex and covalently-bound peptidyl-enzyme, a non-productive analogue of the acyl–enzyme intermediate in proteolytic cleavage. Building on previous work that shows that proteases can be induced to synthesise a peptide bond by careful control of the reaction conditions,[52–55] we reasoned that a linear cyclotide backbone acid bearing the P_1 residue at the C-terminus might be a viable substrate for protease-mediated ligation, thus enabling synthesis of the cyclic backbone without the need for a C-terminal thioester.

An initial proof-of-principle experiment was carried out to test whether this concept could be applied for total synthesis of the naturally-occurring MCoTI-II. The requisite Lys10 C-terminal acid open-chain backbone (*oc*-MCoTI-II) was synthesised in excellent overall yield using Fmoc–^{t}Bu SPPS. As in our previous syntheses of MCoTI cyclotides, aspartimide formation at the DG motif was effectively suppressed by the introduction of Hmb protection at the glycine backbone nitrogen.[42] *oc*-MCoTI-II was refolded to the cystine-knot peptide *rf*-MCoTI-II by exposure to our standard oxidative refolding conditions (Scheme 4), and no misfolded peptide was observed by HPLC. In initial attempts to achieve backbone ligation, mixtures of *rf*-MCoTI-II and trypsin (up to 1 : 1 ratio) were combined in 100 mM phosphate buffer (pH 7.4). In each case, some cyclisation to MCoTI-II was seen by MALDI and HPLC by comparison with authentic MCoTI-II prepared by one-pot TZ-NCL chemistry, but contamination with peptides resulting from protease auto-digestion precluded further analysis and purification.

Trypsin immobilised on Sepharose beads (polymer-supported trypsin, PST) is used widely for sequencing applications, since contamination through autocleavage is greatly suppressed. We considered that PST might be employed as a polymer-supported ligase for clean MCoTI macrolactamisation. Furthermore, the newly formed cyclotide should remain tightly bound to the solid support, enabling facile *in situ* affinity purification.‡ In a typical



Scheme 4 Chemoenzymatic synthesis of MCoTI-II and analogues from open-chain acids (*oc*-MCoTI, prepared by SPPS), *via* refolded cystine–knot intermediates (*rf*-MCoTI). PSP: polymer supported serine protease (trypsin or chymotrypsin); X_3 = K or F. Conditions: (a) 0.1 M ammonium carbonate (pH 7.8)–acetonitrile 1 : 1, 1 mM glutathione (reduced form); (b) PSP (1 equiv.), 100 mM sodium phosphate (pH 7.0); (c) wash (H_2O), then 0.1% $TFA_{(aq)}$.

‡ Subsequent to our first communication of the work presented here, PST was also described as a reagent for affinity purification of MCoTI-II expressed in *E. coli*.[25]

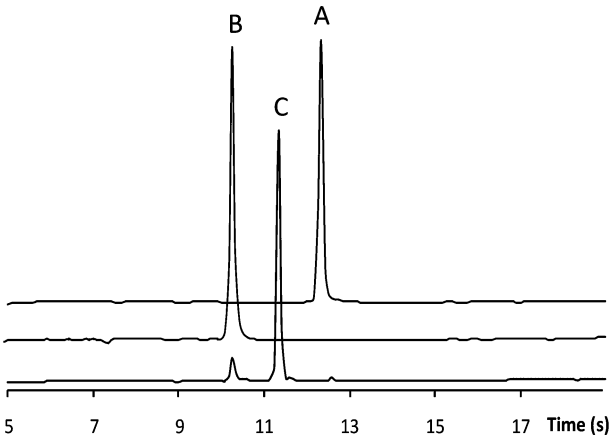


Fig. 2 HPLC traces showing conversion of open-chain acid to refolded backbone to target cyclotide. **A**: purified *oc*-MCoTI-II; **B**: purified *rf*-MCoTI-II; **C**: product after elution of MCoTI-II from PST with 0.1% $TFA_{(aq)}$. Gradient: 0 to 85% MeCN in H_2O (+ 0.1% TFA) over 40 minutes.

binding procedure (Scheme 4) a two-fold excess of *rf*-MCoTI-II was incubated with sequencing grade PST suspended in phosphate buffer (100 mM, pH 7.4) at 37 °C for 15 min, after which the beads were washed several times with buffer. We hypothesised that increased protonation at the trypsin active site might result in release of the cyclotide from the putative cyclotide–enzyme complex in a non-denaturing and pH-dependent manner, and thus allow the relatively costly PST to be recycled. Gratifyingly, upon treatment with 0.1% aqueous TFA (pH ~3) in deionised water MCoTI-II was released from PST in 92% yield, and in high purity (Fig. 2). MCoTI-II synthesised by this chemoenzymatic method was identical in all respects to naturally occurring MCoTI-II.[18,19,29,36] Interestingly, concomitant release of a small amount (8%) of *rf*-MCoTI-II is observed, although it was absent from the flow-through from the wash steps. Incubating pure MCoTI-II with PST under the same conditions yielded an identical mixture, strongly suggesting that this ~10 : 1 ratio reflects the equilibrium mixture of MCoTI-II–trypsin complex to peptidyl-enzyme intermediate, similar to that previously noted by Craik *et al.* in related studies on SFTI-I.[51] It was found that by virtue of the non-denaturing elution conditions, PST could be reused in at least five cycles of protease-mediated ligation with no detectable loss of activity. In summary, PST-mediated ligation effectively combines macrolactamisation with on-bead affinity purification; the cyclotide is bound as a 1 : 1 trypsin–cyclotide complex, as evidenced by the ability of MCoTI-II to titrate the trypsin active site, necessitating a stoichiometric quantity of PST relative to *rf*-MCoTI-II. Remarkably, backbone refolding and PST-mediated ligation/affinity purification may even be combined in a single pot by simple incubation of crude *oc*-MCoTI-II with PST in buffer containing 1 mM glutathione to give MCoTI-II and ~10% residual *rf*-MCoTI-II in very high purity upon elution with 0.1% TFA.

This facile new route to MCoTI-II was explored in a series of experiments that show that PST possesses ligase specificity for positively charged (Arg/Lys) residues at P_1 in the inhibitory loop. The open-chain forms of the naturally occurring cyclotide MCoTI-I (P_1=Lys) and three MCoTI-II analogues in which P_1 was changed to Arg, Gln or Phe (Table 2) were synthesised by SPPS, and subjected to the refolding and PST-mediated ligation conditions described above. Folded macrocyclic MCoTI-I and MCoTI-II[R] were produced in high purity by comparison with authentic samples prepared by TZ-NCL, whilst MCoTI-II[Q] and MCoTI-II[F] appear to be cleaved at one or more positions on the backbone, with multiple peaks observed in the RP-HPLC trace. As for MCoTI-II, ligation reactions yielded MCoTI-I (93%) and MCoTI-II[R] (94%) together with refolded starting material in a 10 : 1 ratio. However, re-engineering the inhibitory loop to contain P_1=Phe enables ligation by chymotrypsin. Thus *oc*-MCoTI-II[F] was refolded and incubated with commercially available sequencing-grade polymer-supported chymotrypsin (PSC) to yield MCoTI-II[F] analogue cyclotide (90%), with 10% residual *rf*-MCoTI-II[F]. Furthermore, the reaction is highly specific: *rf*-MCoTI-II[F] is the only MCoTI analogue susceptible to ligation with PSC; other cyclotides are either unreactive or partially digested under the same conditions. Qualitative selectivity in the ligase reaction is therefore directly related to potency of inhibition: only the most potent inhibitory cyclotides are susceptible to ligation with a given solid-supported protease.

Conclusions

In summary, we have achieved the total synthesis of MCoTI-I, MCoTI-II and MCoTI cyclotide analogues in a strategy that combines optimised SPPS of the peptide backbone bearing an N-terminal cysteine and C-terminal thioester, native chemical ligation/cyclisation *via* an efficient thia-zip reaction, and refolding under mild conditions to yield the native cystine-knot topology. At up to 36 residues in size, these compounds are among the largest synthetic cyclotides reported to date, and their syntheses demonstrate the versatility and efficiency of this synthetic approach. Furthermore, we have developed a biomimetic strategy for the synthesis of MCoTI cyclotides bearing a Lys10 or Phe10 residue,

Table 2 Yield of cyclotides and intermediates synthesised by SPPS, refolding and protease-mediated backbone ligation

	Yield (%)			
Cyclotide Lys10 = [X]	SPPS (*oc*-cyclotide)	Refolding (*rf*-cyclotide)	PST (cyclotide)	PSC (cyclotide)
MCoTI-I	7	72	93[a]	0
MCoTI-II	8	65	92[a]	0
MCoTI-II[R]	13	59	94[a]	0[b]
MCoTI-II[F]	5	64	0[b]	90[a]
MCoTI-II[Q]	10	60	0[b]	0

[a] In each case, residual *rf*-cyclotide was returned in 7–10% yield after *in situ* affinity purification. [b] Starting material partially digested.

utilising commercially available polymer-supported proteases that can act as a ligase for the refolded cyclotide backbone. This chemoenzymatic approach displays even greater efficacy than the non-enzymatic route described above, and provides for *in situ* affinity purification of the target cyclotides. The ease with which *oc*-MCoTI analogues fold and ligate in the presence of an appropriate protease raises the possibility that biosynthesis of MCoTI cyclotides might follow a protease/ligase pathway *in vivo*, in a similar manner to the structurally related Kalata cyclotide family.[32]

To realise the full potential of cyclotides as stable scaffolds for drugs and as tools for chemical genetics it is necessary to demonstrate that their structure may be re-engineered without loss of activity. We have reported here the first instance of redesigning the active loop of a cyclotide to redirect its specificity towards alternative targets whilst retaining moderate to high affinity. Proteases are of central importance as current and potential drug targets in a wide range of diseases. Foot-and-mouth disease, a disease of cloven-hoofed livestock, exacts a severe economic toll on affected communities.[56] In the work presented here, the first cyclotide inhibitors of the foot-and-mouth viral 3C protease are reported,[48,57] based on a re-engineered MCoTI-II cyclotide scaffold. Though the potency of these first generation inhibitors is moderate (low μM), it is notable that cyclotides based on a trypsin inhibitor scaffold exhibit activity against this cysteine protease.

We anticipate that the efficient chemical and chemoenzymatic routes presented here will be readily adapted to ligation, screening and *in situ* purification of cyclotide-based libraries.[2,26] In particular, polymer-supported proteases represent a potentially powerful platform for screening combinatorial libraries of precursor peptides for sequences that can adopt a native cyclotide fold, and for the identification of novel cyclotide-based protease inhibitors with inherent stability *in vivo*.[17,58]

Experimental

Materials and methods

All general laboratory chemicals obtained from chemical suppliers (Novabiochem UK or Sigma-Aldrich Chemical Co.) were used without further purification. Peptides were synthesised using an Advanced ChemTech Apex 396 multiple peptide synthesiser (Advanced Chemtech Europe, Cambridge, UK). Purification of crude peptides was performed on a Gilson semi-preparative RP-HPLC system (Anachem Ltd., Luton, UK) equipped with 306 pumps and a Gilson 155 UV/Vis detector. Analytical RP-HPLC was performed on a Gilson analytical HPLC system (Anachem Ltd., Luton, UK) equipped with a Gilson 151 UV/Vis detector and Gilson 234 auto injector, on a C-18 stationary phase. Peptide purification was achieved using preparative reverse-phase HPLC using a HICHROM C18 Column (250 × 21.2 mm). For both HPLC systems, the peptide bond absorption was detected at 223 nm. The following elution methods were used: Method A: 20 to 50% MeCN in H_2O over 40 min; Method B: 0 to 85% MeCN in H_2O over 40 min. The mobile phases contained 0.1% HPLC-grade TFA as an ion pairing agent and were degassed with helium. All peptides and reaction mixtures were analysed using Method B unless otherwise specified. Solid phase resins, preloaded Wang resin and 4-sulfamylbutyryl AM resin, were purchased from Novabiochem UK. The resin loadings were as follows: Fmoc-Lys(Boc)-Wang: 0.69 mmol g^{-1}; Fmoc-Phe-Wang: 0.74 mmol g^{-1}; Fmoc-Gln(Trt)-Wang: 0.62 mmol g^{-1}; Fmoc-Arg(Pbf)-Wang: 0.51 mmol g^{-1}; 4-sulfamylbutyryl AM resin: 1.1 mmol g^{-1}. Amino acids: *N*-α-9-fluorenylmethoxycarbonyl (*N*-α-Fmoc) protected amino acids were obtained from Novabiochem UK with the following side-chain protecting groups. Ala, Arg(Pbf), Asn(Trt), Asp(tBu), Cys(Trt), Gly, Ile, Leu, Lys(Boc), Pro, Ser(^{t}Bu), Tyr(^{t}Bu), Val and Fmoc-Asp(O^{t}Bu)-(Hmb)Gly-OH were purchased from Novabiochem UK. Reagents: peptide synthesis grade dimethylformamide (DMF) from Rathburn Chemicals UK. Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), *N*-hydroxybenzotriazole (HOBt), diisopropylethylamine (DIPEA) and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU) were purchased from Novabiochem UK and Sigma-Aldrich. Dry tetrahydrofuran (THF) and *N*,*N*-dimethylsulfoxide (DMF), chloroform, dimethylsulfoxide (DMSO), dichloromethane (DCM), *N*-methyl pyrrolidone (NMP), methanol, piperidine, thioanisole, *t*-butylmethylether (TBME), trifluoroacetic acid (TFA), ethanedithiol (EDT), iodoacetonitrile, 3-mercaptoethyl propionate, sodium thiophenolate, monobasic and dibasic sodium phosphate, and trimethylsilylisopropane (TIPS) were regent grade from Sigma-Aldrich. A 'deprotection mixture' of TFA–H_2O–EDT–TIPS (94 : 2.5 : 2.5 : 1) was used for cleavage and deprotection reactions.

General procedures

Loading of sulfamylbutyryl AM resin with Fmoc-Ala-OH. Sulfamylbutyryl AM resin (250 mg, 0.172 mmol) was swollen in DMF (3 mL) for 1 hour at ambient temperature. To this solution were added Fmoc-Ala-OH (268 mg, 0.86 mmol) and DIPEA (264 μL, 1.6 mmol). The reaction mixture was left to stir for 15 minutes followed by cooling to −20 °C for 20 minutes. Then PyBOP (447 mg, 0.86 mmol) was added and the reaction mixture stirred for 8 hours at −20 °C. The reaction mixture was left to warm to ambient temperature overnight. The resin was recovered by filtration and washed with DMF (3 × 2 mL). The coupling was repeated using the same procedure and washed with DMF (3 × 2 mL), DCM (3 × 2 mL) and MeOH (3 × 2 mL), respectively. The loaded resin was dried *in vacuo*. The coupling efficiency was assessed as follows: typically 2 mg of thoroughly-dried resin (or peptidyl resin) was incubated with a solution of 1 mL 20% piperidine in DMF, and left for 1 hour. The solution was then filtered off and the filtrate measured for its absorbance at 290 nm or 301 nm ($\varepsilon_{290\ nm} = 5800\ M^{-1}\ cm^{-1}$, $\varepsilon_{301\ nm} = 7800\ M^{-1}\ cm^{-1}$) against a blank of 20% piperidine in DMF. Loading efficiency was ~81%.

Solid phase peptide synthesis. Peptide synthesis was carried out in peptide synthesis grade DMF. Resin preloaded with appropriate amino acid (25 μmol per well) was swelled in DMF for 60 minutes before each coupling cycle, *N*-α-amino Fmoc group was removed with 20% (v/v) piperidine in DMF for 15 minutes, with two further repetitions. A fivefold excess of amino acid over resin reactive groups (125 μmol, 250 μL of a 0.5 M solution) was used for each coupling. *In situ* activation and coupling was carried out for 45 min with a mixture of HBTU–HOBt (125 μmole, 250 μL of a 0.5 M solution) and DIPEA (125 μmole, 250 μL of 0.5 M solution). The peptide was washed with DMF (3 × 1 mL) between

Table 3 Open-chain acids (*oc*-) and thioesters (*te*-) synthesised by SPPS

	Data			
Cyclotide Lys10 = [X]	Residues double-coupled	Yield	MALDI-MS	Retention time/min (Method B)
oc-MCoTI-I	Ala21 Gly22	7%	3507$[M + H]^+$ 1754$[M + 2H]^{2+}$	12.4
oc-MCoTI-II	Ala21 Gly22 Ile34	8%	3478$[M + H]^+$	12.3
oc-MCoTI-II[R]	Ala21 Gly22 Ile34	13%	3505$[M + H]^+$ 1753$[M + 2H]^{2+}$	12.0
oc-MCoTI-II[F]	Ala21 Gly22 Ile34	10%	3497$[M + H]^+$	14.4
oc-MCoTI-II[Q]	Ala21 Gly22 Ile34	5%	3478$[M + H]^+$ 1739$[M + 2H]^{2+}$	12.3
oc-MCoTI-II[AKQ]	Ala21 Gly22 Ile34	5%	3676$[M + H]^+$	10.2
te-MCoTI-I	Val18	6%	3622$[M + H]^+$	14.8
te-MCoTI-II	Ile14 Lys15 Val18	5%	3594$[M + H]^+$ 1798$[M + 2H]^{2+}$	14.4
te-MCoTI-II[R]	Ile14 Arg15	7%	3622$[M + H]^+$	14.1
te-MCoTI-II[F]	Ile14 Phe15	4%	3613$[M + H]^+$ 1807$[M + 2H]^{2+}$	15.4
te-MCoTI-II[Q]	Ile14 Gln15	4%	3593$[M + H]^+$ 1797$[M + 2H]^{2+}$	14.6
te-MCoTI-II[V]	Ile14 Val15	4%	3566$[M + H]^+$ 1783$[M + 2H]^{2+}$	14.6
te-MCoTI-II[A]	Ile14 Ala15	4%	3537$[M + H]^+$ 1769$[M + 2H]^{2+}$	14.7
te-MCoTI-II[AKQ]	Ile14 Gln15 Lys16 Ala17	5%	3793$[M + H]^+$ 1897$[M + 2H]^{2+}$	14.5

each deprotection and coupling step. A typical cycle consisted of deprotection, DMF wash, coupling and a further DMF wash. The *N*-α-Fmoc protection at the final residue was removed at the end of the synthesis under the usual conditions. After synthesis was complete, the fully protected resin-bound MCoTI was removed from the synthesiser, washed several times (3 × 2 mL DMF, 3 × 2 mL DCM, 3 × 2 mL MeOH) and dried *in vacuo.* Asp7Gly6 was introduced as a dipeptide (Fmoc-Asp(O^{t}Bu)-(Hmb)Gly-OH) and coupled for 2 hours. Cys34 was introduced as Boc-Cys(Trt)-OH for *te*-MCoTI syntheses. Residues were double-coupled where appropriate (see Table 3).

Activation and displacement from sulfamyl resin.

Activation. Fully protected MCoTI bound to sulfamyl resin (25 μmol) was swelled in 1.5 mL of dry NMP for 1 hour. To this solution was added DIPEA (200 μL, 1.1 mmol) followed by excess iodoacetonitrile (180 μL filtered through basic alumina prior to use). The reaction flask was shielded from light and agitated for 24 hours at ambient temperature. The resin was washed with NMP (5 × 5 mL), DCM (5 × 5 mL), MeOH (5 × 5 mL) and dried *in vacuo.*

Displacement. To the activated resin were added a small amount of sodium thiophenolate (1.6 mg), ethyl 3-mercaptopropionate (160 μL, 1.25 mmol) and DMF (500 μL). The reaction mixture was agitated overnight at ambient temperature. The resin was filtered and the filtrate was evaporated to dryness *in vacuo* to yield fully protected MCoTI thioester which was subsequently treated with 2 mL of deprotection mixture over 3 hours to give the corresponding *te*-MCoTI. Precipitation and purification were conducted as described below.

Deprotection and cleavage from Wang resin. Cleavage and deprotection of peptides was achieved by adding 1.5 mL of deprotection mixture to dry peptide bound resin (25 μmol). The mixture was agitated on an orbital shaker for up to 3 hours and then filtered. The resin was washed twice with a small volume of TFA and the combined washings and filtrate were precipitated with 10 mL ice cold TBME. The mixture was centrifuged at 5000 rpm for 20 minutes at 0 °C, the supernatant discarded and the remaining peptide washed with a fresh aliquot of TBME. The process was repeated three times to ensure complete removal of all organic impurities. The crude peptide was dried in a desiccator over silica gel to yield an off-white solid.

Peptide purification. A small sample of crude peptide was analysed by analytical preparative RP-HPLC before purifying by preparative HPLC. Individual fractions from preparative reverse-phase HPLC were analysed using analytical reverse-phase HPLC, and pure fractions combined and diluted to <10% MeCN with deionised water. The peptide was obtained as a white solid after lyophilisation on a Christ Alpha 2–4 freeze dryer (Osterode am Harz Germany). Mass identification by matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS) was performed using α-cyano-4-hydroxycinnamic acid as the matrix.

Synthesis of cyclotides *via* thia-zip native chemical ligation. Glutathione (0.3 mg, 1 μmol) was dissolved in 1 mL of 0.1 M carbonate buffer. This solution was added to *te*-MCoTI (0.28 μmol) which was dissolved in 1 mL of acetonitrile and the reaction mixture left for 24 hours at room temperature. The reaction mixture was then diluted 2-fold in water and purified by preparative RP-HPLC using Method A. Fractions were collected, analysed by analytical HPLC, pure fractions pooled and lyophilized to yield the product cyclotide.

Synthesis of *rf*-MCoTI-I, II and analogues *via* oxidative refolding. *oc*-MCoTI acid (0.28 μmol) was dissolved in 1 mL of 0.1 M ammonium carbonate buffer. To this solution was added glutathione (0.3 mg, 1 μmol), which was dissolved in acetonitrile (1 mL). The reaction mixture was allowed to stand for 48 hours at room temperature, diluted two-fold with water and purified by RP-HPLC using Method A. Fractions were collected, analysed using Method B, pooled and lyophilised.

Synthesis of cyclotides *via* ligation by immobilised protease.

Cyclisation using immobilised trypsin. Immobilised trypsin (500 μL of re-suspended beads) was washed with 0.1 M phosphate buffer (5 × 1 mL, pH 7.4) before use. *rf*-MCoTI peptide (0.03 μmol) was dissolved in 500 μL 0.1 M phosphate buffer pH 7.4 and added to immobilised trypsin. The reaction mixture was incubated at 37 °C for 15 minutes, mixed on a vortex mixer for 10 s and centrifuged at 14 000 rpm for 5 minutes. The supernatant was removed from immobilised trypsin which was subsequently

Table 4 Open-chain refolded (*rf*-) cyclotides synthesised by oxidative refolding and cyclotides synthesised by TZ-NCL and/or immobilised protease-mediated ligation

	Data		
Cyclotide Lys10 = [X]	Yield	MALDI-MS	Retention time/min (Method B)
rf-MCoTI-I	72%	3501$[M + H]^+$	10.5
rf-MCoTI-II	65%	3472$[M + H]^+$ 1737$[M + 2H]^{2+}$	10.4
rf-MCoTI-II[R]	59%	3500$[M + H]^+$ 1751$[M + 2H]^{2+}$	10.5
rf-MCoTI-II[F]	60%	3491$[M + H]^+$ 1746$[M + 2H]^{2+}$	12.2
rf-MCoTI-II [Q]	64%	3473$[M + H]^+$ 1736$[M + 2H]^{2+}$	10.6
rf-MCoTI-II[AKQ]	36%	3672$[M + H]^+$	12.0
MCoTI-I	68%[a]	3482$[M + H]^+$ 1741$[M + 2H]^{2+}$	12.3
MCoTI-II	72%[a]	3454$[M + H]^+$ 1727$[M + 2H]^{2+}$	11.5
MCoTI-II[R]	72%[a]	3483$[M + H]^+$ 1742$[M + 2H]^{2+}$	11.8
MCoTI-II[F]	64%[a]	3474$[M + H]^+$ 1737$[M + 2H]^{2+}$	15.2
MCoTI-II[V]	64%[a]	3427$[M + H]^+$ 1713$[M + 2H]^{2+}$	10.2
MCoTI-II[A]	63%[a]	3399$[M + H]^+$ 1699$[M + 2H]^{2+}$	10.1
MCoTI-II[AKQ]	71%[a]	3653$[M + H]^+$ 1827$[M + 2H]^{2+}$	11.7
MCoTI-I	93%[b]	3482$[M + H]^+$ 1742$[M + 2H]^{2+}$	12.4
MCoTI-II	92%[b]	3454$[M + H]^+$ 1728$[M + 2H]^{2+}$	11.4
MCoTI-II[R]	94%[b]	3481$[M]^+$ 1741$[M + 2H]^{2+}$	11.8
MCoTI-II[F]	90%[b]	3474$[M + H]^+$	15.4

[a] Synthesised by TZ-NCL. [b] Synthesised by immobilised protease-mediated ligation.

washed with water (5 × 1 mL); the reaction mixture was mixed and centrifuged as above at each washing. 0.1% TFA in water was added and the mixture mixed and left to stand for 5 minutes, the supernatant was recovered, and this procedure was repeated. The combined TFA washings were analysed by analytical HPLC and MALDI-MS.

Cyclisation using immobilised chymotrypsin. The reaction was performed exactly as for immobilised trypsin, except that 25 mg immobilised chymotrypsin was weighed out and washed as above.

Peptide concentration determination for MCoTI-I, II and MCoTI-II[R]. The concentration of active MCoTI cyclotide was determined by titration with trypsin (adapted from ref. 12). Microprotein at a range of concentrations (1 μM to 0.1 nM, 50 μL) in TBS pH 7.6 (Tris buffered saline: 50 mM Tris·HCl, 150 mM NaCl, 0.01% Tritron® X-100, 0.02% sodium azide) was incubated with trypsin (25 nM, 50 μL) for 30 minutes at 37 °C in a 96-well plate (Falcon Microtest™ 96). Carbobenzoxy-L-arginine-7-amino-4-methyl coumarin (75 μM, 100 μL) was added to the solution and the absorbance measured at room temperature with excitation and emission wavelengths at 360 and 465 nm respectively. The concentration of active cyclotide was determined from inhibition curves obtained in two separate experiments assuming a 1 : 1 interaction between inhibitor and trypsin.

Peptide concentration determination for MCoTI-II[F] and MCoTI-II[Q]. These MCoTI-II analogues have relatively low inhibition activity against trypsin, and so a standard addition method was used to determine peptide concentration using analytical HPLC. A standard addition graph from MCoTI-I, II and MCoTI-II[R] was plotted by varying the active peptide concentration (200 μM to 10 μM) *versus* peak area measured at 223 nm and, by comparison of peak areas, used to determine the concentration of microproteins MCoTI-II[F] and MCoTI-II[Q].

Inhibition kinetics. All inhibition kinetics were determined by competitive binding assays at 37 °C on a Cytofluor series 400 microplate reader (Perseptive Biosystem, Warrington, UK), using Falcon Microtest™ 96-well plates. TBS pH 7.6 was prepared for trypsin and chymotrypsin assays. The equilibrium dissociation constant (K_i) for the complex between trypsin or chymotrypsin and the cyclotide was determined by using enzyme concentrations lower than the K_i. Thus, proteases (0.005 nM, 50 μL trypsin or 0.5 nM, 50 μL chymotrypsin, 12.5 μM, 50 μL subtilisin and 1 nM, 50 μL thrombin) were incubated at a suitable range of concentrations of inhibitor in TBS for 1 hour. The measurement was started by the addition of substrate: (5 μM, 100 μL *N*-*p*-tosyl-Gly-Pro-Arg-AMC (AMC: 7-amido-4-methyl coumarin)) for trypsin and thrombin assays, or succinimidyl-Ala-Ala-Pro-Phe-AMC (10 μM, 100 μL for chymotrypsin or 19 μM, 100 μL for subtilisin assays). The initial rate of substrate hydrolysis was monitored by the cleavage of AMC from substrate at an excitation of 360 nm and emission of 460 nm. Cyclotide activity against FMDV $3C^{pro}$ was determined as described previously, using 617 nM, 50 μL FMDV $3C^{pro}$, 50 μM, 100 μL FRET substrate.[59] Initial rate data were then fitted to determine K_i using the GraFit Software package (http://www.erithacus.com/grafit). All assays were reproduced 3 times with fresh solutions of all reagents, and the standard deviation is given where appropriate.

Additional characterisation data

Acknowledgements

We are grateful to the the BBSRC for a David Phillips Research Fellowship (to EWT), the Royal Thai Government for a research studentship (to PT), and the European Union for Marie Curie Host Fellowships for Early Stage Research Training (to NR and PT).

Notes and references

1 D. J. Craik, M. Cemazar and N. L. Daly, *Curr. Opin. Drug Discovery Dev.*, 2007, **10**, 176–184.
2 D. J. Craik, M. Cemazar, C. K. Wang and N. L. Daly, *Biopolymers*, 2006, **84**, 250–266.

3 D. C. Ireland, C. K. Wang, J. A. Wilson, K. R. Gustafson and D. J. Craik, *Biopolymers*, 2008, **90**, 51–60.
4 K. R. Gustafson, T. C. McKee and H. R. Bokesch, *Curr. Protein Pept. Sci.*, 2004, **5**, 331–340.
5 N. L. Daly, K. R. Gustafson and D. J. Craik, *FEBS Lett.*, 2004, **574**, 69–72.
6 C. V. Jennings, K. J. Rosengren, N. L. Daly, M. Plan, J. Stevens, M. J. Scanlon, C. Waine, D. G. Norman, M. A. Anderson and D. J. Craik, *Biochemistry*, 2005, **44**, 851–860.
7 C. Jennings, J. West, C. Waine, D. Craik and M. Anderson, *Proc. Natl. Acad. Sci. USA*, 2001, **98**, 10614–10619.
8 D. J. Craik and N. L. Daly, *Protein Pept. Lett.*, 2005, **12**, 147–152.
9 M. Cemazar, N. L. Daly, S. Haggblad, K. P. Lo, E. Yulyaningsih and D. J. Craik, *J. Biol. Chem.*, 2006, **281**, 8224–8232.
10 K. J. Rosengren, N. L. Daly, M. R. Plan, C. Waine and D. J. Craik, *J. Biol. Chem.*, 2003, **278**, 8606–8616.
11 P. Seydel, C. W. Gruber, D. J. Craik and H. Dornenburg, *Appl. Microbiol. Biotechnol.*, 2007, **77**, 275–284.
12 A. D. Gillon, I. Saska, C. V. Jennings, R. F. Guarino, D. J. Craik and M. A. Anderson, *Plant J.*, 2007.
13 D. J. Craik, D. G. Barry, R. J. Clark, N. L. Daly and L. Sando, *J. Toxicol. Toxin Rev.*, 2003, **22**, 555–576.
14 T. Leta Aboye, R. J. Clark, D. J. Craik and U. Goransson, *ChemBioChem*, 2008, **9**, 103–113.
15 D. J. Craik, M. Cemazar and N. L. Daly, *Curr. Opin. Drug Discovery Dev.*, 2006, **9**, 251–260.
16 D. J. Craik, S. Simonsen and N. L. Daly, *Curr. Opin. Drug Discovery Dev.*, 2002, **5**, 251–260.
17 M. Werle, K. Kafedjiiski, H. Kolmar and A. Bernkop-Schnurch, *Int. J. Pharm.*, 2007, **332**, 72–79.
18 M. E. Felizmenio-Quimio, N. L. Daly and D. J. Craik, *J. Biol. Chem.*, 2001, **276**, 22875–22882.
19 J. F. Hernandez, J. Gagnon, L. Chiche, T. M. Nguyen, J. P. Andrieu, A. Heitz, T. Trinh Hong, T. T. Pham and D. Le Nguyen, *Biochemistry*, 2000, **39**, 5722–5730.
20 D. J. Craik, N. L. Daly, T. Bond and C. Waine, *J. Mol. Biol.*, 1999, **294**, 1327–1336.
21 S. M. Simonsen, L. Sando, D. C. Ireland, M. L. Colgrave, R. Bharathi, U. Goransson and D. J. Craik, *Plant Cell*, 2005, **17**, 3176–3189.
22 M. Trabi and D. J. Craik, *Plant Cell*, 2004, **16**, 2204–2216.
23 D. J. Craik and N. L. Daly, *Mol. BioSyst.*, 2007, **3**, 257–265.
24 M. Cemazar and D. Craik, *J. Pept. Sci.*, 2008, DOI: 10.1002/psc.972.
25 J. A. Camarero, R. H. Kimura, Y. H. Woo, A. Shekhtman and J. Cantor, *ChemBioChem*, 2007, **8**, 1363–1366.
26 J. A. Camarero, R. H. Kimura, Y. H. Woo, J. Cantor and E. Steenblock, *Chim. Oggi*, 2007, **25**, 20–23.
27 S. Gunasekera, N. L. Daly, M. A. Anderson and D. J. Craik, *IUBMB Life*, 2006, **58**, 515–524.
28 R. H. Kimura, A. T. Tran and J. A. Camarero, *Angew. Chem., Int. Ed.*, 2006, **45**, 973–976.
29 O. Avrutina, H. U. Schmoldt, D. Gabrijelcic-Geiger, D. Le Nguyen, C. P. Sommerhoff, U. Diederichsen and H. Kolmar, *Biol. Chem.*, 2005, **386**, 1301–1306.
30 O. Avrutina, H. U. Schmoldt, H. Kolmar and U. Diederichsen, *Eur. J. Org. Chem.*, 2004, 4931–4935.
31 E. W. Tate, *Signal Transduction*, 2006, **6**, 144–159.
32 I. Saska, A. D. Gillon, N. Hatsugai, R. G. Dietzgen, I. Hara-Nishimura, M. A. Anderson and D. J. Craik, *J. Biol. Chem.*, 2007, **282**, 29721–29728.
33 C. W. Gruber, M. Cemazar, R. J. Clark, T. Horibe, R. F. Renda, M. A. Anderson and D. J. Craik, *J. Biol. Chem.*, 2007, **282**, 20435–20446.
34 P. Thongyoo, E. W. Tate and R. J. Leatherbarrow, *Chem. Commun.*, 2006, 2848–2850.
35 P. Thongyoo, A. M. Jaulent, E. W. Tate and R. J. Leatherbarrow, *ChemBioChem*, 2007, **8**, 1107–1109.
36 A. Heitz, J. F. Hernandez, J. Gagnon, T. T. Hong, T. T. Pham, T. M. Nguyen, D. Le-Nguyen and L. Chiche, *Biochemistry*, 2001, **40**, 7973–7983.
37 W. L. DeLano, The PyMOL molecular graphics system, http://www.pymol.org.
38 J. P. Tam, Y. A. Lu, J. L. Yang and K. W. Chiu, *Proc. Natl. Acad. Sci. USA*, 1999, **96**, 8913–8918.
39 J. P. Tam and Y. A. Lu, *Protein Sci.*, 1998, **7**, 1583–1592.
40 J. P. Tam and Y. A. Lu, *Tetrahedron Lett.*, 1997, **38**, 5599–5602.
41 P. Heidler and A. Link, *Bioorg. Med. Chem.*, 2005, **13**, 585–599.
42 R. G. Simmonds, *Int. J. Pept. Protein Res.*, 1996, **47**, 36–41.
43 H. H. Niemann, H. U. Schmoldt, A. Wentzel, H. Kolmar and D. W. Heinz, *J. Mol. Biol.*, 2006, **356**, 1–8.
44 J. D. McBride and R. J. Leatherbarrow, *Curr. Med. Chem.*, 2001, **8**, 909–917.
45 J. D. McBride, E. M. Watson, A. B. Brauer, A. M. Jaulent and R. J. Leatherbarrow, *Biopolymers*, 2002, **66**, 79–92.
46 D. Scarpi, J. D. McBride and R. J. Leatherbarrow, *J. Pept.Res.*, 2002, **59**, 90–93.
47 T. R. Sweeney, N. Roque-Rosell, J. R. Birtley, R. J. Leatherbarrow and S. Curry, *J. Virol.*, 2007, **81**, 115–124.
48 S. Curry, N. Roque-Rosell, P. A. Zunszain and R. J. Leatherbarrow, *Int. J. Biochem. Cell Biol.*, 2007, **39**, 1–6.
49 C. Groeger, H. R. Wenzel and H. Tschesche, *Angew. Chem., Int. Ed. Engl.*, 1993, **32**, 898–900.
50 C. Groeger, H. R. Wenzel and H. Tschesche, *Int. J. Pept. Protein Res.*, 1994, **44**, 166–172.
51 U. C. Marx, M. L. Korsinczky, H. J. Schirra, A. Jones, B. Condie, L. Otvos, Jr. and D. J. Craik, *J. Biol. Chem.*, 2003, **278**, 21782–21789.
52 R. V. Ulijn, B. Baragana, P. J. Halling and S. L. Flitsch, *J. Am. Chem. Soc.*, 2002, **124**, 10988–10989.
53 R. V. Ulijn, N. Bisek, P. J. Halling and S. L. Flitsch, *Org. Biomol. Chem.*, 2003, **1**, 1277–1281.
54 D. Y. Jackson, J. Burnier, C. Quan, M. Stanley, J. Tom and J. A. Wells, *Science*, 1994, **266**, 243–247.
55 D. Y. Jackson, J. P. Burnier and J. A. Wells, *J. Am. Chem. Soc.*, 1995, **117**, 819–820.
56 P. Kitching, J. Hammond, M. Jeggo, B. Charleston, D. Paton, L. Rodriguez and R. Heckert, *Vaccine*, 2007, **25**, 5660–5664.
57 S. Curry, N. Roque-Rosell, T. R. Sweeney, P. A. Zunszain and R. J. Leatherbarrow, *Biochem. Soc. Trans.*, 2007, **35**, 594–598.
58 K. P. Greenwood, N. L. Daly, D. L. Brown, J. L. Stow and D. J. Craik, *Int. J. Biochem. Cell Biol.*, 2007, **39**, 2252–2264.
59 A. M. Jaulent, A. S. Fahy, S. R. Knox, J. R. Birtley, N. Roque-Rosell, S. Curry and R. J. Leatherbarrow, *Anal. Biochem.*, 2007, **368**, 130–137.

