Cyclic tetrapeptides *via* the ring contraction strategy: chemical techniques useful for their identification

Douglas A. Horton,†[a] Gregory T. Bourne,†[a,b] Justin Coughlan,[a,b] Sonya M. Kaiser,[a,b] Carolyn M. Jacobs,[a] Alun Jones,[c] Andreas Rühmann,[a] Jill Y. Turner[b] and Mark L. Smythe*[a,b]

Received 10th January 2008, Accepted 5th February 2008
First published as an Advance Article on the web 28th February 2008
DOI: 10.1039/b800464a

Cyclic tetrapeptides are a class of natural products that have been shown to have broad ranging biological activities and good pharmacokinetic properties. In order to synthesise these highly strained compounds a ring contraction strategy had previously been reported. This strategy was further optimised and a suite of techniques, including the Edman degradation and mass spectrometry/mass spectrometry, were developed to enable characterisation of cyclic tetrapeptide isomers. An NMR solution structure of a cyclic tetrapeptide was also generated. To illustrate the success of this strategy a library of cyclic tetrapeptides was synthesised.

Introduction

Cyclic tetrapeptides are a class of naturally occurring privileged substructures, providing many potent molecules in a diverse range of therapeutic areas.[1,2] Typical examples, all derived through extraction from culture broths, are the cytotoxic and antimitogenic agents HC toxin[3,4] and chlamydocin,[5,6] the antitumor agent trapoxin,[7] the tyrosinase inhibitor cyclo-[(L)Pro-(L)Tyr-(L)Pro-(L)Val][8] and the antimalarial apicidins.[9] One compound from the apicidin family is reported to show *in vivo* activity by both parenteral and oral administration in mice against *Plasmodium berghei*.[9,10]

Head-to-tail cyclic tetrapeptides differ from their larger macrocyclic counterparts in that they are highly constrained, to the point that typically the planarity of the amide bonds is twisted.[3,5,11–18] This ensures that they display their substituents in a highly spatially defined manner, which makes them very useful as molecular toolkits to probe a receptor and then develop smaller organics with the requisite functionality.

However, because of these characteristics cyclic tetrapeptides are difficult to synthesise. So-called "difficult" sequences produce linear and cyclic oligomers in preference to the monocyclic target, even when cyclisation is performed at high dilution,[19,20] making them generally inaccessible. The few cyclic tetrapeptides that have been synthesised carry either D-residues, postmodified or unnatural residues, or at least one tertiary amide in the sequence.[11,15,16,21–29] Cyclic tetrapeptides are also difficult to characterize. NMR spectra of these molecules are complicated as they are often a mixture of slowly interconverting conformers, which results in peak broadening.

A ring contraction approach was reported by Meutermans *et al.* that enables the synthesis of several cyclic tetrapeptides from their linear precursor.[19,20] The strategy utilizes an auxiliary, 2-hydroxy-6-nitrobenzylaldehyde (HnB) (Fig. 1). Due to the success of this strategy, the objective of this work was to further optimise this procedure, develop a suite of characterisation techniques for cyclic tetrapeptides, and to synthesise a cyclic tetrapeptide library.

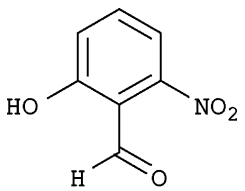


Fig. 1 HnB auxiliary.

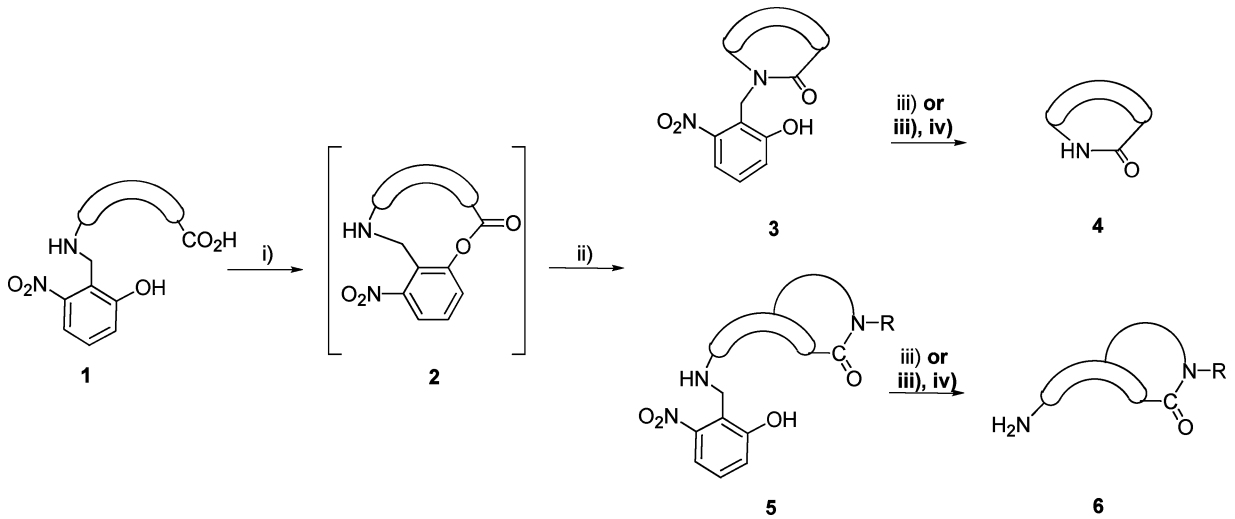
Results and discussion

Synthesis of cyclic tetrapeptides

A photolabile cyclisation auxiliary (HnB, Fig. 1) was recently reported that permits the synthesis of difficult cyclic peptides sequences including cyclic tetrapeptides from their linear precursors.[19,20] The strategy is outlined in Scheme 1. The auxiliary was first reductively aminated onto the *N*-terminus of the resin-bound peptide. After cleavage from resin and concomitant removal of the side-chain protecting groups to produce **1**, cyclisation can be accomplished to produce the cyclic nitrophenyl ester intermediate **2**. Upon heating at 70 °C, this product ring contracts through an *O*-to-*N* acyl transfer to generate the desired cyclic peptide **3**. Photolytic removal of HnB then provides the target cyclic product **4**. Using the described method several tetrapeptide sequences have been successfully synthesized, including cyclo-[Tyr-Arg-Phe-Ala].[20]

The versatility of this synthetic route for each of the synthetic steps was explored for a number of cyclic tetrapeptides. In parallel with this, the development of a suite of analytical tools to aid in the characterisation of these compounds and their intermediates

[a]*Institute for Molecular Bioscience, The University of Queensland, St. Lucia, Qld, 4072, Australia. E-mail: m.smythe@imb.uq.edu.au; Web: http://www.imb.uq.edu.au/; Fax: +61-7-3346-2101; Tel: +61-7-3346-2222*
[b]*Protagonist Pty. Ltd., Level 7 Queensland Bioscience Precinct, 306 Carmody Rd, St Lucia, Qld, 4072, Australia; Web: http://www.protagonist.com.au.; Fax: +61-7-3346-2101; Tel: +61-7-3346-2222*
[c]*ARC Special Research Centre for Functional and Applied Genomics,*
† Co-first authors. Equally contributed



Scheme 1 Synthesis of difficult sequences through a ring contraction strategy.

was very important. Having accomplished this, a library of cyclic tetrapeptides was synthesized.

The initial sequences chosen for the study were based on the (D)Phe-Arg-Trp and Arg-Phe-Phe sequences. The Trp, Arg and Phe residues were selected as they are often found as common recognition motifs. Glycine was appended to the *C*-terminus of these sequences as prior experience had shown that the *C*-terminal residue is capable of racemizing during synthesis.[20] Initially, no side chain protecting groups were used. It was believed that the presence of protecting groups was unnecessary and could possibly hinder cyclisation-ring contraction yields based on steric effects.

A small number of unprotected Phe-Arg-Trp-Gly sequences were synthesized on solid-support (Table 1, **1aa**, **1ac**, **1ae**). After cleavage and purification, the cyclisation and ring contraction steps were performed using 1 equiv. BOP at 70 °C with 10 equiv. of diisopropylethylamine (this method had been used to synthesize other cyclic tetrapeptides).[20]

Although the linear starting material could still be observed two products were also identified by LC/MS for each of the sequences cyclised. In addition, after purification both products appeared to have the mass corresponding to the cyclised material. Characterisation at this point was found to be problematic due to the instability of the HnB cyclised products. Hence, the HnB auxilary was immediately removed by photolysis. NMR characterisation of the final products was found to be very labour intensive, primarily due to the slowly interconverting conformations. It thus became apparent that other characterisation techniques would be necessary in order to quickly identify the products. This is especially important when generating large numbers of cyclic tetrapeptides as a possible discovery project.

Table 1 Cyclisation of linear tetrapeptides **1** and corresponding yields of the cyclic product **3** after photolysis to give product **4**

	Linear peptide	Yield (%)[a]	
	1	3	4
aa	**[HnB]**(D)Phe-(D)Arg-Trp-Gly-OH	46[b]	12[e,g]
ab	**[HnB]**(D)Phe-(D)Arg(Pbf)-Trp(Boc)-Gly-OH	29[b]	18[e,h]
ac	**[HnB]**(D)Phe-Arg-(D)Trp-Gly-OH	77[b]	<5[e,g]
ad	**[HnB]**(D)Phe-Arg(Pbf)-(D)Trp(Boc)-Gly-OH	34[c]	11[h]
ae	**[HnB]**Phe-(D)Arg-(D)Trp-Gly-OH	54[b]	25[e,g]
af	**[HnB]**His-(D)Phe-Arg-Trp-OH[f]	36[c]	72[d,g]
ag	**[HnB]**(D)Phe-(D)Arg(Pbf)-(D)Trp(Boc)-Gly-OH	28[b]	19[e,h]
ah	**[HnB]**Phe-Arg(Pbf)-(D)Trp(Boc)-Gly-OH	40[b]	26[e,h]
ai	**[HnB]**Phe-(D)Arg(Pbf)-Trp(Boc)-Gly-OH	30[b]	19[e,h]
aj	**[HnB]**(D)Phe-Arg(Pbf)-Trp(Boc)-Gly-OH	29[b]	21[e,h]
ak	**[HnB]**Arg(Pbf)-Phe-Phe-Gly-OH	65[b]	10[e,h]

[a] Yield of purified product after RP-HPLC. [b] 1 eq HATU, 2 eq DIEA, 1 mM in DMSO, 3h/rt; ii) 10 eq DIEA, 15 h, 70 °C. [c] 1 eq BOP, 2 eq DIEA, 1 mM in DMSO, 3h/rt; ii) 10 eq DIEA, 15 h, 70 °C. [d] hυ, 1 mM peptide in 1% HOAc in DMSO, 5 h. [e] hυ, 0.1 mM peptide in 1% HOAc in DMSO, 5 h. [f] Chiral amino acid analysis showed that the C-terminal Trp α-carbon inverted 99.7% during cyclic tetrapeptide synthesis. [g] Yield after photolysis. [h] Yield after photolysis of protected peptide followed by TFA treatment. Note: **4aa** and **4ab** are equivalent; **4ac** and **4ad** are equivalent.

Characterisation techniques: Edman degradation and mass-spectrometry/mass-spectrometry (MS/MS)

Characterisation of cyclic tetrapeptides is very challenging. In many cases, multiple conformers of the same molecule may be observed by both NMR and HPLC. Conceptually, there are three possible cyclisation products: the desired *N*- to *C*-terminal cyclised product (**4**, Scheme 1), a side-chain to *C*-terminal cyclised product (for example, when cyclisation occurs through the amino group of lysine or the guanidyl group of arginine) (**6**, Scheme 1) or an amide bond to *C*-terminal cyclised product (for example, producing a diketopiperazine). Two different analytical techniques were selected to assist in characterisation: the Edman degradation and mass-spectrometry/mass-spectrometry (MS/MS).

The Edman degradation was selected as the reaction will proceed for side-chain cyclised peptides, but not head-to tail cyclised peptides. Furthermore, the reaction may be accomplished on very small quantities and analysis merely requires a mass spectrometer.

The Edman degradation proceeds through the reaction of an *N*-terminal amino group with phenyl isothiocyanate under mildly alkaline conditions to form a thiourea. This product then rearranges under acidic conditions to produce the thiohydantoin,

Table 2 Edman degradation of selected cyclic tetrapeptides

Peptide		Observed Mass of Starting Material[a]	Observed Peaks after Treatment[a]			
			Starting Material	Thiohydantoin	Des-R1 Peptide	Peptide-thiourea complex
	ACP (65–74)[b]	1063	—	235.1 (Val)	964.7	—
4aa	Cyclo-[(D)Phe-(D)Arg-Trp-Gly]	547.4	547.4	—	—	—
6aa	(D)Phe-Cyclo[*(D)Arg*-Trp-Gly]	547.3	—	283.1 (Phe)	400.3	—
4ac	Cyclo-[(D)Phe-Arg-(D)Trp-Gly]	547.6	547.6	—	—	—
6ac	(D)Phe-Cyclo-[*Arg*-(D)Trp-Gly]	547.3	547.6	Not observed	400.3	—
4ae	Cyclo-[Phe-(D)Arg-(D)Trp-Gly]	547.6	547.6	—	—	—
6ae	Phe-Cyclo[*(D)Arg*-(D)Trp-Gly]	547.3	—	283.1 (Phe)	400.3	—
	Cyclo[*(D)Arg*-(D)Trp-Gly]	400.3	—	—	—	535.3
4af	Cyclo-[His-(D)Phe-Arg-Trp][c]	627.4	627.4	—	—	—
4ak	Cyclo-[Arg(Pbf)-Phe-Phe-Gly)	760.5	760.5	—	—	—

[a] Peaks are for [M + H] ions, [M + Na] ions were also often observed. [b] Acetyl Carrier Protein ACP(65–74) was used as a positive control. [c] It was later confirmed that (L)Trp had inverted to (D)Trp in 99.7% purity.

leaving the peptide chain without the *N*-terminal residue. If the *N*-terminus is not a primary amine, the reaction will not proceed.

After development on the positive control peptide Acyl Carrier Protein (ACP) (65–74), all of the cyclic peptides prepared above (Table 1) were photolysed and purified by RP-HPLC (Scheme 1). The peptides were then tested (Table 2). As can be observed, ACP (65–74) works very well as a control, producing only the desired thiohydantoin and cleaved peptide. Next, pairs of Phe-Arg-Trp-Gly photolysed products from the [**HnB**]peptide cyclisation were tested (**4** and **6**). In each case, one of these failed to react at all with the phenyl isothiocyanate, confirming its identity as the desired *N*- to *C*-terminal cyclised product. The other compound produced the *N*-terminal thiohydantoin and a cleaved peptide. This product could therefore either be an arginine side-chain cyclised product, or could be cyclised through one of the amide bonds. As a result, the crude Edman degradation mixture from Phe-cyclo[*(D)Arg*-(D)Trp-Gly] was re-subjected to the Edman degradation. Gratifyingly, the cyclo[*(D)Arg*-(D)Trp-Gly] thiourea complex was observed, but no thiohydantoin corresponding to the arginine appeared. Therefore, this product had cyclised through the arginine side-chain. Only one product was obtained from the cyclisation and photolysis of [HnB]His-(D)Phe-Arg-Trp-OH and [HnB]Arg(Pbf)-Phe-Phe-Gly-OH. These were examined by the Edman degradation, which verified that they had cyclised through the *N*- and *C*-termini.

Many of these products were also subjected to mass-spectrometry/mass-spectrometry (MS/MS) analysis. This confirmed the results from the Edman degradation. For example, head-to-tail cyclised products such as cyclo[His-(D)Phe-Arg-Trp] **4af** exhibited fragments corresponding to a *N*- and *C*- terminal residue dipeptide fragment (Trp-His). The side-chain cyclised product (D)Phe-Cyclo[*(D)Arg*-Trp-Gly] **6ac** exhibited no fragment corresponding to Phe-Gly or Arg-Gly (Arg-Gly is unlikely, as three bonds must be broken and cleavage at the guanidyl group would be favoured), although fragments corresponding to the loss of Phe were observed.

Thus, three products were obtained through cyclisation with the HnB auxiliary. The first of these products to elute by RP-HPLC was the linear peptide, followed by the arginine-side chain cyclised product and then the desired *N*- to *C*-termini cyclised product.

Development and optimisation

Having identified all three materials obtained from the cyclisation, optimisation of the yield of the head-to-tail cyclised product was attempted. Five different coupling agents were trialled over the standard conditions (13 h at 70 °C) (Table 3). The ratio of the products did not appreciably change after 13 hours, except in the case of BOP and DIC cyclisations. For cyclisation with activating agent DIC, very little cyclic material (either head to tail or head to side chain) was obtained. Treatment with HATU resulted in a much higher yield of the desired cyclic product.

It was observed that the best coupling agent to form the head-to-tail cyclic material was HATU and the best to form the side-chain cyclised material was BOP. However it became clear that due to hydrolysis and slow decomposition, immediate purification and photolysis was required. Interestingly, while conducting these tests it was observed that **3** could be retreated with coupling agent to obtain **5**.

Analysis using mass spectrometry indicated that by-products of HnB photolysis were reacting with the cyclic peptide. Photolysis of *o*-nitrobenzylamines is known to proceed through the elimination of *o*-nitrosobenzaldehyde, which can then break down to other products when irradiated at wavelengths higher than 350 nm.[42] To try and limit the effect of these products, a number of conditions were tested, including the addition of scavengers, using different solvents, cooling the solution, diluting the solution and utilising different workup conditions. Unfortunately, no improvement on

Table 3 Cyclisation yields and products of [HnB]Phe-(D)Arg-(D)Trp-Gly-OH **1ae**[a]

Coupling	Yield[b] (%)	Yield[c] (%)	Yield[d] (%)
Reagent	(1)	(3)	(5)
BOP	11	37	52
DIC	80	12	8
HBTU	40	27	33
PyBOP	8	51	41
HATU	16	63	21

[a] Relative yields calculated by HPLC Data after 13 hours heating at 70 ° C. [b] [HnB]Phe-(D)Arg-(D)Trp-Gly-OH **1ae** [c] [HnB]Cyclo-[Phe-(D)Arg-(D)Trp-Gly] **3ae**. [d] [HnB]Phe-Cyclo-[*(D)Arg*-(D)Trp-Gly] **5ae**.

the 1% acetic acid/DMF solution could be found, except for the dilution of the DMF solution to 0.1 mM.

As another confirmation of the structure of the head-to-tail cyclic peptides **4aa** and **4ac** these products were re-synthesised with all side-chain protecting groups **4ab** and **4ad** (producing **3**, Scheme 1, Table 1). Overall yields for the cyclisation ring contraction step for the protected peptide were up to half that of the analogous reaction with the unprotected peptide. This illustrates that the presence of protecting groups may hinder cyclisation-ring contraction yields based on steric effects. However, as expected the yields for the photolysis of the protected cyclic peptides were much higher. Subsequent protecting group cleavage and purification reduced the yields for this step as shown in Table 1, so that the overall yield for the complete synthesis of the cyclic tetrapeptide was similar when functional groups were protected or unprotected. Five more peptides (**4ag-4ak**) were also synthesized with all side-chain protecting groups giving comparable yields.

Interestingly, while the photolysis yield normally varied from 3–26%, one cyclic peptide displayed quite different results. Cyclo-[His-(D)Phe-Arg-Trp] **4af** photolysed in 72% yield. As the cyclisation only yielded one product in similar quantity to that obtained for the other peptides and it was known that epimerisation of the *C*-terminal residue of these peptides can be problematic,[20] these results were suspicious, even though Edman degradation proved that the product was a head-to-tail cyclic peptide. Chiral amino acid analysis revealed that the tryptophan had inverted in 99.7% purity.

It was presumed that the stereoinversion must have occurred at the cyclisation step. Cyclisation proceeds through the formation of the *C*-terminal residue activated benzotriazole ester (from BOP), followed by the nucleophilic substitution of the benzotriazole with the phenolic oxygen of HnB. An *O*- to *N*- acyl transfer then occurs, contracting the ring system. This product is known to be unstable and the reaction reversible. Although epimerisation of the *C*-terminal residue can occur prior to the formation of the benzotriazole ester, this is unlikely given the rate of this intermolecular reaction (analogous amide bond forming intermolecular reactions are completed in minutes at room temperature). As a result, stereoinversion most likely occurred from the phenolic or benzotriazole esters or from the amide, *i.e.* immediately prior to or during cyclisation. As cyclisation and stereoinversion are reversible, this indicates that the LDLL cyclic tetrapeptide is significantly more sterically constrained than the LDLD peptide, effectively trapping the reaction after complete stereoinversion.

It therefore seems that the substantial increase in yield for both the cyclisation and photolysis of Hnb-Cyclo[His-(D)Phe-Arg-(D)Trp] is the result of slow hydrolysis of this product. This indicates that the stereoinversion to the (D)Trp product is a thermodynamic sink.

LDLD cyclic peptides are known to behave very differently to other cyclic peptides. Ngu-Schwemlein *et al.* synthesized a series of LDLD cyclic tetrapeptides in which every second residue was (D)-alanine.[11] In this case, the synthesis proceeded at elevated temperature with an activated ester and no ring contraction auxiliary was necessary. It has also been reported that cyclic LD peptides can adopt flat conformations, in which the backbone amides are orientated perpendicular to the side chains and the plane of the ring system.[30,31] The products (head-to-tail cyclo-octapeptides) have also been known to form cylindrical β-sheet peptide assemblies, although a dimerisation is more difficult in smaller cyclic systems and is dependent upon amino acid composition.[30,31]

NMR structure of cyclo[(L)His-(D)Phe-(L)Arg-(D)Trp]

The majority of cyclic tetrapeptides synthesized (**1aa**–**1ak**) exhibited a great deal of line broadening by ^{1}H NMR, making structural analysis very difficult. Cyclo[Phe-(D)Arg-(D)Trp-Gly] was a typical example (Fig. 2). However, cyclo[His-(D)Arg-Phe-(D)Trp] exhibited a very sharp spectrum, and only one set of signals. A solution structure was generated from this product.

A DQF-COSY, TOCSY, ECOSY and ROESY was run on cyclo[His-(D)Arg-Phe-(D)Trp] in d_6-DMSO and a TOCSY and ROESY were run in two mixtures of acetonitrile/water/deuterium oxide, in an effort to resolve all cross-peaks. As can be seen in Table 4 a small change in chemical shift for the NH's, Hα's and Hβ's could be observed. This is due to the change in hydrogen bonding ability of the solvent systems, and may also indicate a change in conformational state in aqueous media. However, a solution structure was only examined in d_6-DMSO. Analysis of the spectrum indicated that the cyclic peptide possessed only *trans* amide bonds. The ROESY spectrum exhibited very strong inter-residue rOe's between all NH (*i*) and Hα (*i*+1), but much weaker intra-residue rOe's between NH and Hα (Fig. 3) indicating that these atoms are close in space, which is illustrative of a *trans* configuration.[3,11,14] In addition, no inter-residue cross-peaks could be observed between any Hα's, which is usually a sign of *cis* amide bonds.[14] According to the Karplus equation, the large repeating $^3J_{HN\text{-}H\alpha}$ pattern of 9.19 Hz and 9.56 Hz indicates an

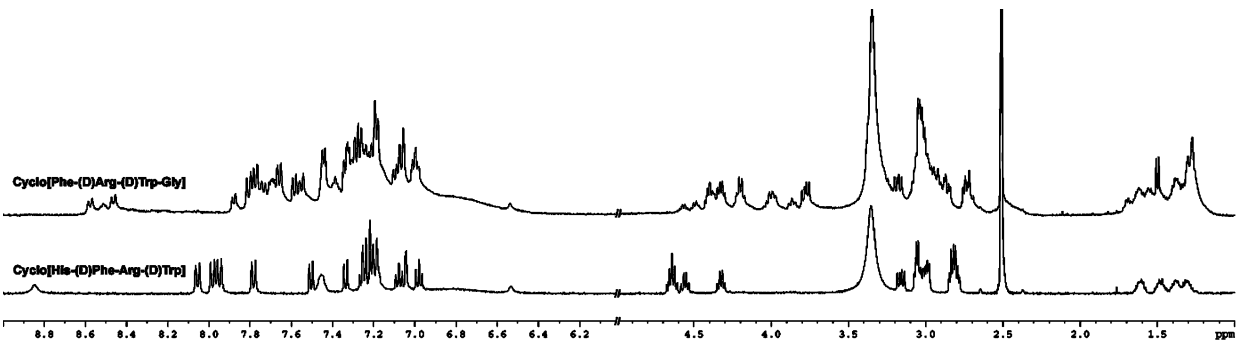


Fig. 2 ^{1}H NMR Spectrum (500 MHz), 9–6 ppm and 5–1 ppm, of Cyclo[Phe-(D)Arg-(D)Trp-Gly] **4ae** and Cyclo]His-(D)Arg-Phe-(D)Trp] **4af**.

Table 4 Proton chemical shifts for cyclo[His-(D)Phe-Arg-(D)Trp]

	Solvent			
Proton	d_6-DMSO	H_2O/d_3-MeCN/D_2O (76.5:13.5:10)	d_3-MeCN/D_2O (9:1)	Random Coil Values[32]
Histidine				
NH	8.034, J 9.56 Hz	8.407		8.41
Hα	4.612	4.865	4.870	4.63
Hβ	2.983, 2.784	3.235, 3.149	3.361, 3.147	3.26, 3.20
ArH	7.165, 8.827	7.290, 8.220		7.14, 8.12
Phenylalanine				
NH	7.962, J 9.19 Hz	8.311		8.23
Hα	4.540	4.917	4.901	4.66
Hβ	2.978, 2.792	3.308, 3.180	3.303, 3.208	3.22, 2.99
2,6H	7.15–7.23	7.46–7.57	7.49–7.57	7.30
3,5H				7.39
4H				7.34
Arginine				
NH	7.761, J 9.56 Hz	8.323		8.27
Hα	4.305	4.580	4.583	4.38
Hβ	1.590, 1.462	1.915, 1.826	1.953, 1.837	1.89, 1.79
Hγ	1.364, 1.284	1.631, 1.526	1.717, 1.619	1.70
Hδ	3.041	3.239	3.331	3.32
NHε	7.429	7.290		7.17, 6.62
Tryptophan				
NH	7.928, J 9.19 Hz	8.373		8.09
Hα	4.634	5.030	4.967	4.70
Hβ	3.139, 2.810	3.491, 3.283	3.525, 3.258	3.32, 3.19
ArH	6.942, 7.025, 7.047, 7.315, 7.476	7.359, 7.470, 7.717, 7.884	7.315,7.333, 7.442, 7.681, 7.858	7.17, 7.24, 7.50, 7.65
NH1	10.717	10.261		10.22

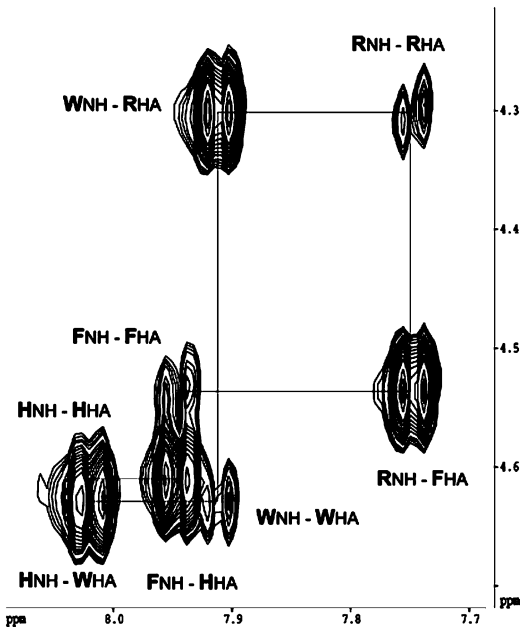


Fig. 3 NH-Hα region of ROESY spectrum in d_6-DMSO.

almost antiperiplanar relationship between NH and Hα, which corresponds to a ϕ angles of around 120° (CO-NH-Hα-CO torsion angle) and also illustrates a symmetry to the peptide backbone. These coupling constants are typical of extended peptide units, such as a β-sheet[32] and is therefore indicative of *trans* amide bonds.

The amino acid side chains in this cyclic tetrapeptide exhibited very few inter-residue rOe's. This would be expected, as the orientation of the backbone forces the side chains to be as far away from each other as possible. In addition, identical ECOSY cross-peaks between Hα and Hβ indicate that rotation about this bond is largely unrestrained.

Variable temperature experiments were conducted on the cyclic tetrapeptide in d_6-DMSO between 298 and 338 K (Fig. 4). It appeared that there are no strong hydrogen bonding interactions as all NH's exhibited a change in chemical shift with temperature of between 3 and 5 ppb/K. When a D_2O exchange experiment was conducted (Fig. 5) it was clear that the amide NH's of Arg, Trp and Phe all exchange very slowly (approximately 11.5, 11 and 4.5 h, respectively for half of the NH's to exchange), but the His NH is fully exchanged after approximately 5 h. When these pieces of data are examined together it becomes clear that none of the amide bond NH's are involved in hydrogen bonding, although they all appear to be quite buried in the molecule. This concurs with a previously reported LDLD cyclic peptide structure (cyclo[Leu-(D)Ala-Leu-(D)Ala]) by Ngu-Schwemlein *et al.*, in

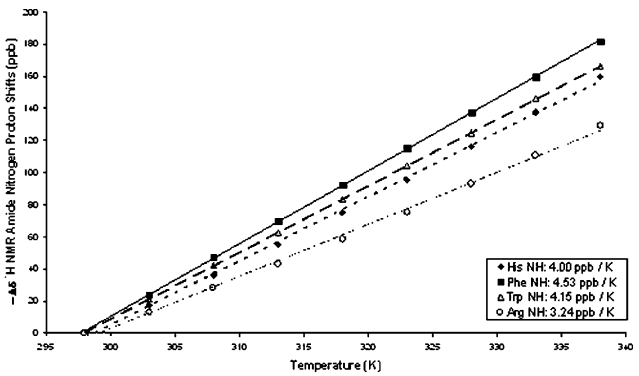


Fig. 4 Variable temperature experiment: Δδ amide nitrogen ^{1}H NMR shift *versus* temperature (d_6-DMSO).

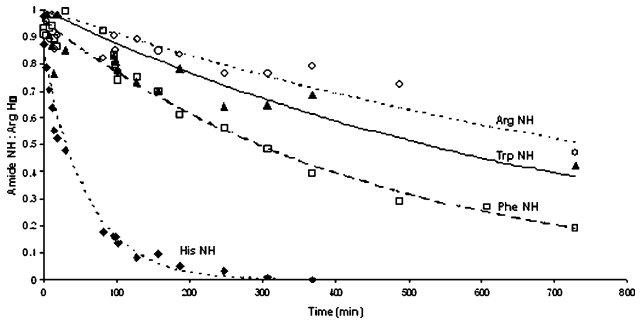


Fig. 5 D_2O exchange experiment (5% D_2O in d_6-DMSO).

which an open β-turn structure not stabilized by intramolecular hydrogen bonding was proposed.[11]

From the information collected, a solution structure was generated using X-PLOR. This resulted in ω angle (C_α-CO-NH-C_α torsion angles) violations. X-ray structures of cyclic tetrapeptides indicate that the ω angles (Cα-CO-NH-Cα torsion angles) are quite commonly twisted from planarity, regardless of whether the amide bonds are *cis* or *trans*.[12,13,15–18,33] In fact, the all-*trans* cyclic tetrapeptides commonly possess a ω angle twist of ± 15–25 °.[3,5,12,13] As a result, all of the energy restraints in parallhdg5.2.pro (the X-PLOR parameter file) on the ω torsion angle were relaxed from their default value of 500. Solution structures were then calculated at energy values of 500, 250, 150 and 50 (Table 5, Fig. 6).

As can be seen in Table 5 and Fig. 6, the ω dihedral angles twist to approximately 20° when the energy restraint is relaxed to 50, which correlates well with twist seen in X-ray structures. This structure also displays a relatively small change in energy over the 20 lowest energy structures, and the smallest RSMD. Another factor of note is that as the ω twist energy restraint is relaxed, the direction of planarity of the amide bond becomes more and more perpendicular to the plane of the peptide backbone.

Synthesis of the cyclic-tetrapeptide library

Having explored the synthesis and characterisation of a small number of cyclic-tetrapeptides, a general synthesis of a 44 member library was conducted (Table 6). This synthesis was carried out using HATU cyclisation and microwave assisted ring contraction.

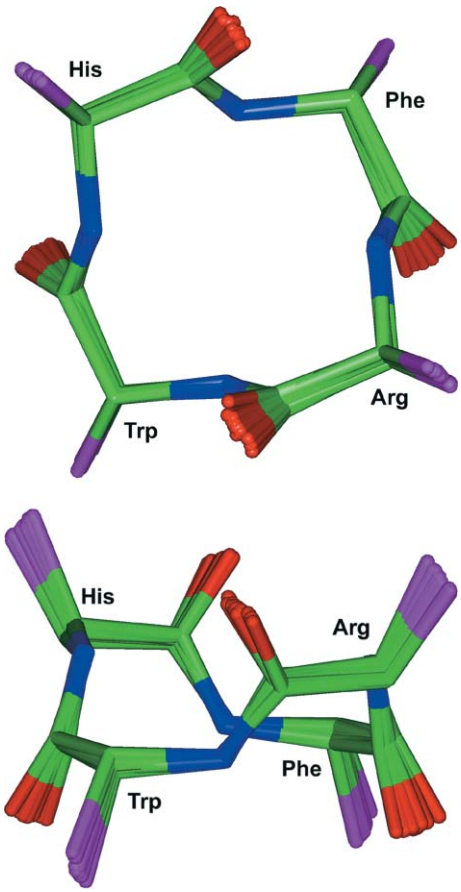


Fig. 6 Superimposition of the peptide backbone and βC (purple) of the 20 lowest energy minimised structures of cyclo-[His-(D)Phe-Arg-(D)Trp], in which the ω torsion angle energy restraint is relaxed to 50.

Replacement of thermal heat with microwave irradiation resulted in shorter reaction times. Isolation of **3** was not carried out, as the crude mixture was photolysed immediately to generate the desired cyclic tetrapeptide **4**.

Due to the difficulties in synthesising these highly constrained macrocyles, in combination with the well known "sequence dependency" on cyclisation, it was not expected that every compound

Table 5 Solution structure information from the relaxation of the ω angle energy restraints on cyclo[His-(D)Phe-Arg-(D)Trp]

	Restraint energy of Omega bond (dihedrals)			
	500 (default)	250	150	50
ΔE for top 20 structures	11.7 kcal/mol	8.4 kcal/mol	10.0 kcal/mol	5.4 kcal/mol
RSMD for backbone atoms (N,C,CA) of top 20 structures	0.069	0.109	0.097	0.075
$\omega_{His\text{-}Phe}$	−174.72	−171.48	−166.88	−160.45
$\omega_{Phe\text{-}Arg}$	175.10	170.47	166.48	159.56
$\omega_{Arg\text{-}Trp}$	−175.53	−171.52	−167.17	−160.49
$\omega_{Trp\text{-}His}$	173.98	169.33	165.66	159.70
ϕ_{His}	−139.19	−131.07	−125.01	−100.70
ϕ_{Phe}	144.37	141.79	129.18	106.11
ϕ_{Arg}	−142.25	−134.13	−125.97	−101.12
ϕ_{Trp}	149.56	143.59	130.90	105.29
ψ_{His}	39.53	40.71	47.87	55.77
ψ_{Phe}	−39.91	−44.60	−49.51	−59.79
ψ_{Arg}	34.96	38.75	46.02	56.75
ψ_{Trp}	−42.35	−47.19	−50.02	−59.95

Table 6 Cyclic tetrapeptide library synthesis

	Linear peptide[a] (1)	Yield (4)	Mass Theory[c]	Mass Found[c]
al	[HnB]Ala-Arg-Tyr-Gly-OH	29%	448.23	448.23
am	[HnB]Ala-Ile-Ile-Gly-OH	5%	355.24	355.22
an	[HnB]Ala-Asn-His-Gly-OH	9%	380.29	380.28
ao	[HnB]Phe-Gly-Asn-Gly-OH	8%	376.16	376.28
ap	[HnB]Phe-His-Ala-Gly-OH	9%	413.20	413.22
aq	[HnB]Phe-Asn-Phe-Gly-OH	4%	466.21	466.34
ar	[HnB]Phe-Arg-His-Gly-OH	12%	498.26	498.39
as	[HnB]Tyr-Ala-Ile-Gly-OH	9%	405.22	405.41
at	[HnB]Tyr-Asn-Ile-Gly-OH	—	448.21	—
au	[HnB]Tyr-Trp-Phe-Gly-OH	4%	554.24	554.40
av	[HnB]Tyr-Trp-Ile-Gly-OH	5%	520.26	520.23
aw	[HnB]Ile-Ala-Val-Gly-OH	—	341.21	—
ax	[HnB]Ile-Trp-Phe-Gly-OH	—	504.39	—
ay	[HnB]Asn-His-Phe-Gly-OH	—	456.20	—
az	[HnB]Asn-Arg-Phe-Gly-OH	6%	475.24	474.50
ba	[HnB]Asn-Ser-Trp-Gly-OH	—	445.19	—
bb	[HnB]Arg-His-Asn-Gly-OH	10%	465.23	465.25
bc	[HnB]Arg-Ile-His-Gly-OH	15%	464.28	464.27
bd	[HnB]Thr-His-Phe-Gly-OH	10%	443.21	444.18
be	[HnB]Trp-Ala-His-Gly-OH	7%	452.21	452.20
bf	[HnB]Trp-Asn-His-Gly-OH	5%	495.21	495.28
bg	[HnB]Trp-Arg-Phe-Gly-OH	11%	547.28	547.29
bh	[HnB]Tyr-Ile-Gln-Gly-OH	—	462.24	—
bi	[HnB]Phe-Tyr-Ala-Gly-OH	11%	439.20	439.22
bj	[HnB]Ala-Arg-Tyr-Gly-OH	19%	448.23	478.23
bk	[HnB]Ile-Arg-Tyr-Gly-OH	11%	490.28	490.30
bl	[HnB]Ile-Trp-Tyr-Gly-OH	—	420.26	—
bm	[HnB]Tyr-Ile-Ile-Gly-OH	—	447.26	—
bn	[HnB]Tyr-Asn-Arg-Gly-OH	—	491.24	—
bo	[HnB]Arg-His-Ile-Gly-OH	11%	464.28	464.31
bp	[HnB]Phe-Arg-Ile-Gly-OH	16%	474.29	474.40
bq	[HnB]Arg-Arg-Gly-Gly-OH	—	427.26	—
br	[HnB]Asn-Arg-Tyr-Gly-OH	7%	491.24	491.32
bs	[HnB]Arg-Tyr-Val-Gly-OH	9%	476.26	476.35
bt	[HnB]Trp-Arg-Gly-Gly-OH	12%	457.23	457.22
bu	[HnB]Gln-Tyr-Trp-Gly-OH	28%	535.23	535.22
bv	[HnB]Arg-Tyr-Arg-Gly-OH	9%	533.30	333.69
bw	[HnB]Ala-Trp-Ile-Gly-OH	14%	428.23	428.25
bx	[HnB]Arg-Trp-Val-Gly-OH	11%	499.28	499.34
by	[HnB]Ser-Ile-Ile-Gly-OH	7%	371.23	371.20
bz	[HnB]Arg-Ser-Ile-Gly-OH	12%	414.25	414.26
ca	[HnB]Phe-Arg-Trp-Gly-OH	5%	547.28	547.33
cb	[HnB]Tyr-Val-Phe-Gly-OH	7%	467.23	467.42
cc	[HnB]Phe-Val-Tyr-Gly-OH	6%	467.23	467.32

[a] All naturally occurring amino acids are the L-isomer. [b] % Yield over two steps from **1** without purification of the intermediate material; i) 1 eq HATU, 2 eq DIEA, 1 mM in DMSO, 3h/rt; ii) 10 eq DIEA, 3h/microwave; iii) AcOH added to make a 1% solution, hυ, 3 h. [c] Mass of the purified cyclic tetrapeptide. Characterisation was based upon mass spectroscopy results.

could be successfully prepared. Surprisingly, around 70% of all of these peptides were successfully synthesised.

Some unsuccessful syntheses contained β-branched amino acids (Val, Ile), indicating that steric problems were a possible cause. Cyclo[Tyr-Ala-Ile-Gly] **4as** was successful, whilst related compound cyclo[Ile-Ala-Val-Gly] **4aw** was not. Further, cyclo[Tyr-Trp-Phe-Gly] **4au** succeeded, whilst cyclo[Ile-Trp-Phe-Gly] **4ax** did not. When sequences were changed, dramatic effects were also observed. Cyclo[Ile-Arg-Tyr-Gly] **4bk** succeeded, whilst cyclo[Ile-Trp-Tyr-Gly] **4bl** could not be prepared. Strikingly cyclo[Gln-Tyr-Trp-Gly] **4bu** was synthesised in relatively high yield, whilst the related compound cyclo[Asn-Ser-Trp-Gly] **4ba** could not be prepared. This sequence dependent effect on cyclisation had been noted previously, and consequently HnB had been incorporated into the backbone of cyclic tetrapeptides to further facilitate cyclisation of these more difficult sequences.[19] Overall, isolated yields were low and this is probably a reflection of the photolysis rather than the ring contraction.

Edman degradation was performed on the successfully synthesised library members. In most cases the correct cyclic material was observed. The only exception was when arginine was present in the 4th position (Table 6). The library contained 7 such sequences **4bb**, **4bc**, **4bo**, **4bq**, **4bs**, **4bv**, **4bx**, **4bz**. Of these only 3 of the cyclised products contained both the head-to-tail cyclic material and the sidechain-to-head cyclic material **4bo**, **4bs**, **4bz**. Interestingly when Asn was placed in the 4th position no side chain cyclic product was observed. In addition, side chain cyclised products were not identified by mass spectrometry when nucleophilic sidechains were positioned in the 2nd or 3rd position. This is probably an indication that 9 to 11 membered rings would not form due to the constrained nature of these systems, and consequently that the formation of the cyclic tetrapeptides would be favoured. This library is currently

being screened against a number of G-protein coupled receptors and protein kinases.

Conclusion

Despite three decades of discovery of numerous biologically active cyclic tetrapeptides from various microbial sources and their reported oral activity, these products have been largely unexplored in the pharmaceutical industry. Primarily this is due to their synthetic inaccessibility.

A photolabile ring-contraction auxiliary was used for the synthesis of number of cyclic tetrapeptides and the cyclisation and photolysis steps were examined and optimised. In parallel with this, a suite of characterisation techniques was developed, including Edman degradation and mass-spectrometry/mass-spectrometry. An NMR solution structure of cyclo[His-(D)Phe-Arg-(D)Trp] was also generated, which displayed all *trans* amide bonds. The alternating L and D amino acids allowed the cyclic peptide to adopt a relatively flat configuration in which the backbone amides are orientated perpendicular to the ring system. This product was synthesised from cyclo[His-(D)Phe-Arg-Trp] and a complete stereoinversion at the *C*-terminal residue occurred during the synthesis, illustrating that the LDLL cyclic peptide is significantly more sterically constrained than the LDLD peptide.

A small number of cyclic tetrapeptides were synthesised to find the optimal conditions for cyclisation. Following this, a larger library of 34 compounds was synthesised and these are currently being tested for biological activity aginst a number of GPCRs.

HnB is an ideal auxiliary for this procedure due to its superior acyl transfer properties, its ready accessibility and its photolability. Using this auxiliary, a range of cyclic tetrapeptides may be synthesised which would be inaccessible by other means.

Materials and methods

Thin Layer Chromatography (TLC) was performed on silica gel 60 F_{254} plates (Merck). The chromatograms were viewed under U.V. light. Flash column chromatography was performed with flash silica gel 60 (0.063–0.200 mm, Merck). Nuclear Magnetic Resonance spectra were recorded at 300 MHz (^{1}H)/75 MHz (^{13}C) or 500 MHz (^{1}H) on a Varian Gemini-300 or a Bruker ARX 500 spectrometer, respectively. ^{1}H and ^{13}C chemical shifts (δ) are given in parts per million (ppm) using residual protonated solvent as an internal standard. Coupling constants are given in Hertz (Hz). The following abbreviations are used: s = singlet, d = doublet, t = triplet, m = multiplet. Low resolution mass spectral data were recorded on a Micromass LCT (TOF MS ES+) instrument. High resolution mass spectral data was obtained on a PE Sciex API QSTAR Pulsar (ES-QqTOF) instrument using ACP (acyl carrier protein) (65–74) ($C_{47}H_{75}N_{12}O_{16}$ (M + H), 1063.5424) and reserpine ($C_{33}H_{40}N_2O_9$ (M + H), 609.2812) as internal references. Resolution for the instrument was set between 10,000 and 12,000 for all standards. Melting points were determined on a Bausch and Lomb hot stage. Chiral amino acid analysis was performed by C.A.T. GmbH & Co. Chromatographie und Analysentechnik KG, Tübingen, Germany. Analytical reversed-phase HPLC was performed on a Vydac C_{18} column (4.6 × 250 mm). Preparative reversed phase HPLC was performed on a Vydac C_{18} column (22 × 250 mm) or Phenomonex Jupiter 10 μ 300 Å C_{18} column (21.2 × 100 mm). Separations were achieved using linear gradients of buffer B in A (A = 0.1% aqueous TFA; B = 90% CH_3CN, 10% H_2O, 0.09% TFA) at a flow rate of 1 mL/min (analytical) and 20 mL/min (preparative). Microwave irradiation of library members was performed sing the Ethos Microwave Labstation (Milestone Inc., CT, USA). Abbreviations: MeCN, acetonitrile; TFA, trifluoroacetic acid; DCM, dichloromethane; petrol, petroleum spirit (bp 40–60 °C); EtOAc, ethyl acetate; DIEA, diisopropylethyl amine; AcOH, acetic acid; MeOH, methanol; Et_2O, diethyl ether; DMSO, dimethylsulfoxide; DMF, *N,N*-dimethylformamide; HBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate; HATU, *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; BOP, benzotriazol-1-yl-oxytris(dimethyamino)phosphonium hexafluorophosphate; DIC, diisopropylcarbodiimide; PyBOP, benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate.

Materials

Aminomethyl polystyrene resin (sv = 0.41 mmol/g) and all N_α-Fmoc-amino acids were peptide synthesis grade purchased from Auspep (Melbourne Australia) or Novabiochem (San Diego, USA). Dichloromethane, diisopropylethylamine, *N,N*-dimethylformamide, and trifluoroacetic acid were obtained from Auspep (Melbourne, Australia). HPLC grade acetonitrile was purchased from BDH (Brisbane, Australia). 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was purchased from Richelieu Biotechnologies (Quebec, Canada).

^{1}H NMR spectrometry

All NMR experiments were recorded on a Bruker ARX 500 spectrometer equipped with a *z*-gradient unit at 298 K except for variable temperature experiments. Peptide concentrations were ~3 mM. Cyclo[His-(D)Arg-Phe-(D)Trp] was examined in d_6-DMSO, H_2O/d_3-MeCN/D_2O (76.5:13.5:10) and d_3-MeCN/D_2O (9:1). ^{1}H NMR experiments recorded were ROESY with mixing time of 400 ms, TOCSY, DQF-COSY and E-COSY. All spectra were run over 5555 Hz with 4 K data points, 16–24 scans. Spectra was processed using XWIN-NMR and ^{1}H chemical shifts are given in parts per million (ppm) using residual protonated solvent as an internal standard. Coupling constants are given in Hertz (Hz). $^3J_{\text{NH-H}\alpha}$ coupling constants were measured on the 1D spectrum.

Distance restraints and structure calculations

Peak volumes in ROESY spectra were classified as strong, medium, weak and very weak, corresponding to upper bounds on interproton distances of 2.7, 3.5, 5.0 and 6.0 Å, respectively. Appropriate pseudoatom corrections were made,[34] and distances of 0.6 and 2.0 Å were added to the upper limits of restraints involving methyl and phenyl protons, respectively. $^3J_{\text{NH-H}\alpha}$ coupling constants were used to determine ϕ dihedral angle restraints,[35] and as all coupling constants exceeded 9.0 Hz, ϕ was restrained to + or-120 ± 30 ° for L- and D- amino acids, respectively.

Structures were calculated using the torsion angle dynamics/simulated annealing protocol in X-PLOR version 3.851 using a modified geometric force field based on parallhdg5.2.pro.[36] Structure refinements were performed using energy minimisation (2000 steps) using parallhdg5.2.pro. Structure modelling,

visualisation and superimpositions were done using Insight II (MSI). RMS deviations, and the quality of the solution structure was determined in X-PLOR.

Edman degradation

Edman degradation studies were performed according to the method of Pitt *et al.*,[37] with the following modifications. Less than 0.01 mg cyclic peptide was treated with 25 μL of a phenylisothiocyanate/pyridine/H_2O (1:1.5:1.5) solution. The solution was left at room temperature for 1.5 h under argon then the aqueous phase was extracted twice with 400 μL hexane/EtOAc (2:1). Trifluoroacetic acid (20 μL) was then added to the aqueous layer, which was subsequently left at room temperature for 1 h. Finally, 60 μL of water was added and the resulting solution analysed by ES-MS.

Synthesis

2-Hydroxy-6-nitrobenzaldehyde (HnB)[38]. *meta*-Nitrophenol (10.0 g, 71.9 mmol) was dissolved in 57.1 mL 75% poly-phosphoric acid and was heated to 100 °C. Hexamine (10.2 g, 72.4 mmol) was then slowly added to this stirred solution. After the addition of the hexamine, the solution was stirred for a further 50 minutes before it was cooled and dissolved in water. This aqueous solution was then extracted several times with ethyl acetate, before drying the organic layer with $MgSO_4$ and removing the ethyl acetate under vacuum. The crude product was then chromatographed over silica gel (10% EtOAc/petrol) and dried to produce a yellow solid (1.25 g, 7.48 mmol). Yield: 10.4%. Melting point: 51–52 °C (lit. 53–54 °C). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.30 (m, 1H), 7.55 (m, 1H), 7.63 (t, 1H), 10.31 (s, 1H), 12.09 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 112.3, 116.0, 124.1, 135.9, 151.2, 163.2, 193.8.

General procedure for the synthesis for peptides

Synthesis of linear peptides on resin. All linear peptides were chemically synthesised stepwise using Fmoc protecting groups and *in situ* HBTU activation protocols as previously described.[39,40] Coupling efficiencies were determined by the quantitative ninhydrin test[41] and recoupled where necessary to obtain >99.5% efficiency. These peptides are displayed in Table 1.

Introduction of HnB

The 2-hydroxy-6-nitrobenzaldehyde (2 eq) was dissolved in MeOH/DMF (1:1) (0.1 M) and added to the amino peptide resin. After 5 min the resin was filtered and a second portion of aldehyde (2 eq) added. After another 5 min the resin was filtered and washed with MeOH/DMF (1:1). 0.1M $NaBH_4$ (10 eq) in MeOH/DMF (1:3) was then added and the reaction mixture left standing for 5 min. The resin was again filtered and washed with MeOH/DMF (1:3), DMF, MeOH/DCM (1:1) and air-dried.

Cleavage

Peptides on chlorotrityl resin were cleaved using TFA (either 95% with 5% H_2O or 1% in DCM, 10 mL/500 mg resin, 1h at RT). The resin was filtered and solvents evaporated *in vacuo.* The residue was washed with ether (2 × 10 mL) and dissolved in aqueous acetonitrile (50%). The product was then purified by reversed-phase HPLC.

Peptide cyclisation

1 equivalent of BOP or HATU and 2 equivalents of DIEA were added to a 1 mM solution of the linear peptide in DMSO and stirred for 3 h at rt. 10 equivalents of DIEA were then added and the solution stirred overnight at 70 °C. The DMSO was then removed under vacuum, the residue dissolved in a solution of aqueous acetonitrile (50%) and lyophilised before purification by reversed-phase HPLC.

General procedure for photolysis

Cyclic peptides were dissolved in 1% acetic acid/DMF to make a 1 to 0.1 mM concentration. The solution was placed in a beaker and photolysis carried out for 5 hours using a UV lamp (350–365 nm, 20 W, Black/White/Blue). The DMF was removed under vacuum, the residue dissolved in a solution of aqueous acetonitrile (50%) and lyophilised, before purification by reversed-phase HPLC.

The Pbf protecting group was then removed by treating the product with 400 μL cleavage mixture (92.5% TFA, 5% H_2O, 2.5% TiPS) for 1 hour. The TFA solution was then blown off with argon and the residue dissolved in a small amount of acetonitrile and lyophilised. The product was then purified by reversed-phase HPLC.

Library synthesis of peptides

All reactions were performed on a Bohdan MiniBlock™ using 1.5 mL of solvent for all reactions and washings. All linear peptides were chemically synthesised stepwise as before using Fmoc protecting groups and *in situ* HBTU activation protocols as previously described.[39,40] Coupling efficiencies were determined by the quantitative ninhydrin test[41] and recoupled where necessary to obtain >99.5% efficiency. HnB was introduced as reported above. Cleavage was performed using TFA 95% with 5% H_2O, 1h at RT. The resin was filtered and filtrate evaporated under reduced pressure. The residue was washed with ether (2 × 10 mL) and dissolved in aqueous acetonitrile (50%). The product was then purified by reverse-phase HPLC (Table 6).

HATU (1 eq) and DIEA (2 eq) were added to a 1 mM solution of the linear peptide in DMSO and stirred for 1 h at RT. Excess DIEA (10 eq) was then added and the solution stirred for 3 h at 100 °C using the Ethos Microwave Labstation. After ring contraction, the solution was placed in a beaker (2 mL). AcOH was added and photolysis was carried out for 3 hours using a UV lamp (350–365 nm, 20 W, Black/White/Blue). The DMSO was removed under vacuum, the residue dissolved in aqueous acetonitrile (50%) and the product purified by reversed-phase HPLC.

Acknowledgements

We would like to acknowledge the Australian Research Council (ARC) and the Biotechnology Infrastructure Fund (BIF) for their financial support. We would also like to thank David Craik, Christina Schroeder, Martin Stoermer, Sue Kydd, Peter Seale, John Murray and Mike Hann.

Notes and references

1 D. A. Horton, G. T. Bourne and M. L. Smythe, *J. Comput. Aided Mol. Des.*, 2002, **16**, 415–31.
2 D. A. Horton, G. T. Bourne and M. L. Smythe, *Chem. Rev.*, 2003, **103**, 893–930.
3 M. Kawai and D. H. Rich, *Biochem. Biophys. Res. Commun.*, 1983, **111**, 398–403.
4 R. B. Pringle, *Plant Physiol.*, 1970, **46**, 45–9.
5 M. Kawai, R. D. Jasensky and D. H. Rich, *J. Am. Chem. Soc.*, 1983, **105**, 4456–62.
6 H. Stähelin and A. Trippmacher, *Eur. J. Cancer*, 1974, **10**, 801–8.
7 M. Kijima, M. Yoshida, K. Sugita, S. Horinouchi and T. Beppu, *J. Biol. Chem.*, 1993, **268**, 22429–35.
8 H. Kawagishi, A. Somoto, J. Kuranari, A. Kimura and S. Chiba, *Tetrahedron Lett.*, 1993, **34**, 3439–40.
9 S. B. Singh, D. L. Zink, J. D. Polishook, A. W. Dombrowski, S. J. Darkin-Rattray, D. M. Schmatz and M. A. Goetz, *Tetrahedron Lett.*, 1996, **37**, 8077–80.
10 S. J. Darkin-Rattray, A. M. Gurnett, R. W. Myers, P. M. Dulski, T. M. Crumley, J. J. Allocco, C. Cannova, P. T. Meinke, S. L. Colletti, M. A. Bednarek, S. B. Singh, M. A. Goetz, A. W. Dombrowski, J. D. Polishook and D. M. Schmatz, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 13143.
11 M. Ngu-Schwemlein, Z. Zhou, T. Bowie and R. Eden, *J. Mol. Struct.*, 2003, **655**, 59–68.
12 J. L. Flippen and I. L. Karle, *Biopolymers*, 1976, **15**, 1081–92.
13 J. P. Declercq, G. Germain, M. van Meerssche, T. Debaerdemaeker, J. Dale and K. Titlestad, *Bull. Soc. Chim. Belg.*, 1975, **84**, 275–87.
14 M. Kawai, R. S. Pottorf and D. H. Rich, *J. Med. Chem.*, 1986, **29**, 2409–11.
15 W. Mästle, U. Link, W. Witschel, U. Thewalt, T. Weber and M. Rothe, *Biopolymers*, 1991, **31**, 735–44.
16 W. Mästle, T. Weber, U. Thewalt and M. Rothe, *Biopolymers*, 1989, **28**, 161–74.
17 W. L. Meyer, L. F. Kuyper, D. W. Phelps and A. W. Cordes, *J. Chem. Soc., Chem. Commun.*, 1974, 339–40.
18 D. Seebach, O. Bezençon, B. Jaun, T. Pietzonka, J. L. Matthews, F. N. M. Kühnle and W. B. Schweizer, *Helv. Chim. Acta*, 1996, **79**, 588–608.
19 W. D. F. Meutermans, S. W. Golding, G. T. Bourne, L. P. Miranda, M. J. Dooley, P. F. Alewood and M. L. Smythe, *J. Am. Chem. Soc.*, 1999, **121**, 9790–6.
20 W. D. F. Meutermans, G. T. Bourne, S. W. Golding, D. A. Horton, M. R. Campitelli, D. Craik, M. Scanlon and M. L. Smythe, *Org. Lett.*, 2003, **5**, 2711–4.
21 D. H. Rich and P. K. Bhatnagar, *J. Am. Chem. Soc.*, 1978, **100**, 2218–24.
22 M. Tamaki, S. Komiya, M. Yabu, E. Watanabe, S. Akabori and I. Muramatsu, *J. Chem. Soc., Perkin Trans. 1*, 1997, 3497–500.
23 E. Pinet, F. Cavelier, J. Verducci, G. Girault, L. Dubart, F. Haraux, C. Sigalat and F. André, *Biochemistry*, 1996, **35**, 12804–11.
24 T. Shimizu, Y. Tanaka and K. Tsuda, *Int. J. Pept. Protein Res.*, 1986, **27**, 554–61.
25 M. Tamaki, M. Yabu, T. Saeki, A. Kaneko, S. Akabori and I. Muramatsu, *Bull. Chem. Soc. Jpn.*, 1999, **72**, 733–9.
26 I. McEwen and K. Ottosson, *Biopolymers*, 1993, **33**, 1377–87.
27 D. H. Rich, P. Bhatnagar and P. Mathiaparanam, *J. Org. Chem.*, 1978, **43**, 296–302.
28 F. Cavelier and J. Verducci, *Tetrahedron Lett.*, 1995, **36**, 4425–28.
29 J. E. Baldwin, R. M. Adlington, C. R. A. Godfrey and V. K. Patel, *Tetrahedron*, 1993, **49**, 7837–56.
30 S. Fernandez-Lopez, H.-S. Kim, E. C. Choi, M. Delgado, J. R. Granja, A. Khasanov, K. Kraehenbuehl, G. Long, D. A. Weinberger, K. M. Wilcoxen and M. R. Ghadiri, *Nature*, 2001, **412**, 452–5.
31 T. D. Clark, J. M. Buriak, K. Kobayashi, M. P. Isler, D. E. McRee and M. R. Ghadiri, *J. Am. Chem. Soc.*, 1998, **120**, 8949–62.
32 K. Wüthrich, *NMR of Proteins and Nucleic Acids*, Wiley-Interscience, USA, 1986.
33 K. Ueno and T. Shimizu, *Biopolymers*, 1983, **22**, 633–41.
34 K. Wüthrich, M. Billeter and W. Braun, *J. Mol. Biol.*, 1983, **169**, 949–61.
35 A. Pardi, M. Billeter and K. Wüthrich, *J. Mol. Biol.*, 1984, **180**, 741–51.
36 A. T. Brünger, *X-PLOR: A System for X-ray Crystallography and NMR*, ver. 3.851, Yale University, New Haven, CT.
37 J. J. Pitt, E. Da Silva and J. J. Gorman, *J. Biol. Chem.*, 2000, **275**, 6469–78.
38 T. Harayama, K. Nakatsuka, H. Nishioka, K. Murakami, Y. Ohmori, Y. Takeuchi, H. Ishii and K. Kenmotsu, *Heterocycles*, 1994, **38**, 2729–38.
39 M. Schnölzer, P. F. Alewood, A. Jones, D. Alewood and S. B. H. Kent, *Int. J. Pept. Protein Res.*, 1992, **40**, 180–93.
40 P. Alewood, D. Alewood, L. Miranda, S. Love, W. Meutermans and D. Wilson, *Methods Enzymol.*, 1997, **289**, 14–29.
41 V. K. Sarin, S. B. H. Kent, J. P. Tam and R. B. Merrifield, *Anal. Biochem.*, 1981, **117**, 147.
42 A. Patchornik, B. Amit and R. B. Woodward, *J. Am. Chem. Soc.*, 1970, **92**, 6333–6335.