Peptides Constrained by an Aliphatic Linkage between Two C^{α} Sites: Design, Synthesis, and Unexpected Conformational Properties of an *i*,(*i* + 4)-Linked Peptide

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A novel route for the synthesis of cyclic peptides constrained by an aliphatic bridge between two C^{α} sites, using a triply orthogonal protecting group strategy, is described. The synthesis of the orthogonally protected bis-amino acid **1**, via an enantioselective route utilizing the Schöllkopf and Evans methodologies, is first described. This is then incorporated into a short, alanine-rich peptide **13**, using a novel triply orthogonal protecting group strategy to couple first one, then the other, amino acid moiety in such a way that an aliphatic bridge is formed between the *i* and *i* + 4 positions. Unexpectedly, the resulting constrained peptide does not adopt a helical conformation: instead, it is shown by CD at low temperature to adopt a left-handed type II β-turn conformation in aqueous media and a right-handed type I β-turn conformation in TFE.

Introduction

The design and synthesis of conformationally constrained peptides and peptidomimetics is of major importance in biological and medicinal chemistry, as they may be used as a basis for the design of low-molecular weight therapeutic agents and as tools for the elucidation of structure−activity relationships.[1] The conformational constraints may be of many types, including conformationally restricted amino acids, templates, and peptide cyclization between N- and C-termini or between side chains. Peptide cyclization has also been exploited for the construction of mimics of protein secondary structure.[2] In particular, the introduction of linkages between the *i* and *i* + 4 positions of peptides has been extensively used to stabilize the helical conformation of short peptides.[3]

Synthesis of cyclic peptides via linkages not encountered in nature is of particular importance, as the resulting peptidomimetics may have increased metabolic stability, reduced immunogenicity, greater affinity, or higher selectivity when compared to naturally occurring linkages such as amide bonds or disulfide links. However, despite the potential importance of cyclic peptides bearing unnatural side-chain to side-chain linkages, the challenges involved in synthesizing such peptides have meant that few examples of such peptides have been described.[4] The most powerful methodology so far reported is the RCM[5] strategy of Grubbs, which has been used to access several families of side-chain to side-chain aliphatic bridged peptides,[6] including *i*, (*i* + 4)-bridged helical peptides.[7]

We have initiated a program to develop a flexible method of synthesizing cyclic peptides with unnatural aliphatic bridges. Our approach involves the synthesis of a differentially protected bis-amino acid **1**,[8,9] in which both amino acid chiral centers and the desired aliphatic

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(1) (a) Haubner, R.; Finsinger, D.; Kessler, H. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 1374. (b) Olson, G. L.; Bolin, D. R.; Bonner, M. P.; Bös, M.; Cook, C. M.; Fry, D. C.; Graves, B. J.; Hatada, M.; Hill, D E.; Kahn, M.; Madison, V. S.; Ruisiecki, V. K.; Sarabu, R.; Sepinwall, J.; Vincent, G. P.; Voss, M. E.; *J. Med. Chem.* **1993**, *36*, 3039. (c) Giannis, A.; Kolter, T. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1244. (d) Hanessian, S.; McNaughton-Smith, G.; Lombart, H.-G.; Lubell, W. D. *Tetrahedron* **1997**, *38*, 12789. (e) Hruby, V. J.; Al-Obeidi, F.; Kazmierski, W. *Biochem. J.* **1990**, *268*, 249. (f) Gante, J. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1699.

(2) (a) Peczuh, M. W.; Hamilton, A. D. *Chem. Rev.* **2000**, *100*, 2479. (b) Schneider, J. P.; Kelly, J. W. *Chem. Rev.* **1995**, *95*, 2169. (c) Gillespie, O.; Cicariello, J.; Olsen, G. L. *Biopolymers* **1997**, *43*, 191. (d) Hruby, V. J.; Li, G.; Haskell-Luevano, C.; Shenderovich, M. *Biopolymers* **1997**, *43*, 219.

(3) (a) Ösapay, G.; Taylor, J. W. *J. Am. Chem. Soc.* **1990**, *112*, 6046. (b) Ösapay, G.; Taylor, J. W. *J. Am. Chem. Soc.* **1992**, *114*, 6966. (c) Campbell, R. M.; Bongers, J.; Felix, A. M.; *Biopolymers* **1995**, *37*, 67. (d) Chen, S.-T.; Chen, H.-J.; Yu, H.-M.; Wang, K.-T. *J. Chem. Res., Synop.* **1993**, 228. (e) Andrews, M. J. I.; Tabor, A. B. *Tetrahedron* **1999**, *55*, 11711.

(4) Fairlie, D. P.; Abbenante, G.; March, D. R. *Curr. Med. Chem.* **1995**, *2*, 654.

(5) Abbreviations: Aloc, allyloxycarbonyl; DIC, diisopropylcarbodiimide; Fmoc, 9-fluorenylmethyloxycarbonyl; hGRF, human growth hormone releasing factor; HATU, *N*-[(dimethylamino)-*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; KHMDS, potassium bis(trimethylsilyl)amide; NMM, *N*-methyl morpholine; PPII, polyproline II; PyAOP, (7-azabenzotriazol-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate; RCM, ring-closing metathesis; TBAF, tetra-*n*-butylammonium fluoride; TBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TES, triethylsilane; TFE, trifluoroethanol; trisyl azide, triisopropylbenzenesulphonyl azide.

(6) (a) Miller, S. J.; Blackwell, H. E.; Grubbs, R. H. *J. Am. Chem. Soc.* **1996**, *118*, 9606. (b) Reichwein, J. F.; Versluis, C.; Liskamp, R. M. J. *J. Org. Chem.* **2000**, *65*, 6187. (c) Reichwein, J. F.; Wels, B.; Kruijtzer, J. A. W.; Versluis, C.; Liskamp, R. M. *Angew. Chem., Int. Ed. Engl.* **1999**, *38*, 3684. (d) Piscopio, A. D.; Miller, J. F.; Koch, K.; *Tetrahedron* **1999**, *55*, 8189. (e) Furstner, A. *Angew. Chem., Int. Ed. Engl.* **2000**, *39*, 3013.

(7) Blackwell, H. E.; Grubbs, R. H. *Angew. Chem., Intl. Ed. Engl.* **1998**, *37*, 3281.

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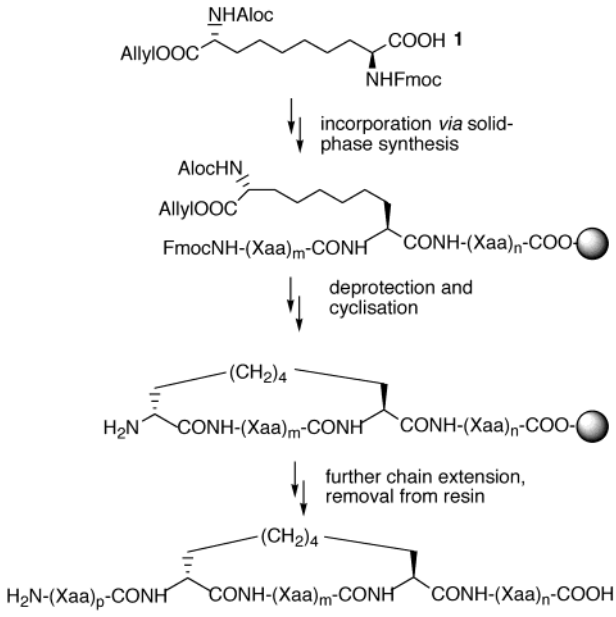
Scheme 1



bridge are present. We envisaged that this could be used to form a cyclic peptide, via solid-phase peptide synthesis, utilizing a triply orthogonal protecting group strategy (Scheme 1). At one end, the amino acid is N^{α}-Fmoc protected, allowing the incorporation of the bis-amino acid into a linear peptide using standard Fmoc-based methodology. At the other end, the amino acid is protected as the N^{α}-Aloc and allyl ester derivatives, enabling these moieties to be selectively deprotected. This is then followed by on-resin cyclization, forming the desired bridged peptide with a free N-terminal amino group for further chain extension.

In this paper, we first describe the synthesis of bis-amino acid **1**. We were interested in exploring the use of such aliphatic linkages as tools for stabilizing α-helical structures and, therefore, incorporated the bis-amino acid **1** into a model peptide, using solid-phase methods, to form a bridge between the *i* and *i* + 4 positions. The design and synthesis of this peptide, and conformational studies by CD, are also presented herein.

Results and Discussion

Synthesis of Bis-amino Acid 1. The synthesis of this differentially protected bis-amino acid requires that two amino acid groups of opposing chirality must be installed. As these chiral centers are remote from each other, this necessitates the use of two separate enantiospecific methodologies. It is then necessary to unmask and protect each functional group selectively and sequentially, in order that like groups can be differentiated. We have previously reported[8a] the synthesis of **1** via the route outlined in Scheme 2. The (*R*)-chiral center was installed via the coupling of the Schöllkopf[10] bis-lactim ether **2**[11] (as the higher order bislactim ether lithium cyanocuprate[12]) with *tert*-butyldimethylsilyl 8-iodooctanoate **3**, to give **4**. The (*S*)-chiral center was then installed using the Evans enolate azidation methodology,[13] giving **6**, in which both amino acid functionalities have been formed in an enantiospecific manner and are present in masked form. The chiral centers were sequentially unmasked and protected in the following manner. The Evans oxazolidinone was first removed using lithium hydroperoxide to give the (*S*)-azido acid **7**, followed by acidic cleavage[14] of the Schöllkopf bislactim ether and reaction with allyl chloroformate to give **8**. Transesterification[15] of ethyl ester **8** to allyl ester **9** and Staudinger[16] reduction of the azide, followed by immediate protection with Fmoc-Cl, gave the desired differentially protected bis-amino acid **1**. However, the yields of the final reduction step were unacceptably low, and were capricious upon scale-up. In addition, removal of the triphenylphosphine oxide byproduct from the desired amino acid proved problematic. Other methods which have been reported to selectively reduce azides in the presence of C=C groups, including polymer bound triphenylphosphine,[17] hydrogenation over Lindlar catalyst,[18] sodium hydrogen telluride[19] and propanedithiol-triethylamine[20] were explored in an attempt to improve the yield and reproducibility of this step; however, these attempts were all unsuccessful.

We therefore investigated alternative routes to the desired amino acid. Staudinger reduction of the key intermediate **6**, followed by Fmoc protection, afforded **10**; however, subsequent attempts to remove the Evans' oxazolidinone were unsuccessful (Scheme 3). Instead, the oxazolidinone auxiliary was first removed from **6** to give the azido acid **7**. The azido group was successfully reduced using a modified Staudinger procedure in which the intermediate iminophosphorane was first formed under anhydrous conditions and then hydrolyzed over 48 h. The resulting amine was immediately protected to give

(8) (a) Andrews, M. J. I.; Tabor, A. B. *Tetrahedron Lett.* **1997**, *38*, 3063. (b) Alexander McNamara, L. M.; Andrews, M. J. I.; Mitzel, F.; Siligardi, G.; Tabor, A. B. *Tetrahedron Lett.* **2001**, *42*, 1591.

(9) Other strategies for the synthesis of α,ω-diaminodicarboxylic acids have been reported: 2,5-diaminoadipic acids: (a) Hiebl, J.; Blanka, M.; Guttman, A.; Kollman, H.; Leitner, K.; Mayrhofer, G.; Rovenszky, F.; Winkler, K. *Tetrahedron* **1998**, *54*, 2059. 2,6-diaminopimelic acids: (b) Gao, Y.; Lane-Bell, P.; Vederas, J. C. *J. Org. Chem.* **1998**, *63*, 2133. (c) Jurgens, A. R. *Tetrahedron Lett.* **1992**, *33*, 4727. (d) Williams, R. M.; Yuan, C. *J. Org. Chem.* **1992**, *57*, 6519. (e) Davis, F. A.; Srirajan, V. *J. Org. Chem.* **2000**, *65*, 3248. 2,7-diaminosuberic acids: (f) Williams, R. M.; Liu, J. *J. Org. Chem.* **1998**, *63*, 2130. (g) Kremminger, P.; Undheim, K. *Tetrahedron* **1997**, *53*, 6925. (h) Nutt, R. F.; Strachan, R. G.; Veber, D. F.; Holly, F. W. *J. Org. Chem.* **1980**, *45*, 3078. (i) O'Leary, D. J.; Miller, S. J.; Grubbs, R. H. *Tetrahedron Lett.* **1998**, *39*, 1689. (j) Lange, M.; Fischer, P. M. *Helv. Chim. Acta* **1998**, *81*, 2053. 2,8-diaminoazelaic acid: (k) Hiebl, J.; Kollman, H.; Rovenszky, F.; Winkler, K. *J. Org. Chem.* **1999**, *64*, 1947.

(10) (a) Schöllkopf, U.; Groth, U.; Deng, C. *Angew. Chem., Int. Ed. Engl.* **1981**, *20*, 798. (b) Schöllkopf, U. *Top. Curr. Chem.*, **1983**, *109*, 65. (c) Schöllkopf, U.; Busse, U.; Lonsky, R.; Hinrichs, R. *Liebigs Ann. Chem.* **1986**, 2150.

(11) Bull, S. D.; Davies, S. G.; Moss, W. O. *Tetrahedron*: *Asymmetry* **1998**, *9*, 321. We have found that this procedure gives significantly higher yields and better purity than the originally reported procedure.

(12) Baldwin, J. E.; Adlington, R. M.; Mitchell, M. B. *Tetrahedron* **1995**, *51*, 5193.

(13) (a) Evans, D. A.; Britton, T. C. *J. Am. Chem. Soc.* **1987**, *109*, 6881. (b) Evans, D. A.; Britton, T. C.; Ellman, J. A.; Dorow, R. L. *J. Am. Chem. Soc.* **1990**, *112*, 4011. (c) Evans, D. A.; Bartroli, J.; Shih, T. L. *J. Am. Chem. Soc.* **1981**, *103*, 2127.

(14) Cleavage of the bislactim ether under the mild acidic conditions previously reported[10,11] resulted in a mixture of the desired **8** and a dipeptide byproduct arising from incomplete hydrolysis. It was therefore necessary to use a higher concentration of HCl in order to effect the desired transformation.

(15) (a) Seebach, D.; Thaler, A.; Blaser, D. *Helv. Chim. Acta* **1989**, *72*, 857. (b) Seebach, D.; Thaler, A.; Blaser, D.; Ko, S. Y. *Helv. Chim. Acta* **1991**, *74*, 1102.

(16) (a) Staudinger, H.; Hauser, E. *Helv. Chim. Acta* **1921**, *4*, 21. (b) Heerding, D.; Bhatnager, P.; Hartmann, M.; Kremminger, P.; LoCastro, S. *Tetrahedron*: *Asymmetry* **1996**, *7*, 237.

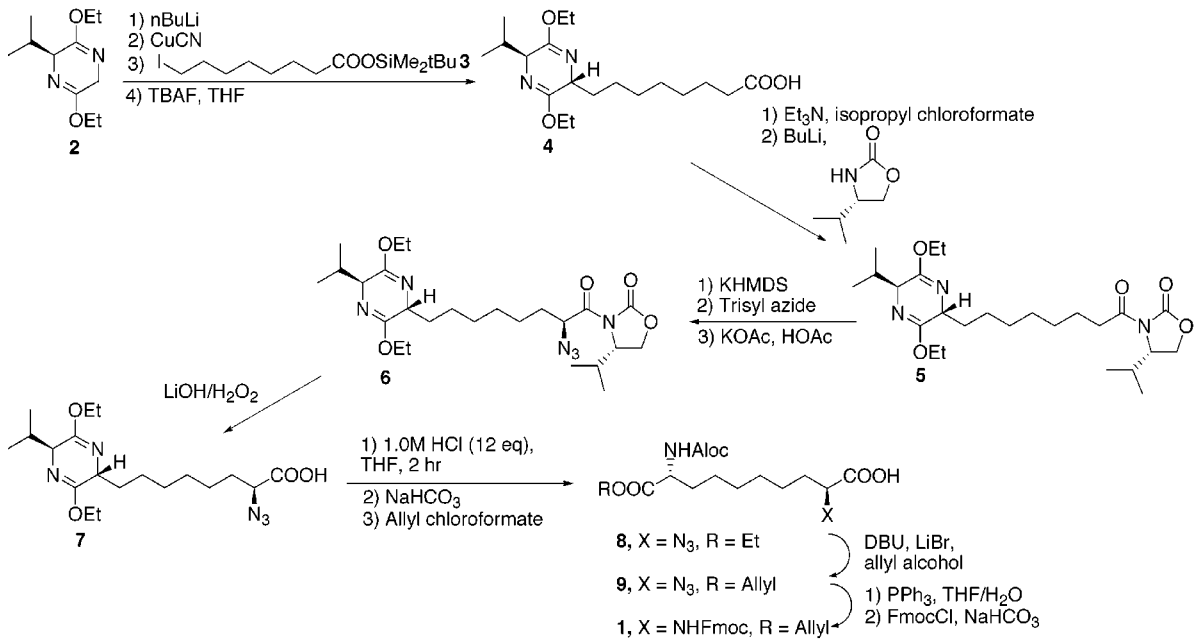
(17) Bernard, M.; Ford, W. T. *J. Org. Chem.* **1983**, *48*, 326.

(18) Nicolaou, K. C.; Corey, E. J.; Balanson, R. D.; Machida, Y. *Synthesis* **1975**, 590.

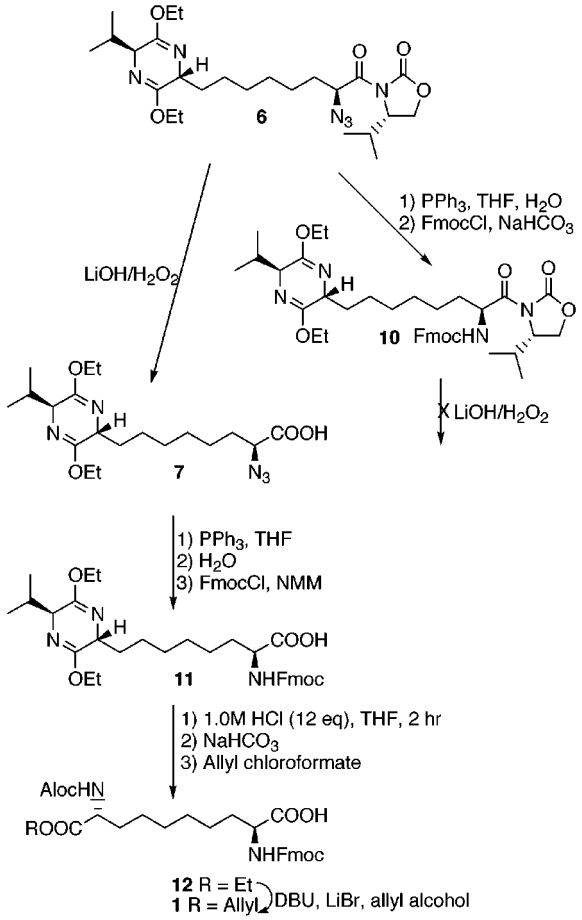
(19) Suzuki, H.; Takaoka, K. *Chem. Lett.* **1984**, 1733.

(20) Bayley, H.; Standring, D. N.; Knowles, J. R. *Tetrahedron Lett.* **1978**, 3633.

Scheme 2



Scheme 3



11 in high yield. Cleavage of the Schöllkopf bis-lactim ether was then carried out, using the conditions previously developed; this was followed by Aloc-protection of the free amino group to give **12**. Transesterification using allyl alcohol/DBU/LiBr proceeded as before, with no loss of the base-labile Fmoc group, to give **1** in high yield.

Design and Synthesis of a Model *i*, (*i* + 4)-Bridged Helical Peptide. The aim of this research was to explore the effects of incorporating an aliphatic bridge between two residues spaced *i*, (*i* + 4) apart on the α-helical conformation of a short peptide. Stabilization of α-helical peptides by formation of *i*, (*i* + 4) lactam bridges between the side chains of residues such as Glu and Lys has been extensively studied in both model systems and in analogues of peptide hormones.[3] The optimal length of lactam bridge required for greatest helix stabilization appears to be context-dependent. For example, in a series of model peptides, shorter lactam bridges formed between Lys and Asp residues (leading to a 20-membered ring) were found to give optimal helix stabilization,[3a,b] whereas in studies of analogues of human growth hormone releasing factor (hGRF), lactam bridges with 21 or 22-membered rings were found to be most stable.[3c] The majority of these studies have been carried out with L-amino acids in both positions, although there is one report of a helical heptapeptide with an *i*, (i + 7) lactam bridge being formed between D-Glu and L-Lys;[3d] in theory, placing a L-amino acid at the *i* position should orient that side chain favorably for lactam formation, allowing a shorter bridge to be used. We designed the aliphatic link between the *i* and (*i* + 4) positions with the aim of producing as short a bridge as possible (forming a 19-membered ring) and, therefore, elected to synthesize a bridge with a L-chiral center at the *i* position. Preliminary modeling studies indicated this approach to be feasible.

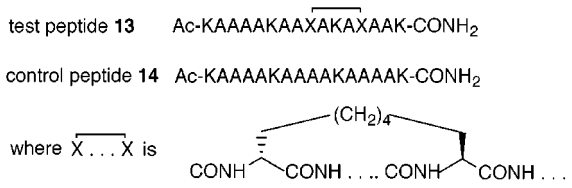
Systematic studies of the factors governing helix stabilization in peptides[21] have been facilitated in recent years by the discoveries of the Baldwin[22,23] and Kallen-

(21) (a) Scholtz, J. M.; Baldwin, R. L. α-Helix Formation by Peptides in Water. In *Peptides: Synthesis, Structures and Applications*; Academic Press: New York, 1995; 171. (b) Chakrabartty, A.; Baldwin, R. L. *Adv. Prot. Chem.* **1995**, *46*, 141. (c) Parthasarthy, R.; Chaturvedi, S.; Go, K. *Prog. Biophys. Mol. Biol.* **1995**, *64*, 1.

(22) (a) Marqusee, S.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5286. (b) Padmanabhan, S.; Marqusee, S.; Ridgeway, T.; Laue, T. M.; Baldwin, R. L. *Nature* **1990**, *344*, 268.

(23) (a) Armstrong, K. M.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11337. (b) Scholtz, J. M.; Qian, H.; Robbins, V. H.; Baldwin, R. L. *Biochemistry* **1993**, *32*, 9668.

Scheme 4



bach[24] groups. These groups have devised a series of short peptides (17−20 residues in length) that have significant helicity in aqueous solution, arising either from the apparent high helix propensity of Ala[22] or from the formation of multiple salt bridges.[23,24] These peptides may be used as models to test the helix-forming propensity of other amino acids: substituting one or more amino acids into these model peptides has a significant effect on the helical content of the peptide, which can be measured using CD.

To test the α-helix stabilization properties of our aliphatic linker, as well as to demonstrate the feasibility of our triply orthogonal protecting group approach to side-chain-bridged peptides, we synthesized an analogue of the Marqusee−Baldwin AK peptide,[22] test peptide **13**, in which amino acid **1** was incorporated as a bridge between positions 9 and 13 (Scheme 4).

The peptide synthesis (Scheme 5) was largely carried out on an automated peptide synthesizer as follows. Linear peptide **15** was synthesized on a PEG-PS Sieber amide resin[25] using standard conditions (HOAt/DIC).[26] The incorporation of bis-amino acid **1** at position 13 proceeded smoothly using HOAt/DIC, although limited solubility of the parent amino acid required that several coupling steps be carried out. The peptide−resin was then transferred to a Merrifield bubbler, and the allyl and Aloc groups were simultaneously removed with Pd-$(PPh_3)_4$ following the method of Kates et al.[27] The removal of the Aloc group from the amine was monitored by qualitative Kaiser test[28] and the completeness of the reaction verified by mass spectrometry. Subsequent removal of the Fmoc group with piperidine afforded peptide **16**; after this step, thorough washing with DMF was followed by an additional wash with 0.4% HOBt in DMF to prevent the undesired conversion of the free carboxy group to a piperidyl amide.[29]

The next stage of the synthesis required on-resin cyclization of peptide **16**, thus forming the aliphatic link between positions 9 and 13. Cyclization of peptides on the solid phase is now established as the optimal method for most peptides, as problems of dimerization and solubility of protected fragments are thereby avoided.[30] Many groups have also observed an acceleration of cyclization on-resin; this has been attributed to a "pseudodilution" of the resin-bound peptide.[31] Reagents such as PyAOP[32] and the so-called uronium reagents HBTU, TBTU,[33] and HATU[26] have been reported to give optimal cyclization yields with minimal racemisation.[34] However, guanylation of free amino groups has been reported as a competing reaction when cyclizing peptides on-resin.[35] As peptide **16** possesses two free amino groups, we elected to use PyAOP in the on-resin cyclization; this proceeded smoothly to give cyclic peptide **17**. The completion of the peptide cyclization was again verified by mass spectrometry; no evidence for dimerization, caused by cross-coupling between two adjacent peptides on the resin, was seen. The free *N*-terminal amino group of peptide **17** was immediately coupled with Fmoc-Ala-OH, and the peptide−resin was then transferred back to the peptide synthesizer. The synthesis was completed by automated coupling of the remaining eight amino acids under standard conditions. This was followed by acetylation and then cleavage from the resin with concomitant removal of the side-chain protecting groups under acidic conditions (TFA/TES/H_2O 86:10:4) to give the desired model peptide **13** in 23% yield after purification by HPLC. As a control for the CD experiments, control peptide **14** was also synthesized using standard conditions.

Circular Dichroism Studies. Control peptide **14**, which is based on the AK peptide, is known to be highly helical in solution.[22] This was confirmed by the CD spectra of the control peptide at 4 °C in H_2O, TFE, ethanediol−H_2O (2:1), and 20 mM SDS respectively (Figure 1), which were dominated by an α-helix conformation. The α-helix content, estimated from CD data, was higher in 20 mM SDS than ethanediol−H_2O (2:1), TFE, and H_2O, respectively (Table 1). As expected, the α-helix conformation increased on lowering the temperature; however, the effect was least pronounced in 20 mM SDS and less pronounced in ethanediol−H_2O (2:1) and TFE than in H_2O (Table 1). On further lowering the temperature to −100 °C, the helical content of control peptide **14** in ethanediol−H_2O (2:1) increased steadily, although the CD profiles also suggested the presence of a distorted α-helix conformation. The addition of 4 M urea caused a reduction of helical content at 25 °C from 37.3% in ethanediol−H_2O (2:1) to 9.1% (Table 1). Repeating the cryogenic study with 4 M urea showed the appearance of a PPII-like structure.[36] Initially, the helical

(24) (a) Lyu, P. C.; Liff, M. I.; Marky, L. A.; Kallenbach, N. R. *Science* **1990**, *250*, 669. (b) Yang, J.; Spek, E. J.; Gong, Y.; Zhou, H.; Kallenbach, N. R. *Protein Sci.* **1997**, *6*, 1264.

(25) (a) Sieber, P. *Tetrahedron Lett.* **1987**, *28*, 2107. (b) Chan, W. C.; White, P. D.; Beythien, J.; Steinauer, R. *J. Chem. Soc., Chem. Commun.* **1995**, 589. (c) Chan, W. C.; Mellor, S. L. *J. Chem. Soc., Chem. Commun.* **1995**, 1475.

(26) (a) Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397. (b) Carpino, L. A.; El-Faham, A.; Minor, C. A.; Albericio, F. *J. Chem. Soc., Chem. Commun.* **1994**, 201.

(27) (a) Kates, S. A.; Solé, N. A.; Johnson, C. R.; Hudson, D.; Barany, G.; Albericio, F. *Tetrahedron Lett.* **1993**, *34*, 1549. (b) Kates, S. A.; Daniels, S. B.; Albericio, F. *Anal. Biochem.* **1993**, *212*, 303.

(28) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. J. *Anal. Biochem.* **1970**, *84*, 595.

(29) McMurray, J. S.; Lewis, C. A.; Obeyesekere, N. U. *Pept. Res.* **1994**, *7*, 195.

(30) (a) Kates, S. A.; Solé, N. A.; Alberico, F.; Barany, G. Solid-Phase Synthesis of Cyclic Peptides. In *Peptides: Design, Synthesis and Biological Activity*; Basava, C., Anantharamaiah, G. M., Eds.; Birkhauser: Boston, 1994; p 39. (b) Blackburn, C.; Kates, S. A. Solid-Phase Synthesis of Cyclic Homodetic Peptides. In *Methods in Enzymology, Vol. 289: Solid-Phase Peptide Synthesis*; Fields, G. B., Ed.; Academic Press: New York, 1997; p 175. (c) Webster, K. L.; Rutherford, T. J.; Gani, D. *Tetrahedron Lett.* **1997**, *38*, 5716. (d) Taylor, C.; Quinn, R. J.; Alewood, P. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2107.

(31) Mazur, S.; Jayalekshmy, P. *J. Am. Chem. Soc.* **1978**, *101*, 677.

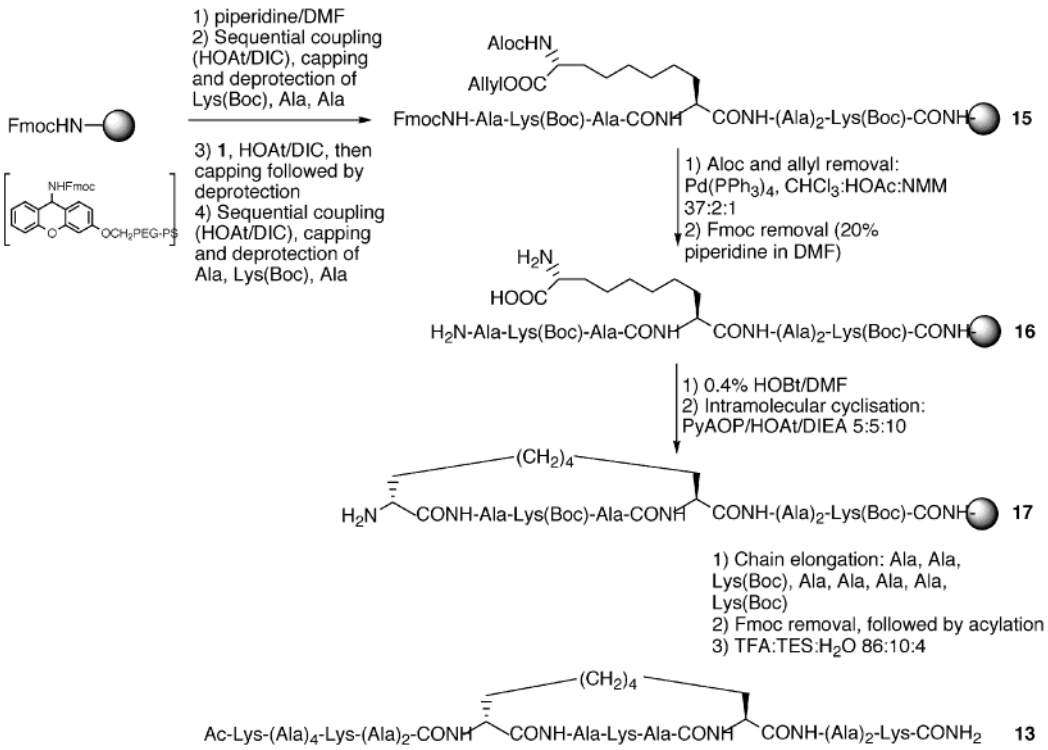
(32) Albericio, F.; Cases, M.; Alsina, J.; Triolo, S. A.; Carpino, L. A.; Kates, S. A. *Tetrahedron Lett.* **1997**, *38*, 4853.

(33) Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillessen, D. *Tetrahedron Lett.* **1989**, *30*, 1927.

(34) (a) Ehrlich, A.; Heyne, H.-U.; Winter, R.; Beyermann, M.; Haber, H.; Carpino, L. A.; Bienert, M. *J. Org. Chem.* **1996**, *61*, 8831. (b) Spatola, A. F.; Darlak, K.; Romanovskis, P. *Tetrahedron Lett.* **1996**, *37*, 591.

(35) (a) Story, S. C.; Aldrich, J. V. *Int. J. Pept. Protein Res.* **1994**, *43*, 292. (b) Delforge, D.; Dieu, M.; Delaive, E.; Art, M.; Gillon, B.; Devreese, B.; Raes, M.; van Beeumen, J.; Remacle, J. *Lett. Pept. Sci.* **1996**, *3*, 89.

Scheme 5



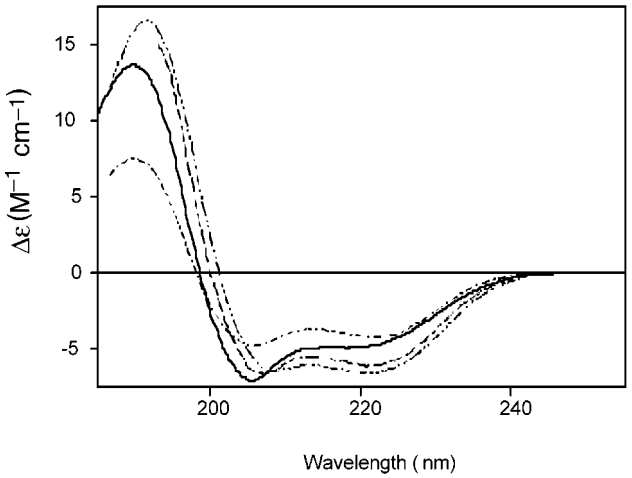


Figure 1. CD spectra of control peptide **14** at 4 °C in H_2O (−·−), TFE (—), ethanediol−H_2O (2:1) (---), and SDS 20mM (−··−).

content increased on lowering the temperature from +21 to −33 °C, then a positive band emerged at about 214 nm on further lowering the temperature to −55 and −75 °C (refer to the Supporting Information). On further lowering the temperature to as low as −115 °C, no further CD changes were observed. The presence of the small positive CD band at about 214 nm together with an apparent negative band at 225 nm are indicative of a conformational equilibrium with small PPII and residual helical contributions being the most thermodynamically stable conformations at low temperature.

Test peptide **13** showed CD spectra completely different from those observed with control peptide **14**. There was no evidence of an induced α-helical conformation in the constrained peptide. Instead, **13** showed a positive CD band at about 211 nm that increased in intensity on lowering the temperature in H_2O, ethanediol−H_2O (2:1), and 20 mM SDS, respectively (Figure 2). The highest CD intensity was observed in the cryogenic solvent mixture ethanediol−H_2O (2:1) at −92 °C; however, the conformation associated with this 211 nm CD band is more stable in 20 mM SDS. This arises because one-third of the ethanedio−-H_2O (2:1) conformational state is already present in 20 mM SDS at 4 °C. Assignment of secondary structure to the CD positive band at 211 nm is dependent on its wavelength maximum.[37] For peptides without aromatic amino acid residues, a positive CD band at about 217 nm has been assigned to a dominant left-handed extended PPII-type conformation. For example, in a study of polylysine·HBr in ethanediol−H_2O (2:1)[36] the 218 nm positive CD band was attributed to a dominant PPII conformation. This latter CD band increased in intensity in the presence of 4 M urea, but the wavelength maximum remained unchanged, revealing

Table 1. Contents of Secondary Structure for Control Peptide 14

solvent	α-helix (%)	*β*-sheet (%)	other (%)
ethanediol−H_2O (2:1) + 4 M urea at 21 °C	9.1	40.2	50.7
ethanediol−H_2O (2:1) + 4 M urea at −33 °C	28.9	27.6	43.5
H_2O at 25 °C	18.8	23.9	57.3
H_2O at 4 °C	37.2	19.0	43.8
TFE at 25 °C	33.2	37.8	29.0
TFE at 4 °C	39.0	35.6	25.4
ethanediol−H_2O (2:1) at 25 °C	37.7	37.0	25.3
ethanediol−H_2O (2:1) at 4 °C	48.3	32.0	19.7
SDS 20 mM at 25 °C	57.3	20.9	21.8
SDS 20 mM at 4 °C	61.3	18.7	20.0

(36) Drake, A. F.; Siligardi, G.; Gibbons, W. A. *Biophys. Chem.* **1988**, *31*, 143.

(37) Siligardi, G.; Drake, A. F. *Biopolymers* (*Peptide Sci.)* **1995**, *37*, 281.

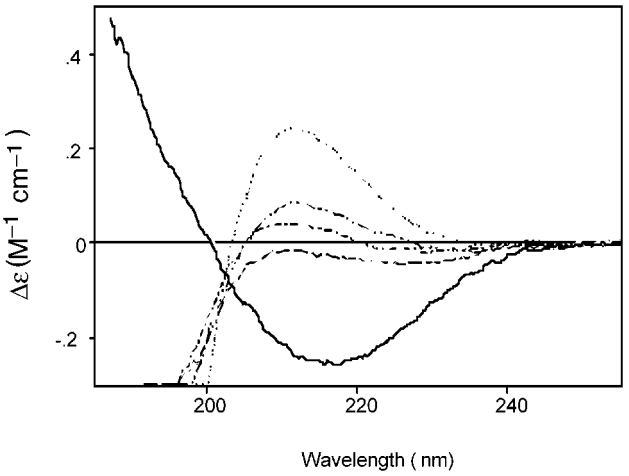


Figure 2. CD spectra of test peptide **13** at 4 °C in H_2O (−•−), ethanediol−H_2O (2:1) (− at 4 °C and • at −92 °C), 20 mM SDS (−••−) and 4 °C in TFE (—).

that the PPII conformation is unexpectedly stabilized by high concentrations of urea. A high concentration of urea is known to substantially disrupt intramolecular hydrogen bonded structures such as α-helix, *β*-sheet, and *β*-turns but it was not known to stabilize PPII. Conversely, in a previous study[38] of a tetradecapeptide in ethanediol−H_2O (2:1) a positive CD band at about 212 nm was attributed to a *β*-turn of type II. At low temperature, this CD band was red-shifted to 218 nm in the presence of 4 M urea, indicating a change in structure from the *β*-turn of type II to the PPII conformation, which was again stabilized by urea.

To discriminate between a *β*-turn of type II and a PPII conformation for test peptide **13**, a cryogenic study with and without 4 M urea was therefore carried out. At −92 °C in ethanediol−H_2O (2:1), the positive CD band at about 211 nm was not affected by 4 M urea (Figure 2). This confirms that **13** adopts a *β*-turn conformation and rules out the alternative PPII assignment. The fact that this band is positive and insensitive to 4 M urea also suggests that the *β*-turn is left-handed and "open", in other words, that it lacks an intramolecular hydrogen bond and therefore arises exclusively from the constraint imposed by the aliphatic bridge between positions 9 and 13.

The conformational properties of test peptide **13** were also studied in TFE. In this medium, a CD spectrum with a negative band at about 218 nm and a cross-over at about 200 nm was observed (Figure 2). Such a spectrum could be interpreted as arising from a *β*-sheet conformation. In general, an association/aggregation-type *β*-sheet conformation is time- and temperature-dependent, and the CD changes induced at high temperatures are nonreversible. In TFE, the CD of **13** was found to be time- and temperature-independent from 4 to 65 °C. This behavior, together with the small overall $\Delta\epsilon$ intensity, is better correlated with a *β*-turn conformation; because the 218 nm CD band is negative, this turn appears to be right-handed skewed.[39]

(38) Siligardi, G.; Drake, A. F.; Mascagni, P.; Neri, P.; Lozzi, L.; Niccolai, N.; Gibbons, W. A. *Biochem. Biophys. Res. Commun.* **1987**, *143*, 1005.

(39) Gierash, L. M.; Deber, C. M.; Madison, V.; Niu, C. H.; Blout, E. R. *Biochemistry* **1981**, *20*, 4730.

Conclusions

An enantioselective and high-yielding route to the differentially protected bis-amino acid **1** has been developed. Amino acid **1** was then incorporated into a short, alanine-rich peptide **13** via a novel solid-phase synthesis approach. This involved the use of a triply orthogonal protecting group strategy to couple first one then the other amino acid moiety in such a way that an aliphatic bridge was formed between the *i* and *i* + 4 positions. Unexpectedly, the presence of this bridge did not lead to an α-helical conformation for peptide **13**; instead, the peptide was constrained to adopt a *β*-turn conformation. At low temperatures, a left-handed *β*-turn of type-II conformation is seen in aqueous media, while a right-handed *β*-turn of type-I conformation is seen in TFE. At room temperature, an equilibrium exists between these two *β*-turn conformations.

In general, peptides and peptidomimetics may be constrained to adopt a stable *β*-turn conformation by the formation of linkages between the *i* and *i* + 3 positions,[40] whereas incorporation of linkages between the *i* and *i* + 4 positions leads to stable α-helical structures.[3] There are a very few examples of *i* and *i* + 4 lactam-bridged peptides in which turn structures appear to be preferentially stabilized;[41] of particular note is a recent study of human calcitonin analogues in which a type-I *β*-turn is observed by CD.[42] This may indicate that the stabilization of helical conformation by bridging two residues is more context-dependent than previously thought. However, it is more probable that the failure of the bridge described in this paper to stabilize a helical conformation may arise from the stereochemistry (*R*(*i*),*S*(*i* + 4)) of the two chiral centers and possibly also from the nature of the aliphatic bridge itself. A recent study by the Verdine group[43] in which a different series of aliphatic-bridged analogues of an α-helical peptide were formed by RCM supports this theory. In that work, attempts to form *R*(*i*),*S*(*i* + 4)-bridged peptides by RCM failed: as the parent peptide was already highly helical, this indicates that the *R*(*i*),*S*(*i* + 4) orientation is simply not suited to the stabilization of an α-helix. Furthermore, although *S*(*i*),*S*(*i* + 4) and *R*(*i*),*R*(*i*+4)-bridged peptides could be synthesized using RCM, unexpectedly neither stabilization nor destabilization of the helix was observed; instead, optimal helix stabilization was encountered with *i*, *i* + 7 linkages. Finally, it is also interesting to note that, although "open *β*-turns" do exist in nature,[44] the majority of turn structures are stabilized by an intramolecular hydrogen bond between the carbonyl group at the *i* position and the amide hydrogen of the *i* + 3 position.

Both the synthetic route to amino acid **1** and the solid-phase peptide synthesis strategy for incorporation of such

(40) (a) Rao, M. H.; Yang, W.; Joshua, H.; Becker, J. M.; Naider, F. *Int. J. Pept. Protein Res.* **1995**, *45*, 418. (b) Marepalli, H. R.; Antohi, O.; Becker, J. M.; Naider, F. *J. Am. Chem. Soc.* **1996**, *118*, 6531. (c) Ripka, A. S.; Bohacek, R. S.; Rich, D. H. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 357.

(41) (a) Rist, B.; Zerbe, O.; Ingenhoven, N.; Scapozza, L.; Peers, C.; Vaughan, P. F. T.; McDonald, R. L.; Wieland, H. A.; Beck-Sickinger, A. G. *FEBS Lett.* **1996**, *394*, 169. (b) Cavallaro, V.; Thompson, P.; Hearn, M. *J. Pept. Sci.* **1998**, *4*, 335.

(42) (a) Kapurniotu, A.; Taylor, J. W. *J. Med. Chem.* **1995**, *38*, 836. (b) Kapurniotu, A.; Kayed, R.; Taylor, J. W.; Voelter, W. *Eur. J. Biochem.* **1999**, *265*, 606.

(43) Schafmeister, C. E.; Po, J.; Verdine, G. L. *J. Am. Chem. Soc.* **2000**, *122*, 5891.

(44) (a) Crawford, J. L.; Lipscomb, W. N.; Schellman, C. G. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 538. (b) Smith, J. A.; Pease, L. G. *CRC Crit. Rev. Biochem.* **1980**, *8*, 314.

bis-amino acids to form cyclic peptides bearing an aliphatic bridge between two C^{α} sites may be generalized. The methodology described may, therefore, be used to construct peptides with a wide variety of unnatural side-chain linkages between a range of positions, opening up exciting possibilities for the design and synthesis of a range of conformationally constrained peptidomimetics.

Experimental Section

General Methods. Unless otherwise noted, solvents and reagents were reagent grade and used without purification. THF was dried by distillation from a sodium/benzophenone suspension under a dry N_2 atmosphere. CH_2Cl_2 was dried by distillation from CaH_2 or P_2O_5 suspension. Et_3N, piperidine, dioxane, acetic anhydride, and $^iPr_2EtN$ were all distilled from calcium hydride under an inert atmosphere. Allyl alcohol was distilled from Mg turnings activated by iodine. LiBr was dried at 140 °C/0.1 mmHg for 14 h immediately prior to use. Melting points are uncorrected. Infrared spectra were recorded in solutions of $CHCl_3$ unless otherwise stated. ^{1}H NMR spectra were obtained in $CDCl_3$. The chemical shift data for each signal is given in parts per million (ppm: δ) relative to TMS. Mass spectra were recorded using FAB, atmospheric pressure chemical ionization positive (APCI+), or electrospray positive (ES+) ionization. Thin-layer chromatography was carried out on precoated 0.25 mm Merck 60 F_{254} plates, visualizing with potassium permanganate, basic bromocresol green, ninhydrin, or UV absorption, as appropriate. Flash column chromatography was performed using silica 60 (BDH).

Amino acids, coupling reagents, and resins were obtained from Novabiochem and Perseptive Biosystems. Analytical-grade DMF was obtained from BDH. Test peptide **13** was synthesized on a Milligen 9050 Plus PepSynthesizer and control peptide **14** on an ABI 430A automated synthesizer, using the Fmoc strategy.

Analytical reversed-phase HPLC was carried out using a Waters 600E quaternary gradient system pump, Rheodyne 7125 valve injector, Gilson 115 variable-wavelength UV detector, and Hewlett-Packard HP3396 A integrator with Vydac column, 4.6 mm × 25 cm. Chiral normal-phase HPLC was carried out using the same system, with a CHIRALCEL OD chiral column (Daicel Chemical Industries, Ltd.), 250 × 4.6 mm. Preparative reversed-phase HPLC was carried out using a Waters 600E quaternary gradient system pump, Rheodyne 7125 valve injector, Gilson Holochrome variable-wavelength UV detector, and Waters 745B Data Module with Vydac column, 21.4 mm × 25 cm. Liquid chromatography−mass spectrometry (LC−MS) was carried using a Hewlett-Packard HP1100 for HPLC and Micromass Quattro LC for mass spectrometry using the Vydac analytical HPLC column. Retention times are given in minutes.

CD spectra were recorded with JASCO J720 and J600 spectropolarimeters flushed with nitrogen to improve performance below 200 nm. Solution concentrations of 0.300 mg/mL were prepared by weighing using a microbalance Mettler Toledo MT5 (sensitivity ± 1 μg), and a 0.05 cm cell path length was used in the far-UV CD region (185−250 nm). All spectra are reported in terms of $\Delta\epsilon = \epsilon_L - \epsilon_R$ (M^{-1} cm^{-1}) using a mean molecular weight (MW = 113) per amino acid residue. Low-temperature measurements were carried out using a JOUAN attachment. The contents of secondary structure were estimated using a principle component regression analysis (P1 plus of Grams/32 suite program, Galactic Industries Corporation) with a calibration data set of 16 proteins.[45]

***tert*-Butyldimethylsilyl 8-Iodooctanoate (3).** To a solution of sodium iodide (30.0 g, 0.200 mol, 3 equiv) in Analar acetone (120 mL) was added a solution of 8-bromooctanoic acid (14.8 g, 0.066 mol) in Analar acetone (90 mL) in one portion. The reaction mixture was stirred for 2 h under N_2 at room temperature, after which time the reaction solution was poured into water (120 mL) and the product extracted with Et_2O (3 × 200 mL). The organic layer was then washed with saturated sodium thiosulfate solution (30 mL) and dried over Na_2SO_4 and the solvent removed in vacuo, giving 8-iodooctanoic acid as a white solid (17.2 g, 63.7 mmol, 97%). Purification by column chromatography (50% Et_2O/hexane and 0.1% formic acid, R_f 0.33) gave a white solid: mp 37−38 °C; ^{1}H NMR ($CDCl_3$ 400 MHz) δ 1.33−1.40 (6H, br m), 1.57−1.64 (2H, br m), 1.76−1.84 (2H, br m) 2.33 (2H, t, *J* 7.4 Hz), 3.16 (2H, t, *J* 7.0 Hz); ^{13}C NMR ($CDCl_3$ 75 MHz) δ 7.6, 24.9, 28.6, 29.2, 30.7, 33.8, 34.4, 180.5; IR ν_{max} ($CHCl_3$) 3013 (weak), 2934, 2860, 1708, 1458 cm^{-1}; HRMS (FAB) calcd for $C_8H_{15}O_2I$ (M + H^+) 271.0195, found 271.0208.

To a cooled (0 °C) solution of 8-iodooctanoic acid (3.50 g, 13.0 mmol) in THF (30 mL) under N_2 was added Et_3N (2.17 mL, 15.6 mmol, 1.2 equiv) in one portion. After 5 min, TBDMS-Cl (1.95 g, 13.0 mmol, 1.0 equiv) in THF (5 mL) was added dropwise over 5 min, giving a white precipitate. The reaction mixture was then allowed to stir for 1 h at 0 °C, after which time filtration and removal of the solvents in vacuo gave a clear pale yellow oil. This was purified by Kugelrohr distillation (ot 160 °C/1 mmHg) to give *tert*-butyldimethylsilyl 8-iodooctanoate (**3**) (4.83 g, 12.6 mmol, 97%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.24 (6H, s), 0.91 (9H, s), 1.28−1.40 (6H, br m), 1.55−1.62 (2H, br m), 1.75−1.83 (2H, m), 2.27 (2H, t, *J* 7.4 Hz), 3.15 (2H, t, *J* 7.0 Hz); ^{13}C NMR ($CDCl_3$,100 MHz) δ 7.1, 17.6, 24.9, 25.5, 25.6, 28.2, 28.9, 30.3, 35.9, 68.0,174.0; mass spectrum *m*/*z* 327 (M − 57, M − *tert*-butyl group, 77) 385 (M + 1, 75) 327 (M − 57, M − *tert*-butyl group, 77) 257 (M − I, 40); HRMS (FAB) calcd for $C_{14}H_{29}O_2SiI$ (M + H^+) 385.1060, found 385.1068.

8-(*R*)-(5(*S*)-Isopropyl-3,6-diethoxy-2,5-dihydropyrazin-2-yl)octanoic Acid (4). To a cooled (−78 °C) solution of 6(*S*)-isopropyl-2,5-diethoxy-3,6-dihydropyrazine (**2**)[11] (2.50 g, 11.8 mmol) in THF (20 mL) under Ar was added *n*-BuLi (1.67 M, 7.05 mL, 1 equiv) dropwise over 10 min. Stirring was continued for a further 20 min. The reaction mixture was then added via Teflon cannula to a cooled (0 °C) suspension of CuCN (0.527 g, 5.89 mmol, 0.5 equiv) in THF (20 mL) over 30 min, ensuring that all the CuCN dissolved over this period. The reaction mixture was then recooled to −78 °C, and to this was added via Teflon cannula a cooled (−78 °C) solution of *tert*-butyldimethylsilyl 8-iodooctanoate (**3**) (3.39 g, 8.83 mmol, 1.5 equiv with respect to CuCN) in THF (20 mL). The reaction mixture was then warmed to −23 °C and stirring continued at this temperature for a further 5 h, after which time the reaction was stored at −23 °C for 18 h. The reaction was then quenched with concentrated NH_3/saturated NH_4Cl solution (1:9, 30 mL), and this was poured into Et_2O (750 mL). The organic layer was separated and washed with concentrated NH_3/saturated NH_4Cl solution (1:9, 3 × 300 mL) and dried (Na_2SO_4). The solvents were removed in vacuo giving a crude yellow oil (1.44 g) that was passed immediately into the next reaction.

To the crude 8-(*R*)-(4(*S*)-isopropyl-3,6-diethoxy-2,5-dihydropyrazin-2-yl)-*tert*-butyldimethylsilyl octanoate (assumed to be 5.89 mmol based on CuCN) under Ar was added tetra-*n*-butylammonium fluoride (TBAF) in THF (1.0M, 17.7 mL, 2 equiv with respect to **3**). The reaction was stirred at room temperature for 75 min and then quenched with saturated NH_4Cl solution (35 mL). The organic layer was extracted with Et_2O (2 × 125 mL), and the combined organic layers were dried (Na_2SO_4). The solvents were removed in vacuo, giving the crude product as a white solid (4.77 g) that was purified by column chromatography (50% Et_2O/hexane and 0.1% formic acid, R_f 0.40) (2.06 g, 5.81 mmol, 98% based on CuCN). The chiral purity of **4** was checked by chiral HPLC: the retention time (isocratic, 2% *i*-PrOH/hexane) was 3.94 min, and no trace of the other diastereoisomer was detected: $[\alpha]^{14}_D$ −3.7 (*c* 1.29, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.67 (3H, d, *J* 6.8 Hz), 1.00 (3H, d, *J* 6.9 Hz), 1.23 (6H, t, *J* 7.1 Hz), 1.25−1.35 (8H, br m), 1.55−1.62 (2H, br m), 1.65−1.75 (2H, br m), 2.20−2.25 (1H, m), 2.30 (2H, t, *J* 7.6 Hz), 3.87 (1H, t, *J* 3.4 Hz), 3.95 (1H, dd, *J* 3.4, 10.2 Hz), 4.0−4.2 (4H, complex m); ^{13}C NMR ($CDCl_3$, 100 Hz) δ 14.76, 14.81, 17.1, 19.5, 24.7, 25.5, 26.2, 29.0, 29.4, 32.2, 34.3, 34.7, 55.7, 60.99, 61.03, 64.7, 163.9, 174.4,

(45) Hennessey, J. P.; Johnson, W., C. Jr. *Biochemistry* **1981**, *20*, 1085.

178.3; IR ν_{max} ($CHCl_3$) 2931, 1689 cm^{-1}; mass spectrum *m*/*z* 355 (M + 1, 55); HRMS (FAB) calcd for $C_{19}H_{34}N_2O_4$ (M + H^+) 355.2597, found 355.2589. Anal. Calcd for $C_{19}H_{33}N_2O_4$: C, 64.38; H, 9.68; N, 7.90. Found: C, 64.07; H, 9.71; N, 7.93.

3-[8(*R*)-(5(*S*)-Isopropyl-3,6-diethoxy-2,5-dihydropyrazin-2-yl)octanoyl]-4-(*S*)-isopropyloxazolidin-2-one (5). To a solution of 8(*R*)-(5(*S*)-isopropyl-3,6-diethoxy-2,5-dihydropyrazin-2-yl)octanoic acid (**4**) (0.560 g, 1.58 mmol) in THF (30 mL) was added Et_3N (0.33 mL, 2.37 mmol, 1.5 equiv) at room temperature. After being stirred for 5 min, the reaction mixture was cooled to −78 °C. Isopropyl chloroformate in toluene (1.0 M, 1.90 mL, 1.90 mmol, 1.2 equiv) was added dropwise to the reaction. Stirring was then continued for a further 30 min at −78 °C, then the reaction was warmed to room temperature for 90 min before recooling to −78 °C. A precooled (−78 °C) solution of 4-(*S*)-isopropyloxazolidin-2-one[13c] (0.224 g, 1.74 mmol, 1.1 equiv) in THF (10 mL), pretreated with *n*-BuLi (1.4 M, 1.24 mL, 1.78 mmol, 1.1 equiv), was then added via a Teflon cannula to the reaction mixture. The resulting mixture was stirred at −78 °C for a further 30 min and was then quenched by addition to $NaHCO_3$ (1% aqueous solution, 66 mL). The THF was then removed in vacuo and the product extracted with EtOAc (4 × 80 mL). The combined organic layers were dried (Na_2SO_4) to give a crude yellow oil. This was purified by column chromatography (40% EtOAc/hexane, R_f 0.63) to give **5** (0.678 g, 1.46 mmol, 92%) as a yellow oil: $[\alpha]^{16}_D$ +27.6 (*c* 1.09, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.67 (3H, d, *J* 6.8 Hz), 0.84 (3H, d, *J* 7.0 Hz), 0.88 (3H, d, *J* 7.0 Hz), 1.0 (3H, d, *J* 7.0 Hz), 1.23 (6H, t, *J* 7.1 Hz) 1.24−1.35 (8H, br m), 1.60−1.70 (4H, br m), 2.20−2.30 (1H, m), 2.34−2.40 (1H, m), 2.75−2.85 (1H, m), 2.90−3.00 (1H, m), 3.86 (1H, t, *J* 3.4 Hz), 3.95 (1H, dd, *J* 3.4, 10.2 Hz), 4.0−4.3 (6H, complex m), 4.4 (1H, dt, *J* 3.8, 7.1 Hz); ^{13}C NMR ($CDCl_3$, 100 Hz) δ 14.77, 14.81, 15.1, 17.0, 18.4, 19.5, 24.7, 25.4, 26.2, 29.00, 29.01, 29.4, 29.6, 32.1, 34.6, 35.9, 55.9, 58.8, 60.82, 60.88, 63.7, 154.5, 163.3, 163.9, 173.8; IR ν_{max} 2934, 1778, 1692, 1463, 1232 cm^{-1}; mass spectrum *m*/*z*: 466 (M + 1, 100); HRMS (FAB) calcd for $C_{25}H_{43}N_3O_5$ (M + H^+) 466.3281, found 466.3274. Anal. Calcd for $C_{25}H_{42}N_3O_5$: C, 64.48; H, 9.31; N, 9.02. Found: C, 64.33; H, 9.43; N, 8.40.

3-[8(*R*)-(5(*S*)-Isopropyl-3,6-diethoxy-2,5-dihydropyrazin-2-yl)-2(*S*)-azidooctanoyl]-4(*S*)-isopropyloxazolidin-2-one (6). To a cooled (−78 °C) solution of KHMDS (0.5M in toluene, 6.7 mL, 3.33 mmol, 1.1 equiv) in THF (3 mL) was added via Teflon cannula a precooled (−78 °C) solution of 3-[8(*R*)-(5(*S*)-isopropyl-3,6-diethoxy-2,5-dihydropyrazin-2-yl)-octanoyl]-4-(*S*)-isopropyloxazolidin-2-one (**5**) (1.41 g, 3.03 mmol) in THF (3 mL). The resulting solution was stirred for a further 30 min at −78 °C. To this reaction was added via Teflon cannula a precooled (−78 °C) solution of trisyl azide[46] (1.12 g, 3.64 mmol, 1.2 equiv) in THF (3 mL). The reaction was allowed to proceed for 2 min before being quenched by saturated KOAc/glacial acetic acid (1.04 g, 10.6 mmol KOAc/0.170 mL, 3.03 mmol glacial acetic acid 3.5:1.0 equiv with respect to **5**, in 0.06 mL water). The reaction mixture was then stirred for 12 h at room temperature. Removal of solvents in vacuo gave a crude product that was purified by flash chromatography (30% EtOAc/hexane, R_f 0.43). This yielded **6** as a thick yellow oil (1.29 g, 2.55 mmol, 84%): $[\alpha]^{17}_D$ +60.1 (*c* 1.08, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.69 (3H, d, *J* 6.9 Hz), 1.02 (3H, d, *J* 6.9 Hz), 0.88 (3H, d, *J* 7.0 Hz), 0.90 (3H, d, *J* 7.0 Hz), 1.22 (6H, t, *J* 6.8 Hz), 1.24−1.40 (8H, br m), 1.40−1.80 (4H, br m), 2.16−2.20 (1H, m), 2.24−2.40 (1H, m), 3.88 (1H, t, *J* 3.5 Hz), 3.95 (1H, dd, *J* 3.4, 10.2 Hz), 4.00−4.15 (6H, complex m), 4.3 (1H, dt, *J* 3.8, 7.1 Hz), 4.90 (1H, dd, *J* 4.4, 9.1 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.6, 17.1, 18.1, 19.5, 21.5, 24.78, 24.79, 24.8, 25.17 (twice), 28.6, 31.4, 31.5, 32.2 (twice), 34.8, 55.8, 59.2, 61.1, 64.7, 124.1, 149.6, 153.9, 163.8, 171.5; IR ν_{max} 2957, 2932, 2109 (strong), 1781 (strong), 1691 (strong), 1465, 1228 cm^{-1}; mass spectrum *m*/*z* 464 (M − 42, M − N_3, 55), 507 (M + 1, 45); HRMS (FAB) calcd for $C_{25}H_{42}N_6O_5$ (M + H^+) 507.3295, found 507.3312.

8(*R*)-(4(*S*)-Isopropyl-3,6-diethoxy-2,5-dihydropyrazin-2-yl)-2(*S*)-azidooctanoic acid (7). A cooled solution (0 °C) of 3-[8(*R*)-(5(*S*)-isopropyl-3,6-diethoxy-2,5-dihydropyrazin-2-yl)-2-(*S*)-azidooctanoyl]-4-(*S*)-isopropyloxazolidin-2-one (**6**) (0.108 g, 0.213 mmol) in THF (3.20 mL) was treated slowly with a precooled (0 °C) solution of hydrogen peroxide (30% w/v, 100 volumes) (0.104 mL, 0.917 mmol, 4.3 equiv) and lithium hydroxide (17.9 mg, 0.426 mmol, 2 equiv) in 1.06 mL of water (final ratio 3:1 THF/water). The reaction was stirred for 1 h, monitoring by TLC (40% EtOAc/hexane and 0.1% formic acid, R_f 0.29, or 100% EtOAc, R_f 0.65). Saturated aqueous NH_4Cl solution (4 mL) was added to the reaction mixture, resulting in a pH 8 solution. This was further acidified to pH 4 with 2 M HCl. The mixture was extracted with CH_2Cl_2 (3 × 100 mL), and the combined organic layers were dried over sodium sulfate and concentrated in vacuo at room temperature. A crude clear oil (0.135 g) was obtained that was purified by flash chromatography (40% EtOAc/hexane, R_f 0.29) giving **7** as a pale yellow solid (60 mg, 0.152 mmol, 71% yield): mp 78 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.83 (3H, d, *J* 6.7 Hz), 1.15 (3H, d, *J* 6.9 Hz), 1.39−1.42 (14H, br m), 1.80−2.10 (4H, br m), 2.36−2.41 (1H, br m), 3.98−4.02 (1H, m), 4.05−4.10 (1H, m), 4.21−4.33 (5H, complex m); ^{13}C NMR ($CDCl_3$, 100 Hz) δ 14.6, 14.75, 14.79, 17.0, 19.5, 25.0, 26.8, 29.8, 32.1, 34.6, 55.8, 60.8, 60.90, 60.91, 61.0, 61.7, 163.4, 163.8, 177.5; IR ν_{max} 2968, 2931, 2109, 1687, 1457, 1243, 1213; mass spectrum *m*/*z* 396 (M + 1, 100) (ES+).

8(*R*)-(5,(*S*)-Isopropyl-3,6-diethoxy-2,5-dihydropyrazin-2-yl)-2(*S*)-(9-fluorenylmethyloxycarbonyl)amidooctanoic Acid (11). A solution of 8(*R*)-(4(*S*)-isopropyl-3,6-diethoxy-2,5-dihydropyrazin-2-yl)-2(*S*)-azidooctanoic acid (**7**) (51 mg, 0.129 mmol) in THF (0.258 mL) was treated with Ph_3P (51 mg, 0.194 mmol, 1.5 equiv). The reaction mixture was stirred for 24 h at room temperature. Water (7 μL, 0.387 mmol, 3 equiv) was then added and the reaction stirred for a further 48 h. The reaction was concentrated in vacuo to give the intermediate amine (*m*/*z* 370 (M + 1, 88)) as a viscous yellow oil. This was taken up in THF (0.129 mL) and treated with NMM (0.03 mL, 0.271 mmol, 2.1 equiv). The reaction mixture was cooled to 0 °C followed by slow addition of 9-fluorenylmethylchloroformate (50 mg, 0.194 mmol, 1.5 equiv) in THF (0.080 mL). After a further 4 h at room temperature, concentration in vacuo yielded a crude viscous oil that was purified by flash chromatography (50% EtOAc/hexane, R_f 0.11) giving **11** as a pale yellow solid (76 mg, 0.128 mmol, 99% yield): mp 55 °C; ^{1}H NMR ($CDCl_3$, 500 MHz, 323K) δ 0.69 (3H, d, *J* 6.9 Hz), 1.00 (3H, d, *J* 7.0 Hz), 1.19−1.25 (14H, br m), 1.56−1.80 (4H, br m), 2.23−2.25 (1H, br m), 3.72 (1H, t, *J* 3.4 Hz), 3.82−3.87 (1H, m) 4.03−4.16 (7H, complex m), 4.25−4.40 (1H, br m), 7.44 (2H, dt, *J* 2.8, 7.5 Hz), 7.50−7.53 (2H, m), 7.64−7.72 (4H, m); ^{13}C NMR ($CDCl_3$, 75 MHz, 273K) δ 14.8, 17.0, 19.5, 22.8, 25.3, 26.2, 29.0, 29.7, 29.9, 30.1, 32.1, 34.7, 55.8, 60.8, 120.2, 127.4, 128.0, 132.5, 141.6, 144.2, 163.9; IR ν_{max} 2933, 2860, 1691, 1444, 1218; mass spectrum *m*/*z* 592 (M + 1, 100) (ES+); HRMS (FAB) calcd for $C_{34}H_{45}N_3O_6Na$ (M + Na^+) 614.3206, found 614.3228.

(10-Ethyl ester)-9(*R*)-allyloxycarbonylamido-2(*S*)-(9-fluorenylmethyloxycarbonyl)amidodecane-1,10-dicarboxylic Acid (12). A solution of 8(*R*)-(5(*S*)-isopropyl-3,6-diethoxy-2,5-dihydropyrazin-2-yl)-2(*S*)-(9-fluorenylmethyloxycarbonyl)amidooctanoic acid (**11**) (0.112 g, 0.189 mmol) in THF (2.27 mL) was treated with 1 M HCl (2.27 mL, 2.27 mmol, 12 equiv). The reaction mixture was stirred for 90 min at room temperature and then concentrated in vacuo to remove the THF. The residual aqueous mixture (pH 2) was diluted with dioxane (1 mL) and neutralized with $NaHCO_3$ (0.191 g, 2.27 mmol, 12 equiv). The resulting solution was stirred for 20 min at room temperature before more $NaHCO_3$ (0.127 g, 1.51 mmol, 8 equiv) in water (1.27 mL) was added. Stirring was continued for a further 10 min. The reaction was then cooled to 0 °C and allyl chloroformate (0.055 mL, 0.514 mmol, 4 equiv) added slowly. The reaction mixture was stirred

(46) (a) Regitz, M.; Hocker, J.; Leidhegener, A. *Organic Syntheses*; Wiley: New York, 1973; Collect. Vol. V, p 179. (b) Leffler, J. E.; Tsuno, Y. *J. Org. Chem.* **1963**, *28*, 902.

for 18 h at 0 °C and then acidified with saturated NH_4Cl (3 mL) followed by 2 M HCl, to pH 3. The mixture was extracted with CH_2Cl_2, and the combined organic layers were dried (Na_2SO_4) and concentrated in vacuo at room temperature. The resulting crude colorless oil was purified by flash chromatography (EtOAc, R_f 0.58) to give **12** as a white solid (82 mg, 0.145 mmol, 77% yield): mp 48 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.23−1.32 (11H, br m), 1.59−1.70 (2H, br m), 1.75−1.87 (2H, br m), 3.95−4.00 (1H, br m), 4.10 (2H, q, *J* 7.1 Hz), 4.14−4.17 (3H, br m), 4.39−4.40 (1H, br m), 4.53 (2H, d, *J* 5.5 Hz), 5.18−5.30 (2H, m), 5.86−5.93 (1H, m), 7.28 (2H, t, *J* 7.3 Hz), 7.37 (2H, t, *J* 7.4 Hz), 7.55 (2H, d, *J* 7.0 Hz), 7.73 (2H, d, *J* 7.4 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 18.8, 25.8, 29.4, 33.0, 47.5, 54.4, 59.8, 61.8, 66.3, 104.0, 118.2, 120.3, 125.7, 127.4, 128.0, 128.9, 129.1, 132.4, 132.6, 141.6, 156.9, 173.1; IR ν_{max} 3438, 3037, 2970, 2937, 2856, 1712, 1427, 1226, 1209; mass spectrum *m*/*z* 589 (M + Na, 13.7) (ES+); HRMS (FAB) calcd for $C_{31}H_{38}N_2O_8Na$ (M + Na^+) 589.2526, found 589.2503.

(10-Allyl ester)-9(*R*)-allyloxycarbonylamido-2(S)-(9-fluorenylmethyloxycarbonylamido)decane-1,10-dicarboxylic Acid (1). To a solution of (10-ethyl ester)-9(*R*)-allyloxycarbonylamido-2(*S*)-(9-fluorenylmethyloxycarbonyl)amidodecane-1,10-dicarboxylic acid (**12**) (0.100 g, 0.177 mmol) in freshly distilled allyl alcohol (0.588 mL) was added dried LiBr (76.6 mg, 0.882 mmol, 5 equiv). The reaction mixture was stirred for 1 h at room temperature and then cooled to 0 °C. Freshly distilled DBU (0.04 mL, 0.265 mmol, 1.5 equiv) was then added, and the reaction mixture was stirred at 2 °C for 24 h. The reaction was then acidified with saturated NH_4Cl solution (5 mL) to pH 7, followed by 2 M HCl to pH 4. The mixture was then extracted with CH_2Cl_2 (3 × 100 mL), and the combined organic layers were dried (Na_2SO_4) and concentrated in vacuo to give **1** as a clear oil (R_f 0.70 in 70% EtOAc/hexane). This was purified by preparative reversed-phase HPLC: gradient 40−80% acetonitrile/water and 0.1% TFA over 35 min; retention time 17.6 min (0.100 g, 0.173 mmol, 98% yield). The chiral purity of **1** was checked by chiral HPLC: the retention time (isocratic, 10% *i*-PrOH/hexane) was 11.9 min and no trace of the other diastereoisomer was detected: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.20−1.80 (12H, br m), 3.46 (1H, dd, *J* 7.1, 14.2 Hz), 4.26−4.36 (3H, m), 4.49 (1H, dd, *J* 2.6, 6.9 Hz), 4.56 4.61 (4H, m, d, *J* 4.9 Hz), 5.18−5.33 (4H, m), 5.85−5.92 (2H, m) 7.30 (2H, t, *J* 7.3 Hz), 7.39 (2H, t, *J* 7.2 Hz), 7.62 (2H, dd, *J* 7.8, 10.3 Hz), 7.75 (2H, d, *J* 7.5 Hz);); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 19.9, 24.3, 25.5, 27.2, 29.4, 32.9, 38.4, 49.2, 55.0, 66.3, 67.4, 118.2, 119.3,120.4, 125.6, 127.5, 128.1, 128.6, 129.1, 132.5, 132.6, 142.2, 144.7, 144.8, 167.2; IR ν_{max} 3600, 3442, 3026, 2937, 2862, 1730, 1674, 1452, 1226, 1207 cm^{-1}; mass spectrum *m*/*z* 579 (M + 1, 75) (APCI+); HRMS (FAB) calcd for $C_{32}H_{38}N_2O_8Na$ (M + Na^+) 601.2526, found 601.2512.

Synthesis of Test Peptide 13. The first amino acid was loaded onto the resin manually, as follows. Novasyn TG Sieber resin was placed in a Merrifield bubbler and the terminal Fmoc group removed with piperidine−DMF (1:4 v/v) (2 mL). After thorough washing with DMF, Fmoc-Lys(Boc)-OH (93.7 mg, 0.20 mmol) was activated for 15 min with HOAt (0.20 M in DMF, 1 mL, 0.20 mmol) and DIC (0.20 M in dioxane, 1 mL, 0.20 mmol) and then coupled to the resin for 30 min with agitation by N_2 bubbling. This coupling procedure was repeated twice. Following a satisfactory qualitative Kaiser test, 10 min capping with 0.5 M acetic anhydride/0.125 M ^{i}Pr$_2$EtN/0.2% HOBt in DMF (2 mL) ensued. The resin loading was then determined to be 0.122 mmol/g by UV absorbance. The resin was then transferred to the peptide synthesizer and linear peptidyl-resin **15** was synthesized by the solid-phase method, as follows. For the Fmoc-Ala-OH and Fmoc-Lys(Boc)-OH residues, the standard automated Fmoc deprotection, washing, amino acid activation, coupling and capping cycles were followed, and the reactions were quantitatively monitored by measuring the UV absorbance of the Fmoc group on and off the solid support. The protected bis-amino acid (**1**) (0.145 g, 0.251 mmol, 3.3 equiv with respect to the determined loading of Fmoc-Lys(Boc)-resin), was activated by HOAt (54.4 mg, 0.40 mmol) and 0.3 M DIC in DMF (1.3 mL, 0.39 mmol) over 7 min. The activated amino acid was then divided between six vials and each was coupled with a coupling cycle of 45 min and further recycling for 15 min. The capping step was followed by a 5 min deblocking of the *N*-terminal Fmoc group. This was followed by coupling of Fmoc-Ala-OH and Fmoc-Lys(Boc)-OH residues, as previously described: the coupling of the final Fmoc-Ala-OH was carried out without Fmoc deprotection.

Peptidyl-resin **15** was then transferred to the Merrifield bubbler and washed with CH_2Cl_2 (1 × 20 mL) to remove any residual DMF. A solution of $Pd(PPh_3)_4$ (0.231 g, 0.20 mmol, 2 equiv with respect to the peptidyl-resin **15**) in $CHCl_3$ (3.25 mL, 37 times the volume of NMM), glacial AcOH (0.176 mL, twice the volume of NMM), and NMM (0.088 mL, 0.80 mmol, 8 equiv) was added to the peptidyl-resin and gently bubbled for 2 h under an argon atmosphere in the dark. The resin was washed with 0.5% *i*-Pr$_2$EtN in CH_2Cl_2 (v/v) (4 × 4 mL) and 0.5% sodium diethyldithiocarbamate trihydrate in DMF (w/v) (4 × 4 mL), followed by a final wash with DMF. The qualitative Kaiser test was pale purple. The peptidyl-resin was then deprotected by bubbling with piperidine-DMF (1:4 v/v) (2 mL) and thorough washing with DMF (6 × 20 mL), followed by a wash with 0.4% HOBt in DMF (w/v) (1 × 5 mL) to prevent the possible side reaction of the undesired conversion of the free carboxy group to a piperidyl amide. The qualitative Kaiser test was dark purple in color.

On-resin cyclization of the resulting peptidyl-resin **16** was then carried out as follows. A 0.5 M solution of PyAOP (0.261 g, 0.50 mmol, 5 equiv) and HOAt (68.1 mg, 0.50 mmol, 5 equiv) in DMF (2 mL) was added to the peptidyl resin, followed by *i*-Pr$_2$EtN (0.174 mL, 1.0 mmol, 10 equiv). The mixture was bubbled for 1 h under an argon atmosphere, after which the qualitative Kaiser test was pale purple. The resultant peptidyl-resin **17** was then thoroughly washed with DMF. Fmoc-Ala-OH (63 mg, 0.20 mmol) was activated for 15 min with HOAt (0.20 M in DMF, 1 mL, 0.20 mmol) and DIC (0.20 M in dioxane, 1 mL, 0.20 mmol) and then coupled to the resin for 30 min with agitation by N_2 bubbling. This coupling procedure was repeated twice, giving a negative qualitative Kaiser test.

The peptidyl-resin was then transferred to the peptide synthesizer and the peptide synthesis completed, using the standard automated Fmoc deprotection, washing, amino acid activation, coupling and capping cycles as before. The final Fmoc deprotection was carried out on the Merrifield bubbler. This was followed by thorough washing with DMF and final acylation with the capping solution by bubbling with 0.5 M Ac_2O (1 mL, 0.50 mmol, 2 equiv)/0.125 M *i*-Pr$_2$EtN (1 mL, 0.125 mmol, 0.5 equiv)/0.2% HOBt in DMF for 10 min, then thorough rinsing with DMF (1 × 20 mL, 1 × 5 mL). The peptide was cleaved from the resin with 95% TFA/water (6 × 10 mL), finally washing with MeOH/CH_2Cl_2. Et_3SiH was added (to 10% (v/v)) and the mixture stirred for 2 h under Ar. The peptide was precipitated by the addition of ice-cold Et_2O, followed by refrigeration overnight. Filtration and lyophilisation with 40% acetic acid in water (v/v) (10 mL) yielded 47 mg of crude peptide. This was purified by preparative reverse phase HPLC: gradient 0−7% MeCN/H_2O with 0.1% TFA over 20 min; retention time 5.13 min. This gave the desired test peptide **13** as a white solid (11 mg, 22.5% yield based on the cleavage of 0.400 g peptidyl-resin with a loading of 0.122 mmol/g). Mass spectrum: *m*/*z* 742 (M^{2+} + 1, 10) (ES+). A deletion sequence, lacking the bis-amino acid, was recovered and purified by preparative reverse phase HPLC: gradient 5−15% MeCN/H_2O with 0.1% TFA over 20 min; retention time 7.3 min (0.003 g). Mass spectrum: *m*/*z* 671 (M^{2+} + 1, 12), 672 (M^{2+} + 2, 8) (ES+).

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Supporting Information Available: ^{1}H NMR spectra of the compounds described in the Experimental Section, together with experimental procedures and full spectral data for novel compounds not described in the Experimental Section; the procedure for the synthesis of peptide **14**; CD spectra for the cryogenic study with control peptide **14** in 4 M urea; analytical HPLC traces and ES+ spectra for control peptide **14**, purified test peptide **13**, and the crude test peptide **13**, showing the deletion sequence byproduct. This material is available free of charge via the Internet at http://pubs.acs.org.

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