Serine–threonine protein phosphatase inhibitors derived from nodularin: role of the 2-methyl and 3-diene groups in the Adda residue and the effect of macrocyclic conformational restraint

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In order to probe the effect upon macrocycle conformation and $PP1_{cat}$ enzyme inhibition of structural changes to nodularin, specific replacements for the Adda residue were introduced. Two new analogues, cyclo[-(3*S*,*E*)-3-phenylethenyl-3-aminopropanoyl-α-(*R*)-Glu-α-OH-γ-Sar-(*R*)-Asp-α-OH-β-(*S*)-Phe-] **19a** and cyclo[-(2*S*,3*S*,*E*)-2-methyl-3-phenylethenyl-3-aminopropanoyl-β-(*R*)-Glu-α-OH-γ-Sar-(*R*)-Asp-α-OH-β-(*S*)-Phe-] **19b** were prepared incorporating previously optimised preparative protocols [see previous article, K. L. Webster, A. B. Maude, M. E. O'Donnell, A. P. Mehrotra and D. Gani, *J. Chem. Soc., Perkin Trans. 1* (DOI: 10.1039/b100401h)], and these differed only at C-2 of the Adda residue. The presence of a (2*S*)-methyl group in compound **19b** stabilised the *trans*-rotameric form of the (2*R*)-Glu-γ-Sar amide bond in solution as determined by NMR spectroscopic analysis (*trans–cis*; 10:1), and enhanced efficacy as a $PP1_{cat}$ inhibitor by 20-fold over compound **19a**. The methyl homologue displayed a competitive mode of inhibition, with respect to the substrate Ac-Arg-Arg-Thr(P)-Val-Ala and displayed a K_i value of 206 ± 30 μmol dm^{-3}. Substitution of the Sar residue in the methyl homologue by (2*S*)-Pro gave a competitive inhibitor of similar efficacy (K_i = 400 ± 75 μmol dm^{-3}). The proline analogue **22** existed as a 6:1 mixture of *trans–cis* rotamers. Evidently the *trans*-rotamer of the (2*S*)-Pro-containing compound differed in conformational structure compared to the sarcosine-containing variant, only close to the site of the substitution. A structural model for the inhibition of $PP1_{cat}$ and a strategy for the selective inhibition of PP1 over PP2A are discussed within the context of the results.

Introduction

Reversible protein phosphorylation plays a major role in the control of a wide range of metabolic processes. In eukaryotic cells, the reversible phosphorylation–dephosphorylation of serine, threonine and tyrosine residues serves as a switch in turning on and off key enzyme activities within cells. This is achieved by inducing changes in protein conformation and charge that alter binding affinities in protein–protein and protein–ligand interactions.[1] The downstream effect is the regulation of a host of cellular processes including glycogen synthesis, cell differentiation and proliferation, gene expression, muscle contraction and the control of signal transduction pathways.[2] While protein kinases as a group have been the focus of attention for decades, recent years have witnessed enormous advances in understanding the role, modulation and structure of protein phosphatases (PPases).[3]

To date, eight types of Ser-Thr protein phosphatase have been identified and four of these, PP1 and PP2A, B and C, see below, have been studied in some detail. The catalytic subunit of such phosphatases, for example $PP1_{cat}$, is usually associated with one of a number of modulating subunits.[4] These modulating proteins are able either to enhance catalytic activity and, simultaneously, modify substrate specificity, or to inhibit activity. Given that the modulating protein may itself be a phosphoprotein substrate for the same or a different phosphatase catalytic subunit, the situation is extremely complex.

In view of the overlapping specificity of different types of catalytic subunit for given substrates and the effects of modulation on altering the substrate specificity of a given catalytic subunit, PPases cannot be classified by substrate alone. It has been necessary to include consideration of the action of the modulating protein subunits and, also, the action of small molecules and/or ions as inhibitors and activators. Type 1 PPase activities (PP1) are those that are inhibited by the small thermostable, acid-resistant proteins inhibitor 1 (I-1), inhibitor 2 (I-2 or modulator) and DARPP-32.[5] Type 2 activities (PP2) are insensitive to inhibitors 1 and 2 and are further sub-divided into three types, PP2A, PP2B (calcineurin) and PP2C, according to their requirement for divalent cations and modulating proteins and their primary structures. PP1, PP2A and PP2B are structurally related to each other and possess almost identical active sites.[6] Each site contains two catalytically essential divalent metal ions, usually two Mn^{2+} ions, and shows considerable similarity to purple acid phosphatase.[7] PP2B is differentiated from PP2A through its activation by calmodulin while PP2C possesses an unrelated primary structure and requires Mg^{2+} ions.[8]

The native catalytic $PP1_{cat}$ and $PP2A_{cat}$ subunits show little substrate specificity and are able to dephosphorylate a range of substrates.[9] However, they are highly regulated *in vivo* by the action of the modulating subunits and endogenous protein inhibitors described above, and by compartmentalisation, through subcellular localisation. Our attentions have been focussed in two major areas. The first has concerned the mode of action of the highly toxic natural product inhibitors typified by microcystin and nodularin, with the objective of developing inhibitory analogues that are differentially selective for $PP1_{cat}$ and $PP2A_{cat}$, see preceding article.[10] The second has striven to understand the mechanism of catalytic hydrolysis and its allosteric modulation. To these ends we have recently developed a new and reliable assay for the catalytic subunits of both PP1 and PP2A using ^{14}C-radiolabelled phosphopeptide substrates, see following article.[11] We have also shown that dephosphorylation occurs *via* a ternary complex mechanism in which water directly attacks the phosphate ester moiety.[12] The new assay

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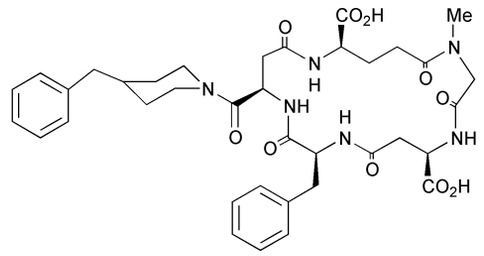
protocol (described in full in the following article[11]) has facilitated a detailed analysis of the binding determinants for substrates and inhibitors and has allowed a direct comparative analysis of the activities of $PP1_{cat}$ and $PP2A_{cat}$. Most pertinent is the low affinity of the dephosphorylated product peptide for the enzyme ($PP1_{cat}$) and the predominant effect of inorganic phosphate as a product inhibitor. In the current study, this information, together with the preliminary data on the structural binding determinants for nodularin analogues, has been utilised in the design of inhibitory probes for $PP1_{cat}$ to test the role of key moieties in the macrocycle. Here we report on the synthesis of such nodularin analogues and on their conformational properties and biological activities. Based upon these results we provide a structural model for inhibitor binding interactions at the active site of $PP1_{cat}$. These interactions suggest a strategy for the preparation of selective inhibitors that can differentiate between $PP1_{cat}$ and $PP2A_{cat}$.

Results and discussion

In previous work,[10,13–15] a number of synthetic strategies for preparing nodularin analogues were considered. The three key requirements concerned facilitating the construction of linear precursors to the nodularin macrocycle, performing macrolactamisation reactions, and introducing suitable lipophilic moieties into Adda [(2*S*,3*S*,4*E*,6*E*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid] surrogates. This work established that strategies based upon the gel-phase construction of fully functionalised linear isopeptidic precursors, already possessing preformed Adda surrogates, and solution-phase, off-resin, macrolactamisation worked best. Given these potentially robust preparative protocols, attention could be turned to the molecular recognition issues underpinning the design of selective inhibitors for $PP1_{cat}$ and for $PP2A_{cat}$.

Before embarking on such, a comment on safety in relation to research strategy is relevant. In previous work in which we had started to explore the potential biological activity of $PP1_{cat}$ inhibitors, we were acutely aware of the severe toxicity of the natural nodularins and microcystins. Such compounds display IC_{50} values for $PP1_{cat}$ of *ca.* 0.1 nM and are potent hepatotoxins in a diverse array of species including mammals and including man.[16] We were concerned to the extent that we chose to deliberately tune-down biological activity. This was achieved by replacing the Adda side-chain in nodularin analogues by smaller lipophilic side-chains, for example, as in compounds **1** and **2**. The Adda side-chain in nodularin is thought to bind to a hydrophobic groove formed by residues Ile-130, Ile-133, Tyr-134 and Tyr-206 in $PP1_{cat}$, as for microcystin. Such analogues **1** and **2** displayed K_i values in the low millimolar range and were expected, on the basis of the correlation of hepatotoxicity with efficacy as a $PP1_{cat}$ inhibitor, to be some 10^7 times less toxic. This millimolar range for inhibitory activity seemed ideal for inhibitor development in that both improvements and impairments in binding could be detected easily without exposing research personnel to highly toxic end products. Thus, for the purposes of refining our understanding of how to differentially inhibit $PP1_{cat}$ and $PP2A_{cat}$ in the present study, we opted to continue to employ tuned-down non-toxic inhibitors. It was also expected that for biochemical studies designed to deconvolute the individual role of each enzyme, access to highly selective inhibitors for each type was more important than absolute inhibitory efficacy. Moreover, it was reasoned that the absolute efficacy of any given inhibitor could be enhanced at any stage simply by re-introducing a larger part of the Adda residue, or all of it, should this be necessary.

With respect to improving efficacy in the tuned-down analogues, it was noted that compounds **1** and **2** did not exist as single conformers or rotamers in solution, as determined by NMR spectroscopy,[10] unlike the natural nodularins.[17,18] The



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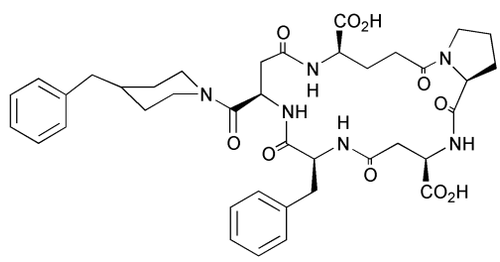


2

synthetic analogues differ from the natural products in that the α-methyl group of the Adda surrogate (at residue position 1) is missing. Thus, we wished first to address the effect of the methyl group on the conformation of the macrocycle and, in turn, the correlation of changes in conformation with biological activity. In order to relate our findings to unbiased macrocycles, in the first instance, we chose to use sarcosine, an unrestained *N*-alkyl amino acid residue, at position 3. We also opted to prepare an Adda surrogate that would more closely resemble the diene moiety appended to the β-position of the Adda residue. The rigid (*E*)-phenethenyl moiety seemed ideal for this purpose and, hence, homochiral 3-phenethenyl-3-aminopropanoic acids were chosen for residue position 1. This strategy was expected to increase the confidence level of modelling the active-site interactions of $PP1_{cat}$ and also $PP2A_{cat}$ with nodularin-type inhibitors on the basis of structure–activity relationships.

Synthesis of 3-phenethenyl-3-aminopropanoic acids

Several groups have reported the total synthesis of Adda and its derivatives.[19] Typically, the construction of the Adda residue has required 19–20 steps and has provided the product in 5% overall yield. However, a very recent synthesis was reported in which Adda was obtained in 40% overall yield over 13 steps.[20] Many of the synthetic schemes share a common connective strategy based on development of the (*E,E*)-diene framework *via* Julia–Wittig type chemistry, which does not allow for complete control of the stereochemistry of the double bonds. Given our desire to produce tuned-down macrocyclic analogues possessing homochiral 3-phenethenyl-3-aminopropanoic acids, we looked to utilise an asymmetric conjugate addition reaction of the type developed by Davies and Waters for the introduction of the 3-amino group.[21] Such reactions use a chiral lithium amide, for example, that derived from (*S*)-benzyl-α-methylbenzylamine, to control the absolute stereochemistry at C-3 in the product. To prepare the 2-methyl analogue it was expected that C-methylation of the enolate derived from the addition product would proceed diastereoselectively to give (2*S*)-absolute stereochemistry.

Accordingly, Horner–Wadsworth–Emmons reaction of methyl diethylphosphonoacetate with cinnamaldehyde gave the required (*E,E*)-dienoate precursor **3** in 93% yield (Scheme 1).[22] Reaction of lithium (*S*)-*N*-benzyl-*N*-α-methylbenzylamide with the dienoate **3** afforded the desired 1,4-addition product **4a** with high diastereoselectivity in 61% yield along with the 1,4- and 1,2-bis-addition products in 15% yield. No 1,6-addition product was detected. For non-methylated target residue **10a**,

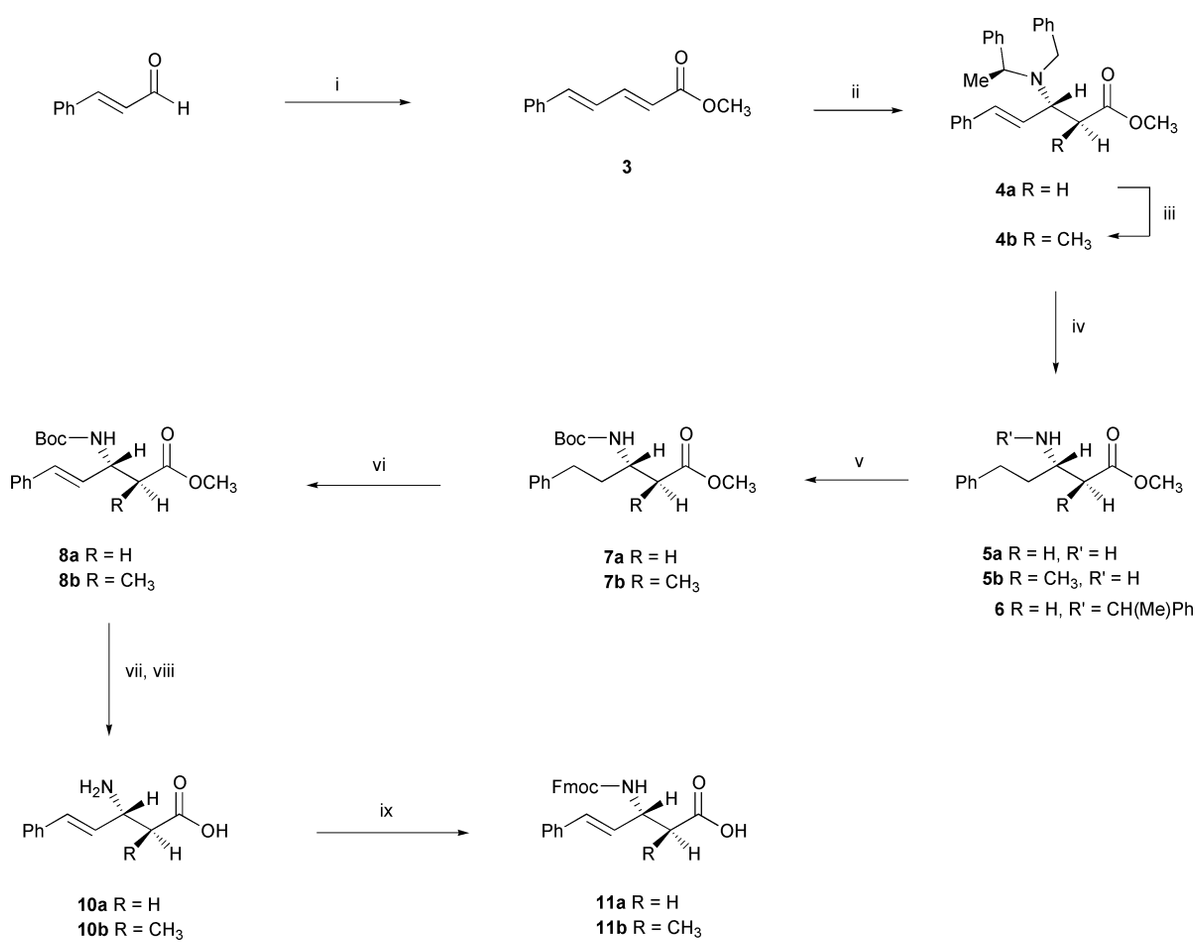
the tertiary amine **4a** was stored pending deprotection as described below.

In order to prepare the 2-methyl derivative **10b**, the tertiary amine **4a** was treated with LDA at −78 °C to generate the lithium enolate and the resulting anion was alkylated with methyl iodide to give a 4:1 ratio of *anti* to *syn* products.[21] The required *anti* product **4b** was obtained in 57% yield (Scheme 1).

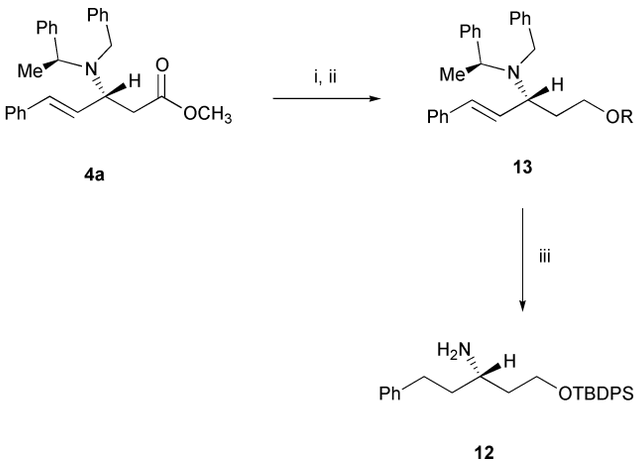
Removal of the benzyl groups, while keeping the double bond intact, was not possible. Standard hydrogenolysis conditions employing $Pd(OH)_2/C$ (Pearlman's catalyst) cleanly removed the *N*-benzyl groups from amino ester **4a** but also hydrogenated the double bond to give the saturated amine **5a**, as expected.[23] Analysis of the reaction products after partial reduction indicated that the double bond was reduced before complete hydrogenolysis occurred. Dissolving metal reductions, which are widely used for debenzylations,[24] were deemed to be unsuitable due to the presence of the conjugated double bond in compound **4a** and were not attempted. Several alternative protocols exist for debenzylation of amines under non-reducing conditions including the use of trichloroethoxycarbonyl chloride (Treoc-Cl).[25] However, when debenzylation was attempted on compound **4a**, only starting material was recovered. Partial success was achieved using ceric ammonium nitrate (CAN)[26] and treatment of the dibenzylamine **4a** with 3 equivalents of CAN in acetonitrile–water resulted in rapid monodebenzylation to give the amine **6** in good yield (80–90%). The partial deprotection was complete in 15–20 min and benzaldehyde was generated as the oxidised by-product. Reactions performed using excess CAN and/or for extended reaction periods were not successful and a variety of oxidised products were formed.

In view of these findings, it was decided to remove the *N*-benzyl groups with concomitant reduction of the double bond, as in formation of **5a**, and to re-introduce the double bond later on in the synthesis, through benzylic bromination followed by the elimination of hydrogen bromide. To avoid potential side reactions, the amine functionality in compounds **5a** and **5b** were first protected as their *tert*-butyl urethane derivatives **7a** and **7b** through reaction with Boc anhydride. The urethanes were then irradiated in the presence of NBS using a tungsten lamp, to afford the intermediate 5-bromides which upon treatment with DBU at 70 °C for 10 min eliminated HBr to give the alkenes **8a** and **8b** in moderate yield.[27] Approximately 20% of the starting material was also recovered in each case. The synthesis of each β-amino acid was completed by removal of the methyl ester and Boc protecting groups, using lithium hydroxide and then TFA, respectively, to give compounds **10a** and **10b**, each in approximately 75% yield. In preparation for using standard solid-phase peptide synthesis protocols, each free amino acid was converted to its *N*-Fmoc derivative **11a** and **11b**.

The absolute stereochemistry at C-3 of the adduct **4a** was confirmed as 3*R* by converting the amino ester **4a** to the known amine, compound **12**,[28] for comparison of optical rotation values (Scheme 2). Treatment of the ester **4a** with DIBAL-H followed by reaction with TBDPSCl in the presence of imidazole afforded the silyl ether **13** in 75% yield and subsequent hydrogenation and debenzylation using Pearlman's catalyst gave amine **12** in 60% yield. Upon comparison of the specific rotation $\{[a]_D^{24}$ −2.4 (*c* 1.45 in $CHCl_3$)} to the literature value $[a]_D^{24}$ −2.81 (*c* 1.63 in $CHCl_3$) it was evident that the compound was the (3*S*)-antipode. Note the priority change following reduction of the double bond that reverses the absolute stereochemical



Scheme 1 *Reagents and conditions*: i) $(EtO)_2POCH_2CO_2CH_3$, NaH, THF, 0 °C, then add cinnamaldehyde at −78 °C, 20 min, 93%; ii) (*S*)-*N*-benzyl-α-methylbenzylamine, BuLi, THF, −78 °C, 2 h, 61%; iii) LDA, −78 °C, 45 min, MeI, −78 °C, 30 min, 57%; iv) $Pd(OH)_2$, H_2, CH_3OH–H_2O–CH_3COOH (20:3:3), rt, 3 h, 71–77%; v) Boc_2O, $NaHCO_3$, dioxane, H_2O, rt, 4 h, 72–82%; vi)NBS, CCl_4, 25 °C, 2 h, then DBU, 60 °C, 10 min, 42–45%; vii) LiOH, THF, CH_3OH, H_2O, 1 h, 20 °C, 71–95%; viii) TFA, DCM, rt, 2 h, 79–83%; ix) Fmoc-Cl, K_2CO_3, dioxane, H_2O, 0 °C→rt, 12 h, 52–66%.

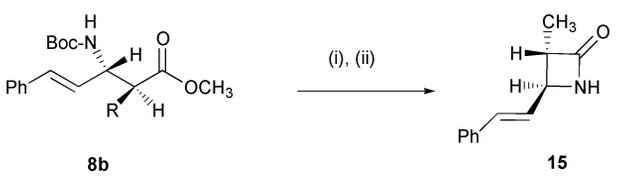


4a 13 12

Scheme 2 *Reagents and conditions*: i)DIBAL-H, THF, −78 °C, 2 h; ii) TBDPSCl, imidazole, THF, rt, 6 h, 75% from **4a**; iii) $Pd(OH)_2$, ammonium formate, CH_3OH, 60 °C, 2 h, 60%.

assignment. ^{1}H-NMR spectra of the crude precursors indicated that the diastereomeric purity was greater than 95%.

The relative stereochemistry at C-2 and C-3 in compound **4b** was assigned by conversion of the Boc protected amine **8b** to a β-lactam, (Scheme 3).[21] The Boc group was removed with TFA



8b 15

Scheme 3 *Reagents and conditions*: i) TFA, DCM, rt, 1 h; ii) LDA, THF, −78 °C, 20 min.

to give the amino ester **14** which was cyclised to the β-lactam **15** through treatment with LDA at −78 °C. The observed H-2/H-3 coupling constant of ~2 Hz indicates a *trans*-relationship, and given that the β-lactam is derived from amino ester **4a** which possesses (3*R*)-stereochemistry before methylation and (3*S*)-stereochemistry after methylation, due to a priority change, it is evident that compound **4b** and, therefore, compound **8b** possess (2*S*,3*R*)-absolute stereochemistry.

Solid-phase peptide synthesis (SPPS) of pentapeptides

The SPPS of the linear pentapeptides **17a** and **17b** from the Fmoc-Asp-(α)-OMe-O-Wang **16** was carried out on a Rainin PS3 automated peptide synthesiser using methods similar to those as described previously (Scheme 4).[10] The crude products **17a** and **17b** were obtained in quantitative recovery after cleavage from the resin. Analysis by HPLC revealed one major peak in each case (approx. 80% of total) with several close running bands which were removed by HPLC. Each pure material eluted as a single peak and gave the required ES mass spectral data (704, $[M + Na]^+$) for **17a** and (718, $[M + Na]^+$) for **17b**. Analysis of the ^{1}H and ^{13}C spectra revealed the presence of two major rotomeric forms, in each case.

The macrocyclisations of compounds **17a** and **17b** were carried out under high dilution conditions (1 mg cm^{-3}), using BOP-Cl in the presence of *N,N*-diisopropylethylamine (DIPEA) in a CH_2Cl_2–DMF mixture over a period of five days using conditions previously optimised in the preparation of earlier analogues (see previous article).[10] The crude products were purified by flash chromatography on silica to give the cyclic pentapeptides **18a** {(ES) 686 ($[M + Na]^+$)} in 28% yield and **18b** {(ES) 700 ($[M + Na]^+$)} in 27% yield. ^{1}H-NMR and mass spectral data indicated that each cyclisation had occurred and analysis of the NMR data indicated that each diester existed as a mixture of *cis–trans* rotamers/conformers. This was in accord with expectations since it had been shown previously that the natural product motuporin, an analogue of nodularin, existed in a single conformation whereas the dimethyl ester existed in multiple forms.[29] Saponification of the methyl ester protecting groups in compounds **18a** and **18b** was performed using LiOH in aqueous THF. The acidified products were purified by reverse-phase HPLC to give the required diacids **19a** {(ES) 658 ($[M + Na]^+$)} in 77% yield and **19b** {(ES) 672 ($[M + Na]^+$)} in 72% yield respectively. As expected, the ^{1}H-NMR spectra of the diacids were much less complex than for the dimethyl ester precursors. The analogue **19a**, which is derived from the non-methylated β-amino acid **5a**, possesses two α-H-atoms. This compound existed as a 2.5:1 mixture of two conformers in solution and detailed analysis revealed that these were *trans*- and *cis*-rotamers at the *N*-acylsarcosine amide bond, respectively. The more restrained macrocyclic methyl homologue **19b** displayed an even simpler ^{1}H-NMR spectrum corresponding to a 10:1 mixture of *trans*- to *cis*-rotamers at the *N*-acylsarcosine amide bond. Thus, each macrocycle existed predominantly in the *trans*-rotameric form. Interestingly, these major forms displayed almost identical coupling constants and chemical shift values to each other in ^{1}H-NMR and ^{13}C-NMR spectra, for all of the conserved residues.

These findings suggest that the solution conformations of the major forms are identical. Moreover, it appeared that the conformations of the dominant *trans*-rotameric forms closely matched that for the solution-phase conformation of nodularin as determined by comparison of ^{1}H-NMR data (see discussion below).

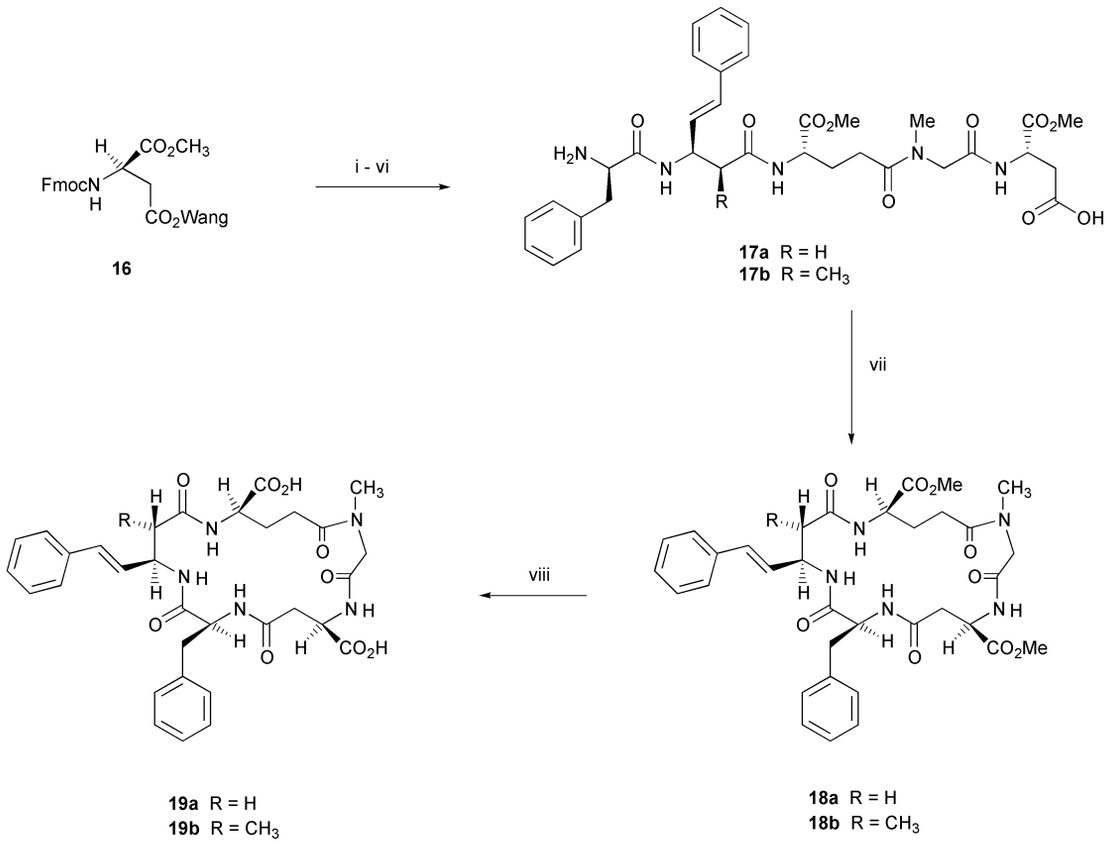
(2*S*)-Proline analogue

The crystal structure of microcystin-LR bound to $PP1_{cat}$ shows that the Adda residue, residue number 1, binds in a hydrophobic groove on the surface of the protein, while the Adda carbonyl group and the glutamate α-carboxy group are involved in water-mediated hydrogen bonding to the metal ion site.[30] Results to date indicate that the solution structure of microcystin is highly preorganised for binding and shows a high degree of similarity to the $PP1_{cat}$-bound conformation. In our earlier work to identify a stripped-down nodularin analogue, we had shown that the *N*-methyldehydroalanine residue in microcystin does not form a covalently bonded Michael adduct with Cys-273 as part of the mechanism for tight-binding inhibition.[13] Indeed, we showed that each of the dihydromicrocystin epimers containing (2*R*)- and (2*S*)-*N*-methylalanine at position 3 were equally potent inhibitors to the parent. We also showed that the introduction of (2*R*)- and (2*S*)-proline at the congruous 3-position in nodularin analogues gave restrained macrocycles that displayed poor but significant inhibitor activity against $PP1_{cat}$.[10] These compounds did not exist as single conformers in solution and conformational analysis was extremely difficult. It was however clear that each macrocycle existed in both the *trans*- and *cis-rotameric* forms and that each of these rotamers existed in more than one conformation that interconverted slowly on the NMR time scale.

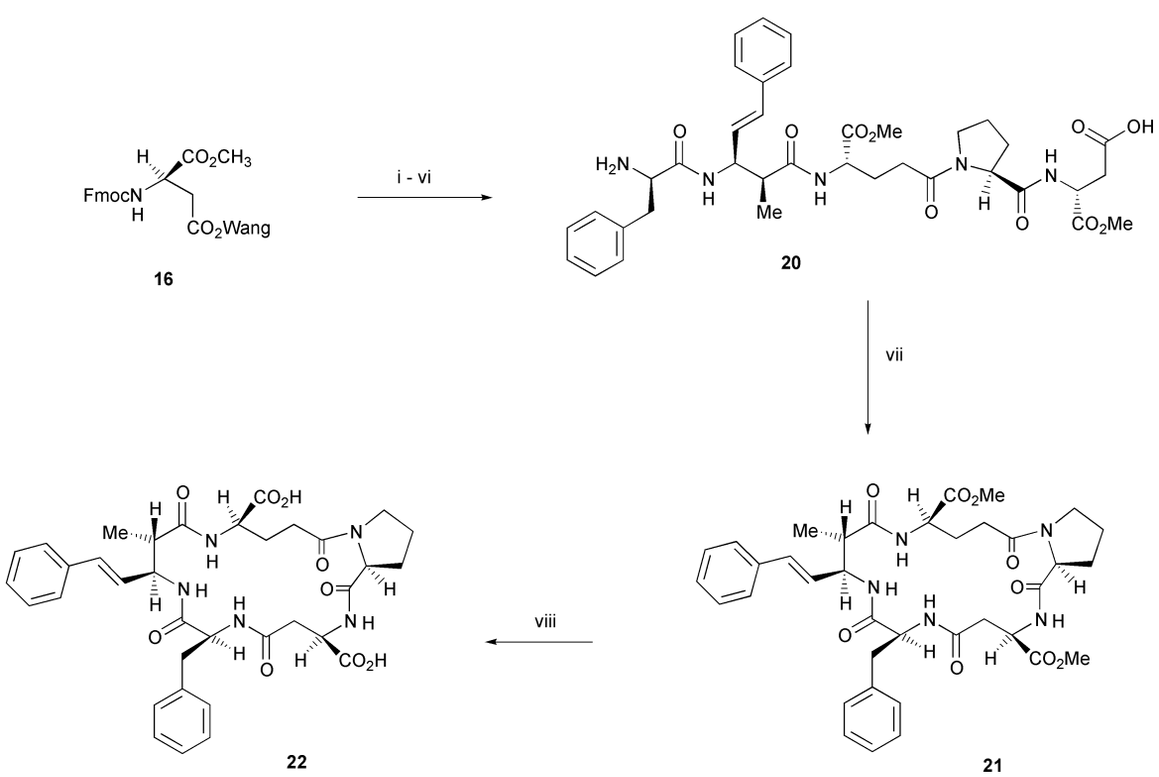
Given these difficulties, a fuller structural analysis was not possible. However, the compounds did not contain an α-methyl group in the Adda surrogate at position 1, the presence of which might have reduced the number of populated conformations and allowed a simplified analysis. In view of the results obtained for the sarcosine containing analogues **19a** and **19b**, above, it was determined that a comparison of the structures of the identically substituted macrocycles possessing α-methylated Adda analogues and differing only in the nature of the residue at position 3 would be highly informative.

Accordingly, the (2*S*)-Pro analogue of macrocycle **19b** was prepared using the now established protocols starting with the SPPS of the linear pentapeptide **20**. Cyclisation to **21** was

performed using BOP-Cl in DMF–CH_2Cl_2 in the presence of DIPEA and the macrocyclic diester **21** was obtained in 23% yield (Scheme 5). Again the ^{1}H-NMR spectrum of the diester revealed the existence of multiple conformations. Gratifyingly, saponification of the methyl ester groups gave diacid **22** which existed in one predominant conformation as judged by ^{1}H-



Scheme 4 *Reagents and conditions*: i) 20% piperidine in DMF; ii) Fmoc-Sar-OH, PyBOP, DMF; then 20% piperidine in DMF; iii) Fmoc-(2*R*)-Glu-α-OMe-γ-OH, PyBOP, DMF; then 20% piperidine in DMF; iv) **11a** or **11b**, PyBOP, DMF; then 20% piperidine in DMF; v) Fmoc-(2*S*)-Phe-OH, PyBOP, DMF; then 20% piperidine in DMF; vi) TFA–TES–H_2O (95:2.5:2.5), 1 h, rt; vii) BOP-Cl, DIPEA, DCM, DMF, rt, 7 days, 27–28%; viii) LiOH, THF, H_2O, 0 °C, 1 h, 68–80%.



Scheme 5 *Reagents and conditions*: i) 20% piperidine in DMF; ii) Fmoc-(2*S*)-Pro-OH, PyBOP, DMF; then 20% piperidine in DMF; iii) Fmoc-(2*R*)-Glu-α-OMe-γ-OH, PyBOP, DMF; then 20% piperidine in DMF; iv) **11b**, PyBOP, DMF; then 20% piperidine in DMF; v) Fmoc-(2*S*)-Phe-OH, PyBOP, DMF; then 20% piperidine in DMF; vi) TFA–TES–H_2O (95:2.5:2.5), 1 h, rt, quantitative recovery from **16**; (vii) BOP-Cl, DIPEA, DCM, DMF, rt, 7 days, 23%; viii) LiOH, THF, H_2O, 0 °C, 1 h, 85%.

NMR spectroscopy. More thorough analysis revealed that the major conformation corresponded to the *trans*-rotameric form of the γ-Glu-Pro amide bond in a 6:1 mixture with a conformer displaying signals consistent with its *cis*-rotameric form. Compared to the *trans*-rotamer of the sarcosine containing macrocycle **19b**, the *trans*-rotamer of macrocyclic diacid **22** showed minor but significant changes in chemical shift values and coupling constants for conserved residues. The differences were most pronounced in the signals for the (2*R*)-Glu and (2*R*)-Asp residues (residues 2 and 4) that flank the variant residue. These results indicate that the introduction of (2*S*)-Pro at position 3 causes local changes, but not global changes, to the conformation of the macrocycle, in accord with expectations. These conclusions are supported by the biological activity of the analogues, as is described below.

Biological activity

Each of the three synthetic macrocyclic diacids, **19a**, **19b**, and **22** were tested as inhibitors for recombinant $PP1_{cat}$ using a new assay protocol involving the dephosphorylation of the synthetic phosphopeptide [*acetyl*-^{14}C]-Ac-Arg-Arg-Ala-Thr(P)-Val-Ala (described in the following article). The assay allows the determination of V and K_M under a wide range of conditions and, importantly, for the purposes of evaluating inhibitors, allows the mode of inhibition to be determined. Previously it has been common practice to report only IC_{50} values and, unfortunately, such values are of limited use in the determination of inhibition mechanism or in allowing comparisons of enzyme activity under differing conditions. A full consideration is given in the following article.[11]

All three nodularin analogues were competitive inhibitors, in accord with the results obtained for nodularin (see following article[11]). The non-methylated macrocycle containing Sar at position 3, **19a** displayed a K_i value of 4000 ± 1000 μmol dm^{-3}. This compares with an IC_{50} value of 2900 μmol dm^{-3} obtained for the 4-benzylpiperidine amide analogue **1**, described previously. Thus, the phenethenyl side chain at C-3 in **19a** appears to confer a similar level of binding affinity to the macrocycle as the 4-benzylpiperidine amide, given that detailed analysis is complicated by differing conformational preferences. The methylated homologue **19b** was 20-fold more active than macrocycle **19a** and gave a K_i value of 206 ± 30 μmol dm^{-3}. Thus, the presence of the methyl group significantly enhances binding to $PP1_{cat}$. The methyl homologue and the non-methylated macrocycle possess very similar solution-phase structures and both exist predominantly in the *trans*-rotameric form at the γ-Glu-Sar amide bond. The large differences in inhibitory efficacy, therefore, appear to be largely due to solvent exclusion, rather than due to conformational preferences alone. Interestingly there is a well-defined hydrophobic methyl group binding pocket at the active site of $PP1_{cat}$ formed by Ile-130 and Tyr-134 in the X-ray crystal structures of microcystin- $PP1_{cat}$ complexes. It would appear, therefore, that nodularin does bind in a highly conserved manner where the hydrophobic Adda contacts, emanating from C-2 and C-3, and the bridged water molecule binding interactions with the carboxylate moieties and the metal ions are similar to those for microcystin. This same methyl binding pocket also appears to be utilised in conferring substrate specificity to the phosphatases PP1 and PP2A. While no structural information is yet available for substrate binding, it is known that phosphothreonine residues are preferred over phosphoserine residues in small peptides.[31] Moreover, our recent mechanistic studies,[12] which indicate that phosphothreonyl peptide hydrolysis should occur by direct attack of a μ-bound hydroxide ion, with inversion of configuration at phosphorus, place the threonine 3-methyl group in the same hydrophobic pocket.

The (2*S*)-proline containing analogue **22** was also a competitive inhibitor and gave a K_i value of 400 ± 75 μmol dm^{-3}. Thus, the perturbations in the conformation of the macrocycle introduced through the inclusion of a pyrrolidine ring, as determined by NMR spectroscopy (see above), did not undermine the enhanced affinity for the protein mediated by the extra methyl group. It is interesting to recall that the macrocyclic benzylpiperidine amides, which differed in the nature of the residue at position 3 (described in the previous article[10]), showed similar IC_{50} values of 2900 and 2700 μmol dm^{-3} respectively. However, it was not possible to deconvolute the rotameric and conformational contributions to the solution structures of the macrocycles. It should be noted that the Sar residue in **19b** cannot adopt a low energy conformation in which the *N*-methyl group and the 2-*pro*-*S* proton are nearly eclipsed, as in a conformation that mimics that for (2*S*)-proline. Thus, the two macrocycles *must* exist in different conformations about residues 2,3 and 4, as was observed by NMR spectroscopy.

These results are exciting for two reasons. First, it would appear that the structures of the Adda and (2*R*)-Glu and (2*R*)-Asp residues, residues 1,2 and 4, respectively, are largely responsible for conferring inhibitory activity. As such, it appears that anything that would fit in the active site at position 3 will allow binding. [It is useful to recall that the structure at position 5 varies considerably in known natural products and is not important for activity.[13]] The fact that microcystin is a heptaisopeptidic macrolactam in which the two extra residues occur between residues 3 and 4 in nodularin is consistent with this analysis. Moreover, the observation that the (2*S*)- and (2*R*)-*N*-methylalanine[13] epimers of dihydromicrocystin were as active as the parent microcystin further suggests that the nature of the residue at and around position 3 is not crucial. Second, the (2*S*)-proline structure itself provides a conformationally stable and well-defined substructure to which additional functional groups can be appended. There are large differences in size and charge between the two target phosphatases, PP1 and PP2A, in the vicinity of the region adjacent to nodularin residue 3 in modelled bound complexes. The results of the work presented here provide confidence to the validity of these modelled complexes and indicate that selectively modified (2*S*)-Pro analogues might give specific inhibitors of both PP1 and PP2A. Work towards these ends is in progress.

Experimental

Elemental microanalyses were performed in the departmental micro-analytical laboratory. NMR spectra were recorded on a Bruker AM-300 spectrometer (^{1}H, 300 MHz; ^{13}C, 75.4 MHz), a Bruker AMX-400 spectrometer (^{1}H, 400 MHz; ^{13}C, 100.6 MHz), or a Bruker DRX 500 spectrometer (^{1}H, 500 MHz; ^{13}C, 125 MHz). Chemical shifts are described in parts per million downfield shift from $SiMe_4$ and are reported consecutively as position (δ_H or δ_C), relative integral, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = double of doublets, sep = septet, m = multiplet, and br = broad), coupling constant (*J*/Hz) and assignment (numbering according to the IUPAC nomenclature for the compound).^{1}H-NMR spectra were referenced internally on ^{2}HOH (δ 4.68), $CHCl_3$ (δ 7.27) or $(CH_3)_2SO$ (δ 2.47). ^{13}C-NMR spectra were referenced on C^2HCl_3 (δ 77.5) or $(CH_3)_2SO$ (δ 39.70). Pyrrolidine ring carbons and hydrogens are assigned in NMR spectra as α,β,γ,δ, going anticlockwise from the ring nitrogen, according to normal convention. Where more than one conformational isomer could be detected in the NMR spectrum due to the presence of a tertiary amide moiety, these are assigned as *c* (*cis*) or *t* (*trans*), according to the rotameric state of the amide bond. Assignments for proline were aided by COSY, TOCSY and HSQC NMR experiments and by comparison with well established ^{13}C-NMR differences for the β- and γ-CH_2 signals.[32,33] Sarcosine *cis*/*trans* isomers were assigned on the basis of their ^{1}H/^{13}C NMR chemical shifts, where the *trans* isomer showed upfield

resonances relative to the *cis* isomer in the ^{1}H-NMR spectra. IR spectra were recorded on Perkin-Elmer 1710 or Nicolet Avatar 360 FT-IR spectrometers. The samples were prepared as Nujol mulls, solutions in chloroform or thin films between sodium chloride discs. The frequencies (ν) as absorption maxima are given in wavenumbers (cm^{-1}) relative to a polystyrene standard. Mass spectra and accurate mass measurements were recorded on a VG 70-250 SE, a Kratos MS-50, VG Zabspec or VG Prospec spectrometer. Fast atom bombardment spectra were recorded using glycerol as a matrix. CI spectra were recorded using ammonia as a reagent. Major fragments were given as percentages of the base peak intensity (100%). Flash chromatography was performed according to the method of Still *et al.*[34] using Fluka Kieselgel C60 (40–60 μm mesh) silica gel. Analytical thin layer chromatography was carried out on 0.25 mm precoated silica gel plates (Whatman PE SIL G/UV) and compounds were visualised using UV fluorescence or ethanolic phosphomolybdic acid. Melting points were taken on an Electrothermal melting point apparatus and are uncorrected. Optical rotations were measured at 24 °C on a Optical Activity AA-1000 polarimeter using 10 or 20 cm path length cells and are given in units of 10^{-1} deg cm^2 g^{-1}.

Preparative RP HPLC was carried out using a PerSeptive BioCAD™ SPRINT™ Perfusion® Chromatography System. Preparative RP HPLC was performed on a Luna C-18(2) 10 μm column (150 × 21.2 mm) or on a Luna C-18(2) 10 μm column (250 × 21.2 mm) fitted with a Luna C-18(2) 10 μm column (60 × 21.2 mm).

Protected amino acid precursors were purchased from Calbiochem-Novabiochem (UK) Ltd (Beeston, Nottingham). All other chemicals were of analytical grade or were recrystallised or redistilled before use. The solvents used were either distilled or of Analar quality and petroleum ether refers to that portion boiling between 40–60 °C. Solvents were dried according to literature procedures. Ethanol and methanol were dried using magnesium turnings. DMF, CH_2Cl_2 and diisopropylamine were distilled over CaH_2. THF and diethyl ether were dried over sodium–benzophenone and distilled under nitrogen.

General solid-phase peptide synthesis and peptide removal from Wang resin

Solid-phase synthesis of the pentapeptides was carried out using a Ranin PPS automated peptide synthesiser. The synthesis employed Fmoc chemistry and the C-terminal and amino acids were linked to α-methyl (2*R*)-*N*-(fluoren-9-ylmethoxycarbonyl)aspartyl-Wang resin. A four-fold excess of the amino acid was used for each coupling procedure. The *N*-α-Fmoc group was deprotected using a 20% piperidine–DMF solution and the activation was achieved using a 5% NMM–DMF solution. Once SPPS was complete the resin was placed in a sintered glass funnel washed sequentially with DMF, acetic acid, CH_2Cl_2 and finally methanol. The resin was then dried *in vacuo* for several hours. The dried resin was then treated with a mixture of 95% TFA–2.5% water–2.5% TES (5 cm^3) for 1 h. The resin was filtered and washed through with TFA (5 cm^3). The filtrate was concentrated under reduced pressure until an oil was obtained. The flask was placed in an ice bath and after the addition of diethyl ether (15 cm^3) the mixture was stirred for 10 min. The suspension was allowed to settle and the diethyl ether was decanted off using a pipette. The last traces of diethyl ether were removed by flushing the flask with argon for a few minutes. The peptide was then lyophilised to yield the product as a white solid.

General cyclisation procedure using BOP-Cl

To a stirred solution of the pentapeptide (350 mg, 0.6 mmol) in CH_2Cl_2–DMF (9:1) (350 cm^3) was added DIPEA (150 mm^3, 1.5 mmol) and the mixture was cooled down to 0 °C. BOP-Cl (156 mg, 0.66 mmol) was added and the mixture stirred at 0 °C for 6 h. The solution was allowed to warm to room temperature and then stirred for a further 7 days. The reaction mixture was concentrated under reduced pressure, redissolved in ethyl acetate (50 cm^3), and then washed with 10% citric acid (2 × 25 cm^3), 5% sodium bicarbonate (2 × 25 cm^3), distilled water (2 × 25 cm^3) and then brine (1 × 25 cm^3). The organic phase was dried ($MgSO_4$) and the solvent removed under reduced pressure to a volume of 1 cm^3. The product was obtained as a white solid after trituration with diethyl ether (10 cm^3).

Methyl (2*E*,4*E*)-5-phenylpenta-2,4-dienoate 3

To an ice-cooled suspension of hexane-washed sodium hydride (60% dispersion; 4 g, 0.1 mol) in THF (150 cm^3) was slowly added methyl diethylphosphonoacetate (19 cm^3, 0.1 mol) and the mixture was then stirred for 15 min. The mixture was cooled to −78 °C and after the dropwise addition of cinnamaldehyde (12.6 cm^3, 0.1 mol) stirring was continued for a further 20 min and then the mixture was allowed to warm up to room temperature. The resulting mixture was quenched with saturated aqueous NH_4Cl (100 cm^3) and then extracted with diethyl ether (2 × 100 cm^3). The organic phase was washed with brine (50 cm^3), dried ($MgSO_4$) and concentrated under reduced pressure to give the crude product as an off-white solid. Purification by flash silica chromatography (petroleum ether–ethyl acetate; 9:1) gave the dienoate **3** as a white solid (17.5 g, 93%), mp 68–70 °C (lit.,[35] 67–68 °C); ν_{max}(thin film)/cm^{-1} 1709 (CO, ester) and 1626 (C=C, alkene); δ_H(300 MHz; C^2HCl_3) 3.78 (3 H, s, OCH_3), 6.02 (1 H, d, *J* 16.0, 2-H), 6.87 (2 H, m, 4-H and 5-H) and 7.25–7.60 (6 H, m, Ar-H and 3-H); δ_C(75.4 MHz; C^2HCl_3) 51.6 (OCH_3), 120.8, 126.2, 127.2, 128.8 and 129.1 (Ar-CH and C=C), 136.0 (Ar-C quaternary), 140.6 and 144.8 (Ar-CH) and 167.5 (CO, ester); *m*/*z* (EI) 188 (60%, M^+), 157 (30, $[M - OCH_3]^+$) and 129 (100, $[M - CO_2CH_3]^+$).

Methyl (3*R*,4*E*,α*S*)-3-(*N*-benzyl-*N*-α-methylbenzylamino)-5-phenylpent-4-enoate 4a

A stirred solution of (*S*)-*N*-benzyl-*N*-α-methylbenzylamine (3.34 cm^3, 16 mmol) in dry THF (40 cm^3) was cooled to −78 °C and butyllithium (15 mmol, 6 cm^3 of 2.5 mol dm^{-3} solution in hexanes) was slowly added. The resulting pink solution of the lithium amide was stirred for 30 min, and then a solution of methyl (2*E*,4*E*)-5-phenylpenta-2,4-dienoate **3** (2.1 g, 11 mmol) in THF (15 cm^3) was added dropwise. Stirring was continued for 2 h at −78 °C and then the reaction was quenched by the slow addition of saturated aqueous NH_4Cl (5 cm^3). The mixture was allowed to warm up to room temperature and then partitioned between brine (30 cm^3) and diethyl ether (30 cm^3). The organic layer was separated, dried ($MgSO_4$), and then concentrated under reduced pressure to furnish the crude conjugate adduct as a 3:1 ratio of the required 1,4 addition product and 1,2-1,4 bis-addition product respectively. Purification by flash silica chromatography (petroleum ether–ethyl acetate; 9:1) gave the conjugate adduct **4a** as a colourless oil (2.7 g, 61%) (Found C, 81.0; H, 7.5; N, 3.3. $C_{27}H_{29}NO_2$ requires C, 81.2; H, 7.3; N, 3.5%) (HRMS: found $[M + Na]^+$, 422.2084. $C_{27}H_{29}NO_2Na$ requires 422.2096); $[\alpha]_D$ +89 (*c* 10 in CH_2Cl_2); ν_{max}(thin film)/cm^{-1} 1733 (CO, ester) and 1626 (C=C, alkene); δ_H(300 MHz; C^2HCl_3) 1.40 (3 H, d, *J* 7.0, CH_3), 2.42 (1 H, dd, *J* 7.7 and 14.3, 1 H of CH_2CO), 2.59 (1 H, dd, *J* 7.2 and 14.4, 1 H of CH_2CO), 3.48 (3 H, s, OCH_3), 3.72 and 3.77 (2 H, AB system, *J* 11.6, PhCH_2N), 3.97 (1 H, q, *J* 7.0, Ph$CH$$CH_3$N), 4.08 (1 H, m, N$CH$$CH_2$), 6.30 (1 H, dd, *J* 7.5 and 16.0, 4-H), 6.43 (1 H, d, *J* 16.0, 5-H) and 7.20–7.40 (15 H, m, Ar-H); δ_C(75.4 MHz; C^2HCl_3) 17.12 (CH_3), 39.11 (CH_2CO), 50.77 (CH_2N), 51.96 (OCH_3), 57.01 and 57.36 (2 × CHN), 121.25 (ArCHCH), 126.80, 127.20, 127.65, 127.99, 128.34, 128.49, 128.65, 128.94, 129.04, 129.26, 129.53, 130.27 and 131.74 (Ar-CH), 137.44, 141.33 and 144.48 (Ar-C quaternary) and 172.39 (CO, ester);

m/*z* (CI) 400 (100%, $[M + H]^+$), 296 (10, $[M + H - PhCHCH_3]^+$).

Methyl (2*S*,3*S*,4*E*,α*S*)-2-methyl-3-(*N*-benzyl-*N*-α-methylbenzylamino)-5-phenylpent-4-enoate 4b

A solution of methyl (3*R*,4*E*,α*S*)-3-(*N*-benzyl-*N*-α-methylbenzylamino)-5-phenylpent-4-enoate **4a** (5.5 g, 14 mmol) in THF (10 cm^3) was added dropwise to a stirred solution of lithium diisopropylamide (18 mmol) in THF (35 cm^3) at −78 °C. The reaction mixture was stirred for 1 h at −78 °C and then methyl iodide (6 cm^3, 90 mmol) was added. The reaction mixture was allowed to warm up to room temperature overnight and then quenched with saturated aqueous NH_4Cl solution (40 cm^3). The mixture was then partitioned between diethyl ether (40 cm^3) and brine (40 cm^3). The organic layer was separated, dried ($MgSO_4$) and solvent removed under reduced pressure to yield the crude product as a 4:1 ratio of the *anti* and *syn* diastereoisomers respectively. Flash silica chromatography (petroleum–ethyl acetate; 30:1) gave the unwanted (2*R*,3*S*) diastereoisomer product (800 mg), followed by product **4b** as a oil (3.2 g, 57%). Recrystallisation from a cold methanol–water (9:1) mixture gave **4b** as a crystalline solid. Major diastereoisomer **4b**: mp 80–82 °C (HRMS: found $[M + Na]^+$, 436.2243. $C_{28}H_{31}NO_2Na$ requires 436.2252); $[\alpha]_D$ +128 (*c* 10 in CH_2Cl_2); ν_{max}(thin film)/cm^{-1} 1733 (CO, ester) and 1626 (C=C, alkene); δ_H(300 MHz; C^2HCl_3) 0.90 (3 H, d, *J* 6.8, CH_3CHCO), 1.37 (3 H, d, *J* 6.8, $PhCHCH_3$), 2.79 (1 H, dq, *J* 6.9 and 10.7, CHCO), 3.43 (3 H, s, OCH_3), 3.47 (1 H, dd, *J* 10.2 and 10.3, NCHCH), 3.60 and 3.84 (2 H, AB system, *J* 11.6, $PhCH_2N$), 4.14 (1 H, q, *J* 6.8, $PhCHCH_3$), 6.11 (1 H, dd, *J* 9.5 and 16.0, 4-H), 6.32 (1 H, d, *J* 16.0, 5-H) and 7.20–7.40 (15 H, m, Ar-H); δ_C(75.4 MHz; C^2HCl_3) 16.20 and 16.52 (2 × CH_3), 43.70 (CHCO), 50.22 (CH_2N), 51.84 (OCH_3), 56.03 (ArCHN), 64.15 ($CHCHNH$), 126.80, 127.23, 127.40, 128.07, 128.27, 128.51, 129.15 and 129.49 (Ar-CH and C=C), 137.36, 140.58 and 144.72 (Ar-C quaternary) and 176.00 (CO, ester); *m*/*z* (CI) 414 (100%, $[M + H]^+$) and 310 (10, $M + H - PhCHCH_3]^+$).

Methyl (3*S*)-3-amino-5-phenylpentanoate 5a

To a stirred solution of methyl (3*R*,4*E*,α*S*)-3-(*N*-benzyl-*N*-α-methylbenzylamino)-5-phenylpent-4-enoate **4a** (6.0 g, 20 mmol) in methanol (100 cm^3) was added Pearlman's catalyst (1.0 g) and ammonium formate (1.5 g, 48 mmol). The mixture was heated under reflux for 45 min, allowed to cool down, filtered through a plug of Celite and the filtrate concentrated under reduced pressure to give a viscous oil. This oil was dissolved in 10% aqueous $NaHCO_3$ (30 cm^3) and extracted with CH_2Cl_2 (50 cm^3). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to yield the product amine **5a** as a colourless liquid (2.4 g, 77%). For analytical purposes a small amount of the amine was chromatographed on silica gel (CH_2Cl_2–methanol; 95:5). R_f = 0.2 (HRMS: found $[M + H]^+$, 208.1343. $C_{12}H_{17}NO_2$ requires 208.1338); $[\alpha]_D$ −1.6 (*c* 1 in $CHCl_3$); ν_{max}(CH_2Cl_2)/cm^{-1} 3375 (NH_2) and 1732 (CO, ester); δ_H(300 MHz; C^2HCl_3) 1.55 (2 H, br s, NH_2), 1.61–1.85 (2 H, m, $PhCH_2CH_2$), 2.29–2.55 (2 H, m, CH_2CO), 2.63–2.83 (2 H, m, $PhCH_2$), 3.18–3.29 (1 H, m, NH_2CH), 3.68 (3 H, s, OCH_3) and 7.20–7.32 (5 H, m, Ar-H); δ_C(75.4 MHz; C^2HCl_3) 32.17 ($ArCH_2CH_2$), 39.06 ($ArCH_2CH_2$), 42.20 (CH_2CO), 47.70 ($CHNH_2$), 51.25 (OCH_3), 125.6, 128.05 and 128.16 (Ar-CH), 141.56 (Ar-C quaternary), and 172.54 (CO, ester); *m*/*z* (FAB) 208 (100%, $[M + H]^+$).

Methyl (2*S*,3*S*)-3-amino-2-methyl-5-phenylpentanoate 5b

This compound was prepared in a manner identical to that described for **5a** using methyl (2*S*,3*S*,4*E*,α*S*)-2-methyl-3-(*N*-benzyl-*N*-α-methylbenzylamino)-5-phenylpent-4-enoate **4b** (2.4 g, 6 mmol) to give the product amine **5b** as a colourless liquid (0.9 g, 71%). For analytical purposes a small amount of the amine was chromatographed on silica gel (CH_2Cl_2–methanol; 95:5) (HRMS: found $[M + Na]^+$, 244.1311. $C_{13}H_{19}NO_2Na$ requires 244.1313); $[\alpha]_D$ −2.4 (*c* 10 in CH_2Cl_2); ν_{max}(thin film)/cm^{-1} 3387 (NH_2) and 1728 (CO, ester); δ_H(300 MHz; C^2HCl_3) 1.18 (3 H, d, *J* 7.0, CH_3CH), 1.40 (2 H, s, NH_2), 1.47–1.65 (1 H, m, 1 H of $PhCH_2CH_2$), 1.75–1.90 (1 H, m, 1 H of $PhCH_2CH_2$), 2.49 (1 H, dq, *J* 6.8 and 7.0, CHCO), 2.65–2.85 (1 H, m, 1 H of $PhCH_2$), 2.89–2.95 (1 H, m, NH_2CH), 3.69 (3 H, s, OCH_3) and 7.20–7.32 (5 H, m, Ar-H); δ_C(75.4 MHz; C^2HCl_3) 14.57 (CH_3), 32.86 ($ArCH_2CH_2$), 37.26 ($ArCH_2CH_2$), 46.45 (CHCO), 51.83 (OCH_3), 53.96 (CHNH), 126.20 and 128.73 (Ar-CH), 142.27 (Ar-C quaternary) and 176.20 (CO, ester); *m*/*z* (CI) 222 (100%, $[M + H]^+$).

Methyl (3*S*)-3-(*N*-*tert*-butoxycarbonylamino)-5- phenylpentanoate 7a

To a stirred solution of methyl (3*S*)-3-amino-5-phenylpentanoate **5a** (4.6 g, 22 mmol) in dioxane (8 cm^3) was a solution of Na_2CO_3 (2.3 g, 0.6 mol) in water (16 cm^3) added. The mixture was cooled on an ice bath and di-*tert*-butyl dicarbonate (5.5 g, 25 mmol) was slowly added. The solution was allowed to warm up to room temperature and stirred for 3 h after which time a heavy white precipitate had appeared. Most of the solvent was removed under reduced pressure and the residue dissolved in ethyl acetate (40 cm^3). The organic layer was washed successively with water (50 cm^3), 0.5 mol dm^{-3} HCl solution (50 cm^3) and brine (50 cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by flash silica chromatography (petroleum ether–ethyl acetate; 9:1) to give the Boc-protected amine **7a** as a colourless oil which solidified upon drying *in vacuo* to a white solid (5.5 g, 82%). R_f = 0.35; mp 55–57 °C (lit.,[36] 64–66 °C) (Found C, 66.7; H, 8.5; N, 4.5. $C_{17}H_{25}NO_4$ requires C, 66.4; H, 8.2; N, 4.6%) (HRMS: found $[M + Na]^+$, 330.1695 $C_{17}H_{25}NO_4Na$ requires 330.1681); $[\alpha]_D$ −9.5 (*c* 1.8 in $CHCl_3$); ν_{max}(CH_2Cl_2)/cm^{-1} 3360 (NH, urethane), 1732 (CO, ester) and 1712 (CO, urethane); δ_H(300 MHz; C^2HCl_3) 1.42 [9 H, s, $C(CH_3)_3$], 1.77–1.91 (2 H, m, $PhCH_2CH_2$), 2.50–2.85 (4 H, m, $PhCH_2CH_2$ and CH_2CO), 3.68 (3 H, s, OCH_3), 3.90–4.01 (1 H, m, NHCH), 4.97 (1 H, d, *J* 9.9, NH) and 7.13–7.31 (5 H, m, Ar-H); δ_C(75.4 MHz; C^2HCl_3) 28.42 [$C(CH_3)_3$], 32.62 ($ArCH_2CH_2$), 36.48 ($ArCH_2CH_2$), 39.21 (CH_2CO), 47.43 (CHNH), 51.70 (OCH_3), 79.34 [$OC(CH_3)_3$], 125.98, 128.38 and 128.45 (Ar-CH), 141.47 (Ar-C quaternary), 155.38 (CO, urethane) and 172.08 (CO, ester); *m*/*z* (ES) 330 (100%, $[M + Na]^+$), 274 (90, $[M + Na + H - C_4H_9]^+$) and 230 (10, $[M + H + Na - C_5H_9O_2]^+$).

Methyl (2*S*,3*R*)-3-(*N*-*tert*-butoxycarbonylamino)-2-methyl-5-phenylpentanoate 7b

This compound was prepared in a manner identical to that described for **7a** using methyl (2*S*,3*S*)-3-amino-2-methyl-5-phenylpentanoate **5b** (2.7 g, 12 mmol) to yield the Boc-protected amine **7b** as a colourless oil which solidified upon drying *in vacuo* to a white solid (2.8 g, 72%), mp 62–64 °C (Found C, 67.2; H, 8.4; N, 4.3. $C_{18}H_{27}NO_4$ requires C, 67.3; H, 8.5; N, 4.4%) (HRMS: found $[M + Na]^+$, 344.1833 $C_{18}H_{27}NO_4Na$ requires 44.1838); $[\alpha]_D$ +2.1 (*c* 4 in CH_2Cl_2); ν_{max}(CH_2Cl_2)/cm^{-1} 3433 (NH, urethane) and 1729 (CO, ester and Boc); δ_H(300 MHz; C^2HCl_3) 1.21 (3 H, d, *J* 7.2, CH_3CH), 1.47 [9 H, s, $C(CH_3)_3$], 1.70–1.80 (2 H, m, $PhCH_2CH_2$), 2.60–2.78 (3 H, m, $PhCH_2CH_2$ and CHCO), 3.67 (3 H, s, OCH_3), 3.75–3.81 (1 H, m, NHCH), 5.21 (1 H, d, *J* 9.9, NH) and 7.20–7.32 (5 H, m, Ar-H); δ_C(75.4 MHz; C^2HCl_3) 15.07 (CH_3), 28.82 [$C(CH_3)_3$], 33.12 ($ArCH_2CH_2$), 36.61 ($ArCH_2CH_2$), 43.27 (CHCO), 52.07 (OCH_3), 52.88 (CHNH), 79.51 [$OC(CH_3)_3$], 126.30 and 128.81 (Ar-CH), 142.12 (Ar-C quaternary), 156.47 (CO, urethane) and 176.20 (CO, ester); *m*/*z* (ES) 344 (100%, $[M + Na]^+$), 288 (48, $[M + Na - C_4H_9]^+$).

Methyl (3*R*,4*E*)-3-(*N*-*tert*-butoxycarbonylamino)-5-phenylpent-4-enoate 8a

Under an atmosphere of argon, a solution of *N*-Boc ester **7a** (4.0 g, 13 mmol) in dry carbon tetrachloride (50 cm^3) was treated with *N*-bromosuccinimide (2.6 g, 15 mmol). The reaction mixture was illuminated for 3 h under a 60 W tungsten lamp held ~2 cm away from the flask while maintaining the temperature at less than 10 °C. The reaction was judged complete when the initial red–brown colour due to bromine faded to a pale yellow colour. The precipitated succinimide was filtered off and the filtrate was concentrated under reduced pressure to give the crude bromide as a mixture of epimers. To the resulting crude bromide was then added DBU (6.0 cm^3, 38 mmol) and the mixture heated to 70 °C for 15 min. The mixture was partitioned between diethyl ether (30 cm^3) and water (30 cm^3). The organic layer was washed successively with water (3 × 40 cm^3) and brine (40 cm^3), dried ($MgSO_4$) and concentrated under reduced pressure to give the alkene as a dark oil. Purification by flash silica column chromatography (ethyl acetate–petroleum ether; 9:1) gave the alkene product **8a** as an oil which slowly solidified to a white solid upon drying *in vacuo* (1.70 g, 42%), mp 65–67 °C (Found C, 67.0; H, 7.7; N, 4.4. $C_{17}H_{23}NO_4$ requires C, 66.9; H, 7.6; N, 4.6%) (HRMS: found $[M + Na]^+$, 328.1531. $C_{17}H_{23}NO_4Na$ requires 328.1525); $[\alpha]_D$ +22.7 (*c* 2.2 in $CHCl_3$); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3353 (NH, urethane), 1736 (CO, ester) and 1712 (CO, urethane); δ_H(300 MHz; C^2HCl_3) 1.46 [9 H, s, $C(CH_3)_3$], 2.67–2.74 (2 H, m, CH_2CO), 3.70 (3 H, s, OCH_3), 4.62–4.78 (1 H, m, NHC*H*), 5.23–5.37 (1 H, m, NH), 6.19 (1 H, dd, *J* 6.2 and 15.7, 4-H), 6.55 (1 H, d, *J* 15.7, 5-H) and 7.20–7.38 (5 H, m, Ar-H); δ_C(75.4 MHz; C^2HCl_3) 28.41 [$C(CH_3)_3$], 39.54 (CH_2CO), 49.13 (CHNH), 51.83 (OCH_3), 79.68 [$OC(CH_3)_3$], 126.52, 127.75, 128.55, and 130.89 (Ar-CH and alkene C), 136.46 (Ar-C quaternary), 155.07 (CO, urethane) and 171.63 (CO, ester); *m*/*z* (ES) 328 (100%, $[M + Na]^+$) and 272 (45, $[M + Na + H - C_4H_9]^+$).

Methyl (2*S*,3*S*,4*E*)-3-(*N*-*tert*-butoxycarbonylamino)-2-methyl-5-phenylpent-4-enoate 8b

This compound was prepared in a manner identical to that described for **7a** using methyl (2*S*,3*S*)-3-(*N*-*tert*-butoxycarbonylamino)-2-methyl-5-phenylpentanoate **7b** to give the product alkene **8b** as an oil which slowly solidified upon drying *in vacuo* to a white solid (1.25 g, 45%), mp 52–56 °C (HRMS: found $[M + Na]^+$, 342.1687. $C_{18}H_{27}NO_4Na$ requires 342.1681); $[\alpha]_D$ +38.8 (*c* 10 in CH_2Cl_2); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3433 (NH, urethane) and 1712 (CO, ester and urethane); δ_H(300 MHz; C^2HCl_3) 1.27 (3 H, d, *J* 7.0, CH_3CH), 1.46 [9 H, s, $C(CH_3)_3$], 2.75–2.85 (1 H, m, CHCO), 3.70 (3 H, s, OCH_3), 4.47 (1 H, m, NHC*H*), 5.41–5.45 (1 H, m, NH), 6.11 (1 H, dd, *J* 6.4 and 16.0, 4-H), 6.56 (1 H, d, *J* 16.0, 5-H) and 7.20–7.35 (5 H, m, Ar-H); δ_C(75.4 MHz; C^2HCl_3) 15.02 (CH_3), 28.87 [$C(CH_3)_3$], 44.24 (*C*HCO), 52.26 (OCH_3), 55.03 (CHNH), 79.96 [$OC(CH_3)_3$], 126.96, 127.10, 128.72, 128.98 and 131.57 (Ar-CH and alkene C), 137.04 (Ar-C quaternary), 156.02 (CO, urethane) and 175.66 (CO, ester); *m*/*z* (ES) 342 (100%, $[M + Na]^+$) and 286 (31, $[M + Na - C_4H_9]^+$).

(3*R*,4*E*)-3-(*N*-*tert*-Butoxycarbonylamino)-5-phenylpent-4-enoic acid 9a

To a cooled solution of *N*-Boc unsaturated ester **8a** (1.4 g, 4.5 mmol) in methanol–water–THF (3:1:1) (45 cm^3) was added a solution of lithium hydroxide (800 mg, 31 mmol) in water (3 cm^3) and the mixture stirred at 0 °C for 3 h. The solution was concentrated under reduced pressure to 2–3 cm^3, diluted with water (40 cm^3), acidified to pH 2 with HCl (0.1 mol dm^{-3}) and then extracted with ethyl acetate (45 cm^3). The organic layer was washed with brine (30 cm^3), dried ($MgSO_4$) and solvent removed under reduced pressure to give an oily residue. Purification by flash silica column chromatography (ethyl acetate–petroleum ether; 70:30) gave the free acid **9a** as a white solid (0.95 g, 71%), R_f = 0.15, mp 123–125 °C (Found C, 66.0; H, 7.3; N, 4.75. $C_{16}H_{21}NO_4$ requires C, 65.95; H, 7.25; N, 4.8%) (HRMS: found $[M + Na]^+$, 314.1374. $C_{16}H_{21}NO_4Na$ requires 314.1368); $[\alpha]_D$ +36.6 (*c* 1.1 in $CHCl_3$); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3322 (NH, urethane) and 1711 (CO, acid and urethane); δ_H(300 MHz; C^2HCl_3) 1.46 [9 H, s, $C(CH_3)_3$], 2.65–2.82 (2 H, m, CH_2CO), 4.62–4.78 (1 H, m, NHC*H*), 5.20–5.38 (1 H, m, NH), 6.21 (1 H, dd, *J* 6.25 and 15.8, 4-H), 6.57 (1 H, d, *J* 15.8, 5-H) and 7.20–7.42 (5 H, m, Ar-H); δ_C(75.4 MHz; C^2HCl_3) 28.40 [$C(CH_3)_3$], 39.48 (CH_2CO), 49.50 (CHNH), 79.78 [$OC(CH_3)_3$], 126.55, 127.81, 128.23, 128.58 and 131.11 (Ar-CH and CH=CH), 136.39 (Ar-C quaternary), 155.34 (CO, urethane) and 174.63 (CO, acid); *m*/*z* (ES) 338 (20%, $[M + 2Na - H]^+$), 314 (100, $[M + Na]^+$) and 258 (45, $[M + Na + H - C_4H_9]^+$).

(2*S*,3*S*,4*E*)-3-(*N*-*tert*-Butoxycarbonylamino)-2-methyl-5-phenylpent-4-enoic acid 9b

This compound was prepared in a manner identical to that described for **9a** using methyl (2*S*,3*S*,4*E*)-3-(*N*-*tert*- butoxycarbonylamino)-2-methyl-5-phenylpent-4-enoate **8b** (1.2 g, 11 mmol) to give the free acid **9b** as a colourless oil (1.1 g, 95%) (HRMS: found $[M + Na]^+$, 328.1523. $C_{17}H_{23}NO_4Na$ requires 328.1525); $[\alpha]_D$ +50.4 (*c* 4 in CH_2Cl_2); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3421 (NH, urethane) and 1706 (CO, acid and urethane); δ_H(300 MHz; C^2HCl_3) 1.28 (3 H, d, *J* 7.17, CH_3CH), 1.47 [9 H, s, $C(CH_3)_3$], 2.79–2.90 (3 H, m, CH_3CH), 4.45–4.55 (1 H, m, NHC*H*), 5.45 (1 H, br d, NH), 6.13 (1 H, dd, *J* 9.0 and 15.8, 4-H), 6.57 (1 H, d, *J* 15.8, 5-H) and 7.20–7.35 (5 H, m, Ar-H); δ_C(75.4 MHz; C^2HCl_3) 15.01 (CH_3CH), 28.82 [$C(CH_3)_3$], 43.25 (*C*HCO), 56.25 (CHNH), 79.85 [$OC(CH_3)_3$], 126.98, 128.16, 128.99 and 131.84 (Ar-CH and CH=CH), 136.94 (Ar-C quaternary), 156.66 (CO, urethane) and 180.44 (CO, acid); *m*/*z* (ES) 350 (30%, $[M + 2Na - H]^+$), 328 (100, $[M + Na]^+$) and 272 (20, $[M + Na - C_4H_8]^+$).

(3*R*,4*E*)-3-Amino-5-phenylpent-4-enoic acid 10a

To an ice-cooled solution of the *N*-Boc unsaturated acid **9a** (1.8 g, 6.2 mmol) in dry CH_2Cl_2 (30 cm^3) was added TFA (20 cm^3). The solution was allowed to warm up to room temperature, stirred for a further 4 h and then concentrated under reduced pressure to an oil. CH_2Cl_2 (25 cm^3) was added and the mixture concentrated again under reduced pressure and the resulting residue was thoroughly dried *in vacuo* for several hours. The residue was dissolved in water (30 cm^3) and the resulting aqueous solution was washed with ether (30 cm^3). The aqueous layer was lyophilised to give the β-amino acid **10a** as a foamy white solid (1.55 g, 83%); mp 123–125 °C; $[\alpha]_D$ −7.6 (*c* 1 in water); ν_{max}(KBr disc)/cm^{-1} 1698 (NH) and 1650 (CO, acid); δ_H(300 MHz; ^2H_2O) 2.79 (2 H, d, *J* 6.6, CH_2CO), 4.20–4.30 (1 H, m, NHC*H*), 6.12 (1 H, dd, *J* 8.1 and 16.0, 4-H), 6.69 (1 H, d, *J* 16.0, 5-H) and 7.18–7.40 (5 H, m, Ar-H); δ_C(75.4 MHz; ^2H_2O) 39.30 (CH_2CO), 52.54 ($CHNH_2$), 125.11 (4-C), 129.43 and 131.54 (Ar-CH), 137.80 (Ar-C quaternary), 138.29 (5-C) and 176.08 (CO, acid); *m*/*z* (CI) 192 (100 %, $[M + H]^+$), 175 (45, $[M - NH_2]^+$) and 132 (40, $[M - CO_2H - NH_2]^+$).

(2*S*,3*S*,4*E*)-3-Amino-2-methyl-5-phenylpent-4-enoic 10b

This compound was prepared in a manner identical to that described for **10a** using (2*S*,3*S*,4*E*)-3-*N*-(*tert*-butoxycarbonylamino)-2-methyl-5-phenylpent-4-enoic acid **9b** (1.1 g, 5.3 mmol) to give the β-amino acid **10b** as a foamy white solid after lyophilisation (870 mg, 79%) (HRMS: found $[M + Na]^+$, 206.1188. $C_{12}H_{16}NO_2$ requires 206.1181); $[\alpha]_D$ −24.9 (*c* 8.6 in water); ν_{max}(KBr disc)/cm^{-1} 1696 (NH) and 1650 (CO, acid);

δ_H(300 MHz; ^2H_2O) 1.08 (3 H, d, *J* 7.17, C*H*$_3$CH), 2.75–2.95 (3 H, m, CH$_3$C*H*), 4.00–4.05 (1 H, m, NHC*H*), 6.03 (1 H, dd, *J* 9.0 and 15.8, 4-H), 6.67 (1 H, d, *J* 15.8, 5- H) and 7.20–7.35 (5 H, m, Ar-H); δ_C(75.4 MHz; $C^2H_3O^2H$) 14.21 (CH_3), 42.87 (*C*HCO), 56.45 (CHNH), 122.40, 127.91 and 130.00 (Ar-CH and ArCHCH), 138.24 (Ar-C quaternary) and 178.01 (CO, acid); *m*/*z* (CI) 206 (100%, $[M + H]^+$), 160 (17, $[M - CO_2H]^+$) and 145 (15, $[M - CO_2H - NH_2]^+$).

(3*R*,4*E*)-3-[*N*-(Fluoren-9-ylmethoxycarbonyl)amino]-5-phenylpent-4-enoic acid 11a

To an ice-cooled solution of (3*R*,4*E*)-3-amino-5-phenylpent-4-enoic **10a** (1.7 mg, 5.6 mmol) in dioxane–water (60 cm^3; 3:1) was added potassium carbonate (1.4 g, 10 mmol). A solution of Fmoc-Cl (2.0 g, 6.6 mmol) in dioxane (10 cm^3) was added. The resulting solution was warmed to room temperature and stirred for 12 h. The solution was concentrated under reduced pressure to one third of the original volume and water (35 cm^3) was added. The aqueous phase was acidified to pH 2 with 1 mol dm^{-3} HCl and then extracted with ethyl acetate (2 × 25 cm^3). The organic layers were combined, washed with brine (5 cm^3), dried ($MgSO_4$) and the solvent was removed under reduced pressure to give the crude product as an oil. Purification by flash silica chromatography (petroleum ether–ethyl acetate; 60:40) gave the Fmoc protected amine **11a** as a white solid (1.2 g, 52%), mp 167–171 °C (HRMS: found $[M + Na]^+$, 436.1529. $C_{26}H_{23}NO_4Na$ requires 436.1525); $[\alpha]_D$ +22.4 (*c* 8.6 in $CHCl_3$); $\nu_{max}(CH_2Cl_2)$/cm^{-1} 3310 (NH, urethane) and 1690 (CO, urethane); δ_H(300 MHz; C^2HCl_3) 2.60–2.85 (2 H, m, CH_2CO), 4.17 (1 H, t, *J* 6.60, Fmoc-C*H*), 4.30–4.60 (2 H, m, Fmoc-CHC*H*$_2$), 4.61–4.75 (1 H, m, NHC*H*), 5.39–5.47 (1 H, m, NH), 6.08–6.20 (1 H, m, 4-H), 6.50 (1 H, d, *J* 15, 5-H) and 7.15–7.75 (13 H, m, Ar-H); *m*/*z* (ES) 436 (100%, $[M + Na]^+$).

(2*S*,3*S*,4*E*)-3-[*N*-(Fluoren-9-ylmethoxycarbonyl)amino]-2-methyl-5-phenylpent-4-enoic acid 11b

This compound was prepared in a manner identical to that described for **11a** using (2*S*,3*S*,4*E*)-3-amino-2-methyl-5-phenylpent-4-enoic **10b** (800 mg, 3.2 mmol) to give the Fmoc-protected amine **11b** as a white solid (1.1 g, 66%), mp 147–151 °C (Found C, 75.8; H, 6.0; N, 3.2. $C_{27}H_{25}NO_4$ requires C, 75.9; H, 5.9; N, 3.3%) (HRMS: found $[M + Na]^+$, 704.2903. $C_{27}H_{25}NO_4Na$ requires 704.2908); $[\alpha]_D$ +41.5 (*c* 6.5 in EtOH); $\nu_{max}(CH_2Cl_2)$/cm^{-1} 3321 (NH, urethane) and 1712 (CO, ester and urethane); δ_H(300 MHz; C^2HCl_3) 1.30 (3 H, d, *J* 6.80, C*H*$_3$CH), 2.85–2.95 (1 H, m, CH$_3$C*H*), 4.17 (1 H, t, *J* 6.62, Fmoc-C*H*), 4.30–4.60 (3 H, m, Fmoc-CHC*H*$_2$ and NHC*H*), 5.56 (1 H, d, *J* 10.0, NH), 6.08 (1 H, dd, *J* 6.40 and 15.7, 4-H), 6.50 (1 H, d, *J* 15.63, 5-H) and 7.15–7.55 (15 H, m, Ar-H); δ_C(75.4 MHz; C^2HCl_3) 15.22 (CH_3), 44.10 (*C*HCO), 47.70 (Fmoc-C*H*CH$_2$), 55.47 (CHNH), 67.36 (Fmoc-CHC*H*$_2$), 120.43, 125.49, 127.04, 127.53, 127.75, 128.15, 128.34, 129.01 and 132.38 (Ar-CH and 2 × alkene), 136.64, 141.75 and 144.30 (Ar-C quaternary), 156.69 (CO, urethane) and 178.01 (CO, acid); *m*/*z* (ES) 450 (100%, $[M + Na]^+$).

(2*S*)-Phenylalanyl-[(3*S*,*E*)-3-phenylethenyl-3-aminopropanoyl]-[α-methyl (2*R*)-glutamyl]-γ-sarcosyl-[α-methyl (2*R*)-aspartate] diester 17a

This compound was synthesised on the peptide synthesiser from Fmoc-Asp-(α)-OMe-O-Wang **16** (1 g, 0.54 mmol loading) using the general SPPS method described above, followed by cleavage of the peptide from the resin to give the crude peptide **17a** as a white solid after lyophilisation (280 mg). For analytical purposes a small amount (~10 mg) was further purified by preparative RP HPLC using a Luna C-18 10 μm column (150 × 21.1 mm). The column was eluted under gradient conditions [100%A–0%B to 50%A–50%B (solvent A: 0.1% aqueous TFA; solvent B: 80:20 CH_3CN–H_2O with 0.1% TFA) over 25 minutes; flow rate 2 cm^3 min^{-1}] and the peak corresponding to retention time 19.6 min was collected and the solvent removed under reduced pressure and by lyophilisation to give the desired peptide. Mp 100–105 °C (softening point) (HRMS: found $[M + Na]^+$, 704.2903. $C_{34}H_{43}N_5O_{10}Na$ requires 704.2908); $[\alpha]_D$ +20 (*c* 0.2 in water); ν_{max}(KBr disc)/cm^{-1} 3411 br (COOH), 1737 (CO, esters) and 1672 (amides); δ_H[500 MHz; $(C^2H_3)_2SO$ mixture of rotamers] 1.78–2.01 [2 H, m, β-CH$_2$(Glu)], 2.25–2.45 [4 H, m, γ-CH$_2$ (Glu) and α-CH$_2$ (propanoyl)], 2.60–2.88 [2 H, m, β-CH$_2$(Asp)], 2.77 and 2.85 (3 H, 2 × s, NCH$_3$), 2.97–3.12 [2 H, m, β-CH$_2$(Phe)], 3.55, 3.57 and 3.65 (6 H, 3 × s, 2 × CH$_3$), 3.89–4.02 [2 H, m, CH$_2$ (Sar)], 4.05–4.12 [1 H, m, α-H, (Phe)], 4.23–4.32 [1 H, m, α-H, (Glu)], 4.61–4.70 [1 H, m, α-H, (Asp)], 4.80–4.89 [1 H, m, β-H, (propanoyl)], 6.20–6.27 [1 H, m, γ-H (propanoyl)], 6.50 [1 H, d, *J* 18.0, δ-H (propanoyl)], 7.16–7.44 (10 H, m, Ar-H), 8.18–8.24 [1 H, m, NH (Phe)], 8.34 [0.6 H, d, *J* 8.5, NH (Asp)], 8.42 [0.4 H, d, *J* 8.5, NH (Glu)], 8.46 [0.6 H, d, *J* 8.5, NH (Glu)], 8.56 [0.4 H, d, *J* 8.5, NH (Asp)] and 8.60 [0.6 H, d, *J* 8.5, NH (Asp)]; δ_C[125 MHz; $(C^2H_3)_2SO$ mixture of rotamers] 26.4 [β-CH$_2$ (Glu)], 28.3 and 28.5 [γ-CH$_2$ (Glu)], 35.7 [β-CH$_2$ (Asp)], 37.0 [β-CH$_2$ (Asp)], 39.6 [α-CH(propanoyl)], 34.1 and 35.5 (NCH$_3$), 48.4 [α-C (Asp)], 47.8 [β-C (propanoyl)], 49.5 and 51.3 [α-C (Sar)], 51.4 [α-C (Glu)], 51.4 and 51.6 (2 × CH$_3$), 53.5 [α-C (Phe)], 126.1, 127.1, 127.5, 128.1, 128.4 and 128.7 (Ar-CH and Ar-C quaternary), 128.6 and 129.4 [γ-and δ-C (propanoyl)], 134.8 [γ-C (Phe)] and 165.0, 166.9, 168.2, 169.3, 171.3, 171.8 and 172.3 (CO, amides and esters); *m*/*z* (ES) 726 (17%, $[M + 2Na - H]^+$), 704 (100, $[M + Na]^+$) and 682 (30, $[M + H]^+$).

(2*S*)-Phenylalanyl-[(2*S*,3*S*,*E*)-2-methyl-3-phenylethenyl-3-aminopropanoyl]-[α-methyl (2*R*)-glutamyl]-γ-sarcosyl-[α-methyl (2*R*)-aspartate] diester 17b

This compound was synthesised on the peptide synthesiser from Fmoc-Asp-(α)-OMe-O-Wang **16** (0.75 g, ~0.6 mmol loading) using the general SPPS method described above, followed by cleavage of the peptide from the resin to give the crude peptide **17b** as a white solid in quantitative yield after lyophilisation. For analytical purposes a small amount (~10 mg) was further purified by preparative RP HPLC using a Luna C-18 10 μm column (150 × 21.1 mm). The column was eluted under gradient conditions [100%A– 0%B to 40%A–60%B (solvent A: 0.1% aqueous TFA; solvent B: 80:20 CH_3CN–H_2O with 0.1% TFA) over 25 min; flow rate 2 cm^3 min^{-1}] and the peak corresponding to retention time 20.4 min was collected and the solvent removed under reduced pressure to give the desired peptide, mp 90–95 °C (softening point) (HRMS: found $[M + Na]^+$, 718.3054. $C_{35}H_{45}N_5O_{10}Na$ requires 718.3064); ν_{max}(KBr disc)/cm^{-1} 3426 br (COOH), 1737 (CO, esters) and 1672 (amides); δ_H[500 MHz; $(C^2H_3)_2SO$ mixture of rotamers] 0.90–0.95 [3 H, m, CH$_3$ (propanoyl)], 1.79–1.98 [2 H, m, β-CH$_2$ (Glu)], 2.22–2.40 [2 H, m, γ-CH$_2$ (Glu)], 2.60–2.80 [3 H, m, α-H (propanoyl) and β-CH$_2$ (Asp)], 2.73 (3 H, s, NCH$_3$), 2.93–3.13 [2 H, m, β-CH$_2$ (Phe)], 3.65 (6 H, s, 2 × CH$_3$), 3.85–4.00 [2 H, m, CH$_2$ (Sar)], 4.20–4.28 [2 H, m, α-H (Phe) and α-H, (Glu)], 4.62 [2 H, m, α-H (Asp) and β-H (propanoyl)], 6.20–6.26 [1 H, m, γ-H (propanoyl)], 6.55 [1 H, d, *J* 18.0, δ-H (propanoyl)], 7.23–7.44 (10 H, m, Ar-H), 8.16–8.20 [2 H, m, NH$_2$ (Phe)], 8.32 [1 H, d, *J* 8.5, NH (Asp)], 8.38 [1 H, d, *J* 8.5, NH (Glu)] and 8.46 [1 H, d, *J* 8.5, NH (propanoyl)]; δ_C[125 MHz; $(C^2H_3)_2SO$ mixture of rotamers] 14.3 [CH$_3$ (propanoyl)], 26.1 [β-CH$_2$ (Glu)], 28.7 [γ-CH$_2$ (Glu)], 35.8 [β-CH$_2$ (Asp)], 35.0 (NCH$_3$), 37.1 [β-CH$_2$ (Phe)], 42.8 [α-CH (propanoyl)], 48.4 [α-C (Asp)], 49.4 and 52.0 [α-C (Sar)], 51.5 [α-C (Glu)], 52.1 (OCH$_3$), 53.0 [β-C (propanyl)], 53.4 [α-C (Phe)], 126.1, 127.1, 127.5, 127.9, 128.5, 128.6 and 129.4 (Ar-CH and Ar-C quaternary), 134.8 [γ-C (Phe)], 136.3 [γ-C (propanoyl)] and 167.3, 171.3, 172.0 and 173.3 (CO, amides and esters); *m*/*z* (ES) 718 (100%, $[M + Na]^+$) and 696 (30, $[M + H]^+$).

Cyclo[-(3*S*,*E*)-3-phenylethenyl-3-aminopropanoyl-α-(*R*)-Glu-α-OMe-γ-Sar-(*R*)-Asp-α-OMe-β-(*S*)-Phe-] 18a

This compound was synthesised according to the general cyclisation procedure using **17a** (210 mg, 0.3 mmol) as starting material to give the desired cyclic peptide **18a**, after flash silica chromatography (methanol–CH_2Cl_2; 2:98), as a white solid (45 mg, 28%), $R_f = 0.5$, mp >230 °C (decomp.) (HRMS: found $[M + Na]^+$, 686.2797. $C_{34}H_{41}N_5O_9Na$ requires 686.2802); $[\alpha]_D$ +5.6 (*c* 1 in CH_3OH); ν_{max}(KBr disc)/cm^{-1} 3318 br (NH), 1742 (CO, esters) and 1652 (amides); δ_H[500 MHz; $(C^2H_3)_2SO$ mixture of rotamers] 1.75–2.02 [2 H, m, β-CH_2 (Glu)], 2.15–2.35 [2 H, m, γ-CH_2 (Glu)], 2.40–2.80 [4 H, m, β-CH_2 (Asp) and α-CH_2 (propanoyl)], 2.78 and 2.85 (3 H, 2 × s, NCH_3), 3.10–3.30 [2 H, m, β-CH_2 (Phe)], 3.60, 3.63 and 3.65 (6 H, 3 × s, 2 CH_3), 3.89–4.18 [2 H, m, CH_2 (Sar)], 4.23–4.32 [1 H, m, α-H, (Glu)], 4.37–4.50 [2 H, m, α-H, (Phe) and α-H (Asp)], 4.78–4.86 [1 H, m, β-H (propanoyl)], 6.28–6.33 [1H, m, γ-H (propanoyl)], 6.52 [1 H, d, *J* 18.0, δ-H (propanoyl)], 7.18–7.44 (10 H, m, Ph) and 7.80–8.74 [4 H, m, NH of Phe, Asp, Glu and propanoyl]; δ_C[125 MHz; $(C^2H_3)_2SO$ mixture of rotamers] 26.2 [β-CH_2 (Glu)], 28.0 [γ- CH_2 (Glu)], 34.1 (NCH_3), 35.1 [β-CH_2 (Asp)], 36.4 [β-CH_2 (Phe)], 38.6 [α-CH (propanoyl)], 47.3 [β-C (propanoyl)], 48.7 [α-C (Asp)], 51.1 [α-C (Glu)], 51.1 [α-C (Sar)], 51.4 and 51.6 (2 × CH_3), 53.6 [α-C (Phe)] and 125.8, 127.1, 127.5, 128.1 and 128.7 (Ar-CH and Ar-C quaternary); *m*/*z* (ES) 686 (100%, $[M + Na]^+$).

Cyclo[-(2*S*,3*S*,*E*)-2-methyl-3-phenylethenyl-3-aminopropanoyl-α-(*R*)-Glu-α-OMe-γ-Sar-(*R*)-Asp-α-OMe-β-(*S*)-Phe-] 18b

This compound was synthesised according to the general cyclisation procedure using **17b** (310 mg, 0.45 mmol) as starting material to give the desired cyclic peptide **18b** after flash silica chromatography (methanol–CH_2Cl_2; 2:98), as a white solid (80 mg, 27%), $R_f = 0.5$, mp > 150 °C (softening point) (HRMS: found $[M + Na]^+$, 700.2964. $C_{35}H_{43}N_5O_9Na$ requires 700.2958); ν_{max}(KBr disc)/cm^{-1} 3446 br (NH), 1740 (CO, esters) and 1645 (amides); δ_H[500 MHz; $(C^2H_3)_2SO$ mixture of rotamers] 1.00–1.10 [3 H, m, CH_3 (propanoyl)], 1.30–1.36 [1 H, m, 1 H of β-CH_2 (Glu)], 1.70–1.75 [1 H, m, 1 H of β-CH_2 (Glu)], 1.90–1.96 [1 H, m, 1 H of γ-CH_2 (Glu)], 2.15–2.20 [1 H, m, 1 H of γ-CH_2 (Glu)], 2.63 and 2.72 (3 H, 2 × s, NCH_3), 2.72– 2.80 [3 H, m, β-CH_2 (Asp) and α-H (propanoyl)], 2.96–3.14 [2 H, m, β-CH_2 (Phe)], 3.20–3.24 [1 H, m, 1 H of CH_2 (Sar)], 3.50, 3.58, 3.62 and 3.67 (6 H, 4 × s, 2 × OCH_3), 4.25–4.31 [1 H, m, α-H, (Phe)], 4.32–4.45 [2 H, m, α-H, (Glu) and α-H, (Asp)], 4.48–4.55 [1 H, m, 1 H of CH_2 (Sar)], 4.64–4.68 [1 H, m, β-H (propanoyl)], 6.22 [1 H, dd, *J* 7.5, 18.0, γ-H (propanoyl)], 6.52 [1 H, d, *J* 18.0, γ-H (propanoyl)], 7.18–7.44 (10 H, m, Ar-H) and 8.00–8.82 (4 H, m, NH of Phe, Asp, Glu, propanoyl); δ_C[125 MHz; $(C^2H_3)_2SO$ mixture of rotamers] 14.83 and 15.97 [CH_3 (propanoyl)], 26.24, 26.69, 27.91 and 29.36 [β-CH_2 (Glu) and γ-CH_2 (Glu)], 33.61 and 35.54 (NCH_3), 35.78, 36.43 and 37.15 [β-CH_2 (Asp) and β-CH_2 (Phe)], 41.53 and 41.95 [α-CH (propanoyl)], 48.86 [α-C (Asp)], 49.99 [α-C (Glu)], 50.08 [α-C (Sar)], 51.25, 51.35, 51.76, 52.02, 52.18 and 52.24 (2 × OCH_3), 52.56 [β-C (propanoyl)], 54.78 and 55.79 [α-C (Phe)] and 126.19, 126.30, 128.05, 128.22, 128.61, 128.66, 129.01, 129.09, 129.82 and 129.89, [Ar-CH, γ-C (propanoyl) and δ-C (propanoyl)], 136.17, 136.47, 138.24 and 128.34 (Ar-C quaternary) and 167.57, 168.15, 169.32, 170.13, 170.52, 171.13, 171.56, 171.67, 171.92, 172.33, 174.82 and 175.52 (CO, amides and esters); *m*/*z* (ES) 700 (100%, $[M + Na]^+$).

Cyclo[-(3*S*,*E*)-3-phenylethenyl-3-aminopropanoyl-α-(*R*)-Glu-α-OH-γ-Sar-(*R*)-Asp-α-OH-β-(*S*)-Phe-] 19a

To a cooled solution of diester **18a** (11 mg, 0.16 mmol) in a mixture of THF (2 cm^3) and water (0.75 cm^3) was added 10 drops of 1 mol dm^{-3} LiOH solution.The mixture was vigorously stirred for 1 h and then partitioned between ethyl acetate (3 cm^3) and 1 mol dm^{-3} $NaHSO_4$ solution (1.5 cm^3). The organic layer was separated, dried ($MgSO_4$) and concentrated under reduced pressure to give the diacid as a white solid in quantitative recovery. The crude diacid was then purified by preparative RP HPLC using a Luna C-18 10 μm column (150 × 21.1 mm). The column was eluted under gradient conditions [100%A–0%B to 40%A–60%B (solvent A: 0.1% aqueous TFA; solvent B: 100% CH_3CN) over 25 min; flow rate 2 cm^3 min^{-1}] and the peak corresponding to retention time 20.4 min was collected and the solvent removed under reduced pressure to give the desired peptide (7 mg, 68%), mp ~130 °C (softening point); ν_{max}(KBr disc)/cm^{-1} 3444 br (NH), 1737 (CO, esters) and 1639 (amides); δ_H[500 MHz; $(C^2H_3)_2SO$ mixture of rotamers] 1.35–1.85 [2 H, m, β-CH_2(Glu)], 1.95–2.60 [4 H, m, α-CH_2 (Glu) and β-CH_2 (Asp)], 2.65 and 2.72 (3 H, 2 × s, NCH_3), 2.75–2.97 [2 H, α-CH_2 (propanoyl)], 3.05–3.30 [2 H, m, β-CH_2 (Phe)], 3.78–4.18 [2 H, m, CH_2 (Sar)], 4.39–4.44 [1 H, m, α-H, (Glu)], 4.51–4.59 [1 H, m, α-H, (Phe)], 4.78–4.84 [1 H, m, α-H, (propanoyl)], 4.88–4.92 [1 H, m, α-H, (Asp)], 6.18–6.24 [1 H, m, γ-H (propanoyl)], 6.46 [1 H, d, *J* 18.0, δ-H (propanoyl)], 7.16–7.44 (10 H, m, Ar-H), 7.76 [1 H, d, *J* 8.5, NH (Asp)], 8.19 [1 H, m, NH (Phe)], 8.40 [1 H, d, *J* 8.5, NH (propanoyl)] and 8.78 [1 H, d, *J* 8.5, NH (Glu)]; δ_C[125 MHz; $(C^2H_3)_2SO$ mixture of rotamers] 26.3 [β-CH_2 (Glu)], 27.5 [γ-CH_2 (Glu)], 35.4 (NCH_3), 35.7 [β-CH_2 (Phe)], 35.9 [β-CH_2 (Asp)], 37.0 [α-CH (propanoyl)], 46.4 [β-C (propanoyl)], 48.2 [α-C (Asp)], 49.8 [α-C (Glu)], 50.9 [α-C (Sar)], 53.6 [α-C (Phe)], 125.8, 127.1, 128.1 and 128.7 (Ar-CH and Ar-C quaternary), 129.0 and 129.4 [γ- and δ-C (propanoyl)] and 168.4, 169.2, 170.6, 171.5 and 172.3 (CO, amides and esters); *m*/*z* (ES) 658 (100%, $[M + Na]^+$).

Cyclo[-(2S,3*S*,*E*)-2-methyl-3-phenylethenyl-3-aminopropanoyl-β-(*R*)-Glu-α-OH-γ-Sar-(*R*)-Asp-α-OH-β-(*S*)-Phe-] 19b

This compound was prepared in a manner identical to that described for **19a** using **18b** (5 mg, 0.077 mmol) as starting material to give the crude product as a white solid. The crude diacid **19b** was then purified by preparative RP HPLC using a Luna C-18 10 μm column (150 × 21.1 mm).The column was eluted under gradient conditions [100%A–0%B to 30%A–70%B (solvent A: 0.1% aq. TFA; solvent B: 100% CH_3CN) over 25 min; flow rate 2 cm^3 min^{-1}] and the peak corresponding to retention time 16.9 min was collected and the solvent removed under reduced pressure and by lyophilisation to give the desired peptide (4 mg, 80%), mp >150 °C (decomp.); $[\alpha]_D$ +24 (*c* 0.1 in CH_3OH); ν_{max}(KBr disc)/cm^{-1} 3430 br (NH), 1732 (CO, esters) and 1637 (amides); δ_H[500 MHz; $(C^2H_3)_2SO$] 1.04–1.08 [3 H, m, CH_3 (propanoyl)], 1.25–1.33 [1 H, m, 1 H of β-CH_2 (Glu)], 1.80–1.83 [1 H, m, 1 H of β-CH_2 (Glu)], 2.08–2.16 [2 H, m, 1 H of γ-CH_2 (Glu) and 1 H of β-CH_2 (Asp)], 2.22–2.32 [1 H, m, 1 H of γ-CH_2 (Glu)], 2.56 [1 H, m, 1 H of β-CH_2 (Asp)], 2.63 (3 H, s, NCH_3), 3.02–3.10 [1 H, α-CH (propanoyl)], 3.14–3.24 [3 H, m, β-CH_2 (Phe) and 1 H of CH_2 (Sar)], 4.39–4.24 [1 H, m, α-H, (Glu)], 4.56–4.62 [2 H, m, α-H, (Phe) and β-H (propanoyl)], 4.63–4.68 [1 H, m, 1 H of α-CH_2 (Sar)], 4.88–5.01 [1 H, m, α-H, (Asp)], 6.25 (1 H, dd, *J* 4.5 and 16.0, γ-H (propanoyl)], 6.38 (1 H, d, *J* 16.0, δ-H (propanoyl)], 7.26–7.42 (10 H, m, Ar-H), 7.68 [1 H, d, *J* 8.5, NH (Asp)], 8.02 [1 H, d, *J* 8.5, NH (propanoyl)], 8.16 [1 H, m, *J* 8.5, NH (Phe)] and 8.80 [1 H, d, *J* 8.5, NH (Glu)]; δ_C[125 MHz; $(C^2H_3)_2SO$] 14.3 [CH_3 (propanoyl)], 26.3 [β-CH_2 (Glu)], 27.5 [γ-CH_2 (Glu)], 35.2 (NCH_3), 35.6 [β-CH_2 (Phe)], 35.9 [β-CH_2 (Asp)], 37.0 [α-CH (propanoyl)], 48.0 [α-C (Asp)], 49.7 [α-C (Glu)], 50.7 [α-C (Sar)], 52.1 [β-C (propanoyl)], 53.6 [α-C (Phe)], 125.8, 127.1, 128.1 and 128.7 (Ar-CH and Ar-C quaternary), 129.0 [γ- and δ-C (propanoyl)] and 168.0, 170.2, 170.6, 171.5 and 176.0 (CO, amides and esters); *m*/*z* (ES) 672 (100%, $[M + Na]^+$).

(2*S*)-Phenylalanyl-[(2*S*,3*S*,*E*)-2-methyl-3-phenylethenyl-3-aminopropanoyl]-[α-methyl (2*R*)-glutamyl]-γ-[(2*S*)-prolyl]-[α-methyl (2*R*)-aspartate] diester 20

This compound was synthesised on the peptide synthesiser from Fmoc-Asp-(α)-OMe-O-Wang **16** (0.8 g, ~0.6 mmol loading) using the general SPPS method described above, followed by cleavage of the peptide from the resin to give the crude peptide **20** as a white solid in quantitative recovery (433 mg) after lyophilisation. ^{1}H NMR spectroscopy showed that the crude peptide was at least 90% pure. For analytical purposes a small amount (~10 mg) was further purified by preparative RP HPLC using a Luna C-18 10 μm column (150 × 21.1 mm). The column was eluted under gradient conditions [100%A–0%B to 40%A–60%B (solvent A: 0.1% aq. TFA; solvent B: 80:20 CH_3CN–H_2O with 0.1% TFA) over 25 min; flow rate 2 cm^3 min^{-1}] and the peak corresponding to retention time of approx. 21 min was collected and the solvent removed under reduced pressure and by lyophilisation to give the desired peptide. Mp 114–117 °C (softening point); ν_{max}(KBr disc)/cm^{-1} 1740 (CO, esters) and 1672 (amides); δ_H[500 MHz; $(C^2H_3)_2SO$ mixture of rotamers] 0.86–0.90 [3 H, m, CH_3 (propanoyl)], 1.68–1.96 [6 H, m, β-CH_2 (Glu), β-CH_2 (Pro) and γ-CH_2 (Pro)], 2.12–2.26 [2 H, m, γ-CH_2 (Glu)], 2.60–2.80 [3 H, m, α-H (propanoyl) and β-CH_2 (Asp)], 2.90–3.13 [3 H, m, 1 H of δ-CH_2 (Pro) and β-CH_2 (Phe)], 3.20–3.26 [1 H, m, 1 H of δ-CH_2 (Pro)], 3.55–3.64 (6 H, m, 2 × CH_3), 4.16–4.26 [3 H, m, α-H (Phe), α-H (Glu) and α-H (Pro)], 4.55–4.60 [1 H, m, α-H (Asp)], 4.64–4.69 [1 H, m, β-H (propanoyl)], 6.18–6.24 [1 H, m, γ-H (propanoyl)], 6.50–6.56 [1 H, m, δ-H (propanoyl)], 7.21–7.40 (10 H, m, Ar-H), 8.06–8.16 [2 H, m, NH_2 (Phe)], 8.18 [1 H, d, *J* 8.5, NH (Asp)], 8.38 [1 H, d, *J* 8.5, NH (Glu)] and 8.44 [1 H, d, *J* 8.5, NH (propanoyl)]; δ_C[125 MHz; $(C^2H_3)_2SO$ mixture of rotamers] 14.06 and 14.30 [CH_3 (propanoyl)], 22.01 and 23.82 [γ-CH_2 (Pro)], 26.05 [β-CH_2 (Glu)], 29.29 [β-CH_2 (Pro)], 30.19 [γ-CH_2 (Glu)], 35.98 [β-CH_2 (Asp)], 37.47 [β-CH_2 (Phe)], 42.77 [α-CH (propanoyl)], 46.37 [δ-CH_2 (Pro)], 48.65 [α-C (Asp)], 51.68 [α-C (Glu)], 51.85, 52.02 and 52.08 (OCH_3), 53.01 [β-C (propanyl)], 53.42 [α-C (Phe)], 59.00 [α-CH_2 (Pro)], 126.13, 127.14, 127.57, 127.99, 128.56, 128.61, 129.42, 130.34, 134.86 and 136.34 [Ar-CH, Ar-C quaternary, γ-C (propanoyl) and δ-C (propanoyl)] and 169.76, 171.20, 171.45, 172.24 and 173.74 (CO, amides and esters); *m*/*z* (ES) 766 (20%, [M + 2Na − H]$^+$), 744 (100, [M + Na]$^+$) and 722 (35, [M + H]$^+$).

Cyclo[-(2*S*,3*S*,*E*)-2-methyl-3-phenylethenyl-3-aminopropanoyl-β-(*R*)-Glu-α-OMe-γ-(*S*)-Pro-(*R*)-Asp-α-OMe–β-(*S*)-Phe-] 21

This compound was synthesised according to the general cyclisation procedure using pentapeptide **20** (250 mg, 0.35 mmol) as starting material to yield the cyclic peptide **21** as a white solid after column chromatography (55 mg, 23%) (HRMS: found [M + Na]$^+$, 726.3123. $C_{37}H_{45}N_5O_9Na$ requires 726.3115); δ_H[500 MHz; $(C^2H_3)_2SO$] 1.11 [3 H, d, *J* 7, CH_3 (propanoyl)], 1.30–1.36 [1 H, m, 1 H of β-CH_2 (Glu)], 1.55–1.62 [1 H, m, 1 H of β-CH_2 (Glu)], 1.72–1.98 [5 H, m, 1 H of γ-CH_2 (Glu), β-CH_2 (Pro), γ-CH_2 (Pro)], 2.15–2.24 [1 H, m, 1 H of γ-CH_2 (Glu)], 2.66–2.78 [3 H, m, 1 H of β-CH_2 (Asp) and δ-CH_2 (Pro)], 2.86–2.98 [2 H, m, 1 H of β-CH_2 (Asp) and δ-H (propanoyl)], 3.16–3.30 [2 H, m, β-CH_2 (Phe)], 3.57, 3.58 and 3.66 [6 H, 3 × s, OCH_3 (Asp) and OCH_3 (Asp)], 4.05–4.65 [5 H, α-H of Glu, Asp, Phe and Pro, and β-CH (propanoyl)], 6.19 [1 H, dd, *J* 3.5 and 16, γ-H (propanoyl)], 6.41 [1 H, dd, *J* 16, δ-H (propanoyl)], 7.15–7.42 (10 H, m, Ar-H), 7.75–8.50 (3 H, m, NH of propanoyl, Phe and Asp) and 8.67 [1 H, d, *J* 9.5, NH (Glu)]; δ_C[125.8 MHz; $(C^2H_3)_2SO$, predominately *trans* rotamer] 15.8 [β-CH_3 (propanoyl)], 23.6 [γ-CH_2 (Pro)], 26.9 [β-CH_2 (Glu)], 28.9 and 30.0 [β-CH_2 (Pro) and γ-CH_2 (Glu)], 34.8 [β-CH_2 (Phe)], 36.7 [β-CH_2 (Asp)], 41.7 [α-CH (propanoyl)], 45.8 [δ-CH_2 (Pro)], 49.6 [α-CH (Asp)], 50.2 [α-CH (Glu)], 52.1 and 52.2 [OCH_3 (Asp) and (Glu)], 52.6 [β-CH (propanoyl)], 56.7 [α-CH (Phe)], 59.6 [α-CH (Pro)], 126.1, 126.2, 127.6, 129.9, 128.1, 128.5, 128.6, 126.9, 129.0, 129.1, 129.7 and 128.1 (γ- and δ-C propanoyl, and Ar-CH), 136.4 and 138.8 (Ar-C quaternary) and 169.6, 170.1, 170.7, 171.3, 171.6, 172.0 and 175.5 (ester and amides); *m*/*z* (ES) 726 (100%, [M + Na]$^+$).

Cyclo[-(2*S*,3*S*,*E*)-2-methyl-3-phenylethenyl-3-aminopropanoyl-β-(*R*)-Glu-α-OH-γ-(*S*)-Pro-(*R*)-Asp-α-OH–β-(*S*)-Phe-] 22

This compound was prepared in a manner identical to that described for **19a** using **21** (4.5 mg, 0.07 mmol) as starting material to give the crude product as a white solid. The crude product was purified by preparative RP HPLC using a Luna C-18 10 μm column (150 × 21.1 mm). The column was eluted under gradient conditions [100%A–0%B to 40%A–60%B (solvent A: 0.1% aq. TFA; solvent B: 100% CH_3CN) over 25 min; flow rate 2 cm^3 min^{-1}] and the peak corresponding to retention time 17.4 min was collected and the solvent removed under reduced pressure and by lyophilisation to give the desired peptide as a white solid (3.5 mg, 85%), mp ~130 °C (softening point) (HRMS: found [M + Na]$^+$, 698.2822. $C_{35}H_{41}N_5O_9Na$ requires 698.2802); ν_{max}(KBr disc)/cm^{-1} 1742 (CO, esters) and 1635 (amides); δ_H[500 MHz; $(C^2H_3)_2SO$] 1.08–1.12 [3 H, m, CH_3 (propanoyl)], 1.25–1.35 [2 H, m, β-CH_2 (Glu)], 1.56–1.75 [2 H, m, γ-CH_2 (Pro)], 1.76–1.80 [1 H, m, 1 H of γ-CH_2 (Glu)], 1.92–2.06 [2 H, m, β-CH_2 (Pro)], 2.08–2.20 [1 H, m, 1 H of β-CH_2 (Asp)], 2.21–2.26 [1 H, m, 1 H of γ-CH_2 (Glu)], 2.51 [1 H, m, 1 H of β-CH_2 (Asp)], 2.77–2.82 [1 H, m, 1 H of δ-CH_2 (Pro)], 2.92–2.98 [1 H, m, 1 H of δ-CH_2 (Pro)], 3.00–3.05 [1 H, α-H (propanoyl)], 3.20–3.23 [2 H, m, β-CH_2 (Phe)], 4.25–4.30 [1 H, m, α-H (Pro)], 4.40–4.50 [1 H, m, α-H, (Glu)], 4.54–4.62 [2 H, m, α-H, (Phe) and β-H (propanoyl)], 4.88–4.92 [1 H, m, α-H, (Asp)], 6.15–6.20 (1 H, m, γ-H (propanoyl)], 6.38 (1 H, d, *J* 16.0, δ-H (propanoyl)], 7.26–7.42 (10 H, m, Ar-H), 7.80–7.85 [2 H, m, NH (Asp and Phe)], 8.07 [1 H, d, *J* 8.5, NH (propanoyl)] and 8.62–8.65 [1 H, m, NH (Glu)]; δ_C[100.6 MHz; $(C^2H_3)_2SO$, predominately *trans* rotamer] 14.4 [β-CH_3 (propanoyl)], 21.8 [γ-CH_2 (Pro)], 25.5 [β-CH_2 (Glu)], 26.5 and 27.0 [β-CH_2 (Pro) and γ-CH_2 (Glu)], 32.9 [β-CH_2 (Phe)], 33.8 [β-CH_2 (Asp)], 39.5 [α-CH (propanoyl)], 44.0 [δ-CH_2 (Pro)], 46.9 [α-CH (Asp)], 48.0 [α-CH (Glu)], 50.1 [β-CH (propanoyl)], 51.9 [α-CH (Phe)], 57.7 [α-CH (Pro)], 124.1, 124.2, 124.6, 125.5, 125.7, 126.2, 126.4, 126.7, 126.9, 127.1, 127.4 and 127.8 (γ and δ-C propanoyl, and Ar-CH), 134.1 and 135.7 (Ar-C quaternary), 167.9, 168.6, 168.8, 169.5, 169.7, 170.9 and 174.5 (amides and acids); *m*/*z* (ES) 698 (100%, [M + Na]$^+$).

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