Design and preparation of serine–threonine protein phosphatase inhibitors based upon the nodularin and microcystin toxin structures: Part 1. Evaluation of key inhibitory features and synthesis of a rationally stripped-down nodularin macrocycle

Amit P. Mehrotra, Kerri L. Webster and David Gani *

School of Chemistry and Centre for Biomolecular Sciences, The Purdie Building, The University, St. Andrews, Fife, UK KY16 9ST

The natural nodularin and microcystin toxins are powerful but non-selective inhibitors of the ubiquitous and structurally related eukaryotic Ser-Thr protein phosphatases, PP1 and PP2A, enzymes that are intimately involved in controlling cellular metabolism. Both families of toxin are cyclic tri-isopeptides typified by the presence of two free carboxylic acid groups, a dehydroamino acid moiety, and a large, rigid exocyclic lipophilic side-chain. To learn how to design specific inhibitors for each enzyme, the nature of specific interactions with potential inhibitor-conferring moieties in the toxin was considered. Borohydride reduction of the dehydroalanine residue present in microcystin-LR, a potential Michael acceptor, gave two diastereoisomeric dihydromicrocystin products. Each of these displayed subnanomolar activities as inhibitors for PP2A, as for the parent compound, indicating that the dehydroamino acid residue in microcystin and, probably, in nodularin, is not essential for activity. Other conserved features appeared to be required to confer activity, hence strategies towards the synthesis of simplified non-dehydroamino acid-containing analogues of each macrocycle type were considered. In each case it was planned to elaborate the lipophilic side-chain functionality after the formation of the macrocyclic ring. In order to synthesize the precursor nodularin-type macrolactam, two peptide-bond disconnections of the ring were investigated using a model system, *cyclo*-[β-Ala-(*R*)-Glu-α-OMe-γ-Sar-(*R*)-Asp-α-OMe-β-(*S*)-Phe-], one bond disconnecting between the sarcosine carboxy group and the (2*R*)-Asp N-atom and the other disconnecting between the (2*R*)-Asp β-carboxy group and the (2*S*)-Phe N-atom. Preparation of the linear precursors was achieved using solution-phase chemistry without incident. Macrolactamisation *via* the displacement of the β-pentafluorophenyl ester of the (2*R*)-Asp α-methyl ester residue by the free amino group of the (2*S*)-phenylalanine residue proceeded in excellent yield (89%), but the alternative strategy failed. Application of the successful macrolactamisation strategy to other nodularin macrocycles and to the construction of the microcystin-type macrocycle is described in the following article.

Introduction

It is now widely accepted that modulation of the reversible phosphorylation of proteins on serine, threonine and tyrosine residues, as catalysed by protein kinases and phosphatases, is the principal mechanism by which eukaryotic cells respond to external stimuli.[1,2] The phosphorylation–dephosphorylation process acts as a switch in turning on and off, or off and on, key enzyme activities within cells such that the degree of phosphorylation at certain sites within a population of given protein molecules determines the activity. Thus, the balance between phosphorylated and dephosphorylated proteins, which is controlled by the protein kinase and phosphatase activities, is crucial to maintaining proper cellular function. Moreover, modulation of the opposing kinase and phosphatase activities is an efficient way to amplify a stimulus response (Fig. 1). The intricacies of the system become even more apparent when it is appreciated that this ubiquitous intracellular control mechanism regulates not one but myriad metabolic processes. For the Ser-Thr protein kinase/phosphatase system alone, important examples include the control of glycogen metabolism, muscle contraction, membrane transport and cell differentiation and division.[2–5]

The four most abundant Ser-Thr protein phosphatases are PP1, PP2A, PP2B and PP2C. PP1, PP2A and PP2B (calcineurin) are structurally related, possess similar catalytic subunits and display overlapping substrate activities. However, for every type of catalytic subunit, there are many different types of regulatory subunit that can bind to the catalytic domain to modulate its activity.[5–8]

The natural cyclic isopeptides microcystin **1**[9–12] and nodularin **2a**,[12–15] previously established as potent hepatotoxins,[16] are now known to inhibit the catalytic subunit of mammalian PP1 and PP2A (but not PP2B or PP2C) with subnanomolar inhibition constants.[17] These enzymes are highly homologous and display ~50% amino acid sequence identity.[2] There has been heightened awareness of the extremely toxic properties of microcystins (cyanoginosins) produced by the genus *Microcystis*, as extensive wildlife fatalities have been attributed to reservoir water contaminated with cyanobacteria (blue-green algae). In countries where drinking water supplies are contaminated with cyanobacteria, adverse effects on humans have also been recorded.[18] Indeed, these hepatotoxins have been linked to a higher incidence of liver cancer among populations in Third World countries that depend on surface drinking water.[16,19] Liver cells possess a special ability to take-up microcystins *via* a transport system known as the bile acid carrier salt transport system,[20,21] which explains their susceptibility to such toxins. Other naturally occurring competitive inhibitors of cat-PP1 and cat-PP2A include the powerful tumour promotor okadaic acid, responsible for diarrhetic shellfish poisoning, and the calyculins, tautomycin and cantharidin.[22]

While there has been much interest in understanding how such diverse toxin structures might interact with the enzymes, until very recently[23,24] there was no 3-D structural information for the protein [for a recent review on the structure and mechan-

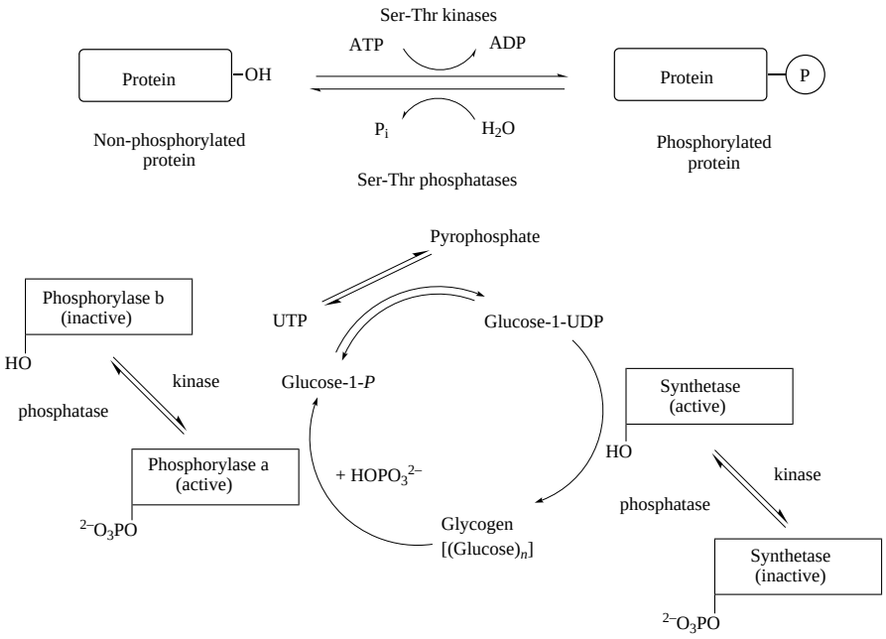
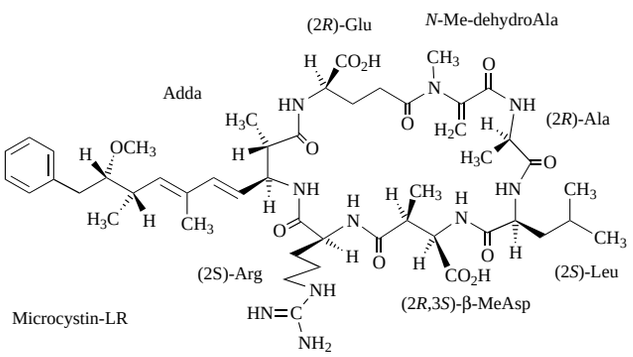
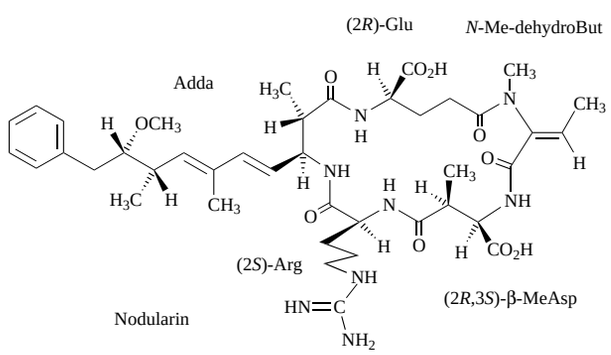


Fig. 1



1a



2a

1b Microcystin-LA [(2*S*)-Ala replaces (2*S*)-Arg in **1a**] **1c** Microcystin-RR [(2*S*)-Arg replaces (2*S*)-Leu in **1a**] **1d** Dihydromicrocystin-LR [(2*S*)- or (2*R*)-*N*-methylalanine replaces the N-methyldehydroalanine residue in **1a** (see text and Experimental section)] **2b** Motuporin [(2*S*)-Val replaces (2*S*)-Arg in **2a**]

ism of phosphatases, see Gani and Wilkie[25]]. Our starting point was to identify which parts of the nodularin/microcystin toxin were essential for inhibitory activity and then design a stripped-down and simpler macrocycle than that present in each of the natural products, which would serve as a framework for attaching specific functionalities. The longer term interests were to synthesize minimal analogues to probe the active-site binding interactions and then identify specific inhibitors for each catalytic subunit type. Small molecule inhibitors that are specific for either PP1 or PP2A are not available but are required to delineate the individual physiological roles of the enzymes. Here we describe the results of experiments designed to identify fully active but simpler synthetic target inhibitors, our strategies towards synthesizing the macrocyclic frame of such compounds, and the successful preparation of a stripped-down nodularin-type macrocycle. The following article describes the application of this chemistry to the preparation of a functionalised nodularin macrocycle and the construction of a microcystin-type macrocycle.

Results and discussion

Identification of a minimal macrocyclic framework

Both families of toxin, microcystins and nodularins, are cyclic tri-isopeptides and contain two free carboxylic acid groups, an *N*-methyl dehydroamino acid moiety, and a large rigid lipophilic side-chain. These five principal features are the only highly conserved structural motifs. For both families replacement of the (2*S*)-Arg residue in structures **1a** and **2a** by a hydrophobic residue, for example, as occurs in the natural (2*S*)-Ala-containing variant microcystin-LA **1b**[9] or the natural (2*S*)-Val-containing nodularin variant, motuporin **2b**,[26] gives highly efficacious PP1 inhibitors. In order to identify the important inhibitory interactions between the protein and the toxin, each conserved potential interaction was evaluated in turn with reference to the known inhibitory activity of published natural variants. While microcystins and nodularins are cyclic tri-isopeptides of different size, it was expected that there was sufficient flexibility in the microcystin system such that the key functional groups in each type could occupy similar enzyme-bound positions in space, simultaneously. This analysis (which is in accord with more recent protein-toxin X-ray crystal data[23] and toxin NMR spectroscopic data[27,28]) allowed us to assume that the similar groups in microcystins and nodularins were functionally equivalent.

The two α-carboxylic acid groups from (2*R*)-Glu and (2*R*,3*S*)-3-methylaspartic acid residues in microcystin were known to be important since the ester derivatives of active toxins were inactive.[29,30] Thus, both of these would need to be retained in the target macrocycle. Nevertheless, since it had been shown that the 3-methyl group of (2*R*,3*S*)-3-methylaspartic acid was absent in several variants which retained their biological activity, it was apparent that (2*R*)-aspartic acid should serve as a functional surrogate for (2*R*,3*S*)-3-methylaspartic acid in synthetic inhibitors.

The lipophilic side-chain moiety in the Adda residue, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, is also highly conserved in both families of toxin. However, it was reported that the stereochemistry of the double bond at C-6 of the Adda residue in both microcystin-LR **1a** and microcystin-RR **1c** is not crucial for biological activity and that the 6*Z*-isomer is only 100-fold less active against PP2A (ED_{50} = 80 nM for both isomers) than the more common 6*E*-isomer (ED_{50} = 0.28 nM for microcystin-LR and 0.78 nM for microcystin-RR).[31,32] The Adda residue itself, unsurprisingly, was totally inactive[33] but the experiment provided an important control. A microcystin variant possessing a C-9 hydroxy group in place of the methoxy group in the Adda side-chain was fully active,[29] as were their O-acetylated derivatives.[34] However, the catalytic hydrogenation product of microcystin-LR showed little or no activity as a hepatotoxin.[33] Since the hydrogenation reaction reduced not only the diene but presumably also the dehydroamino acid residue [to the corresponding alanine-containing derivative(s)] this latter result indicated that either a rigid lipophilic Adda side-chain or a Michael-type acceptor is required for biological activity, or, possibly, both. The latter finding[33] also indicates that the macrolactam structure itself, together with the two carboxylic acid groups, is not sufficient to confer good inhibitory properties as has been re-discovered by others more recently.[35]

Some information regarding the importance of the potential Michael acceptor came from the finding that the *N*-methylserine analogue of microcystin-LR, which does not contain a conjugated electrophilic site, showed full activity as a hepatotoxin.[29] However, while this molecule, which may be a biosynthetic precursor to microcystin, appears not to be able to act as a Michael acceptor, it is well known that β-hydroxy groups and other leaving groups in serine/threonine residues are extremely prone to undergo α,β-elimination reactions.[36,37] Therefore, we could not rule out that the conjugated system might form in the incubation medium or upon interaction with the enzyme.

In order to resolve the issue, 1 mg of microcystin-LR **1a** was treated with an excess of sodium borohydride in wet methanol. The reduced product displayed the expected olefinic protons for the diene moiety (δ 5.41–6.26) in the ^{1}H NMR spectrum, but no dehydroalanine signals. These occur at δ 5.44 and 5.89 in the parent compound. It was also evident that the reduced product was a mixture of two compounds or conformers, one major and one minor, possessing very similar signals. A portion of this sample was purified by reversed-phase HPLC and two new product bands of ratio ~5:1 (now ascribed as diastereoisomers **1d**[38]), were identified. These were separated and each compound was tested for biological activity using cat-PP2A. The major diastereoisomer gave an IC_{50} value of 0.1 nmol dm^{-3} and the minor diastereoisomer also gave an IC_{50} value of 0.1 nmol dm^{-3}. These values were indistinguishable from that for the non-reduced material which also gave an IC_{50} value of 0.1 nmol dm^{-3}.[39] Thus, it is evident that the conjugated double bond is not important for inhibition and, furthermore, that the stereochemistry at C-α of the reduced product has little or no effect on the potency of microcystin as a PP2A inhibitor. In conjunction with the finding that the fully saturated (catalytically reduced) microcystin analogue displayed no activity as a hepatotoxin,[33] see above, it appears that the lipophilic side-chain of the Adda residue must be rigid to confer activity. Note that in apparent contrast to our own findings, the recently reported X-ray crystal structure of a cat-PP1–microcystin complex shows that the thiol moiety of Cys-273 in cat-PP1 does form a Michael adduct with the *N*-methyldehydroalanine residue of microcystin.[23,40] However, there is, in fact, no dichotomy and other workers have reached similar conclusions to our own by showing that the replacement of Cys-273 by alanine, which cannot form a Michael adduct, does not significantly compromise the inhibitory properties of microcystin.[40] How the crystallographic result fits in with our own analysis is easily rationalised by considering the fact that microcystin and nodularin are not irreversible inhibitors of PP1 and show linear plots for time *versus* substrate hydrolysis. Thus, the recorded values of K_i or IC_{50} represent the dual effects of non-covalent binding and reversible covalent binding where the two forms of the enzyme-inhibitor complex are in equilibrium (Fig. 2).

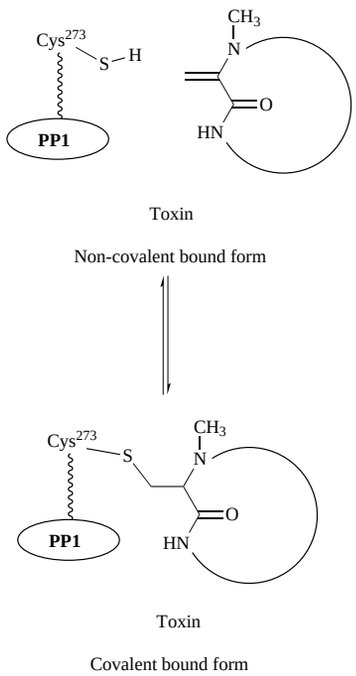
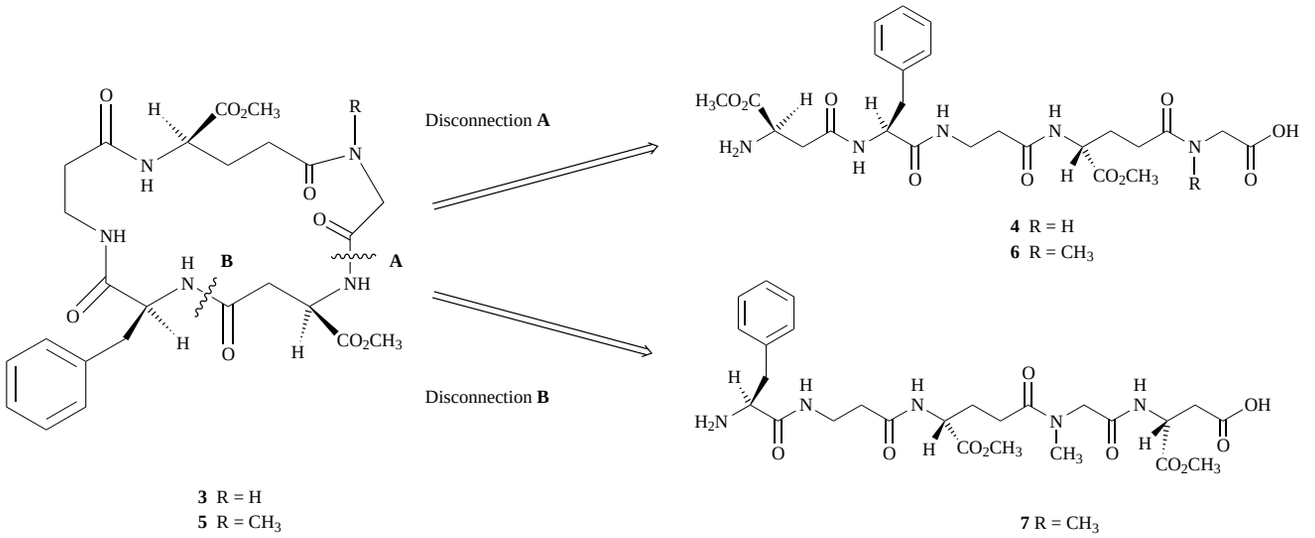


Fig. 2

Returning to the identification of a simpler macrocyclic target for synthesis, it was now evident that four of the five principal conserved features would need to be retained in order to confer biological activity; *viz*., the cyclic structure, the two carboxylic acid groups and the rigid lipophilic appendage. However, the requirement for a dehydroamino acid residue could be relaxed. From the synthetic point of view and from considerations of compound stability, being able to relax the requirement for a dehydroamino acid residue without comprising activity was considered to be a highly useful result. Enamides derived from dehydroalanine[41,42] react quickly with amines and thiols and are sensitive to hydrolysis in aqueous solution.

Given that the stereochemistry at the C-α centre of the *N*-methylalanine residue in the dihydromicrocystin samples had had no effect on biological activity, we chose to use glycine and *N*-methylglycine (sarcosine) as the replacement residue in the synthesis of the stripped-down macrocycles. Focussing now on the nodularin system, it was evident, see above, that the (2*S*)-Arg residue could be replaced by a less polar hydrophobic residue. We chose to introduce (2*S*)-Phe in view of its low polarity and because it possessed a useful chromophore that would aid the chromatographic separation of intermediates during the synthesis of the macrocycle. The (2*R*)-Glu residue in the nodularin presented no special problems but the requirement to incorporate the (2*R*,3*S*)-3-methylaspartic acid residue, which is not readily available, needed further evaluation. Both the (2*R*,3*S*)-3-methylaspartic acid residue itself and the β-amino acid portion of the Adda residue share the same relative stereochemistry and could be derived from the same precursor. The priority rules change the apparent (absolute) stereochemistry of the Adda residue at C-3 but, in fact, the first double bond of the diene moiety occupies the same position in 3-D space as the α-carboxylic acid group of (2*R*,3*S*)-3-methylaspartic acid. Enantiomerically pure (2*S*,3*S*)-3-methylaspartic acid has been prepared in our laboratory through the enzymic amination of mesaconic acid with methylaspartase in excellent yield on a



Scheme 1

multigram scale[43] and we reasoned that it would be possible to invert the C-2 centre to give the required (2*R*,3*S*)-diastereoisomer, if the C-3-methyl group was really essential for conferring biological activity to the nodularin macrocycle. While it was expected that the deletion of the methyl group might alter the population of active conformer(s) in the macrocyclic ring, we did not expect the deletion of a methyl group to prevent access to such conformers. The reported finding that nodularin/microcystin containing a (2*R*)-Asp residue in place of the (2*R*,3*S*)-3-methylaspartic acid residue showed biological activity[15,29] persuaded us that a similar deletion would be acceptable in our stripped-down target macrocycle (see above). Furthermore, because it was intended to introduce several β-amino acid replacement residues for the Adda residue, see below, decisions on the final structure of the replacement β-amino acids could be deferred until after the macrolactamisation chemistry and conformational analysis had been completed for model systems.

Synthesis of the stripped-down nodularin macrocycle

The preliminary target replacing the Adda residue by β-alanine was thus identified as *cyclo*-[β-Ala-(*R*)-Glu-α-OMe-γ-Gly-(*R*)-Asp-α-OMe-β-(*S*)-Phe-], compound **3**.

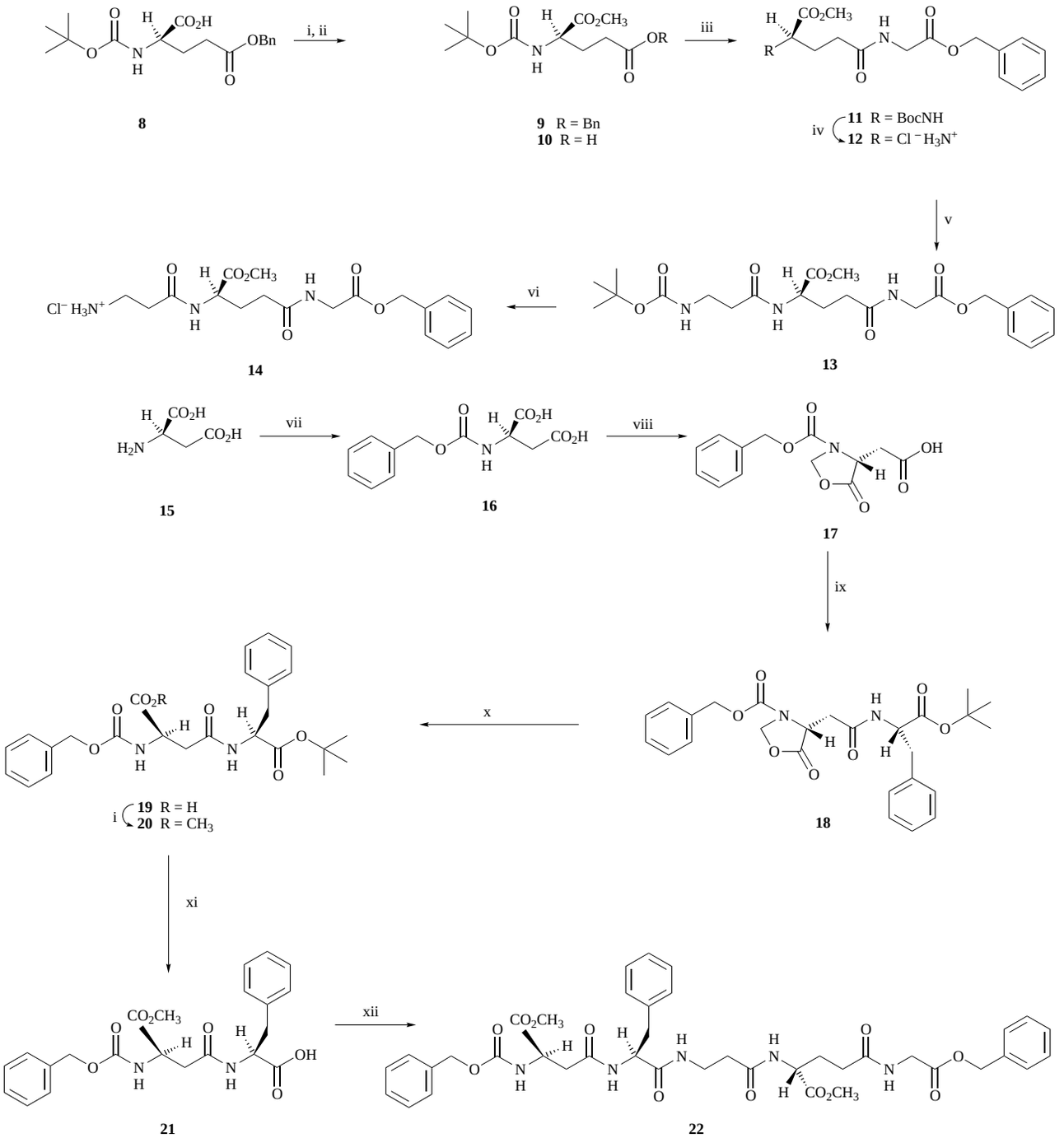
Two peptide-bond disconnections of the macrolactam ring were investigated, one disconnecting between the glycine carboxy group and the (2*R*)-aspartic acid N-atom (scission **A**, Scheme 1), and the other disconnecting between the (2*R*)-aspartic acid β-carboxy group and the (2*S*)-phenylalanine N-atom (scission **B**, Scheme 1).

Disconnection **A** of compound **3** (Scheme 1) gives the linear pentapeptide precursor (2*R*)-NH_2-Asp-α-OMe-β-(2*S*)-Phe-β-Ala-(2*R*)-Glu-α-OMe-γ-Gly-OH **4**, which we intended to prepare initially as its *N*-benzyloxycarbonyl-protected C-terminal benzyl ester derivative **22** so that both protecting groups could be simultaneously removed, as shown in Scheme 2.

Accordingly, N-Boc (2*R*)-glutamic acid γ-benzyl ester **8** was treated with ethereal diazomethane to give the methyl ester **9** as an oil. Catalytic hydrogenolysis of the benzyl ester group followed by activation of the γ-carboxy group of compound **10** using *N*-methylmorpholine (NMM) and isobutyl chloroformate (IBCF) to give the mixed anhydride, facilitated reaction with glycine benzyl ester toluenesulfonate salt to afford the required amide **11** in 96% overall yield {mp 80–82 °C; $[\alpha]_D^{23}$ +18.6 (MeOH)}. The *N*-Boc protection was removed from compound **11** by using hydrogen chloride gas in ethyl acetate to give the amine salt **12** in 90% yield. This was treated with *N*-Boc-β-Ala to give the tripeptide ester **13** in 91% yield {mp 74–76 °C; $[\alpha]_D^{23}$ +4.6 (MeOH)} which displayed the expected spectroscopic and analytical properties. Subsequent removal of the N-Boc protecting group by using hydrogen chloride gas afforded the hydrochloride salt **14** in 90% yield as an amorphous hygroscopic solid which gave the required NMR and mass spectral data.

The required N-terminal β-aspartylphenylalanyl dipeptide fragment of target **4** was prepared using the *N*-(benzyloxycarbonyl)oxazolidin-5-one moiety for the selective protection of (2*R*)-aspartic acid **15** (Scheme 2). Treatment of *N*-Z-(2*R*)-aspartic acid **16** with paraformaldehyde at reflux in toluene in the presence of a catalytic amount of *p*-TsOH in a Dean–Stark apparatus gave the oxazolidin-5-one **17** in 91% yield which displayed the expected properties.[44] The free β-carboxy group of compound **17** was then activated by using NMM and IBCF and the resulting anhydride was treated with (2*S*)-Phe-OBu^t hydrochloride and an extra mole equivalent of NMM to give the required dipeptide **18** in 91% yield {mp 117 °C; $[\alpha]_D^{23}$ −92.9 (MeOH)}. The oxazolidin-5-one **18** was hydrolysed with methanolic aq. NaOH to give the free acid **19**. Treatment of this acid with ethereal diazomethane gave the required diester **20**. The *tert*-butyl ester protecting group was removed by using TFA in dichloromethane and the resulting free acid was activated as the mixed anhydride and this was treated with the tripeptide hydrochloride **14** and NMM to afford the fully protected pentapeptide **22** in 86% yield after crystallisation from aq. acetone {mp 133–135 °C; $[\alpha]_D^{23}$ −1.6 (MeOH)}. Simultaneous deprotection of the *N*-Z and benzyl ester groups by catalytic hydrogenolysis gave the required deprotected pentapeptide **4** {mp 116–118 °C} as judged by ^{1}H and ^{13}C NMR spectroscopy, which showed that the aromatic and benzylic signals were no longer present.

The deprotected peptide (2*R*)-NH_2-Asp-α-OMe-β-(2*S*)-Phe-β-Ala-(2*R*)-Glu-α-OMe-γ-Gly-OH **4** was now ready for cyclisation. Accordingly, compound **4** was treated with DPPA and Et_3N in several sets of experiments at a range of temperatures (−15 to 25 °C) under high-dilution conditions in dry DMF for 12–48 h, but all of these reaction conditions failed to give any of the desired macrocycle **3**. The replacement of Et_3N by solid $NaHCO_3$ in experiments performed under similar conditions also failed to give compound **3**.[45] The utility of TBTU and EDCI in attempts to mediate cyclisation was also investigated, (both in the absence and presence of HOBt), again employing a range of different bases including Et_3N or DIPEA, at a range of temperatures (0 to 25 °C) under conditions of high dilution for 12–48 h but all without success. Pentapeptide **4** was also treated with PyBOP and solid $NaHCO_3$ at various temperatures (−15 to 25 °C) under high-dilution conditions in dry DMF, and allowing the possible reaction to proceed for 12–48 h. However,

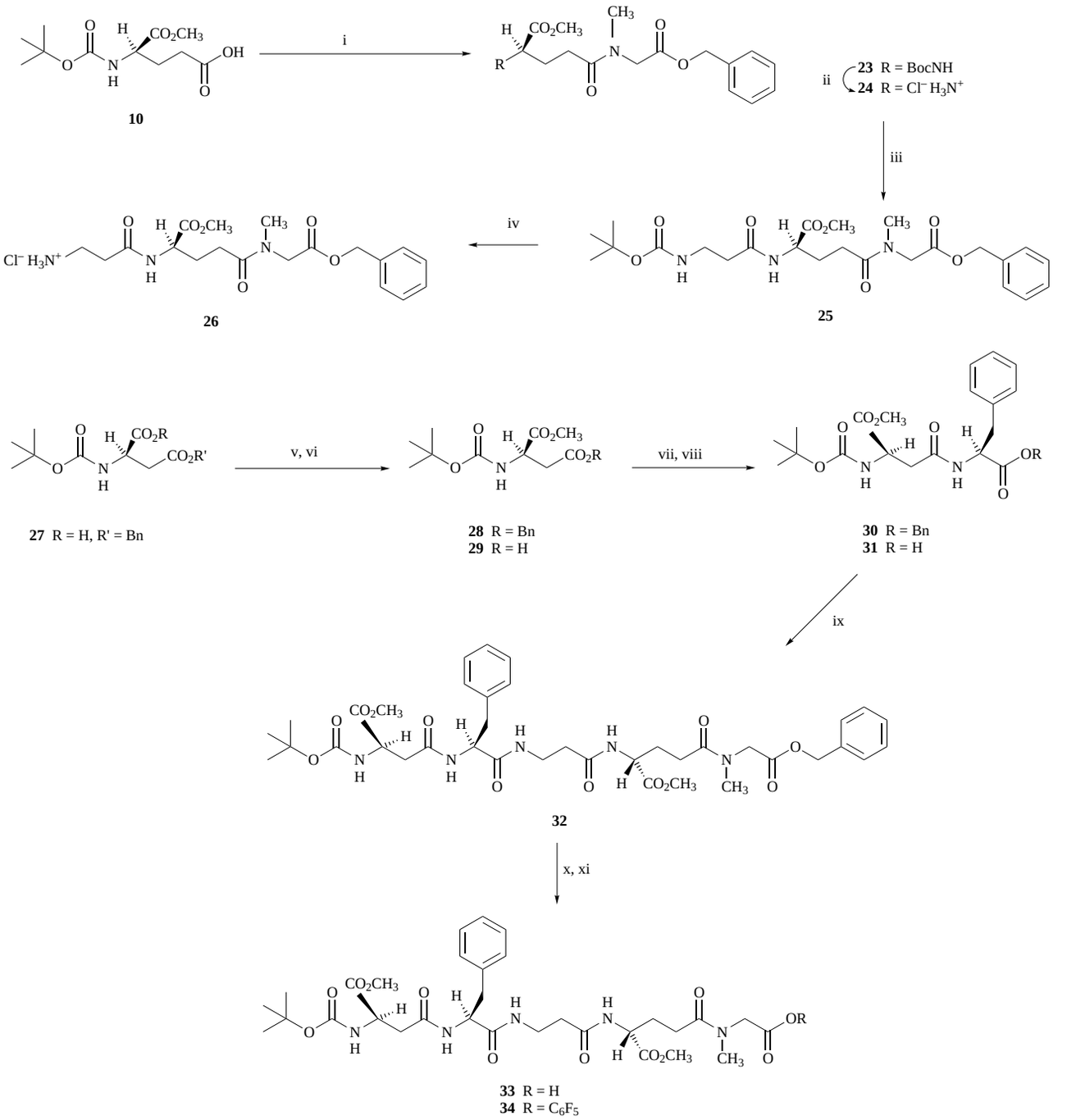


Scheme 2 *Reagents, conditions* (*and yields*): i, CH_2N_2, Et_2O, 0 °C, 30 min (100%); ii, H_2, 10% Pd/C, EtOH, room temp., 3 h (100%); iii, NMM, Bu^{i}OCOCl, Gly-OBn; *p*-TsOH, THF–DMF, −15 °C ⟶ room temp., 30 min (96%), iv, $HCl_{(g)}$, EtOAc, 0 °C ⟶ room temp., 1.5 h (90%); v, NMM, Bu^{i}OCOCl, Boc-β-Ala, THF–DMF, −15 °C ⟶ room temp., 30 min (91%); vi, $HCl_{(g)}$, EtOAc, 0 °C ⟶ room temp., 1.5 h (90%); vii, BnOCOCl, $NaHCO_{3(aq.)}$, room temp., 18 h (79%); viii, paraformaldehyde, *p*-TsOH, toluene, reflux, 2 h (91%); ix, NMM, Bu^{i}OCOCl, (2*S*)-Phe-OBu^t·HCl, THF, −15 °C ⟶ room temp., 30 min (91%); x, 1 mol dm^{-3} aq. NaOH, EtOH, room temp., 1 h (87%); xi, TFA, CH_2Cl_2, room temp., 4 h (73%); xii, NMM, Bu^{i}OCOCl, **14**, THF–DMF, −15 °C ⟶ room temp., 2.5 h (86%).

all of these reaction conditions failed to give the desired macrocycle **3**. In a final attempt the reagent FDPP, which had been used successfully to achieve ring closure in the synthesis of cyclotheonamide A,[46] was applied to the cyclisation of isopentapeptide **4** again without success.

Although it is difficult to understand why the model cyclisation did not occur, it was conceivable that the NH moiety of the glycine residue, which is replaced by an NMe moiety in the natural toxins, was stabilising an unreactive conformation through transannular H-bond formation. Accordingly, the synthesis was repeated using sarcosine in place of glycine. At the same time, the strategy for the use of protecting groups was altered so that the N-terminal and C-terminal functionalities could be unmasked separately in order to take advantage of a wider range of activated ester cyclisation protocols, including the pentafluorophenyl ester method.

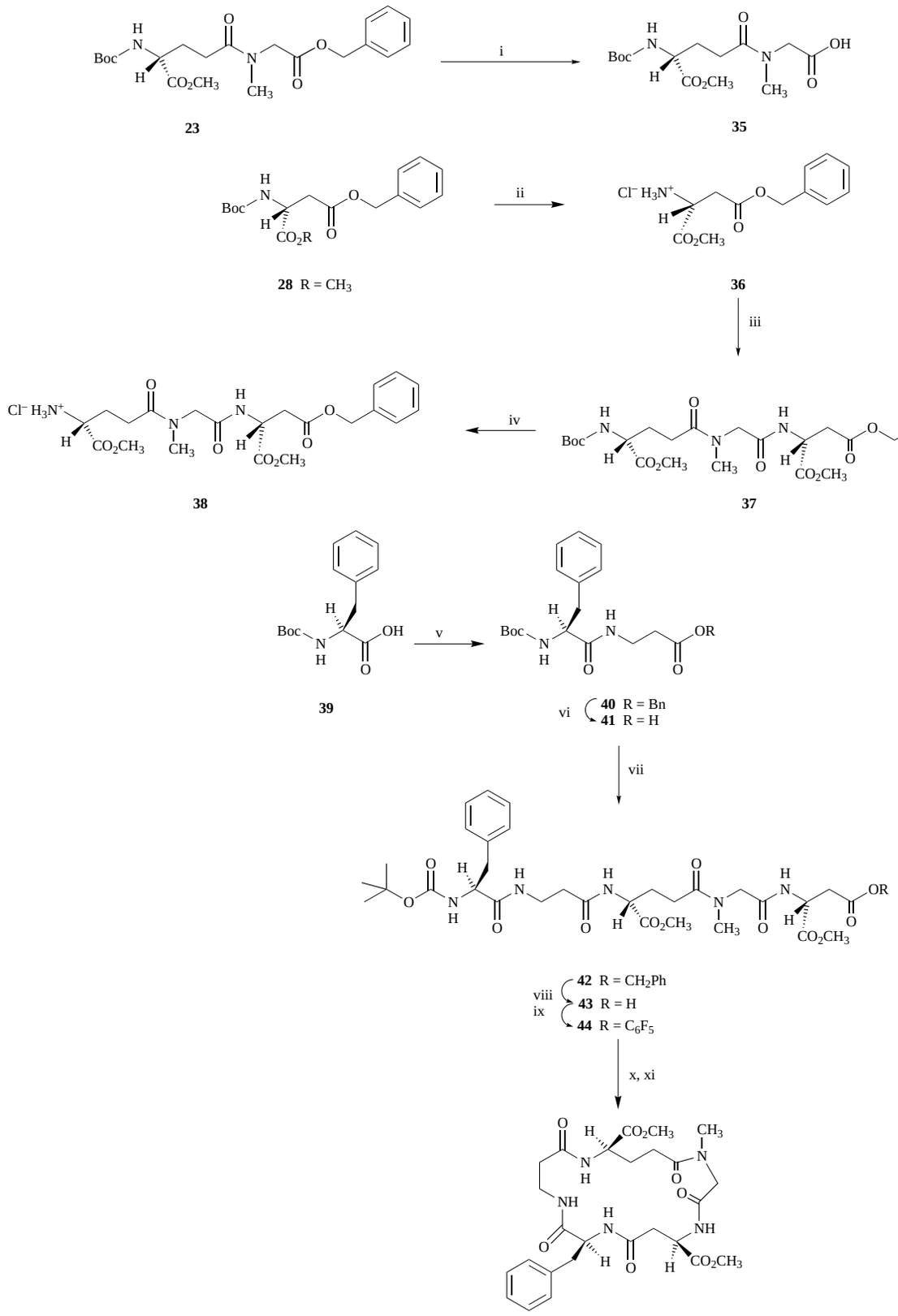
Disconnection **A** of *cyclo*-[β-Ala-(*R*)-Glu-α-OMe-γ-Sar-(*R*)-Asp-α-OMe-β-(*S*)-Phe-] **5** gives the linear tri-isopentapeptide precursor **6** (see Scheme 1). For the synthesis of the linear pentapeptide *N*-Boc-(2*R*)-Asp-α-OMe-β-(2*S*)-Phe-β-Ala-(2*R*)-Glu-α-OMe-γ-Sar-OBn (see Scheme 3) we opted to use orthogonal protecting groups for the N- and C-termini in order to have access to a wider range of macrolactamisation protocols, as mentioned above. The sarcosine-containing tripeptide ester **25** was prepared in 63% overall yield, *via* compound **23**, in an identical manner to that for the glycine analogue **13** (Scheme



Scheme 3 *Reagents, conditions* (*and yields*): i, NMM, Bu^{i}OCOCl, Sar-OBzl·*p*-TsOH, THF–DMF, −15 °C ⟶ room temp., 1 h (94%), ii, $HCl_{(g)}$, EtOAc, 0 °C ⟶ room temp., 1.5 h (82%); iii, NMM, Bu^{i}OCOCl, Boc-β-Ala, THF–DMF, −15 °C ⟶ room temp., 45 min (82%); iv, $HCl_{(g)}$, EtOAc, 0 °C ⟶ room temp., 1.5 h (85%); v, CH_2N_2, Et_2O, 0 °C, 30 min (100%); vi, H_2, 10% Pd/C, EtOH, room temp., 3 h (100%); vii, NMM, Bu^{i}OCOCl, (2*S*)-Phe-OBn·*p*-TsOH, THF, −15 °C ⟶ room temp., 45 min (99%); viii, H_2, 10% Pd/C, MeOH, room temp., 3 h (100%); ix, NMM, Bu^{i}OCOCl, THF, −15 °C ⟶ room temp., 2 h (72%); x, H_2, 10% Pd/C, EtOH, room temp., 12 h (100%); xi, EDCI, C_6F_5OH, CH_2Cl_2, 0 °C ⟶ room temp., 12 h (63%).

2), starting from compound **10**. Compound **25** {mp 78–79 °C; $[\alpha]_D^{23}$ +26.0 (MeOH)} and precursor **23** gave satisfactory analytical data but showed two sets of signals in the ^{1}H NMR spectrum, one set for each of the *cis*- and *trans*-rotameric forms of the γ-Glu–Sar amide bond. The N-Boc protection in compound **25** was removed by treatment with HCl gas in ethyl acetate to give the amine salt **26** in 85% yield. Reaction of this amine salt with the mixed anhydride of *N*-Boc-(2*R*)-Asp-α-OMe-β-(2*S*)-Phe-OH **31**, itself prepared from *N*-Boc-(2*R*)-aspartic acid β-benzyl ester **27** as shown in Scheme 3, afforded the pentapeptide **32** in 72% yield. This compound {mp 82–83 °C; $[\alpha]_D^{23}$ +6.22 (MeOH)} displayed the expected spectral and analytical properties and was now ready for activation for cyclisation.

In order to effect cyclisation, the pentafluorophenyl ester method was first considered. Thus, pentafluorophenyl ester **34** was prepared in three steps through catalytic hydrogenolysis of the benzyl ester group of pentapeptide **32**, activation of the unmasked carboxylic acid group with EDCI, and, finally, reaction of the activated ester with pentafluorophenol. The required PFP ester **34** was obtained in 63% yield after chromatographic purification and displayed the expected spectral properties. However, when the N-protection was removed with TFA and the amine salt was treated with Hünig's base (DIPEA), none of the required cyclic peptide **5** was formed as judged by ^{1}H NMR spectroscopy. DPPA-mediated macrolactamisation was also investigated, as described for the Gly-containing pentapeptide **4**, but again none of the required macrocycle **5** was obtained.



Scheme 4 *Reagents, conditions* (*and yields*): i, H_2, 10% Pd/C, EtOH, room temp., 3 h (98%); ii, $HCl_{(g)}$, EtOAc, 0 °C ⟶ room temp., 1.5 h (89%); iii, NMM, Bu^iOCOCl, **35**, THF–DMF, −15 °C ⟶ room temp., 50 min (92%); iv, $HCl_{(g)}$, EtOAc, 0 °C ⟶ room temp., 2 h (89%); v, NMM, Bu^iOCOCl, β-Ala-OBn·*p*-TsOH, THF, −15 °C ⟶ room temp., 1 h (85%); vi, H_2, 10% Pd/C, EtOH, room temp., 3 h (99%); vii, NMM, Bu^iOCOCl, **38**, THF, −15 °C ⟶ room temp., 3.5 h (81%); viii, H_2, 10% Pd/C, MeOH, room temp., 5 h (98%); ix, EDCI, C_6F_5OH, CH_2Cl_2, 0 °C ⟶ room temp., 12 h (69%); x, TFA, DCM, room temp., 45 min; xi, DIPEA, CH_2Cl_2, room temp., 9 days (89%).

We cannot offer an explanation as to why this reaction has repeatedly failed to work. However, it is evident that a ground-state conformation for macrocyclisation is not accessible enough to facilitate reaction *via* disconnection strategy **A**, Scheme 1, because (i) both longer reaction times and more forcing conditions gave side-products and/or some polymeric material, and (ii) identical activating reagents worked well for an alternative cyclisation of the same macrocyclic target, compound **5**, see below.

In view of the failure to effect macrolactamisation *via*

disconnection **A** (Scheme 1), disconnection **B** was examined. Disconnection **B** of compound **5** (Scheme 1), gives the linear pentapeptide precursor (2*S*)-NH_2-Phe-β-Ala-(2*R*)-Glu-α-OMe-γ-Sar-(2*R*)-Asp-α-OMe-β-OH, **7.** This was prepared from the N-*tert*-butoxycarbonyl-protected C-terminal benzyl ester, which was itself constructed as shown in Scheme 4. The γ-glutamylsarcosine diester **23** was prepared as shown in Scheme 3, and as expected, displayed NMR spectral signals corresponding to both amide rotamers as noted earlier. Diester **23** was subjected to hydrogenolysis to uncover the sarcosine carboxy group to give compound **35**. The carboxy group was activated as the mixed isobutyl carbonic anhydride which was then treated with the (2*R*)-aspartate α-methyl β-benzyl diester hydrochloride **36** [derived from the deprotection of compound **28** with dry HCl] to give the fully protected intermediate Glu-γ-Sar-Asp tripeptide triester **37** in 92% yield which displayed the expected properties. Compound **37** was treated with HCl gas in ethyl acetate to give the amine salt **38** which was now ready for incorporation into the target pentapeptide.

In order to prepare the N-terminal dipeptide of target **7**, benzyl β-alaninate ester toluenesulfonate salt was treated with mixed anhydride–activated *N*-Boc-(2*S*)-phenylalanine to give the dipeptide benzyl ester **40** in 85% yield. Removal of the benzyl ester protection by catalytic hydrogenolysis gave the required acid **41**. Activation of the free acid as the mixed anhydride, followed by treatment with the N-terminal unmasked tripeptide triester **38** and NMM, gave the pentapeptide triester **42** in 81% yield {mp 73–76 °C; $[\alpha]_D^{23}$ −18.33 (MeOH)} which displayed the expected spectral and analytic properties.

In order to activate the peptide for cyclisation, the C-terminal benzyl ester of compound **42** was removed through catalytic hydrogenolysis and was replaced by a PFP group, using EDCI and pentafluorophenol in dichloromethane as described previously. Compound **44** was obtained in 69% yield after chromatographic purification and displayed the expected properties. Deprotection of the N-Boc pentapeptide PFP ester **44** with TFA in dichloromethane gave the amine TFA salt which was thoroughly dried under high vacuum. The amine TFA salt was then treated with DIPEA in dichloromethane under conditions of high dilution, at room temperature.

The cyclisation reaction could be conveniently followed by TLC on silica gel with 94% CH_2Cl_2–MeOH as eluent, and after seven days at 20 °C the appearance of a new less polar band was judged to be almost complete. After nine days the reaction was stopped and after concentration of the reaction mixture under reduced pressure and trituration of the residue with diethyl ether, a solid was obtained. This was filtered and washed on the pad to afford the required cyclic isopeptide **5** in 89% yield. Macrocycle **5** was found to be insoluble in common organic solvents but was soluble in DMSO. The compound {mp >250 °C (decomp.)} displayed the expected mass spectral and analytical data, but NMR spectra showed the existence of three stable populated conformations. These have been assigned using TOCSY, HSQC and COSY NMR experiments, and 1-D ^{1}H and ^{13}C NMR spectral data in support of the composition of structure **5** are given in the experimental section. NOESY and ROESY spectra have also been recorded for the compound and 3-D conformational structures consistent with these spectra will be reported on in due course together with data for several other synthetic nodularin macrocycles.[47] [Note that it was not possible to assign specific NMR signals to conformational structures for the single macrocycle **5**, due to the presence of so many diastereotopic methylene protons until analogues containing (2*S*)- and (2*R*)-proline in place of the sarcosine residue had been prepared.[47]] Thus, it is possible to prepare non-functionalised nodularin-type macrocycles in high yield using solution-phase protocols.

A small sample of the macrocyclic diester **5** was quantitatively converted into the dilithium salt by using lithium hydroxide in aq. methanol. ^{1}H and ^{13}C NMR spectra of this salt also indicated that, as for the diester **5**, the macrocycle existed in more than one conformational or rotameric form. Note that while this property has been observed in many other nodularin-type macrocycles[47,48] and a microcystin-type macrocycle[48] that we have now prepared using the methodology described here, multiple conformers are not observed in nodularin,[26] motuporin,[36] microcystin[27] or dihydronodularin or dihydromicrocystin.[38] These latter compounds all possess both free carboxylic acid groups and the lipophilic exocyclic side-chain. Since Schreiber's group observed that the dimethyl ester of motuporin also existed as a mixture of rotamers in chloroform,[36] it is likely that the minimum requirements for favouring the single rotameric form of the natural tri-isopentapeptide macrocycles are free acid/or salt groups, which can possibly stabilise a transannular H-bond between the Asp carboxy group and the Glu NH, and a large rigid exocyclic lipophilic moiety. Work currently in progress on synthetic tri-isopentapeptide macrocycles bearing lipophilic side-chains should significantly resolve the issues.

Since our own work commenced, many syntheses have been reported for the unusual β-amino acid, Adda.[49] The first reported total synthesis of a complete macrocycle was that for motuporin **2b**.[36] This appeared in 1995 and used a similar cyclisation strategy to that described here and in the following article for the preparation of a hydroxymethyl-group-functionalised nodularin macrocycle,[48] except for the fact that the lipophilic side-chain was introduced prior to cyclisation. Thus, cyclisation through the reaction of an activated β-carboxy group of the Asp residue with the N-terminal amino group of the linear tri-isopentapeptide appears to be reliable for a range of structures. While we have used a similar disconnection to prepare successfully a microcystin model (see the following article), a recent paper describes the synthesis of microcystin-LA **1b** in which cyclisation was effected between the amino group of the Adda residue and the activated carboxy group of alanine.[50]

Experimental

NMR spectra were recorded on a Bruker AM-300 spectrometer (^{1}H, 300 MHz; ^{13}C, 75.4 MHz), a Varian Gemini spectrometer (^{1}H, 200 MHz; ^{13}C, 50.3 MHz), a Varian Gemini spectrometer (^{1}H, 300 MHz; ^{13}C, 75.4 MHz) and a Varian Unity Plus 500 spectrometer (^{1}H, 500 MHz; ^{13}C, 125.6 MHz). ^{1}H NMR spectra were referenced internally to $(C^2H_3)_2SO$ (δ_H 2.47), $C^2H_3O^2H$ (δ_H 3.35), ^{2}HOH (δ_H 4.68) or C^2HCl_3 (δ_H 7.27). ^{13}C NMR spectra were referenced to $(C^2H_3)_2SO$ (δ_C 39.70), $C^2H_3O^2H$ (δ_C 49.9) or C^2HCl_3 (δ_C 77.5). *J*-Values are given in Hz. IR spectra were recorded using a Perkin-Elmer 1710 FT-IR spectrometer. The samples were prepared as Nujol mulls or thin films between sodium chloride discs. Absorption maxima are given in wavenumbers (cm^{-1}) relative to a polystyrene standard. Mps were measured using an electrothermal mp apparatus and are uncorrected. Optical rotations were measured on an Optical Activity Ltd. AA-1000 polarimeter using 10 cm path length cells at room temperature and $[\alpha]_D$-values are given in units of 10^{-1} deg cm^2 g^{-1}. Mass spectra were recorded on a VG AutoSpec. Major fragments are given as percentages of the base-peak intensity. Solvents and common reagents were purified according to the methods of Perrin, Armarego and Perrin.[51] Analytical TLC was carried out on 0.25 mm precoated silica gel plates (MN SIL G/UV_{254}), and compounds were visualised by UV fluorescence, iodine vapour, ethanolic phosphomolybdic acid or aq. potassium permanganate. Light petroleum refers to the fraction boiling at 40–60 °C. Protected amino acid precursors were purchased from Calbiochem-Novabiochem (UK) Ltd (Beeston, Nottingham). Microcystin-LR was purchased from Life Technologies Ltd (Paisley, UK). All other chemicals were of analytical grade or were recrystallised or redistilled before use.

Dihydromicrocystin-LR diastereoisomers 1d

To a solution of microcystin-LR (1 mg, 0.001 mmol) in MeOH–water (4:1; 0.5 cm^3) was added sodium borohydride (6 mg) in 1 mg portions, and the mixture was stored at room temperature with periodic shaking. After 48 h, the reaction was quenched with 100 mm^3 of 1 mol dm^{-3} aq. KH_2PO_4 and the pH was adjusted to 6.8 using the same acidic buffer. The solution was lyophilised and the residue was subjected to analysis by ^{1}H NMR spectroscopy: δ_H(500 MHz; ^2H_2O) 0.81 [3 H, d, *J* 6.6, CH_3 (Leu)], 0.87 [3 H, d, *J* 6.4, CH_3 (Leu)], 0.92–1.03 [9 H, m, CH_3 (β-MeAsp), CH_3 (C-2 Adda) and CH_3 (C-8 Adda)], 1.26 [3 H, d, *J* 7.3, CH_3 (Ala)], 1.31 [3 H, d, *J* 7.1, CH_3 (*N*-MeAla)], 1.42–1.58 [7 H, m, CH_2CH (Leu), β- and γ-H_2 (Arg)], 1.65 [3 H, s, CH_3 (C-6 Adda)], 1.93–2.39 [4 H, m, β- and γ-CH_2 (Glu)], 2.52–3.08 [7 H, m, H-2 (Adda), CH_2 (C-10 Adda), δ-H_2 (Arg), β-H (β-MeAsp) and H-9 (Adda)], 3.16 [3 H, s, NCH_3 (*N*-MeAla)], 3.25 [3 H, s, OCH_3 (C-9 Adda)], 3.77–3.83 [1 H, m, α-H (*N*-MeAla)], 4.05–4.43 [6 H, m, α-H (Glu), α-H (Arg), α-H (Leu), α-H (β-MeAsp), H-3 (Adda) and α-H (Ala)], 5.47 [1 H, d, *J* 10.0, H-7 (Adda)], 5.52 [1 H, dd, *J* 15.6 and 8.7, H-4 (Adda)] and 7.20–7.30 [5 H, m, Ph (Adda)], which showed that complete reduction had occurred and that the product was a ~ 5:1 mixture of diastereoisomers presumably differing in configuration at C-2 of the *N*-methylalanine residue. In particular, the spectrum showed that the olefinic protons expected for the diene moiety (δ 5.41–6.26) remained, and that the signals for the dehydroalanine moiety (which occur as two singlets at δ 5.44 and 5.89, respectively, in the parent compound) were absent. Analysis of a portion of the product by reversed-phase HPLC verified that the ratio of diastereoisomers was 5:1. The diastereoisomers were separated by HPLC on a C-18 column or, alternatively, a Poros 10 R2 reversed-phase column [using isocratic reversed-phase conditions, eluting with 17% acetonitrile–83% aq. ammonium acetate (0.1% v/v) as eluent at a flow rate of 2 cm^3 min^{-1}, with the detector set at 254 nm] and were collected as two separated bands with retention times of 7.14 min (8.6 column volumes, integral 5.0) and 9.04 (10.8 column volumes, integral 1.0) (data for Poros 10 R2 column). Each fraction was subjected to analysis by ^{1}H NMR spectroscopy which verified that the separation was complete. Each fraction was tested as an inhibitor for cat-PP2A by Dr C. MacKintosh using established protocols (which measure the release of [^{32}P]phosphate from radiolabelled phosphorylated substrate)[52] and each diastereoisomer gave an IC_{50}-value of 0.1 nmol dm^{-3}. Note that similar findings have been reported by Rinehart and coworkers.[38]

α-Methyl γ-benzyl (2*R*)-*N*-(*tert*-butoxycarbonyl)glutamate 9

γ-Benzyl (2*R*)-*N*-(*tert*-butoxycarbonyl)glutamate ester **8** (5.00 g, 11.5 mmol) was dissolved in stirred diethyl ether (15 cm^3) at 0 °C and diazomethane (25 cm^3) was added dropwise. After 30 min the solution was purged with nitrogen to remove excess of diazomethane and was then concentrated under reduced pressure to give a pale yellow oil. Upon storage the oil solidified to give the *title compound* as an amorphous solid in almost quantitative recovery (4.02 g), mp 33–34 °C (Found: C, 61.2; H, 6.9; *N*, 3.8. $C_{18}H_{25}NO_6$ requires C, 61.5; H, 7.2; N, 4.0%) (HRMS: Found: $[M - CO_2CH_3]^+$, 292.1549. $C_{16}H_{22}NO_4$ requires *m/z* 292.1549); $[\alpha]_D^{23}$ +10.48 (*c* 1.0, MeOH) {lit.,[53] −18.0 (*c* 5.2, MeOH) (for the *S*-isomer)}; $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3376 (NH), 1740 (CO, urethane) and 1699 and 1694 (CO, esters); δ_H(200 MHz; C^2HCl_3) 1.43 [9 H, s, $(CH_3)_3$], 1.86–2.29 (2 H, m, β-H_2), 2.31–2.59 (2 H, m, γ-H_2), 3.72 (3 H, s, CH_3), 4.33 (1 H, q, *J* 5.1, α-H), 5.11 (2 H, s, PhCH_2), 5.20 (1 H, d, *J* 8.0, NH) and 7.35 (5 H, s, Ph); δ_C(50.3 MHz; C^2HCl_3) 28.04 (β-CH_2), 28.75 [$(CH_3)_3$], 30.74 (γ-CH_2), 52.83 (CH_3), 53.29 (α-C), 66.90 (PhCH_2), 80.32 [$C(CH_3)_3$], 128.72 and 129.02 (ArCH), 136.26 (ArC quaternary), 155.89 (CO, urethane) and 172.97 and 173.18 (CO, esters); *m/z* (EI) 292 (10%, $[M - CO_2CH_3]^+$), 236 (14, $[M - C_4H_9 - CO_2CH_3 + H]^+$), 192 (47, $[M - Boc - CO_2CH_3 + H]^+$), 108 (12, $PhCH_2OH^+$) and 91 (100, $PhCH_2^+$).

α-Methyl (2*R*)-*N*-(*tert*-butoxycarbonyl)glutamate ester 10

To a solution of benzyl ester **9** (5.00 g, 14.8 mmol) in ethanol (50 cm^3) was added 10% palladium on carbon (0.25 g) and the mixture was stirred under an atmosphere of hydrogen for 3 h. The catalyst was removed by filtration through a pre-washed Celite pad and the filtrate was concentrated under reduced pressure to give the *title compound* as an oil in quantitative recovery which was refractory to crystallisation (3.85 g) (Found: C, 50.8; H, 7.5; N, 5.5. $C_{11}H_{19}NO_6$ requires C, 50.6; H, 7.3; N, 5.4%) (HRMS: Found: $[M + H]^+$, 262.1291. $C_{11}H_{20}NO_6$ requires *m/z*, 262.1291); $[\alpha]_D^{23}$ +27.23 (*c* 5.2, MeOH) {lit.,[53] −27.7 (*c* 4.8 in MeOH) (for the *S*-isomer)}; $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3560–2850br (OH and NH), 1717 (CO, urethane) and 1699 and 1695 (CO, ester and acid); δ_H(200 MHz; C^2HCl_3) 1.43 [9 H, s, $(CH_3)_3$], 1.85–2.20 (2 H, m, β-H_2), 2.42–2.51 (2 H, m, γ-H_2), 3.75 (3 H, s, CH_3), 4.36 (1 H, q, *J* 5.1, α-H), 5.19 (1 H, d, *J* 8.1, NH) and 6.74–7.47 (1 H, br s, CO_2H); δ_C(50.3 MHz; C^2HCl_3) 28.02 (β-CH_2), 28.69 [$(CH_3)_3$], 30.54 (γ-CH_2), 52.91 (CH_3), 53.25 (α-C), 80.64 [$C(CH_3)_3$], 155.99 (CO, urethane), 172.35 (CO, ester) and 173.25 (CO, acid); *m/z* (CI) 262 (8%, $[M + H]^+$), 205 (34, $[M + H - C_4H_9 + H]^+$), 162 (100, $[M + H - C_5H_9O_2 + H]^+$) and 144 (42, $[M + H - C_5H_{10}NO_2 + H]^+$).

Benzyl [(2*R*)-*N*-(*tert*-butoxycarbonyl)-α-methyl glutamyl]-γ-glycinate diester 11

To a stirred soultion of α-methyl (2*R*)-*N*-(*tert*-butoxycarbonyl)glutamate ester **10** (1.55 g, 5.92 mmol) in dry THF (30 cm^3) at −15 °C was added NMM (651 mm^3, 5.92 mmol). IBCF (805 mm^3, 5.92 mmol) was added and the suspension was stirred at −15 °C for 5 min. A mixture of benzyl glycinate toluene-*p*-sulfonate salt (2.00 g, 5.92 mmol) and further NMM (651 mm^3, 5.92 mmol) in a dry mixture of DMF (10 cm^3) and THF (25 cm^3) was then added. The reaction mixture was allowed to warm up to room temperature and then stirred for a further 25 min. The hydrochloride salts were removed by filtration and the solution was concentrated under reduced pressure to yield a pale yellow oil. The residue was re-dissolved in ethyl acetate (20 cm^3), and the solution was washed successively with water (15 cm^3), 5% aq. $NaHCO_3$ (15 cm^3), 10% aq. citric acid (15 cm^3) and brine (20 cm^3) and then was dried ($MgSO_4$), and concentrated under reduced pressure to give an oil which upon storage solidified to give the *title compound* as a crystalline solid (2.32 g, 96%), mp 80–82 °C (Found: C, 59.1; H, 7.2; N, 7.0. Calc. for $C_{20}H_{28}N_2O_7$: C, 58.8; H, 6.9; N, 6.9%) (HRMS: Found: $[M + H]^+$, 409.1975. $C_{20}H_{29}N_2O_7$ requires *m/z* 409.1975); $[\alpha]_D^{23}$ +18.6 (*c* 1.0, MeOH); ν_{max}(Nujol)/cm^{-1} 3334 (NH), 1743 (CO, urethane), 1714 and 1698 (CO, esters) and 1665 (CO, amide); δ_H(200 MHz; C^2HCl_3) 1.44 [9 H, s, $(CH_3)_3$], 1.86–2.28 (2 H, m, β-H_2), 2.31–2.39 (2 H, m, γ-H_2), 3.74 (3 H, s, CH_3), 4.10 [2 H, m, CH_2 (Glu)], 4.35 (1 H, br, α-H), 5.19 (2 H, s, PhCH_2), 5.31 [1 H, d, *J* 5.9, NH (Glu)], 6.63 [1 H, br, NH (Gly)] and 7.36 (5 H, s, Ph); δ_C(50.3 MHz; C^2HCl_3) 28.78 (β-CH_2), 29.48 [$(CH_3)_3$], 32.70 (γ-CH_2), 41.81 [CH_2 (Gly)], 52.97 (CH_3), 53.32 (α-C), 67.48 (PhCH_2), 80.61 [$C(CH_3)_3$], 128.36, 128.51 and 128.63 (ArCH), 135.70 (Ar C quaternary), 156.35 (CO, urethane), 170.39 (CO, amide), 172.84 and 173.28 (CO, esters); *m/z* (CI) 409 (11%, $[M + H]^+$), 353 (15, $[M + H - C_4H_9 + H]^+$) and 309 (100, $[M + H - C_5H_9O_2 + H]^+$).

Benzyl [(2*R*)-α-methyl glutamyl]-γ-glycinate diester hydrochloride 12

Hydrogen chloride gas was bubbled into dry ethyl acetate (50 cm^3) at 0 °C and the solution was stirred for 1 h. To this was added a solution of the glutamyl-glycyl dipeptide **11** (2.04 g, 5 mmol) in ethyl acetate (20 cm^3) and the mixture was stirred for 1.5 h at room temperature. The solution was concentrated under reduced pressure to give the *title salt* as a hygroscopic

solid, which was suspended in diethyl ether and collected by filtration (1.55 g, 90%) (HRMS: Found $[M + H - HCl]^+$, 309.1450. $C_{15}H_{21}N_2O_5$ requires *m/z*, 309.1450); δ_H(200 MHz; ^2H_2O) 2.09–2.28 (2 H, m, β-H_2), 2.44–2.51 (2 H, m, γ-H_2), 3.76 (3 H, s, CH_3), 3.95 [2 H, s, CH_2 (Gly)], 4.07 (1 H, t, *J* 6.6, α-H), 5.13 (2 H, s, PhCH_2) and 7.36 (5 H, s, Ph); δ_C(50.3 MHz; ^2H_2O) 26.38 (β-CH_2), 31.62 (γ-CH_2), 42.42 [CH_2 (Gly)], 53.10 (α-C), 54.64 (CH_3), 68.62 (PhCH_2), 128.96, 129.73 and 129.82 (ArCH), 136.16 (ArC quaternary) and 171.06, 172.39 and 175.52 (CO, amide and esters); *m/z* (CI) 309 (5%, $[M + H - HCl]^+$) and 166 (100, $[M + H - HCl - Gly{\cdot}OBzl + H]^+$).

Benzyl *N*-(*tert*-butoxycarbonyl)-β-alanyl-[α-methyl (2*R*)-glutamyl]-γ-glycinate ester 13

This compound was prepared in a manner identical with that described for the glutamyl-glycyl dipeptide **11**, using *N*-(*tert*-butoxycarbonyl)-β-alanine (0.93 g, 4.91 mmol) and hydrochloride **12** (1.69 g, 4.91 mmol) to give an oil, which was triturated with diethyl ether to afford an amorphous solid which was collected by filtration (2.14 g, 91%), mp 74–76 °C (Found: C, 57.4; H, 7.0; N, 8.8. Calc. for $C_{23}H_{33}N_3O_8$: C, 57.6; H, 7.0; N, 8.8%) (HRMS: Found $[M + H]^+$, 480.2346. $C_{23}H_{34}N_3O_8$ requires *m/z*, 480.2346); $[\alpha]_D^{23}$ +4.6 (*c* 0.5, MeOH); ν_{max}(Nujol)/cm^{-1} 3318 (NH), 1735 (CO, urethane), 1689 and 1683 (CO, esters) and 1651 and 1645 (CO, amides); δ_H(300 MHz; C^2HCl_3) 1.40 [9 H, s, $(CH_3)_3$], 1.94–2.26 [2 H, m, β-H_2 (Glu)], 2.32 [2 H, m, γ-H_2 (Glu)], 2.42 [2 H, t, *J* 6.0, CH_2 (β-Ala)], 3.37 [2 H, q, *J* 6.1, CH_2 (β-Ala)], 3.71 (3 H, s, CH_3), 3.99 [1 H, dd, *J* 5.8 and 18.1, 1 H of CH_2 (Gly)], 4.11 [1 H, dd, *J* 5.2 and 18.3, 1 H of CH_2 (Gly)], 4.57 (1 H, m, α-H), 5.15 (2 H, s, PhCH_2), 5.35 [1 H, t, *J* 5.9, NH (β-Ala)], 7.01 [1 H, br, NH (Gly)], 7.07 [1 H, d, *J* 6.8, NH (Glu)] and 7.33 (5 H, s, Ph); δ_C(75.4 MHz; C^2HCl_3) 27.49 [β-CH_2 (Glu)], 28.20 [$(CH_3)_3$], 31.87 [γ-CH_2 (Glu)], 35.89 and 36.53 [2 × CH_2 (β-Ala)], 41.18 [CH_2 (Gly)], 51.73 (α-C), 52.33 (CH_3), 67.00 (PhCH_2), 79.11 [$C(CH_3)_3$], 128.12, 128.32 and 128.43 (ArCH), 134.97 (ArC quaternary), 155.90 (CO, urethane), 169.80 and 171.84 (CO, amides) and 172.15 and 172.38 (CO, esters); *m/z* (CI) 480 (32%, $[M + H]^+$), 424 (8, $[M + H - C_4H_9 + H]^+$), 406 (10, $[M + H - C_4H_9O + H]^+$), 380 (100, $[M + H - C_5H_9O_2 + H]^+$), 309 (2, [M + H − Boc-β-Ala + H]$^+$) and 108 (5, $PhCH_2OH^+$).

Benzyl β-alanyl-[α-methyl (2*R*)-glutamyl]-γ-glycinate diester hydrochloride 14

The hydrochloride **14** was prepared in a manner identical with that described for the hydrochloride **12**, by using the tripeptide **13** (0.60 g, 1.25 mmol) as the starting material to give the *salt* **14** as a hygroscopic solid which was not purified further (0.47 g, 90%) (HRMS: Found $[M + H - HCl]^+$, 380.1820. $C_{18}H_{26}N_3O_6$ requires *m/z*, 380.1821); δ_H(300 MHz; ^2H_2O) 1.91–2.23 (2 H, m, β-H_2), 2.39 (2 H, m, γ-H_2), 2.72 [2 H, m, CH_2 (β-Ala)], 3.23 [2 H, t, *J* 6.4, CH_2 (β-Ala)] 3.73 (3 H, s, CH_3), 4.00 [2 H, s, CH_2 (Gly)], 4.07 (1 H, m, α-H), 5.19 (2 H, s, PhCH_2) and 7.42 (5 H, s, Ph); δ_C(75.4 MHz; ^2H_2O) 24.47 (β-CH_2), 29.69 (γ-CH_2), 33.59 and 39.18 [CH_2 (β-Ala)], 39.55 [CH_2 (Gly)], 50.21 (α-C), 51.15 (CH_3), 65.68 (PhCH_2), 125.56, 126.34 and 126.91 (ArCH) and 133.33 (ArC quaternary); *m/z* (CI), 380 (30%, $[M + H - HCl]^+$), 304 (31, $[M - Cl - C_6H_5 + H]^+$), 233 (7, $[M + H - C_9H_9O_2 - Cl + H]^+$) and 91 (100, $PhCH_2^+$).

(4*R*)-3-Benzyloxycarbonyl-5-oxooxazolidine-4-acetic acid 17

A mixture containing *N*-Z-(2*R*)-aspartic acid **16** (1.34 g, 5.00 mmol), paraformaldehyde (0.30 g, 10.0 mmol) and toluene-*p*-sulfonic acid (0.06 g, 0.30 mmol) in toluene (50 cm^3) was refluxed for 2 h using a Dean–Stark trap to remove water. The mixture was concentrated under reduced pressure to give an oily residue, which was dissolved in ethyl acetate (30 cm^3), and the solution was washed with water (20 cm^3) and extracted with 5% aq. $NaHCO_3$ (3 × 20 cm^3). The combined aqueous extracts were acidified with 6 mol dm^{-3} HCl at 0 °C and were then re-extracted with ethyl acetate (3 × 25 cm^3). The pooled organic phase was washed with water, dried ($MgSO_4$), and then concentrated under reduced pressure to give an oil. Crystallisation from diethyl ether–light petroleum gave the title oxazolidineacetic acid as a crystalline solid (1.27 g, 91%), mp 81–83 °C [lit.,[44] 85–87 °C (for the *S*-isomer)] (Found: C, 55.8; H, 4.6; N, 4.9. Calc. for $C_{13}H_{13}NO_6$: C, 55.9; H, 4.7; N, 5.0%) (HRMS: Found: $[M + H]^+$, 280.0821. $C_{13}H_{14}NO_6$ requires *m/z*, 280.0821); $[\alpha]_D^{23}$ −130.6 (*c* 1.0, MeOH) [lit.,[44] +125.7 (*c* 3.53, MeOH) (for the *S*-isomer)]; $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3000br (OH), 1805 (CO, oxazolidinone), 1719 (CO, urethane) and 1710 (CO, acid); δ_H(200 MHz; C^2HCl_3) 3.04–3.45 (2 H, m, β-H_2), 4.37 (1 H, br, 4-H), 5.13 (2 H, s, PhCH_2), 5.31 (1 H, d, *J* 3.0, 2-H), 5.51 (1 H, br, 2-H) and 7.37 (5 H, s, Ph); δ_C(50.3 MHz; C^2HCl_3) 34.62 (β-C), 51.85 (4-C), 68.74 (PhCH_2), 78.72 (2-C), 128.87 and 129.24 (ArCH), 135.59 (ArC quaternary), 153.28 (CO, urethane), 171.98 (CO, oxazolidinone) and 175.65 (CO, acid); *m/z* (EI) 279 (4%, M^+), 235 (14, $[M - CO_2]^+$), 108 (5, $PhCH_2OH^+$) and 91 (100, $PhCH_2^+$).

tert-Butyl *N*-[(4*R*)-3-benzyloxycarbonyl-5-oxooxazolidin-4-yl-acetyl]-(2*S*)-phenylalaninate 18

To a stirred solution of the oxazolidinone **17** (2.23 g, 8 mmol) in dry THF (50 cm^3) at −15 °C was added NMM (880 mm^3, 8 mmol). IBCF (1.09 cm^3, 8 mmol) was added and the suspension was stirred at −15 °C for 5 min. A mixture of (2*S*)-phenylalanine *tert*-butyl ester hydrochloride (2.06 g, 8 mmol) and NMM (880 mm^3, 8 mmol) in dry THF (25 cm^3) was then added and the stirred reaction mixture was allowed to warm up to room temperature and was then left for a further 30 min. The hydrochloride salts were removed by filtration and the solvents were removed under reduced pressure to yield an oil, which was crystallised from diethyl ether–light petroleum to afford compound **18** as a crystalline solid (3.51 g, 91%), mp 117 °C (Found: C, 64.6; H, 6.15; N, 5.8. Calc. for $C_{26}H_{30}N_2O_7$: C, 64.7; H, 6.3; N, 5.8%) (HRMS: Found $[M + H]^+$, 483.2131. $C_{26}H_{31}N_2O_7$ requires *m/z* 483.2131); $[\alpha]_D^{23}$ −92.9 (*c* 1.0, MeOH); ν_{max}(Nujol)/cm^{-1} 3331 (NH), 1812 (CO, oxazolidinone), 1716 (CO, urethane), 1706 (CO, ester) and 1665 (CO, amide); δ_H(300 MHz; C^2HCl_3) 1.41 [9 H, s, $(CH_3)_3$], 2.85–3.00 (2 H, m, β-H_2), 3.00–3.11 [2 H, m, PhCH_2 (Phe)], 4.27 [1 H, br, α-H (Asp)], 4.70 [1 H, q, *J* 6.6, α-H (Phe)], 5.18 (2 H, s, PhCH_2), 5.21 (1 H, d, *J* 5.8, 2-H), 5.51 (1 H, br, 2-H), 6.02–6.14 (1 H, br, NH) and 7.07–7.38 (10 H, m, Ph); δ_C(75.4 MHz; C^2HCl_3) 27.79 [$(CH_3)_3$], 35.82 (β-CH_2), 38.14 [PhCH_2 (Phe)], 51.69 [α-C (Asp)], 53.59 [α-C (Phe)], 67.70 [PhCH_2 (Asp)], 78.12 (2-C), 82.62 [$C(CH_3)_3$], 126.78, 128.11, 128.19, 128.50, 128.61 and 129.28 (ArCH), 135.32 and 135.87 (ArC quaternary), 152.52 (CO, urethane), 167.80 (CO, amide) and 170.19 and 171.90 (CO, oxazolidinone and ester); *m/z* (CI) 483 (16%, $[M + H]^+$), 427 (100, $[M + H - C_4H_9 + H]^+$), 383 (9, $[M + H - C_5H_9O_2 + H]^+$), 263 (18, [M + H − Phe·OBu^t]$^+$), 108 (14, $PhCH_2OH^+$) and 91 (7, $PhCH_2^+$).

tert-Butyl [(2*R*)-*N*-benzyloxycarbonyl-α-methyl aspartyl]-β-(2*S*)-phenylalaninate diester 20

To a stirred solution of dipeptide **18** (1.00 g, 2.07 mmol) in methanol (35 cm^3) was added 1 mol dm^{-3} aq. NaOH (5 cm^3). After 1 h, the reaction mixture was concentrated under reduced pressure to give a solid, which was then dissolved in water (10 cm^3) and partitioned carefully between 10% aq. citric acid–diethyl ether (1:1; 50 cm^3) at 0 °C. The aqueous phase was extracted with diethyl ether (3 × 25 cm^3) and the combined ethereal extracts were dried ($MgSO_4$), and concentrated under reduced pressure to yield the acid **19** as a solid (0.88 g, 91%).

The crude acid **19** (0.81 g, 1.73 mmol) was dissolved in diethyl ether (15 cm^3) and an excess of ethereal diazomethane was then added at 0 °C. After 30 min the solution was purged with nitrogen to remove the excess of diazomethane. The solution was then concentrated under reduced pressure to give the ester **20** as

an oil in quantitative recovery which was refractory to crystallisation (0.91 g) (Found: C, 64.7; H, 6.9; N, 5.8. Calc. for $C_{26}H_{32}N_2O_7$: C, 64.45; H, 6.7; N, 5.8%) (HRMS: Found $[M + H]^+$, 485.2288. $C_{26}H_{33}N_2O_7$ requires *m/z*, 485.2288); $[\alpha]_D^{23}$ +2.34 (*c* 1.11, MeOH); ν_{max}(Nujol)/cm^{-1} 3340 (NH), 1729 (CO, urethane), 1700 and 1692 (CO, esters) and 1670 (CO, amide); δ_H(300 MHz; C^2HCl_3) 1.41 [9 H, s, $(CH_3)_3$], 2.65–2.75 (1 H, m, 1 H of β-H_2), 2.92–3.02 (1 H, m, 1 H of β-H_2), 3.06 [2 H, d, *J* 5.9, PhCH_2 (Phe)], 3.74 (3 H, s, CH_3), 4.55–4.63 [1 H, m, α-H (Asp)], 4.69 [1 H, q, *J* 6.5, α-H (Phe)], 5.13 [2 H, d, *J* 5.9, PhCH_2 (Asp)], 5.91 [1 H, d, *J* 8.4, NH (Asp)], 6.04 [1 H, br d, NH (Phe)] and 7.09–7.39 (10 H, m, Ph); δ_C(75.4 MHz; C^2HCl_3) 27.81 [$(CH_3)_3$], 37.75 (β-CH_2), 38.05 [PhCH_2 (Phe)], 50.51 [α-C (Asp)], 52.60 (CH_3), 53.47 [α-C (Phe)], 66.89 [PhCH_2 (Asp)], 82.52 [$C(CH_3)_3$], 126.90, 127.92, 128.28, 128.37, 128.39 and 129.36 (ArCH), 135.79 and 136.14 (ArC quaternary), 155.99 (CO, urethane), 168.90 (CO, amide) and 170.26 and 171.36 (CO, esters); *m/z* (CI) 485 (6%, $[M + H]^+$), 429 (74, $[M + H - C_4H_9 + H]^+$), 414 (100), 385 (13, $[M + H - C_5H_9O_2 + H]^+$), 263 (68, $[M - Phe{\cdot}OBu^t]^+$), 108 (71, $PhCH_2OH^+$) and 91 (18, $PhCH_2^+$).

[(2*R*)-*N*-Benzyloxycarbonyl-α-methyl aspartyl]-β-(2*S*)-phenylalanine ester 21

To a stirred solution of dipeptide **20** (1.36 g, 2.81 mmol) in dichloromethane (15 cm^3) was added TFA (15 cm^3). After 4 h, when no starting material remained, as judged by TLC, the reaction mixture was concentrated under reduced pressure. The residual oil was re-dissolved in ethyl acetate (30 cm^3), the solution was washed with water (30 cm^3) and then extracted with 10% aq. $NaHCO_3$ (3 × 40 cm^3). The combined aqueous extracts were carefully acidified with 6 mol dm^{-3} HCl and then re-extracted with ethyl acetate (3 × 40 cm^3). The organic extracts were washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure to give the *title compound* as a solid which was not purified further (0.88 g, 73%) (HRMS: Found $[M + H]^+$, 429.1660. $C_{22}H_{25}N_2O_7$ requires *m/z*, 429.1662); ν_{max}(Nujol)/cm^{-1} 3432 (NH), 3050br (OH) and 1747–1680br (CO, urethane, acid, ester and amide); δ_H(300 MHz; C^2HCl_3) 2.65–2.82 (1 H, m, 1 H of β-H_2), 2.84–2.91 (1 H, m, 1 H of β-H_2), 2.99–3.20 [2 H, m, PhCH_2 (Phe)], 3.73 (3 H, s, CH_3), 4.52–4.58 [1 H, m, α-H (Asp)], 4.77–4.83 [1 H, m, α-H (Phe)], 5.11 [2 H, d, *J* 6.3, PhCH_2 (Asp)], 6.05 [1 H, dd, *J* 8.2 and 36.3, NH (Asp)], 6.35 [1 H, dd, *J* 7.7 and 18.0, NH (Phe)] and 7.10–7.36 (10 H, m, Ph); δ_C(75.4 MHz; C^2HCl_3) 37.10 (β-CH_2), 37.53 [PhCH_2 (Phe)], 50.68 [α-C (Asp)], 52.71 (CH_3), 53.05 [α-C (Phe)], 67.10 [PhCH_2 (Asp)], 127.65, 128.51, 128.74, 128.81, 129.08 and 129.82 (ArCH), 135.53 and 135.88 (ArC quaternary), 156.32 (CO, urethane), 169.71 (CO, amide) and 171.36 (CO, ester) and 173.82 (CO, acid); *m/z* (CI) 429 (100%, $[M + H]^+$), 411 (32, $[M - H_2O]^+$), 385 (35, $[M - CO_2 + H]^+$) and 91 (29, $PhCH_2^+$).

Benzyl [(2*R*)-*N*-benzyloxycarbonyl-α-methyl aspartyl]-β-(2*S*)-phenylalanyl-β-alanyl-[α-methyl (2*R*)-glutamyl]-γ-glycinate triester 22

To a stirred solution of the aspartyl-phenylalanyl dipeptide **21** (0.36 g, 0.84 mmol) in dry THF (10 cm^3) at −15 °C was added NMM (92 mm^3, 0.84 mmol). IBCF (114 mm^3, 0.84 mmol) was added and the suspension was stirred at −15 °C for a further 5 min. A mixture of tripeptide hydrochloride **14** (0.35 g, 0.84 mmol) and further NMM (92 mm^3, 0.84 mmol) in a dry mixture of DMF (1 cm^3) and THF (8 cm^3) was then added and the reaction mixture was allowed to warm to room temperature and then was stirred for a further 2 h. The hydrochloride salts were removed by filtration and the solvents were removed under reduced pressure to give a pale yellow oil. The residue was re-dissolved in ethyl acetate (15 cm^3), and the solution was washed successively with water (10 cm^3), 5% aq. $NaHCO_3$ (10 cm^3), 10% aq. citric acid (10 cm^3) and then (10 cm^3), and then dried ($MgSO_4$), and concentrated under reduced pressure to give an oil. Crystallisation from acetone–water gave the pentapeptide **22** as a microcrystalline solid (0.57 g, 86%), mp 133–135 °C (Found: C, 60.8; H, 6.0; N, 9.0. Calc. for $C_{40}H_{47}N_5O_{12}$: C, 60.8; H, 6.0; N, 8.9%) (HRMS: Found $[M + H]^+$, 790.3297. $C_{40}H_{48}N_5O_{12}$ requires *m/z*, 790.3299); $[\alpha]_D^{23}$ −1.6 (*c* 0.5, MeOH); ν_{max}(Nujol)/cm^{-1} 3279 (NH), 1738br (CO, urethanes), 1685br (CO, esters) and 1645 (CO, amides); δ_H(500 MHz; C^2HCl_3) 1.90–1.95 [2 H, br, β-H_2 (Glu)], 2.23–2.39 [6 H, br, γ-H_2 (Glu), CH_2 (β-Ala) and β-H_2 (Asp)], 2.59–3.23 [4 H, m, PhCH_2 (Phe) and CH_2 (β-Ala)], 3.72 (3 H, s, CH_3), 3.73 (3 H, s, CH_3), 3.89–3.97 [1 H, dd, *J* 4.8 and 13.3, 1 H of CH_2 (Gly)], 4.12- 4.17 [1 H, m, 1 H of CH_2 (Gly)], 4.54–4.66 (3 H, m, 3 × α-H), 5.14 (2 H, s, PhCH_2), 5.17 (2 H, s, PhCH_2), 6.04–6.10 (1 H, m, NH), 6.72–6.85 (3 H, m, 3 × NH) and 7.10–7.32 (16 H, m, Ph and NH); δ_C(125.7 MHz; C^2HCl_3) 27.42 [β-CH_2 (Glu)], 31.71 [γ-CH_2 (Glu)], 35.57 and 35.73 [2 × CH_2 (β-Ala)], 37.51 [β-CH_2 (Asp)], 37.88 [PhCH_2 (Phe)], 41.23 [CH_2 (Gly)], 51.67 (α-C), 52.49 (α-C), 52.57 and 52.67 (2 × CH_3), 54.44 (α-C), 66.95 and 67.26 [2 × PhCH_2 (Asp and Gly)], 126.81, 127.99, 128.19, 128.31, 128.50, 128.58, 128.65, 129.16 and 129.23 (ArCH), 134.96, 136.10 and 136.44 (ArC quaternary), 155.99 (CO, urethane) and 170.25, 170.91, 171.40, 171.59, 171.86, 172.40 and 172.50 (CO, amides and esters); *m/z* (FAB) 812 (9%, $[M + Na]^+$), 790 (27, $[M + H]^+$) and 154 (100).

[α-Methyl (2*R*)-aspartyl]-β-(2*S*)-phenylalanyl-β-alanyl-[α-methyl (2*R*)-glutamyl]-γ-glycine diester 4

To a solution of pentapeptide **22** (0.6 g, 0.76 mmol) in methanol–ethanol (2 : 1; 75 cm^3) was added 10% palladium on carbon (80 mg) and the mixture was stirred under hydrogen for 24 h. The catalyst was then removed by filtration through a pre-washed Celite pad and the filtrate was concentrated under reduced pressure to give compound **4** as a solid which was not purified further (0.36 g, 83%), mp 116–118 °C (HRMS: Found: $[M + H]^+$, 566.2467. $C_{25}H_{36}N_5O_{10}$ requires *m/z*, 566.2462); δ_H(300 MHz; ^2H_2O) 1.93–2.03 [2 H, m, 1 H of β-H_2 (Glu)], 2.11–2.21 [2 H, m, 1 H of β-H_2 (Glu)], 2.29–2.50 [4 H, m, γ-H_2 (Glu) and CH_2 (β-Ala)], 2.79–3.09 [4 H, m, β-H_2 (Asp) and PhCH_2 (Phe)], 3.70 (3 H, s, CH_3), 3.71 (3 H, s, CH_3), 3.66–3.80 [2 H, m, CH_2 (Gly)], 4.30–4.40 (2 H, m, 2 × α-H), 4.48 (1 H, q, *J* 6.4, α-H) and 7.17–7.37 (5 H, m, Ph); δ_C(75.4 MHz; ^2H_2O) 27.46 [β-CH_2 (Glu)], 29.79 [γ-CH_2 (Glu)], 32.00 and 32.71 [2 × CH_2 (β-Ala)], 33.66 [β-CH_2 (Asp)], 35.46 [PhCH_2 (Phe)], 41.12 [CH_2 (Gly)], 47.39 (α-C), 50.39 (α-C), 51.07 and 51.86 (2 × CH_3), 53.22 (α-C), 125.29, 126.85 and 127.24 (ArCH), 134.96 (ArC quaternary), 167.66, 168.18, 170.92, 171.80, 171.92 and 172.75 (CO, amides and esters) and 174.29 (CO, acid); *m/z* (FAB) 588 (9%, $[M + Na]^+$), 566 (6, $[M + H]^+$), 91 (36, $PhCH_2^+$) and 59 (100).

Benzyl [(2*R*)-*N*-(*tert*-butoxycarbonyl)-α-methyl glutamyl]-γ-sarcosinate diester 23

To a stirred solution of the α-methyl (2*R*)-*N*-(*tert*-butoxycarbonyl)glutamate **10** (2.45 g, 6.00 mmol) in dry THF (35 cm^3) at −15 °C was added NMM (660 mm^3, 6.00 mmol). IBCF (816 mm^3, 6.00 mmol) was added and the suspension was stirred at −15 °C for a further 5 min. A mixture of benzyl sarcosinate toluene-*p*-sulfonate (2.11 g, 6.00 mmol) and more NMM (660 mm^3, 6.00 mmol) in a dry mixture of DMF (10 cm^3) and THF (25 cm^3) was added and the the reaction mixture was allowed to warm to room temperature and was then stirred for a further 45 min. The hydrochloride salts were removed by filtration and the solution was concentrated under reduced pressure to give a pale yellow oil, which was re-dissolved in ethyl acetate (15 cm^3), and the solution was washed successively with water (10 cm^3), 5% aq. $NaHCO_3$ (15 cm^3), 10% aq. citric acid (15 cm^3) and brine (15 cm^3), and then was dried ($MgSO_4$), and concentrated under reduced pressure to give an oil which crystallised on storage to give the *title compound*, (2.38 g, 94%), mp 75–77 °C (Found: C, 59.6; H, 7.1; N, 6.5. $C_{21}H_{30}N_2O_7$

requires C, 59.6; H, 7.2; N, 6.6%) (HRMS: Found $[M + H]^+$, 423.2132. $C_{21}H_{31}N_2O_7$ requires *m/z*, 423.2131); $[\alpha]_D^{23}$ +13.4 (*c* 1.5, MeOH); ν_{max}(Nujol)/cm^{-1} 3340 (NH), 1745 (CO, urethane), 1713br (CO, esters) and 1658 (CO, amide); δ_H(200 MHz; C^2HCl_3, mixture of rotamers) 1.42 [9 H, s, $(CH_3)_3$], 1.90–2.32 (2 H, m, β-H_2), 2.38–2.59 (2 H, m, γ-H_2), 3.02 and 3.10 (3 H, 2 × s, NCH_3), 3.75 (3 H, s, CH_3), 4.05–4.19 [2 H, m, CH_2 (Sar)], 4.23–4.36 (1 H, br, α-H), 5.18 (2 H, s, PhCH_2), 5.30 (1 H, br, NH) and 7.37 (5 H, s, Ph); δ_C(50.3 MHz; C^2HCl_3, mixture of rotamers) 28.13 (β-CH_2), 28.79 [$(CH_3)_3$], 29.47 (γ-CH_2), 35.58 and 36.95 (NCH_3), 50.08 [CH_2 (Sar)], 52.88 (CH_3), 53.64 (α-C), 67.45 (PhCH_2), 80.37 [$C(CH_3)_3$], 128.83, 128.94 and 129.21 (ArCH), 135.82 (ArC quaternary), 156.84 (CO, urethane), 169.68 (CO, amide) and 173.13 and 173.41 (CO, esters); *m/z* (CI) 409 (11%, $[M + H]^+$), 353 (15, $[M + H - C_4H_9 + H]^+$) and 309 (100, $[M + H - C_5H_9O_2 + H]^+$).

Benzyl [α-methyl (2*R*)-glutamyl]-γ-sarcosinate diester hydrochloride 24

This compound was prepared in a manner identical with that described for the hydrochloride **12**, using the dipeptide **23** (2.11 g, 5 mmol) as the starting material to give the required *salt* as an essentially pure hygroscopic solid which was not purified further (1.47 g, 82%) (HRMS: Found: $[M + H - HCl]^+$, 323.1615. $C_{16}H_{23}N_2O_5$ requires *m/z*, 323.1607); $[\alpha]_D^{23}$ +13.50 (*c* 0.64, MeOH); $\nu_{max}(CH_2Cl_2)$/cm^{-1} 3323 (NH), 1759 (CO, ester) and 1633 (CO, amide); δ_H(200 MHz; ^2H_2O, mixture of rotamers) 2.02–2.20 (2 H, m, β-H_2), 2.38–2.69 (2 H, m, γ-H_2), 2.85 and 3.02 (3 H, 2 × s, NCH_3), 3.73 (3 H, s, CH_3), 4.02–4.18 [3 H, m, CH_2 (Sar) and α-H], 5.11 (2 H, s, PhCH_2) and 7.35 (5 H, s, Ph); δ_C(50.3 MHz; ^2H_2O) 27.74 (β-CH_2), 31.25 (γ-CH_2), 39.51 [NCH_3 (Sar)], 53.03 [CH_2 (Sar)], 54.97 (CH_3), 56.43 (α-C), 70.32 (PhCH_2), 131.12, 131.20, 131.53 and 131.63 (ArCH), 137.97 (ArC quaternary) and 172.97, 173.83 and 176.95 (CO, amide and esters); *m/z* (CI) 323 (13%, $[M + H - HCl]^+$), 263 (31, $[M - CO_2CH_3]^+$), 91 (94, $PhCH_2^+$), 84 (100, CH_2CH-$CO_2CH_3^+$) and 56 (52, $CH_2CH_2CO^+$).

Benzyl *N*-(*tert*-butoxycarbonyl)-β-alanyl-[α-methyl (2*R*)-glutamyl]-γ-sarcosinate diester 25

This compound was prepared in a manner identical with that described for the glutamyl-glycyl dipeptide **11**, using *N*-(*tert*-butoxycarbonyl)-β-alanine (0.95 g, 5.00 mmol) and the hydrochloride **24** (1.79 g, 5.00 mmol) to give the required product as an oil, which upon trituration with diethyl ether afforded a solid (2.02 g, 82%), mp 78–79 °C (Found: C, 58.1; H, 6.9; N, 8.4. Calc. for $C_{24}H_{35}N_3O_8$: C, 58.4; H, 7.15; N, 8.5%) (HRMS: Found: M^+, 493.2428. $C_{24}H_{35}N_3O_8$ requires M, 493.2424); $[\alpha]_D^{23}$ +26.0 (*c* 0.25, MeOH); ν_{max}(Nujol)/cm^{-1} 3324 (NH), 1732 (CO, urethane), 1694 and 1685 (CO, esters) and 1651 and 1647 (CO, amides); δ_H(300 MHz; C^2HCl_3, mixture of rotamers) 1.42 [9 H, s, $(CH_3)_3$], 2.04–2.25 [2 H, m, β-H_2 (Glu)], 2.40 [2 H, m, γ-H_2 (Glu)], 2.49 [2 H, m, CH_2 (β-Ala)], 2.98 and 3.06 (3 H, 2 × s, NCH_3), 3.39 [2 H, q, *J* 5.8, CH_2 (β-Ala)], 3.74 (3 H, s, CH_3), 4.17 [2 H, dd, *J* 12.1 and 17.6, CH_2 (Sar)], 4.48 (1 H, q, *J* 5.1, α-H), 5.16 (2 H, s, PhCH_2), 5.29 [1 H, br, NH (β-Ala)], 6.83 [1 H, d, *J* 7.0, NH (Glu)] and 7.36 (5 H, s, Ph); δ_C(75.4 MHz; C^2HCl_3) 26.66 [β−CH_2, (Glu)], 28.63 [$(CH_3)_3$], 29.73 [γ-CH_2 (Glu)], 35.55 and 36.20 [2 × CH_2 (β-Ala)], 36.90 (NCH_3), 50.11 [CH_2 (Sar)], 51.93 (α-C), 52.79 (CH_3), 67.36 (PhCH_2), 79.50 [$C(CH_3)_3$], 128.72, 128.91 and 129.08 (ArCH), 135.75 (ArC quaternary), 156.43 (CO, urethane), 169.50 and 172.75 (CO, amides) and 172.81 and 173.57 (CO, esters); *m/z* (EI) 494 (3%, $[M + H]^+$), 493 (7, M^+), 437 (17, $[M + H - C_4H_9]^+$), 393 (30, $[M + H - C_4H_9O_2]^+$), 323 (44, [M + H − Boc-β-Ala + H]$^+$), 144 (85, $C_7H_{14}NO_2^+$), 91 (100, $PhCH_2^+$) and 57 (58, $C_4H_9^+$).

Benzyl β-alanyl-[α-methyl (2*R*)-glutamyl]-γ-sarcosinate diester hydrochloride 26

This compound was prepared in a manner identical with that described for the hydrochloride **12**, using the tripeptide **25** (0.60 g, 1.22 mmol) as the staring material to give the required, essentially pure, product as a hygroscopic solid which was not purified further (0.44 g, 85%); $[\alpha]_D^{23}$ +8.53 (*c* 0.95 in MeOH); $\nu_{max}(CH_2Cl_2)$/cm^{-1} 3327 (NH), 1752 (CO, ester) and 1653 (CO, amides); δ_H(200 MHz; ^2H_2O, mixture of rotamers) 1.72–2.14 [2 H, m, γ-H_2 (Glu)], 2.39–2.52 [2 H, m, β-H_2 (Glu)], 2.53–2.68 [2 H, m, CH_2 (β-Ala)], 2.82 and 2.98 (3 H, 2 × s, NCH_3), 3.07–3.18 [2 H, m, CH_2 (β-Ala)], 3.62 (3 H, s, CH_3), 4.18–4.38 [3 H, m, α-H (Glu) and CH_2 (Sar)], 5.11 (2 H, s, PhCH_2) and 7.32 (5 H, s, Ph); δ_C(50.31 MHz; ^2H_2O) 28.48 [β-CH_2 (Glu)], 31.58 [γ-CH_2 (Glu)], 34.41 and 38.26 [2 × CH_2 (β-Ala)], 39.66 (NCH_3), 52.81 [CH_2 (Sar)], 53.22 [α-C (Glu)], 55.02 (CH_3), 70.43 (PhCH_2), 130.35, 131.57 and 131.82 (ArCH) and 173.93 and 178.09 (CO, ester and amide); *m/z* (CI) 394 (45%, $[M + H - HCl]^+$), 318 (97, $[M + H - HCl - C_6H_5 + H]^+$) and 180 (100, $[M + H - HCl - C_9H_{14}N_2O_4]^+$).

α-Methyl β-benzyl (2*R*)-*N*-(*tert*-butoxycarbonyl)aspartate diester 28

To a stirred solution of β-benzyl (2*R*)-*N*-(*tert*-butoxycarbonyl)aspartate ester **27** (4.00 g, 12.38 mmol) in diethyl ether (25 cm^3) at 0 °C was added an excess of ethereal diazomethane (25 cm^3) and the solution was stirred for a further 30 min. The solution was then purged with nitrogen to remove excess of diazomethane and was then concentrated under reduced pressure to afford the pure compound as a crystalline solid in quantitative yield, which did not require further purification; mp 64–66 °C {lit.,[54] 67–68 °C (for the 2*S*-isomer)} (Found: C, 60.8; H, 6.9; N, 4.1. $C_{17}H_{23}NO_6$ requires C, 60.5; H, 6.9; N, 4.2%); $[\alpha]_D^{23}$ +8.13 (*c* 1.0, MeOH) {lit.,[54] −7.1 (*c* 1.0, acetone) (for the *S*-isomer)}; ν_{max}(Nujol)/cm^{-1} 3401 (NH), 1737 (CO, urethane) and 1708br (CO, esters); δ_H(200 MHz; C^2HCl_3) 1.45 [9 H, s, $(CH_3)_3$], 2.81–3.09 (2 H, m, β-H_2), 3.70 (3 H, s, CH_3), 4.55–4.64 (1 H, m, α-H), 5.13 (2 H, s, PhCH_2), 5.49 (1 H, d, *J* 8.1, NH) and 7.36 (5 H, s, ArH); δ_C(50.3 MHz; C^2HCl_3) 28.78 [$(CH_3)_3$], 37.39 (β-CH_2), 50.47 (α-C), 53.17 (CH_3), 67.30 (PhCH_2), 80.67 [$C(CH_3)_3$], 128.81, 128.92 and 129.09 (ArCH), 135.86 (ArC quaternary), 155.83 (CO, urethane) and 171.29 and 171.98 (CO, esters); *m/z* (CI) 338 (4%, $[M + H]^+$), 282 (22, $[M + H - C_4H_9 + H]^+$), 238 (8, $[M + H - C_5H_9O_2 + H]^+$), 91 (100, $PhCH_2^+$) and 57 (90, $C_4H_9^+$).

α-Methyl (2*R*)-*N*-(*tert*-butoxycarbonyl)aspartate ester 29

To a solution of α-methyl β-benzyl (2*R*)-*N*-(*tert*-butoxycarbonyl)aspartate diester **28** (1.00 g, 2.97 mmol) in ethanol (30 cm^3) was added 10% palladium on carbon (0.1 g) and the mixture was stirred under hydrogen for 3 h. The catalyst was then removed by filtration through a pre-washed Celite pad and the filtrate was concentrated under reduced pressure to give the *title compound* as a solid in quantitative recovery (0.73 g), mp 98–99 °C; $\nu_{max}(CH_2Cl_2)$/cm^{-1} 3510–2840 br (OH and NH), 1745 (CO, urethane) and 1690br (CO, ester and acid); δ_H(300 MHz; C^2HCl_3) 1.44 [9 H, s, $(CH_3)_3$], 2.86 (1 H, dd, *J* 17.7 and 4.1, 1 H of β-H_2), 3.04 (1 H, dd, *J* 17.1 and 4.1, 1 H of β-H_2), 3.74 (3 H, s, CH_3), 4.58 (1 H, t, *J* 4.2, α-H), 5.55 (1 H, d, *J* 8.3, NH) and 8.30 (1 H, br, OH); δ_C(75.4 MHz; (C^2HCl_3) 28.35 [$(CH_3)_3$], 36.73 (β-CH_2), 49.78 (α-C), 53.92 (CH_3), 80.54 [$C(CH_3)_3$], 155.62 (CO, urethane), 171.66 (CO, ester) and 176.24 (acid CO); *m/z* (EI) 247 (1%, M^+), 188 (30, $[M - CO_2CH_3]^+$) and 57 (100, $C_4H_9^+$).

Benzyl [(2*R*)-*N*-(*tert*-butoxycarbonyl)-α-methyl aspartyl]-β-(2*S*)-phenylalaninate diester 30

This compound was prepared in a manner identical with that described for the glutamyl-glycyl dipeptide **11**, using α-methyl (2*R*)-*N*-(*tert*-butoxycarbonyl)aspartate **29** (0.91 g, 3.68 mmol) and benzyl (2*S*)-phenylalaninate toluene-*p*-sulfonate (1.52 g, 3.68 mmol) to give an oil, which was crystallised from ethyl acetate–hexane to yield the *title compound* as a solid (1.94 g,

99%), mp 132–133 °C (Found: C, 64.85; H, 6.55; N 5.5. Calc. for $C_{26}H_{32}N_2O_7$: C, 64.45; H, 6.6; N, 5.8%) (HRMS: Found: $[M + H]^+$, 485.228. $C_{26}H_{33}N_2O_7$ requires 485.229); $[\alpha]_D^{23}$ −4.4 (*c* 1.1, MeOH); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3350 (NH), 1746 (CO, urethane), 1720 (CO, esters) and 1675 (CO, amide); δ_H(300 MHz; (C^2HCl_3) 1.49 [9 H, s, $(CH_3)_3$], 2.66 [1 H, dd, *J* 15.9 and 4.6, 1 H of β-H_2 (Asp)], 2.91 [1 H, dd, *J* 17.1 and 16.1, 1 H of β-H_2 (Asp)], 3.03–3.16 [2 H, m, PhCH_2 (Phe)], 3.73 (3 H, s, CH_3), 4.49 [1 H, q, *J* 4.2, α-C (Asp)], 4.88 [1 H, q, *J* 5.7, α-C (Phe)], 5.14 (2 H, AB, *J* 12.3 and 6.0, PhCH_2), 5.62 [1 H, d, *J* 6.0, NH (Asp)], 6.04 [1 H, d, *J* 7.8, NH (Phe)] and 6.96–7.42 (10 H, m, Ph); δ_C(75.4 MHz; C^2HCl_3) 28.42 [$(CH_3)_3$], 37.93 [br, β-CH_2 (Asp) and PhCH_2 (Phe)], 50.32 [α-C (Phe)], 52.74 [α-C (Asp)], 53.29 (CH_3), 67.49 (PhCH_2), 80.1 [*C*$(CH_3)_3$], 127.26, 128.71, 128.77 and 129.41 (ArCH), 135.05 and 135.62 (ArC quaternary), 155.74 (CO, urethane) and 169.52, 171.27 and 171.91 (CO, ester); *m/z* (CI) 485 (12%, $[M + H]^+$), 429 (30, $[M + H - C_4H_9 + H]^+$), 385 (38, $[M + H - C_5H_9O_2 + H]^+$), 356 (100), 256 (22, $C_{16}H_{18}NO_2^+$), 91 (20, $PhCH_2^+$) and 57 (32, $C_4H_9^+$).

[(2*R*)-*N*-(*tert*-Butoxycarbonyl)-α-methyl aspartyl]-β-(2*S*)-phenylalanine ester 31

This compound was prepared in a manner identical with that described for α-methyl (*R*)-*N*-(*tert*-butoxycarbonyl)aspartate **29**, using α-methyl (2*R*)-*N*-(*tert*-butoxycarbonyl)aspartyl-β-(2*S*)-phenylalaninate benzyl ester **30** (1.00 g, 2.14 mmol) to give a solid in quantitative recovery (0.81 g), mp 64–65 °C (HRMS: Found: $[M + H]^+$, 395.181. $C_{19}H_{27}N_2O_7$ requires 395.182); $[\alpha]_D^{23}$ +21.25 (*c* 1.2, MeOH); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3333 (NH), 1749 (CO, urethane), 1710br (CO, ester and acid) and 1664 (CO, amide); δ_H(300 MHz; C^2HCl_3) 1.37 [9 H, s, $(CH_3)_3$], 2.61–3.60 [4 H, m, β-H_2 (Asp), PhCH_2 (Phe)], 3.60 (3 H, s, CH_3), 4.37 [1 H, br, α-H (Phe)], 4.71 [1 H, br, α-H (Asp)], 5.62 [1 H, d, *J* 8.3, NH (Asp)], 6.74 [1 H, br, NH (Phe)] and 7.06–7.17 (5 H, m, Ph); δ_C(50.31 MHz; C^2HCl_3) 28.33 [$(CH_3)_3$], 38.01 [br, β-CH_2 (Asp) and PhCH_2 (Phe)], 50.81 [α-C (Asp)], 52.99 (CH_3), 53.86 [α-C (Phe)], 80.56 [*C*$(CH_3)_3$], 127.32, 128.82 and 129.81 (ArCH), 136.71 (ArC quaternary), 156.35 (CO, urethane), 170.43 and 172.60 (CO, ester and amide) and 174.10 (CO, acid); *m/z* (CI) 395 (5%, $[M + H]^+$), 339 (15, $[M + H - C_4H_9 + H]^+$), 295 (17, $[M + H - C_5H_9O_2 + H]^+$), 266 (100) and 57 (14, $C_4H_9^+$).

Benzyl [(2*R*)-*N*-(*tert*-butoxycarbonyl)-α-methyl aspartyl]-β-(2*S*)-phenylalanyl-β-alanyl-[α-methyl (2*R*)-glutamyl]-γ-sarcosinate triester 32

To a stirred solution of (2*R*)-*N*-(*tert*-butoxycarbonyl-aspartyl)-α-methyl-β-(2*S*)-phenylalanine **31** (583 mg, 1.36 mmol) in dry THF (20 cm^3) at −15 °C was added NMM (149 mm^3, 1.36 mmol). IBCF (184 mm^3, 1.36 mmol) was added and the suspension was stirred at −15 °C for a further 5 min. A mixture of the benzyl β-alanyl-α-methyl-(2*R*)-glutamyl-γ-sarcosinate diester hydrochloride **26** (513 mg, 1.36 mmol) and NMM (149 mm^3, 1.36 mmol) in THF (10 cm^3) was then added. The reaction mixture was allowed to warm up to room temperature and was then stirred for a further 2 h. The hydrochloride salts were removed by filtration and the solvents were removed under reduced pressure to give an oil, which was purified by silica chromatography (94% CH_2Cl_2–MeOH) to yield compound **32** as a crystalline solid (750 mg, 72%), mp 82–83 °C (Found: C, 59.25; H, 6.55; N 8.9. Calc. for $C_{38}H_{51}N_5O_{12}$: C, 59.3; H, 6.7; N, 9.1%); $[\alpha]_D^{23}$ +6.22 (*c* 1.24, MeOH); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3316 (NH), 1756 (CO, urethane), 1710br (CO, esters) and 1658 (CO, amides); δ_H(500 MHz; C^2HCl_3, mixture of rotamers) 1.37 and 1.39 [9 H, 2 × s, $(CH_3)_3$], 1.96–2.03 [1 H, m, 1 H of β-H_2 (Glu)], 2.11–2.53 [6 H, m, 1 H of β-H_2 (Glu), γ-H_2 (Glu), 1 H of β-H_2 (Asp) and CH_2 (β-Ala)], 2.75–3.04 [3 H, m, 1 H of β-H_2 (Asp), PhCH_2 (Phe)], 3.04 and 3.06 (3 H, 2 × s, NCH_3), 3.35–3.48 [2 H, m, CH_2 (β-Ala)], 3.63, 3.65, 3.67 and 3.69 (6 H, 4 × s, 2 × CH_3), 3.97–4.12 [2 H, m, CH_2 (Sar)], 4.10–4.44 [2 H, m, 2 × α-H (Glu and Asp)], 4.57 [1 H, br, α-H (Phe)], 5.08 and 5.13 (2 H, 2 × s, PhCH_2), 5.58 [0.4 H, d, *J* 8.35, 0.4 NH (Asp)], 5.66 [0.6 H, d, *J* 8.36, 0.6 NH (Asp)], 6.61–6.91 (3 H, m, 3 × NH) and 7.11–7.3 (10 H, m, Ph); δ_C(75.4 MHz; C^2HCl_3, mixture of rotamers) 27.98 [β-CH_2 (Glu)], 28.32 [$(CH_3)_3$], 29.46 [γ-CH_2 (Glu)], 35.24 (NCH_3), 35.52 and 35.92 [2 × CH_2 (β-Ala)], 36.67 (NCH_3), 37.6 [β-CH_2 (Asp)], 38.22 [PhCH_2 (Phe)], 49.77 [α-C (Asp)], 50.38 [CH_2 (Sar)], 51.62 [α-C (Glu)], 52.46 and 52.55 (2 × CH_3), 54.53 [α-C (Phe)], 67.09 (PhCH_2), 79.88 [*C*$(CH_3)_3$], 126.91, 128.36, 128.57, 128.73, 128.84 and 129.39 (ArCH), 135.41 and 136.94 (ArC quaternary) and 155.72 (CO, urethane), 169.22, 170.21, 171.21, 171.83, 172.47 and 173.28 (CO, amides and esters); *m/z* (FAB) 792 (5%, $[M + Na]^+$), 770 (1, $[M + H]^+$), 670 (17, $[M + H - C_5H_9O_2 + H]^+$) and 120 (100, $[C_8H_9N + H]^+$).

[(2*R*)-*N*-(*tert*-Butoxycarbonyl α-methyl aspartyl]-β-(2*S*)-phenylalanyl-β-alanyl-[α-methyl (2*R*)-glutamyl]-γ-sarcosine diester 33

To a solution of triester **32** (250 mg, 0.325 mmol) in ethanol (30 cm^3) was added 10% palladium on carbon (25 mg) and the mixture was stirred under hydrogen for 12 h. The catalyst was removed by filtration through a pre-washed Celite pad and the filtrate was concentrated under reduced pressure to afford the required acid as a solid in quantiative recovery (0.22 g), mp 55–56 °C (HRMS: Found: $[M + Na]^+$, 702.2975. $C_{31}H_{45}N_5NaO_{12}$ requires *m/z*, 702.2962); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3296 (NH), 1745 (CO, ester) and 1649 (CO, amides); δ_H(300 MHz; C^2HCl_3, mixture of rotamers) 1.35 [9 H, s, $(CH_3)_3$], 1.91–2.14 [2 H, m, β-H_2 (Glu)], 2.21–2.59 [6 H, m, γ-H_2 (Glu), β-H_2 (Asp) and CH_2 (β-Ala)], 2.76–2.82 [2 H, m, PhCH_2 (Phe)], 2.82 and 2.99 (3 H, 2 × s, NCH_3), 2.37 [2 H, br, CH_2 (β-Ala)], 3.60, 3.61, 3.62 and 3.63 (6 H, 4 × s, 2 × CH_3), 3.93–4.18 [2 H, m, CH_2 (Sar)], 4.40 [2 H, br, 2 × α-H (Glu and Asp)], 4.57 [1 H, br, α-H (Phe)], 5.71 [0.7 H, d, *J* 7.0, NH (Asp)] 5.82 [0.3 H, br d, NH (Asp)] and 7.03–7.58 (8 H, m, Ph and 3 × NH); δ_C(75.4 MHz; (C^2HCl_3) 26.19 [β-CH_2 (Glu)], 28.36 [$(CH_3)_3$], 29.58 [γ-CH_2 (Glu)], 35.29 (NCH_3), 35.96 and 36.83 [2 × CH_2 (β-Ala)], 37.64 [β-H_2 (Asp)], 38.26 [CH_2 (Phe)], 50.08 [α-C (Asp)], 50.38 [CH_2 (Sar)], 52.52 [α-C (Glu)], 52.58 and 54.62 (2 × CH_3), 57.96 [α-C (Phe)], 80.00 [*C*$(CH_3)_3$)], 126.85, 127.12, 128.51, 128.58, 129.31 and 129.38 (ArCH), 136.66 (ArC quaternary), 155.81 (CO, urethane), 170.41, 171.50, 171.67, 171.99, 172.40 and 172.57 (CO, esters and amides) and 173.50 (CO, acid); *m/z* (FAB) 702 (40%, $[M + Na]^+$), 680 (12, $[M + H]^+$), 580 (45, $[M + H - C_5H_9O_2 + H]^+$) and 120 (100, $[C_8H_9N + H]^+$).

β-Pentafluorophenyl [(2*R*)-*N*-(*tert*-butoxycarbonyl)-α-methyl aspartyl]-β-(2*S*)-phenylalanyl-β-alanyl-[α-methyl (2*R*)-glutamyl]-γ-sarcosinate triester 34

To a stirred solution of the pentapeptide carboxylic acid **33** (160 mg, 0.22 mmol) in CH_2Cl_2 (20 cm^3) at 0 °C was added pentafluorophenol (122 mg, 0.66 mmol) followed by EDCI (99 mg, 0.33 mmol). The reaction mixture was allowed to warm to room temperature and was then stirred for a further 12 h. The solution was concentrated under reduced pressure and the residual oil was purified by flash chromatography on silica and eluted with 95% CH_2Cl_2–MeOH to give triester **34** as a crystalline solid (119 mg, 63%), mp 109–110 °C; $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3322 (NH), 1757 (CO, urethane), 1715br (CO, esters) and 1656 (CO, amides); δ_H(300 MHz; C^2HCl_3, mixture of rotamers) 1.41 [9 H, s, $(CH_3)_3$], 2.02–2.61 [8 H, m, β-H_2 (Glu), γ-H_2 (Glu), β-H_2 (Asp) and CH_2 (β-Ala)], 2.79–3.04 [2 H, m, CH_2 (Phe)], 3.01 and 3.12 (3 H, 2 × s, NCH_3), 3.14–3.59 [2 H, m, CH_2 (β-Ala)], 3.67 and 3.68 (6 H, 2 × s, 2 × CH_3), 4.09–4.62 [5 H, m, CH_2 (Sar) and 3 × α-H (Glu, Asp and Phe)], 5.78 [1 H, d, *J* 7.2, NH (Asp)], 6.58, 6.75 and 6.90 (3 H, 3 × br, 3 × NH) and 7.08–7.34 (5 H, m, Ph); δ_C(75.4 MHz; C^2HCl_3, mixture of rotamers) 26.77 [β-CH_2 (Glu)], 28.75 [$(CH_3)_3$], 29.92 [(γ-CH_2 (Glu)], 35.97 (NCH_3), 36.2 and 37.08 [2 × CH_2 (β-Ala)], 38.01 [β-CH_2 (Asp)], 38.59 [PhCH_2 (Phe)], 50.07 [α-C (Asp)], 50.71 [CH_2 (Sar)], 51.87

[α-C (Glu)], 52.11 and 52.75 (2 × CH_3), 54.78 [α-C (Phe)], 80.3 [*C*$(CH_3)_3$], 127.3, 128.99 and 129.69 (ArCH), 137.2 (ArC quaternary), 156.01 (CO, urethane), 171.28, 171.98 and 172.73 (CO, esters and amides) and 173.61 (CO, acid); *m/z* (ES) 884 (2%, $[M + K]^+$), 868 (4, $[M + Na]^+$), 846 (7, $[M + H]^+$), 213 (100, $C_7H_2F_5O_2^+$), 157 (57, $C_6H_7NO_4^+$) and 101 (25, $C_5H_9O_2^+$).

[(2*R*)-*N*-(*tert*-Butoxycarbonyl)-α-methyl glutamyl]-γ-sarcosine ester 35

This compound was prepared in a manner identical with that described for α-methyl (2*R*)-*N*-(*tert*-butoxycarbonyl)aspartate ester **29**, using the dipeptide benzyl ester **23** (1.52 g, 3.60 mmol) as the starting material to give the *title product* as a solid in quantitative recovery (1.19 g), mp 68–69 °C (HRMS: Found: $[M + H]^+$, 333.1654. $C_1H_{25}N_2O_7$ requires *m/z*, 333.1662); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3374br (OH and NH), 1725 (CO, urethane) and 1714br (CO, ester and acid); δ_H(200 MHz; C^2HCl_3) 1.41 [9 H, s, $(CH_3)_3$], 1.93–2.00 [1 H, m, 1 H of β-H_2 (Glu)], 2.03–2.58 [3 H, m, 1 H of β-H_2 (Glu) and γ-H_2 (Glu)], 2.96 and 3.05 (3 H, 2 × s, NCH_3), 3.71 and 3.72 (3 H, 2 × s, CH_3), 4.12 [2 H, s, CH_2 (Sar)], 4.29 [1 H, br s, α-H (Glu)], 5.38 (1 H, d, *J* 8.0, NH) and 9.07 (1 H, br s, OH); δ_C(75.4 MHz; C^2HCl_3) 27.32 (β-CH_2), 28.07 [$(CH_3)_3$], 29.03 (γ-CH_2), 35.04 and 36.39 (NCH_3), 49.45 [CH_2 (Sar)], 52.25 (CH_3), 52.94 (α-C), 79.92 [*C*$(CH_3)_3$], 155.76 (CO, urethane) and 172.13, 172.97 and 173.35 (CO, amide, ester and acid); *m/z* (CI) 333 (30%, $[M + H]^+$), 277 (34, $[M + H - C_4H_9 + H]^+$), 233 (59, $[M + H - C_5H_9O_2 + H]^+$) and 144 (100).

α-Methyl β-benzyl (2*R*)-aspartate diester hydrochloride 36

This compound was prepared in a manner identical with that described for the hydrochloride **12**, using α-methyl β-benzyl (2*R*)-*N*-(*tert*-butoxycarbonyl)aspartate diester **28** (1.54 g, 4.80 mmol) as the starting material, to give the *title compound* **36** as a solid, which was recrystallised from methanol–diethyl ether (1.17 g, 89%), mp 139–140 °C [lit.,[55] 137.5–138.0 °C (for the *S*-isomer)]; $[\alpha]_D^{23}$ −17.9 (*c* 1.3, MeOH) [lit.,[55] +19.2 (*c* 4.2, water) (for the *S*-isomer)]; $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3405 (NH), 1739 (CO, ester) and 1634 (CO, amide); δ_H(200 MHz; ^2H_2O) 3.08 (1 H, dd, *J* 5.0 and 18.2, 1 H of β-H_2), 3.21 (1 H, dd, *J* 5.7 and 18.1, 1 H of β-H_2), 3.65 (3 H, s, CH_3), 4.45 (1 H, t, *J* 5.3, α-H), 5.18 (2 H, d, *J* 2.6, PhCH_2) and 7.39 (5 H, s, Ph); δ_C(50.3 MHz; C^2HCl_3) 36.68 (β-CH_2), 51.90 (α-C), 56.64 (CH_3), 70.71 (Ph*C*H_2), 131.37, 131.53 and 131.69 (ArCH), 137.78 (ArC quaternary), and 172.08 and 173.59 (CO, esters); *m/z* (CI) 238 (100%, $[M + H - HCl]^+$).

β-Benzyl [(2*R*)-*N*-(*tert*-butoxycarbonyl)-α-methyl glutamyl]-γ-sarcosyl-[α-methyl (2*R*)-aspartate] triester 37

This compound was prepared in a manner identical with that described for the glutamyl-γ-sarcosyl dipeptide **23**, using the dipeptide **35** (1.20 g, 3.60 mmol) and α-methyl β-benzyl (2*R*)-aspartate diester hydrochloride **36** (0.99 g, 3.60 mmol) to give a pale yellow foam. Recrystallisation from diethyl ether–hexane gave the required tripeptide as a crystalline solid (1.82 g, 92%), mp 83–85 °C (HRMS: Found: $[M + H]^+$, 552.2543. $C_{26}H_{38}N_3O_{10}$ requires *m/z*, 552.2557); ν_{max}(Nujol)/cm^{-1} 3372 (NH), 1725br (CO, urethane and esters) and 1685br (CO, amides); δ_H[300 MHz; $(C^2H_3)_2SO$, mixture of rotamers] 1.35 and 1.36 [9 H, 2 × s, $(CH_3)_3$], 1.70–2.02 [2 H, m, β-CH_2 (Glu)], 2.22–2.42 [2 H, m, γ-H_2 (Glu)], 2.72–2.92 [2 H, m, β-H_2 (Asp)], 2.75 and 2.89 (3 H, 2 × s, NCH_3), 3.59, 3.60 and 3.60 (6 H, 3 × s, 2 × CH_3), 3.89–4.07 [1 H, m, α-H (Glu)], 3.95 [2 H, s, CH_2 (Sar)], 4.67–4.76 [1 H, m, α-H (Asp)], 5.10 (2 H, s, PhCH_2), 7.22 (0.5 H, d, *J* 7.7, 0.5 NH), 7.26–7.39 (0.5 H, m, 0.5 NH), 7.35 (5 H, s, Ph), 8.37 (0.5 H, d, *J* 8.0, 0.5 NH) and 8.62 (0.5 H, d, *J* 8.0, 0.5 NH); δ_C[75.4 MHz; $(C^2H_3)_2SO$, mixture of rotamers] 28.25 [β-CH_2 (Glu)], 28.52 [$(CH_3)_3$], 28.94 and 29.11 [γ-CH_2 (Glu)], 34.61 and 36.15 (NCH_3), 36.27 [β-CH_2 (Asp)], 48.89 [α-C (Glu)], 50.18 [CH_2 (Sar)], 52.16 and 52.62 (2 × CH_3), 53.47 [α-C (Asp)], 66.36 (PhCH_2), 78.57 [*C*$(CH_3)_3$], 128.52, 128.56 and 128.99 (ArCH), 136.48 (ArC quaternary), 156.25 (CO, urethane) and 168.95, 169.08, 170.49, 171.52, 171.65, 172.44, 172.67 and 173.59 (CO, amides and esters); *m/z* (FAB) 574 (13%, $[M + Na]^+$), 552 (19, $[M + H]^+$) and 452 (100, $[M + H - C_5H_9O_2 + H]^+$).

β-Benzyl [α-methyl (2*R*)-glutamyl]-γ-sarcosyl-[α-methyl (2*R*)-aspartate] triester hydrochloride 38

This compound was prepared in a manner identical with that described for the hydrochloride **12**, using the tripeptide triester **37** (1.69 g, 3.06 mmol) as the starting material, to yield *title compound* **38** as a hygroscopic solid (1.33 g, 89%) (HRMS: Found: $[M + H - HCl]^+$, 452.2042. $C_{21}H_{30}N_3O_8$ requires *m/z*, 452.2033); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3419 (NH), 1746br (CO, urethane), 1695 (CO, esters) and 1645 (CO, amides); δ_H(300 MHz; $C^2H_3O^2H$, mixture of rotamers) 2.09–2.32 [2 H, m, β-H_2 (Glu)], 2.58–2.71 [2 H, m, γ-H_2 (Glu)], 2.84–3.02 [2 H, m, β-H_2 (Asp)], 2.90 and 3.04 (3 H, 2 × s, NCH_3), 3.67, 3.68, 3.82 and 3.84 (6 H, 4 × s, 2 × CH_3), 4.04–4.20 [3 H, m, CH_2 (Sar) and α-H (Asp)], 4.82–4.90 [1 H, m, α-H (Glu)], 5.14 and 5.17 (2 H, 2 × s, PhCH_2) and 7.29–7.39 (5 H, m, Ph); δ_C(75.4 MHz; $C^2H_3O^2H$) 26.61 and 26.85 [β-CH_2 (Glu)], 29.87 and 30.09 [γ-CH_2 (Glu)], 35.52 [β-CH_2 (Asp)], 37.10 (NCH_3), 50.46 (α-C), 50.63 [CH_2 (Sar)], 51.72, 53.40 and 53.73 (2 × CH_3), 53.93 (α-C), 67.91 (Ph*C*H_2), 129.59 and 129.85 (ArCH), 137.55 (ArC quaternary), 170.95, 171.11, 171.36, 172.01, 172.64, 174.74 and 174.85 (CO, esters and amides); *m/z* (CI) 452 (36%, $[M + H - HCl]^+$), 309 (27, $[M + H - HCl - C_6H_{10}NO_3 + H]^+$), 238 (24, $[C_{12}H_{15}NO_4 + H]^+$) and (100, $C_6H_{10}NO_3^+$).

Benzyl (2*S*)-*N*-(*tert*-butoxycarbonyl)phenylalanyl-β-alaninate ester 40

This compound was prepared in a manner identical with that described for the glutamyl-γ-sarcosyl dipeptide **23**, using *N*-Boc-(2*S*)-phenylalanine **39** (2.65 g, 10 mmol) and benzyl β-alaninate toluene-*p*-sulfonate salt (3.51 g, 10 mmol) to give a solid. Recrystallisation from ethyl acetate–hexane gave the required pure dipeptide **40** as a crystalline solid (3.62 g, 85%), mp 92–93 °C (Found: C, 67.3; H, 7.1; N, 6.6. Calc. for $C_{24}H_{30}N_2O_5$: C, 67.6; H, 7.1; N, 6.6%) (HRMS: Found: $[M + H]^+$, 427.2227. $C_{24}H_{31}N_2O_5$ requires *m/z*, 427.2233); $[\alpha]_D^{23}$ +0.63 (*c* 0.71, MeOH); ν_{max}(Nujol)/cm^{-1} 3325 (NH), 1746 (CO, urethane), 1710br (CO, ester) and 1673 (CO, amide); δ_H(300 MHz; C^2HCl_3) 1.41 [9 H, s, $(CH_3)_3$], 2.35–2.58 [2 H, m, CH_2 (β-Ala)], 3.01 [2 H, m, PhCH_2 (Phe)], 3.35–3.56 [2 H, m, CH_2N (β-Ala)], 4.26 [1 H, br, α-H (Phe)], 5.01 [1 H, br, NH (Phe)], 5.08 (2 H, s, PhCH_2), 6.27 [1 H, br, NH (β-Ala)] and 7.16–7.40 (10 H, m, Ph); δ_C(75.4 MHz; C^2HCl_3) 28.14 [$(CH_3)_3$], 33.71 [CH_2 (β-Ala)], 34.60 [Ph*C*H_2 (Phe)], 38.72 [CH_2N (β-Ala)], 55.91 [α-C (Phe)], 66.44 (Ph*C*H_2), 80.06 [*C*$(CH_3)_3$], 126.89, 128.27, 128.42, 128.64 and 129.29 (ArCH), 135.60 and 136.72 (ArC quaternary), 155.33 (CO, urethane) and 171.26 [CO (Phe)] and 172.06 [CO, (β-Ala)]; *m/z* (CI) 427 (100%, $[M + H]^+$), 371 (63, $[M + H - C_4H_9 + H]^+$) and 327 (21, $[M + H - C_5H_9O_2 + H]^+$).

(2*S*)-*N*-(*tert*-Butoxycarbonyl)phenylalanyl-β-alanine 41

To a solution of benzyl ester **40** (2.13 g, 5.00 mmol) in ethanol (40 cm^3) was added 10% palladium on carbon (0.1 g) and the mixture was stirred under hydrogen for 6 h. The catalyst was then removed by filtration through a pre-washed Celite pad and the filtrate was concentrated under reduced pressure to give a solid (1.65 g, 98%), mp 89–90 °C (Found: C, 60.9; H, 7.4; N, 8.3. Calc. for $C_{17}H_{24}N_2O_5$: C, 60.7; H, 7.2; N, 8.3%) (HRMS: Found: $[M + H]^+$, 337.1747. $C_{17}H_{25}N_2O_5$ requires *m/z*, 337.1763); $[\alpha]_D^{23}$ +1.00 (*c* 0.65, MeOH); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3326 (NH), 1727 (CO, ester) and 1673 (CO, amide); δ_H(200 MHz; C^2HCl_3) 1.37 [9 H, s, $(CH_3)_3$], 2.36–2.49 [2 H, m, CH_2 (β-Ala)], 2.97 [2 H, br, CH_2 (Phe)], 3.30–3.57 [2 H, m, CH_2N (β-Ala)],

4.55 [1 H, br, α-H (Phe)], 5.52 [1 H, d, NH (Phe)], 7.02 [1 H, br, NH (β-Ala)] and 7.14–7.27 (5 H, m, Ph); δ_C(75.4 MHz; C^2HCl_3) 28.35 [$(CH_3)_3$], 33.61 [CH_2 (β-Ala)], 34.65 [PhCH_2 (Phe)], 39.24 [CH_2N (β-Ala)], 55.73 [α-C (Phe)], 80.72 [$C(CH_3)_3$], 127.04, 128.65 and 129.41 (ArCH), 137.61 (ArC quaternary), 156.71 (CO, urethane) and 171.94 [CO (Phe)] and 175.58 [CO (β-Ala)]; *m/z* (CI) 337 (32%, $[M + H]^+$), 281 (100, $[M + H - C_4H_9 + H]^+$) and 237 (41, $[M + H - C_5H_9O_2 + H]^+$).

β-Benzyl (2*S*)-*N*-(*tert*-butoxycarbonyl)phenylalanyl-β-alanyl-[α-methyl-(2*R*)-glutamyl]-γ-sarcosyl-[α-methyl (2*R*)-aspartate] triester 42

To a stirred solution of (2*S*)-*N*-Boc-Phe-β-Ala-OH **41** (0.69 g, 2.05 mmol) in dry THF (15 cm^3) at −15 °C was added NMM (226 mm^3, 2.05 mmol). IBCF (279 mm^3, 2.05 mmol) was added to the stirred mixture and the suspension was stirred at −15 °C for 10 min. A mixture of the tripeptide **38** (1.00 g, 2.05 mmol) and more NMM (226 mm^3, 2.05 mmol) in a dry mixture of DMF (5 cm^3) and dry THF (10 cm^3) was then added. The reaction mixture was allowed to warm to room temperature and was then stirred for a further 3 h. The hydrochloride salts were removed by filtration and the solvents were removed under reduced pressure. The crude residue was re-dissolved in ethyl acetate (25 cm^3), and the solution was then washed successively with water (15 cm^3), 5% aq. $NaHCO_3$ (20 cm^3), 10% aq. citric acid (20 cm^3) and then brine (20 cm^3) and then was dried ($MgSO_4$), and concentrated under reduced pressure. The resulting yellow solid was purified by flash chromatography on silica and eluted with 94% CH_2Cl_2–MeOH to give the pentapeptide as a foam. Recrystallisation from ethyl acetate–hexane gave the pure pentapeptide **42** as a crystalline solid (1.28 g, 81%), mp 73–76 °C (Found: C, 59.2; H, 6.4; N, 8.8. Calc. for $C_{38}H_{51}N_5O_{12}$: C, 59.3; H, 6.7; N, 9.1%) (HRMS: Found: $[M + H]^+$, 770.3642. $C_{38}H_{52}N_5O_{12}$ requires *m/z*, 770.3612); $[\alpha]_D^{23}$ −18.33 (*c* 0.15, MeOH); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3311 (NH), 1741 (CO, urethane), 1716br (CO, esters) and 1658 (CO, amides); δ_H(500 MHz; C^2HCl_3, mixture of rotamers) 1.35 [9 H, s, $(CH_3)_3$], 1.89–1.95 [2 H, br, β-H_2 (Glu)], 2.27–2.52 [4 H, m, γ-H_2 (Glu) and CH_2 (β-Ala)], 2.86–3.05 [4 H, m, β-H_2 (Asp) and PhCH_2 (Phe)], 2.89 and 3.00 (3 H, 2 × s, NCH_3), 3.42 [2 H, br, CH_2N (β-Ala)], 3.65, 3.70, 3.71 and 3.72 (6 H, 4 × s, 2 × CH_3), 3.78–4.00 [1 H, m, 1 H of CH_2 (Sar)], 4.32 [2 H, br, α-H (Glu) and 1 H of CH_2 (Sar)], 4.47 [0.2 H, br, 0.2 α-H (Phe)], 4.55 [0.8 H, br, 0.8 α-H (Phe)], 4.89–4.93 [1 H, m, α-H (Asp)], 5.10 and 5.11 (2 H, 2 × s, PhCH_2), 5.19 (0.6 H, d, *J* 8.2, 0.6 NH), 5.26 (0.4 H, d, *J* 8.2, 0.4 NH), 6.80 (1.5 H, br, 1.5 NH), 6.95 (0.5 H, br, 0.5 NH) and 7.16–7.37 (11 H, m, Ph and NH); δ_C(75.4 MHz; C^2HCl_3, mixture of rotamers) 27.02 [β-CH_2 (Glu)], 28.24 [$(CH_3)_3$], 29.04 [γ-CH_2 (Glu)], 34.91 (NCH_3), 35.51 and 35.54 [2 × CH_2 (β-Ala)], 36.28 (NCH_3), 36.50 [β-CH_2 (Asp)], 38.97 [PhCH_2 (Phe)], 48.62 [α-C (Asp)], 51.67 [CH_2 (Sar)], 52.02 [α-C (Glu)], 52.59 and 52.78 (2 × CH_3), 55.94 [α-C (Phe)], 66.92 [PhCH_2 (Asp)], 79.83 [$C(CH_3)_3$], 126.90, 128.45, 128.63, 128.73 and 129.52 (ArCH), 135.56 and 137.11 (ArC quaternary), 155.48 (CO, urethane) and 168.77, 170.70, 171.41, 171.72, 171.88, 172.74 and 173.07 (CO, amides and esters); *m/z* (FAB) 808 (7%, $[M + K]^+$), 792 (29, $[M + Na]^+$), 770 (18, $[M + H]^+$), 692 (5, $[M + Na - C_5H_9O_2 + H]^+$), 670 (70, $[M + H - C_5H_9O_2 + H]^+$) and 91 (100, $PhCH_2^+$).

(2*S*)-*N*-(*tert*-Butoxycarbonyl)phenylalanyl-β-alanyl-[α- methyl (2*R*)-glutamyl]-γ-sarcosyl-[α-methyl (2*R*)-aspartate] diester 43

To a solution of benzyl ester **42** (0.54 g, 0.7 mmol) in methanol–ethanol (15 : 5 cm^3) was added 10% palladium on carbon (50 mg) and the mixture was stirred under hydrogen for 5 h. The catalyst was then removed by filtration through a pre-washed Celite pad and the filtrate was concentrated under reduced pressure to give the required acid diester **43** as a solid (0.47 g, 98%), mp 64–67 °C (HRMS: Found: $[M + H]^+$, 680.3121. $C_{31}H_{46}N_5O_{12}$ requires *m/z*, 680.3143); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3530–3150br (OH and NH), 1781 (CO, urethane) and 1712–1658br (CO, esters and amides); δ_H[300 MHz; $(C^2H_3)_2SO$, mixture of rotamers] 1.27 [9 H, s, $(CH_3)_3$], 1.78–1.98 [2 H, m, β-H_2 (Glu)], 2.26–2.37 [4 H, m, γ-H_2 (Glu) and CH_2 (β-Ala)], 2.60–2.71 [4 H, m, β-H_2 (Asp) and PhCH_2 (Phe)], 2.74 and 2.87 (3 H, 2 × s, NCH_3), 3.32 [2 H, br, CH_2N (β-Ala)], 3.55, 3.57, 3.59 and 3.60 (6 H, 4 × s, 2 × CH_3), 3.93–3.96 [2 H, m, CH_2 (Sar)], 4.03–4.12 [1 H, m, α-H (Glu)], 4.20–4.27 [1 H, m, α-H (Phe)], 4.56–4.63 [1 H, m, α-H (Asp)], 6.86 (0.7 H, d, *J* 8.5, 0.7 NH), 7.16–7.24 (5.3 H, m, Ph and 0.3 NH), 7.93 [1 H, br, NH (β-Ala)], 8.26–8.33 (1.6 H, m, 1.6 NH) and (0.4 H, d, *J* 8.0, 0.4 NH); δ_C[75.4 MHz; $(C^2H_3)_2SO$, mixture of rotamers] 28.25 [β-CH_2 (Glu)], 28.54 [$(CH_3)_3$], 28.54 and 29.00 [γ-CH_2 (Glu)], 34.63 (NCH_3), 35.20 and 35.58 [2 × CH_2 (β-Ala)], 36.18 (NCH_3), 36.38 [β-CH_2 (Asp)], 38.06 [PhCH_2 (Phe)], 48.93 [α-C (Asp)], 50.12 [CH_2 (Sar)], 51.92, 52.06, 52.29 and 52.38 (2 × CH_3), 52.57 [α-C (Glu)], 56.22 [α-C (Phe)], 78.44 [$C(CH_3)_3$], 126.71, 128.56 and 129.75 (ArCH), 138.86 (ArC quaternary), 155.80 (CO, urethane) and 169.00, 171.30, 171.39, 172.24, 172.42 and 173.17 (CO, amides, esters and acid); *m/z* (FAB) 702 (66%, $[M + Na]^+$), 680 (47, $[M + H]^+$), 602 (7, $[M + Na - C_5H_9O_2 + H]^+$), 580 (85, $[M + H - C_5H_9O_2 + H]^+$), 91 (100, $PhCH_2^+$) and 57 (100, $C_4H_9^+$).

β-Pentafluorophenyl (2*S*)-*N*-(*tert*-butoxycarbonyl)phenylalanyl-β-alanyl-[α-methyl (2*R*)-glutamyl]-γ-sarcosyl-[α-methyl (2*R*)-aspartate] triester 44

To a stirred solution of the pentapeptide carboxylic acid diester **43** (627 mg, 0.92 mmol) in CH_2Cl_2 (20 cm^3) at 0 °C was added pentafluorophenol (510 mg, 2.77 mmol) followed by EDCI (412 mg, 1.39 mmol). The reaction mixture was allowed to warm to room temperature and was stirred for a further 12 h before being concentrated under reduced pressure. The resulting cream coloured foam was purified by flash chromatography on silica and eluted with 94% CH_2Cl_2–MeOH to give *triester* **44** as a foam (536 mg, 69%), mp 62–65 °C (softening point, did not exhibit a distinct mp) (Found: C, 51.25; H, 5.15; N, 8.00. $C_{37}H_{44}F_5N_5O_{12}\cdot H_2O$ requires C, 51.45; H, 5.35; N, 8.10%) (HRMS: Found: $[M + H - C_5H_9O_2 + H]^+$, 746.2473. $C_{32}H_{37}F_5N_5O_{12}$ requires *m/z*, 746.2461); $[\alpha]_D^{23}$ −2.19 (*c* 0.16, CH_2Cl_2); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3311 (NH), 1714 (CO, esters) and 1660 (CO, amides); δ_H(300 MHz; C^2HCl_3, mixture of rotamers) 1.38 [9 H, s, $(CH_3)_3$], 1.92–2.08 [2 H, m, β-H_2 (Glu)], 2.23–2.56 [4 H, m, γ-H_2 (Glu) and CH_2 (β-Ala)], 2.83–3.12 [2 H, m, PhCH_2 (Phe)], 2.95 and 3.10 (3 H, 2 × s, NCH_3), 3.19–3.30 [2 H, m, β-H_2 (Asp)], 3.42–3.50 [2 H, br, CH_2N (β-Ala)], 3.75, 3.76, 3.77 and 3.78 (6 H, 4 × s, 2 × CH_3), 3.82–4.17 [1 H, m, 1 H of CH_2 (Sar)], 4.25–4.38 [(1 H, m, α-H (Glu)], 4.41–4.63 [2 H, m, 1 H of CH_2 (Sar) and α-H (Phe)], 4.98–5.10 [1 H, m, α-H (Asp)], 5.15 ([1 H, br, NH (Phe)], 6.76 (1.4 H, br, 1.4 NH), 7.16–7.29 (5.6 H, m, Ph and 0.6 NH) and 7.52 (1 H, d, *J* 8.5, NH); δ_C(75.4 MHz; C^2HCl_3, mixture of rotamers) 27.30 [β-CH_2 (Glu)], 28.19 [$(CH_3)_3$], 28.92 [γ-CH_2 (Glu)], 35.01 (NCH_3), 35.48 and 35.64 [2 × CH_2 (β-Ala)], 35.72 (NCH_3), 36.50 [β-CH_2 (Asp)], 38.81 [PhCH_2 (Phe)], 48.61 [α-C (Asp)], 48.72 [CH_2 (Sar)], 51.92, 52.03, 52.63 and 53.11 (2 × CH_3), 53.45 [α-C (Glu)], 55.98 [α-C (Phe)], 80.05 [$C(CH_3)_3$], 127.00, 128.69 and 129.48 (ArCH), 138.89 (ArC quaternary), 155.61 (CO, urethane) and 166.80, 169.02, 169.21, 170.80, 172.00, 172.73 and 173.04 (CO, amides, esters); *m/z* (FAB) 868 (4%, $[M + Na]^+$), 846 (5, $[M + H]^+$), 746 (57, $[M + H - C_5H_9O_2 + H]^+$), 219 (68, $[C_{12}H_{14}N_2O_2 + H]^+$) and 120 (100, $[C_8H_9N + H]^+$).

cyclo-[β-Ala-(*R*)-Glu-α-OMe-γ-Sar-(*R*)-Asp-α-OMe-β-(*S*)-Phe] 5

To a stirred solution of the *N*-(*tert*-butoxycarbonyl)-protected pentafluorophenyl ester **44** (559 mg, 0.66 mmol) in CH_2Cl_2 (15 cm^3) was added TFA (15 cm^3). The reaction mixture was stirred for 45 min, when the reaction was judged to be complete by TLC. The solution was diluted with toluene (5 cm^3), and was

then concentrated under reduced pressure and thoroughly dried under high vacuum for 6 h. The resulting residue was dissolved in CH_2Cl_2 (800 cm^3), treated with DIPEA (2.67 cm^3, 19.8 mmol) and then stirred at room temperature for nine days. The reaction mixture was concentrated under reduced pressure and was then triturated with diethyl ether to give a solid, which was filtered off, and washed on the pad with diethyl ether (4 × 25 cm^3) to afford the *pure macrocycle* **5** (330 mg, 89%), mp >250 °C (decomp.) (Found: C, 55.5; H, 6.4; N, 12.1. $C_{26}H_{35}N_5O_9$ requires C, 55.6; H, 6.3; N, 12.5%) (HRMS: Found: $[M + Na]^+$, 584.2316. $C_{26}H_{35}N_5NaO_9$ requires *m/z*, 584.2332); ν_{max}(Nujol)/cm^{-1} 3290 (NH), 1737 (CO, esters) and 1667 and 1643 (CO, amides); δ_H[500 MHz; $(C^2H_3)_2SO$, mixture of rotamers] 1.67–2.01 [2 H, m, β-H_2 (Glu)], 2.06–2.34 [3 H, m, γ-H_2 (Glu) and 1 H of CH_2 (β-Ala)], 2.40–2.58 [3 H, m, β-H_2 (Asp) and 1 H of CH_2 (β-Ala)], 2.64–2.77 [1 H, m, 1 H of PhCH_2 (Phe)], 2.79 and 2.89 (3 H, 2 × s, NCH_3), 2.90–3.00 [1 H, m, 1 H of PhCH_2 (Phe)], 3.04–3.10 [1 H, m, 1 H of CH_2 (β-Ala)], 3.37–3.48 [1 H, m, 1 H of CH_2 (β-Ala)], 3.61, 3.62 and 3.63 (6 H, 3 × s, 2 × CH_3), 3.96 [2 H, AB, *J* 17.8, CH_2 (Sar)], 4.20–4.57 [2 H, m, 2 × α-H (Glu and Phe)], 4.47–4.51 [1 H, m, α-H (Asp)], 7.16–7.34 (5.5 H, m, Ph and 0.5 NH), 7.82–7.86 (0.5 H, m, 0.5 NH), 8.01–8.10 (1.5 H, m, 1.5 NH) and 8.19–8.27 (1.5 H, m, 1.5 NH); δ_C[125.7 MHz; $(C^2H_3)_2SO$, mixture of rotamers] 26.32 and 26.57 [β-CH_2 (Glu)], 28.46 and 28.58 [γ-CH_2 (Glu)], 34.43 (NCH_3), 34.89 and 34.90 [2 × CH_2 (β-Ala)], 35.20 [CH_2 (β-Ala)], 35.43 (NCH_3), 35.86 [β-CH_2 (Asp)], 36.98 and 37.62 [PhCH_2 (Phe)], 49.19 [α-C (Asp)], 49.61 (α-C), 50.60 [CH_2 (Sar)], 50.82 (α-C), 51.64 [α-C (Glu)], 51.66, 51.82, 51.97 and 53.11 (2 × CH_3), 53.73 [α-C (Phe)], 54.71 (α-C), 126.30, 128.07 and 129.27 (ArCH), 137.97 (ArC quaternary) and 168.23, 168.56, 169.41, 171.00, 171.17, 171.34, 171.76, 172.12, 172.36 and 172.72 (CO, amides, esters); *m/z* (FAB) 584 (100%, $[M + Na]^+$) and 562 (22, $[M + H]^+$).

Acknowledgements

We thank the BBSRC and EPSRC for BMS grant GR/J55243, the Wellcome Trust for grant 040331, Dr J. Wilkie, Dr T. Rutherford and Dr M. Akhtar for computational, NMR and scientific support, respectively, Dr C. MacKintosh for testing the dihydromicrocystins, and Zeneca Pharmaceuticals for an EPSRC CASE studentship to K. L. W.

Abbreviations

ADP, Adenosine diphosphate; ATP, adenosine triphosphate; COSY, 2-D homonuclear chemical-shift correlation spectroscopy; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DPPA, diphenylphosphoryl azide; EDCI, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide; FDPP, pentafluorophenyl diphenylphosphinate; HOBt, 1-hydroxybenzotriazole hydrate; HSQC, heteronuclear correlation spectroscopy; IBCF, isobutyl chloroformate; NMM, 4-methylmorpholine; NOESY, nuclear Overhauser enhancement; PFP, pentafluorophenyl; PyBOP, benzotriazol-1-yl oxy(trispyrrolidino)phosphonium hexafluorophosphate; ROESY, NOESY in a rotating frame of reference; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; THF, terahydrofuran; TFA, trifluoroacetic acid; TOCSY, phase sensitive 2-D total correlation spectroscopy; UDP, uridine diphosphate; UTP, uridine triphosphate.

References

1 T. Hunter, *Cell*, 1995, **80**, 225.
2 P. Cohen, *Annu. Rev. Biochem.*, 1989, **58**, 453; P. T. W. Cohen, N. D. Brewis, V. Hughes and D. Mann, *FEBS Lett.*, 1990, **268**, 355.
3 S. Shenolikar and A. C. Nairn, in *Advances in Second Messenger and Phosphoprotein Research*, ed. P. Greengard and G. A. Robinson, Raven Press, Ltd., New York, 1991, vol. 23, pp. 1–121.
4 M. Bollen and W. Stalmans, *Crit. Rev. Biochem. Mol. Biol.*, 1992, **27**, 227.
5 A. A. Depaoli-Roach, I.-K. Park, V. Cerovsky, C. Csortos, S. D. Durbin, M. J. Kuntz, A. Sitikov, P. M. Tang, A. Verin and S. Zolnierowicz, *Adv. Enzyme Regul.*, 1994, **34**, 199.
6 M. J. Hubbard and P. Cohen, *Trends Biochem. Sci.*, 1993, **18**, 172.
7 B. McCright and D. Virshup, *J. Biol. Chem.*, 1995, **270**, 26 123.
8 M. C. Faux and J. D. Scott, *Trends Biochem. Sci.*, 1996, **21**, 312.
9 D. P. Botes, A. A. Tuinman, P. L. Wessels, C. C. Wiljoen, H. Kruger, D. H. Williams, S. Santikarn, R. J. Smith and S. J. Hammond, *J. Chem. Soc., Perkin Trans. 1*, 1984, 2311.
10 D. P. Botes, P. L. Wessels, H. Kruger, M. T. C. Runnegar, S. Santikarn, R. J. Smith, J. C. J. Barna and D. H. Williams, *J. Chem. Soc., Perkin Trans. 1*, 1985, 2747.
11 W. W. Carmichael, in *Natural Toxins; Characterisation, Pharmacology and Therapeutics*, Pergamon Press, London, 1988, pp. 3–16.
12 W. W. Carmichael, V. R. Beasley, D. L. Bunner, J. N. Eloff, I. R. Falconer, P. Gorham, K.-I. Harada, M.-J. Yu, T. Krishnamurthy, R. E. Moore, K. L. Rinehart, M. T. C. Runnegar, O. M. Skulberg and M. Watanabe, *Toxicon*, 1988, **26**, 971.
13 K. L. Rinehart, K.-I. Harada, M. Namikoshi, C. Chen, C. A. Harvis, M. H. G. Munro, J. W. Blunt, P. E. Mulligan, V. R. Beasley, A. M. Dahlem and W. W. Carmichael, *J. Am. Chem. Soc.*, 1988, **110**, 8557.
14 K. Sivonen, K. Konen, W. W. Carmichael, A. M. Dahlem, K. L. Rinehart, J. Kiviranta and S. Niemela, *Appl. Environ. Microbiol.*, 1989, **55**, 1990.
15 M. Namikoshi, B. W. Choi, R. Sakai, K. L. Rinehart, W. W. Carmichael, W. R. Evans, P. Cruz, M. H. G. Munro and J. W. Blunt, *J. Org. Chem.*, 1994, **59**, 2349.
16 W. W. Carmichael, *J. Appl. Bacteriol.*, 1992, **72**, 445.
17 R. E. Honkanen, J. Zwiller, S. L. Daily, B. S. Khatra, M. Dukelow and A. L. Boynton, *J. Biol. Chem.*, 1991, **266**, 6614.
18 I. R. Falconer, A. M. Beresford and M. T. C. Runnegar, *Med. J. Aust.*, 1983, **1**, 511.
19 S.-Z. Yu, in *Primary Liver Cancer*, eds. Z. Y. Tang, M. C. Wu and S. S. Xia, Springer-Verlag, Berlin, 1989, pp. 30–37.
20 M. T. C. Runnegar, I. R. Falconer and J. Silver, *Naunyn–Schmiedeberg's Arch. Pharmacol.*, 1981, **317**, 268.
21 M. T. C. Runnegar, R. G. Gerdes and I. R. Falconer, *Toxicon*, 1991, **29**, 43.
22 C. F. B. Holmes and M. P. Boland, *Curr. Opin. Struct. Biol.*, 1993, **3**, 934.
23 J. Goldberg, H.-B. Huang, Y.-G. Kwon, P. Greengard, A. C. Nairn and J. Kuriyan, *Nature*, 1995, **376**, 745.
24 M.-P. Egloff, P. T. W. Cohen, P. Reinemer and D. Barford, *J. Mol. Biol.*, 1995, **254**, 942.
25 D. Gani and J. Wilkie, *Metal Ions in the Mechanism of Enzyme Catalysed Phosphate Monoester Hydrolyses* in *Structure and Bonding*, ed. H. A. Hill, P. J. Sadler and A. J. Thomson, Springer-Verlag, Berlin, Heidelberg, 1997, **89**, pp. 133–175.
26 E. D. de Silva, D. E. Williams, R. J. Andersen, H. Klix, C. F. B. Holmes and T. Allen, *Tetrahedron Lett.*, 1992, **33**, 1561.
27 S. Rudolph-Bohner, D. F. Mierke and L. Moroder, *FEBS Lett.*, 1994, **349**, 319; D. F. Mierke, S. Rudolph-Bohner, G. Muller and L. Moroder, *Biopolymers*, 1995, **36**, 811.
28 A. Annila, J. Lehtimaki, K. Mattila, J. E. Eriksson, K. Sivonen, T. T. Rantala and T. Drakenberg, *J. Biol. Chem.*, 1996, **271**, 16 695.
29 M. Namikoshi, K. L. Rinehart, R. Sakai, R. R. Stotts, A. M. Dahlem, V. R. Beasley, W. W. Carmichael and W. R. Evans, *J. Org. Chem.*, 1992, **57**, 866.
30 K. L. Rinehart, M. Namikoshi and B. W. Choi, *J. Appl. Phycology*, 1994, **6**, 159.
31 R. Nishiwaki-Matsushima, S. Nishiwaki, T. Ohta, S. Yoshizawa, M. Suganuma, K.-I. Harada, M. F. Watanabe and H. Fujiki, *Jpn. J. Cancer Res.*, 1991, **82**, 993.
32 K.-I. Harada, K. Ogawa, K. Matsuura, H. Murata, M. Suzuki, M. F. Watanabe, Y. Itezono and N. Nakayama, *Chem. Res. Toxicol.*, 1990, **3**, 473.
33 M. Namikoshi, K. L. Rinehart, V. R. Beasley and W. W. Carmichael, *Tetrahedron Lett.*, 1989, **30**, 4349.
34 M. Namikoshi, K. L. Rinehart, R. Sakai, K. Sivonen and W. W. Carmichael, *J. Org. Chem.*, 1990, **55**, 6135.
35 C. Taylor, R. J. Quinn, M. Suganuma and H. Fujiki, *Bioorg. Med. Chem. Lett.*, 1996, **6**, 2113.
36 R. J. Valentekovich and S. L. Schreiber, *J. Am. Chem. Soc.*, 1995, **117**, 9069.
37 P. Hormozdiari and D. Gani, *Tetrahedron Lett.*, 1996, **37**, 8227.
38 M. Namikoshi, B. W. Choi, F. Sun, K. L. Rinehart, W. R. Evans and W. W. Carmichael, *Chem. Res. Toxicol.*, 1993, **6**, 151.
39 A. P. Mehrotra, Ph.D. Thesis, University of St. Andrews, 1995.

40 R. W. MacKintosh, K. N. Dalby, D. G. Campbell, P. Cohen and C. MacKintosh, *FEBS Lett.*, 1995, **371**, 236.
41 G. Moorhead, R. W. MacKintosh, N. Morrice, T. Gallagher and C. MacKintosh, *FEBS Lett.*, 1994, **356**, 46.
42 M. S. Gulzar, K. B. Morris and D. Gani, *J. Chem. Soc., Chem. Commun.*, 1995, 1061.
43 M. Akhtar, N. P. Botting, M. A. Cohen and D. Gani, *Tetrahedron*, 1987, **43**, 5899.
44 J. M. Scholtz and P. A. Bartlett, *Synthesis*, 1989, 542.
45 S. F. Brady, R. M. Freidinger, W. J. Paleveda, C. D. Colton, C. F. Homnick, W. L. Whitter, P. Curley, R. F. Nutt and D. F. Veber, *J. Org. Chem.*, 1987, **52**, 764.
46 P. Wipf and H. Kim, *J. Org. Chem.*, 1993, **58**, 5592.
47 K. L. Webster, A. P. Mehrotra, A. B. Maude, D. Gani and T. J. Rutherford, parts 3 and 4 in this series, in preparation. Also see K. L. Webster, T. J. Rutherford and D. Gani, *Tetrahedron Lett.*, 1997, **38**, 5713.
48 A. B. Maude, A. P. Mehrotra and D. Gani, following paper.
49 T. K. Chakraborty and S. P. Joshi, *Tetrahedron Lett.*, 1990, **31**, 2043; M. F. Beatty, C. Jennings-White and M. A. Avery, *J. Chem. Soc., Perkin Trans. 1*, 1992, 1637; H. Y. Kim and P. L. Toogood, *Tetrahedron Lett.*, 1996, **37**, 2349; F. D'Aniello, A. Mann and M. Taddei, *J. Org. Chem.*, 1996, **61**, 4870; N. Sin and J. Kallmerten, *Tetrahedron Lett.*, 1996, **37**, 5645; F. D'Aniello, A. Mann, A. Schoenfelder and M. Taddei, *Tetrahedron*, 1997, **53**, 1447.
50 J. M. Humphrey, J. B. Aggen and A. R. Chamberlain, *J. Am. Chem. Soc.*, 1996, **118**, 11 759.
51 D. D. Perrin, W. L. F. Armarego and D. R. Perrin, in *Purification of Laboratory Chemicals*, Pergamon Press, Oxford, 1980.
52 C. MacKintosh, K. A. Beattie, S. Klumpp, P. Cohen and G. A. Codd, *FEBS Lett.*, 1990, **264**, 187.
53 E. Kato, K. Yamamoto, Y. Kawashima, T. Watanabe, M. Oya, T. Iso and J. Iwao, *Chem. Pharm. Bull.*, 1985, **33**, 4836.
54 S. Kim, J. I. Lee and Y. C. Kim, *J. Org. Chem.*, 1985, **50**, 560.
55 E. Wünsch, in *Methoden der Organischen Chemi: Synthese Von Peptiden I*, ed. M. Deffner, K.-H. Deimer, E. Jaeger, P. Stelzel, P. Thamm, G. Wendlberger and E. Wünsch, Georg Thieme Verlag, Stuttgart, 1974, p. 685.

Paper 7/02407J
Received 8*th April* 1997
Accepted 29*th April* 1997