

# The synthesis and properties of Gla- and Phe-containing analogues of cyclic RGD pentapeptides

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Cyclopentapeptides containing the Arg-Gly-Asp motif have been synthesised using solid-phase assembly of side-chain-protected linear precursors, followed by solution-phase cyclisation. The replacement of the Asp residue by  $\gamma$ -carboxyglutamic acid (Gla) is a novel feature which gives rise to an analogue which inhibits cell adhesion, yet its congeners do not show activity in binding assays with recombinant integrin receptors. NMR techniques support a  $\beta/\gamma$ -turn conformation in most of the analogues.

## Introduction

There has been much interest recently in the development of mimetics of the -Arg-Gly-Asp- (RGD) domain which features at the core of surface interactions involving cell adhesion<sup>1</sup> and fibrinogen–blood-platelet-receptor interactions.<sup>2</sup> The former studies have led to development of anticancer agents,<sup>3</sup> while the latter are providing potential therapeutics<sup>4</sup> in antithrombotic therapy.

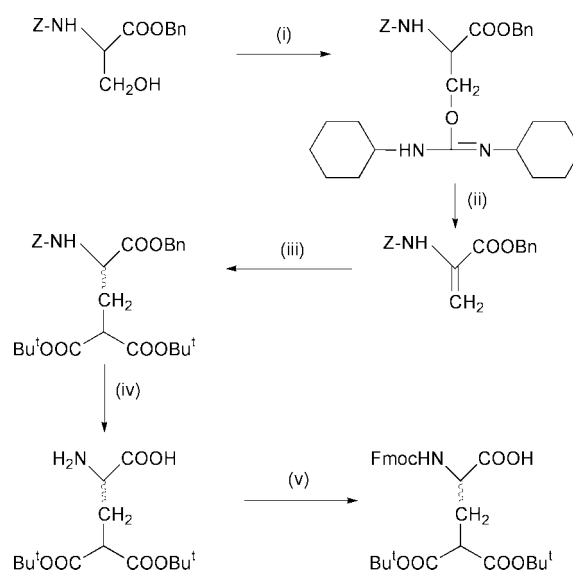
Restricting the conformation of the RGD domain in a cyclic peptide structure has contributed<sup>5</sup> greatly to an understanding of the pharmacophoric conformational requirements of the domain and, with few exceptions<sup>4</sup> in the peptide field, the cyclopentapeptide structure is the most favoured template for maintaining biological activity. Within this template, conformation and analogue studies have revealed that, for biological activity, the arginyl residue, R, can be replaced by basic side-chains, but the -Gly-Asp- duo of residues has been reported<sup>6</sup> to be invariant, except for an example from our own studies<sup>7</sup> where we found cyclo(-Arg-Gly-DL-Gla-Ser-Lys-) **1** to be amongst the best inhibitors of cell adhesion. The choice of the Gla ( $\gamma$ -carboxyglutamic acid) residue was rationalised at the time as a means of securing better binding, due to the close proximity of a  $\text{Ca}^{2+}$ -binding site quite near to the recognition domain.<sup>8</sup> However, the Gla residue in chirally pure form, and derivatised in a form suitable for the Fmoc-protocol in solid-phase synthesis,<sup>9</sup> is not readily available in the academic environment, so one theme of this paper for the Gla-analogue describes attempts to achieve the synthesis of cyclo(-Arg-Gly-L-Gla-Ser-Lys-) **2** and cyclo(-Arg-Gly-D-Gla-Ser-Lys-) **3** in chirally pure form for biological testing. We also report on the synthesis of cyclo(-Arg-Gly-Asp-Phe-) **4**, which is an attempt to introduce more lipophilic character around the RGD domain, as inclusion of aromatic side-chains has proved successful in other studies.<sup>10</sup>

**Abbreviations:** Generally, the three-letter amino acid nomenclature is used, except in Table 1 where single-letter nomenclature, R = Arg, G = Gly, D = Asp, F = Phe, S = Ser, K = Lys, is used. Lower case in single-letter nomenclature implies D-configuration. EDC = 1-ethyl-3-(3-dimethylamino)propylcarbodiimide·HCl, HATU = O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, TBTU = 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate, HOBT = N-hydroxybenzotriazole, Mtr = 2,3,6-trimethyl-4-methoxyphenylsulfonfyl.

## Discussion

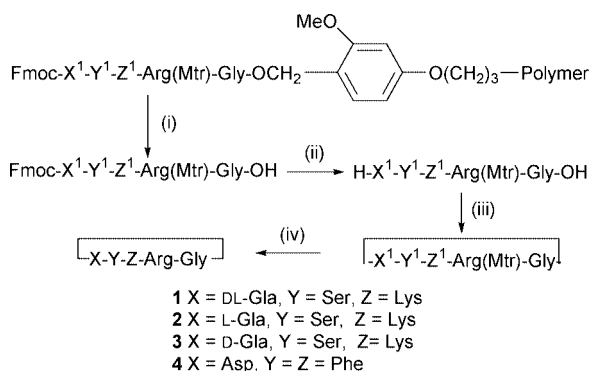
### Synthetic studies

Our first attempt at the synthesis of analogues **2** and **3** was based on the strategy of synthesising **1** with both diastereoisomers present, and to use separation on an HPLC column to achieve the separation into analogues **2** and **3**. Therefore the efficient synthesis of the racemic derivative, Fmoc-DL-Gla(OBu')<sub>2</sub>-OH, as summarised in Scheme 1 provided the



**Scheme 1** Reagents and conditions: (i) DCC,  $\text{CH}_2\text{Cl}_2$  (ii)  $\text{CuCl}$  (iii) di-*tert*-butyl malonate, NaH, DMF (iv)  $\text{H}_2$ , Pd/C, MeOH (v) Fmoc-Cl,  $\text{NaHCO}_3$ , 1,4-dioxane.

required building block for incorporation into the solid-phase protocol, summarised in Scheme 2. The benzylation of *N*-(benzyloxycarbonyl)serine was achieved in high yield by reaction with *O*-benzyl-*N,N'*-diisopropylisourea according to the procedure of Goodman and co-workers,<sup>11</sup> applied initially for  $\alpha$ -hydroxy acids. As shown in Scheme 1, the intermediate synthesis of the dehydroserine derivative was carried out by an isourea-mediated  $\beta$ -elimination<sup>12</sup> using *N,N'*-diisopropylcarbodiimide with 0.3 mole equivalents of  $\text{CuCl}$ . The dehydroserine derivative and di-*tert*-butyl malonate anion generated

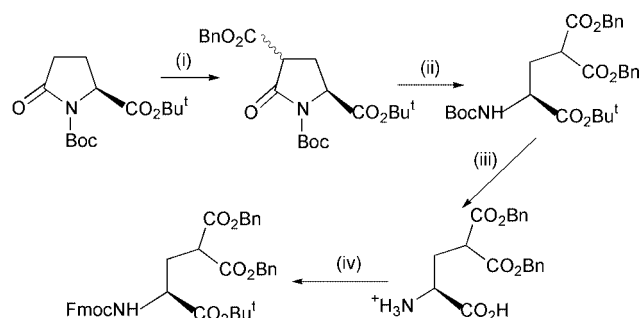


**Scheme 2** (a)  $X^1 = \text{DL-Gla}(\text{OBu}^t)_2$ ,  $Y^1 = \text{Ser}(\text{Bu}^t)$ ,  $Z^1 = \text{Lys}(\text{Boc})$ ; (b)  $X^1 = \text{L-Gla}(\text{OBn})_2$ ,  $Y^1 = \text{Ser}(\text{Bu}^t)$ ,  $Z^1 = \text{Lys}(\text{Boc})$ ; (c)  $X^1 = \text{L-Gla}(\text{OBu}^t)_2$ ,  $Y^1 = \text{Ser}(\text{Bu}^t)$ ,  $Z^1 = \text{Lys}(\text{Boc})$ ; (d)  $X^1 = \text{D-Gla}(\text{OBu}^t)_2$ ,  $Y^1 = \text{Ser}(\text{Bu}^t)$ ,  $Z^1 = \text{Lys}(\text{Boc})$ ; (e)  $X^1 = \text{L-Asp}(\text{OBu}^t)$ ,  $Y^1 = Z^1 = \text{Phe}$ . **Reagents and conditions:** (i) 1% TFA (ii) 10% piperidine-DMF (iii) EDC, DMAP, *N*-methylmorpholine for Schemes 2(a), 2(b) and 2(e), HATU, DIPEA, DMF,  $\text{CH}_2\text{Cl}_2$  for Schemes 2(c) and 2(d) (iv) TFA.

using sodium hydride gave  $\text{Z-DL-Gla}(\text{OBu}^t)_2\text{-OBn}$  in 51% yield, which was converted by standard methodology to  $\text{Fmoc-DL-Gla}(\text{OBu}^t)_2\text{-OH}$ .

The protocol summarised in Scheme 2 represents our optimised sequence of solid-phase steps using a highly acid-labile linker group previously reported,<sup>13</sup> which releases a protected linear pentapeptide precursor from the resin, and is then available for cyclisation in the solution phase. To form  $\text{cyclo[Arg(Mtr)-Gly-DL-Gla}(\text{OBu}^t)_2\text{-Ser}(\text{Bu}^t)\text{-Lys}(\text{Boc})\text{-}]$ , the cyclisation conditions chosen were water-soluble carbodiimide (EDC)-4-(dimethylamino)pyridine (DMAP) under high-dilution conditions. A crude yield of 82% for the cyclisation step was reduced to 40% by gradient elution on HPLC. The HPLC trace showed a single sharp peak with a  $t_R$  of 9.6 min on a semi-preparative C-18 HPLC cartridge, but there was no evidence of diastereoisomeric separation of the peak under a variety of HPLC conditions. The possibility that there had been diastereoisomeric selection during ring closure, with one form of Gla-containing linear precursor being easier to cyclise than the other, appeared unlikely as two sets of sharp signals were evident in the NMR spectrum of the purified protected cyclopentapeptide. The protecting groups were removed by treatment with trifluoroacetic acid (TFA) and contaminants removed by semi-preparative HPLC. The trifluoroacetate salt of **1** was counter-ion exchanged with acetate ions for biological assay. The results of the analysis, discussed later, gave us our most active analogue to date, but having failed to resolve the material into separate diastereoisomers, we made attempts to synthesise each one from chirally pure Gla-building blocks.

In recent years there have been several reports of enantioselective synthesis of Gla derivatives,<sup>14–21</sup> many requiring multi-step synthesis, but having received a generous gift of a key intermediate (from Roche Research Centre, Welwyn Garden City), the  $\text{Fmoc-L-Gla}(\text{OBn})_2\text{-OH}$  was synthesised as summarised in Scheme 3 following the published procedure<sup>16</sup> as far as



**Scheme 3** **Reagents and conditions:** (i)  $\text{BnO}_2\text{C}$ , LDA, THF,  $-78^\circ\text{C}$  (ii)  $\text{BnOH}$ ,  $\text{BnONa}$  (iii)  $\text{HCl}$ ,  $\text{EtOAc}$  (iv)  $\text{Fmoc-OSu}$ , DIPEA.

the formation of  $\text{Boc-L-Gla}(\text{OBn})_2\text{-OBu}^t$ . A check on optical purity was made by deprotection of this derivative with ethyl acetate saturated with HCl, followed by hydrogenation, to give a sample of  $\text{H-Gla-OH}$  with  $[\alpha]_D +41.5^\circ$  ( $c$  0.27, 6 M HCl), compared with the literature value<sup>16</sup> of  $[\alpha]_D +40.4^\circ$  ( $c$  1, 6 M HCl). The conversion of  $\text{H-L-Gla}(\text{OBn})_2\text{-OH}$  to  $\text{Fmoc-L-Gla}(\text{OBn})_2\text{-OH}$ , for some reason (probably solubility problems), became far from trivial, and several attempts were made before finally achieving a 47% yield, using  $\text{Fmoc-OSu}$ -diisopropylethylamine (DIPEA) (1.5 equiv.) in 1,4-dioxane solution.

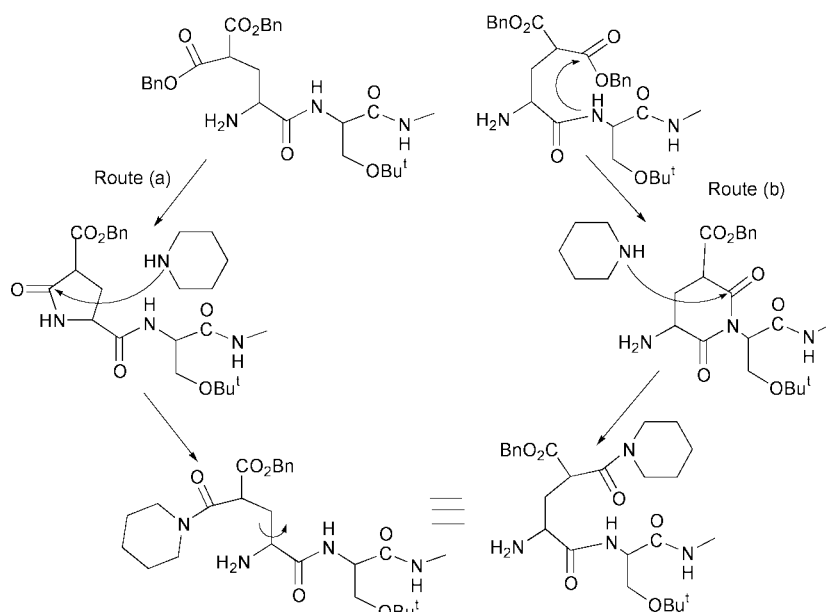
With  $\text{Fmoc-L-Gla}(\text{OBn})_2\text{-OH}$  in hand, the derivatised pentapeptide precursor  $[\text{Fmoc-L-Gla}(\text{OBn})_2\text{-Ser}(\text{Bu}^t)\text{-Lys}(\text{Boc})\text{-Arg(Mtr)-Gly-OH}]$  was assembled as summarised in Scheme 2(b). However, when the Fmoc group was removed from this pentapeptide derivative using piperidine, and the product cyclised using EDC-DMAP, the cyclic pentapeptide derivative did not give the expected molecular mass ion of  $m/z$  1150. Instead a strong ion at  $m/z$  1127 was found. On re-analysing the stages, it was found that one of the benzyl ester side-chains of Gla had been replaced by piperidine, and this had occurred during the deprotection of the Fmoc group. So rather than having the precursor molecule  $\text{H-L-Gla}(\text{OBn})_2\text{-Ser}(\text{Bu}^t)\text{-Lys}(\text{Boc})\text{-Arg(Mtr)-Gly-OH}$  to cyclise, the starting material used was  $\text{H-Gla}(\text{OBn}, \text{NC}_5\text{H}_{10})\text{-Ser}(\text{Bu}^t)\text{-Lys}(\text{Boc})\text{-Arg(Mtr)-Gly-OH}$ , the  $\text{NC}_5\text{H}_{10}$  representing a piperidine. There are two possible mechanisms (Scheme 4) which can be put forward to explain this result, but we favour route (a), since by experimenting with  $\text{Fmoc-L-Gla}(\text{OBn})_2\text{-OH}$  in the presence of piperidine, removal of the Fmoc group was accompanied by replacement of one benzyl ester group, even if the piperidine was in excess. As  $\text{Fmoc-L-Gla}(\text{OBn})_2\text{-OH}$  does not have an amide NH it eliminates the aspartimide mechanism (b). [A referee of this paper favoured mechanism (b) on the basis that the 5-membered-ring amide in route (a) would be rather stable.]

This side-reaction forced us to reconsider our strategy, so we opted for the  $\text{Fmoc-Gla}(\text{OBu}^t)_2\text{-OH}$  building block again, but this time seeking to resolve the racemic Gla derivative into its enantiomers. Two recent methods<sup>22,23</sup> were considered, but we opted for chemical resolution,<sup>22</sup> as summarised in Scheme 5. Samples of  $\text{Z-DL-Gla}(\text{OBu}^t)_2\text{-OH}$  resolved in this manner gave  $\text{Z-D-Gla}(\text{OBu}^t)_2\text{-OH}$  quinine salt,  $[\alpha]_D -87^\circ$  ( $c$  0.16 in MeOH) [lit.,<sup>22</sup>  $[\alpha]_D -86.8^\circ$  ( $c$  1 in MeOH)], and  $\text{Z-L-Gla}(\text{OBu}^t)_2\text{-OH}$ , ephedrine salt  $[\alpha]_D -15^\circ$  ( $c$  0.15 in MeOH) {lit.,<sup>22</sup>  $[\alpha]_D -15.3^\circ$  ( $c$  1 in MeOH)}. Removal of the auxiliary salts, gave samples of  $\text{Z-D-Gla}(\text{OBu}^t)_2\text{-OH}$  and  $\text{Z-L-Gla}(\text{OBu}^t)_2\text{-OH}$  with  $[\alpha]_D$ -values of  $+11.4$  and  $-11.8^\circ$  ( $c$  0.135 in MeOH) respectively.

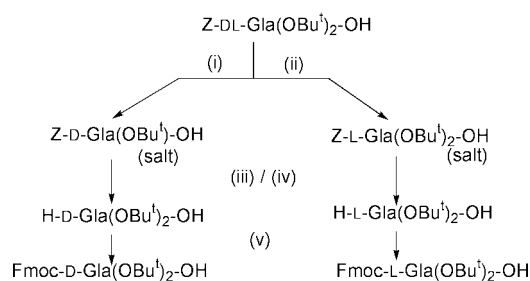
Routine conversion of these resolved products to their Fmoc derivatives gave the necessary building blocks for incorporation into Schemes 2(c) and 2(d). Cyclisation, at high dilution, of the appropriate linear precursors released from the resin was carried out using HATU-DIPEA in dimethylformamide (DMF)-dichloromethane solution<sup>24</sup> to yield  $\text{cyclo[Arg(Mtr)-Gly-L-Gla}(\text{OBu}^t)_2\text{-Ser}(\text{Bu}^t)\text{-Lys}(\text{Boc})\text{-}]$  and  $\text{cyclo[Arg(Mtr)-Gly-D-Gla}(\text{OBu}^t)_2\text{-Ser}(\text{Bu}^t)\text{-Lys}(\text{Boc})\text{-}]$ . Crude cyclisation yields of 75% were obtained. Each of the crude products needed extensive purification by preparative- and semi-preparative-scale HPLC. There was evidence that some of the cyclised D-Gla analogue was present in the L-Gla analogue but could be removed by HPLC. The cyclised D-Gla analogue did not seem to contain any of the L-Gla form as perceived from HPLC analysis. Deprotection of the derivatised cyclopentapeptides using TFA gave pure samples of the cyclic pentapeptides **2** and **3**, as their trifluoroacetate salts, which were bioassayed as described later.

The constraining of the RGD domain into a more hydrophobic environment has been explored through the synthesis of  $\text{cyclo(-Arg-Gly-Asp-Phe-Phe-)}$ , chosen on the basis of previous

<sup>†</sup>  $[\alpha]_D$ -Values are given in units of  $10^{-1} \text{ deg cm}^2 \text{ g}^{-1}$  throughout this paper.



Scheme 4



**Scheme 5** Reagents and conditions: (i) (–)-quinine (ii) (–)-ephedrine (iii) NaHSO<sub>4</sub> (iv) H<sub>2</sub>, Pd/C (v) Fmoc-OSu.

studies<sup>25</sup> where aromatic residues enhanced adhesive interaction. Work had been started before the announcement<sup>10</sup> that a Phe residue seems advantageous in enhancing the selective inhibition of the  $\alpha_v\beta_3$  receptors. The protocol for synthesis of the linear pentapeptide precursor followed the standard schedule of Scheme 2(e). After resin release and removal of the Fmoc group, H-Asp(OBu<sup>t</sup>)-Phe-Phe-Arg(Mtr)-Gly-OH was cyclised under high dilution conditions, using EDC-DMAP. After purification using HPLC, pure cyclo[-Arg(Mtr)-Gly-Asp(OBu<sup>t</sup>)-Phe-Phe-] was obtained in 55% purified yield. Treatment of this purified material with TFA for 5 h gave a quantitative yield of cyclo(-Arg-Gly-Asp-Phe-Phe-), which was counter-ion exchanged with acetate ions before biological testing.

### Bioassays

Cell-adhesion assays on cyclopentapeptide **1** and cyclo(-Arg-Gly-Asp-Phe-Phe-) were carried out at the Randall Institute, King's College, London using conditions and techniques previously reported<sup>13</sup> for other analogues. These analogues were tested on NIH 3T3 fibroblasts which are enriched in  $\alpha_5\beta_1$  integrin receptors, and without any peptide added 98% of the fibroblasts in suspension adhered to 'lawns' of fibronectin. This adhesion was reduced to  $15 \pm 7\%$  in the presence of cyclopentapeptide **1** (cf.  $65 \pm 6\%$  for cyclo(-Arg-Gly-Asp-Ser-Lys-)) and remains the best we have obtained. When the cyclo(-Arg-Gly-Asp-Phe-Phe-) was analysed in the same manner the fibroblasts died during the experiment, implying that this compound was cytotoxic.

During the bioassays it was noticed that stock solutions (kept at pH 7.4) of cyclic analogue **1** gradually lost their initial cell-adhesion inhibition. On analysing solutions of **1** by HPLC and electrospray MS, it was discovered that there was a slow

conversion of **1** to cyclo(-Arg-Gly-Glu-Ser-Lys-), which appeared as a peak in HPLC at  $t_R$  2.68 min (compared with  $t_R$  3.18 min for **1**). Obviously, decarboxylation of the Gla residue was occurring since the MS analysis of a solution of **1** left for 4 weeks showed that the molecular ion at  $m/z$  602 had almost disappeared, being replaced by that of the Glu analogue with  $m/z$  558.

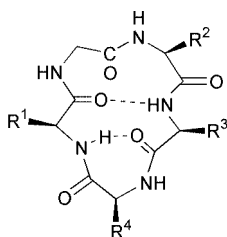
The analogues **2** and **3** were analysed by Merck KgaA, Darmstadt Germany, in association with Professor Horst Kessler (Technische Universität, Munich). In binding assays, again carried out in fresh solutions of dimethyl sulfoxide (DMSO) to avoid loss of CO<sub>2</sub> from the Gla residue, **2** and **3** were found to be inactive at concentrations of 10  $\mu$ M against recombinant integrins  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$ , and GP2b3a. This result in one way confirms the general hypothesis that substitution of the Asp residue cannot be tolerated. However, it does not explain the activity of the racemate **1** against 3T3 fibroblast cells, which recognise fibronectin *via* the  $\alpha_5\beta_1$  integrin. It is possible that **2** and **3** would react likewise, but unfortunately a direct comparison of the chirally pure analogues on whole cells was not available to us.

### Conformational analysis

Our approach to the conformational analysis of cyclopentapeptides follows procedures already reported,<sup>26</sup> and we presumed that the easiest to study first would be cyclo[-Arg(Mtr)-Gly-Asp(OBu<sup>t</sup>)-Phe-Phe-]. However, after dissolution in CDCl<sub>3</sub>, the initial clear solution turned into a gel. Solvent gelation has been noticed<sup>27</sup> before with cyclic depsipeptides, but is unusual in peptides. So subsequent studies had to revert to d<sub>6</sub>-DMSO solutions. Detailed correlation of peaks from 2D-NMR spectra appear in the Experimental section, and on carrying out temperature dependence correlations for the amide NHs in the range 25–40 °C, values lower than 3 ppb K<sup>–1</sup>, indicative of intramolecular H-bonding,<sup>28</sup> were found for the Arg and Phe NHs with values of  $\delta/T$  –2.6 and –2.0 ppb K<sup>–1</sup> respectively. These results confirm the  $\beta/\gamma$  turn conformation as depicted in Fig. 1.

The cyclic analogue **1** was soluble only in D<sub>2</sub>O, and to maintain solubility the NMR spectrum had to be determined at 40 °C. Under these conditions only weak signals were obtained for the amide protons, which became partly exchanged with D<sub>2</sub>O, so temperature dependence studies could not be carried out. The 1D spectrum supported the correct structure, but the presence of diastereoisomeric signals confused the interpre-





$R^1 = (\text{Arg})$ ;  $R^2 = (\text{Asp})$ ;  $R^3 = R^4 = (\text{Phe})$

$R^1 = (\text{Arg})$ ;  $R^2 = (\text{Gla})$ ;  $R^3 = (\text{Ser})$ ;  $R^4 = (\text{Lys})$

Fig. 1

tation. With the availability of the chirally pure forms **2** and **3** NMR studies were first carried out on cyclo[Arg(Mtr)-Gly-L-Gla(OBu')<sub>2</sub>-Ser(Bu')-Lys(Boc)-] and its D-Gla analogue. The 2D-NMR studies carried out in CDCl<sub>3</sub> showed a reasonable number of cross-peak correlations which confirmed the structure, and in temperature dependence studies on the amide NHs it was found that these protected Gla derivatives showed differences in their H-bonding patterns. The D-Gla analogue followed the same pattern of intramolecular H-bonds as the molecule bearing two Phe residues, but this time the NHs of Arg and Ser are involved. In the L-Gla analogue, strong H-bonding could be detected in the Ser-NH, supporting a typical  $\beta$ -turn, but only the figures for the Gla NH get anywhere near supporting an intramolecular H-bond interaction elsewhere. NMR studies on the deprotected L-analogue **2** in d<sub>6</sub>-DMSO confirmed H-bonding at the Arg and Ser NHs. Insufficient quantities of pure material prevented us from securing good spectra for the D-Gla analogue.

Within this series of studies it seems that the  $\beta/\gamma$ -turn conformations typical of cyclopentapeptides in general have been maintained, so the lack of activity with the recombinant integrins does not seem to be dictated by drastic conformational changes in the Gla analogues.

## Experimental

NMR spectra were recorded on a Bruker AC-400 FT spectrometer at 400 MHz for protons and 100 MHz for carbon, using CDCl<sub>3</sub> or d<sub>6</sub>-DMSO as solvent and tetramethylsilane as internal reference. FAB-MS spectra were run on a VG-Autospec instrument using Cs<sup>+</sup> ions as bombarding ions using thioglycerol or NOBA as matrix. Positive-mode electrospray MS was carried out on a VG Quattro II 'triple' quadrupole from Micromass. Solvent used was usually MeCN–water (1 : 1) with formic acid.

Flash chromatography was carried out on Merck Matrex silica 60. TLC was performed on Merck silica gel mounted on aluminium backing with fluorescent indicator (254 nm). Mps were measured on a Gallenkamp hot-stage apparatus and are uncorrected. Optical rotations were measured using a Perkin-Elmer 141 polarimeter using a sodium lamp ( $\lambda = 589$  nm) at ambient temperature using a path length of 1 dm.

HPLC analyses were made using an LDC–Milton Roy apparatus consisting of a spectromonitor D, constametric pumps and a CI-4100 or CI-10 $\beta$  integrator. A Spherisorb-ODS C<sub>18</sub> analytical column (25  $\times$  0.43 cm) at a flow rate of 1 cm<sup>3</sup> min<sup>-1</sup> was used to check purity, while preparative separations were either carried on a Prep-10 cartridge (RP-18 stationary phase with spherical 20  $\mu$ m silica of 300 Å pore size) from Applied Biosystems, or an RP-18 column (PL-Reversed Phase) at flow rates of 2 cm<sup>3</sup> min<sup>-1</sup> or up to 10 cm<sup>3</sup> min<sup>-1</sup> respectively. Solvents for HPLC were filtered before hand through 0.45  $\mu$ m filters followed by ultrasonic degassing. Detector wavelengths were set at 220 nm unless otherwise stated.

A Vega Biochemicals Peptide synthesiser, Model 250 (inherited from Wellcome Research Laboratories) was available for solid-phase synthesis.

DMF for solution-phase synthesis was Analar Grade (Aldrich), purified by distillation under reduced pressure after treatment with ninhydrin, and stored over 4 Å molecular sieves. HPLC-grade DMF (Fisons) from freshly opened bottles was directly used in solid-phase synthesis. THF was purified by, first, passage through dry alumina under nitrogen, stirring over calcium hydride for 16 h, and distillation. Finally, sodium (2 g per dm<sup>3</sup>) and benzophenone (8 g per dm<sup>3</sup>) were then added to the THF in a still and the mixture was stirred under argon to give a purple solution of the sodium benzophenone ketal. The THF was then distilled from the ketal under argon as required.

Solutions of *n*-butyllithium in hexane were obtained from Aldrich and standardised before use by direct titration of the carbon–lithium bond with butan-2-ol using 2,2'-bipyridyl as indicator. Piperidine, DIPEA and *N*-methyldmorpholine, from Aldrich, were all distilled over KOH before use. TFA (99%) was used directly from the manufacturer (Fisons). All other solvents were routinely distilled before use. Light petroleum (40–60) refers to the fraction with distillation range 40–60 °C.

HMPB–MBHA the highly acid-labile resin (theoretical loading 0.57 mmol g<sup>-1</sup>), Fmoc amino acid derivatives, and peptide synthesis reagents were purchased from Novabiochem. All reagents were dried in a desiccator under reduced pressure over P<sub>2</sub>O<sub>5</sub> for at least 4 h before use.

### *N*-(Benzyloxycarbonyl)dehydroalanine benzyl ester

A solution of dried *N*-benzyloxycarbonyl-L-serine<sup>29</sup> (9.56 g, 40 mmol) in DMF (20 mL) was added to freshly prepared *O*-benzyl-*N,N'*-diisopropylisourea<sup>11</sup> (9.37 g, 40 mmol) at room temperature. After stirring for 1 h 30 min, the reaction mixture became turbid and viscous, so more DMF (10 mL) was added. After completion of reaction (24 h), *N,N'*-diisopropylurea was filtered off and the filtrate was concentrated under high vacuum to give a gelatinous solution which yielded more precipitated diisopropylurea on cooling in the fridge. The final DMF filtrate was evaporated to dryness to give a yellow gum, which was dissolved in chloroform and washed twice with water. The dried organic layer, on removal of solvent, yielded a solid which crystallised as *N*-benzyloxycarbonyl-L-serine benzyl ester from CHCl<sub>3</sub>–light petroleum (40–60) in 87% yield, and had *t*<sub>R</sub> 14.6 min on the analytical column in 60% aq. MeCN ( $\lambda$  254 nm), mp 82–84 °C (lit.,<sup>30</sup> 82–84 °C);  $\delta_{\text{H}}$  (400 MHz; d<sub>6</sub>-DMSO) 2.87 (1 H, br, OH), 3.86–4.0 (2 H, 2q,  $\beta$ CH<sub>2</sub>), 4.45–4.47 (1 H, m,  $\alpha$ CH), 5.09 (2 H, s, CH<sub>2</sub>Ph), 5.19 (2 H, s, CH<sub>2</sub>Ph), 5.95–5.96 (1 H, d, NH), 7.29–7.39 (10 H, m, ArH); *m/z* (EI/CI) 330 (M + H)<sup>+</sup>.

CuCl (77 mg, 0.78 mmol) was added to a solution of dicyclohexylcarbodiimide (DCC) (0.73 g, 3.5 mmol). The *N*-benzyloxycarbonyl-L-serine benzyl ester prepared above (0.99 g, 3.0 mmol) was dissolved in dry dichloromethane (10 mL) and added to the CuCl–DCC suspension at room temperature, the reaction mixture turning at once from green to orange. After stirring of the mixture for 2 h, the green colour returned and dicyclohexylurea precipitated. The reaction mixture was filtered over an alumina pad to remove the copper salts and the urea. The dehydroalanine derivative was washed through with dichloromethane (20 mL), and on work-up using the usual extraction processes gave a crude solid on evaporation of the dried solvent. This solid was dissolved in hot light petroleum (40–60), and after removal of more precipitated urea, on cooling, *N*-(benzyloxycarbonyl)dehydroalanine benzyl ester crystallised out as white needles (90%), *t*<sub>R</sub> 18.5 min (60% aq. MeCN) on the analytical column at  $\lambda$  254 nm, mp 45–47 °C (lit.,<sup>31</sup> an oil),  $\delta_{\text{H}}$  (250 MHz; CDCl<sub>3</sub>) 5.15 (2 H, s, CH<sub>2</sub>Ph), 5.24 (2 H, s, CH<sub>2</sub>Ph), 5.84 (1 H, s, =CHH), 6.26 (1 H, s, =CHH), 7.36 (10 H, m, ArH); *m/z* (EI/CI) 329 (M + NH<sub>4</sub>)<sup>+</sup>, 312 (M + H)<sup>+</sup>.

**$\gamma,\gamma'$ -Di-*tert*-butyl-DL- $\gamma$ -carboxyglutamic acid  
[H-DL-Gla(OBu')<sub>2</sub>-OH]**

Sodium hydride (80% suspension in mineral oil; 0.23 g, 7.7 mmol) was washed with dry light petroleum (40–60) under nitrogen, then suspended in dry DMF (10 mL). Di-*tert*-butyl malonate (1.7 mL, 7.6 mmol) was added dropwise and the reaction mixture was stirred for 24 h at room temperature. Dry *N*-(benzyloxycarbonyl)dehydroalanine benzyl ester (1.9 g, 6.1 mmol) was dissolved in dry DMF (10 mL) and added to the malonate anion solution. The reaction mixture was stirred at room temperature, and after 24 h the DMF was removed under high vacuum to produce a yellow oil, which was chromatographed on silica gel, and eluted with a gradient from dichloromethane to 5% ethyl acetate in dichloromethane. Fractions containing product were combined and evaporated to dryness to produce Z-DL-Gla(Bu')<sub>2</sub>-OBn as a gum (1.64 g, 51%), *t*<sub>R</sub> 12.01 min (70% MeCN–water) on analytical HPLC;  $\delta_{\text{H}}$  (250 MHz; CDCl<sub>3</sub>) 1.40 [9 H, s, C(CH<sub>3</sub>)<sub>3</sub>], 1.42 [9 H, s, C(CH<sub>3</sub>)<sub>3</sub>], 2.06–2.46 (2 H, m,  $\beta$ CH<sub>2</sub>), 3.22–3.28 (1 H, t,  $\gamma$ CH), 4.44–4.51 (1 H, m,  $\alpha$ CH), 5.09 (2 H, s, CH<sub>2</sub>Ph), 5.17 (2 H, s, CH<sub>2</sub>Ph), 5.36–5.38 (1 H, d, NH), 7.26–7.37 (10 H, m, ArH); *m/z* (EI/CI) 545 (M + NH<sub>4</sub>)<sup>+</sup>, 528 (M + H)<sup>+</sup>.

Z-DL-Gla(Bu')<sub>2</sub>-OBn (2.4 g, 4.6 mmol) was dissolved in dry methanol (40 mL) and 10% Pd/C (0.25 g) was added to this solution. This suspension was stirred at room temperature under hydrogen, and after 20 h the catalyst was removed by filtration, and the methanol removed under reduced pressure to give a waxy solid, which on recrystallisation from methanol–diethyl ether gave H-DL-Gla(OBu')<sub>2</sub>-OH (0.88 g, 63%), mp 151–153 °C (lit.,<sup>32</sup> 148–150 °C);  $\delta_{\text{H}}$  (400 MHz; d<sub>6</sub>-DMSO) 1.41 [9 H, s, C(CH<sub>3</sub>)<sub>3</sub>], 1.42 [9 H, s, C(CH<sub>3</sub>)<sub>3</sub>], 1.76–1.95 and 2.01–2.20 (2 H, 2m,  $\beta$ CH<sub>2</sub>), 3.07–3.10 (1 H, t,  $\gamma$ CH), 3.75–3.79 (1 H, t,  $\alpha$ CH);  $\delta_{\text{C}}$  (100 MHz; d<sub>6</sub>-DMSO) 27.59 (CH<sub>3</sub>), 30.27 (CH<sub>2</sub>), 50.35 and 51.58 (2  $\times$  CH), 80.80 and 80.91 [2  $\times$  C(CH<sub>3</sub>)<sub>3</sub>], 167.96 and 168.10 [2  $\times$  CO<sub>2</sub>C(CH<sub>3</sub>)<sub>3</sub>], 169.58 (CO<sub>2</sub>H); *m/z* (EI/CI) 304 (M + H)<sup>+</sup>.

**$\gamma,\gamma'$ -Di-*tert*-butyl-N-fluoren-9-ylmethoxycarbonyl)-DL- $\gamma$ -carboxyglutamic acid [Fmoc-DL-Gla(OBu')<sub>2</sub>-OH]**

To a solution of H-DL-Gla(Bu')<sub>2</sub>-OH (0.75 g, 2.5 mmol) in 10% aq. sodium bicarbonate (pH 8; 75 mL) and 1,4-dioxane (30 mL) was added a solution of fluoren-9-ylmethyl chloroformate (0.77 g, 3.0 mmol) in 1,4-dioxane (30 mL) at room temperature with stirring, which was continued for 24 h. The reaction mixture was extracted with diethyl ether (2  $\times$  50 mL) to remove unchanged chloroformate. The aqueous phase (pH 8) was then extracted with ethyl acetate (2  $\times$  100 mL), and after this extraction and drying of the organic phase, a colourless gum was obtained (0.98 g, 75%), which partially crystallised in the fridge; *t*<sub>R</sub> 11.36 min (50% aq. MeCN) on analytical HPLC;  $\delta_{\text{H}}$  (100 MHz; CDCl<sub>3</sub>) 1.41 [18 H, s, 2  $\times$  C(CH<sub>3</sub>)<sub>3</sub>], 2.06–2.50 (2 H, m,  $\beta$ CH<sub>2</sub>), 3.30–3.51 (1 H, m,  $\gamma$ CH), 4.00–4.42 (4 H, m,  $\alpha$ CH overlapping with Fmoc CH and CH<sub>2</sub>), 5.0–5.6 (1 H, br, COOH), 5.7–5.8 (1 H, d, NH), 7.14–7.82 (8 H, m, ArH); *m/z* (FAB) 548 (M + Na)<sup>+</sup>, 526 (M + H)<sup>+</sup>, 468 (M – Bu')<sup>+</sup>.

**$\gamma,\gamma'$ -Dibenzyl-N-(*tert*-butoxycarbonyl)-L-carboxyglutamic acid *tert*-butyl ester [Boc-L-Gla(OBn)<sub>2</sub>-OBu'] (Scheme 3)<sup>16</sup>**

To a solution of distilled diisopropylamine (5 mL, 35.57 mmol) in dry THF (35 mL) was added dropwise standardised *n*-butyllithium (2.53 M; 14 mL, 35.42 mmol) over a period of 15 min, under nitrogen, at –78 °C. The mixture was vigorously stirred for 45 min. A solution of 1,5(*S*)-bis(*tert*-butoxycarbonyl)-2-pyrrolidone (gift from Roche Research Centre) (8.66 g, 30.37 mmol) in dry THF (45 mL) was then added dropwise to the freshly made lithium diisopropylamine (LDA), over a period of 55 min. The solution was kept at –78 °C, under an inert atmosphere and vigorous stirring, for a further 30 min. Benzyl chloroformate (5 mL, 35.02 mol) was then added dropwise, and

the reaction mixture was allowed to warm to room temperature overnight and was stirred for a further 24 h. The yellow solution was partitioned between ethyl acetate (60 mL) and water (40 mL), followed by further washing of the organic layer with water. After drying and removal of the ethyl acetate solvent under reduced pressure, the resulting orange oil could be partly crystallised from hot light petroleum. More compound could be obtained after chromatography of the mother liquor on silica gel, using gradient elution (ethyl acetate–light petroleum 20:80 to 80:20). Overall yield of pure compound was 5.64 g (45%); mp 105–110 °C (mixture of diastereoisomers). On being left in solution for NMR determination the two sets of diastereoisomeric signals became more prominent, and were assigned to the mixture of the 3*S*,5*S* and 3*R*,5*S* diastereoisomers. It seemed that only the 3*R*,5*S* diastereoisomer was present in a freshly prepared solution. This was also confirmed by HPLC on an analytical column (MeCN–H<sub>2</sub>O 60:40). The fresh solution had a *t*<sub>R</sub> of 15.88 min which changed after 3 h to *t*<sub>R</sub> 15.65 and 18.90 min. NMR of major diastereoisomer (probably 3*R*,5*S* form)  $\delta_{\text{H}}$  (400 MHz; CDCl<sub>3</sub>) 1.47 [9 H, s, C(CH<sub>3</sub>)<sub>3</sub>], 1.51 [9 H, s, C(CH<sub>3</sub>)<sub>3</sub>], 2.53 (2 H, 2d, *J*, 7.2 and 3.2, H<sub>2</sub>-4), 3.57 (1 H, m, H-3), 4.47 (1 H, m, H-5), 5.20 (2 H, m, CH<sub>2</sub>Ph), 7.35 (5 H, m, ArH);  $\delta_{\text{C}}$  (100 MHz; CDCl<sub>3</sub>) 24.5 (C-4), 27.8 [C(CH<sub>3</sub>)<sub>3</sub>], 27.9 [C(CH<sub>3</sub>)<sub>3</sub>], 48.7 (C-5), 58.1 (C-3), 67.7 (CH<sub>2</sub>Ph), 82.6 [C(CH<sub>3</sub>)<sub>3</sub>], 83.8 [C(CH<sub>3</sub>)<sub>3</sub>], 128.2–128.6, 135.2 (aromatic carbons), 149.1, 167.3, 167.6, and 169.3 (carbonyl carbons). NMR of other diastereoisomer,  $\delta_{\text{H}}$  (400 MHz; CDCl<sub>3</sub>) 1.46 [9 H, s, C(CH<sub>3</sub>)<sub>3</sub>], 1.51 [9 H, s, C(CH<sub>3</sub>)<sub>3</sub>], 2.23 (1 H, m, H-4), 2.69 (1 H, m, H-4), 3.70 (1 H, m, H-3), 4.53 (1 H, dd, *J*, 9.6, 2.1, H-5), 5.20 (2 H, m, CH<sub>2</sub>Ph), 7.35 (5 H, m, ArH);  $\delta_{\text{C}}$  (100 MHz; CDCl<sub>3</sub>) 25.6 (C-4), 27.9 [C(CH<sub>3</sub>)<sub>3</sub>], 27.9 [C(CH<sub>3</sub>)<sub>3</sub>], 48.5 (C-5), 57.8 (C-3), 67.7 (CH<sub>2</sub>Ph), 82.8 [C(CH<sub>3</sub>)<sub>3</sub>], 84.0 [C(CH<sub>3</sub>)<sub>3</sub>], 128.2–128.6, 135.1 (aromatic carbons), 149.0, 168.0, 168.3 and 169.9 (carbonyl carbons); FAB MS (NOBA) *m/z* 442 (M + Na)<sup>+</sup>, 420 (M + H)<sup>+</sup>, 342 (M – Boc + H + Na)<sup>+</sup>, 320 (M – Boc + 2H)<sup>+</sup>, 308 (M – CO<sub>2</sub>Bz + H + Na)<sup>+</sup>, 286 (M – CO<sub>2</sub>Bz + 2H)<sup>+</sup>.

Sodium metal (0.26 g, 11.27 mmol) was made to react, under nitrogen, with dry benzyl alcohol (10 mL) (a highly exothermic reaction occurred) under vigorous stirring, until no sodium lumps were left. 3(*R**S*)-Benzyloxycarbonyl-1,5(*S*)-bis(*tert*-butoxycarbonyl)-2-pyrrolidone prepared as above (1.59 g, 3.80 mmol) was dissolved in anhydrous benzyl alcohol (8 mL). Freshly made sodium benzoxide (1.1 M; 1.2 mL, 1.3 mmol) was added in small portions (0.1 mL) over a period of 30 h. The mixture was left to react for 5 days before being quenched with glacial acetic acid (0.15 mL, 2.62 mmol). The benzyl alcohol was removed by Kugelrohr distillation under reduced pressure (0.2 mbar and oven temp. 80 °C). The crude product was purified by silica gel column chromatography using isocratic elution with ethyl acetate–light petroleum 30:70. Boc-L-Gla(OBn)<sub>2</sub>-OBu' was obtained as a transparent oil (1.2 g, 64%),<sup>14</sup> [ $\alpha$ ]<sub>D</sub> +11.7 (*c* 1.4 in CHCl<sub>3</sub>);  $\delta_{\text{H}}$  (400 MHz; CDCl<sub>3</sub>) 1.41 [9 H, s, C(CH<sub>3</sub>)<sub>3</sub>], 1.44 [9 H, s, C(CH<sub>3</sub>)<sub>3</sub>], 2.15 (1 H, m,  $\beta$ CH<sub>2</sub>), 2.35 (1 H, m,  $\beta$ CH<sub>2</sub>), 3.60 (1 H, dd, *J*, 8.0, 6.2,  $\gamma$ CH), 4.31 (1 H, br m,  $\alpha$ CH), 5.05 (1 H, br d, NH), 5.11–5.19 (4 H, m, 2  $\times$  CH<sub>2</sub>Ph), 7.25–7.35 (10 H, m, ArH);  $\delta_{\text{C}}$  (100 MHz; CDCl<sub>3</sub>) 27.9 [C(CH<sub>3</sub>)<sub>3</sub>], 28.3 [C(CH<sub>3</sub>)<sub>3</sub>], 31.7 ( $\beta$ C), 48.7 ( $\gamma$ C), 52.3 ( $\alpha$ C), 66.4, 67.4 (benzyl CH<sub>2</sub>), 79.9 [C(CH<sub>3</sub>)<sub>3</sub>], 82.5 [C(CH<sub>3</sub>)<sub>3</sub>], 128.2–128.5, 135.1, 135.2 (aromatic carbons), 155.4, 168.4, 168.9 and 170.9 (carbonyl carbons); FAB-MS (NOBA) *m/z* 550 (M + Na)<sup>+</sup>, 528 (M + H)<sup>+</sup>, 416 (M – 2Bu' + 2H)<sup>+</sup>, 372 (M – Boc – Bu' + 2H)<sup>+</sup>.

**$\gamma,\gamma'$ -Dibenzyl-N-(fluoren-9-ylmethoxycarbonyl)-L- $\gamma$ -carboxyglutamic acid [Fmoc-L-Gla(OBn)<sub>2</sub>-OH]**

Ethyl acetate (45 mL) was saturated with hydrogen chloride gas (from conc. H<sub>2</sub>SO<sub>4</sub> and NH<sub>4</sub>Cl). Boc-L-Gla(OBn)<sub>2</sub>-OBu' prepared above (0.65 g, 1.23 mmol) was treated with a solution

of HCl in ethyl acetate (10 mL). A white precipitate formed, and the solution was stirred at room temperature for 3 days. The ethyl acetate was removed under reduced pressure, and the solid residue was triturated several times with fresh ethyl acetate. After filtration, and drying under high vacuum,  $\text{H}_2^+(\text{Cl}^-)\text{-L-Gla}(\text{OBn})_2\text{-OH}$  was obtained as a white solid (0.45 g, 89%);  $[\alpha]_{\text{D}} +24.8$  ( $c$  1.6 in MeOH);  $\delta_{\text{H}}$  (400 MHz;  $\text{d}_6\text{-DMSO}$ ) 2.40 (2 H, 2m,  $\beta\text{CH}_2$ ), 3.87 (1 H, br s,  $\alpha\text{CH}$ ), 4.10 (1 H, t,  $J$ , 6.7–7.7,  $\gamma\text{CH}$ ), 5.13 (4 H, m,  $\text{CH}_2\text{Ph}$ ), 7.33 (10 H, m, ArH), 8.70 (2.6 H, br s,  $\text{NH}_3^+$ );  $\delta_{\text{C}}$  (100 MHz;  $\text{d}_6\text{-DMSO}$ ) 28.7 ( $\beta\text{C}$ ), 47.6 ( $\gamma\text{C}$ ), 49.9 ( $\alpha\text{C}$ ), 66.8 ( $\text{CH}_2\text{Ph}$ ), 127.8–128.3, 135.2 (aromatic carbons), 167.6–167.7 and 169.9 (carbonyl carbons); FAB-MS (NOBA)  $m/z$  394 ( $\text{M} - \text{Cl}^- + \text{Na}^+$ ), 372 ( $\text{M} - \text{Cl}^- + \text{H}^+$ ).

This dibenzyl derivative (0.31 g, 0.75 mmol) in methanol (8 mL) was hydrogenated in the presence of Pd/C (30.5 mg) to give, on standard work-up, H-L-Gla-OH, (20 mg, 15%) as white crystals from MeOH–water, mp 168–169 °C (lit.,<sup>16</sup> 168–169 °C),  $[\alpha]_{\text{D}} +41.5$  ( $c$  0.27 in 6 M HCl) [lit.,<sup>16</sup> +40.4 ( $c$  1 in 6 M HCl)].

$\text{H}_2^+(\text{Cl}^-)\text{-L-Gla}(\text{OBn})_2\text{-OH}$  (2.28 g, 5.61 mmol) as a solution in 1,4-dioxane (80 mL) was placed in an ultrasonic bath for 30 min, but complete dissolution was not obtained. After addition of DIPEA (1.5 mL, 8.61 mmol), the solution became transparent and was then stirred until a white suspension (amine salt) was formed. A solution of Fmoc-OSu (2.09 g, 6.19 mmol) in 1,4-dioxane (12 mL) was then added slowly over a period of 50 min, and the mixture was stirred for another 24 h. 10% Aq.  $\text{NaHCO}_3$  (90 mL) was added before extraction of the excess of Fmoc-OSu into diethyl ether ( $2 \times 75$  mL). The aqueous phase (pH 8) was acidified to pH 2–3 with 10% citric acid (100 mL) before further extraction into ethyl acetate ( $2 \times 125$  mL). After drying of the organic layer, and removal of the solvent, the residue was chromatographed on silica gel using ethyl acetate–0.4% acetic acid as eluent. Pooled fractions were evaporated under reduced pressure to yield Fmoc-L-Gla(OBn)<sub>2</sub>-OH as a white glassy solid (1.56 g, 47%) (Found: C, 70.9; H, 5.2; N, 2.45.  $\text{C}_{35}\text{H}_{31}\text{NO}_8$  requires C, 70.9; H, 5.1; N, 2.4%);  $[\alpha]_{\text{D}} +8.7$  ( $c$  1.2 in  $\text{CHCl}_3$ );  $\delta_{\text{H}}$  (400 MHz;  $\text{CDCl}_3$ ) 2.29 (1 H, m,  $\beta\text{CH}$ ), 2.64 (1 H, m,  $\beta\text{CH}$ ), 3.65 (1 H, t,  $J$  7.0,  $\gamma\text{CH}$ ), 4.15 (1 H, t,  $J$ , 7.1,  $\alpha\text{CH}$ ), 4.35 (2 H, 2dd,  $J$ , 10.5, 7.0, Fmoc  $\text{CH}_2\text{O}$ ), 4.50 (1 H, m, Fmoc CH), 5.0–5.20 (4 H, d,  $J$ , 7.9,  $\text{PhCH}_2$ ), 7.1–7.7 (18 H, m, ArH);  $\delta_{\text{C}}$  (100 MHz;  $\text{CDCl}_3$ ) 30.9 ( $\beta\text{C}$ ), 47.0 ( $\gamma\text{C}$ ), 48.7 ( $\alpha\text{C}$ ), 52.1 (fluorene CH), 67.3 (Fmoc  $\text{CH}_2\text{O}$ ), 67.6 ( $\text{PhCH}_2$ ), 120.0–128.6, 134.9, 135.0, 141.3, 143.6 (aromatic carbons), 156.3, 168.4–168.7, 175.3 (carbonyl carbons); FAB-MS (NOBA)  $m/z$  616 ( $\text{M} + \text{Na}^+$ ), 594 ( $\text{M} + \text{H}^+$ ), 178 (fulvene).

**Preparation of  $\gamma,\gamma'$ -di-*tert*-butyl-*N*-(fluoren-9-ylmethoxycarbonyl)-L- $\gamma$ -carboxyglutamic acid [Fmoc-L-Gla(OBu')<sub>2</sub>-OH] and  $\gamma,\gamma'$ -di-*tert*-butyl-*N*-(fluoren-9-ylmethoxycarbonyl)-D- $\gamma$ -carboxyglutamic acid [Fmoc-D-Gla(OBu')<sub>2</sub>-OH] by chemical resolution<sup>22</sup>**

(a) **Addition of (–)-quinine.** Z-DL-Gla(OBu')<sub>2</sub>-OH<sup>22</sup> (8.1 g, 18.4 mmol) was dissolved in ethyl acetate (55 mL) and (–)-quinine (9 g) was added. The suspension was heated until all the quinine had dissolved, and then the solution was left for 1 day in the refrigerator to crystallise. The precipitate was collected by filtration, and recrystallised from ethyl acetate to give the (–)-quinine salt of Z-D-Gla(OBu')<sub>2</sub>-OH (2.65 g, 47%), mp 137 °C (lit.,<sup>22</sup> 141–143 °C);  $[\alpha]_{\text{D}} -87.3$  ( $c$  0.157 in MeOH) [lit.,<sup>22</sup> –86.8 ( $c$  1 in MeOH)]. This quinine salt was suspended in ethyl acetate and the quinine removed by addition of 10% aq.  $\text{NaHSO}_4$ . The organic phase was dried over anhydrous sodium sulfate, and then the solvent was evaporated to give crystalline Z-D-Gla(OBu')<sub>2</sub>-OH,  $[\alpha]_{\text{D}} +11.4$  ( $c$  0.132 in MeOH); FAB-MS (NOBA) 438 ( $\text{M} + \text{H}^+$ ), 461 ( $\text{M} + \text{H} + \text{Na}^+$ ).

(b) **Addition of (–)-ephedrine to filtrate from (a).** The ethyl acetate mother liquor from (a) was treated with (–)-ephedrine (1.68 g) in ethyl acetate (18.5 mL), the solution was cooled to

–15 °C in a refrigerator, then hexane ( $\approx 50$  mL) was added. The product separated as an oil, which partly crystallised out after 4 days in the freezer. The crystalline product was filtered off together with an oily precipitate, and both were dried thoroughly before being re-dissolved in hot hexane–ethyl acetate (3:1 v/v). On cooling, and addition of more hexane, the (–)-ephedrine salt of Z-L-Gla(OBu')<sub>2</sub>-OH separated out and on collection gave  $[\alpha]_{\text{D}} -15.0$  ( $c$  0.12 in MeOH) [lit.,<sup>22</sup> –15.3 ( $c$  1 in MeOH)]. The ephedrine salt was suspended in ethyl acetate and the ephedrine was removed by addition of 10% aq.  $\text{NaHSO}_4$ . The organic phase was dried over anhydrous sodium sulfate, and the solvent was evaporated to give crystalline Z-L-Gla(OBu')<sub>2</sub>-OH (3.2 g),  $[\alpha]_{\text{D}} -11.8$  ( $c$  0.135 in MeOH), with similar physical data to its D-enantiomer.

The *N*-benzyloxycarbonyl groups of both enantiomers were removed using the standard procedure as follows. The appropriate Z-Gla(OBu')<sub>2</sub>-OH enantiomer (1.67 g, 3.8 mmol) and Pd/C (340 mg) were added to 10% acetic acid (30 mL) and, while the suspension was stirred, hydrogen gas was slowly bubbled through for 1 day. The catalyst was filtered off and the solvent was removed under reduced pressure, to yield white crystalline samples of the corresponding H-Gla(OBu')<sub>2</sub>-OH compounds [ $\delta_{\text{H}}$  (400 MHz,  $\text{d}_6\text{-DMSO}$ ) 1.55 (18 H, s,  $2 \times \text{C}(\text{CH}_3)_3$ ), 2.25–2.15 (2 H, m,  $\beta\text{CH}_2$ ), 3.2 (1 H, t,  $\gamma\text{CH}$ ), 3.9 (1 H, t,  $\alpha\text{CH}$ ), 5.2 (1 H, m, NH)], which were used directly to make Fmoc derivatives.

H-D-Gla(OBu')<sub>2</sub>-OH (0.94 g, 2.4 mmol) and Fmoc-OSu (0.99 g, 3.08 mmol) were added to a suspension of  $\text{NaHCO}_3$  (0.52 g, 6.2 mmol) in 50/50 aq. 1,4-dioxane (22 mL). After stirring of the mixture for 5 h, the solvent was evaporated off and the residue was treated with 10% aq.  $\text{NaHSO}_4$  (pH 2). This solution was extracted with ethyl acetate (25 mL), which was then washed with water, dried over anhydrous sodium sulfate, and evaporated to give an oily residue, which solidified under high vacuum (1.23 g, 75%). Crystals of Fmoc-D-Gla(OBu')<sub>2</sub>-OH, mp 118–120 °C (lit.,<sup>22</sup> 118–121 °C), were obtained from ethyl acetate–hexane,  $[\alpha]_{\text{D}} +8.6$  ( $c$  0.173 in MeOH) [lit.,<sup>22</sup> +7.4 ( $c$  1 in MeOH)];  $\delta_{\text{H}}$  (400 MHz;  $\text{CDCl}_3$ ) 1.5 [18 H, s,  $2 \times \text{C}(\text{CH}_3)_3$ ], 2.2–2.55 (2 H, 2m,  $\beta\text{CH}_2$ ), 3.35 (1 H, t,  $\gamma\text{CH}$ ), 4.25 (1 H, t,  $\alpha\text{CH}$ ), 4.38 (1 H, t, Fmoc CH), 4.45 (2 H, m, Fmoc  $\text{CH}_2$ ), 5.55 (1 H, d, NH), 7.3–7.9 (8 H, m, ArH).

Similar data were obtained for Fmoc-L-Gla(OBu')<sub>2</sub>-OH.

**Preparation of side-chain-protected linear pentapeptide precursors using solid-phase peptide synthesis (Scheme 2)**

[All syntheses were carried out on a resin (HMPB–MBHA) bearing an acid-labile linker which allows removal of the pentapeptide with side-chain protection intact.]

**Loading of resin: first amino acid attachment.** All pentapeptides synthesised had glycine at the C-terminal position. (Resin and Fmoc-Gly-OH were dried over  $\text{P}_2\text{O}_5$  under high vacuum for 24 h prior to use.) Fmoc-Gly-OH (2.04 g, 6.84 mmol) was dissolved in dry dichloromethane (5 mL), but a further 1 mL of DMF was required for complete dissolution. DCC (0.71 g, 3.43 mmol) as a solution in dichloromethane (2 mL) was added and the reaction mixture was stirred at room temperature until co-precipitation of Fmoc-Gly-anhydride and dicyclohexylurea occurred. The dichloromethane was removed *in vacuo*, and the residual solution was diluted with more DMF (5 mL). The remaining precipitate of dicyclohexylurea was filtered off and subsequently washed with DMF (3 mL). The filtrates were collected in a flask containing the dry resin (1.00 g, 0.57 mmol of free OH). A catalytic amount of DMAP (0.06 g) dissolved in DMF (2 mL) was then added to the resin–glycine symmetrical anhydride mixture, and the mixture was swirled periodically over a period of 2 h. The loaded resin was then filtered off, washed successively with DMF ( $6 \times 10$  mL), dichloromethane ( $6 \times 10$  mL) and diethyl



ether (6 × 10 mL) and finally dried under high vacuum over P<sub>2</sub>O<sub>5</sub>.

The loading of the resin was assessed either by our own HPLC methodology<sup>13</sup> or by the published colorimetric method.<sup>33</sup> For the former method, a reversed-phase column, eluted with MeOH–water 80:20 with detection at  $\lambda_{\max}$  266 nm was used, giving Fmoc-Gly-OH at a  $t_R$  of 3.0 min. Standard calibration of the column was carried out with 6 standard solutions of Fmoc-Gly-OH, each solution injected five times to give an average area plotted against concentration. To determine the loading, dry loaded resin (14 mg) was deprotected by treatment with 1% TFA in dichloromethane, at room temperature for 30 min. It was then filtered off and washed with dichloromethane, and the combined filtrates were evaporated on a rotary evaporator to give a residue, which was dissolved in MeOH (5.0 mL). Five injections of this solution onto the HPLC column gave an average figure for reading off the calibration graph. Confirmation of loadings in the range 80–98% were achieved by this process.

**Solid-phase assembly of the protected pentapeptides.** A semi-automatic Vega synthesiser was used which effected assembly under a nitrogen stream which was also used as pressure to transfer solvents and reagents in and out of the reaction vessel. The Fmoc-Gly-loaded resin (0.98 g) and all the selected Fmoc-derivatised amino acids and reagents were dried over P<sub>2</sub>O<sub>5</sub> under high vacuum for 4 h prior to use. The loaded resin in the synthesiser reaction vessel was subjected to a series of coupling–Fmoc-deprotection cycles in the order summarised in Scheme 2. Each coupling cycle involved the addition of the Fmoc-protected amino acid (3 equiv.), previously mixed in DMF (20 mL), with the coupling agents TBTU (0.48 g, 3 equiv.), HOBt hydrate (0.69 g, 9 equiv.) and DIPEA (0.80 mL, 9 equiv. added just before addition to reaction vessel). Then the mixture was shaken in the vessel for 1 h. The addition of Fmoc-Arg(Mtr)-OH was carried out twice. After wash cycles with DMF (10 × 20 mL) to remove reagents the Fmoc-group at each stage was removed using a solution of 20% piperidine in DMF (v/v) and allowing two resin exposures of 3 and 5 min (2 × 20 mL) for the deprotection, followed by extensive washing cycles with DMF. Once the solid-phase assembly was completed, the resin was removed from the reaction vessel, washed successively with dichloromethane (2 × 20 mL) and diethyl ether (2 × 20 mL), and dried over P<sub>2</sub>O<sub>5</sub> under high vacuum for 4 h.

**Peptide–resin linkage cleavage.** The dried peptide–resin was placed in a small sintered funnel and gently stirred for 5 min in a solution of 1% TFA–dichloromethane (15 mL). The solution was then sucked directly into a flask containing DMF (5 mL) cooled in an ice–salt-bath, to prevent any cleavage of the *tert*-butyl side-chains. This process was repeated 5 times, followed by washing with dichloromethane (6 × 10 mL). The combined filtrates were washed successively with 10% aq. citric acid (75 mL) and distilled water (2 × 75 mL). The organic layers were then dried over anhydrous sodium sulfate and concentrated to give the appropriate Fmoc-derivatised pentapeptides with characteristics as follows:

Fmoc-DL-Gla(OBu')<sub>2</sub>-Ser(Bu')-Lys(Boc)-Arg(Mtr)-Gly-OH, white solid (90% crude yield integrating for 81% on HPLC analysis),  $t_R$  4.66 min (50% aq. MeCN isocratic elution), FAB-MS  $m/z$  1345 (M + Na)<sup>+</sup>, 1322 (M + H)<sup>+</sup>, 1110 (M – Mtr)<sup>+</sup>, 888 (M – Mtr – Fmoc)<sup>+</sup>. C<sub>65</sub>H<sub>95</sub>N<sub>9</sub>O<sub>18</sub>S requires  $M$ , 1321.65158.

Fmoc-L-Gla(OBn)<sub>2</sub>-Ser(Bu')-Lys(Boc)-Arg(Mtr)-Gly-OH, white powder (85% crude), FAB-MS (thioglycerol)  $m/z$  1413 (M + Na)<sup>+</sup>, 1391 (M + H)<sup>+</sup>. Electrospray MS  $m/z$  1390.4 M<sup>+</sup>, C<sub>71</sub>H<sub>91</sub>N<sub>9</sub>O<sub>18</sub>S requires  $M$ , 1390.6;  $\delta_H$  (400 MHz; d<sub>6</sub>-DMSO) 1.05 (9 H, s, Bu'), 1.36 (9 H, s, Boc), 1.2–1.8 (10 H, br, Lys  $\beta\gamma\delta$ CH<sub>2</sub>, Arg  $\beta\gamma$ CH<sub>2</sub>), 2.05 (3 H, s, Mtr CH<sub>3</sub>), 2.2–2.4 (2 H, 2m,

Gla  $\beta$ CH<sub>2</sub>), 2.5, 2.6 (6 H, 2s, Mtr CH<sub>3</sub>), 2.9 (2 H, m, Lys  $\epsilon$ CH<sub>2</sub>), 3.0 (2 H, m, Arg  $\delta$ CH<sub>2</sub>), 3.4 (2 H, m, Ser CH<sub>2</sub>), 3.6–3.9 (6 H, overlapping, Lys  $\alpha$ CH, Gly  $\alpha$ CH, Gla  $\gamma$ CH, Mtr OCH<sub>3</sub>), 4.1–4.3 (6 H, overlapping, Arg  $\alpha$ CH, Ser  $\alpha$ CH, Gla  $\alpha$ CH, Fmoc CH<sub>2</sub>, Fmoc CH), 5.0–5.2 (4 H, 2m, CH<sub>2</sub>Ph), 6.4 (1 H, br s, Arg  $\epsilon$ NH), 6.7 (2 H, m/s, Lys  $\epsilon$ NH, Mtr ArH), 7.2–8.2 (24 H, m, ArH and 6 × amide NH), 12.6 (0.7 H, br s, COOH).

Fmoc-L-Gla(OBu')<sub>2</sub>-Ser(Bu')-Lys(Boc)-Arg(Mtr)-Gly-OH, white solid (85% crude yield integrating for 71% on HPLC),  $t_R$  5.68 min (isocratic MeOH–water 80:20, analytical column); FAB-MS  $m/z$  1345 (M + Na)<sup>+</sup>, 1322 (M + H)<sup>+</sup>. Calc. for C<sub>65</sub>H<sub>95</sub>N<sub>9</sub>O<sub>18</sub>S:  $M$ , 1321.65.

Fmoc-D-Gla(OBu')<sub>2</sub>-Ser(Bu')-Lys(Boc)-Arg(Mtr)-Gly-OH, white solid (89% crude yield, integrating for 68% on HPLC),  $t_R$  5.18 min (same system as for L-analogue); FAB-MS  $m/z$  1345 (M + Na)<sup>+</sup>, 1322 (M + H)<sup>+</sup>.

Fmoc-Asp(OBu')-Phe-Phe-Arg(Mtr)-Gly-OH, as a white powder (0.638 g, 99% crude, integrating for 77% on HPLC),  $t_R$  10.96 min (linear gradient 5% MeCN to 80% aq. MeCN over a period of 15 min); FAB-MS  $m/z$  1131 (M + H)<sup>+</sup>, 1075 (M – Bu'). Calc. for C<sub>59</sub>H<sub>70</sub>N<sub>8</sub>O<sub>13</sub>S:  $M$ , 1130.478.

**Removal of N-terminal Fmoc group from each protected pentapeptide.** Fmoc-protected pentapeptide (0.49 mmol) was dissolved in distilled DMF (10 mL) and 10% piperidine in DMF (v/v) (2 mL) at room temperature. After 1 h the solvent was removed under high vacuum to produce a residue, which was repeatedly washed with diethyl ether until no more fulvene–piperidine adduct could be detected in the washings. Characteristics of the protected pentapeptides obtained were as follows:

H-DL-Gla(OBu')<sub>2</sub>-Ser(Bu')-Lys(Boc)-Arg(Mtr)-Gly-OH, as a white solid (0.53 g, 98%, integrating for 87% on HPLC),  $t_R$  4.73 min (50% aq. MeCN); FAB-MS  $m/z$  1100 (M + H)<sup>+</sup>, 889 (M – Mtr)<sup>+</sup>. C<sub>50</sub>H<sub>85</sub>N<sub>9</sub>O<sub>16</sub>S requires  $M$ , 1099.5835.

H-L-Gla(OBn,NC<sub>5</sub>H<sub>10</sub>)-Ser(Bu')-Lys(Boc)-Arg(Mtr)-Gly-OH, as a white solid (92%),  $t_R$  9.6 (21%) and 9.8 (49%) min (linear gradient 5% to 70% MeCN in 15 min). Electrospray MS  $m/z$  1145 (M<sup>+</sup>). C<sub>54</sub>H<sub>84</sub>N<sub>10</sub>O<sub>15</sub>S requires  $M$ , 1145.38;  $\delta_H$  (400 MHz; d<sub>6</sub>-DMSO) 1.09 and 1.35 (18 H, 2s, Boc and Bu'), 1.20, 1.50, 1.60 (16 H, 3 br signals, piperidide CH<sub>2</sub>, Lys  $\beta\gamma\delta$ CH<sub>2</sub>, Arg  $\beta\gamma$ CH<sub>2</sub>), 2.04 (3 H, s, Mtr CH<sub>3</sub>), 2.2–2.3 (2 H, 2m, Gla  $\beta$ CH<sub>2</sub>), 2.5 (3 H, s, Mtr CH<sub>3</sub>), 2.6 (3 H, s, Mtr CH<sub>3</sub>), 2.9–3.0 [8 H, m, Lys  $\epsilon$ CH<sub>2</sub>, Arg  $\delta$ CH<sub>2</sub>, 2 × CH<sub>2</sub> (NC<sub>5</sub>H<sub>10</sub>)], 3.4–3.6 (6 H, overlapping, Lys  $\alpha$ CH, Gla  $\gamma$ CH, Ser CH<sub>2</sub>, 2 unidentified H), 3.78 (3 H, s, Mtr OCH<sub>3</sub>), 4.2–4.4 (3 H, overlapping, Arg  $\alpha$ CH, Ser  $\alpha$ CH, Gla  $\alpha$ CH), 5.1–5.2 (2 H, m, CH<sub>2</sub>Ph), 6.7 (2 H, m/s, Lys  $\epsilon$ NH, Mtr ArH), 7.2–7.4 (5 H, m, ArH), 7.2–7.4 (5 H, m, amide NH).

H-L-Gla(OBu')<sub>2</sub>-Ser(Bu')-Lys(Boc)-Arg(Mtr)-Gly-OH as a yellowish solid (90% crude yield, integrating as 63% on HPLC),  $t_R$  8.32 min (analytical column, in MeOH–water, 80:20 isocratic); FAB-MS  $m/z$  1122 (M + Na)<sup>+</sup>, 1100 (M + H)<sup>+</sup>. Calc. for C<sub>50</sub>H<sub>85</sub>N<sub>9</sub>O<sub>16</sub>S:  $M$ , 1099.58.

H-D-Gla(OBu')<sub>2</sub>-Ser(Bu')-Lys(Boc)-Arg(Mtr)-Gly-OH as an off-white solid (98% crude yield, integrating for 59% on HPLC),  $t_R$  8.48 min (analytical column in MeOH–water, 80:20 isocratic); FAB-MS  $m/z$  1122 (M + Na)<sup>+</sup>, 1100 (M + H)<sup>+</sup>.

H-Asp(OBu')-Phe-Phe-Arg(Mtr)-Gly-OH, as a white solid (98% crude yield integrating for 79% on HPLC),  $t_R$  8.68 min (linear gradient from 5% to 80% aq. MeCN in 15 min); FAB-MS  $m/z$  909 (M + H)<sup>+</sup>, 853 (M – Bu'), 697 (M – Mtr)<sup>+</sup>. Calc. for C<sub>44</sub>H<sub>60</sub>N<sub>8</sub>O<sub>11</sub>S:  $M$ , 908.410.

**Cyclisation of side-chain-protected linear pentapeptides.** *Method A.*<sup>13</sup> A mixture of the linear pentapeptide precursor ( $\approx$ 0.5 mmol) in dry DMF (75 mL)–dry dichloromethane (500 mL) was cooled to –5 °C with an ice–salt-bath. A solution of DMAP (10.2 mmol) in dry DMF (10 mL), and *N*-methylmorpholine (0.5 mL), were added to the stirred reaction mixture. A solution of EDC (10 mmol) in DMF (20 mL)–

dichloromethane (15 mL) was added dropwise to the reaction mixture. After being kept at  $-2^{\circ}\text{C}$  for 4 h the temperature was allowed to rise to ambient and stirring was continued for 2 days. The reaction mixture was then recooled to  $-5^{\circ}\text{C}$  and a second batch of DMAP (5.0 mmol) and EDC (5.0 mmol) in dry DMF (8 mL)–dry dichloromethane (20 mL) was added. After 3 h, the reaction mixture was allowed to warm to room temperature and stirring was continued for 4 days. After removal of solvent the yellow–brown residue was dissolved in a mixture of ethyl acetate (100 mL) and distilled water (150 mL). After separation of layers, the aqueous phase was washed a second time with ethyl acetate (100 mL). The combined organic layers were washed successively with 10% aq. citric acid (50 mL), 10% aq.  $\text{NaHCO}_3$ , and distilled water ( $2 \times 50$  mL). After drying over anhydrous magnesium sulfate, filtration, and removal of solvent, a yellowish solid was usually obtained. The protected cyclic pentapeptides synthesised by this method were as follows:

Cyclo[Arg(Mtr)-Gly-DL-Gla(OBu')<sub>2</sub>-Ser(Bu')-Lys(Boc)-] as a yellow solid (82%),  $t_R$  14.51 min (78% by integration), on HPLC (isocratic 50% aq. MeCN) ( $t_R$  = 9.6 min, linear gradient 5–100% MeCN over a period of 15 min). FAB-MS  $m/z$  1104 ( $M + \text{Na}$ )<sup>+</sup>, 1082 ( $M + \text{H}$ )<sup>+</sup>. Accurate mass Found: ( $M + \text{Na}$ )<sup>+</sup>, 1104.56180.  $\text{C}_{50}\text{H}_{83}\text{N}_9\text{O}_{15}\text{S} \cdot \text{Na}^+$  requires  $m/z$ , 1104.562705.

Cyclo[Arg(Mtr)-Gly-L-Gla(OBn,NC<sub>5</sub>H<sub>10</sub>)-Ser(Bu')-Lys-(Boc)-] as a yellow solid (94% crude yield),  $t_R$  split peak 17.4–17.7 min (73% by integration) on HPLC (gradient 5–70% MeCN over a period of 15 min). Electrospray MS  $m/z$  1127.  $\text{C}_{54}\text{H}_{82}\text{N}_{10}\text{O}_{14}\text{S}$  requires  $M$ , 1127.4;  $\delta_H$  (400 MHz; d<sub>6</sub>-DMSO) 1.09 and 1.35 (18 H, 2s, Boc and Bu'), 1.2–1.7 (16 H, br m, piperidide CH<sub>2</sub>, Lys  $\beta\gamma\delta\text{CH}_2$ , Arg  $\beta\gamma\text{CH}_2$ ) 2.04, 2.49, 2.58 (9 H, m,  $3 \times$  Mtr CH<sub>3</sub>), 2.50 (2 H, m, Gla  $\beta\text{CH}_2$ ), 2.8 (2 H, m, Lys  $\epsilon\text{CH}_2$ ), 3.0 [6 H overlapping signals Arg  $\delta\text{CH}_2$ ,  $2 \times \text{CH}_2$  (NC<sub>5</sub>H<sub>10</sub>)], 3.43 (2 H, m, Ser CH<sub>2</sub>), 3.46 (1 H, m, Gly  $\alpha\text{CH}$ ), 3.78 (3 H, s, Mtr OCH<sub>3</sub>), 4.2–4.4 (3 H, overlapping, Arg  $\alpha\text{CH}$ , Ser  $\alpha\text{CH}$ , Gla  $\alpha\text{CH}$ ), 5.1–5.2 (2 H, m, CH<sub>2</sub>Ph), 6.4 (1 H, br s, Arg  $\epsilon\text{NH}$ ), 6.7 (3 H, m/s, Lys  $\epsilon\text{NH}$ , Mtr CH), 7.2–7.4 (5 H, m, ArH), 7.8–8.4 (5 H, 5m, amide NH). Exact positions of a Gly  $\alpha\text{CH}$  and Gla  $\gamma\text{CH}$  could not be correlated.

Cyclo[Arg(Mtr)-Gly-Asp(OBu')-Phe-Phe-] as a yellow solid (68% yield),  $t_R$  5.65 min (59% by integration) on HPLC (gradient 50–100% MeCN over a period of 15 min), further purified (55% yield) to 98% purity by repeated collection of fractions at  $t_R$  5.63 min; FAB-MS Found: ( $M + \text{H}$ )<sup>+</sup>, 891.404913.  $\text{C}_{44}\text{H}_{59}\text{N}_8\text{O}_{10}\text{S}$  requires  $m/z$  891.407488;  $\delta_H$  (400 MHz; d<sub>6</sub>-DMSO) 1.39 (9 H, s, Bu'), 1.35–1.52 (4 H, m,  $2 \times$  Arg CH<sub>2</sub>), 2.0, 2.55, 2.6 (9 H, 3s,  $3 \times$  Mtr CH<sub>3</sub>), 2.4–2.6 (2 H, m, Asp CH<sub>2</sub>), 3.15 (3 H, m, Arg  $\delta\text{CH}_2$ ), 3.8 (3H, s, Mtr OCH<sub>3</sub>), 3.15, 3.34 (2 H, m, PhCH<sub>2</sub>), 3.45, 3.9 (2 H, m,  $2 \times$  Gly  $\alpha\text{H}$ ), 3.88–3.92 (2 H, m, PhCH<sub>2</sub>), 4.05 (1 H, m, Phe  $\alpha\text{CH}$ ), 4.1 (1 H, m, Arg  $\alpha\text{CH}$ ), 4.25 (1 H, m, Phe  $\alpha\text{CH}$ ), 4.5 (1 H, m, Asp  $\alpha\text{CH}$ ), 6.68 (1 H, s, Mtr CH), 7.1–7.4 (10 H, m, ArH), 7.9 (1 H, m, Phe NH), 8.26 (1 H, m, Arg NH), 8.32 (1 H, m, Asp NH), 8.4 (1 H, m, Phe NH), 8.7 (1H, m, Gly NH).

**Method B.**<sup>24</sup> The linear pentapeptide precursor (0.1 mmol), HATU (0.11 mmol) and DIPEA (0.3 mmol) in DMF (100 mL) – dichloromethane (300 mL) (to bring overall concentration to 2.5  $\mu\text{M}$ ) were stirred at  $0^{\circ}\text{C}$  for 1 h and then at room temperature for 1 h. The solvent was removed *in vacuo*, and the residual yellow product was re-dissolved in dichloromethane (100 mL), which solution was washed successively with 10% aq. citric acid (50 mL), aq. sodium bicarbonate (1 M; 50 mL) and water ( $2 \times 50$  mL). After drying, the organic layer on evaporation yielded a yellowish product in approx. 75% yield.

Purification of both protected cyclic peptides produced by this method was carried out by preparative HPLC using a reversed-phase column (PL-32  $\times$  2.75 cm), flow rate 10 mL min<sup>-1</sup>, with gradient elution 31–70% aq. MeCN during 90 min. Pure cyclic pentapeptides were collected from fractions corresponding to peaks at  $t_R$  76.5 min for the all-L-form and  $t_R$  81

min for the D-Gla analogue. Evaporation of the eluent solvent initially gave oils, but, on trituration with acetonitrile, white solids were obtained, with the following physical data.

Cyclo[Arg(Mtr)-Gly-L-Gla(OBu')<sub>2</sub>-Ser(Bu')-Lys(Boc)-], ES-MS  $m/z$  1082 ( $M + \text{H}$ )<sup>+</sup>, 1105 ( $M + \text{Na}$ )<sup>+</sup>;  $\delta_H$  (400 MHz; CDCl<sub>3</sub>) 1.3 (9 H, s, Bu'), 1.42 (9 H, s, Boc), 1.45–1.46 (4 H, overlapping, Arg and Lys CH<sub>2</sub>), 1.47 (18 H, s, Gla Bu'), 1.5–1.6 (4 H, overlapping, Arg and Lys CH<sub>2</sub>), 1.83 (2 H, m, Lys CH<sub>2</sub>), 2.13 (3 H, s, Mtr CH<sub>3</sub>), 2.2–2.4 (2 H, m, Gla CH<sub>2</sub>), 2.64 (3 H, s, Mtr CH<sub>3</sub>), 2.7 (3 H, s, Mtr C<sub>3</sub>), 3.1 (2 H, br s, Lys  $\epsilon\text{CH}_2$ ), 3.22–3.33 (2 H, m, Arg  $\delta\text{CH}_2$ ), 3.5 (2 H, br m, Ser CH<sub>2</sub>), 3.7 (2 H, overlapping peaks, Gly  $\alpha\text{CH}$  and Gla  $\gamma\text{CH}$ ), 3.83 (3 H, s, Mtr OCH<sub>3</sub>), 3.9 (1 H, br s, Ser  $\alpha\text{CH}$ ), 4.0 (1 H, s, Gly  $\alpha\text{CH}$ ), 4.4 (1 H, s, Gla  $\alpha\text{CH}$ ), 4.5 (2 H, br s, Arg and Lys  $\alpha\text{CH}$ ), 5.05 (1 H, s, Lys  $\epsilon\text{NH}$ ), 6.32 (3 H, br s,  $3 \times$  Arg NH), 6.5 (1 H, s, Mtr CH), 7.16 (1 H, s, Ser NH), 7.51–7.54 (2 H, d, Arg and Lys NH), 7.96 (1 H, s, Gla NH), 8.51 (1 H, br s, Gly NH).

Cyclo[Arg(Mtr)-Gly-D-Gla(OBu')<sub>2</sub>-Ser(Bu')-Lys(Boc)-], ES-MS  $m/z$  1082 ( $M + \text{H}$ )<sup>+</sup>, 1105 ( $M + \text{Na}$ )<sup>+</sup>;  $\delta_H$  (400 MHz; CDCl<sub>3</sub>) 1.23 (9 H, s, Bu'), 1.42 (9 H, s, Boc), 1.45–1.46 (4 H, overlapping, Arg and Lys CH<sub>2</sub>), 1.47 (18 H, s, Gla Bu'), 1.53–1.63 (4 H, overlapping, Arg and Lys CH<sub>2</sub>), 1.83 (2 H, d, Lys CH<sub>2</sub>), 2.13 (3 H, s, Mtr CH<sub>3</sub>), 2.2–2.4 (2 H, m, Gla CH<sub>2</sub>), 2.64 (3 H, s, Mtr CH<sub>3</sub>), 2.7 (3 H, s, Mtr CH<sub>3</sub>), 3.1 (2 H, br s, Lys  $\epsilon\text{CH}_2$ ), 3.22–3.33 (2 H, m, Arg  $\delta\text{CH}_2$ ), 3.5 (2 H, br m, Ser CH<sub>2</sub>), 3.7 (2 H, overlapping peaks, Gly  $\alpha\text{CH}$  and Gla  $\gamma\text{CH}$ ), 3.83 (3 H, s, Mtr OCH<sub>3</sub>), 3.9 (1 H, br s, Ser  $\alpha\text{CH}$ ), 4.0 (1 H, s, Gly  $\alpha\text{CH}$ ), 4.35 (1 H, s, Gla  $\alpha\text{CH}$ ), 4.5 (2 H, br s, Arg and Lys  $\alpha\text{CH}$ ), 5.05 (1 H, s, Lys  $\epsilon\text{NH}$ ), 6.32 (3 H, br s,  $3 \times$  Arg NH), 6.5 (1 H, s, Mtr CH), 7.16 (1 H, s, Ser NH), 7.51–7.54 (2 H, 2s, Arg and Lys NH), 7.96 (1 H, s, Gla NH), 8.51 (1 H, br s, Gly NH).

**Removal of side-chain-protecting groups.** The protected cyclopentapeptides (0.2 mmol) were dissolved, either in 99% TFA or 95% TFA (10 mL), for periods of between 2 and 12 h at room temperature, until all protecting groups (especially the Mtr group which was the most difficult to remove) had been removed as monitored by HPLC. Removal of TFA on a rotary evaporator usually gave a brown gum, which was then washed several times with diethyl ether to give quantitative yields of the crude cyclopentapeptides as solid, off-white residues. Each of the residues had to be purified by collecting fractions off the HPLC columns at  $t_R$ -values reported for each compound.

Cyclo[Arg-Gly-DL-Gla-Ser-Lys-] as an off-white solid (36%),  $t_R$  3.61 min on semi-preparative HPLC (linear gradient 5–70% aq. MeCN during 15 min); FAB-MS  $m/z$  602 ( $M + \text{H}$ )<sup>+</sup>.  $\text{C}_{23}\text{H}_{39}\text{N}_9\text{O}_{10}$  requires  $M$ , 601.28;  $\delta_H$  (400 MHz; D<sub>2</sub>O) 1.48 (2 H, Lys CH<sub>2</sub>), 1.64–1.66 (4 H, m, Arg CH<sub>2</sub>), 1.72–1.89 (4 H, m, Lys CH<sub>2</sub>), 2.5 (2 H, m, Gla CH<sub>2</sub>), 3.02 (2 H, m, Lys  $\epsilon\text{CH}_2$ ), 3.09 (1 H, m Lys  $\alpha\text{CH}$ ), 3.24 (2 H, m, Arg  $\delta\text{CH}_2$ ), 3.94 (2 H, br m, Ser CH<sub>2</sub>), 3.7–4.3 (2 H, overlapping peaks, Gly  $\alpha\text{CH}_2$ ), 4.07 (1 H, br s, Ser  $\alpha\text{CH}$ ), 4.12 (1 H, m, Arg  $\alpha\text{CH}$ ), 4.40 (2 H, m, Gla  $\alpha$ - and  $\gamma$ -CH) (NH signals very weak as sample was in D<sub>2</sub>O).

Cyclo[Arg-Gly-L-Gla(OBn,NC<sub>5</sub>H<sub>10</sub>)-Ser-Lys-] as a white solid, (30%),  $t_R$  4.1 min on semi-preparative HPLC (non-linear, exponent 2 gradient 5–40% aq. MeCN during 15 min). Electrospray MS  $m/z$  759 ( $M + \text{H}$ )<sup>+</sup>, 380 ( $M + 2\text{H}$ )<sup>2+</sup>.  $\text{C}_{35}\text{H}_{54}\text{N}_{10}\text{O}_9$  requires  $M$ , 758.4;  $\delta_H$  (400 MHz; d<sub>6</sub>-DMSO) 1.10–1.70 (16 H, m, piperidide CH<sub>2</sub>, Lys  $\beta\gamma\delta\text{CH}_2$ , Arg  $\beta\gamma\text{CH}_2$ ), 2.2 (1 H, m, Gla  $\beta\text{CH}$ ), 2.8 (4 H, m,  $2 \times \text{CH}_2$  from NC<sub>5</sub>H<sub>10</sub>), 3.0 (4 H, overlapping signals, Arg  $\delta\text{CH}_2$ , Lys  $\epsilon\text{CH}_2$ ) 3.5 (2 H, m, Ser CH<sub>2</sub>), 3.9 (2 H, m, Gly  $\alpha\text{CH}$ ,  $1 \times$  unknown H), 4.2–4.4 (3 H, overlapping, Arg  $\alpha\text{CH}$ , Ser  $\alpha\text{CH}$ , Gla  $\alpha\text{CH}$ ), 5.1–5.2 (2 H, m, CH<sub>2</sub>Ph), 7.2–7.4 (5 H, m, ArH), 7.8–8.4 (5 H, br signals, amide NH). Some peaks were obscured by DMSO/H<sub>2</sub>O signals).

Cyclo[Arg-Gly-Asp-Phe-Phe-] as a white solid (97%),  $t_R$  9.7 min on semi-preparative HPLC (linear gradient 5–50% aq. MeCN over 15 min); FAB-accurate mass MS Found: ( $M + \text{H}$ )<sup>+</sup>, 623.294200.  $\text{C}_{30}\text{H}_{39}\text{N}_8\text{O}_7$  requires  $m/z$ , 623.294171. For



Table 1

Cyclopentapeptide	$\alpha\text{NH } \delta/T$				
	Arg	Gly	Asp (Gla)	Ser (Phe)	Lys (Phe)
Cyclo[-R(Mtr)GD(Bu')FF]	-2.6	-5.4	-5.8	-2.0	-3.4
Cyclo[-R(Mtr)GGla(Bu') <sub>2</sub> S(Bu')K(Boc)-]	-3.9	-5.4	-3.2	-0.2	-4.1
Cyclo[-R(Mtr)Ggla(Bu') <sub>2</sub> S(Bu')K(Boc)-]	-1.7	-2.9	-5.2	-2.3	-3.3
Cyclo[-RGGlaSK-]	-2.6	-3.3	-3.6	-2.7	-3.7

the biological analysis of this cyclic peptide and the DL-Gla analogue above, counter-ion exchange was carried out using an Amberlyst resin IRA-400 in acetate form. The cyclopentapeptide (0.043 mmol) in 50% aq. MeCN (2 mL) was applied to the resin set up in a column. The first fraction collected from the resin contained the acetate salt of the cyclopentapeptides.

Cyclo[-Arg-Gly-L-Gla-Ser-Lys-], as a white solid,  $t_R$  16.21 min on preparative-scale HPLC ( $32 \times 2.75$  cm),  $7 \text{ mL min}^{-1}$  (linear gradient 100%  $\text{H}_2\text{O}$ –40% aq. MeCN during 70 min); ES-MS  $m/z$  602 ( $\text{M} + \text{H}$ )<sup>+</sup>, 624 ( $\text{M} + \text{Na}$ )<sup>+</sup>;  $\delta_H$  (400 MHz;  $d_6$ -DMSO) 1.19 (2 H, br s, Lys  $\delta\text{CH}_2$ ), 1.73 (4 H, overlapping, Lys  $\text{CH}_2$ ), 2.17 (2 H, br d, Arg  $\gamma\text{CH}_2$ ), 2.18 (2 H, m, Arg  $\text{CH}_2$ ), 3.05 (2 H, br s, Lys  $\epsilon\text{CH}_2$ ), 3.15 (2 H, br m, Arg  $\delta\text{CH}_2$ ), 3.7 (2 H, br, Gla  $\beta\text{CH}_2$ ), 4.18 (3 H, br, Lys, Ser, Gly  $\alpha\text{CH}$ ), 4.29 (1 H, s, Arg  $\alpha\text{CH}$ ), 4.3 (1 H, s, Gla  $\alpha\text{CH}$ ), 7.29 (6 H, br, guanidyl NH, Lys  $\epsilon\text{NH}_2$ ), 7.82 (3 H, br, COOH/OH), 8.01 (1 H, s, Lys NH), 8.37 (1 H, s, Gla NH), 8.43 (1 H, s, Ser NH), 8.57 (1 H, s, Arg NH), 8.72 (1 H, br s, Gly NH). A broad  $\text{H}_2\text{O}$  signal obscured the region  $\delta$  3.2–3.6, so Gly  $\alpha$  CH and Gla  $\gamma\text{CH}$  could not be correlated.

Cyclo[-Arg-Gly-D-Gla-Ser-Lys-], as a white solid,  $t_R$  19.0 min on preparative-scale HPLC ( $32 \times 2.75$  cm),  $7 \text{ mL min}^{-1}$  (linear gradient 100%  $\text{H}_2\text{O}$ –40% aq. MeCN during 70 min); ES-MS  $m/z$  602 ( $\text{M} + \text{H}$ )<sup>+</sup>, 624 ( $\text{M} + \text{Na}$ )<sup>+</sup>.

**Chemical-shifts temperature dependence studies on selected cyclic peptides.** After identification of the relevant amide NH signals using 2D COSY techniques, 1D spectra of the cyclic peptides were run over the temperature range 298–333 K, collecting chemical-shift data to plot against temperature change. From the slopes of the graphs for each amide proton  $\delta/T$ -values were calculated and are given in Table 1. A value less than 3 ppb  $\text{K}^{-1}$  was taken as evidence of intramolecular H-bonds.

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