Synthesis and Cell-Adhesion Properties of cyclo(-Arg-Gly-Asp-Ser-Lys-), a Constrained Analogue of the Active Domain of Fibronectin†

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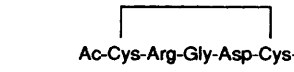
Details of the synthesis of the protected pentapeptide linear precursor on a resin with an acid-labile linker, and its subsequent cyclisation to cyclo(-Arg-Gly-Asp-Ser-Lys-) are presented. The cyclopentapeptide, which is known to exist in solution in a β- and γ-turn conformation, shows specific differences in its effect on adhesion and cell spreading for fibroblast 3T3 and macrophage Bac 1 cells.

The seminal observation of Pierschbacher and Ruoslahti[1] in 1984, that the cell-adhesive function of the extracellular matrix protein, fibronectin, was associated with a small tetrapeptide sequence -Arg-Gly-Asp-Ser (or RGDS as it has become universally known in single-letter nomenclature) held in a β-loop, has initiated intense exploration of the molecular mechanism of cell adhesion. Within the last decade significant inroads have been made in the understanding of the way a family of cell-surface proteins, known as the integrins, act as receptors in recognising the RGD-motif. There are a number of reviews available[2–4] which highlight the significance of the RGD sequence. The central role of the RGD sequence has spurred on a quest for active peptides and peptidomimetics which can provide insights into the fundamental mechanism of cell adhesion as well as potential therapeutic agents for the treatment of diseases such as thrombosis and cancer. It is not surprising therefore that, with the larger pharmaceutical companies involved, the literature is rich in structural/activity data, and criteria for pharmacophoric activity are being formulated. The necessity for a fairly precise conformational relationship between the side-chains of the basic argininyl residue and the acidic aspartyl residue is widely recognised, and the use of constrained peptides in tandem with high-field NMR and molecular dynamics calculations has assisted in highlighting the most likely conformational parameters. Representative of this type of approach is the work on the constrained RGD analogues represented by compound **1**,[5] and by compound **2**.[6] Some general views about the spatial arrangements can be drawn from these studies, but even with these cyclic constrictions there are still an ensemble of low-energy conformations which have to be considered as being representative of receptor-binding conformations.

Our approach at the outset (preliminary results revealed earlier through conference proceedings)[7] was to consider a cyclic pentapeptide ring as a means of giving a reasonable level of constraint as well as offering an effective opportunity of studying its conformation using high-field NMR techniques established for cyclic analogues of the thymopoietins.[8] Our decision to concentrate on the cyclopentapeptide format has been fully vindicated as it is now known[9] that cyclohexapeptides



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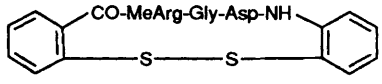

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containing the RGD sequence are less biologically active. Some cycloheptapeptides, however, do show activity.[10] A detailed NMR study on the conformation of our cyclo(-Arg-Gly-Asp-Ser-Lys-) **4** has already been published,[11] and showed that the preferred conformation in dimethyl sulfoxide (DMSO) solution is a type II′β-turn spanning Gly-Asp and a slightly less stable γ-turn at the Lys residue. There has also been a detailed conformational study[9] and structure/activity relationships worked out on a family of cyclopentapeptides based on cyclo-(-Arg-Gly-Asp-Phe-X-). Significant selectivity between vitronectin and laminin receptors was shown by some of the analogues, and when the chirality of each of the amino acid residues was changed in turn, all the peptides adopted a βII′ γ-turn conformation with the D-residue in the $i + 1$ position of the βII′-turn.

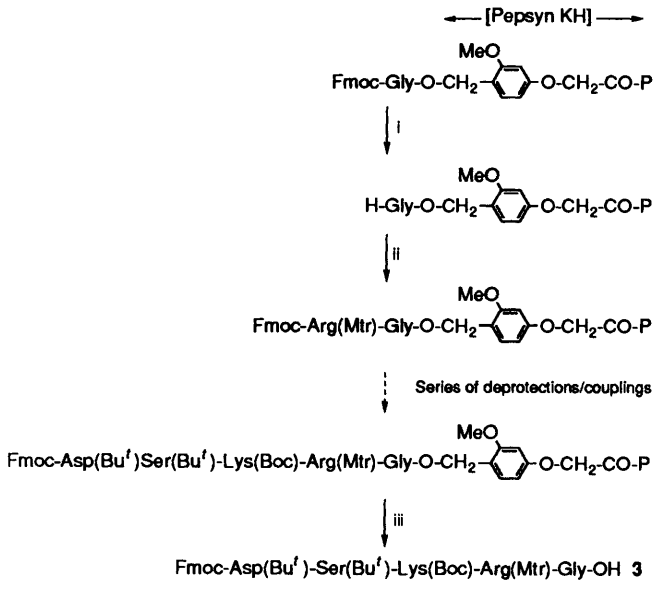
This paper reports the optimised synthesis of cyclo(-Arg-Gly-Asp-Ser-Lys-) **4** and the details of the cell-adhesion and cell-spreading inhibition studies carried out on the compound.

Results and Discussion

Synthetic Studies.—Since the cyclisation of linear peptide precursors to yield cyclic analogues tends to be a low yielding step, usually requiring high-dilution conditions, the need for reasonable quantities of side-chain-protected linear precursors had made solution-phase synthesis a preferable prospect for many years. That is, until solid-phase technology became available utilising a highly acid-labile linker group,[12] which allows release of protected peptides from the resin. Once released these protected peptides can be cyclised in the solution phase. It is the latter approach which is described in this paper, according to the synthetic sequence summarised in Scheme 1.

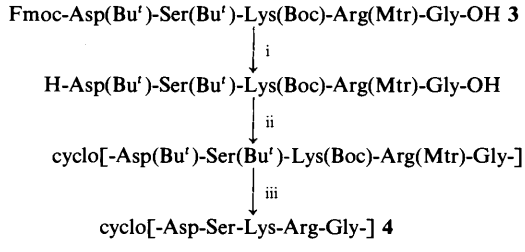
The standard Fmoc/polyamide solid-phase approach[12] provided the desired sequence anchored *via* glycine to the highly acid-labile Pepsyn KH resin to produce the fully protected linear precursor **3** on C-terminal cleavage. After removal of the N-terminal Fmoc group, cyclisation and side-chain deprotection as summarised in Scheme 2, cyclopeptide **4** had to be

† *Abbreviations used:* R = Arg, G = Gly, D = Asp, S = Ser, E = Glu, K = Lys; DCCI = *N*,*N*′-dicyclohexylcarbodiimide; TBTU = 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroboranuide; DIEA = diisopropylethylamine; DMF = *N*,*N*-dimethylformamide; TFA = trifluoroacetic acid; Mtr = 4-methoxy-2,3,6-trimethylbenzenesulfonyl; DMAP = 4-(dimethylamino)pyridine; EDCI = 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; Fmoc = fluoren-9-ylmethoxycarbonyl; HOBt = *N*-hydroxybenzotriazole.



Scheme 1 *Reagents:* i, 20% piperidine in DMF; ii, Fmoc-Arg(Mtr)-OH, TBTU, DIEA, HOBt, DMF; iii, 1% TFA in CH_2Cl_2 [P = polydimethylacrylamide-Kieselguhr (Macrosorb SPR)]

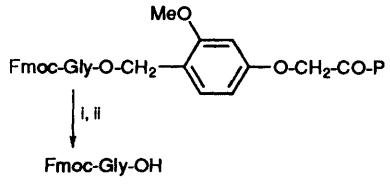
counter-ion exchanged from its trifluoroacetate form to its acetate form, because the cells used in the biological tests could not tolerate the trifluoroacetate form. Some of the preliminary attempts at building up the sequence on the resin were carried out for us by Cambridge Research Biochemicals, but this report summarises our own attempts at optimisation of the synthesis of the protected linear precursor.



Scheme 2 *Reagents:* i, 10% piperidine in DMF; ii, EDCI, DMAP; iii, TFA, thio-4-cresol, thioanisole

Pepsyn KH is a Kieselguhr-beaded resin[13] most suitable for low-pressure continuous-flow synthesis with a loading of 0.09 mmol g^{-1}. It is the 4-hydroxymethyl-3-methoxyphenoxyacetic acid linker for the attachment of the first residue to the resin that enables cleavage of the final peptide from the resin with only 1% TFA in dichloromethane, these conditions leaving the side-chain-protecting groups intact. The anchoring of the first Fmoc-amino acid residue to this type of *para*-alkoxybenzyl alcohol linker is not straightforward but is usually achieved using preformed symmetrical anhydrides and catalytic amounts of DMAP.[14] There have been reports[15] that such conditions catalyse racemisation if the first residue is chiral, and conditions need to be controlled to minimise this problem. In our approach, however, choosing glycine to be the first residue eliminated the problem, but special problems have been noted also for this residue;[16] it is prone to give low loading yields, and symmetrical anhydride rearrangement can occur to give inadvertent attachment of diglycyl units.

To assess the efficiency of the first residue's attachment we have developed an approach based on HPLC analysis, which not only measures the loading (usually carried out[17] by quantitative UV analysis of the fulvene–piperidine adduct released from Fmoc-amino acyl resin), but also detects any overreaction by analysing for the Fmoc-dipeptide as well. The essential steps in the analysis are summarised in Scheme 3. An aliquot of the loaded resin was treated with 1% TFA in dichloromethane and the release of Fmoc-glycine and Fmoc-glycylglycine was followed by HPLC. Quantities of released products can be deduced by comparison with calibration runs. For the current pentapeptide five runs were processed after treatment of separate batches (each 1 g) of resin with a 6-fold excess of symmetrical anhydride. To obtain between 87 and 95% loading of the available sites, the acylation process had to be repeated in each case with 6-fold excess of the anhydride. However, further acylations introduced increasing amounts of dipeptide attachment.



(quantitative analysis by reversed phase HPLC, detection at 265 nm)

Scheme 3 *Reagents and conditions:* i, 1% TFA in CH_2Cl_2; ii, filtration and removal of solvent

The only other predicted problem in the synthesis was the coupling of Fmoc-Arg(Mtr)-OH to the first residue, which has been reported[18] to be troublesome. Using just one coupling of the Fmoc-Arg(Mtr)-OH with TBTU,[19] DIEA and three mole equivalents of HOBt the overall yield of peptide **3** never exceeded 26%. However, repetition of the Fmoc-Arg(Mtr)-OH coupling conditions gave twice the overall yield of compound **3** (to an average of 55%). It has been our general experience that the combination of TBTU, excess of base and HOBt as additive for the coupling steps gave the most pure end product. During the course of the optimisation of the synthesis it was also revealed that stringently purified, amine-free DMF could be replaced by commerically available HPLC-grade DMF without obvious detriment.

The conditions chosen[20] to carry out the cyclisation (Scheme 2) were dictated by the decision to use EDCI in the presence of DMAP under the conditions of high dilution. The optimised yield of protected cyclopentapeptide obtained from the cyclisation was 83% with reversed-phase HPLC showing 84% purity. For the deprotection step the 'scavenger mixture', thiocresol and thioanisole (4-methylbenzenethiol and methylsulfanylbenzene), was added to the TFA to avoid any unexpected cleavage of the Mtr group which could lead to sulfonated arginines.[21] Exchange of the counterions on the purified cyclopentapeptide **4** was carried out by using IRA Amberlyte resin in the acetate form, to give a final overall yield of almost 19%.

Biological Tests on the Cyclopentapeptide **4**.—Experiments were carried out using a 96-well microtitre plate format to test the ability of the peptide to inhibit both cell attachment and spreading upon prepared lawns of human serum fibronectin.[22] The results obtained have been summarised in histogram form in Fig. 1, which also compares the results with RGES, a control analogue. As shown in Fig. 1, murine 3T3 fibroblasts do not attach to plasma fibronectin in the presence of RGDS in its linear form, while our synthesized cyclo(RGDSK) is ineffective as an adhesion blocker in this system. However, the result of using a murine cell line, Bac-1, shows that the cyclo(RGDSK) can block cell attachment. There seems therefore to be a subtle difference in the molecular-recognition characteristics of our

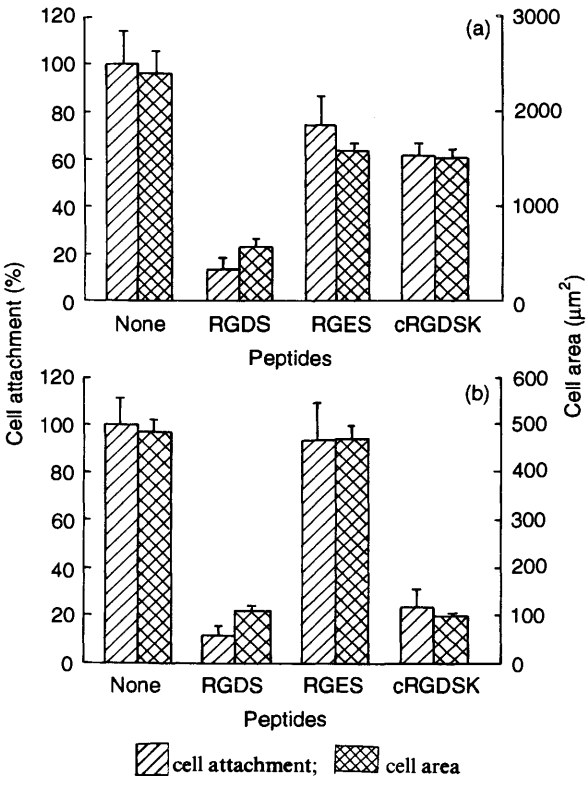


Fig. 1 Adhesion and cellular spreading of NIH 3T3 fibroblasts (a) and Bac-1 macrophages (b) onto lawns of fibronectin.

(a) In the absence of added peptides, the seeding efficiency of 3T3 cells is very high, with ~98% of cells in the suspension adhering to fibronectin within 60 min. Similarly, once attached, the rounded cells begin to spread such that by 60 min the initial profile of the cells has grown from a starting value of 200 μm^2 to ~2300 μm^2. The presence of a control peptide (RGES) has an effect on both adhesion and spreading of fibroblasts, probably due to the use of extra buffering agents required as detailed in the Experimental section. Linear RGDS has the dramatic effect of reducing the efficiency of cell attachment to minimal levels, and those few cells which do manage to adhere to fibronectin fail to spread successfully. In contrast, cyclo-RGDSK elicits only a slight reduction in the efficiency of cell adhesion and has no effect whatsoever on the rate of cell spreading in comparison to control RGES.

(b) Bac-1 macrophages also attach and spread well on fibronectin, in both the presence of RGES peptide and in the total absence of added reagents. In contrast to the results seen for 3T3 fibroblasts, both linear and cyclo versions of RGDS effectively inhibit cell adhesion and cell spreading upon fibronectin lawns.

In all cases, histograms display the mean ± standard deviation of values obtained from $n = 4$ (cell attachment) or $n = 50$ (cell area) for each treatment.

cyclic peptide towards integrins in 3T3 cells that usually recognise fibronectin *via* the $\alpha_5\ \beta_1$ integrin heterodimer and the integrins of the Bac-1 cells.

Further studies to correlate conformational effects and receptor recognition are in hand, using the cyclopentapeptide ring as constraint and utilising integrin-specific antibodies to identify the major fibronectin-binding integrin of Bac-1 cells.

Experimental

A Vega Biochemicals Peptide Synthesiser, Model 250 (kindly donated by Wellcome Research Labs) was available for the solid-phase synthesis.

HPLC measurements were carried out on a LDC/Milton Roy apparatus consisting of a spectromonitor D, constametric pumps and a Cl-10β integrator, and a Spherisorb-ODS C-18 column (25 × 0.43 cm) at a flow rate of 1.0 $cm^3\ min^{-1}$. The solvents were filtered through Nylon-66 filters, 0.45 μm pore size, followed by ultrasonic degassing. Acetonitrile–water mixtures [containing a small amount (0.1%) of TFA] were used for the peptide-derivative analysis using a detector wavelength of 220 nm. Methanol–water mixtures were preferred for Fmoc-Gly-OH calibration at 265 nm with a sensitivity of 0.5 at an attenuation of 4.

Fast-atom bombardment (FAB) mass spectra were determined by using thioglycerol (3-sulfanylpropane-1,2-diol) as matrix at the SERC Mass Spectrometry Unit, Swansea.

Analar DMF (from Aldrich Chemicals) was distilled under reduced pressure after treatment with ninhydrin. HPLC-grade DMF (from Fisons) was used directly without re-distillation. Dichloromethane was distilled twice, once over NaOH and once over P_2O_5. Piperidine, DIEA and *N*-methylmorpholine (from Aldrich) were all distilled over KOH under nitrogen prior to use. Pepsyn KH (loading 0.09 mmol g^{-1}), Fmoc-amino acid derivatives, TBTU and EDCI were purchased from Novabiochem. HOBt and DMAP (from Aldrich) were recrystallised prior to use.

First Amino Acid Attachment.—Pepsyn KH (0.09 mmol of free OH) (1 g) and the corresponding 12 mol equiv. of Fmoc-Gly-OH were dried *in vacuo* over P_2O_5 for 3 h prior to use. The Fmoc-Gly-OH was dissolved in dry CH_2Cl_2 (5 cm^3) and freshly distilled DMF (1 cm^3) to provide total dissolution. DCCI (6 mol equiv.) was dissolved in dry CH_2Cl_2 (1 cm^3) and added to the stirred reaction solution at room temperature. After 1 min, co-precipitation of dicyclohexylurea and Fmoc-Gly-anhydride occurred, to give a slurry. The dichloromethane was removed after 15 min under reduced pressure and the residue was swirled in DMF (5 cm^3) and filtered directly into the flask containing the dry resin. The dicyclohexylurea precipitate was further washed with DMF (5 cm^3) directly sucked up into the reaction flask. A catalytic amount of DMAP was dissolved in DMF and added to the resin–symmetrical anhydride mixture. After the mixture had been swirled several times, the resin was left for 1.5 h at room temperature without being stirred, then was filtered off, and washed successively with DMF, CH_2Cl_2 and finally diethyl ether.

Quantification of Loading Levels.—Eight standard solutions of Fmoc-Gly-OH in methanol with concentrations ranging from 1.3468 to 1.0522 × 10^{-3} mol dm^{-3} were analysed by UV spectroscopy and HPLC at λ_{max} 265 nm. The same calibration was repeated with solutions of Fmoc-Gly-OH previously treated with 1% TFA in CH_2Cl_2 to mimic the peptide–resin cleavage conditions. Identical results were obtained for the two sets of solutions. The λ_{max} at 265 nm had a molar extinction coefficient of 15.5 × 10^3 $dm^3\ mol^{-1}\ cm^{-1}$ obtained by linear regression ($R = 0.9858$). HPLC analysis using this detector wavelength, with 80% MeOH/20% water as eluent, showed the Fmoc-Gly-OH and Fmoc-Gly-Gly-OH eluting at t_R 3.1 and 4.4 min, respectively. Four consecutive injections (each 20 mm^3) of each solution gave an average area which was plotted against concentration. By linear regression ($R = 0.998\ 97$), the response factor was 6.3 × 10^8, allowing more accurate measurements to be made compared with UV results. After drying of the loaded resin *in vacuo* over P_2O_5 for 1 h an aliquot (5 mg) was placed in a 1% TFA–CH_2Cl_2 solution at room temperature for 30 min. The resin was then filtered off, and washed with neat CH_2Cl_2. After removal of the solvent, the residue was dissolved in MeOH (5 cm^3) and analysed by UV spectrocopy and HPLC under standard conditions. The esterification was repeated until the theoretical loading was reached, which usually required three consecutive reactions.

Solid-phase Synthesis Procedures.—The loaded resin was dried prior to use over P_2O_5 *in vacuo* for 3 h and placed in the

peptide synthesizer vessel. Deprotection of the Fmoc group was carried out with a solution of 20% piperidine in DMF (v/v) by allowing two resin exposures of 3 and 7 min, respectively. For each coupling, 3-fold excesses of TBTU, and the Fmoc-amino acid derivatives and HOBt hydrate were used in DMF which had been freshly distilled from ninhydrin. The DIEA was added to the solution just prior to use. Between each step, extensive washing cycles were carried out with re-distilled Analar DMF or HPLC-grade DMF.

Peptide–Resin Linkage Cleavage.—At the end of the assembly, the resin was removed from the vessel, washed successively with CH_2Cl_2 and diethyl ether, and dried *in vacuo* over P_2O_5 for 3 h. A 250 cm^3 round-bottomed flask containing freshly distilled DMF was cooled with an ice–salt-bath. The dry resin was placed in a small sintered funnel and repeatedly treated with 15 cm^3 of a solution of 1% TFA in dry CH_2Cl_2 by gently stirring the beads with a small spatula for 5 min; then the solvent was sucked directly into the cold DMF to quench the TFA. This process was repeated six times, followed by washing with neat CH_2Cl_2. The resultant DMF/CH_2Cl_2 solution was washed successively with 10% aq. citric acid (75 cm^3) and distilled water (2 × 75 cm^3), then was dried over anhydrous $MgSO_4$, filtered and evaporated to dryness to produce a yellow gum, which decolourised upon addition of diethyl ether. Under these optimised conditions Fmoc-Asp(Bu^t)-Ser(Bu^t)-Lys(Boc)-Arg(Mtr)-Gly-OH **3** was obtained in 55% yield, t_R 3.8 min (MeCN–0.1% TFA, 50:50) [Found: (FAB) $(M + Na)^+$ 1230, $(M + H)^+$ 1208; and *m/z* 997 corresponding to loss of the Mtr group. $C_{59}H_{85}N_9O_{10}S$ requires M, 1207].

Removal of the Fmoc Group.—Protected linear precursor **3** (50 mg, 0.041 mmol) was dissolved in freshly distilled DMF (3 cm^3), and 10% piperidine in DMF (v/v) (1 cm^3) was added at room temperature. After 45 min, the solvent was removed to produce a yellow gum, which was repeatedly washed with diethyl ether until no more fulvene–piperidine adduct could be detected in the washings by HPLC (intense absorbance at 301 nm). A solid, H-Asp(Bu^t)-Ser(Bu^t)-Lys(Boc)-Arg(Mtr)-Gly-OH, was obtained in 94% yield, with t_R 3.1 min (MeCN–0.1% TFA, 50:50) [Found: (FAB) $(M + H)^+$, 986.6 and *m/z* 774.8 (corresponding to loss of Mtr group). $C_{44}H_{75}N_9O_{14}S$ requires (M + H), 986.5238].

Cyclisation.—H-Asp(Bu^t)-Ser(Bu^t)-Lys(Boc)-Arg(Mtr)-Gly-OH (40 mg, 0.039 mmol) was dissolved in pure DMF (6 cm^3)–dry CH_2Cl_2 (30 cm^3) and the solution was cooled to −5 °C with an ice–salt-bath. A solution of DMAP (98 mg, 0.82 mmol) in pure DMF (1 cm^3), and *N*-methylmorpholine (22 mg), were added to the stirred reaction solution. A solution of EDCl (0.1556 g, 0.82 mmol) in DMF (4 cm^3)–dry CH_2Cl_2 (3 cm^3) was added dropwise to the reaction solution. The temperature was kept at −2 °C for 3 h, then was allowed to rise. After being stirred at room temperature for 2 days the reaction mixture was cooled again to −5 °C and a second batch of DMAP (99 mg) and EDCl (0.1642 g) was added. Afer 3 h the reaction mixture was allowed to warm up to room temperature and was stirred for 2 days. After removal of solvent, the brownish residue was dissolved in ethyl acetate (30 cm^3) and distilled water (30 cm^3). The two layers were transferred into a separating funnel; the aqueous phase was washed a second time with ethyl acetate (40 cm^3). The combined organic layers were then washed successively with 10% aq. citric acid, 10% aq. sodium hydrogen carbonate, and distilled water. After drying of the solution over anhydrous magnesium sulfate, filtration, and removal of solvent, a brown oil (33 mg) was obtained, which had t_R 10.81 min (MeCN–0.1% TFA, 70:30) on HPLC analysis (integrating for 84%). No starting material could be detected in the product. Overall yield was 83% for the cyclisation. Repeated collection of the main fraction on the analytical HPLC column, followed by evaporation of solvent, and trituration with diethyl ether, gave cyclo[-Arg(Mtr)-Gly-Asp(Bu^t)-Ser(Bu^t)-Lys(Boc)-] as a powder with identical analytical data with those reported in ref. 11 [Found: (FAB) 968 $(M + H)^+$ and *m/z* 756, corresponding to loss of the Mtr group. $(C_{44}H_{73}N_9O_{13}S + H)$ requires *m/z* 968].

Removal of Side-chain Protection and Counterion Exchange.—Cyclo[-Arg(Mtr)-Gly-Asp(Bu^t)-Ser(Bu^t)-Lys(Boc)-] (30 mg, 0.031 mmol) was dissolved in a solution of TFA–thioanisole–thiocresol (20:1.25:1.25) (5 cm^3) and was left at room temperature for 3 h. After removal of solvent, the gum obtained was washed with diethyl ether to remove the 'scavenger molecules'. The residual solid, which still contained traces of thiocresol, was further purified by ion exchange: analytical-grade Amberlite resin IRA-400 (Cl^--form) was processed prior to use by being washed respectively with 4% aq. NaOH, distilled water, 10% aq. acetic acid, and distilled water until pH 6 (pH meter) was reached; the cyclopentapeptide was dissolved in methanol (3 cm^3)–distilled water (3 cm^3) and applied to the resin. The first fraction collected contained the acetate salt of the cyclopentapeptide. After removal of solvent, a solid (8 mg) was obtained in 43% overall yield. HPLC analysis showed one peak, t_R 3.4 min (MeCN–0.1%TFA, 50:50) [Found: (FAB) $(M + H)^+$, 544. $(C_{21}H_{37}N_9O_8 + H)$ requires *m/z* 544.2843].

Cell-adhesion Assay.—Both NIH 3T3 fibroblasts and Bac-1.2F5 macrophage cell lines were maintained in culture following routine protocols. For cell-adhesion assays, near-confluent monolayer cultures of cells were dissociated by using trypsin to bring cells into suspension. After repeated washes in trypsin-free buffer, cell suspensions were made up to a concentration of 4 × 10^5 cells cm^{-3} in minimal essential medium with HEPES buffer (Delbecco's MEM-HEPES). The viability of the cells was tested by using the Trypan Blue exclusion test after a 2 h recovery period and only suspensions with >90% cell viability were used in the assays (for details of protocols, see refs. 23 and 24).

Preparation of Fibronectin Lawns.—Human serum fibronectin, purified by affinity chromatography as previously described,[22] was diluted to a concentration of 5 μg cm^{-3} in Delbecco's phosphate-buffered saline (PBS), pH 7.2. Aliquots (100 mm^3) were dispensed into the wells of sterile 96-well flat-bottomed microtitre plates (ICN Flow) and the plates were incubated for 18 h at 4 °C to allow adsorption of fibronectin onto the plastic substratum. The plates were brought to room temperature (22 °C) and washed 5 times in PBS to remove unbound fibronectin. Haemoglobin, which prevents the non-specific adherence of cells to plastic, was added to wells in aliquots (100 mm^3) and was incubated at room temperature for 60 min. The wells were then washed in PBS as before and were used for cell-adhesion assays within 3 h.

Addition of Peptides.—Test peptides were made up from lyophilates to stock solutions of 100 mmol dm^{-3} in Milli-Q (Millipore) water. All subsequent peptide manipulations were done at 4 °C. All peptides were diluted with Delbecco's MEM-HEPES to a concentration of 10 mmol dm^{-3}. The pH of test media was then adjusted to 7.4 (pH meter) with saturated aq. sodium hydrogen carbonate.

For each peptide tested, a 1:1 ratio of peptide solution:cell suspension was prepared to give a final cell concentration of 2 × 10^5 cells cm^{-3} and a working peptide concentration of 5 mmol dm^{-3}. Where no peptide was employed, the cell

suspension was diluted in an equal volume of MEM-HEPES. Cells were incubated on ice for 15 min before aliquots (100 mm^3) were plated out onto the fibronectin-coated wells. The cells were then incubated at 37 °C for 60 min, after which the unattached cells were removed by three washes in MEM-HEPES. Fibronectin-bound cells were then aldehyde-fixed and stained as described previously.[24]

Analysis of Cell Attachment and Cell Spreading.—Attached cells were counted by using an Olympus IMT-2 research microscope in conjunction with VIDS III image analysis software. For each peptide treatment and relevant controls, the mean number of cells from 4 microtitre wells was expressed as counts per unit area and from these values a percentage of cell attachment was determined and plotted as shown in Fig. 1 (a and b). The average cell area of 50 cells per peptide treatment was also measured (see Fig. 1) in order to confirm that cells could spread onto the fibronectin substratum once attached.

Acknowledgements

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