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Potent Cyclic Peptide Inhibitors of VLA-4 ($\alpha_4\beta_1$ Integrin)-mediated Cell Adhesion. Discovery of Compounds like cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg) (ZD7349) Compatible with Depot Formulation

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Abstract: Additional structure–activity relationship studies on potent cyclic peptide inhibitors of very late antigen-4 (VLA-4) are reported. The new *N*- to *C*-terminal cyclic hexa-, hepta- and octapeptide inhibitors like cyclo(MeIle/MePhe-Leu-Asp-Val-X) (X = 2–4 amino acids containing hydrophobic and/or basic side chains) were synthesized using solid phase peptide synthesis methods. The peptides were evaluated in *in vitro* cell adhesion assays and in *in vivo* inflammation models. Many of the peptides like cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg) (ZD7349) (**17**), cyclo(MeIle-Leu-Asp-Val-D-Arg-D-Arg-D-Phe) (**20**), cyclo(MeIle-Leu-Asp-Val-D-Arg-D-Arg-MePhe) (**21**) and cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg-D-Ala-D-Ala) (**23**) were potent inhibitors of VLA-4-mediated cell adhesion and inhibited ovalbumin-induced delayed type hypersensitivity (DTH) response in mice. The more potent compounds were highly selective and did not affect U937 cell adhesion to fibronectin (VLA-5), phorbolmyristate acetate or PMA-differentiated U937 cell adhesion to intercellular cell adhesion molecule-1 (ICAM-1)-expressing Chinese hamster ovary cells (LFA-1) and adenosine diphosphate (ADP)-induced platelet aggregation (GPIIb/IIIa). In contrast to the inhibitors like Ac-cyclo(D-Lys-D-Ile-Leu-Asp-Val) and cyclo(CH_2CO-Ile-Leu-Asp-Val-Pip-CH_2CO-Ile-Leu-Asp-Val-Pip) described earlier, the new compounds were much more compatible with the depot formulations based on poly(DL-lactide-co-glycolide) polymers. The hexapeptide cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg) (ZD7349) (**17**) inhibited MOLT-4 cell adhesion to fibronectin and vascular cell adhesion molecule-1 (VCAM-1) with IC_{50} values of 260 and 330 nM, respectively, and did not show any significant effect against other integrins ($IC_{50} > 300$ μM). ZD7349 inhibited ovalbumin-induced DTH response in mice when administered continuously using a mini-pump (ED_{50} 0.01 mg/kg/day) or when given as an s.c. or i.v. bolus injection at a dose of 1–10 mg/kg. ZD7349 was also active in type II collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) tests at a dose of 3–10 mg/kg. The peptide was released from some formulations over a period of 10–20 days. ZD7349 is currently undergoing pre-clinical investigation.

Keywords: $\alpha_4\beta_1$ integrin; cyclic peptides; VCAM-1; VLA-4; fibronectin; ZD7349

Abbreviations: Pip, piperazine; VLA-4, very late antigen-4; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular cell adhesion molecule-1; DTH, delayed type hypersensitivity; CIA, type II collagen-induced arthritis; EAE, experimental autoimmune encephalomyelitis.

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INTRODUCTION

The interactions between an integrin family of heterodimeric cell surface receptors and their protein ligands are fundamental for maintaining cell function, for example by tethering cells at a particular location, facilitating cell migration or by providing survival signals to cells from their environment. Ligands recognized by integrins include: extracellular matrix proteins, such as collagen and fibronectin; plasma proteins, such as fibrinogen; and cell surface molecules, such as transmembrane proteins of the immunoglobulin superfamily and cell-bound complement. Various aspects of integrin research have been reviewed [1–5]. The integrin very late antigen-4 (VLA-4, $\alpha_4\beta_1$) [6] is expressed on human lymphocytes, monocytes, eosinophils, basophils and mast cells and binds to two main ligands, vascular cell adhesion molecule-1 (VCAM-1) and an alternatively spliced form of fibronectin containing the type III connecting segment (CS-1 fibronectin). The interactions between VLA-4 and its ligands are thought to play an important role in leukocyte recruitment and activation during inflammation.

Previously reported inhibitors of VLA-4-ligand interaction were highlighted in our earlier publications [7,8]. These include compounds based around the Leu-Asp-Val binding motif present in the 25-amino acid fibronectin peptide CS-1 and a similar binding epitope present in domain 1 of VCAM-1 contained within the region Arg-Thr-Gln-Ile-Asp-Ser-Pro-Leu-Asn. Some cyclic peptides like cyclo(Ser-D-Leu-Asp-Val-Pro) (85% inhibition at 2.5 mM) and a disulphide bridge containing cyclic peptide, Cys-Trp-Leu-Asp-Val-Cys, also demonstrated weak inhibitory activity. In addition, inhibitors unrelated to the Leu-Asp-Val tripeptide, e.g. adamantaneacetyl-Cys-Gly-Arg-Gly-Asp-Ser-Pro-Cys, Arg-Cys-Asp-thioproline-Cys, Tyr-Cys-Asp-Pro-Cys and Ac-Tyr-Cys-Ser-Pro-Cys (all containing a disulphide bridge) have been reported. However, many of these peptides were not selective for VLA-4-mediated interaction.

In our search for inhibitors of VLA-4-mediated cell adhesion, we were interested in developing novel and potent inhibitors with longer duration of action. Because the intention was to administer the peptides by slow release formulations capable of releasing the drug over a period of 15–30 days, the compatibility of the synthetic peptides with the polymers used in depot formulation and the release of the peptide from the polymer depot were also important issues. Our initial efforts to discover novel cyclic peptides starting from the 25-amino acid peptide CS-1 and to formulate some of the more potent compounds have been reported previously [7,8]. For example, compounds like cyclo(MeIle-Leu-Asp-Val-D-Ala-D-Ala), Ac-cyclo(D-Lys-D-Ile-Leu-Asp-Val) (Lys side chain amino and the Val carboxyl groups linked by an amide bond) and cyclo(CH_2CO-Ile-Leu-Asp-Val-Pip-CH_2CO-Ile-Leu-Asp-Val-Pip) were active in an *in vivo* inflammation model at a dose of < 0.1 mg/kg/day when administered continuously by a subcutaneous mini-pump. However, the compounds did not show extended duration of action after a bolus subcutaneous injection. In addition, monomeric compounds like Ac-cyclo(D-Lys-D-Ile-Leu-Asp-Val) and dimeric compounds like cyclo(CH_2CO-Ile-Leu-Asp-Val-Pip-CH_2CO-Ile-Leu-Asp-Val-Pip) were not completely stable at pH 3 for 30 days and were released from poly(DL-lactide-co-glycolide)-based polymer formulations relatively quickly. The work reported here has led to more stable inhibitors of VLA-4-mediated cell adhesion with much improved formulation characteristics. In addition to demonstrating activity in some *in vivo* models when administered by continuous infusion (mini-pump studies), some of the compounds were active when administered by a bolus s.c. or i.v. injection. One of the compounds, cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg) (**17**) (ZD7349) [9], is undergoing pre-clinical investigation.

MATERIALS AND METHODS

Peptide Synthesis

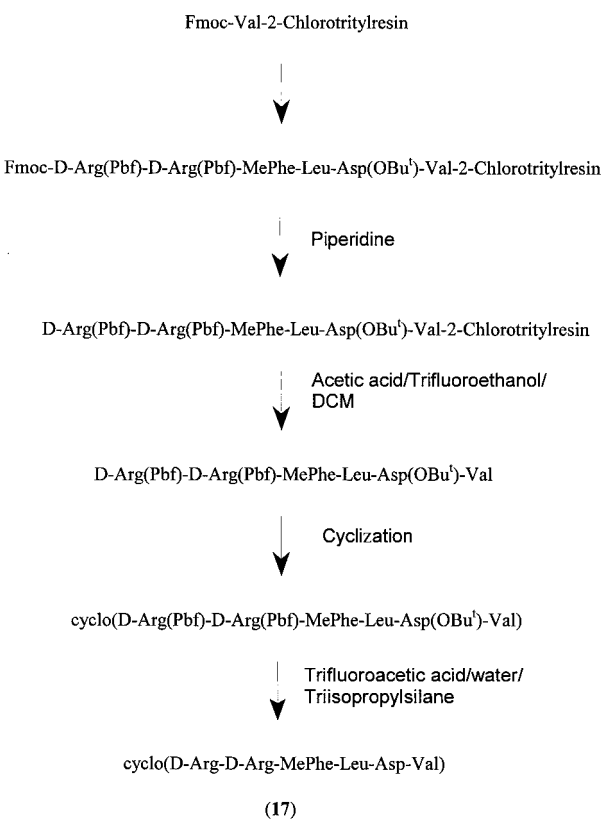
The cyclic peptides listed in Table 1 were obtained by the solid phase peptide synthesis methods using commercially available 2-chlorotritylchloride resin and various coupling and deblocking reagents. Fmoc group was used for N^{α}-amino group protection. The side chain protecting groups were Pmc and Pbf for Arg, OBu^t for Asp, trityl for His and Boc for the side chain amino groups of Orn and Lys. The partially protected linear peptides cleaved from the resin were used in the cyclization and deprotection steps without purification. The crude deprotected cyclic peptides were extensively purified by preparative RP-HPLC on a Vydac 218TP1022 column using a gradient of acetonitrile–water containing 0.1% TFA over a period of 60–65 min at a flow rate of 10.0 ml/min. The homogeneity of the peptides was

Table 1 Inhibition of Cell Adhesion-mediated by VLA-4, VLA-5 and LFA-1 *In Vitro* and DTH Responses in Mice by Cyclic Peptides

No.	Compound	Inhibition of cell adhesion (IC_{50} μM)				DTH ED^{a}_{50}
		VLA-4/ fibronectin	VLA-4/ VCAM-1	VLA-5/ fibronectin	LFA-1/ ICAM-1	(mg/kg/day)
2	cyclo(Ile-Leu-Asp-Val-NH-$(CH_2)_5$-CO)	4.3	9.4	>300	>300	1
3	cyclo(MeIle-Leu-Asp-Val-D-Ala-D-Ala)	0.17	0.11	>300	>300	0.004
4	cyclo(MeIle-Leu-Asp-Val-D-Ala-D-Arg)	0.032	0.17	>300	>300	0.02
5	cyclo(MeIle-Leu-Asp-Val-D-Phe-D-Arg)	0.60	0.86	>300	>300	0.01
6	cyclo(MeIle-Leu-Asp-Val-D-Trp-D-Arg)	0.49	0.60	>300	>300	0.009
7	cyclo(MePhe-Leu-Asp-Val-D-Trp-D-Arg)	0.20	0.30	>300	>300	0.01
8	cyclo(MeIle-Leu-Asp-Val-D-Ala-D-hArg$(Et)_2$)	0.15	0.30	>300	>300	0.02
9	cyclo(MeIle-Leu-Asp-Val-D-Phe-D-hArg$(Et)_2$)	0.97	0.30	NT	NT	0.2
10	cyclo(MeIle-Leu-Asp-Val-D-Trp-D-hArg$(Et)_2$)	0.38	0.25	>300	>300	0.05
11	cyclo(MeIle-Leu-Asp-Val-D-Arg-D-Ala)	0.14	0.40	>300	>300	0.02
12	cyclo(MeIle-Leu-Asp-Val-D-Arg-D-Arg)	0.38	0.51	>300	>300	0.04
13	cyclo(MeIle-Leu-Asp-Val-D-hArg$(Et)_2$-D-hArg$(Et)_2$)	0.62	1.2	NT	NT	0.1
14	cyclo(MeIle-Leu-Asp-Val-D-His-D-Arg)	1.3	0.66	>300	>300	0.05
15	cyclo(MeIle-Leu-Asp-Val-D-Arg-D-His)	0.21	0.27	>300	>300	0.03
16	cyclo(MePhe-Leu-Asp-Val-D-Arg-D-His)	0.24	0.26	>300	>300	0.007
17	cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg)	0.26	0.33	>300	>300	0.01
18	cyclo(MeIle-Leu-Asp-Val-D-Ala-D-Arg-D-Arg)	0.60	1.0	NT	NT	0.008
19	cyclo(MePhe-Leu-Asp-Val-D-Ala-D-Arg-D-Arg)	0.68	1.2	>300	>300	0.01
20	cyclo(MeIle-Leu-Asp-Val-D-Arg-D-Arg-D-Phe)	0.55	0.96	>300	>300	0.009
21	cyclo(MeIle-Leu-Asp-Val-D-Arg-D-Arg-MePhe)	3.0	6.9	NT	NT	0.009
22	cyclo(MeIle-Leu-Asp-Val-D-Arg-D-Arg-D-Ala-D-Ala)	1.1	2.8	NT	NT	0.008
23	cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg-D-Ala-D-Ala)	0.80	2.5	>300	>300	0.008
24	cyclo(MeIle-Leu-Asp-Val-D-Orn$(CHMe_2)$-D-Orn$(CHMe_2)$)	1.7	1.7	>300	>300	0.1
25	cyclo(MeIle-Leu-Asp-Val-D-Lys$(CHMe_2)$-D-Lys$(CHMe_2)$)	0.72	1.0	>300	>300	0.07
26	cyclo(MeIle-Leu-Asp-Val-D-Orn$(CHMe_2)$-D-Ala)	0.44	0.95	300	>300	0.01
27	cyclo(MeIle-Leu-Asp-Val-D-Orn(cyclohexyl)-D-Ala)	0.45	0.37	300	>300	0.07
28	cyclo(MeIle-Leu-Asp-Val-D-Orn(4-chlorobenzyl)-D-Ala)	0.52	1.3	300	>300	0.07
29	cyclo(MeIle-Leu-Asp-Val-D-Lys$(CHMe_2)$-D-Ala)	0.50	0.99	NT	NT	0.04
30	cyclo(MeIle-Leu-Asp-Val-D-Orn-D-Orn)	0.68	0.13	>300	>300	0.01
31	cyclo(MeIle-Leu-Asp-Val-D-Lys-D-Lys)	0.25	0.49	>300	>300	0.04

[a] Dose of cyclic peptide required to inhibit the DTH response by half the maximal inhibition ($36 \pm 1.4\%$, $n = 24$) induced by the anti-mouse α_4 monoclonal antibody PS/2 (7.5 mg/kg i.v.).
NT: not tested.

checked by analytical HPLC before characterization by amino acid analysis and mass spectroscopy. The general route used for the synthesis of cyclic peptides listed in Table 1 is illustrated in Scheme 1 using the synthesis of cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg) (**17**) as an example. The first amino acid (Fmoc-valine) was coupled to the 2-chlorotritylchloride resin. Removal of the Fmoc group (piperidine treatment) followed by sequential coupling [Fmoc-Asp(*O*Bu^t), Fmoc-Leu, Fmoc-MePhe, Fmoc-D-Arg(Pbf) and Fmoc-D-Arg(Pbf)] and deblocking steps gave the fully protected peptide attached to the resin. Cleavage of the *N*-terminal Fmoc group followed by cleavage of the peptide from the resin (acetic acid and TFE in DCM) gave the partially protected hexapeptide D-Arg(Pbf)-D-Arg(Pbf)-MePhe-Leu-Asp(*O*Bu^t)-Val, which was cyclized using HATU. The side chain protecting groups were then cleaved (TFA–water–triisopropylsilane) and the crude cyclic peptide was purified using preparative RP-HPLC.



Scheme 1 Synthesis of c(MePhe-Leu-Asp-Val-D-Arg-D-Arg) (**17**).

In Vitro Cell Adhesion Assays

The effects of cyclic peptides on MOLT-4 cell adhesion to fibronectin, MOLT-4 cell adhesion to VCAM-1, VLA-5 and LFA-1-mediated U937 cell adhesion and on adenosine diphosphate (ADP)-induced aggregation of human platelets were studied using previously described procedures [7,8].

In Vivo Models: Ovalbumin-induced DTH Response, Type II Collagen-induced Arthritis (CIA) and Experimental Autoimmune Encephalomyelitis (EAE)

Animal welfare and experimental procedures were carried out strictly in accordance with the UK Animals (Scientific Procedures) Act, 1986 and the Zeneca International Policy on the Use of Animals. Details of ovalbumin-induced DTH were reported previously [7,8]. CIA was induced in male DBA-1 mice by subcutaneous injection at the base of the tail with 0.1 ml of an emulsion of equal volumes of 2 mg/ml bovine type II collagen in 0.05 M acetic acid and Complete Freund's Adjuvant. Three weeks later, each mouse received an intraperitoneal booster injection of 0.1 ml type II collagen in acetic acid. The development of arthritis over the following 4 weeks was assessed by visual scoring of redness and swelling in each limb joint. Fourteen-day osmotic mini-pumps containing compound **17** (10 mg/kg/day) or saline were implanted subcutaneously under halothane anaesthesia on the day before the booster collagen injection. Development of arthritis was compared in animals treated with **17** and control animals infused with saline (ten mice/group). EAE was induced in B10.PL mice by subcutaneous injection at the base of the tail with 0.1 ml of an emulsion of equal volumes of a rat spinal cord homogenate (20 mg/ml) and Complete Freund's Adjuvant. Two days later, the mice were injected intraperitoneally with 400 ng pertussis toxin. Development of disease was scored by visual assessment of the severity of symptoms of EAE using a 4-point scale (0, no symptoms; 1, flaccid tail; 2, hind limb paralysis with impairment of righting reflex; 3, bilateral hind limb paralysis). Fourteen-day osmotic mini-pumps containing either compound **17** (3 mg/kg/day) or saline were implanted subcutaneously under halothane anaesthesia on the day before injection of spinal cord homogenate and the onset of disease was compared (ten mice/group).

Peptide Stability and Formulation Studies

Details have been reported previously [7,8].

RESULTS AND DISCUSSION

The role of VLA-4-mediated cell adhesion has been highlighted in several publications included in our earlier paper [8]. Based on these studies, it is likely that agents which block this cell adhesion process may be useful in various inflammatory diseases. We were, therefore, interested in identifying novel peptides which may be used as therapeutic agents. It is well known that due to the problems associated with peptides (e.g. lack of oral absorption and short half-life), these agents require frequent administration by parenteral routes. However, significant advances have been made in the delivery of peptides. For example, in our earlier work on an luteinising hormone releasing hormone (LHRH) agonist analogue (Zoladex®, goserelin) (AstraZeneca) [10], we reported on the development of depot formulations [11,12]. Such formulations allow the peptides to be administered only once over a period of 1–3 months, thus avoiding the need for continuous administration to the patients. The design of the formulations was based on drug incorporation in biodegradable polymers like poly(DL-lactide-co-glycolide). Therefore, in addition to finding a potent inhibitor of the VLA-4-mediated cell adhesion process, an additional objective of the current work was to identify peptides which were compatible with the biodegradable poly(DL-lactide-co-glycolide)-based formulations. In our earlier work, we identified potent inhibitors of VLA-4-mediated cell adhesion which were active in *in vitro* and *in vivo* tests. However, the peptides [Ac-cyclo(D-Lys-D-Ile-Leu-Asp-Val) (Lys side chain amino and the Val carboxyl groups linked by an amide bond) and cyclo(CH_2CO-Ile-Leu-Asp-Val-Pip-CH_2CO-Ile-Leu-Asp-Val-Pip) did not appear to be suitable for such formulations [8]. These peptides were not completely stable at pH 3 for 30 days and, in addition, were released from poly(DL-lactide-co-glycolide)-based polymer formulations relatively quickly (< 5 days). We have now attempted to modify the cyclic monomeric compounds in order to overcome some of the problems encountered with the earlier compounds.

The design of the new compounds was based on the structure–activity relationship studies on the earlier compounds like cyclo(Ile-Leu-Asp-Val-NH$(CH_2)_5$CO) and cyclo(Ile-Leu-Asp-Val-D-Ala-D-Ala), which indicated that, in comparison to the Ile and the linking group residues like -NH$(CH_2)_5$CO- and -D-Ala-D-Ala-, the Leu, Asp and Val residues were much more important for the biological activity. We, therefore, decided to keep the Leu, Asp and Val residues and to replace the rest of the amino acid and linking group residues with various hydrophobic and basic amino acid residues. This was based on the assumptions that replacements in these positions may allow the biological activity to be retained or increased and, at the same time, that such hydrophobic and basic side chain groups may also have additional interactions with the polymer chain and the free carboxyl end groups of the polymer. These additional interactions may allow slower release from the polymer as it is degraded *in vivo*.

All of the cyclic peptides were tested as inhibitors of the adhesion of VLA-4-expressing MOLT-4 cells to fibronectin-coated plates. The activity of a selected number of compounds was then investigated in various other integrin-dependent assays for selectivity: MOLT-4 cell adhesion to VCAM-1 (alternative VLA-4 ligand); U937 cell adhesion to fibronectin (VLA-5); PMA-treated U937 cell adhesion to ICAM-1 transfected CHO cells (LFA-1); and ADP-induced platelet aggregation (GPIIbIIIa). Finally, compounds were tested *in vivo* in a model of T-cell-dependent inflammation (mouse DTH) by continuous subcutaneous infusion from osmotic mini-pumps, starting 24 h before antigen challenge. The results are summarized in Table 1. The activity of a selected number of compounds after intravenous or subcutaneous bolus dosing was also investigated in the DTH model. The activity of one of the compounds [cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg)] (ZD7349) (**17**) was investigated in greater detail in mouse models of rheumatoid arthritis (type II CIA) and multiple sclerosis (EAE).

In Vitro Activity

As is shown in Table 1, most of the compounds were much more potent inhibitors of VLA-4-dependent cell adhesion of MOLT-4 cells to fibronectin than one of the earlier cyclic peptides, cyclo(Ile-Leu-Asp-Val-NH$(CH_2)_5$CO) (**2**), which was included as a standard in each assay. In comparison with another cyclic peptide, cyclo(Ile-Leu-Asp-Val-D-Ala-D-Ala) (**3**), which did not have any amino acids containing basic groups in the side chains, all of the new compounds retained most of their cell adhesion inhibitory activity. From a set of compounds

containing a single basic amino acid residue [D-Arg or D-hArg(Et)$_2$] (**4**–**11**), the D-Ala-D-Arg containing hexapeptide [cyclo(MeIle-Leu-Asp-Val-D-Ala-D-Arg) (**4**)] was the most potent (IC_{50} 32 nM). It was about 5-fold more potent than the D-Ala-D-Ala (**3**), D-Trp-D-Arg (**7**), D-Ala-D-hArg(Et)$_2$ (**8**) and D-Arg-D-Ala (**11**) containing hexapeptides and was 10–30-fold more potent than other compounds containing D-Phe-D-Arg (**5**), D-Trp-D-Arg (**6**), D-Phe-D-hArg(Et)$_2$ (**9**) and D-Trp-D-hArg(Et)$_2$ (**10**) residues (IC_{50} 380–970 nM). These compounds also inhibited MOLT-4 cell adhesion to the alternate VLA-4 ligand, VCAM-1 with similar potencies. None of these compounds showed any significant activity in VLA-5 and LFA-1-mediated cell adhesion assays ($IC_{50} > 300$ μM).

In a set of MeIle-Leu-Asp-Val or MePhe-Leu-Asp-Val hexapeptides containing two basic amino acid residues (**12**–**17**), the cyclic peptides containing D-Arg-D-Arg (**12**, **17**) and D-Arg-D-His (**15**, **16**) dipeptides inhibited MOLT-4 cell adhesion to fibronectin with similar potency (IC_{50} 210–380 nM). In comparison with cyclo(MeIle-Leu-Asp-Val-D-Arg-D-Arg) (**12**), the corresponding D-hArg(Et)$_2$-D-hArg(Et)$_2$ (**13**) and D-His-D-Arg (**14**) were less potent in the fibronectin adhesion assay. The cyclic hepta- and octapeptides containing two Arg residues (**18**–**23**) (IC_{50} 550–3000 nM) were 2–10-fold less potent than the most potent D-Arg-D-Arg hexapeptide analogue **17**. As in the case of peptides containing a single basic amino acid residue (**4**–**11**), the hexa-, hepta- and octapeptides containing two basic amino acid residues (**12**–**23**) had similar potency as inhibitors of MOLT-4 cell adhesion to VCAM-1. However, none showed any significant activity in VLA-5 and LFA-1-mediated cell adhesion assays ($IC_{50} > 300$ μM). Results for **17** in VLA-4, VLA-5 and LFA-1 adhesion assays are shown in Figure 1(A).

Comparison of the MeIle and MePhe containing sets of compounds (**6**, **7**), (**15**, **16**), (**12**, **17**) and (**18**, **19**) showed a slight advantage in favour of the MePhe compounds. For example, cyclo(MePhe-Leu-Asp-Val-D-Trp-D-Arg) (**7**) and cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg) (**17**) were somewhat more potent than the corresponding MeIle compounds **6** and **12**, whereas the other two sets of compounds were equipotent. Selected MeIle and MePhe compounds containing D-Trp-D-Arg (**6, 7**), D-Arg-D-His (**15, 16**) and D-Arg-D-Arg (**12, 17**) had no effect on ADP-induced aggregation of human platelets when tested at 100 μM. Results for **17** are shown in Figure 1(B).

The cyclic peptides containing D-Orn and D-Lys-residues (**24**–**31**) were prepared in order to investigate the effect of these residues on the biological

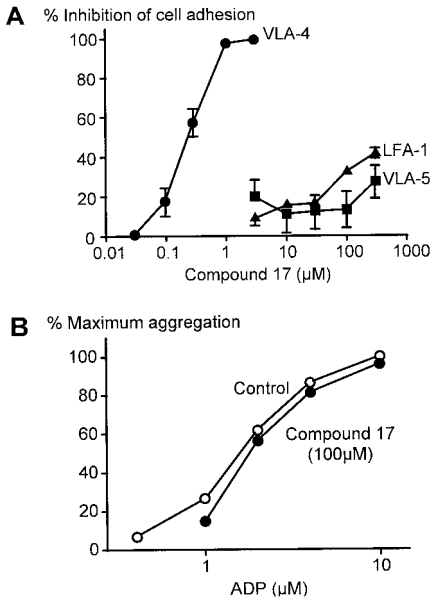


Figure 1 *In vitro* cell adhesion studies with compound **17**. (A) Effect of **17** on VLA-4, VLA-5 and LFA-1-mediated adhesion on 96-well plates (mean ± S.E.M. of four to five separate experiments). (B) Effect of **17** on ADP-induced aggregation of human platelet *in vitro*. Results are expressed as a percentage of the maximal change in light transmission induced by 10 μM ADP in control samples (100%).

activity as a replacement for the D-His and D-Arg residues. These compounds are not likely to be suitable for the poly(DL-lactide-co-glycolide)-based polymer formulations because the amino groups might react with the ester linkages. If these replacements resulted in significant improvements in potency and/or duration of effect, additional formulations would have to be developed to deliver these compounds in depot formulations. However, with the exception of cyclo(MeIle-Leu-Asp-Val-D-Lys-D-Lys) (**31**), which displayed potency similar to the D-Arg-D-Arg containing analogues (**12** and **17**) in inhibiting the VLA-4-mediated adhesion to fibronectin, all of the other analogues were less potent (IC_{50} 440–1700 nM).

In Vivo Activity

The cyclic peptides were tested in a mouse model of T-cell-dependent inflammation, ovalbumin-induced DTH (Table 1). All of the peptides were administered by continuous infusion from a subcutaneous osmotic mini-pump, starting 24 h before the ovalbumin challenge. The ovalbumin DTH response is only partially VLA-4 dependent [7] and ED_{50} values for

the peptides represent the dose required for half the maximal inhibition by the anti-mouse α_4 monoclonal antibody PS/2 (7.5 mg/kg, i.v.). We previously showed that cyclic peptides that are inactive *in vitro* are also inactive in the DTH model [7,8]. The 25-amino acid peptide, CS-1, was active in the DTH test (ED_{50} 1 mg/kg/day) but the maximal inhibition at 3–10 mg/kg/day was only 60% that of the antibody PS/2.

In contrast to CS-1 peptide, the maximum inhibition of the DTH response by the cyclic peptides was similar to PS/2. As is shown in Table 1, all of the compounds containing basic amino acids were more potent than CS1 peptide and the cyclic pentapeptide cyclo(Ile-Leu-Asp-Val-NH-$(CH_2)_5$-CO) (**2**) and were similar in potency to cyclo(MeIle-Leu-Asp-Val-D-Ala-D-Ala) (**3**) in inhibiting the ovalbumin-induced DTH in mice when dosed by continuous subcutaneous infusion. The ED_{50} values ranged between 0.004 to 0.2 mg/kg/day. A dose response for compound **17** (ED_{50} 0.01 mg/kg/day) is compared with CS-1 peptide in Figure 2(A). The peptide is >100-fold more potent than CS-1 in this *in vivo* test. The size of the peptide did not seem to make a significant difference on the *in vivo* potency. Several of the hexa- (**5**–**7**, **16**, **17**, **26** and **30**), hepta- (**18**–**21**) and octapeptides (**22** and **23**) inhibited foot swelling at a dose of <0.01 mg/kg/day. Most of the analogues containing a substituent at the side chain basic functional group (**9**, **10**, **13**, **24**, **25**, **27** and **28**) were amongst the less potent analogues. For example, cyclic peptides cyclo(MeIle-Leu-Asp-Val-D-Phe-D-hArg(Et)$_2$) (**9**), cyclo(MeIle-Leu-Asp-Val-D-Trp-D-hArg(Et)$_2$) (**10**) and cyclo(MeIle-Leu-Asp-Val-D-hArg(Et)$_2$-D-hArg(Et)$_2$) (**13**) were about 3–20-fold less potent than the parent D-Arg analogues. A similar trend was also observed in the case of D-Orn and D-Lys containing analogues. For example, in comparison with cyclo(MeIle-Leu-Asp-Val-D-Lys-D-Lys) (**31**), cyclo(MeIle-Leu-Asp-Val-D-Lys(CHMe$_2$)-D-Lys(CHMe$_2$)) (**25**) was about 2-fold less potent and cyclic peptides cyclo(MeIle-Leu-Asp-Val-D-Orn(CHMe$_2$)-D-Orn(CHMe$_2$)) (**24**), cyclo(MeIle-Leu-Asp-Val-D-Orn(cyclohexyl)-D-Ala) (**27**) and

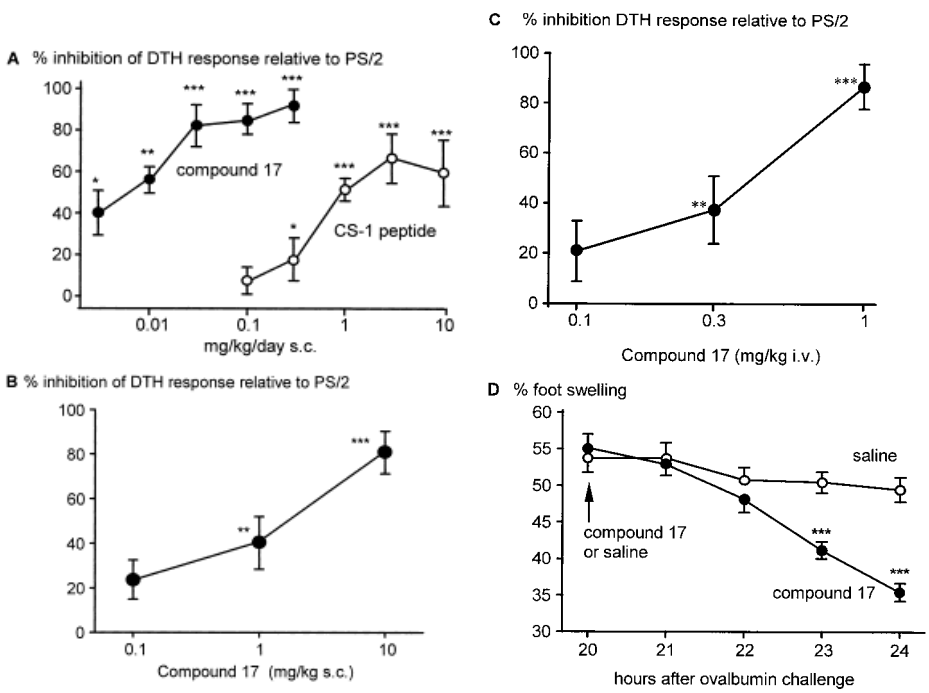


Figure 2 Inhibition of ovalbumin-induced DTH responses by **17** in Balb/c mice (expressed as a percentage of the maximal inhibition induced by the anti-mouse α_4 monoclonal antibody PS/2 (7.5 mg/kg i.v.)). (A) Continuous infusion of **17** and CS-1 from s.c. osmotic mini-pumps implanted 24 h before ovalbumin injection into the footpad (six to ten mice/dose group). (B) Dosing of **17** by bolus s.c. injection, 4 h before ovalbumin injection into the footpad (eight mice/dose group). (C) Dosing of **17** by bolus i.v. injection 20 h after ovalbumin injection into the footpad (ten mice/dose group). (D) Time course of reversal of established inflammation by dosing **17** (3 mg/kg) by bolus i.v. injection 20 h after ovalbumin injection into the footpad (10–15 mice/dose group). Statistical significance of difference from saline-dosed controls was determined using Student's *t*-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

cyclo(MeIle-Leu-Asp-Val-D-Orn(4-chlorobenzyl)-D-Ala) (**28**) were about 7–10-fold less potent than cyclo(MeIle-Leu-Asp-Val-D-Orn-D-Orn) (**30**).

In addition to continuous mini-pump administration, selected hexa- (**6**, **7**, **12**, **15**, **16** and **17**), hepta- (**19**) and octapeptides (**23**) were also dosed by bolus subcutaneous injection (0.1, 1.0 and 10 mg/kg, respectively), 4 h before ovalbumin challenge, in the DTH model (Table 2). The dose response for compound **17** is shown in Figure 2(B). Four of the compounds (**7**, **15**, **17** and **19**) did not show significant inhibition of DTH response at the lowest dose but were effective at the two higher dose levels. The remaining compounds were effective at all three dose levels. However, in comparison with the mini-pump experiments, the selected compounds were less potent when dosed by bolus subcutaneous injection. The ED_{50} values (> 0.1 mg/kg, bolus) after bolus s.c. dosing were at least 10-fold higher than the values (range 0.007–0.04 mg/kg/day) obtained from the continuous administration experiments. Two of the D-Arg-D-Arg containing compounds, cyclo(MeIle-Leu-Asp-Val-D-Arg-D-Arg) (**12**) and cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg-D-Ala-D-Ala) (**23**), appeared to be the most potent after bolus dosing ($ED_{50} < 1$ mg/kg).

In order to get some idea about the duration of action of the compounds, selected hexa- (**12**, **15**, **16** and **17**), hepta- (**19**) and octapeptides (**23**) were also dosed by bolus intravenous injection (1 mg/kg), either 4 h before or 20 h after ovalbumin challenge (Table 3). None of the compounds inhibited the DTH response when dosed before ovalbumin challenge (on the day before measurement of foot swelling) but all showed activity (50–80% of the PS/2 response) when dosed 20 h post-ovalbumin challenge (4 h before foot swelling measurement). An intravenous dose response for compound **17**, dosed 20 h post-ovalbumin challenge, is shown in Figure 2(C). Reduction of foot swelling after intravenous dosing of compound **17** (3 mg/kg) was gradual and was statistically significant at 3 and 4 h post-injection (Figure 2(D)). Inhibition of foot swelling observed 4 h after intravenous injection represented reversal of an established DTH response because foot swelling did not change over this 4-h period in control mice injected with saline (Figure 2(D)).

Compound **17** was further tested in two mouse models of human disease: type II CIA and EAE. A monoclonal antibody to the α_4 integrin subunit of VLA-4 was previously reported to inhibit the autoimmune responses in both models [13,14]. When dosed by continuous infusion from subcutaneous osmotic mini-pumps for 2 weeks, compound **17** significantly reduced the severity of CIA and delayed the onset of EAE (Figure 3).

Table 2 Inhibition of DTH Responses by Selected Cyclic Peptides after Bolus Subcutaneous Injection

Compound	% Inhibition of DTH response (relative to PS/2)[a]		
	0.1 mg/kg	1 mg/kg	10 mg/kg
cyclo(MeIle-Leu-Asp-Val-D-Trp-D-Arg) (**6**)	24 ± 13	44 ± 7.5	50 ± 14
	$p < 0.05$	$p < 0.001$	$p < 0.001$
cyclo(MePhe-Leu-Asp-Val-D-Trp-D-Arg) (**7**)	28 ± 16	47 ± 7.6	56 ± 24
	NS	$p < 0.05$	$p < 0.05$
cyclo(MeIle-Leu-Asp-Val-D-Arg-D-Arg) (**12**)	31 ± 14	88 ± 15	99 ± 6.4
	$p < 0.001$	$p < 0.001$	$p < 0.001$
cyclo(MeIle-Leu-Asp-Val-D-Arg-D-His) (**15**)	12 ± 5.9	43 ± 7.0	60 ± 4.5
	NS	$p < 0.01$	$p < 0.001$
cyclo(MePhe-Leu-Asp-Val-D-Arg-D-His) (**16**)	40 ± 13	50 ± 8.4	89 ± 16
	$p < 0.01$	$p < 0.001$	$p < 0.001$
cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg) (**17**)	24 ± 8.8	40 ± 12	81 ± 9.5
	NS	$p < 0.01$	$p < 0.001$
cyclo(MePhe-Leu-Asp-Val-D-Ala-D-Arg-D-Arg) (**19**)	1.9 ± 13	21 ± 7.2	36 ± 9.6
	NS	$p < 0.05$	$p < 0.01$
cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg-D-Ala-D-Ala) (**23**)	37 ± 6.4	71 ± 15	98 ± 6.4
	$p < 0.001$	$p < 0.001$	$p < 0.001$

[a] Mean PS/2 (7.5 mg/kg i.v.) inhibition for the experiments in this table was $34 \pm 2.0\%$ ($n = 6$).

Table 3 Inhibition of DTH Responses by Selected Cyclic Peptides after Bolus Intravenous Injection (1 mg/kg)

Compound	% Inhibition of DTH response (relative to PS/2)[a]	
	Dosing before ovalbumin challenge	Dosing 20 h post-ovalbumin challenge
cyclo(MeIle-Leu-Asp-Val-D-Arg-D-Arg) (**12**)	37 ± 10	75 ± 7.4
	NS	$p < 0.01$
cyclo(MeIle-Leu-Asp-Val-D-Arg-D-His) (**15**)	3.3 ± 24	66 ± 4.0
	NS	$p < 0.001$
cyclo(MePhe-Leu-Asp-Val-D-Arg-D-His) (**16**)	21 ± 23	80 ± 7.4
	NS	$p < 0.001$
cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg) (**17**)	3 ± 21	83 ± 6.5
	NS	$p < 0.001$
cyclo(MePhe-Leu-Asp-Val-D-Ala-D-Arg-D-Arg) (**19**)	15 ± 5.5	60 ± 12
	NS	$p < 0.001$
cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg-D-Ala-D-Ala) (**23**)	5.7 ± 13	50 ± 7.0
	NS	$p < 0.01$

[a] Mean PS/2 (7.5 mg/kg i.v.) inhibition for the experiments in this table was $34 \pm 2.0\%$ ($n = 6$).

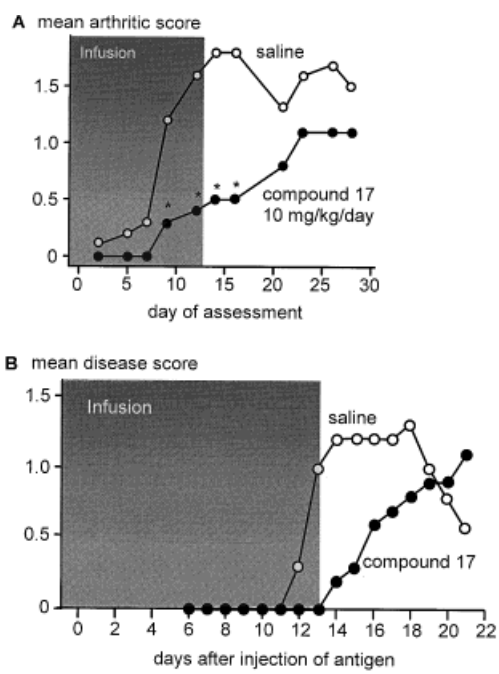


Figure 3 (A) Inhibition of type II CIA by **17** (10 mg/kg/day for 2 weeks by continuous infusion from s.c. osmotic mini-pumps implanted 24 h before the collagen injection. * $p < 0.05$, Wilcoxon rank sum test. (B) Inhibition of EAE by **17** (3 mg/kg/day for 2 weeks, continuous infusion from s.c. osmotic mini-pumps implanted 24 h before injection of spinal cord homogenate) (Log-rank test, $p < 0.0001$ in the animals treated with **17** compared with animals infused with saline).

Formulation Studies

For successful delivery of the peptide from depot formulations over an extended period of time, several issues have to be considered. Depending on the required period of delivery, enough compound has to be incorporated into the polymer. Once incorporated into the polymer, the peptide needs to be stable for several months before it is administered to the patients. In addition, the peptide has to be stable to the experimental conditions used to prepare the depots. Some of our earlier peptides, Ac-cyclo(D-Lys-D-Ile-Leu-Asp-Val) and cyclo(CH_2CO-Ile-Leu-Asp-Val-Pip-CH_2CO-Ile-Leu-Asp-Val-Pip), although stable at pH 7.6 over a period of 1 month, were degraded significantly (69 and 50%, respectively, remaining intact after 1 month) at pH 3. In addition, when incorporated into a 20-kDa 50:50 poly(DL-lactide-co-glycolide)-polymer, the *in vitro* release profiles of the two peptides were not compatible with extended release. Both peptides were released nearly quantitatively in < 4 days.

The onset and duration of release of four cyclic peptides containing at least one D-Arg from 20 kDa 50:50 poly(DL-lactide-co-glycolide) depots at 20% loading are dependent on the structure of the peptide (Figure 4(A)). Compounds **4** and **16**, which contain one D-Arg residue, had a shorter onset of release (3–4 days) than compounds **12** and **17**, which contain two D-Arg residues (5–7 days). The rate of release was faster in compounds **12** and **16** than in compounds **4** and **17**. Nearly 90% of the two peptides (**12** and **16**) was released in about 4 days

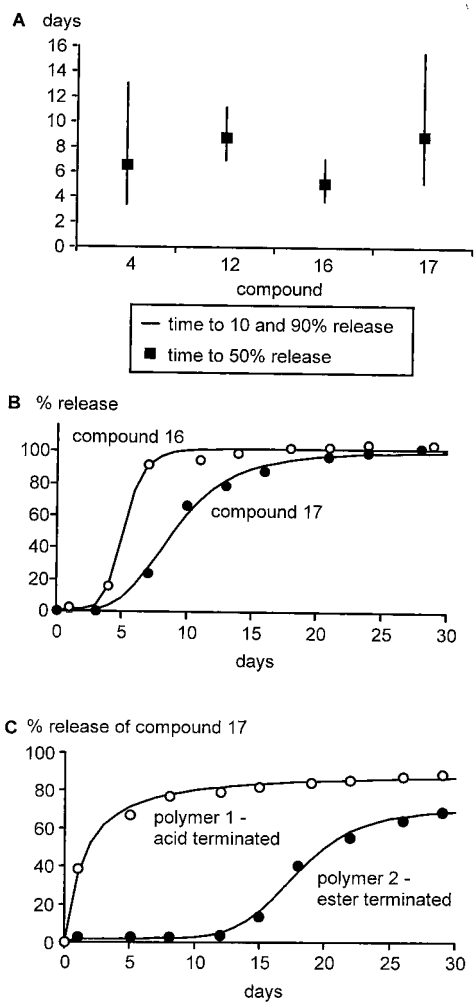


Figure 4 *In vitro* release of cyclic peptides from poly(DL-lactide-co-glycolide) depots into McIlvaine's buffer, pH 7.6 at 37°C. (A) The onset and duration of release of compounds **4**, **12**, **16** and **17** from 20 kDa 50:50 poly(DL-lactide-co-glycolide) monolithic depots at 20% loading. (B) Release profiles of compounds **16** and **17** from 20 kDa 50:50 poly(DL-lactide-co-glycolide) monolithic depots at 20% loading. (C) Release profiles of compound **17** from acid or ester terminated polymers. Polymer 1 is a 10.7-kDa 50:50 poly(DL-lactide-co-glycolide) which is acid terminated while Polymer 2 is a 13-kDa ester terminated 50:50 poly(DL-lactide-co-glycolide) (Boehringer Ingelheim polymer RG502).

once the release had started. Release profiles of compounds **16** and **17** are shown in Figure 4(B). The cyclic peptide with the longest duration of release (about 15 days), **17**, was stable in aqueous solution at pH 3 and 7.6 for 1 month at 37°C. Changing the nature of the polymer changed the release profile of compound **17**. The onset of release from a 10.7-kDa acid terminated 50:50 poly(DL-lactide-co-glycolide) depot was considerably shorter than that from an ester ended polymer of similar molecular weight. The rate of release after onset was not significantly altered but release from the ester terminated polymer was incomplete (Figure 4(C)). Thus, the release of compound **17** from monolithic poly(DL-lactide-co-glycolide) depots is controlled with no burst of release and the release profile is determined by the formulation composition. Further work on other formulations is required to extend the period of release.

Conclusions

Cyclic hexa-, hepta- and octapeptides incorporating a MePhe/MeIle-Leu-Asp-Val tetrapeptide, obtained by linking the *N*- and *C*-terminal ends using a linking group comprising two to four additional amino acids (e.g. -D-Arg-D-Arg- or D-Ala-D-Ala-D-Arg-D-Arg-), are potent inhibitors of VLA-4-mediated MOLT-4 cell adhesion *in vitro* and ovalbumin DTH *in vivo* when dosed continuously from a subcutaneous osmotic mini-pump. In addition, some of the peptides display an extended duration of action in the DTH model when administered as a bolus s.c. or i.v. injection. The increased duration of action may, in part, be due to greater stability of these cyclic peptides owing to the presence of at least three unnatural (*N*-methyl and D-) amino acids in the sequence. The maximum inhibition of the DTH response by the peptides was equivalent to that of the anti-α_4 monoclonal antibody PS/2. This suggests that the *in vivo* activity of the peptides was due to the inhibition of the recruitment and/or activation of VLA-4-expressing leukocytes, although a contribution of additional effects on the integrin $\alpha_4\beta_7$ cannot be excluded. One of the peptides, cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg) (ZD7349) (**17**), demonstrated good *in vivo* activity in three (DTH, CIA and EAE) inflammation models. The peptide was also stable at pH 3 and 7 (at 37°C) for a period of >30 days and could be formulated in poly(DL-lactide-co-glycolide)-based depot formulations. The current formulations allow the peptide to be released over a period of 15 days and further work is required to extend this release period to 30 days. ZD7349 (**17**) is currently undergoing pre-clinical development and is a potential treatment for a number of inflammatory diseases, including rheumatoid arthritis and multiple sclerosis.

EXPERIMENTAL SECTION

Details of the experimental procedure are given below using the synthesis of cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg) (**17**) as an example. In the case of the remaining cyclic peptides, only differences from the standard procedure are highlighted.

Synthesis of cyclo(MePhe-Leu-Asp-Val-D-Arg-D-Arg) (17, Scheme 1)

2-chlorotritylchloride resin (Alexis Corporation; 1.31 mmole Cl/g; 10 g) was swollen in DCM (40 ml) (dried over molecular sieve) for 5 min. A solution of Fmoc-Val (3.39 g, 10 mmole) and DIPEA (5.6 ml, 32 mmole) in DCM (20 ml) was added and the suspension was shaken mechanically for 45 min. Methanol (9 ml) and DIPEA (1 ml) were added and the shaking was continued for a further 5 min. The Fmoc-Val resin was collected by filtration, washed successively with DCM, DMF and DCM and placed in a reaction vessel fitted with a sintered glass disc. The following series of reactions were then carried out manually to obtain the desired peptide resin: (a) removal of the Fmoc group with two treatments (1×5 min and 1×15 min) of 20% piperidine in DMF followed by five washes with DMF to remove excess reagents and cleavage products; and (b) acylation with Fmoc-Asp(*O*Bu^t) (6.17 g, 15 mmole), activated with HBTU (5.70 g, 15 mmole) and DIPEA (5.25 ml, 30 mmole) in DMF (22 ml) for 1 h. The resin was again washed five times with DMF to remove excess reagents. The deprotection and coupling cycles were repeated using Fmoc-Leu (5.29 g, 15 mmole), Fmoc-MePhe (6.01 g, 15 mmole), Fmoc-D-Arg(Pbf) (11.27 g, 15 mmole) and Fmoc-D-Arg(Pbf) (11.27 g, 15 mmole) to give Fmoc-D-Arg(Pbf)-D-Arg-(Pbf)-MePhe-Leu-Asp(*O*Bu^t)-Val-chlorotrityl resin. Coupling of the Fmoc-D-Arg derivative was achieved using HATU and DIPEA. The *N*-terminal Fmoc group was cleaved with 20% piperidine in DMF (1×5 min and 1×15 minutes) and the peptide resin, D-Arg(Pbf)-D-Arg(Pbf)-MePhe-Leu-Asp(*O*Bu^t)-Val-chlorotrityl resin, was washed successively with DMF and DCM.

The peptide resin, D-Arg(Pbf)-D-Arg(Pbf)-MePhe-Leu-Asp(*O*Bu^t)-Val-chlorotrityl resin, was suspended in a mixture of acetic acid:TFE:DCM (2:2:6) (100 ml) for 1 h. The resin was removed by filtration and retreated with the same mixture for a further 1 h. The combined filtrates were evaporated and the residue triturated with ether to give D-Arg(Pbf)-D-Arg(Pbf)-MePhe-Leu-Asp(*O*Bu^t)-Val as an acetate salt (11.66 g). The acetate salt was then converted to a hydrochloride salt by dissolving it in a mixture of water:acetonitrile (2:1; 350 ml), cooling to 0°C, adding 1.05 equivalents of 1N HCl and freeze drying the contents (weight 11.5 g). The linear peptide hydrochloride, D-Arg(Pbf)-D-Arg(Pbf)-MePhe-Leu-Asp(*O*Bu^t)-Val (HCl) (11.5 g, 8.1 mmole), was dissolved in DMF (8000 ml) and HATU (4.62 g, 12.15 mmole) and DIPEA (5.67 mL, 32.4 mmole) were added to the solution. The cyclization reaction was monitored by analytical HPLC. On completion of the reaction (2 h at room temperature), the reaction mixture was evaporated to dryness in vacuum. The residue was triturated with 10% aqueous $NaHCO_3$ solution. The solid was collected and washed with 10% $NaHCO_3$, water, 10% $KHSO_4$ solution and, finally, with water. The solid was dried over P_2O_5 at 45°C in a vacuum oven (retention time 24.80 min on a Vydac 218TP54 column using a gradient of acetonitrile–water containing 0.1% TFA (40–80%) over a period of 30 min at a flow rate of 1.0 ml/min). The protected cyclic peptide, cyclo(D-Arg(Pbf)-D-Arg(Pbf)-MePhe-Leu-Asp(*O*Bu^t)-Val), was treated for 90 min with a mixture of TFA:water (95:5; 80 ml) and triisopropylsilane (3 ml) to remove the Arg and Asp side chain protecting groups. The reaction mixture was evaporated to a small volume and partitioned between water and ether. The aqueous layer was washed four times with ether and freeze dried to give 10.9 g crude product. The crude product was purified by preparative RP-HPLC on a Vydac C_{18} 218TP1015100 column (4 in. $\times$ 25 cm) using a gradient of acetonitrile–water containing 0.1% TFA (15–35%) over a period of 80 min at a flow rate of 180 ml/min. The product containing fractions were combined and freeze dried to give the purified cyclic peptide (3.63 g). The peptide was characterized by amino acid analysis and mass spectroscopy (Table 4).

Syntheses of Compounds 4–7

Cyclic peptides **4**–**7** were synthesized on the 2-chlorotritylchloride resin starting with Fmoc-Val. The procedure was similar to that of compound **17** except that the Arg in **4**–**6** was incorporated using the Fmoc-Arg(Pmc) derivative. In compound **7**, Fmoc-D-Arg(Pbf) was used. Because the Arg Pmc protecting group is more stable to deprotection than the Pbf group, the Pmc-protected cyclic peptides were treated for 4 h with a mixture of TFA:water (95:5; 30 ml) and triisopropylsilane (1 ml) to remove the Arg and Asp side chain protecting groups.

Table 4 Synthesis, Purification and Characterization of the Cyclic Peptides

No.	Linear or cyclic precursor peptide	Amino acid analysis (acid hydrolysis: 6N HCl containing 1% phenol, 24 h, 130°C)	Preparative HPLC (gradient system and time)	Analytical HPLC retention time (min)	Mass Spectrometry $(M+H)^+$
4	D-Ala-D-Arg(Pmc)-MeIle-Leu-Asp(*O*Bu^t)-Val	Asp 1.0, Val 1.0, Leu 1.0, Arg 1.02, Ala 0.96	10–50% (60 min)	15.3	
				20–40% (40 min)	682.5
5	D-Phe-D-Arg(Pmc)-MeIle-Leu-Asp(*O*Bu^t)-Val	Asp 1.0, Val 0.98, Leu 0.96, Arg 0.96, Phe 0.97	10–50% (60 min)	14.1	
				20–80% (40 min)	758.6
6	D-Trp-D-Arg(Pmc)-MeIle-Leu-Asp(*O*Bu^t)-Val	Asp 1.0, Val 0.98, Leu 0.96, Arg 0.96, Trp 0.82	10–50% (60 min)	21.0	
				20–50% (40 min)	797.4
7	D-Trp-D-Arg(Pbf)-MePhe-Leu-Asp(*O*Bu^t)-Val	Asp 0.99, Val 0.97, Leu 1.0, Arg 1.03, Trp 0.59	25–35% (90 min)	7.89	
				30–45% (15 min)	831.0
8	D-hArg(Et)$_2$-MeIle-Leu-Asp(*O*Bu^t)-Val-D-Ala	Asp 1.0, Ala 1.06, Val 0.95, Leu 0.99, hArg(Et)$_2$, 0.97	10–50% (60 min)	13.34	
				20–80% (40 min)	752.4
9	D-hArg(Et)$_2$-MeIle-Leu-Asp(*O*Bu^t)-Val-D-Phe	Asp 1.0, Phe 0.96, Val 0.95, Leu 0.99, hArg(Et)$_2$, 0.98	10–50% (60 min)	16.49	
				20–80% (40 min)	828.9
10	D-hArg(Et$_2$)-MeIle-Leu-Asp(*O*Bu^t)-Val-D-Trp	Asp 1.05, Val 0.96, Leu 1.0, hArg 0.95, Trp 0.48	30–40% (90 min)	13.7	
			4 in. Vydac column	30–45% (15 min)	867.6
11	D-Arg(Pmc)-D-Ala-MeIle-Leu-Asp(*O*Bu^t)-Val	Asp 1.0, Val 0.97, Leu 0.97, Arg 0.95, Ala 0.99	10–50% (60 min)	13.4	
				20–80% (40 min)	682.5
12	D-Arg(Pmc)-D-Arg(Pmc)-MeIle-Leu-Asp(*O*Bu^t)-Val	Asp 1.0, Val 0.98, Leu 1.0, D-Arg 1.94	10–30% (60 min)	10.7	
				20–40% (40 min)	767.3
13	D-hArg(Et)$_2$-D-hArg(Et)$_2$-MeIle-Leu-Asp(*O*Bu^t)-Val	Asp 1.0, Val 0.97, Leu 0.98, hArg(Et)$_2$, 2.1	10–50% (60 min)	12.03	
				20–80% (40 min)	907.6
14	D-His(Trt)-D-Arg(Pmc)-MeIle-Leu-Asp-Val	Asp 0.96, Val 0.98, Leu 1.01, Arg 0.96, His 0.97	15–30% (60 min)	18.86	
				10–40% (40 min)	749.0
15	D-Arg(Pmc)-D-His(Trt)-MeIle-Leu-Asp(*O*Bu^t)-Val	Asp 1.0, Val 0.98, Leu 0.97, Arg 0.97, His 0.96	15–30% (60 min)	17.80	
				10–40% (40 min)	749.0
16	D-Arg(Pbf)-D-His(Trt)-MePhe-Leu-Asp(*O*bu^t)-Val	Asp 1.03, Val 1.01, Leu 1.0, Arg 1.0, His 0.99	15–30% (100 min)	10.59	
				20–35% (15 min)	782.3
17	D-Arg(Pbf)-D-Arg(Pbf)-MePhe-Leu-Asp(*O*bu^t)-Val	Asp 1.00, Val 0.96, Leu 1.0, Arg 1.96	15–30% (100 min)	21.19	
				10–40% (30 min)	801.4
18	D-Ala-D-Arg(Pmc)-D-Arg(Pmc)-MeIle-Leu-Asp(*O*bu^t)-Val	Asp 1.01, Ala 1.07, Val 1.0, Leu 1.0, Arg 1.90	10–30% (60 min)	23.68	
				10–40% (40 min)	838.5
19	D-Ala-D-Arg(Pbf)-D-Arg(Pbf)-MePhe-Leu-Asp(*O*Bu^t)-Val	Asp 1.05, Ala 1.0, Val 0.97, Leu 0.98, Arg 2.0	10–35% (60 min)	26.48	
				10–40% (40 min)	871.5
20	D-Arg(Pbf)-D-Arg(Pbf)-D-Phe-MeIle-Leu-Asp(*O*Bu^t)-Val	Asp 1.04, Val 1.0, Leu 1.02, Arg 2.07, Phe 1.01	20–40% (100 min) 4 in. Vydac column	26.37	
				10–50% (40 min)	914.6

Table 4 Synthesis, Purification and Characterization of the Cyclic Peptides

No.	Linear or cyclic precursor peptide	Amino acid analysis (acid hydrolysis: 6N HCl containing 1% phenol, 24 h, 130°C)	Preparative HPLC (gradient system and time)	Analytical HPLC retention time (min)	Mass Spectrometry $(M+H)^+$
21	D-Arg(Pbf)-D-Arg(Pbf)-MePhe-MeIle-Leu-Asp(*O*Bu^t)-Val	Asp 1.0, Val 0.96, Leu 1.0, Arg 1.98	10–35% (60 min) 1 in. Vydac column	22.74 10–50% (40 min)	928.6
22	Arg(Pbf)-D-Arg(Pbf)-D-Ala-D-Ala-D-MeIle-Leu-Asp(*O*Bu^t)-Val	Asp 1.0, Ala 2.08, Val 0.96, Leu 1.05, Arg 1.95	Vydac column 15–35% (100 min)	24.33 10–40% (30 min)	909.5
23	D-Arg(Pbf)-D-Arg(Pbf)-D-Ala-D-Ala-MePhe-Leu-Asp(*O*Bu^t)-Val	Asp 1.0, Ala 2.18, Val 0.96, Leu 1.01, Arg 2.02	15–30% (100 min) 4 in. Vydac column	25.8 10–40% (40 min)	943.7
24	cyclo(MeIle-Leu-Asp-Val-D-Orn-D-Orn)	Asp 0.99, Val 0.97, Leu 1.04, Orn 2.01	10–40% (70 min) (12 ml/min)	23.45 10–40% (30 min)	767.5
25	cyclo(MeIle-Leu-Asp-Val-D-Lys-D-Lys)	Asp 1.03, Val 1.0, Leu 0.99	10–40% (60 min)	16.0 10–70% (40 min)	795.5
26	cyclo(MeIle-Leu-Asp-Val-D-Orn-D-Ala)	Asp 0.97, Val 0.95, Leu 1.01, Orn(Pr^i) 0.96, Ala 1.05	25–40% (60 min)	17.70 25–40% (30 min)	682.5
27	cyclo(MeIle-Leu-Asp-Val-D-Orn-D-Ala)	Asp 0.99, Val 0.98, Leu 1.0, Ala 1.02	25–40% (60 min)	23.03 25–40% (30 min)	722.6
28	cyclo(MeIle-Leu-Asp-Val-D-Orn-D-Ala)	Asp 1.01, Val 0.98, Leu 0.99, Ala 1.02	10–40% (60 min)	14.36 30–70% (30 min)	764.4
29	cyclo(MeIle-Leu-Asp-Val-D-Lys-D-Ala)	Asp 1.0, Ala 1.0, Val 0.96, Leu 0.97	Dynamax column 10–50% (60 min)	13.21 20–80% (40 min)	696.5
30	D-Orn(Boc)-D-Orn(Boc)-MeIle-Leu-Asp-Val	Asp 1.0, Val 0.98, Leu 0.96, Orn 1.95		20.40 10–40% (30 min)	683.5
31	D-Lys(Boc)-D-Lys(Boc)-MeIle-Leu-Asp(*O*Bu^t)-Val	Asp 1.05, Val 0.99, Leu 1.0, Lys 2.07	10–30% (60 min)	27.57 10–30% (40 min)	711.5

Preparative HPLC was carried out using a reverse phase (C_{18}) 1 in. diameter Vydac column (218TP1022, 22 × 250 mm). In some cases (mentioned in the table), a Deltapak column (30 × 300 mm) was used. The solvent system consisted of water and acetonitrile (each containing 0.1% TFA). The column was eluted using a gradient (solvent ratio and time shown in the table) with increasing concentrations of acetonitrile run at a rate of 10 ml/min for the Vydac column and 30 ml/min for the Deltapak column.

Syntheses of Compounds 8–10 and 13

The linear peptides, D-hArg(Et)$_2$-MeIle-Leu-Asp-(*O*Bu^t)-Val-D-Ala, D-hArg(Et)$_2$-MeIle-Leu-Asp(*O*Bu^t)-Val-D-Phe, D-hArg(Et$_2$)-MeIle-Leu-Asp(*O*Bu^t)-Val-D-Trp and D-hArg(Et)$_2$-D-hArg(Et)$_2$-MeIle-Leu-Asp-(*O*Bu^t)-Val, required for compounds **8**–**10** and **13** were obtained using the procedures described for compound **17**. Boc-D-hArg(Et)$_2$, prepared using the route previously reported [15,16], was converted to Fmoc-D-hArg(Et)$_2$ by the standard procedures and used in the syntheses of the above linear peptides. The linear peptides were then cyclized using the methods described for compound **17**.

Syntheses of Compounds 11, 12, 14, 15 and 18

Synthetic details are similar to those of compound **17** except that the Arg residue in these compounds was incorporated using the Fmoc-Arg(Pmc) derivative. The linear peptides D-Arg(Pmc)-D-Ala-MeIle-Leu-Asp(*O*Bu^t)-Val, D-Arg(Pmc)-D-Arg(Pmc)-MeIle-Leu-Asp(*O*Bu^t)-Val, D-His(Trt)-D-Arg(Pmc)-MeIle-Leu-Asp(*O*Bu^t)-Val, D-Arg(Pmc)-D-His(Trt)-MeIle-Leu-Asp(*O*Bu^t)-Val and D-Ala-D-Arg(Pmc)-D-Arg-(Pmc)-MeIle-Leu-Asp(*O*Bu^t)-Val were cyclized and deprotected to give the expected products. As in the case of compounds **4**–**6**, a longer TFA treatment (TFA:water (95:5; 30 ml) and triisopropylsilane (1 ml), 4 h) was required to deprotect the cyclic peptides.

Syntheses of Compounds 16 and 19–23

The partially protected linear peptides, D-Arg(Pbf)-D-His(Trt)-MePhe-Leu-Asp(*O*Bu^t)-Val, D-Ala-D-Arg (Pbf)-D-Arg(Pbf)-MePhe-Leu-Asp(*O*Bu^t)-Val, D-Arg-(Pbf)-D-Arg(Pbf)-D-Phe-MeIle-Leu-Asp(*O*Bu^t)-Val, D-Arg(Pbf) - D - Arg(Pbf) - MePhe - MeIle - Leu - Asp(*O*Bu^t)-Val, Arg(Pbf)-D-Arg(Pbf)-D-Ala-D-Ala-D-MeIle-Leu-Asp(*O*Bu^t)-Val, and D-Arg(Pbf)-D-Arg(Pbf)-D-Ala-D-Ala-MePhe-Leu-Asp(*O*Bu^t)-Val, were synthesized, cyclized and deprotected using the methods described above for compound **17** to give the desired products.

Syntheses of Compounds 24–31

The precursors to compounds **24** and **25** [cyclo(D-Orn-D-Orn-MeIle-Leu-Asp-Val) (**30**) and cyclo(D-Lys-D-Lys-MeIle-Leu-Asp-Val) (**31**)] and the precursors to compounds **26**–**29** [cyclo(D-Orn-D-Ala-MeIle-Leu-Asp-Val) and cyclo(D-Lys-D-Ala-MeIle-Leu-Asp-Val)] were synthesized using the procedures described above for compound **17**. The deprotected cyclic peptides were then reacted with the required aldehyde or ketone and sodium cyanoborohydride. For example, compound **26** was prepared by dissolving the cyclic peptide cyclo(D-Orn-D-Ala-MeIle-Leu-Asp-Val) (64 mg, 100 μM) in dry acetone (2 ml) and reaction with sodium cyanoborohydride (63 mg, 10 equivalents). After 1 h, the reaction mixture was evaporated to dryness and the residue, dissolved in water (5 ml), was acidified with acetic acid and evaporated under high vacuum. The crude peptide was purified using HPLC.

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