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Actuación	519
Oficio	10928
Folios	2

Patente de Invención	☑		Patente de Modelo de Utilidad
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Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 46 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

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De acuerdo con el Art 46 D486, según el cual: "*La Oficina Nacional competente podrá requerir el informe de expertos o de organismos científicos o tecnológicos que se consideren idóneos para que emitan opinión sobre la patentabilidad de la invención. Asimismo, cuando lo estime conveniente, podrá requerir informes de otras oficinas de propiedad industrial. De ser necesario, a efectos del examen de patentabilidad y a requerimiento de la oficina nacional competente, el solicitante proporcionará, en un plazo que no excederá de 3 meses, uno o más documentos relativos a una o más solicitudes extranjeras referidas total o parcialmente a la misma invención que se examina: ...b) copia de los resultados de exámenes de novedad o de patentabilidad efectuados respecto a esa solicitud extranjera...Si el solicitante no presentara los documentos requeridos dentro del plazo señalado en el presente artículo la oficina nacional competente denegará la patente*"; y considerando que no ha sido posible para esta Oficina encontrar los documentos siguientes, atentamente se requiere su presentación:

- LI BING ET AL; "Minimization of a polypeptide hormone" 1995, SCIENCE (WASHINGTON DC), vol. 270, nr. 5242. pages 1657-1660, ISSNN: 0036-8075.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 22 JUN. 2012

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Potent Cyclic Monomeric and Dimeric Peptide Inhibitors of VLA-4 ($\alpha_4\beta_1$ Integrin)-Mediated Cell Adhesion Based on the Ile-Leu-Asp-Val Tetrapeptide

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Abstract: Potent monomeric and dimeric cyclic peptide very late antigen-4 (VLA-4) inhibitors have been designed based on a tetrapeptide (Ile Leu Asp Val) sequence present in a 25 amino acid peptide (CS 1) reported in the literature. The peptides synthesized by the SPPS techniques, were evaluated in the *in vitro* cell adhesion assays and in the *in vivo* inflammation models. The *N*- to *C*-terminal cyclic peptides such as cyclo(Ile Leu Asp Val NH $(CH_2)_3$ S $(CH_2)_2$ CO) **(28)** and cyclo(Melle Leu Asp Val D Ala D Ala) **(31)**, monomeric and dimeric peptides containing piperazine (Pip) or homopiperazine (hPip) residues as linking groups, e.g. cyclo(Melle Leu Asp Val Pip CH_2CO NH-$(CH_2)_3$ S CH_2 CO) **(49)** and cyclo(Melle Leu Asp Val hPip CH_2CO Melle Leu Asp Val hPip CH_2CO) **(58)** and cyclic peptides containing an amide bond between the side chain amino group of an amino acid such as Lys and the *C* terminal Val carboxyl group, e.g. Ac cyclo(D Lys D Ile Leu Asp Val) **(62)** and β Ala cyclo(D Lys D Leu Leu Asp-Val) **(68)** were more potent than CS 1 in inhibiting the adhesion of the VLA-4-expressing MOLT-4 cells to fibronectin. The more potent compounds were highly selective and did not affect U937 cell adhesion to fibronectin (VLA 5). PMA differentiated U937 cell adhesion to intercellular cell adhesion molecule-1 expressing Chinese hamster ovary cells (LFA 1) and ADP induced platelet aggregation (GPIIb/IIIa). A number of the more potent compounds inhibited ovalbumin induced delayed type hypersensitivity in mice and some were 100–300 times more potent (ED_{50} = 0.003–0.009 mg kg/day, s.c.) than CS-1. Two peptides, Ac-cyclo(D Lys D Ile Leu Asp Val) **(62)** and cyclo(CH_2CO-Ile-Leu Asp Val Pip CH_2CO Ile Leu Asp Val Pip) **(55)**, were formulated in polylactide-co-glycolide) depots and the release profile was investigated *in vitro* over a 30-day period. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

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

INTRODUCTION

Many cell–cell and cell–extracellular matrix interactions are mediated by integrin receptors and their protein ligands. Various aspects of integrin research have been reviewed [1–3]. Very late antigen-4 (VLA-4) ($\alpha_4\beta_1$) is an integrin expressed on human lymphocytes, monocytes, eosinophils, basophils and mast cells that binds to vascular cell adhesion molecule-1 (VCAM-1) and an alternatively spliced form of the extracellular matrix protein, fibronectin containing the type III connecting segment. Evidence for the involvement of VLA-4 in inflammation has been derived from animal models of autoimmune and allergic diseases. For example, CS 1 or monoclonal antibodies specific for the α_4 integrin subunit or VCAM-1 suppressed antigen induced arthritis in rats [4] and reduced contact hypersensitivity responses [5], arthritis [6,7], autoimmune encephalomyelitis [8–10], bronchial hyperreactivity [11,12], insulin dependent diabetes [13–15], acute colitis [16] and allograft rejection [17] in experimental animals. Agents that specifically inhibit cell adhesion mediated by VLA-4 may be useful for the treatment of a number of inflammatory and autoimmune diseases.

VLA-4 binds the variable region of fibronectin via the Leu Asp Val tripeptide [18–20]. Based on this information, peptide inhibitors of VLA-4 binding to both fibronectin and VCAM-1 have been reported [21–24]. Cyclo(Ser D Leu Asp Val-Pro) inhibited VLA-4–VCAM-1 interaction in a scintillation proximity assay (85% inhibition at 2.5 mM) and the disulphide bridge containing cyclic peptide Cys Trp Leu-Asp-Val Cys inhibited VLA-4 dependent binding of lymphocytes to VCAM-1 and CS-1 (IC_{50} 1–3 μM) [23]. A selective inhibitor, Ph(2-Me)-NH-CO-NH-$C_6H_4CH_2CO$-Leu-Asp-Val-Pro (BIO-1211), blocked Jurkat cell binding to VCAM-immunoglobulin fusion protein (IC_{50} 1 nM) and was active in an antigen-induced bronchoconstriction and airway hyperresponsiveness model in sheep [24]. Several inhibitors without the Leu Asp Val sequence, 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 (each with a disulphide bridge) and analogues of a binding epitope in domain 1 of VCAM-1 (Arg^{76}-Thr-Gln-Ile-Asp-Ser-Pro-Leu-Asn^{83} region) have also been reported [25–28]. In an alternative approach, a conformationally restricted analogue of the VCAM-1 (37–43) peptide (Thr-Glu-Ile-Asp-Ser-Pro-Leu) was synthesized as VCAM-1 mimetic using a naphthalene-derived template [29]. However, some of these were not selective for VLA-4. Screening a β-turn mimetic library has resulted in weak inhibitors of VLA-4 mediated cell adhesion to the CS-1 peptide [30].

In our search for more potent and selective inhibitors, we have reported a series of inhibitors such as cyclo(Ile-Leu-Asp-Val-NH-$(CH_2)_n$-CO) **(1)** based upon the Leu-Asp-Val sequence present in CS-1. Compound **1** is a 5-fold more potent inhibitor of VLA-4-mediated cell adhesion than CS-1 [31]. The potency of this series varied only 3-fold when the linker in the cyclizing functionality varied from four to seven methylene groups. However, compounds containing two and three methylene groups were much less potent. The current study was undertaken to develop the structure–activity relationships of the cyclic peptide series represented by compound **1**. In addition, new cyclic monomeric peptides obtained by linking the side chain of an amino acid with the *C*-terminal carboxyl group and some dimeric peptides containing the Leu-Asp-Val sequence are reported. Improvement upon the original series was reflected in VLA-4 mediated cell adhesion assays and in a murine model of delayed type hypersensitivity (DTH). Because it was intended to administer the peptide based drug candidates by depot formulations capable of releasing the drugs over a period of 15–30 days, an additional aim was to investigate the compatibility of the more potent compounds with depot formulations based on poly(DL-lactic glycolic) acid polymers.

Abbreviations: Dip, piperazine; hDip, homopiperazine (1,4-diazacycloheptane); VLA-4, very late antigen-4; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular cell adhesion molecule-1; DTH, delayed type hypersensitivity.

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MATERIALS AND METHODS

Peptide Synthesis

The cyclic peptides listed in Tables 1 and 2 were obtained by the SPPS methods using 2-chlorotritylchloride resin. For the synthesis of cyclic peptides **1**–**27**, the linker group was attached to the resin and the synthesis completed by the general procedure exemplified in Scheme 1 for the synthesis of cyclo(Ile-Leu-Asp-Val-NH-$(CH_2)_3$-CO) **(1)**. Peptides were cyclized using HBTU. For the preparation of **28** and **32**, Fmoc NH $(CH_2)_2$ S CH_2 COOH and Fmoc NH $(CH_2)_2$-S-$(CH_2)_2$-COOH were synthesized by reacting 2- or 3-bromopropionic acid with 2-aminoethanethiol followed by a reaction with Fmoc-OSu. The Fmoc-amino acids were attached to the 2-chlorotritylchloride resin and the syntheses completed by the route shown in Scheme 1. Compounds **29**–**31** and **33**–**44** containing a dipeptide unit as a linking group were synthesized by standard SPPS procedures.

N^{α}-Fmoc-N^{τ}-(CH_2COOH) histamine, required for the preparation of **45**, was synthesized starting from histamine. N^{τ},N^{τ}-di-Boc-histamine, prepared by the reaction of di Bu^t dicarbonate and histamine, was reacted with DIPEA to give N^{τ}-Boc-histamine, which was then reacted with Bu^t-bromoacetate to give N^{τ} Boc-N^{τ}(CH_2COOBu^t) histamine. Similar reactions with this have been previously reported to produce N^{τ}-Boc and N^{τ}-CH_2-$COOBu^t$ derivatives [32–34]. Treatment of the protected histamine derivative with TFA gave N^{τ}(CH_2-COOH) histamine, which was converted to N^{α}-Fmoc N^{τ}-(CH_2 COOH) histamine and attached to the 2-chlorotritylchloride resin. Cyclic peptide **45** was then obtained using the procedure shown in

Table 1 Activity of the *N*- to *C*-terminally Linked Cyclic Peptides *In Vitro* (VLA-4 mediated MOLT-4 Cell Adhesion to Fibronectin or VCAM-1) and *In Vivo* (Murine Oval somal induced DTH)

Number	Compound	*in vitro* IC_{50} (μM)[a]		*in vivo* ID_{50}
		Fibronectin	VCAM-1	(mg/kg/day s.c.)[b]
CS1	DILPQLVTLPHPNLHGPGILDVPST	19	50	1.0
1	cyclo(Ile-Leu-Asp-Val-NH-$(CH_2)_5$-CO)	3.9	11	1.0
2	cyclo(Cha-Leu-Asp-Val-NH-$(CH_2)_4$-CO)	2.3	5.4	0.3
3	cyclo(t-Leu-Leu-Asp-Val-NH-$(CH_2)_5$-CO)	1.4	7	nt
4	cyclo(t-BuAla-Leu-Asp-Val-NH-$(CH_2)_5$-CO)	2.1	13	nt
5	cyclo(D-Ile-Leu-Asp-Val-NH-$(CH_2)_5$-CO)	1.0	1.9	nt
6	cyclo(D-Leu-Leu-Asp-Val-NH-$(CH_2)_5$-CO)	3.1	11	nt
7	cyclo(D-Val-Leu-Asp-Val-NH-$(CH_2)_5$-CO)	8.6	nt	nt
8	cyclo(Gly-Leu-Asp-Val-NH-$(CH_2)_5$-CO)	29	nt	nt
9	cyclo(Pro-Leu-Asp-Val-NH-$(CH_2)_5$-CO)	4.0	nt	nt
10	cyclo(MeAla-Leu-Asp-Val-NH-$(CH_2)_5$-CO)	3.5	21	nt
11	cyclo(MeLeu-Leu-Asp-Val-NH-$(CH_2)_5$-CO)	1.9	2.7	nt
12	cyclo(D-Ile-Leu-Asp-Val-NH-$(CH_2)_5$-CO)	1.1	5.6	0.2
13	cyclo(D-Leu-Leu-Asp-Val-NH-$(CH_2)_4$-CO)	4	17	nt
14	cyclo(MeIle-Leu-Asp-Val-NH-$(CH_2)_4$-CO)	0.21	0.63	0.03
15	cyclo(Ile-Nle-Asp-Val-NH-$(CH_2)_4$-CO)	6.3	11	nt
16	cyclo(Ile-t-buAla-Asp-Val-NH-$(CH_2)_4$-CO)	7.3	15	nt
17	cyclo(Ile-Cha-Asp-Val-NH-$(CH_2)_4$-CO)	14	13	nt
18	cyclo(Ile-Ile-Asp-Val-NH-$(CH_2)_4$-CO)	>100	nt	nt
19	cyclo(Ile-Val-Asp-Val-NH-$(CH_2)_4$-CO)	>100	nt	nt
20	cyclo(Ile-tertLeu-Asp-Val-NH-$(CH_2)_4$-CO)	>100	nt	nt
21	cyclo(Ile-D-Leu-Asp-Val-NH-$(CH_2)_4$-CO)	>100	nt	nt
22	cyclo(Ile-MeLeu-Asp-Val-NH-$(CH_2)_4$-CO)	>30	nt	nt
23	cyclo(Ile-Leu-Asn-Val-NH-$(CH_2)_4$-CO)	>100	nt	nt
24	cyclo(Ile-Leu-D-Asp-Val-NH-$(CH_2)_4$-CO)	>100	nt	nt
25	cyclo(Ile-Leu-Asp-Leu-NH-$(CH_2)_4$-CO)	4.5	7.3	nt
26	cyclo(Ile-Leu-Asp-Phe-NH-$(CH_2)_4$-CO)	>30	nt	nt
27	cyclo(Ile-Leu-Asp-D-Val-NH-$(CH_2)_4$-CO)	>100	nt	nt
28	cyclo(Ile-Leu-Asp-Val-NH-$(CH_2)_2$-S-$(CH_2)_2$CO)	4.0	7.5	0.003
29	cyclo(MeIle-Leu-Asp-Val-β-Ala-Pro)	0.17	0.57	0.009
30	cyclo(MeIle-Leu-Asp-Val-β-Ala-D-Ala)	0.37	0.56	0.2

Table 1 (Continued)

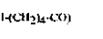
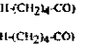
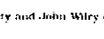
31	cyclo(MeIle-Leu-Asp-Val-D-Ala-D-Ala)	0.17	0.1	0.004
32	cyclo(D-Leu-Leu-Asp-Val NH-$(CH_2)_2$-S-CH_2CO)	5.8	3.1	0.01
33	cyclo(D-Leu-Leu-Asp-Val-Gly-Gly)	44	54	nt
34	cyclo(D-Leu-Leu-Asp-Val-β-Ala-β-Ala)	32	38	nt
35	cyclo(D-Leu-Leu-Asp-Val-β-Ala-Gly)	11	17	nt
36	cyclo(D-Leu-Leu-Asp-Val-β-Ala-D-Ala)	0.73	0.61	0.1
37	cyclo(D-Leu-Leu-Asp-Val-β-Ala-MeAla)	1.2	3.8	nt
38	cyclo(D-Leu-Leu-Asp-Val-β-Ala-Pro)	0.89	2.5	0.03
39	cyclo(D-Leu-Leu-Asp-Val-β-Ala-D-Pro)	0.56	nt	0.1
40	cyclo(D-Phe-Leu-Asp-Phe-β-Ala-D-Pro)	9.9	nt	nt
41	cyclo(D-Leu-Leu-Asp-Val-D-Ala-D-Ala)	1.9	5.9	0.03
42	cyclo(D-Phe-Leu-Asp-Val-D-Ala-D-Ala)	13	22	nt
43	cyclo(D-Leu-Leu-Asp-Val-Pro-Pro)	>30	nt	nt
44	cyclo(D-Leu-Leu-Asp-Val-Pro-D-Pro)	>30	nt	nt
45	cyclo(Ile-Leu-Asp-Val-NH-CH_2-CH_2-  CH_2-CO)	28	nt	0.3
46	cyclo(Ile-Leu-Asp-Val-NHCH_2-  CONH$(CH_2)_2$CO)	62	nt	nt
47	cyclo(Ile-Leu-Asp-Val-  CH_2CONH$(CH_2)_2$CO)	3.3	5.1	0.03
48	cyclo(Ile-Leu-Asp-Val-  CH_2CONH$(CH_2)_2$CO)	10	13	0.007
49	cyclo(MeIle-Leu-Asp-Val-  -CH_2CONH$(CH_2)_2$SCH$_2$CO)	0.69	2.0	0.006
50	cyclo(MePhe-Leu-Asp-Val-  CH_2CONH$(CH_2)_2$SCH$_2$CO)	0.22	0.24	0.01
51	cyclo(MeIle-Leu-Asp-Val-D-Arg-  -CH_2CO)	0.20	0.26	0.07
52	cyclo(MeIle-Leu-Asp-Val-  CH_2CO-D-Arg)	0.26	1.6	0.02
53	cyclo(MeIle-Leu-Asp-Val-  -CH_2CONH$(CH_2)_2$CO-D-Arg)	0.32	1.1	0.02
54	cyclo(MeIle-Leu-Asp-Val-  CH_2CONH$(CH_2)_2$CO-D-Arg)	1.2	1.4	0.03
55	cyclo(Ile-Leu-Asp-Val-  CH_2CO-Ile-Leu-Asp-Val- CH_2CO)	17	15	0.009
56	cyclo(MeIle-Leu-Asp-Val-  -CH_2CO-Ala-Ile-Leu-Asp-Val- CH_2CO)	2.9	5.4	0.008

Table 1 (Continued)

57	cyclo(MePhe-Leu-Asp-Val-N⌒N-CH_2CO-MePhe-Leu-Asp-Val-N⌒N-CH_2CO)	0.52	0.47	0.01
58	cyclo(MePhe-Leu-Asp-Val-N⌒N-CH_2CO-MePhe-Leu-Asp-Val-N⌒N-CH_2CO)	0.44	2.9	0.007

[a] Each value represents the midpoint of a curve fitted to a concentration response for each compound carried out in triplicate.
[b] Dose required to give half the maximal inhibition. 40 ± 2%, mean ± S.E.M. (n = 24) obtained with anti α_4 integrin monoclonal antibody PS 2 (7.5 mg/kg i.v.)
Each value represents the midpoint of a curve fitted to a dose response for each compound with each dose group containing between four and ten mice. The peptides were administered by subcutaneous osmotic mini-pimps.
nt = not tested.

Scheme 1. For the synthesis of **46**, Fmoc aminomethylbenzoic acid was first attached to the 2 chlorotritylchloride resin and the synthesis completed by the route shown in Scheme 1.

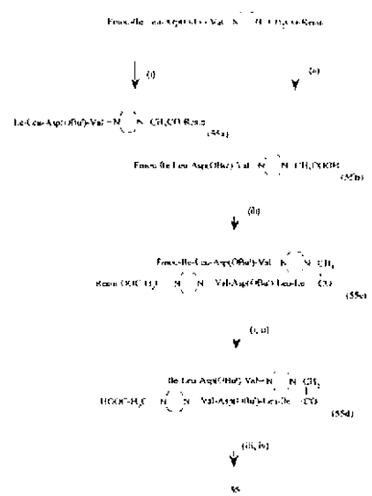
The piperazine (Pip) containing compounds **47–54** were synthesized starting from 1 Pip CH_2COOH. Commercially available *N*-Boc-Pip was reacted with Bu^t-bromoacetate and the resulting Boc Pip CH_2COOBu^t derivative was deprotected and converted to Fmoc Pip-CH_2COOH. The Fmoc derivative was attached to the 2 chlorotritylchloride resin and the synthesis completed by the route shown in Scheme 1. The dimeric compounds containing a Pip or a homopiperazine (hPip) linking group (**55–58**) were prepared either by the cyclization of linear monomeric peptides (**55**) or by the cyclization of linear dimeric derivatives (**55**, **56–58**). In the first route, cyclization of Ile Leu Asp(OBu^t) Val Pip

Table 2 Activity of Cyclic Peptides Containing an Amide Bond between the Side Chain Amino Group of the Lys, Orn or Dab Residues and the Carboxyl Group of the Val Residue *In Vitro* (VLA-4-mediated MOLT-4 cell Adhesion to Fibronectin or VCAM-1) and *In Vivo* (Murine Ovalbumin-induced DTH).

No.	Compound	*In vitro* IC_{50} (μM)[a]		*In vivo* ED_{50}
		Fibronectin	sVCAM-1	(mg/kg/day s.c.)[b]
59	Ac-cyclo(Lys-Ile-Leu-Asp-Val)	3.3	7.9	nt
60	Ac-cyclo(D-Lys Ile-Leu-Asp-Val)	4.3	19	nt
61	Ac-cyclo(Lys-D-Ile-Leu Asp Val)	9.3	8.8	nt
62	Ac-cyclo(D-Lys-D-Ile-Leu-Asp-Val)	2.8	6.9	0.005
63	Ac-cyclo(D-Orn-D-Ile-Leu-Asp-Val)	2.3	nt	nt
64	Cyclo(D-Lys-D-Ile-Leu-Asp-Val)	1.9	4.3	nt
65	Z-cyclo(D-Lys-D-Ile-Leu-Asp-Val)	2.4	4.3	nt
66	Propionyl-cyclo(D Lys D-Ile-Leu-Asp-Val)	1.6	2.5	0.3
67	HOOC-$(CH_2)_4$-CO-cyclo(D-Lys-D Ile Leu-Asp Val	1.3	2.0	nt
68	H_2N-$(CH_2)_4$-CO-cyclo(D-Lys-D-Leu-Leu-Asp-Val)	7.8	16	0.02
69	N^1 benzyl cyclo(D Lys D Leu-Leu-Asp Val)	2.3	2.8	nt
70	Pyr-cyclo(D Lys D-Leu Leu Asp Val)	6.8	16	0.03
71	3-pyridylcarbonyl-cyclo(D-Lys D Leu Leu Asp Val)	7.9	nt	nt
72	Ac-cyclo(Orn Leu Asp Val)	>200	nt	>0.3

[a] Each value represents the midpoint of a curve fitted to a concentration response for each compound carried out in triplicate.
[b] Dose required to give half the maximal inhibition. 40 ± 2%, mean ± S.E.M. (n = 24), obtained with anti α_4 integrin monoclonal antibody PS/2 (7.5 mg/kg i.v.)
Each value represents the midpoint of a curve fitted to a dose response for each compound with each dose group containing between four and ten mice. The peptides were administered by subcutaneous osmotic mini-pimps.
nt = not tested.

CH_2COOH (Scheme 1. Starting from Fmoc Pip CH_2COOH] gave only the dimeric peptide **55**. In the second route (Scheme 2), one part of the Fmoc-Ile Leu Asp(OBu^t) Val Pip CH_2CO resin was treated with piperidine to give Ile-Leu Asp(OBu^t) Val Pip CH_2CO resin (**55a**) and another was treated with a mixture of acetic acid trifluoroethanol to give Fmoc Ile-Leu-Asp(OBu^t)-Val Pip-CH_2COOH (**55b**). Coupling of **55b** to **55a** gave the fully protected octapeptide derivative **55c**, which was deprotected at the *N* terminal end and cleaved from the resin to give **55d**. Cyclization and deblocking gave **55**. An alternative route avoiding the synthesis of Fmoc hPip CH_2COOH was devised for the preparation of **58**. 3-Bromopropionic acid was attached to the chlorotritylchloride resin, followed by a treatment with excess hPip to generate hPip-CH_2CO-resin. Additional reactions similar to those used for **55** (Scheme 2) gave Melle Leu Asp(OBu^t)-Val hPip CH_2CO Melle Leu Asp(OBu^t) Val hPip CH_2COOH.



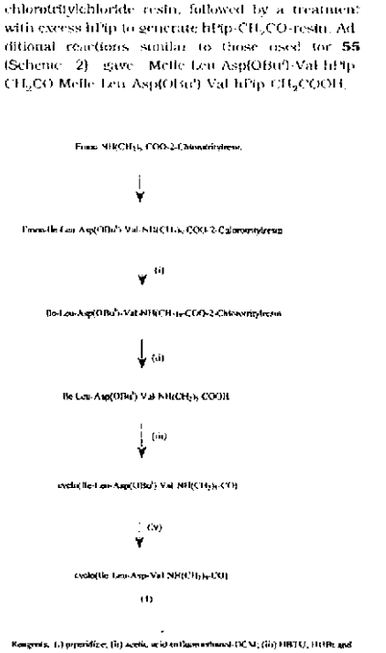
Scheme 2 Synthesis of compound **55**. Route 2.

which was cyclized, deprotected and purified to give the dimeric compound **58**.

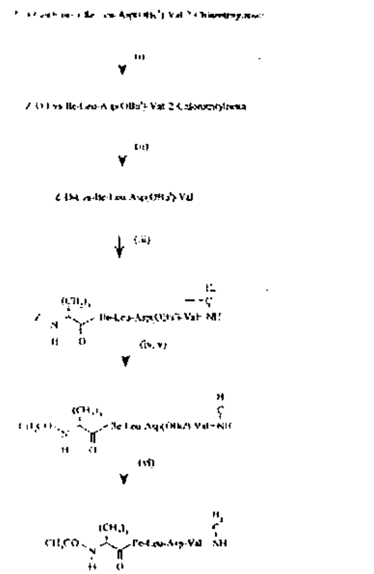
Cyclic peptides containing an amide bond between the Val carboxyl group and the side chain amino group (**59–72**) were synthesized by the route shown in Scheme 3. Following chain assembly and deprotection of the side chain amino group, the peptide was cleaved from the resin and cyclized. The *Z* group was then cleaved by hydrogenolysis and the amino group was converted to the required acyl, alkyl or aryl group. Finally, the Asp(OBu^t) was cleaved to give the desired peptide.

In Vitro Cell Adhesion Assays

The effects of peptides on MOLT-4 cell adhesion to fibronectin were measured using a colorimetric acid phosphatase assay on 96 well plates as was previously described except that the plates were coated with 10 μg/ml fibronectin (prepared from human



Scheme 3 Synthesis of compound **1**



Reagents: [illegible]

Scheme 3 Synthesis of compound **60**.

plasma by D. Barratt, AstraZeneca) [31]. MOLT-4 cell adhesion to VCAM-1 was carried out using the same method except that the 96-well plates were coated with 0.05 μg/ml recombinant human VCAM-1 (D. Barratt, AstraZeneca). Assays to measure the effects of peptides on VLA-5- and LFA-1-mediated cell adhesion and ADP-induced aggregation of human platelets were carried out as previously described [31,35] except that, in the VLA-5 assay, U937 cell adhesion to wells coated with 10 μg/ml fibronectin was measured.

Ovalbumin DTH

Animal welfare and experimental procedures were carried out strictly in accordance with the Animals (Scientific Procedures) Act, 1986 and the Zeneca International Policy on the Use of Animals. Ovalbumin DTH responses were induced and measured in Balb/c mice as previously described [31].

Peptide Stability Studies

Solutions (0.1 mg/ml) of the peptides were prepared in pH 7.6 and pH 3 MacIlvaines buffer and filter sterilized in a class II cabinet. Aliquots were transferred to sterile 2 ml screw cap HPLC vials in two sets. One set was stored at 37°C and the other at −18°C. At suitable intervals, the peptide content was analysed by HPLC using Hewlett Packard's 1050 HPLC machine and a Vydac 218TP54 C_{18} column equipped with 218TP 5 micron guard column. The stability of the peptide was assessed after a suitable period, typically 4 weeks, and is presented as percentage of peptide remaining. The columns were eluted using a gradient from 100% solvent A (20% Far UV acetonitrile (HPLC grade), 80% milli-Q water plus 0.1% TFA) to 100% solvent B (60% Far UV acetonitrile (HPLC grade), 40% milli-Q water plus 0.1% TFA) over a 20 min period. Reversal was then carried out to establish 100% A. The columns were run at 50°C and the peptide was detected at 230 nm.

Preparation of Monolithic Depots and Determination of the Dissolution Profile

Both the peptide (acetate salt, 20 mg) and the polymer (80 mg) were dissolved in anhydride free glacial acetic acid (1 ml) and the solution was frozen dropwise into liquid nitrogen. The frozen material was freeze dried and stored under vacuum over silica gel and anhydrous K_2CO_3 for 3 days prior to extrusion. The freeze dried material was loaded into the barrel of a 100 mg laboratory extruder with a 1-mm nozzle. The temperature was raised slowly to around 90°C and the sample was extruded when it was possible to achieve this with a reasonable pressure being applied (usually between 92 and 95°C). The extrudate was cut into small depots and stored under vacuum. A weighed (5–10 mg) sample of the depot was placed in sterile filtered MacIlvaines buffer (pH 7.6, 2 ml) and the vial was sealed and stored at 37°C. At regular intervals, a part of the supernatant (1.8 ml) was removed and replaced with 1.8 ml of fresh buffer. The concentration of peptide in the supernatant was analysed by HPLC.

RESULTS AND DISCUSSION

VLA-4-mediated Cell Adhesion (Tables 1 and 2)

We previously reported that screening for inhibition of VLA-4 mediated MOLT-4 cell adhesion to

fibronectin *in vitro* led to the discovery that cyclic tetrapeptides (**1** and **2**) containing a relatively flexible linking group were about 5–10 fold more potent than CS-1 [31]. However, replacing the -NH(CH$_2$)$_5$CO- moiety in **1** with β-Ala gave an inactive compound cyclo(Ile-Leu-Asp-Val-NHCH$_2$CH$_2$CO) (IC_{50} > 200 μM). The contribution of the amino acid side chains to the inhibition of MOLT-4 cell adhesion to fibronectin by this series of compounds was investigated by amino acid substitutions in compounds **1** and **2**. Compound **1** was used as a positive control and its potency was similar to that previously reported [31]. Replacement of the Ile by L-, D- and *N*-Me amino acids (**3**–**14**) showed that the *t*-Leu, Bu^t-Ala, Pro, MeAla, MeLeu, MeIle, D-Leu, D-Ile and D-Val containing analogues had similar potency or were more potent than the parent peptides **1** and **2**. The Gly analogue **8** lacking the side chain was 7-fold less potent than **1**. In comparison with the D-Ile analogues **5** and **12**, the corresponding D-Leu (**6**, **13**) and D-Val (**7**) analogues were 4–8-fold less potent. The most potent compound of the series, cyclo(MeIle-Leu-Asp-Val-NH(CH$_2$)$_4$CO) (**14**), was about 10-fold more potent than **2**. In comparison with the Ile, the Leu, Asp and Val were more important for activity. Replacement of the Leu by norleucine (Nle), Bu^t-Ala and cyclohexylalanine (Cha) (**15**–**17**) led a to 3–6 fold loss in potency. The Ile, Val, *t*-Leu, D-Leu and MeLeu analogues (**18**–**22**) were much less potent (IC_{50} > 30 μM). Replacement of the Asp by Asn (**23**) or D-Asp (**24**) also led to a significant loss in activity. Although activity was retained when Val was replaced by Leu (**25**), the Phe (**26**) or D-Val (**27**) analogues were much less potent.

To induce further conformational changes, the -NH-(CH$_2$)$_n$-CO- groups in the cyclic peptides were replaced by natural and unnatural amino acids and non-peptidic residues. Compounds with -NH-(CH$_2$)$_2$S(CH$_2$)$_2$-CO- and -NH(CH$_2$)$_2$SCH$_2$CO- linking groups (**28**, **32**), which allow structures with similar size and flexibility, had similar potency to the parent peptides **1** and **13**, respectively. Dipeptide, e.g. Gly-Gly, β-Ala-β-Ala and β-Ala-Gly (**33**–**35**), containing analogues with an additional amide bond in the linker and no amino acid side chains were 3–10-fold less potent. More potent compounds (**29**–**31**, **37**–**39** and **41**) were obtained when conformationally restricting Pro, D- or *N*-Me amino acids were introduced in the linking groups. The analogues cyclo(MeIle-Leu-Asp-Val-β-Ala-Pro) (**29**), cyclo(MeIle-Leu-Asp-Val-β-Ala-D-Ala) (**30**) and cyclo(MeIle-Leu-Asp-Val-D-Ala-D-Ala) (**31**) were comparable in potency with cyclo(MeIle-Leu-Asp-Val-NH-(CH$_2$)$_4$-CO) (**14**). The D-Leu containing analogues **36**–**39** were at least 3-fold more potent than **13** and at least 40-fold more potent than the corresponding peptide containing a Gly-Gly linker (**33**). The most potent of these peptides (**29** and **31**) were approximately 100 times more potent than CS-1. Replacing D-Leu with D-Phe in the analogues containing β-Ala-Pro (**40**) and D-Ala-D-Ala (**41**) linking groups produced compounds that were 7–18-fold less potent than the parent compounds. More conformationally restricted peptides containing two Pro residues (**43** and **44**) were inactive at 30 μM.

Although peptides containing either a histamine derivative (**45**) or *p*-aminomethylbenzoic acid (**46**) as the linking group were less potent than CS-1, incorporation of a Pip-CH$_2$CO- residue in the linking group along with a -NH-(CH$_2$)$_n$-CO- group (**47**, **48**), a -NH(CH$_2$)$_2$-S-CH$_2$CO- group (**49**, **50**) or additional amino acid residues (**51**–**54**) resulted in more potent compounds. Compound **47**, which contained a -Pip-CH$_2$CO-NH(CH$_2$)$_2$-CO- linking group, was equipotent to **1**. Further ring enlargement by incorporating a -Pip-CH$_2$-CO-NH(CH$_2$)$_4$-CO- group (**48**) resulted in a 3-fold reduction in potency. Replacement of the Ile with *N*-Me amino acids in the Pip containing compounds resulted in more potent compounds such as cyclo(MeIle-Leu-Asp-Val-Pip-CH$_2$-CO-NH(CH$_2$)$_2$-S-CH$_2$-CO) (**49**) and cyclo(MePhe-Leu-Asp-Val-Pip-CH$_2$-CO-NH(CH$_2$)$_2$-S-CH$_2$-CO) (**50**). Comparison of cyclo(Ile-Leu-Asp-Val-Pip-CH$_2$CO-NH(CH$_2$)$_4$-CO) (**48**) and cyclo(MeIle-Leu-Asp-Val-Pip-CH$_2$CO-NH-(CH$_2$)$_2$-S-CH$_2$CO) (**49**), both with the same size ring, showed the MeIle compound to be 14-fold more potent than the Ile analogue. The Pip and hPip containing cyclic dimeric compounds (**55**–**58**) also inhibited VLA-4-mediated cell adhesion. Although the Ile analogue (**55**) inhibited MOLT-4 cell adhesion to fibronectin with a similar potency to CS-1, the MeIle (**56**) and MePhe (**57**) analogues were 6- and 30-fold more potent than CS-1, respectively. Replacement of the Pip ring in the MeIle analogue (**56**) with a hPip ring (**58**) further improved potency 6-fold.

The structure–activity relationship (SAR) in the side chain to C-terminal cyclic peptides (Table 2) was broadly similar to the *N*- to *C*-terminal cyclic peptides. Most of the compounds containing 18–19 atoms in the ring inhibited VLA-4 mediated cell adhesion. Deletion of the Ile led to an inactive compound (**72**, IC_{50} > 200 μM). The chirality of the Lys, ornithine (Orn) or diaminobutyric acid (Dab) residues did not have any significant effect on the activity (**59**–**65**). Analogues containing D- or L-Lys, Orn or Dab residues were similar in potency, e.g. Ac-cyclo(Lys-Ile-Leu-Asp-Val) (**59**) and Ac-cyclo(D-Lys-Ile-

Leu Asp Val) (60). The N-terminal Ac group in the Ac-cyclo(D-Lys-D-Ile-Leu-Asp-Val) and Ac-cyclo(D-Lys-D-Leu-Leu-Asp-Val) series of compounds could be eliminated or replaced by a number of substituents (64–71) without loss of activity (IC_{50} 1.6–7.9 μM).

A number of compounds were tested to see whether they also inhibited VLA-4-mediated MOLT-4 cell adhesion to the vascular endothelial ligand VCAM-1. Compounds that inhibited MOLT-4 cell adhesion to fibronectin also inhibited adhesion to VCAM-1. Most compounds were, like CS-1, slightly more potent against fibronectin than VCAM-1. This probably reflects the higher affinity of VLA-4 for VCAM-1 than for fibronectin. While a few compounds were >3 fold more potent against fibronectin, no compound was significantly more potent against VCAM-1. The SAR in the N- to C-terminal cyclized monomeric and dimeric peptides and the side chain to C-terminal cyclized peptides was similar to that for adhesion to fibronectin. The most potent inhibitor of MOLT-4 cell adhesion to VCAM-1 (cyclo(MeIle-Leu-Asp-Val-D-Ala-D-Ala) (31)) was 500 fold more potent than CS-1.

Selectivity Against Various Other Integrins (Table 3)

The selectivity of several of the cyclic monomeric (12, 31, 51–53 and 62) and dimeric peptides (55–57)

Table 3 Effect of Selected Compounds on Cell Adhesion-mediated by VLA-5 (U937 Cell Adhesion to Fibronectin), LFA-1 (PMA treated U937 Cell Adhesion to ICAM-1 Transfected CHO Cells) and GPIIb/IIa (ADP-induced Human Platelet Aggregation)

Compound	% Inhibition at 300 μM		GPIIb/IIa
	VLA-5	LFA-1	Dose ratio shift at 100 μM[a]
12	20	28	1.1
31	38	0	nt
51	29	31	nt
52	29	8	nt
53	12	19	nt
55	38	33	1.3
56	9	26	1.5
57	1	4	1.5
62	27	37	1.1

[a] Ratio of the concentration of ADP required for half maximal change in light transmission (EC_{50}) in the presence and absence of the compound.
nt not tested.

was assessed by measuring their effects on cell adhesion mediated by another β_1 integrin, VLA-5 ($\alpha_5\beta_1$), which binds to an Arg-Gly-Asp motif in fibronectin (U937 cell adhesion to fibronectin), and the β_2 integrin, LFA-1 ($\alpha_L\beta_2$), which binds to the vascular endothelial cell ligand intercellular cell adhesion molecule 1 (ICAM-1) (PMA differentiated U937 cell adhesion to ICAM-1 expressing CHO cells). While these compounds inhibited MOLT-4 cell adhesion to fibronectin in the range 0.17–17 μM, each compound gave less than 50% inhibition of VLA-5 and LFA-1 mediated cell adhesion at concentrations up to 300 μM. The most potent compound (31), cyclo(MeIle-Leu-Asp-Val-D-Ala-D-Ala), was more than 1000 fold selective. In addition, compounds 12, 55–57 and 62 had no effect on ADP-induced human platelet aggregation when tested at 100 μM, indicating that they do not block the interaction of the platelet integrin GPIIb/IIIa ($\alpha_{IIb}\beta_3$) with its ligand fibrinogen.

In Vivo Activity (Tables 1, 2 and 4)

Selected peptides were tested in vivo in a mouse inflammation model, ovalbumin-induced DTH. DTH responses are initiated by activation of memory T-lymphocytes by specific antigens, leading to an inflammatory response 24–48 h after antigen exposure. Although the cellular infiltrate in human DTH responses are predominantly mononuclear, most leukocytes recruited to the site of inflammation in mouse ovalbumin-induced DTH are neutrophils. The recruitment of these neutrophils is associated with an acute inflammatory response to ovalbumin, which is characterized by oedema that has subsided by 24 h. We previously showed that the oedema measured at 24 h post-ovalbumin injection is largely dependent on T-lymphocyte priming [31]. We used the anti-mouse α_4 integrin monoclonal antibody PS/2 to demonstrate that approximately 40% of the oedema measured at 24 h is dependent on α_4 integrins (10 ± 2% inhibition at 7.5 mg/kg i.v.).

Initially, the peptides were administered by continuous infusion from subcutaneous osmotic minipump starting 24 h before ovalbumin challenge. The potency of the cyclic peptides was compared with CS-1. Potent inhibitors of VLA-4-mediated cell adhesion in vitro were also potent inhibitors of mouse ovalbumin DTH (Tables 1 and 2 and Figure 1). The maximal inhibition achieved with each peptide was similar to the maximum inhibition by PS/2 in the same experiments. In addition, cyclic peptides cyclo(Ile-Leu-Asp-Val-NH(CH$_2$)$_5$CO) [31] and Ac-cyclo(Orn-Leu-Asp-Val) (72), which did not inhibit

Table 4 Inhibition of Mouse Ovalbumin DTH by Cyclic Peptides after Dosing at 1 mg/kg, i.v. 4 h Pre-antigen Challenge or 20 h Post-antigen Challenge

Compound	Inhibition of foot swelling (% of the maximal PS/2 response)			
	Dosing 4 h pre-antigen challenge		Dosing 20 h post-antigen challenge	
1	10 ± 8.9	NS	52 ± 24	p < 0.01
28	17 ± 9.8	NS	33 ± 22	NS
48	41 ± 19	p < 0.05	62 ± 23	p < 0.01
56	52 ± 31	NS	63 ± 11	p < 0.01
57	30 ± 14	NS	71 ± 12	p < 0.01
62	11 ± 16	NS	82 ± 7.3	p < 0.001
68	26 ± 13	NS	19 ± 11	p < 0.05

Each group contained five mice.
NS not significantly different from saline dosed control group.

VLA-4-mediated cell adhesion in vitro at concentrations up to 200 μM, were inactive in vivo at the highest doses tested (10 and 0.3 mg/kg/day, respectively). These findings suggest that the inhibitory effects in DTH are a consequence of VLA-4 inhibition, although a contribution from inhibiting the integrin $\alpha_4\beta_7$, also found on some leukocytes,

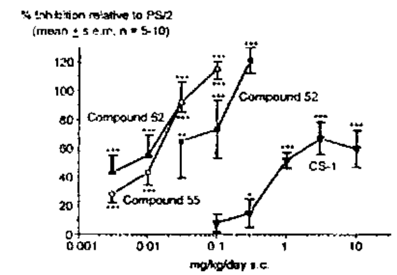


Figure 1 Inhibition of mouse ovalbumin DTH by representative N- to C-terminally linked monomeric (52) dimeric (55) and side chain linked (62) VLA-4 inhibitor cyclic peptides. Each compound was dosed continuously from osmotic mini pumps implanted subcutaneously in mice that had been immunized with ovalbumin 6 days earlier. After a further 24 h, the mice were challenged with ovalbumin into the right footpad and the inhibition of foot swelling was measured 24 h later. Results are expressed as mean ± S.E.M. relative to the inhibition by the anti-mouse α_4 integrin monoclonal antibody, PS/2 (7.5 mg/kg, i.v.), used as a positive control in each experiment. A dose response to CS-1 peptide is included for comparison. Each dose group contained five to ten mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

cannot be ruled out. The most potent compounds in vivo (ED_{50} < 0.02 mg/kg/day) included examples from both the N- to C-terminal and the side chain to C-terminal series of cyclic peptides (28, 29, 31, 32, 48–50, 52, 53, 55–58, 62 and 68) and were more potent than CS-1 both in vitro and in vivo. For example, cyclo(MeIle-Leu-Asp-Val-β-Ala-Pro) (29) and cyclo(MeIle-Leu-Asp-Val-D-Ala-D-Ala) (31), which were 100–500 fold more potent than CS-1 in vitro, were 100–250 fold more potent than CS-1 in vivo (ED_{50} 0.009 and 0.004 mg/kg/day, respectively). Similarly, compounds 38, 49, 50, 52, 53, 57 and 58, which were 20–100-fold more potent than CS-1 in vitro, were 50–200-fold more potent in ovalbumin DTH. However, some compounds such as cyclo(Ile-Leu-Asp-Val-NH(CH$_2$)$_2$-S-(CH$_2$)$_2$CO) (28), cyclo(Ile-Leu-Asp-Val-Pip-CH$_2$CO-Ile-Leu-Asp-Val-Pip-CH$_2$CO) (55) and Ac-cyclo(D-Lys-D-Ile-Leu-Asp-Val) (62) were more potent in vivo (100–300-fold that of CS-1) than would be predicted from the in vitro potency (equipotent to 7 fold that of CS-1). In these cases, the differences in the in vitro and the in vivo profiles are likely to be the result of the differences in bioavailability and pharmacokinetics of the peptides. Despite the peptides being administered continuously, factors such as stability, protein binding and rates of clearance will lead to different steady-state concentrations of the peptides in the blood.

In order to obtain information about the duration of action of the cyclic peptides, selected compounds were also dosed by bolus intravenous injection at different time intervals before measuring the DTH response. When dosed at 1 mg/kg, 20 h after ovalbumin challenge (4 h before measurement of oedema), each peptide inhibited the inflammatory

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response (40–80% of the maximal PS-2 response) and Ac-cyclo(Lys-D-Ile-Leu-Asp-Val), (**62**) was the most potent (Table 4). In contrast, compounds **1**, **28**, **58**, **57**, **62** and **68** did not inhibit the inflammatory response when dosed at 1 mg/kg, 4 h before oval bumin challenge. Only cyclo(Ile-Leu-Asp-Val-Dip-CH_2CO-NH-$(CH_2)_4$-CO) (**48**) showed statistically significant activity when dosed at this time. Thus, most of the cyclic peptides do not appear to display extended duration of action and may require dosing by continuous infusion if they are to be clinically effective as anti-inflammatory agents.

Formulation Studies

The possibilities of achieving sustained delivery systems based on the lactide/glycolide co-polymers were investigated using cyclic peptides Ac-cyclo(D-Lys-D-Ile-Leu-Asp-Val) (**62**) and cyclo(CH$_2$CO-Ile-Leu-Asp-Val-Dip-CH_2CO-Ile-Leu-Asp-Val-Dip) (**55**). Extended release of peptides from such depot formulations is dependent on several factors [36–40]. Depending on the period of delivery, enough compound has to be incorporated into the polymer. This has implications for the total amount of the drug/polymer combination that can be administered in a single injection. The peptide also has to be stable to the experimental conditions used to prepare the depots formulation and for several months afterwards before it is administered to the patients. Although **55** and **62** could be incorporated (20%) into a 20 kDa 1:1 poly(DL-lactide-co-glycolide) polymer without significant degradation and both were stable in pH 7.6 buffer (37°C, 1 month), both were degraded significantly (50 and 69%, respectively, remaining intact after 1 month) at pH 3. The release profiles (Figure 2) indicated that both peptides were released nearly quantitatively in < 4 days. Owing to the instability of the peptides at pH 3, no further work was carried out to modify the release profile by altering the nature of the polymer (e.g. molecular weight and composition). Stability at pH 3 is very important because the preparation of the depots requires acetic acid and because lactic and glycolic acids are generated *in vivo* in the vicinity of the peptide when the poly(DL-lactide-co-glycolide) polymer is degraded.

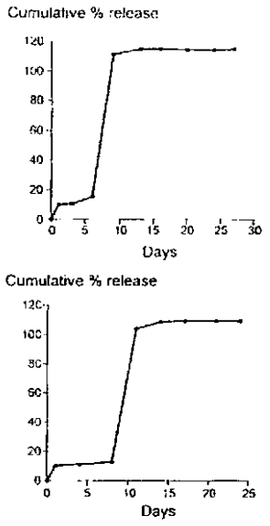

Figure 2. Release profiles of (a) Ac-cyclo(D-Lys-D-Ile-Leu-Asp-Val) (**62**) and (b) cyclo(CH$_2$CO-Ile-Leu-Asp-Val-Dip-CH_2CO-Ile-Leu-Asp-Val-Pip) (**55**) from poly(DL-lactide-co-glycolide) depots *in vitro*

CONCLUSIONS

Cyclic peptides containing one or two Ile-Leu-Asp-Val tetrapeptides – obtained either by linking the *N*- and C-terminal ends by using a linking group, e.g. -NH$(CH_2)_4CO$- or -Pip-CH_2CO-, or by forming an amide bond between the C-terminal Val carboxyl group and the side chain amino groups of the *N*-terminal Lys, Orn or Dab residues – are potent inhibitors of VLA-4 mediated MOLT-4 adhesion *in vitro* and ovalbumin DTH *in vivo* when dosed continuously from a subcutaneous osmotic mini pump. However, the peptides do not display an extended duration of action in the DTH model when administered as a bolus injection. Replacement of the Ile by *N*-methyl or D-amino acids (e.g. MeIle, D-Ile) leads to more potent compounds, however, the Leu, Asp and Val residues can only be replaced by amino acids with similar side chains. *In vitro* studies indicate that only peptides that inhibit VLA-4-mediated cell adhesion *in vitro* are active in the DTH model, which is dependent on antigen induced activation of T lymphocytes. This, in addition to the observation that the maximum inhibition of the DTH response by the peptides was equivalent to that of the anti-α_4 monoclonal antibody PS-2, suggests that the *in vivo* activity of the peptides was the result of 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 ruled out. Based on this evidence, the peptides may be useful for the treatment of a number of inflammatory and autoimmune diseases. However, further improvement in stability (pH 3) and other formulation characteristics will be required if the compounds are to be administered in slow release formulations. Studies in progress have resulted in more potent and stable compounds with much improved release profiles from the depot formulations [41].

EXPERIMENTAL SECTION

The cyclic monomeric and dimeric peptides were prepared by the SPPS procedure using 2-chlorotrityl-chloride resin. Details of the synthetic procedures are only given for a few compounds. In other cases, variations from the standard route are highlighted. Following assembling on the resin, the linear peptides were cleaved from the resin and used in subsequent steps without purification. The final products were purified by RP-HPLC before characterization. The homogeneity of the purified peptides, obtained as freeze-dried powders, was checked using analytical HPLC and, in many cases, using CZE. The gradient systems used in the preparative and analytical RP-HPLC (C_{18}) were mixtures of water and acetonitrile containing 0.1% TFA. TLC was carried out on silica gel plates.

Asp-Glu-Leu-Pro-Gln-Leu-Val-Thr-Leu-Pro-His-Pro-Asn-Leu-His-Gly-Pro-Glu-Ile-Leu-Asp-Val-Pro-Ser-Thr (CS-1). This was synthesized on the Wang resin using N^{α}-Fmoc protected amino acid derivatives and standard deblocking and coupling procedures. The Asp and Glu side chains were protected as But esters and the His side chain was protected by a trityl group. The crude peptide was purified first by ion exchange chromatography (Hydropore SCX column, Rainin Instruments Company, USA, pH 3.0) and then by HPLC (Vydac column, The Separations Group, USA, 218TP1022, 22 × 250 mm) using a gradient system (10–50%, 45 min, flow rate 10 ml/min). The purified product was checked for homogeneity using analytical RP-HPLC (Vydac 218TP54, 4.6 × 250 mm column; retention time 26.87 min (10–40% gradient, 30 min) and 19.39 min (10–60%, 30 min)). Amino acid analysis following acid hydrolysis: Asp 4.06 (4), Thr 1.60 (2), Ser 0.75 (1), Glu 3.02 (3), Pro 5.0 (5), Gly 0.99 (1), Val 1.95 (2), Ile 0.94 (1), Leu 5.07 (5), His 1.99 (2). (M + H)$^+$ 2732.7. The peptide was also characterized by full sequence analysis using an Applied Biosystems instrument.

Cyclo(Ile-Leu-Asp-Val-NH-$(CH_2)_5$-CO) (1) (Scheme 1). 2-Chlorotritylchloride resin (Nova Biochem; 1.6 mmole Cl/g; 1 g), swollen in dry DCM (10 ml) for 5 min was reacted with Fmoc-NH-$(CH_2)_5$-COOH (355 mg, 1 mmole) and DIPEA (560 µl, 3.2 mmole) in DCM (5 ml) for 15 min. Methanol (0 ml) and DIPEA (1 ml) were added and, after 5 min, the resin was collected by filtration, washed successively with DCM, DMF, DCM, isopropanol and ether, dried at 50°C in a vacuum oven (weight 1.33 g) 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. (b) Acylation with Fmoc-Val (678 mg, 2 mmole) activated with HBTU (760 mg, 2 mmole) and DIPEA (700 µl, 4 mmole) in DMF (4 ml) for 1 h followed by five washes with DMF to remove excess reagents. The deprotection and coupling cycles were repeated using Fmoc-Asp(OBu^t) (822 mg, 2 mmole), Fmoc-Leu (700 mg, 2 mmole) and Fmoc-Ile (700 mg, 2 mmole) to give Fmoc-Ile-Leu-Asp(OBu^t)-Val-NH-$(CH_2)_5$-COO-resin. The Fmoc group was cleaved (as above) and the peptide resin was washed successively with DMF, DCM and ether and dried in a vacuum oven at 50°C (weight 1.51 g).

The Ile-Leu-Asp(OBu^t)-Val-NH-$(CH_2)_5$-CO_2 resin was suspended in a mixture of acetic acid: trifluoromethanol:DCM (2:2:6, 25 ml) for 2 h to cleave the peptide from the resin. The resin was filtered off, washed with the above solvent mixture and the combined filtrates were evaporated. The residue was triturated with ether to give Ile-Leu-Asp(OBu^t)-Val-NH-$(CH_2)_5$-COOH as an acetate salt (428 mg). (M + H)$^+$ 628.4, (M + Na)$^+$ 650.5. The acetate salt was converted to a HCl salt by dissolving it in a mixture of water:acetonitrile (2:1, 60 ml), cooling to 0°C, adding 1.05 equivalents of 1N HCl and freeze-drying the contents. A part of the linear peptide hydrochloride (190 mg, 0.288 mmole) was dissolved in DMF (300 ml) and HBTU (109 mg, 0.288 mmole), HOBt (45 mg, 0.288 mmole) and

DIPEA (1.17 µl, 0.86 mmole) were added to the solution. After stirring for 3 h at room temperature (RT) the solvent was evaporated to dryness *in vacuo* and the residue, in ethyl acetate, was washed successively with 1 M citric acid, saturated NaCl, 10% $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$ and the solvent evaporated to dryness *in vacuo*. The crude product was collected, dried over P_2O_5/KOH (125 mg; retention time 20.71 min [Vydac 218TP54 column] (20–80% gradient, 30 min at a flow rate of 1.0 ml/min)) and used in the next step without any purification.

The peptide, cyclo(Leu-Asp(OBu^t)-Val-NH-$(CH_2)_6$-CO) (125 mg, 0.2 mmole), was treated for 30 min with a mixture of TFA:water (95:5, 15 ml) and triisopropylsilane (200 µl) to remove the Asp side chain protecting group. Evaporation to a small volume followed by trituration with ether yielded the crude cyclic peptide (75 mg), which was purified by RP-HPLC (Vydac 218TP1022 column) (15–55% gradient, 60 min) at a flow rate of 10 ml/min. The product containing fractions were combined and freeze dried to give the purified cyclic peptide (50 mg). The peptide (retention time 18.03 min on a Novapak C_{18} column (Waters Ltd., UK) (10–60% gradient, 30 min, 1.0 ml/min flow rate)) was characterized by amino acid analysis and mass spectroscopy (Table 5).

Compounds 2–27, 29–31 and 33–44. Synthesized by the procedure used for compound **1**. Structures of the linear peptides used in the final cyclization reaction are shown in Table 5.

Compound 28. Synthesized as compound **1** starting from Fmoc-NH$(CH_2)_2$-S-$(CH_2)_2$-COOH. The Fmoc derivative was obtained by the procedure described below for Fmoc-NH$(CH_2)_2$-S-CH_2-COOH (**32**) by using 3-bromopropionic acid and 2-aminoethanethiol. $(M+H)^+$ 372.

Compound 32. Synthesized as compound **1** starting from Fmoc-NH$(CH_2)_2$-S-CH_2-COOH. For the synthesis of Fmoc-NH$(CH_2)_2$-S-CH_2-COOH, 2-aminoethanethiol [HCl] (5.68 g, 50 mmole) was dissolved in water (200 ml) and $NaHCO_3$ (25.2 g, 300 mmole) and 2-bromoacetic acid (6.95 g, 50 mmole) in acetonitrile (100 ml) was added to the stirred solution in portions over 30 min. After 1 h at RT, a solution of Fmoc-OSu (16.85 g, 50 mmole) in acetonitrile (150 ml) was added and the stirring was continued for 16 h. The slightly turbid solution was evaporated to remove most of the acetonitrile and the remaining aqueous solution was extracted with ethyl acetate (3 × 50 ml). It was then acidified (pH 2) by the addition of HCl. The white solid was collected, washed with water and dried *in vacuo* at 45 °C. Yield 17 g (95%); $(M+H)^+$ 358.0.

Compound 45. Di-Bu^t dicarbonate (24 g, 110 mmoles) in methanol (50 ml) was added to a solution of histamine dihydrochloride (10 g, 54.3 mmoles) and TEA (15.3 ml, 109 mmoles) in methanol (100 ml) over 15 min with stirring. After 24 h at RT, the solvent was removed by evaporation and the residue was partitioned between DCM and water. The organic phase was washed twice with M citric acid solution and saturated NaCl solution, dried over $MgSO_4$ and evaporated to a solid. Recrystallization from ethyl acetate gave N^{τ},N^{im}-di-Boc-histamine (12.99 g, 76%, m.p. 125–126 °C; TLC, R_f 0.25 in ethyl acetate-isohexane (1:1) and 0.62 in methanol-chloroform (1:9)). DIPEA (1 ml) was added to a solution of N^{τ},N^{im}-bis-Boc-histamine (8.3 g, 26.7 mmoles) in methanol (150 ml) and, after stirring for 24 h at RT, the solvent was removed by evaporation. The residue was precipitated from ethyl acetate-isohexane to give N^{τ}-Boc-histamine (5.46 g, 97%, m.p. 93–95 °C; TLC, R_f 0.53 in acetonitrile:water (3:1), 0.23 in methanol:chloroform (1:9) and 0.73 in chloroform:methanol:water (55:40:10)).

Bu^t-bromoacetate (2.31 g, 11.80 mmoles) in DCM (5 ml) was added to a stirred solution of N^{τ}-Boc-histamine (2.5 g, 11.8 mmoles) and DIPEA (2.06 ml, 11.8 mmoles) in DCM (50 ml). After 24 h at RT, additional DCM (200 ml) was added and the solution was washed with M citric acid solution and saturated NaCl solution, dried over $MgSO_4$ and evaporated to dryness. The residual oil, dissolved in TFA:water (95:5, 100 ml) containing triisopropylsilane (1 ml), was left at RT for 90 min. The solvent was removed by evaporation under reduced pressure and the remaining oil was triturated with ether and dried under high vacuum over P_2O_5 and KOH to give $N^{\tau}CH_2$-COOH-histamine as a solid (3.9 g, MH$^+$ 170, TLC R_f 0.35 in acetonitrile:water (3:1), and 0.37 in chloroform:methanol:water (55:40:10)). The histamine derivative (3.9 g, 14 mmoles) was dissolved in water (50 ml), acetone (30 ml) and 1 M Na_2CO_3 (30 ml) and the solution was cooled in an ice bath. Fmoc-OSu (4.76 g) in acetone (30 ml) was added dropwise with stirring over a period of 20 min (pH maintained at 9 by the addition of 1 M Na_2CO_3 solution) and the stirring was continued overnight. Acetone was removed by evaporation and the aqueous solution was acidified with 1 M $KHSO_4$. The product was then extracted into ethyl acetate. The organic phase was washed with water and saturated

Table 5. Characterization of the Cyclic Peptides

Cpnd. No.	Linear or Cyclic Precursor	Amino Acid Analysis (Acid hydrolysis: 6N HCl with 1% phenol, 24 hours, 130 °C)	Mass Spec. M$^+$H	Anal. HPLC[a] Retention Time (Min.) Gradient system/time
1	Ile-Leu-Asp(OBu^t)-Val-NH-$(CH_2)_6$-COOH	Asp 1.01, Val 0.97, Ile 1.0, Leu 1.03	554	18.03, 10-60% (30 min)
2	Ile-Leu-Asp(OBu^t)-Val-NH-$(CH_2)_5$-COOH	Asp 1.01, Val 0.95, Ile 1.0, Leu 1.05	540	16.9, 10-60% (30 min)
3	cyclo(Leu-Leu-Asp(OBu^t)-Val-NH-$(CH_2)_5$-CO)	Asp 0.97, Val 0.99, Leu 2.01, Abx 1.05, D-Leu	554	21.57, 10-60% (30 min)
4	t-Butyl-Ala-Leu-Asp(OBu^t)-Val-NH-$(CH_2)_6$-COOH	Asp 0.97, Val 0.99, Leu 1.01, Abx 1.05, t-butyl-Ala	568	22.66, 10-60% (30 min)
5	D-Ile-Leu-Asp(OBu^t)-Val-NH-$(CH_2)_6$-COOH	Asp 1.01, Val 0.98, Ile 1.0, Leu 1.01	554	19.8, 10-60% (30 min)
6	D-Leu-Leu-Asp(OBu^t)-Val-NH-$(CH_2)_6$-COOH	Asp 1.01, Val 0.97, Leu 2.03	554	16.28, 10-60% (30 min)
7	D-Val-Leu-Asp(OBu^t)-Val-NH-$(CH_2)_5$-COOH	Asp 1.03, Val 1.95, Leu 1.02, Abx 1.01	540	17.81, 10-60% (30 min)
8	Gly-Leu-Asp(OBu^t)-Val-NH-$(CH_2)_6$-COOH	Asp 1.00, Gly 1.04, Val 0.95, Leu 2.02, Leu 1.04, Abx 0.97	498	12.11, 10-60% (30 min)
9	Pro-Leu-Asp(OBu^t)-Val-NH-$(CH_2)_6$-COOH	Asp 1.00, Pro 1.05, Val 0.95, Leu 2.01, Leu 1.04, Abx 0.97	538	16.81, 10-60% (30 min)
10	MeAla-Leu-Asp(OBu^t)-Val-NH-$(CH_2)_6$-COOH	Asp 1.02, Val 1.0, Leu 1.0, Abx 0.98	526	16.83, 10-60% (30 min)
11	MeLeu-Leu-Asp(OBu^t)-Val-NH-$(CH_2)_6$-COOH	Asp 1.03, Val 1.03, Leu 1.02, Abx 1.03	568	23.55, 10-60% (30 min)
12	D-Ile-Leu-Asp(OBu^t)-Val-NH-$(CH_2)_4$-COOH	Asp 1.02, Val 0.98, Ile 1.03, Leu 1.01	560	19.02, 10-60% (30 min)
13	D-Leu-Leu-Asp(OBu^t)-Val-NH-$(CH_2)_4$-COOH	Asp 1.03, Val 0.96, Leu 2.01	540	19.56, 10-60% (30 min)
14	MeIle-Leu-Asp(OBu^t)-Val-NH-$(CH_2)_6$-COOH	Asp 0.98, Val 0.95, Leu 1.04, Ava 1.05	(M+H) 552.4	21.83, 10-60% (30 min)
15	Ile-Nle-Asp(OBu^t)-Val-NH-$(CH_2)_6$-COOH	Asp 1.00, Val 0.95, Ile 0.99, Nle 0.90, Ava 0.94	540	21.36, 10-60% (30 min)
16	Ile-t-butAla-Asp(OBu^t)-Val-NH-$(CH_2)_6$-COOH	Asp 0.97, Val 0.95, Ile 0.96, Ava 1.04	554	23.47, 10-60% (30 min)
17	Ile-Cha-Asp(OBu^t)-Val-NH-$(CH_2)_6$-COOH	Asp 1.02, Val 0.98, Ile 1.00, Ava 1.04	580	25.37, 10-60% (30 min)
18	Ile-Ile-Asp(OBu^t)-Val-NH-$(CH_2)_6$-COOH	Asp 1.05, Val 0.96, Ile 1.95, Ava 1.04	540	18.88, 10-60% (30 min)
19	Ile-Val-Asp(OBu^t)-Val-NH-$(CH_2)_6$-COOH	Asp 0.95, Val 1.93, Ile 0.97, Ava 1.05	526	17.30, 10-60% (30 min)

Table 5 (Continued)

20	Ile-cyclo[Leu-Asp(OBu^t)-Val-NH-(CH$_2$)$_4$-CO]-OH	Asp 0.97, Val 0.95, Ile 0.96, Ava 1.04	540	15.45, 10-60% (40 min)
21	Ile-D-Leu-Asp(OBu^t)-Val-NH-(CH$_2$)$_4$-COOH	Asp 0.99, Val 0.96, Ile 0.98, Leu 1.02, Ava 0.98	540	20.71, 10-60% (30 min)
22	2e-MeLeu-Asp(OBu^t)-Val-NH-(CH$_2$)$_4$-COOH	Asp 1.03, Val 0.98, Ile 1.01, Ava 0.98	555	25.35, 10-60% (30 min)
23	Ile-Leu-Asn-Val-NH-(CH$_2$)$_4$-COOH	Asp 1.01, Val 0.95, Ile 0.96, Leu 1.05, Ava 1.06	539	19.80, 10-60% (30 min)
24	Ile-Leu-D-Asp(OBu^t)-Val-NH-(CH$_2$)$_4$-COOH	Asp 1.01, Val 0.95, Ile 0.95, Leu 1.04, Ava 1.05.	540	19.70, 10-60% (30 min)
25	Ile-Leu-Asp(OBu^t)-Leu-NH(CH$_2$)$_4$COOH	Ile 0.96, Asp 1.01, Leu 2.02, Ava 1.01.	554.4	22.05, 10-60% (30 min)
26	Ile-Leu-Asp(OBu^t)-Phe-NH(CH$_2$)$_4$COOH	Ile 0.96, Asp 1.04, Leu 1.01, Phe 0.98, Ava 1.04.	588.4	23.46, 10-60% (30 min)
27	Ile-Leu-Asp(OBu^t)-D-Val-NH-(CH$_2$)$_4$-COOH	Asp 1.03, Val 0.95, Ile 0.97, Leu 1.01, Ava 1.04	540.5	18.44, 10-60% (30 min)
28	Ile-Leu-Asp(OBu^t)-Val-NH-(CH$_2$)$_2$-S-(CH$_2$)$_2$-COOH	Asp 1.00, Val 0.96, Ile 0.99, Leu 1.02	(M-H)$^-$ 569.5	13.37, 20-80% (40 min)
29	NH(CH$_2$)$_5$-CO-Pro-MeIle-Leu-Asp(OBu^t)-Val	Asp 1.05, Val 1.00, Leu 1.00, Pro 0.95, β-Ala 0.98.	623.5	16.66, 20-80% (40 min)
30	NH(CH$_2$)$_5$-CO-D-Ala-MeIle-Leu-Asp(OBu^t)-Val	Asp 1.05, Val 1.00, Leu 0.98, Ala 1.00, β-Ala 0.96	(M-H)$^-$ 595.4	15.98, 20-80% (40 min)
31	D-Ala-D-Ala-MeIle-Leu-Asp(OBu^t)-Val	Asp 1.04, Val 0.98, Leu 1.00, Ala 1.96,	(M-H)$^-$ 595.2	16.24, 20-80% (40 min)
32	D-Leu-Leu-Asp(OBu^t)-Val-NH-(CH$_2$)$_2$-S-CH$_2$-COOH	Asp 1.00, Val 0.95, Leu 2.03.	(M-H) 556	14.23, 20-80% (40 min)
33	Gly-D-Leu-Leu-Asp(OBu^t)-Val-Gly	Asp 1.04, Gly 2.04, Val 0.95, Leu 2.0	(M-H) 553	14.07, 20-80% (40 min.)
34	β Ala D-Leu-Leu-Asp(OBu^t)-Val-β-Ala	Asp 1.04, Val 0.96, Leu 1.96, β-Ala 2.0.	(M-H) 581.6	12.20, 20-80% (40 min)
35	Gly-D-Leu-Leu-Asp(OBu^t)-Val-β-Ala	Asp 1.05, Gly 1.04, Val 1.0, Leu 1.05, β-Ala 0.96.	(M-H) 567.4	12.20, 20-80% (40 min)
36	D-Ala-D-Leu-Leu-Asp(OBu^t)-Val-β-Ala	Asp 1.01, Ala 1.02, Val 1.0, Leu 2.02, β-Ala 0.99	(M-H)$^-$ 581.8	9.1, 20-80% (40 min)
37	Leu-Asp(OBu^t)-Val-β-Ala-MeAla-D-Leu	Asp 1.03, Val 1.0, Leu 1.98, β-Ala 0.96.	(M-H)$^-$ 595.8	11.52, 20-80% (40 min)

Table 5 (Continued)

38	Leu-Asp(OBu^t)-Val-β-Ala-Pro-D-Leu	Asp 1.0, Pro 1.01, Val 0.95, Leu 2.0, β-Ala 1.03	(M-H)$^-$ 607.5	14, 20-80% (40 min)
39	D-Leu-Leu-Asp(OBu^t)-Val-β-Ala-D-Pro	Asp 1.0, Val 0.97, Leu 1.98, Pro 1.02, β-Ala 0.98	609.5	13.72, 20-80% (40 min)
40	D-Phe-Leu-Asp(OBu^t)-Phe-β-Ala-D-Pro	Asp 1.0, Phe 1.99, Leu 0.99, Pro 1.01, β-Ala 0.93	691.3	18.55, 20-80% (30 min)
41	D-Ala-D-Leu-Leu-Asp(OBu^t)-Val-D-Ala	Asp 1.05, Ala 2.05, Val 1.0, Leu 1.98	583.6	9.1, 20-80% (40 min)
42	D-Ala-D-Ala-D-Phe-Leu-Asp(OBu^t)-Val	Asp 1.05, Ala 1.95, Val 1.0, Leu 0.98, Phe 0.93	(M-H)$^-$ 613.3	12.34, 20-80% (40 min)
43	Leu-Asp(OBu^t)-Val-Pro-Pro-D-Leu	Asp 1.05, Pro 2.0, Val 0.95, Leu 1.97	(M-H)$^-$ 613.5	10.6, 20-80% (40 min)
44	Leu-Asp(OBu^t)-Val-Pro-D-Pro-D-Leu	Asp 1.04, Pro 2.0, Val 0.96, Leu 2.0.	(M-H)$^-$ 613.4	10.35, 20-80% (30 min)
45	Ile-Leu-Asp(OBu^t)-Val-NHCH$_2$-CH$_2$- 	Asp 1.02, Val 0.96, Ile 0.95, Leu 1.05	593.6	18.01, 10-60% (30 min)
46	β-Ala-Ile-Leu-Asp(OBu^t)-Val-*p*-aminomethylbenzoic acid	Asp 1.02, Val 1.00, Ile 0.98, Leu 1.02	645.6	19.21, 10-60% (30 min)
47	NH$_2$-(CH$_2$)$_5$-CO-Ile-Leu-Asp(OBu^t)-Val-Pip-CH$_2$-COOH	Asp 1.00, Val 1.04, Ile 0.97, Leu 0.99, β-Ala 0.96	638.4	16.82, 10-60% (30 min)
48	NH$_2$-(CH$_2$)$_6$-CO-Ile-Leu-Asp(OBu^t)-Val-Pip-CH$_2$-COOH	Ile 0.97, Asp 1.01, Leu 1.02, Val 0.99, Ava 1.02.	666.4	20.97, 10-40% (30 min)
49	NH$_2$(CH$_2$)$_2$-S-CH$_2$-CO-MeIle-Leu-Asp(OBu^t)-Val-Pip-CH$_2$-COOH	Asp 1.04, Val 0.99, Leu 1.0	698.5	22.75, 10-50% (40 min)
50	NH$_2$(CH$_2$)$_2$-S-CH$_2$-CO-MePhe-Leu-Asp(OBu^t)-Val-Pip-CH$_2$-COOH	Asp 1.04, Val 0.95, Leu 1.0	732.3	25.3, 10-50% (40 min)
51	MeIle-Leu-Asp(OBu^t)-Val-D-Arg(Pmc)-Pip-CH$_2$-COOH	Asp 1.02, Val 0.98, Leu 1.04, Arg 0.96.	737.4	21.49, 10-40% (30 min)
52	D-Arg(Pmc)-MeIle-Leu-Asp(OBu^t)-Val-Pip-CH$_2$-COOH	Asp 1.0, Val 0.96, Leu 0.99, Arg 1.04.	737.4	18.47, 10-40% (30 min)
53	NH$_2$-(CH$_2$)$_2$-CO-D-Arg(Pbf)-MeIle-Leu-Asp(OBu^t)-Val-Pip-CH$_2$ COOH	Asp 1.0, Val 0.96, Leu 1.04, β-Ala 0.96, Arg 1.04	808.5	18.91, 10-40% (30 min)
54	NH$_2$-(CH$_2$)$_5$-CO-D-Arg(Pbf)-MeIle-Leu-Asp(OBu^t)-Val-Pip-CH$_2$-COOH	Asp 1.02, Val 0.96, Leu 1.04, γ-Abu 0.95, Arg 0.99	822.5	19.41, 10-40% (30 min)

Table 5 (Continued)

55	Ile-Leu-Asp(OBu^t)-Val-Pip-CH$_2$-COOH or Ile-Leu-Asp(OBu^t)-Val-Pip-CH$_2$-CO-Ile-Leu-Asp(OBu^t)-Val-Pip-CH$_2$-COOH	Asp 1.05, Val 0.95, Ile 1.97, Leu 2.03	1133.6	23.26, 10-60%* (30 min)
56	MeIle-Leu-Asp(OBu^t)-Val-Pip-CH$_2$-CO-MeIle-Leu-Asp(OBu^t)-Val-Pip-CH$_2$-COOH	Asp 0.98, Val 1.01, Leu 2.0	1161.7	29.68, 30-40% (30 min)
57	MePhe-Leu-Asp(OBu^t)-Val-Pip-CH$_2$-CO-MePhe-Leu-Asp(OBu^t)-Val-Pip-CH$_2$-COOH	Asp 1.02, Val 0.96, Leu 1.02	1229.9 (M+H)$^+$ 615.3	28.27, 10-60% (30 min)
58	MeIle-Leu-Asp(OBu^t)-Val-hPip-CH$_2$-CO-MeIle-Leu-Asp(OBu^t)-Val-hPip-CH$_2$-COOH	Asp 1.02, Val 0.95, Leu 1.03,	(M+2H)$^{2+}$ 595.8	25.54, 20-45%* (30 min)
59	Z-Lys-Ile-Leu-Asp(OBu^t)-Val-OH	Asp 1.01, Val 0.98, Ile 1.01, Leu 1.01, Lys 0.97	611	17.94, 10-60% (30 min)
60	Z-D-Lys-Ile-Leu-Asp(OBu^t)-Val-OH	Asp 1.02, Val 0.98, Leu 1.01, Ile 0.99, Lys 0.99	611	18.91, 10-60%* (30 min)
61	Z-Lys-D-Ile-Leu-Asp(OBu^t)-Val-OH	Asp 1.02, Val 0.97, Ile 1.04, Leu 1.01, Lys 0.97	611	17.96, 10-60% (30 min)
62	Z-D-Lys-D-Ile-Leu-Asp(OBu^t)-Val-OH	Asp 1.00, Val 0.95, Leu 1.03, Ile 1.00, Lys 0.93	611	17.71, 10-60% (30 min)
63	Z-D-Orn-D-Ile-Leu-Asp(OBu^t)-Val-OH	Asp 1.03, Val 0.95, Ile 0.99, Leu 0.98, Orn 1.00	597	16.85, 10-60% (30 min)
64	Z-cyclo(D-Lys-D-Ile-Leu-Asp(OBu^t)-Val)	Asp 1.05, Val 0.98, Ile 0.99, Leu 1.02, Lys 0.97	569.3	15.83, 10-60% (30 min)
65	Z-D-Lys-D-Ile-Leu-Asp(OBu^t)-Val	Asp 1.04, Val 0.98, Ile 0.95, Leu 0.99, Lys 1.02	703	26.65, 10-60% (30 min)
66	cyclo(D-Lys-D-Ile-Leu-Asp(OBu^t)-Val)	Asp 1.04, Val 1.01, Ile 0.96, Leu 1.03, Lys 0.98	625.5	19.23, 10-60% (30 min)
67	cyclo(D-Lys-D-Ile-Leu-Asp(OBu^t)-Val)	Asp 1.02, Val 0.97, Ile 0.99, Leu 1.02, Lys 0.99	669.5	17.51, 10-60% (30 min)
68	cyclo(D-Lys-D-Leu-Leu-Asp(OBu^t)-Val)	Asp 1.04, Val 0.98, Leu 2.01, Lys 0.95	640.5	16.31, 10-60% (30 min)
69	cyclo(D-Lys-D-Leu-Leu-Asp(OBu^t)-Val)	Asp 1.05, Val 0.96, Leu 2.01, Lys 0.95	659.6	21.22, 10-60% (30 min)
70	cyclo(D-Lys-D-Leu-Leu-Asp(OBu^t)-Val)	Asp 1.04, Glu 1.00, Val 0.95, Leu 2.03, Lys 0.97	680.6	17.50, 10-60% (30 min)

Table 5 (Continued)

71	cyclo(D-Lys-D-Ile-Leu-Asp(OBu^t)-Val)	Asp 1.02, Val 0.97, Leu 1.99, Lys 0.96	625	17.77, 10-60% (30 min)
72	Z-Orn-Leu-Asp(OBu^t)-Val-OH	Asp 0.99, Val 0.97, Leu 1.00, Orn 1.02	484	10.98, 10-60%* (30 min)

* Analytical HPLC was carried out using a RP-HPLC (C_{18}) Vydac column (218TP54, 4.6 × 250 mm) except in the case of compounds **2**–**6**, when a Novapak column (3.9 × 150 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 1 ml/min.

NaCl solution, dried over $MgSO_4$ and evaporated to an oil. Trituration with ether and ether/isohexane gave *N*′-Fmoc-*N*-(CH_2-COOH)-histamine as a solid (3.66 g, 66%; MH$^+$ 392). A part of the histamine derivative (782 mg, 2 mmole) was attached to 2-chlorotritylchloride resin (2 g) and the required linear peptide was assembled by the procedure used for compound **1**. Cleavage from the resin followed by cyclization and deprotection gave the final product (**45**).

Compound 46. *p*-aminomethylbenzoic acid was attached to the resin and, after coupling the required amino acids, the final product was obtained by the procedures used for compound **1**.

Compound 47 (*Scheme 2*). But-bromoacetate (4.88 g, 25 mmoles) in DCM (50 ml) was added to a solution of But-4-Pip carboxylate (4.65 g, 25 mmoles) and TEA (3.5 ml, 25 mmoles) in DCM (30 ml). The reaction mixture was stirred overnight, filtered to remove the solids and the filtrate evaporated to dryness. The residue, in ethyl acetate, was washed with water, dried over $MgSO_4$ and the solvent evaporated *in vacuo*. The solid was crystallized from ether–isohexane to yield Boc-Pip-CH_2COOBu^t (5.66 g, 75%, m.p. 99–100°C) (found C 59.8%, H 9.6%, N 9.1%; $C_{16}H_{29}N_2O_4$ requires C 60.0%, H 9.4%, N 9.33%). TLC: R_f 0.38 in ethyl acetate:isohexane (1:1) and 0.68 in CH_3OH:$CHCl_3$ (1:9)). The Pip derivative (5 g, 16.6 mmoles) was treated with TFA–water (95:5; 50 ml) for 1 h. The acid was removed by evaporation *in vacuo* and the residual oil was triturated with ether to give a solid, which was collected, washed with ether, dried over P_2O_5/KOH under vacuum (6.25 g, m.p. 177–182°C) and dissolved in a mixture of water and acetone (1:1, 150 ml) containing K_2CO_3 (6.92 g, 3 equivalents). Fmoc-OSu (5.66 g, 16.7 mmoles) in acetone (30 ml) was added over a 20 min period with stirring while maintaining the pH of the solution at 9 by the addition of M K_2CO_3 solution. After 24 h, acetone was removed by evaporation under vacuum and the aqueous solution was acidified with $KHSO_4$ solution. Fmoc-Pip-CH_2-COOH was extracted into ethyl acetate, washed with water (six times) and saturated NaCl solution and dried over $MgSO_4$. Evaporation of the solvent left an oil, which solidified on trituration with isohexane and ether (yield 3.72 g, 60%). A sample was recrystallized from ethanol–ether, m.p. 179–182°C, MH$^+$ 367.

Fmoc-Pip-CH_2-COOH (366 mg, 1 mmole) in CH_2Cl_2 (15 ml) was attached to the resin (1 g) by the procedure used for **1** (weight 1.13 g). Following cleavage of the Fmoc group, Fmoc-Val (678 mg, 2 mmole) was coupled to the resin (1 h) using HBTU (760 mg, 2 mmole) and DIPEA (700 µl, 4 mmole) in DMF (4 ml). The deprotection and coupling cycles were repeated using Fmoc-Asp(OBu^t), Fmoc-Leu and Fmoc-Ile (2 mmoles each) to give the protected peptide resin. Cleavage of the Fmoc group followed by cleavage from the resin gave the linear pentapeptide derivative β-Ala-Ile-Leu-Asp(OBu^t)-Val-Pip-CH_2-COOH as an acetate salt, which was converted to a HCl salt, cyclized, deprotected and purified as for compound **1** to give **47**.

Compounds 48–54. Synthesized by the route described above for compound **47**. The synthetic route for 4-Fmoc-NH-(CH_2)$_5$-S-CH_2-COOH is described above.

Compound 55. The dimeric peptide **55** was prepared by two routes. In the first, Ile-Leu-Asp(OBu^t)-Val-Pip-CH_2-COOH (HCl) (388 mg, 0.57 mmole), prepared by the route described for compound **47**, was dissolved in DMF (600 ml). HBTU (218 mg, 0.57 mmoles) was then added followed by DIPEA (300 µl, 1.72 mmoles). The reaction mixture was stirred (24 h) at RT, evaporated to dryness under vacuum and treated for 1 h with a mixture of TFA:water (95:5, 25 ml) and triisopropylsilane (200 µl) to remove the Asp(OBu^t) group. Evaporation to a small volume, followed by trituration with ether yielded the crude

472

cyclic dimeric peptide **55**, which was purified by RP HPLC (Vydac 218TP1022 column; using a gradient of acetonitrile water (20–50% 65 min, flow rate 10.0 ml/min). Yield 46 mg. No monomeric peptide could be detected.

The second route used for the synthesis of **55** (Scheme 2) required the synthesis of Fmoc Ile Leu Asp(OBu^t) Val-Pip CH_2 CO-Ile-Leu Asp(OBu^t) Val Pip CH_2 COOH on the resin. After assembling Fmoc Ile Leu Asp(OBu^t) Val Pip CH_2 COO resin by the procedure used for compound **47**, one part of the resin was treated with piperidine to give Ile Leu Asp(OBu^t) Val Pip CH_2 COO resin (**55a**) and the other part was treated with a mixture of acetic acid:trifluoroethanol:DCM (2:2:6) to give Fmoc Ile Leu-Asp(OBu^t) Val Pip CH_2 COOH (**55b**). Coupling of the two fragments by HBTU, followed by deprotection of the *N*-terminal Fmoc group and cleavage of the peptide from the resin gave Ile-Leu Asp(OBu^t) Val Pip CH_2 CO Ile Leu Asp(OBu^t) Val Pip CH_2 COOH (**55d**). The linear peptide **55d** was cyclized, deprotected and purified as compound **47** to yield the dimeric peptide **55**.

Compounds 56 and 57. The precursors to MeIle and MePhe analogues **56** and **57**, MeIle Leu-Asp(OBu^t) Val-Pip-CH_2 CO MeIle-Leu Asp(OBu^t)-Val-Pip CH_2 COOH and MePhe Leu Asp(OBu^t) Val Pip CH_2 CO MePhe-Leu Asp(OBu^t)-Val Pip-CH_2-COOH were synthesized on the resin, cyclized and deprotected by the procedures used for compound **55** (Scheme 2).

Compound 58. 2 Chlorotritylchloride resin (5 g, 1.35 mmol/g), suspended in dry DCM (50 ml), was reacted with 3-bromopropionic acid (765 mg, 5 mmol) and DIPEA (2.62 ml, 15 mmol) for 45 min. A 10% solution of DIPEA in methanol (10 ml) was then added and, after 5 min, the resin was collected and washed with methanol, DMF, DCM and ether. The resin was resuspended in DMF (20 ml), treated with hPip (2 g) in a mixture of DMF/DCM (25 ml) for 1 h, collected and washed with DMF and DCM. The resin, suspended in DCM (60 ml), was treated with Fmoc-OSu (3.77 g, 10 mmol) followed by DIPEA (3.5 ml, 20 mmol) and shaken mechanically for 24 h. The Fmoc hPip CH_2 CO-resin was collected by filtration, washed with DCM, DMF, DCM, ether and dried *in vacuo* at 45°C for 16 h (5.99 g). The Fmoc hPip CH_2 COOH was cleaved from the resin (4 g) by two treatments with a mixture of acetic acid trifluoroethanol:DCM (1:1:3, 25 ml). Evaporation of the solvent left an oil, which gave a solid from ether-isohexane (723 mg).

Using the Fmoc hPip CH_2 CO resin (1 mmol) MeIle Leu-Asp(OBu^t)-Val hPip CH_2 CO resin was synthesised. The second hPip unit was then added by reacting Fmoc hPip CH_2 COOH to the resin and the synthesis was continued to give the dimer MeIle Leu Asp(OBu^t)-Val hPip CH_2-CO MeIle Leu Asp (OBu^t) Val hPip CH_2 COOH (720 mg). The peptide was converted to hydrochloride (0.49 mmol) and cyclized in DMF (500 ml) using HATU as a coupling agent. The crude peptide was deprotected with 95% TFA (25 ml) and triisopropylsilane (2 ml) at RT for 1 h (692 mg) and purified by RP HPLC (64 mg).

Ac-cyclo(D-Lys-Ile-Leu-Asp-Val) (60) (Scheme 3). Starting from Fmoc-Val, Z D Lys(Fmoc) Ile Leu Asp(OBu^t) Val was assembled on the resin by a procedure similar to that described above for Ile Leu Asp(OBu^t) Val NH(CH_2)$_5$ COO resin (used in compound 1). The resin was treated with piperidine to cleave the Fmoc group and the peptide was then cleaved from the resin to give Z D Lys-Ile-Leu Asp(OBu^t)-Val, which was converted to the HCl salt. The hydrochloride (236 mg, 0.291 mmole) was dissolved in DMF (350 ml) and, after adding HBTU (110.5 mg, 0.291 mmole), HOBt (39.3 mg, 0.291 mmole) and DIPEA (152 µl, 0.873 mmole), the reaction mixture was stirred for 2 h at RT. The solvent was removed under vacuum and the residue was partitioned between ethyl acetate and water. The organic phase, after washing with 1N citric acid, saturated NaCl solution, 10% aqueous $NaHCO_3$ and saturated NaCl solution, was dried over $MgSO_4$ and the solvent removed in vacuum. The crude product, Z-cyclo(D Lys-Ile-Leu-Asp(OBu^t)-Val) (157 mg, 0.207 mmole, retention time 25.2 min, Vydac column, 20–80% gradient (30 min)) was dissolved in a mixture of ethanol:water:acetic acid (40:6 10 ml) and Pd/C (200 mg) was added. Hydrogen gas was bubbled through the stirred reaction mixture for 4 h to cleave the Z group. The catalyst was removed by filtration and the filtrate evaporated to dryness. The residue, in DMF (10 ml), was treated with an excess of acetic anhydride and the solution was left at RT for 16 h. The solvent was removed *in vacuo* and the acetyl derivative was collected with ether, washed with ether and dried. The crude cyclic peptide was dissolved in a mixture of TFA:water (95:5, 20 ml) and, after adding triisopropylsilane (200 µl), the mixture was stirred at RT for 30 min. The solvent was evaporated off *in vacuo* and the peptide was purified by RP HPLC (yield 28 mg).

Compounds 59 61 68 and 70 72. All of these compounds were prepared by procedures similar to those described for **60**. The structures of the corresponding linear peptides assembled on the resin are shown in Table 5.

Compound 69. The precursor peptide cyclo(D Lys D-Leu Leu-Asp(OBu^t) Val) (120 mg), prepared by the route used for **60**, was dissolved in DMF (10 ml) and benzaldehyde (20 µl) was added. The mixture was stirred at RT for 5 min and sodium cyanoborohydride (11.1 mg) was then added along with a drop of acetic acid; stirring was continued for 30 min. The solvent was then removed by evaporation and the crude product deblocked and purified by HPLC.

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Solid-phase synthesis of polymyxin B1 and analogues via a safety-catch approach

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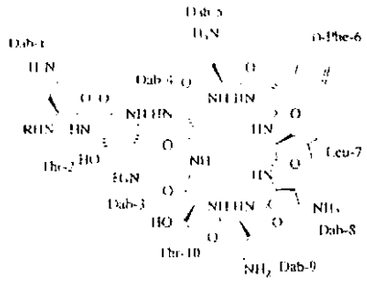
Key words: antibiotics; cationic peptide; polymyxin; safety-catch linker; solid-phase synthesis

Abstract: As part of a program towards the development of novel antibiotics, a convenient method for solid phase synthesis of the cyclic cationic peptide polymyxin B1 and analogues thereof is described. The methodology, based on cleavage by cyclization using Kenner's safety-catch linker, yields crude products with purities ranging from 37–67%. Antibacterial assays revealed that analogues 23–26, in which the (*S*)-6-methyloctanoic acid moiety is replaced with shorter acyl chains, exhibit distinct antimicrobial activity. The results suggest that the length of the acyl chain is rather critical for antimicrobial activity. On the other hand, substitution of the hydrophobic ring-segment D-Phe-6/Leu-7 in polymyxin B1 with dipeptide mimics (i.e. analogues 27–33) resulted in almost complete loss of antimicrobial activity.

Abbreviations: Boc, *t* butyloxycarbonyl; BOP, benzotriazol 1-yloxy tris(dimethylamino)phosphonium hexafluorophosphate; Dab, L-α,γ diaminobutyric acid; DCC, dicyclohexylcarbodiimide; DIC, *N,N*-diisopropylcarbodiimide, DiPEA, *N,N*-diisopropylethylamine; DPPA, diphenylphosphoryl azide; Fmoc, *N* fluorenylmethoxycarbonyl, HOBt, 1 hydroxybenzotriazole; LPS, lipopolysaccharide; MMT, monomethoxytrityl, Mtt, methyltrityl, NMP, 1 methyl-2-pyrrolidone, PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate, TFA, trifluoroacetic acid; TIS, triisopropylsilane

Introduction

Polymyxin B is a group of highly potent cationic peptide antibiotics isolated from *Bacillus polymyxa* (1–3). The general structure comprises (see Fig. 1) a cyclic heptapeptide bound to a linear tripeptide, both of which contain a high percentage of the rare amino acid L-α,γ diaminobutyric acid (Dab). Moreover, the γ-amino group of Dab-4 is linked via an amide bond to the C-terminus of Thr-10, while its α-amino group is connected to Dab-5 of the linear tripeptide. The α-amino group of the N-terminal Dab residue is acylated with a distinctive hydrophobic chain (4). Within the polymyxin B group, polymyxin B1 (1) is the most abundant component.



Polymyxin	R
B1 (1)	(*S*)-6-methyloctanoyl
B2 (2)	6-methylheptanoyl
B3 (3)	Octanoyl
B4 (4)	Heptanoyl
B5 (5)	Nonanoyl
B6 (6)	3-hydroxy-6-methyloctanoyl[†]

[†] Conformations of C3 and C6 have not been established

Figure 1 Structures of polymyxins B1–B6

The presence of positively charged residues (i.e. five Dab units) in 1, as well as the amphiphilic nature are crucial for its activity against Gram negative bacteria. Although the exact mode of action is still a matter of debate, various stages can be distinguished in the membrane permeabilization process (5). Initially, binding of 1 to the anionic lipid A domain leads to disruption of the lipopolysaccharide (LPS) lamellar phase. Next, the hydrophobic tail is inserted into the outer membrane, followed by self-promoted uptake of the remainder of the molecule. After the internalization process, the antibiotic causes disruption of the cytoplasmic membrane and eventually leads to cell death (6). It is also well established that polymyxin B1 decreases the effects of sepsis by binding and neutralizing LPS that is released in the course of Gram negative bacterial infections (7,8).

These properties make polymyxin B1 a good candidate as antibiotic for therapeutic purposes. However, the severe toxicity to humans has thus far limited the clinical use of this antibiotic to topical treatment of infections (9). This hurdle might be cleared by the development of nontoxic analogues. As part of a program towards the design and synthesis of novel antibiotics, we here report a convenient synthesis of 1 and analogues thereof, based on Kenner's safety-catch approach (10). Furthermore, the biological evaluation of these compounds will be addressed.

Results and discussion

The first synthesis of 1 was reported by Vogler and co-workers (11,12) who constructed the linear peptide by fragment condensation in solution, which was successively cyclized using dicyclohexylcarbodiimide (DCC) as activator. Later on, Sharma *et al.* (13) showed that the linear peptide, obtained via solid-phase peptide synthesis, could be cyclized under the influence of the condensing agent diphenylphosphoryl azide (DPPA) (14) in the presence of *N,N*-diisopropylethylamine (DiPEA). Unfortunately, in our hands incomplete DPPA-mediated cyclization of the linear peptide led to an inseparable mixture of target compound 1 and its undesired linear counterpart.

It was expected that the release of linear fragments could be prevented by executing the cyclization (15,16) with concomitant cleavage from the solid support (17). An essential element in this approach is the use of the safety-catch sulphonamide linker, originally developed by Kenner and recently modified by Backes *et al.* (18,19). Attractive features of this type of linker are the stability under acidic and nucleophilic conditions and the cleavage by nucleophilic displacement after *N*-alkylation of the sulphonamide moiety. We reasoned that use of a low substituted resin was desirable in order to prevent any cross coupling during on-resin cyclization. Thus, the safety-catch linker 3-carboxypropanesulphonamide was quantitatively coupled to low loaded polystyrene aminomethyl (AM) resin

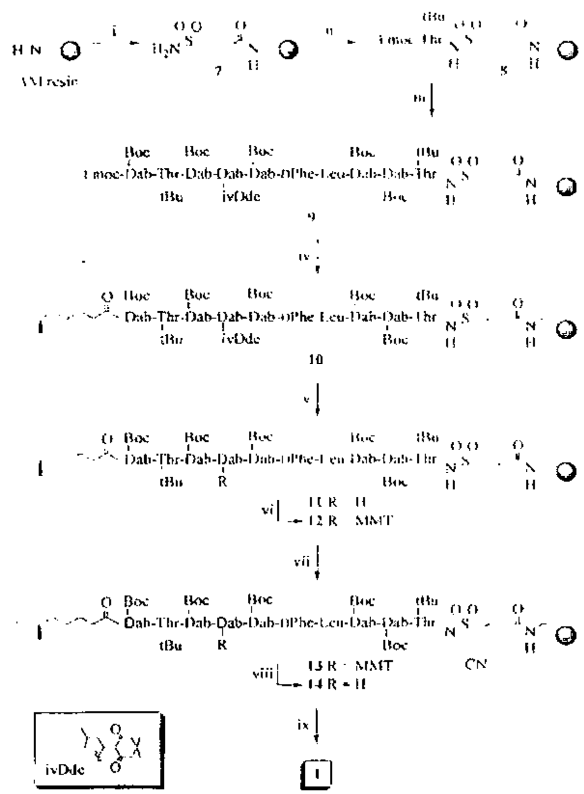


Figure 2. Conditions: (i) 4-sulphamoylbenzoic acid, N,N'-diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt), DMF, quantitative; (ii) Fmoc-Thr(tBu)-F, N,N-diisopropylethylamine (DIPEA), CH_2Cl_2, 5 h, 64%; (iii) repeated sequence: (a) 20% piperidine/1-methyl-2-pyrrolidone (NMP); (b) Fmoc-AA-OH, benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP), HOBt, DIPEA, NMP; (c) Ac_2O, HOBt, DIPEA, NMP; (iv) (a) 20% piperidine/NMP; (b) (S)-6-methyloctanoic acid, BOP, HOBt, DIPEA, NMP, 2 h; (v) 2% H_2NNH_2, H_2O/DMF, 3 × 3 min; (vi) monomethoxytrityl (MMT)-Cl, DIPEA, NMP, 2 h; (vii) ICH_2CN, DIPEA, NMP, 16 h; (viii) trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/CH_2Cl_2, 3 : 5 : 92 (v/v/v); (ix) (a) DIPEA, THF, 24 h; (b) TFA/TIS/H_2O, 95 : 2.5 : 2.5 (v/v/v).

by using N,N'-diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt), to give 7 (see Fig. 2). Subsequent (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP)-mediated [19] coupling of Fmoc-Thr(tBu)-OH afforded 8 in low yields (<20%), which is probably caused by the relatively low reactivity of the threonine residue and/or the sulphonamido group. Alternatively, compound 8 can be obtained via coupling of Fmoc-Thr(tBu)-F to 7, as described by Ingenito *et al.* [20]. In our hands, the highest yield (i.e. 64%) was obtained after prolonging the reaction time to 5 h, and increasing the excess of Fmoc-Thr(tBu)-F and base to 9 and 18 equivalents, respectively. Sequential elongation of 8, resulting in the immobilized peptide 9, was effected by benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP)/HOBt mediated condensation with the suitably protected amino acids in the presence of DiPEA. With respect to residue Dab-4, we initially focused our efforts on the inclusion of the amino acid having a methyltrityl (Mtt) functionality, as the mild acidic removal of this group would be fully compatible with our approach. However, the synthesis of Fmoc-Dab(Mtt)-OH gave unsatisfactory low yields[1]. This problem could be avoided by incorporation of a 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde) protected Dab residue. Compared with the Dde group, the ivDde group

[1] At the moment of preparing this manuscript, the building block Fmoc-Dab(Mtt)-OH became commercially available (NovaBiochem).

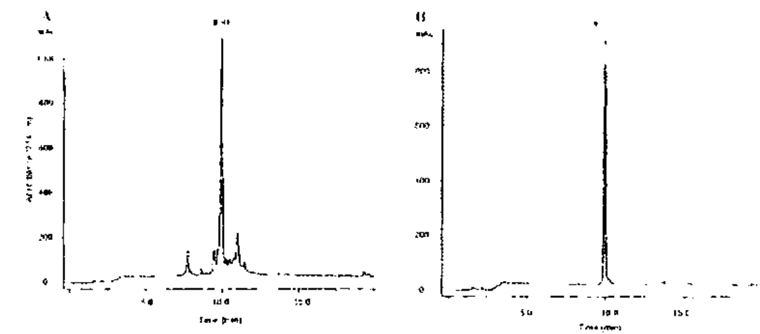


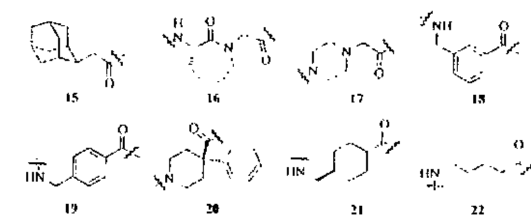
Figure 3. High-pressure liquid chromatography (HPLC) traces of polymyxin B₁ synthesized according to the cleavage by cyclization methodology procedure; (A) crude and (B) after semi-preparative HPLC purification.

shows an increased stability towards piperidine treatment and is less prone to migration. The assembly of the linear polymyxin molecule 10 was completed by removal of the Fmoc-group in 9 and subsequent coupling of (S)-6-methyloctanoic acid using BOP/HOBt in the presence of DiPEA. (S)-6-methyloctanoic acid was prepared as follows: hydrogenolysis of commercially available (R)-citronellyl bromide with $LiAlH_4$, followed by ozonolysis and reductive work-up ($NaBH_4$) afforded S-(+)-4-methyl-1-hexanol [21,22]. Subsequently, S-(+)-4-methyl-1-hexanol was converted into (S)-6-methyloctanoic acid as described by Sharma *et al.* [13].

In order to secure the final cyclization of the amino group of Dab-4 with Thr-10 in activated 14, the ivDde group in 10 was removed by hydrazinolysis and replaced by the monomethoxytrityl (MMT) group. Alkylation of 12 with iodoacetonitrile according to the procedure of Backes *et al.* [19] afforded activated sulphonamide 13. Removal of the MMT group of Dab-4 in 13 by treatment with trifluoroacetic acid (TFA) in the presence of the cation scavenger triisopropylsilane (TIS) yielded the partially protected and immobilized 14. Cyclization of 14 with concomitant release of the cyclic peptide from the solid support was effected with DiPEA. Removal of the remaining side chain protecting groups (i.e. Boc and tBu) by acidolysis furnished 1 (see Fig. 3A). The high purity of the crude product is indicative of the potential of the safety-catch approach. Semi-preparative reversed-phase high pressure liquid chromatography (HPLC) purification afforded pure 1 (see Fig. 3B). The respective linear polymyxin B₁ that was not present, as indicated by LC-MS analysis of the crude product. The synthetic compound co-eluted with polymyxin B₁ that was isolated from a commercial sample of polymyxin B sulphate (4). In addition, MS/MS analysis revealed that the fragmentation pattern of 1 was, in every aspect, identical to the one reported recently [23].

Having developed a convenient synthetic route towards polymyxin B₁, we then directed our attention towards the synthesis of nontoxic analogues. It has been proposed that hydrophobic segments (i.e. the acyl chain and D-Phe-6/Leu-7) may contribute to the toxicity of polymyxin B₁ [24,25]. Based on this simple concept, we synthesized two series of analogues. The first series of analogues comprised those in which the (S)-6-methyloctanoic acid moiety was substituted with shorter acyl chains, i.e. 1-adamantane acetic acid (15, Fig. 4), hexanoic acid, pentanoic acid and

Figure 4. Acyl chain (15) and dipeptide mimics (16–22) used for incorporation into polymyxin B₁ analogues.



butanoic acid. In the second series, the tripeptide D-Phe-6, Leu-7, Xaa-8 was replaced with unnatural, commercially available dipeptide mimics: the β-turn inducing anthranilic acid [8], 1-amino-1-carboxymethyl cyclobutane [10] [20, 21], the three rigid moieties *N*-carboxymethylpiperazine [11], *m*-aminomethylbenzoic acid [18] [26] and *p*-aminomethylbenzoic acid [19], the two extended conformation inducers 4-phenyl-3-carboxymethylpiperidine [20] and *trans*-4-aminomethylcyclohexane carboxylic acid [21] [27], and finally the flexible ω-aminovaleric acid [22].

The synthesis of these analogues was performed as described in Fig. 2. However, double couplings were applied for the inclusion of the dipeptide mimics 16, 18, 19 and 20. Application of the cleavage-cyclization strategy afforded analogues 23–33 in 17–67% purity, based on HPLC analysis of the crude peptides (see Fig. 3 for the HPLC traces of two representative examples (i.e. crude 29 and 33). These results nicely illustrate that the cyclization reaction appears to be deprived of structural requirements and is generally applicable, in contrast to the on-resin cyclization reaction reported recently [30].

After semi-preparative reversed-phase HPLC purification, polymyxin B1 and analogues were evaluated for their antibiotic activity against *Escherichia coli* ATCC 11775. As summarized in Table 1, the acyl chain modified derivatives 23 and 24 exhibit antimicrobial activities similar to 1. Remarkably, pentanoyl and butanoyl derivatives 25 and 26 are considerably less potent than analogues 23 and 24, implying that stepwise shortening of the acyl chain results in gradual increase in the minimal inhibitory concentration. However, both analogues containing rigid dipeptide mimics (i.e. compounds 27–30), and those bearing extended conformation-constraining elements (i.e. compounds 31 and 32) are devoid of any significant antibiotic activity up to concentrations of 50 μg/mL. The only polymyxin B1 analogue with a D-Phe-7-Leu-8 replacement that displays very modest antibiotic activity is compound 33, containing the flexible ω-aminovaleric acid moiety (100% inhibition at 100 μg/mL). The results presented above are in accordance with the findings reported by Tsubery *et al.* [24], indicating that both the size and conformation of the cyclic peptide moiety of polymyxin B1 are crucial for its mode of action.

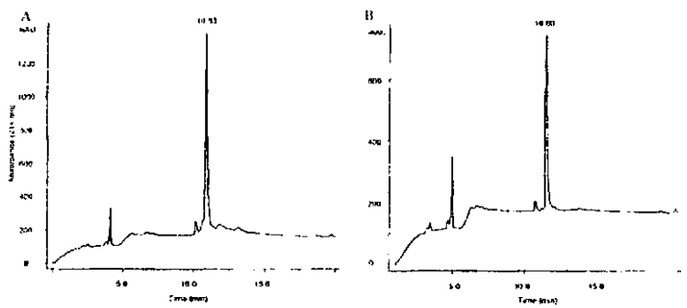
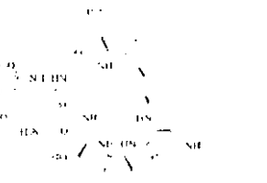


Figure 3. Representative high pressure liquid chromatography (HPLC) traces of crude polymyxin B1 analogues, synthesized according to the cleavage-by-cyclization methodology: (A) *m*-aminomethyl benzoic acid containing peptide 29 and (B) ω-aminovaleric acid-containing peptide 33.

Table 1. Data on polymyxin B1 and analogues in which positions R, X, and Y are varied



Compound	R[a]	X-Y[a]	MIC[b] (μg/mL)
1	(S)-6-methyloctanoyl	Phe-Leu	0.5
23	Nonanoyl	Phe-Leu	0.9
24	Heptanoyl	Phe-Leu	0.7
25	Pentanoyl	Phe-Leu	11
26	Butanoyl	Phe-Leu	21
27	(S)-6-methyloctanoyl	16	
28	(S)-6-methyloctanoyl	17	
29	(S)-6-methyloctanoyl	18	
30	(S)-6-methyloctanoyl	19	
31	(S)-6-methyloctanoyl	20	
32	(S)-6-methyloctanoyl	21	
33	(S)-6-methyloctanoyl	22	500

[a]See Figure 4 for chemical structures. [b]Minimal inhibitory concentration with no detectable bacterial growth. [c]No inhibition detected at peptide concentrations up to 50 μg/mL.

Fmoc-amino acids except for Fmoc-Dab(Fmoc)-OH were obtained from NovaBiochem (Läufelfingen, Switzerland). Fmoc-Dab(Boc)-OH, the Fmoc-protected dipeptide mimics and HOBt (anhydrous) were obtained from Neosystem (Strasbourg, France). All other reagents were obtained from Acros (Geel, Belgium). All reagents were used as supplied by the manufacturer unless stated otherwise.

Analytical and semi-preparative HPLC was performed on an ÄKTA™ Explorer chromatography system (Amersham Pharmacia Biotech, Uppsala, Sweden). The peptides were analysed using a Zorbax SB-C18 column (4.6 × 150 mm, 5 μm particle size, denoted as column 1) or an Alltech Alltima C-18 column (Deerfield, IL, USA) (2.0 × 150 mm, 5 μm particle size, denoted as column 2). Semi-preparative reversed-phase HPLC purification was performed using an Alltech Alltima C-18 column (10.0 × 250 mm, 5 μm particle size). The following buffers were used: (A) 5% CH_3CN in 0.1% aq. TFA and (B) 80% CH_3CN in 0.1% aq. TFA. Maldi-TOF MS analyses were performed on a Bruker Biflex III mass spectrometer (MSI (Billerica, MA, USA)). Electrospray tandem mass analyses were performed on a Q-TOF mass spectrometer (Micromass, Altrincham, UK) at a cone voltage of 20 V.

Fmoc-Thr(tBu)-4-sulphonamidobutyrylaminomethyl resin (8)

Loading of the aminomethyl resin with 3-carboxypropane sulphonamide and subsequent coupling of Fmoc-Thr(tBu)-F was accomplished following the procedure of Ingenito *et al.* [20], with some modifications. Loading of safety catch resin (2 g, 0.72 mmol) was accomplished with DIPEA (2.25 mL, 13 mmol) and Fmoc-Thr(tBu)-F (2.65 g, 7.7 mmol) in CH_2Cl_2 (12 mL) and the reaction time was increased to 5 h, to give 8 in a yield of 63%.

MOA-Dab(Boc)-Thr(tBu)-Dab(Boc)-Dab(ivDde)-Dab(Boc)-D-Phe-Leu-Dab(Boc)-Dab(Boc)-Thr(tBu)-4-sulphonamidobutyrylaminomethyl resin (10)

The acylated decapeptide 10 was synthesized on an ABI 433A (Applied Biosystems, Foster City, CA, USA) peptide synthesizer. The synthesis was performed on 100 μmol scale starting from 8 (0.43 g, 0.23 mmol/g). Cleavage of the Fmoc group from the resin was effected with 20% piperidine in DMF (2 × 2 min and 1 × 1 min). Single couplings were performed using Fmoc-amino acids (0.5 mmol) in NMP (1.0 mL), BOP (0.2 g, 0.5 mmol) and HOBt (67 mg, 0.5 mmol) in DMF/NMP (1 mL, 1/1, v/v) and DIPEA (0.23 mL, 1.0 mmol) in NMP (0.8 mL) and shaking the resulting suspension for 1 h. Residues 3, 4 and 5 were coupled using amino acids (0.4 mmol) in NMP (0.8 mL), BOP (0.16 g, 0.4 mmol) and HOBt (54 mg, 0.4 mmol) in DMF/NMP (0.8 mL, 1/1, v/v) and DIPEA (0.18 mL, 0.8 mmol) in NMP (0.64 mL). Capping was performed after each step using Ac_2O/HOBt/DIPEA/NMP (2 mL, 0.5/0.1/1.5/17.5, v/w/v/v) for 1 min.

MOA-Dab(Boc)-Thr(tBu)-Dab(Boc)-Dab(MMT)-Dab(Boc)-D-Phe-Leu-Dab(Boc)-Dab(Boc)-Thr(tBu)-4-sulphonamidobutyrylaminomethyl resin (12)

Immobilized peptide 10 (100 μmol) was suspended into a solution of 2% hydrazine monohydrate in DMF (5 mL) and shaken for 3 min. The mixture was filtered and the resin was rinsed with DMF and CH_2Cl_2. This procedure was repeated twice to give resin 11. Subsequently, the resin was suspended in NMP (1 mL), DIPEA (0.10 mL, 0.5 mmol) and

MMT chloride [illegible] 0.35 mmol) were added and the mixture was shaken for [illegible] h. The resin was filtered, washed with CH_2Cl_2 and MeOH to give 12.

MOA-Dab(Boc)-Thr(tBu)-Dab(Boc)-Dab-Dab(Boc)-D-Phe-Leu-Dab(Boc)-Dab(Boc)-Thr(tBu)-N-cyanomethyl-N-acylsulphonamide resin (14)

A solution of iodoacetonitrile (0.57 mL, 7.9 mmol) in NMP (3 mL) was passed through a basic alumina plug under the exclusion of light. The resin 12 (100 µmol) was suspended in the obtained solution and subsequently DiPEA (0.2 mL, 1 mmol) was added. The suspension was shaken for 16 h under the exclusion of light. The activated resin 13 was filtered and washed with NMP and CH_2Cl_2. Subsequently, 13 was suspended in TFA/TIS/CH_2Cl_2 (5 mL, 3/5/92, v/v/v) and shaken for 10 min. This procedure was repeated with 10 min periods until the filtrate became colourless. After washing with CH_2Cl_2, resin 14 was washed with CH_2Cl_2 and used immediately in the cyclization reaction.

Polymyxin B1 (1)

To a suspension of 14 (100 µmol) in THF (3 mL; freshly distilled), DiPEA (0.17 mL, 0.85 mmol) was added. The obtained cyclization/cleavage mixture was shaken for 24 h at room temperature. The resin was filtered and washed with CH_2Cl_2, MeOH and CH_2Cl_2, respectively. The filtrate and washing solutions were concentrated in vacuo to afford the protected cyclic peptide. The residue was suspended in a mixture of TFA/TIS/H_2O (5 mL, 95/2.5/2.5, v/v/v) and shaken for 2 h. The resulting oil was precipitated from diethyl ether. The precipitate was centrifuged and the solvent was decanted to give 22.0 mg of crude polymyxin B1 (Rt 9.02 min, column 1, purity 50%). The crude cyclic peptide was purified using reversed-phase HPLC (linear gradient of 0–100% buffer in 20 min) and lyophilized to furnish pure 1. Yield after purification: 1.85 mg (1.54 µmol, 1.5%). MS (ESI): calc. for $C_{56}H_{98}N_{16}O_{13}$ 1202.75, found m/z 402.3 $[M + 3H]^{3+}$, 602.4 $[M + 2H]^{2+}$, 1203.8 $[M + H]^+$.

Adamantyl polymyxin analogue (23)

The synthesis was performed on 50 µmol scale. Crude yield 18.4 mg (Rt 9.50 min, column 1, purity 44%). Yield after purification [illegible] MS (MALDI): calc. for [illegible], found m/z 1240.8 $[M + H]^+$, [illegible] $[M + Na]^+$, [illegible] $[M + K]^+$.

Hexanoyl polymyxin analogue (24)

The synthesis was performed on 50 µmol scale. Crude yield [illegible] mg (Rt [illegible] min, column 1, purity [illegible]). Yield after purification [illegible] mg ([illegible] µmol, 4.3%). MS (MALDI): calc. for [illegible], found m/z 1161.7 $[M + H]^+$, 1183.7 $[M + Na]^+$, 1199.7 $[M + K]^+$.

Pentanoyl polymyxin analogue (25)

The synthesis was performed on 50 µmol scale. Crude yield [illegible] mg (Rt [illegible] min, column 1, purity 26%). Yield after purification [illegible] mg ([illegible] µmol, [illegible]%). MS (MALDI): calc. for [illegible] 1146.69, found m/z 1147.7 $[M + H]^+$, 1169.8 $[M + Na]^+$, [illegible] $[M + K]^+$.

Butanoyl polymyxin analogue (26)

The synthesis was performed on 30 µmol scale. Crude yield 6.5 mg (Rt 7.05 min, column 1, purity 38%). Yield after purification 1.18 mg (1.04 µmol; 3.5%). MS (MALDI): calc. for $C_{51}H_{88}N_{16}O_{13}$ 1132.69, found m/z 1133.9 $[M + H]^+$, 1155.9 $[M + Na]^+$, 1171.9 $[M + K]^+$.

Caprolactame-containing PMB1 analogue (27)

The compound was synthesized on 45 µmol scale using nonoptimized conditions; yield not determined (Rt 9.00 min, column 2, purity 42%); MS (ESI): calc. for $C_{50}H_{86}N_{16}O_{13}$ 1110.69, found m/z 556.6 $[M + 2H]^{2+}$, 1112.2 $[M + H]^+$.

N-Carboxymethylpiperazine-containing PMB1 analogue (28)

The compound was synthesized on 45 µmol scale using nonoptimized conditions; yield not determined (Rt 8.72 min, column 2, purity 51%), MS (ESI): calc. for [illegible] 1068.65, found m/z 268.2 $[M + 4H]^{4+}$, 357.2 $[M + 3H]^{3+}$, 535.4 $[M + 2H]^{2+}$, 1068.7 $[M + H]^+$.

m-Aminomethyl benzoic acid-containing PMB1 analogue (29)

The synthesis was performed on 100 µmol scale. Crude yield [illegible] mg (Rt 10.85 min, column [illegible], purity [illegible]). Yield after purification 9.76 mg (9.08 µmol, [illegible]). MS (MALDI): calc. for [illegible] 1075.65, found m/z 1076.6 $[M + H]^+$, 1098.[illegible] $[M + Na]^+$, 1114.[illegible] $[M + K]^+$.

p-Aminomethyl benzoic acid-containing PMB1 analogue (30)

The compound was synthesized on 40 µmol scale using nonoptimized conditions, crude yield 4.7 mg (Rt 8.43 min, column 1, purity 37%). Yield after purification not determined. MS (MALDI): calc. for [illegible] 1075.65, found m/z 1076.6 $[M + H]^+$, 1098.6 $[M + Na]^+$, 1114.6 $[M + K]^+$.

4-Phenyl-4-carboxymethylpiperidine-containing PMB1 analogue (31)

The synthesis was performed on 40 µmol scale. Crude yield 10.9 mg (Rt 8.45 min, column 1, purity 40%). Yield after purification: 1.78 mg (1.56 µmol, 3.9%). MS (MALDI): calc. for [illegible] 1129.7, found m/z 1130.7 $[M + H]^+$, 1152.7 $[M + Na]^+$, 1168.6 $[M + K]^+$.

Tranexamic acid-containing PMB1 analogue (32)

The compound was synthesized on 30 µmol scale using nonoptimized conditions, crude yield 3.9 mg (Rt 8.33 min, column 1, purity 49%). Yield after purification not determined. MS (MALDI): calc. for [illegible] 1081.65, found m/z 1082.6 $[M + H]^+$, 1104.7 $[M + Na]^+$, 1120.7 $[M + K]^+$.

δ-Aminovaleric acid-containing PMB1 analogue (33)

The synthesis was performed on 100 µmol scale. Crude yield 21.4 mg (Rt 10.6 min, column 2, purity 67%). Yield after purification, 2.80 mg (2.69 µmol; 2.7%). MS (MALDI): calc. for [illegible] 1041.7, found m/z 1042.6 $[M + H]^+$, 1064.6 $[M + Na]^+$, 1080.6 $[M + K]^+$.

Antibacterial assay

The bacteria (E. coli ATCC 11775) were grown on [illegible] agar plates and kept at 4 °C. Lyophilized peptides were dissolved in Luria-Bertani (LB) broth to give a concentration of 80 µM and filtered using 0.22 µm filter discs (Millex GV, Durapore, Millipore, Bedford, MA, USA). An overnight culture in LB broth was adjusted to 5 × 10[illegible] CFU/mL and inoculated into the micro titre plate wells (polystyrene, U bottom, Costar) containing each 100 µL of a serial two-fold dilution (50–0.1 µg/mL) of the tested peptide in LB broth. After incubation for 24 h at 37 °C absorbance was measured at 600 nm using a µQuant micro plate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). The minimal inhibitory concentration of δ-aminovaleric acid-containing PMB1 analogue (33) was determined separately. Hereto, the serial two-fold dilution of 33 in the assay above was adjusted to concentrations ranging from 1000 to 2 µg/mL. Analogue 26 was tested in quadruplo, all other peptides were assayed in duplo.

Conclusion

In this paper an efficient solid-phase synthesis of the cyclic cationic peptide antibiotic polymyxin B1 and analogues thereof has been presented, which is based on a safety-catch strategy. The method has the advantage that relatively pure polymyxins are acquired after the final cyclization/cleavage process, thus obviating extensive purification procedures. Antibacterial assays revealed that analogues 23–26, in which the (S)-6-methyloctanoic acid moiety is replaced with shorter acyl chains, exhibit distinct antimicrobial activity. However, analogues 27–33, in which the hydrophobic ring-segment D-Phe-6/Leu-7 is substituted with dipeptide mimics, were basically devoid of antimicrobial activity. The ability of the analogues to sequester LPS or lipid A, as well as further toxicological evaluation of the analogues, will be the subject of forthcoming studies.

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