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Bioorganic & Medicinal Chemistry Letters 15 (2005) 41–45
Bioorganic & Medicinal Chemistry Letters

Identified a morpholinyl-4-piperidinylacetic acid derivative as a potent oral active VLA-4 antagonist

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Received 18 August 2004; revised 12 October 2004; accepted 13 October 2004
Available online 2 November 2004

Abstract—An investigation into the structure–activity relationship of a lead compound, prolyl-5-aminopentanoic acid **4**, led to the identification of a novel series of 4-piperidinylacetic acid, 1-piperazinylacetic acid, and 4-aminobenzoic acid derivatives as potent VLA-4 antagonists with low nanomolar IC_{50} values. A representative compound morpholinyl-4-piperidinylacetic acid derivative (**13d**: IC_{50} = 4.4 nM) showed efficacy in the *Ascaris*-antigen sensitized murine airway inflammation model by oral administration.

VLA-4 (very late antigen 4; $\alpha_4\beta_1$ integrin; CD49d/CD29) is a key cell receptor expressed on most leukocytes.[1] The natural ligands include VCAM-1 (vascular cell adhesion molecule-1) expressed on cytokine-stimulated endothelial cells and the alternatively spliced connecting segment-1 (CS-1) domain of fibronectin (FN) on the extracellular matrix.[2,3] Recently, it has been reported that junctional adhesion molecule 2 (JAM2) on endothelial cells also interacts with VLA-4.[4] Through the VLA-4/ligands interaction, VLA-4 plays an important role in the process of adhesion, migration, and activation of inflammatory leukocytes at sites of inflammation. It has been shown that *anti*-VLA-4 antibodies or VLA-4 antagonists[5] inhibit leukocyte infiltration to extravascular tissue and prevent tissue damage in inflammatory disease models of asthma,[6] multiple sclerosis (MS),[7] rheumatoid arthritis (RA),[8] and inflammatory bowel disease (IBD).[9] In addition, a humanized monoclonal *anti*-α_4 antibody (natalizumab,[10] Elan Pharmaceuticals Inc.) has revealed efficacies for MS and Crohn's disease in phase II clinical trials. Accordingly, orally active small molecule VLA-4 antagonists should represent an attractive target to enhance the therapeutic benefit.

It has been known that VLA-4 recognizes the sequences Ile-Asp-Ser (IDS) in VCAM and Leu-Asp-Val (LDV) in FN. Therefore, VLA-4 antagonists based on the LDV sequence have been extensively explored by a number of research groups.[5] Among them, LDV mimics incorporated with the 4-(phenylureido)phenylacetyl moiety (diphenylurea portion, Fig. 1) at the N-terminus of the sequence have been reported to show efficacy in animal models, and some representatives such as Bio-1211 (**1**)[11] (Merck/Biogen, Fig. 1) and IVL-745 (**2**)[12] (Aventis, Fig. 1) have advanced into clinical trials as *anti*-asthmatic agents administered as inhalants. These compounds have poor pharmacokinetic profiles, however, such as low oral availability and high plasma clearance because of their residual peptidic character.[5] Therefore, in our efforts to obtain an orally available VLA-4 antagonists, we identified a proline derivative, **PS181895** (**3**, Fig. 2), with an IC_{50} value of 4.7 nM in the VLA-4/VCAM-1 binding assay. In addition, it was found that the 3-methylbutylaminocarbonyl group in **3** could be removed

Keywords: VLA-4; Integrin; Asthma.
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doi:10.1016/j.bmcl.2004.10.041

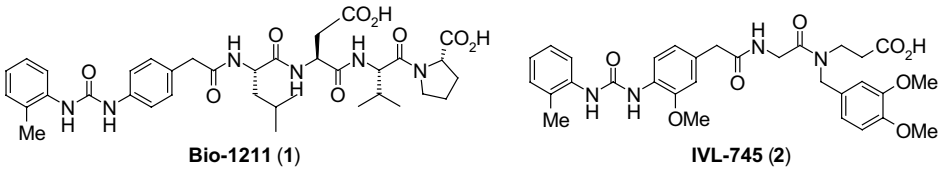


Figure 1. The structures of Bio-1211 and IVL-745.

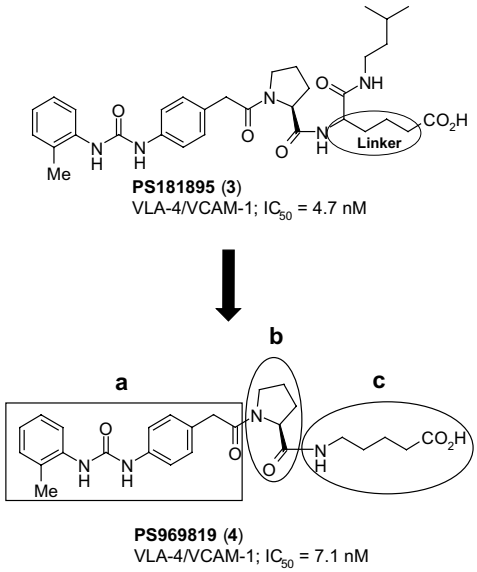


Figure 2. The structures of identified lead compounds.

without significant loss of the activity, **PS969819** (**4**, Fig. 2) showed an IC_{50} value of 7.1 nM. At this point, we considered that this simplified prolyl-5-aminopentanoic acid moiety should be useful as an LDV mimic structure for further optimization. This result led us to select the compound (**4**) as a lead compound for studies toward obtaining a new series of VLA-4 antagonists to show the more potent activity. Compound **4** can be divided into three portions (**a**–**c**) from its structural features (Fig. 2). To explore the structure–activity relationship (SAR), we focused several modifications on the **b** and **c** portions.

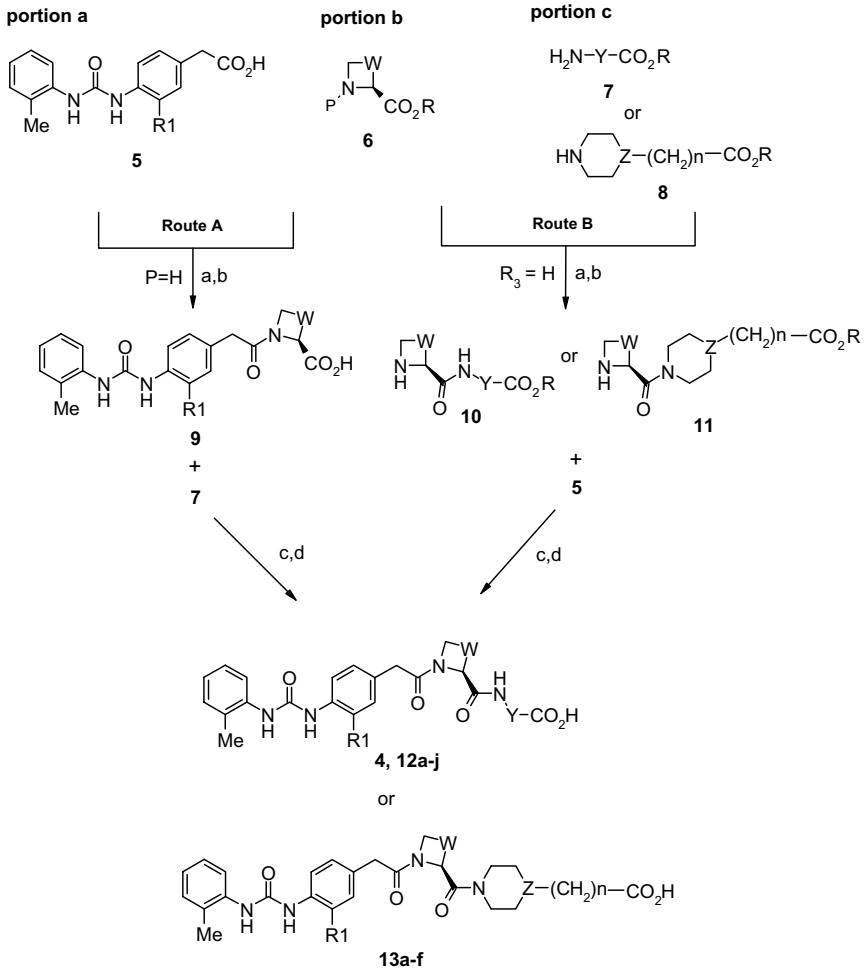
We report herein the identification of a novel series of 4-piperidinylacetic acid, 1-piperazinylacetic acid, and 4-aminobenzoic acid derivatives as potent VLA-4 antagonists and present the results of evaluating representative compounds in the murine asthma model.

Preparation of new compounds was carried out through route A or B in Scheme 1 by coupling three portions **a**–**c**, which were easily prepared from commercially available compounds. In route A, **5** (portion a) was condensed with **6** (P = H, R = *tert*-Bu, portion **b**) by the standard amide bond-forming method (EDC and HOBt) followed by deprotection of the *tert*-butyl ester group with TFA to give **9**. Subsequent coupling with **7** and hydrolysis under basic condition provided **4**, **12a**–**f**, and **13a**. When portion **c** was methyl 4-aminobenzoate, the condensation procedure failed due to lower nucleophilicity of the NH_2 group in the aniline. In this case, the condensation was accomplished via acid chloride treatment of **9**. In a manner similar to that of route A, the other compounds (**12g**–**j** and **13b**–**f**), in which the **a**–**c** portions were linked with amide bonds, were prepared through route B starting from **6**[13] (P = Boc, or Z, R = H, portion **b**). In addition, we synthesized the compounds without the amide oxygen atom between portions **b** and **c** as shown in Scheme 2. Commercially available *N*-Boc-*L*-prolinal (**14**) was subjected to reductive amination utilizing $NaBH_3CN$ with each amine, followed by deprotection of the Boc group, condensation with **5**, and saponification to give the amide bond reduced analogues **13g** and **13h**.

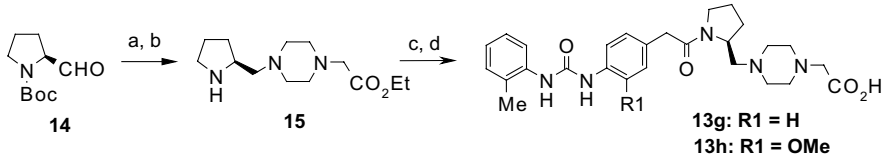
Compounds were evaluated for their VLA-4 inhibitory activity in a receptor binding assay in which CHO cells expressing VLA-4 and an europium (Eu)-labeled human VCAM-1/Fc chimera were used.[14] The evaluation results are summarized in Table 1.

To examine the optimal atom length (distance) between the pyrrolidine nitrogen and the carboxylic acid group in **4**, we prepared proline analogues with a linear chain linker of 5–9 bonds (**12a**–**d**, Table 1). The optimal chain distance was found to be 8 bonds, as in **4**, and the other chain distances resulted in a significant loss of inhibitory activity. Interestingly, the optimal distance was two bonds longer than those of the comparable positions in **Bio-1211** (**1**) [6 bonds (11 bonds counted from proline terminal carboxylic acid group)] and **IVL-745** (**2**) (6 bonds) of Figure 3.

A further increase of potency was achieved by introducing a benzene ring (**12f**, IC_{50} = 3.8 nM) or piperidine ring (**13b**, IC_{50} = 2.9 nM) into this linker, while a similar piperazine analogue **13c** retaining the same distance showed about a 2-fold decrease of potency (IC_{50} = 13 nM) compared with **4** (Table 1). As for transforming the pyrrolidine ring of **4** into other hetero-rings, thiazoline analogue **12h** was slightly less potent (IC_{50} = 10 nM) than **4**, and the others (3,4-dehydropyrrolidine, piperidine, and morpholine analogues) showed a 3–4-fold decrease of potency (Table 1). Replacement of the linear linker of morpholine analogue **13d** with a piperidine ring linker improved the potency again with an IC_{50} value of 4.4 nM (Table 1), suggesting that the piperidine ring could restrict the compound to the preferable conformation in this portion. Then, to further increase potency a methoxy group was introduced at the 3-position on the inner benzene ring of 4-phenylureidophenylacetic acid (portion **a**) based on the SAR previously reported by Biogen.[6] In this case, even a combination of the 3,4-dehydropyrrolidine ring and piperazine linker (**13f**)



Scheme 1. Reagents and conditions: (a) EDC·HCl, HOBt, Et_3N, DMF; (b) TFA, CH_2Cl_2 or H_2, Pd/C, EtOH; (c) EDC·HCl, HOBt, Et_3N, cat. DMAP, DMF; (d) 1 N NaOH, THF/MeOH.



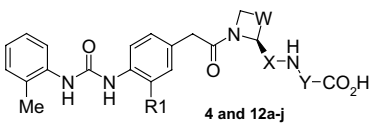
Scheme 2. Reagents and conditions: (a) ethyl 4-piperazinylacetate, $NaBH_3CN$, MeOH–AcOH; (b) TFA, CH_2Cl_2; (c) EDC·HCl, HOBt, DMAP, DMF; (d) 0.25 N NaOH, THF–MeOH.

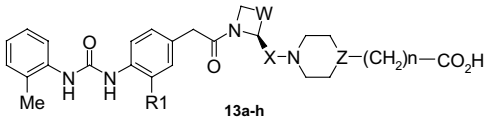
showed high potency with an IC_{50} value of 1.4 nM, as well as did a combination of pyrrolidine ring and piperazine linker (**13e**, IC_{50} = 1.6 nM, Table 1). For the purpose of moving away from the peptidic character by reducing H-bond acceptor, compounds were prepared by removing the amide oxygen atom between the **b** and **c** portions. Piperazinylacetic acid analogues **13g** and **13h** revealed equipotency to the corresponding compound amide-type **13c** and **13e**, revealing that this oxygen atom was unnecessary for retaining the potency.

As for **13b**, **13d**, and **13h** with low nanomolar IC_{50} values, we evaluated these compounds in the *Ascaris*-antigen sensitized murine airway inflammation model[15] by oral administration. Compound **13d** was found to inhibit eosinophils infiltration into bronchial alveolar lavage (BAL) fluid by 36% (50%; total cell count) at a dosage of 30 mg/kg twice a daily for 2 days compared with the vehicle alone (Fig. 4). However, the others did not show effectiveness (**13b**, 30 mg/kg b.i.d.; **13h**, 50 mg/kg b.i.d., data not shown).

On the other hand, pharmacokinetic properties in rats and physicochemical properties of **4**, **13b**, and **13d** were determined (Table 2). Unfortunately, these compounds showed terribly low oral availability. We considered that because of rapid plasma clearance and poor membrane permeability due to lack of lipophilicity, these compounds showed poor availability. Consequently, the

Table 1. Inhibition of VLA-4/VCAM-1 by VLA-4 antagonists





Compd	R_1	W	X	Y	Z	*n*	IC_{50} (nM)
Bio-1211	—	—	—	—	—	—	<0.5
4	H	–$(CH_2)_2$–	C=O	–$(CH_2)_4$–	—	—	7.1
12a	H	–$(CH_2)_2$–	C=O	–CH_2–	—	—	>1000
12b	H	–$(CH_2)_2$–	C=O	–$(CH_2)_2$–	—	—	82
12c	H	–$(CH_2)_2$–	C=O	–$(CH_2)_3$–	—	—	76
12d	H	–$(CH_2)_2$–	C=O	–$(CH_2)_5$–	—	—	295
12e	H	–$(CH_2)_2$–	C=O	–CH_2-*trans*-1,4-cyclohexyl–	—	—	121
12f	H	–$(CH_2)_2$–	C=O	–Ph (4-yl)–	—	—	3.8
12g	H	–CH=CH–	C=O	–$(CH_2)_4$–	—	—	20
12h	H	–S–CH_2–	C=O	–$(CH_2)_4$–	—	—	10
12i	H	–$(CH_2)_3$–	C=O	–$(CH_2)_4$–	—	—	32
12j	H	–CH_2–O–CH_2–	C=O	–$(CH_2)_4$–	—	—	26
13a	H	–$(CH_2)_2$–	C=O	—	C	0	148
13b	H	–$(CH_2)_2$–	C=O	—	C	1	2.9
13c	H	–$(CH_2)_2$–	C=O	—	N	1	13
13d	H	–CH_2–O–CH_2–	C=O	—	C	1	4.4
13e	MeO	–$(CH_2)_2$–	C=O	—	N	1	1.6
13f	MeO	–CH=CH–	C=O	—	N	1	1.4
13g	H	–$(CH_2)_2$–	–CH_2–	—	N	1	14
13h	MeO	–$(CH_2)_2$–	–CH_2–	—	N	1	1.6

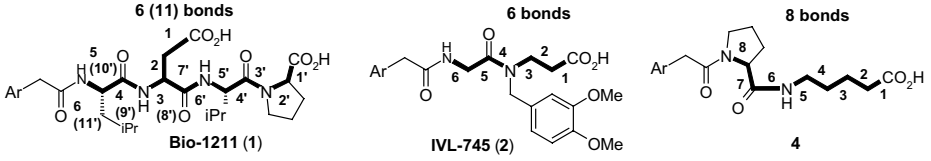


Figure 3. Comparison of optimal bond distance.

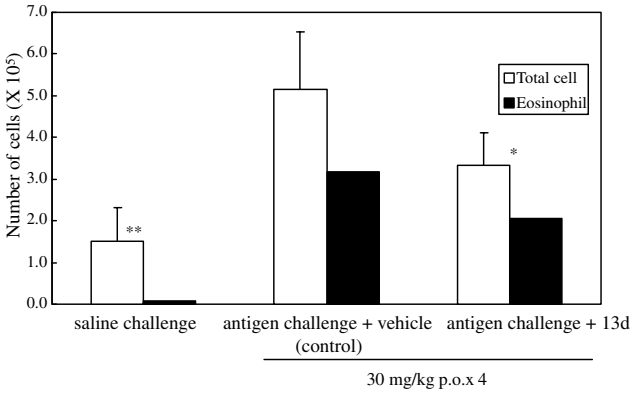


Figure 4. Effect of VLA-4/VCAM-1 antagonist on the leukocyte infiltration in BAL fluid 48 h after allergen challenge in *Ascaris suum* sensitized mice at an oral dosage 30 mg/kg twice a daily for 2 days. $^{*}p < 0.05$, $^{**}p < 0.05$ versus control (Student's *t*). Total numbers of cells in BAL fluid were counted, separately. Eosinophils numbers were expressed as the mean from each treatment group.

structural modification described above did not lead to significant improvement of pharmacokinetic profile.

Starting from lead compound **4**, we had made modifications of the portions **b** and **c** corresponding to the LDV sequence interacting with VLA-4 to explore the SAR. In particular, the atom length (distance) between the nitrogen atom in portion **b** and the carboxylic acid group was found to be important for the inhibitory potency (the optimal distance is 8 bonds). Furthermore, as a useful result for moving away from the peptidic character by reducing H-bond acceptor, we found that the amide bond between portions **b** and **c** is replaceable. This optimization study has led to the identification of a novel

Table 2. Pharmacokinetic and physicochemical properties of selected VLA-4 antagonists

Compd	F[a,b] (%)	Cl (i.v.) (mL/min/kg)	$t_{1/2}$ λ1 (min)	$t_{1/2}$ λ2 (min)	AT ratio[c]	log D[d]
4	<1	75.1	2.8	9.1	0.6	−0.9
13b	<1	41.3	5.8	20.2	0.6	−0.3
13d	<1	69.3	6.8	42.2	0.6	−0.7

[a] Male Sprague–Dawley rats.
[b] Dose: i.v. infusion (2 h) at 1.2 mg/kg; p.o. at 5 mg/kg.
[c] The value of AT ratio means the ratio of Papp (test compound) to Papp (Atenolol) in Caco-2 cell permeability assay. Atenolol has been reported to show oral bioavailability of 50% in human.[16]
[d] 1-Octanol to the Japanese Pharmacopoeia second fluid (pH 6.8) partition coefficient.

series of 4-piperidinylacetic acid, 1-piperazinylacetic acid, and 4-aminobenzoic acid derivatives as potent VLA-4 antagonists with IC_{50} values of less than 10 nM. In addition, a representative compound morpholinyl-4-piperidinylacetic acid derivative **13d** demonstrated efficacy in vivo asthma model by oral administration. Further pharmacokinetic improvements and structural modifications of these leading compounds will be presented in forthcoming publications.

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13. (*S*)-Morpholinic acid derivative (**6**; P = Z, see Scheme 1) was synthesized as reported by: Kogami, Y.; Okawa, K. *Bull. Chem. Soc. Jpn.* **1987**, *60*, 2963.
14. A human VLA-4-expressing cell line, 4B4, was established at Pharmacopeia (New Jersey, USA). The 4B4 cell line was established by transfecting both the α4 gene and β1 gene of VLA-4 into CHO-K1 cells and maintained in F-12 medium (Ham's F-12) supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (Invitrogen Corporation, Carlsbad, USA) and 1 mg/mL G-418 (Geneticin, Invitrogen Corporation, Carlsbad, USA). Human VCAM-1/Fc Chimera (R&D Systems Inc., Minneapolis, USA) was labeled with Eu as follows. The protein was reconstituted in labeling buffer (150 mM NaCl, 50 mM sodium carbonate, pH 8.5). Eu-Labeling Reagent (Perkin–Elmer Inc., Wellesley, USA) reconstituted with the labeling buffer was added to the protein solution. The mixture was then incubated at room temperature for 4 days. The Eu-labeled protein was purified with a PD-10 column (Amersham Biosciences KK, Tokyo, Japan) and stored at −80 °C until use. All assays were performed in duplicate. Prior to the assay, 4B4 cells were suspended at 3×10^5 cells/mL in F-12 medium (Ham's F-12) supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine (Invitrogen Corporation, Carlsbad, USA). One hundred microliters of the 4B4 cell suspension at 3×10^5 cells/mL was distributed into each well of 96 well-culture plates (Costar, 3599, USA). The plates were incubated in a 5% CO_2 humidified incubator at 37 °C (Themo Forma, model: 3120, Forma Scientific, Inc. USA) for 2 days. The medium was discarded and each well was washed twice with 300 μL of chilled Wash Buffer (25 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 4 mM $MnCl_2$). Then 50 μL of compound solution was distributed to the wells, followed by 50 μL of 2 nM of Eu-labeled Human VCAM-1/Fc Chimera diluted with the Wash Buffer (final concentration: 1 nM). The plates were incubated for 60 min at room temperature, and the wells were washed four times with 300 μL of chilled Wash Buffer. Finally 100 μL of the enhancement solution (Perkin–Elmer Inc., Wellesley, USA) was added to each well. The plates were placed on a shaker for 5 min. Eu fluorescence was then measured in a time-resolved fluorometer (DELFIA Research fluorometer, model: 1234-001, Perkin–Elmer Inc., Wellesley, USA). The concentration of compound required for 50% inhibition in the assay was determined.
15. Female mice received cyclophosphamide dissolved in water administered orally at a dose of 150 mg/10 mL/kg (day 0). An extract of 500 μg of *Ascaris suum* protein as antigen in 0.2 mL saline containing 4.5 mg aluminum hydroxide as adjuvant were prepared. On day 2, 0.2 mL of the antigen solution was injected intraperitoneally. On day 14, a booster dose of the same antigen solution was injected intraperitoneally. Finally on day 22, mice were anesthetized by intraperitoneal injection with 65 mg/10 mL/kg of pentobarbital sodium solution. After 10 min, mice were challenged intratracheally with 300 μg protein of the *Ascaris suum* extract. The test compound was given to the mice at 15 min before and 8, 24, and 32 h after the antigen challenge. Forty-eight hours later, the lungs of mice were lavaged via a tracheal polyethylene cannula (outside diameter 1.33 mm, Hibiki No. 4, Hibiki Co., Tokyo, Japan) with 2 × 0.5 mL Hank's balanced buffered salt solution supplemented with 0.05 mM potassium EDTA. The bronchial alveolar lavage (BAL) was performed, and the total numbers of cells in BAL fluid were counted, separately. Eosinophils numbers were expressed as the mean from each treatment group.
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