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Squaric Acid Derivatives as VLA-4 Integrin Antagonists

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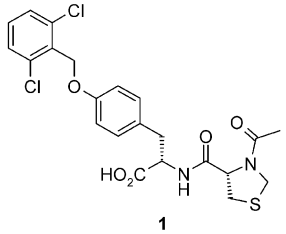
Abstract—SAR studies aimed at improving the rate of clearance by the incorporation of a 3,4-diamino-3-cyclobutene-1,2-dione group as an amino acid isostere in a series of VLA-4 integrin antagonists are described.

The integrin Very Late Antigen-4 (VLA-4) is a heterodimeric ($\alpha4\beta1$) adhesion molecule, expressed on the surface of many leukocytes, including T-lymphocytes and eosinophils, but not neutrophils. The binding of VLA-4 to ligands such as Vascular Cell Adhesion Molecule-1 (VCAM-1) expressed on endothelial cells is recognised as a key step in the processes of adhesion, migration and activation of inflammatory leukocytes at sites of inflammation.[1] Blocking such an interaction would be expected to be of therapeutic benefit in a variety of inflammatory and autoimmune diseases. Anti-$\alpha4$ antibodies have shown efficacy in animal models of a number of inflammatory diseases and the results from early stage clinical trials are promising.[2] Thus, small molecule VLA-4 receptor antagonists represent an attractive target for the treatment of various chronic inflammatory diseases such as asthma, multiple sclerosis and rheumatoid arthritis.[3]

We have recently described the discovery of the thioproline CT5219,[4] **1**, a small molecule VLA-4 antagonist that showed excellent efficacy in inhibiting antigen-induced airway responses in the allergic sheep model. The potency of **1** could be further enhanced by the replacement of the 2,6-dichlorobenzyl ether with a 3,5-dichloropyridylamide group, **2**. Unfortunately both **1** and **2** displayed rapid clearance in a number of species, including the rat, thereby preventing further development. The rapid biliary clearance was shown to be the result of an active transport mechanism by the presence in the bile of only minor amounts of metabolites compared to the parent compounds.[5]

Postulating that the α-amido group, although apparently essential for potency, was responsible for the rapid clearance in this series, we investigated a number of methods for its replacement. The 3,4-diamino-3-cyclobutene-1,2-dione group has been recognised as an isostere for α-amino acids[6] as well as other functional groups such as cyanoguanidines.[7] We now wish to report that the replacement of the α-amido carbonyl moiety in compounds such as **2** with 3-cyclobutene 1,2-dione analogues gave compounds such as **3** that are potent VLA-4 antagonists and have an improved pharmacokinetic profile.



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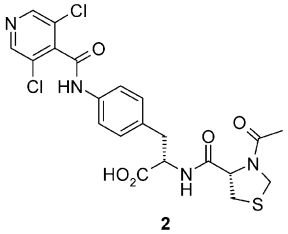
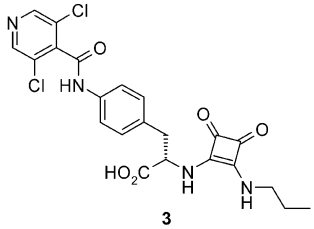


2



3

The compounds were readily prepared as outlined in Scheme 1. The lithiated 3,5-dichloropyridine **4** was

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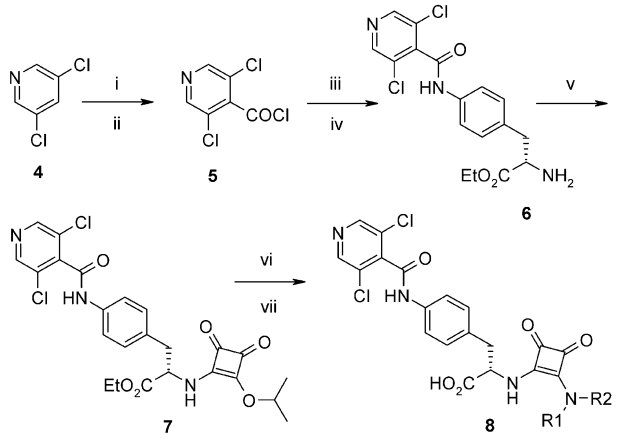
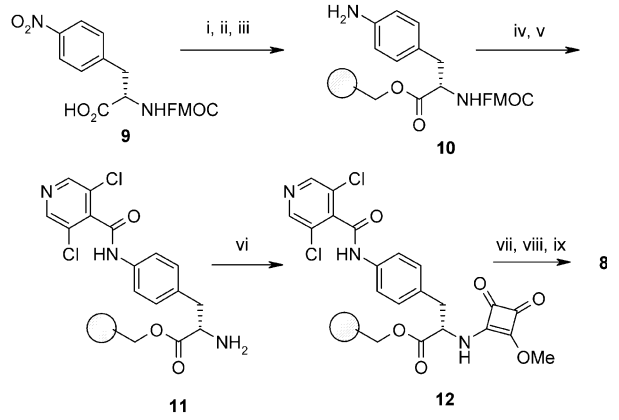
quenched with CO_2 to generate the carboxylic acid that was converted to the acid chloride **5** by treatment with thionyl chloride. Coupling of **5** to the α-N-Boc protected ethyl ester of 4-aminophenylalanine in the presence of base gave the amine **6** after removal of the Boc group with TFA. Heating with a solution of 3,4-diisopropoxy-3-cyclobuten-1,2-dione in ethanol gave the key intermediate **7** that was further functionalised with a variety of amines. Aqueous LiOH hydrolysis of the ethyl ester gave the target acid **8**.

This method was adapted for parallel synthesis as shown in Scheme 2. α-N-FMOC protected 4-nitrophenylalanine **9** was coupled to Wang resin and the nitro group reduced with $SnCl_2$ in DMF to give the resin bound 4-aminophenylalanine **10**. Addition of the acid chloride **5** followed by FMOC deprotection gave the amine **11**, heating with a DMF solution of 3,4-dimethoxy-3-cyclobuten-1,2-dione gave the key intermediate **12**. Derivatisation with a range of amines was followed by cleavage from the resin and HPLC purification to give the target acids **8**.



Scheme 1. (i) LDA, THF, −78 °C, 30 min then CO_2; (ii) $SOCl_2$, DCM; (iii) α-*N*-Boc 4-aminophenylalanine ethyl ester, *N*-methyl morpholine, DCM; (iv) 3 M HCl in EtOAc; (v) 3,4-diisopropoxy-3-cyclobuten-1,2-dione, DIPEA, EtOH; (vi) R_1R_2NH, EtOH; (vii) LiOH, THF, H_2O.



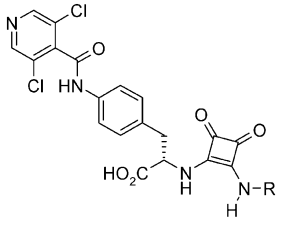
Scheme 2. (i) Paramax Wang resin, 2,6-dichlorobenzoyl chloride, pyridine, DMF; (ii) 20% acetic anhydride, DMF; (iii) $SnCl_2(H_2O)_2$, DMF; (iv) 5, DIPEA, DCM; (v) 20% piperidine, DMF; (vi) 3,4-dimethoxy-3-cyclobuten-1,2-dione, DMF, 70 °C; (vii) R_1R_2NH, DCM; (viii) 60% TFA, DCM; (ix) HPLC purification.

In order to gain some understanding of the SAR for clearance, it was considered essential to have access to a relatively high throughput screen that could provide this data. The method chosen was the isolated perfused rat liver[8] (IPRL), whereby five compounds (including a reference compound) could be dosed as a cassette. The elimination of each compound from the perfusate is expressed in terms of a rate constant, *k*, and normalised to the reference compound. The higher the value of *k* the more rapidly the compound was cleared. Compounds were assayed for their ability to inhibit the binding of VLA-4 to VCAM-1 in a protein-based, ligand binding[9] and a cell-based, adhesion[10] assay.

The initial preparation of a library of 23 compounds, based on the scaffold **12**, with a diverse range of amines identified the methoxyethylamine analogue **13** as a promising lead with good potency in both the protein and cell-based assays. Compound **13** also had a moderate rate of clearance as measured by the IPRL assay, that translated to a clearance of 5 mL/min/kg in the rat.

Encouraged by these results, we set about making further libraries based upon this scaffold initially using primary unbranched amines, a representative selection of which are shown in Table 1. Although most of these compounds had a relatively flat SAR in the protein-based assay there were noticeable trends in the cell-based assay. The methyl analogue, **14**, displays poor potency but as the length of the carbon chain increases an increase in potency is observed that reaches a maximum at propyl **3**, and butyl **16**, before appearing to drop off again with pentyl **17**. The clearance rate constant of all of these compounds is reasonably low but appears to reach a minimum of 0.1 h^{-1} with the propyl analogue **3**. This translated to a clearance of 0.1 mL/min/kg in the rat. Interestingly the more lipophilic trifluoromethyl analogue **19** is cleared at a faster rate. The rate of clearance is not dependent on the stereochemistry of the α-centre is shown by comparison of the

Table 1. Potency and rate of clearance for secondary unbranched amines



Compd	R	VLA-4 protein[9] (IC_{50}, nM)	VLA-4 cell[10] (IC_{50}, nM)	IPRL k h^{-1}
13	2-Methoxyethyl	1.2	75	1.8
14	Methyl	0.9	990	0.9
15	Ethyl	0.6	425	2.0
3	*n*-Propyl	1.5	120	0.1
16	*n*-Butyl	0.4	130	1.3
17	*n*-Pentyl	1.0	225	0.9
18	Allyl	1	120	0.4
19	2-Trifluoromethylethyl	nd	90	1.4
20	3-Trifluoromethylpropyl	1	370	1.1

propyl analogue **3** with its distomer **3a** (VLA-4 protein IC_{50} 102 nM; VLA-4 cell $IC_{50} > 10{,}000$ nM; IPRL k 0.1 h^{-1}).

The next library of compounds was based around branched primary amines and a representative selection is shown in Table 2. This series displayed similar levels of potency to the unbranched analogues with the isopropyl **21** and isobutyl **25** compounds being the most active in the cell-based assay, though they have a clearance rate constant higher than the essentially equipotent propyl analogue **3**. Increasing the steric bulk, for example the *t*-butyl analogue **28**, has a detrimental effect on potency and the rate of clearance.

The preparation of a library of compounds based on secondary amines shows that the amino hydrogen is not required for potency (Table 3). Although the dimethyl analogue **31** has poor activity in the cell based assay (as may be expected from the result with the methyl compound **14**) the diethyl **32** and the di-*n*-butyl **34** analogues display excellent levels of activity. This trend is continued with the cyclic amines, notably the thiomorpholine **37** and the piperidine **38**. The presence of a basic centre, for example the piperazine **39**, is less well tolerated. However, despite their enhanced potency, all of these tertiary amine derivatives display very high rates of clearance.

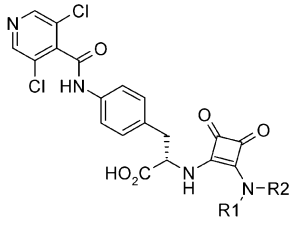
This correlation between the degree of amine substitution, potency and rate of clearance extended to replacements for the dichloropyridyl amide. We prepared a series of these analogues as either the *n*-propylamino or diethylamino substituted squarates, a representative sample of which are shown in Table 4. The 2,6-dimethoxyphenyl substituent has been used in a number of phenylalanine-based VLA4 antagonist analogues,[11] but the squarate analogues **43** and **44** display less potency than their dichloropyridylamide counterparts **32** and **3**, respectively. However the relative potencies (tertiary amine being more potent than secondary) and clearance rates (secondary amine being cleared more slowly than tertiary) remain the same regardless of the 4-phenylalanine substituent for all of the examples prepared. Obviously the squarate amino substituent has a profound effect upon clearance, and although we were unable, at this stage, to rule out plasma protein binding, we speculated that conformational differences may have some influence.

Opportunities for probing the local structural environment around the squarate using NMR methods such as

Table 2. Potency and rate of clearance for secondary branched amines

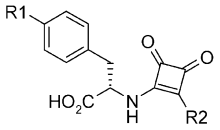
Compd	R	VLA-4 protein[9] (IC_{50}, nM)	VLA-4 cell[10] (IC_{50}, nM)	IPRL k h^{-1}
21	*i*-Propyl	1.0	95	2.6
22	*c*-Propyl	1.5	250	1.6
23	*c*-Butyl	2.6	155	0.5
24	*c*-Pentyl	9	290	0.5
25	*i*-Butyl	0.5	85	1.3
26	1-Methylpropyl	0.5	160	3.1
27	1-Methylbutyl	0.6	50	2.4
28	*t*-Butyl	3.5	690	3.5
29	Phenyl	50	845	1.1
30	Benzyl	3.7	320	4.2

Table 3. Potency and rate of clearance for tertiary amines



Compd	R^1	R^2	VLA-4 protein[9] (IC_{50}, nM)	VLA-4 cell[10] (IC_{50}, nM)	IPRL k h^{-1}
31	Methyl	Methyl	0.5	590	4.6
32	Methyl	Ethyl	0.2	100	4.9
32	Ethyl	Ethyl	0.4	3.5	5.8
34	Methyl	*n*-Propyl	0.2	13	4.5
35	*n*-Butyl	*n*-Butyl	2.0	5.7	6.6
36	Morpholino		0.5	11	5.0
37	Thiomorpholino		0.3	3.1	5.7
38	Piperidine		0.4	8.4	4.7
39	Piperazine		0.4	40	4.5
40	*N*-Ethyl piperazine		0.5	60	3.7

Table 4. Dichloropyridyl amide replacements



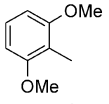
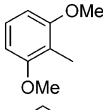
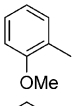
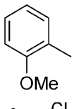
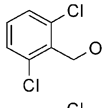
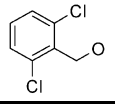
Compd	R^1	R^2	VLA-4 protein[9] (IC_{50}, nM)	VLA-4 cell[10] (IC_{50}, nM)	IPRL k h^{-1}
41		NEt_2	1.5	2300	2.7
42		NHPr	6.9	8200	0.6
43		NEt_2	0.6	180	3.9
44		NHPr	2.1	710	0.7
45		NEt_2	1.2	1160	2.2
46		NHPr	6.7	10,800	nd
47		NEt_2	25	12,400	3.9
48		NHPr	67	18,300	nd

Table 5. Comparison of NHCHα chemical shift temperature dependence gradient against IPRL

Compd	Gradient (ppb/K)	IPRL k h^{-1}
41	−7.7	2.7
42	−4.4	0.6
45	−7.2	2.2
46	−4.2	nd
43	−7.6	3.9
42	−4.5	0.7
32	−7.7	5.8
3	−4.3	0.1
3a	−4.2	0.1
18	−4.4	0.4
16	−4.3	0.8
36	−6.3	1.3
47	−7.9	3.9
48	−4.7	nd

NOEs are limited as there are so few protons associated with this moiety. The closest protons to the squarate moiety are those attached to the nitrogen atoms on either side. One technique that has been used successfully in the past is the study of the amide NH chemical shift temperature dependencies in non-exchanging solvents.[12] The gradients obtained (in ppb/K) give an indication of the extent of exposure that the NH proton has to its surroundings. Values > -2.0 ppb/K suggest hydrogen bonding, intermediate values < -3.0 ppb/K but > -6.0 ppb/K suggest some degree of shielding from the environment while values < -6.0 ppb/K indicate almost total exposure. The gradients are always negative, that is, there is an increase in magnetic shielding (lower δ) for the NH resonance as temperature increases.

The data are summarised in Table 5. In each case compounds with a tertiary amine have a steeper temperature gradient (typically −6.0 to −9.0 ppb/K than those with a secondary amine (typically −4.0 to −5.0 ppb/K). This effect appears to be independent of the nature of the phenylalanine substituent. In the secondary amine series the gradients for the two squarate NH protons are consistently similar, in many cases their resonances being isochronous across the temperature range. We postulate that it may be the presence or absence of a hydrogen atom on the amine nitrogen that influences the degree of exposure of the NHCHα to solvent. In terms of potency and clearance, in the secondary amine series where the NHCHα is more shielded, it may be that the ligand's interaction with the protein is slightly weakened by this apparent shielding effect, however this shielding may also be responsible for the slower clearance. Conversely, in the tertiary amine series the greater exposure of the NHCHα may relate to the improved activity but may also make this series more prone to the observed rapid clearance.

As the *n*-propyl analogue, **3**, appears to have the optimum combination of reasonable potency and low rate of clearance it was selected for further study in animal models of inflammatory disease and optimisation for improved oral bioavailability. The results of these studies will be reported at a later date.

Acknowledgements

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References and Notes

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9. VLA-4 (from HL60 lysate) was immobilised on a plate with a non-blocking anti-β1 antibody (TS2/16). The test compounds were titrated into a solution of 2-domain VCAM-Fc-Ig in a separate plate and added to the wells. The assay was carried out in TBS, 1% BSA, 1 mM $MnCl_2$, 0.1% Tween. After incubation for 2 h at room temperature the plates were washed and residual VCAM visualised with peroxidase coupled anti-human Fc.
10. A Jurkat cell line expressing VLA-4 was incubated at 37 °C for 30 min with human 2-domain VCAM-1-FC immobilised on a plate with anti-human FC in the presence of the test compounds. The plates were washed and residual cells were stained with Rose Bengal.
11. For examples see ref 3.
12. Wright, P. E.; Dyson, H. J.; Lerner, R. A. *Biochem.* **1988**, *27*, 7167.