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NOVEL INHIBITORS OF α4β1 INTEGRIN RECEPTOR INTERACTIONS THROUGH LIBRARY SYNTHESIS AND SCREENING

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Abstract: A library of 2302 small molecule β-turn mimetics was screened for inhibition of the α4β1 integrin-CS1 splice variant binding interaction. Preliminary data revealed several active ligands, and validation with purified material culminated in the identification of some of the first small molecule ligands (**1**, IC_{50} = 5 μM, and **2**, IC_{50} = 8 μM) to be reported for this class of integrins.

Events such as cell adhesion and migration are dependent on receptor-mediated recognition between cell-adhesive proteins and the extracellular domains of membrane proteins. One such cell-adhesive protein is the extracellular matrix protein fibronectin, a component of connective tissues and body fluids. It has been shown that fibronectin binds to a range of integrins, including α4β1 (VLA-4). This integrin is expressed on most leukocytes, including lymphocytes, monocytes, eosinophils, and basophils.[1] The firm adhesion of leukocytes to activated endothelium and their subsequent extravasation into the surrounding tissues are critical events of inflammation and are mediated in part by this class of integrins.[2] α4β1 has also been shown to support initial lymphocyte attachment and rolling.[3] The efficacy of anti-α4 antibodies in animal models of inflammatory diseases such as asthma,[4] multiple sclerosis,[5] diabetes,[6] and inflammatory bowel disease[7] suggests that the α4β1 integrin receptor may be a good target for therapeutic intervention.

Several sites for α4β1 binding have been located on fibronectin, including the alternatively spliced connecting segment 1 (CS1).[8] It has been shown that the minimal sequence of the CS1 fragment required for α4β1 binding is Leu-Asp-Val (LDV),[9] and a turn structure has been implicated for these residues.[10]

Vanderslice and coworkers identified a cyclic hexapeptide antagonist of the α4 integrins based on the CS1 region of fibronectin, providing a good lead structure, but no truly nonpeptidal ligands have been reported to date.[11,12] In this communication, we report the synthesis and biological evaluation of a library of small molecule β-turn mimetics whose side chain displays are based on the LDV sequence. An initial screening of the library and subsequent reevaluation with purified material resulted in the identification of two antagonists of VLA-4 binding to the CS1 splice variant.

Earlier developments of small molecule β-turn mimetics in our laboratory culminated in the general scaffold shown in Figure 1. An α-bromo acid supplies the i+1 side chain, an α-amino acid supplies the i+2 side

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chain, a primary amine furnishes the i+3 side chain, and a hydroxyalkyl thiol provides the backbone component.[13]

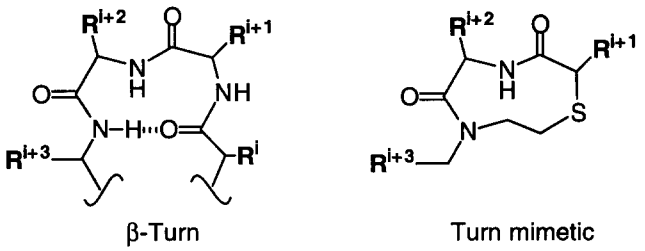
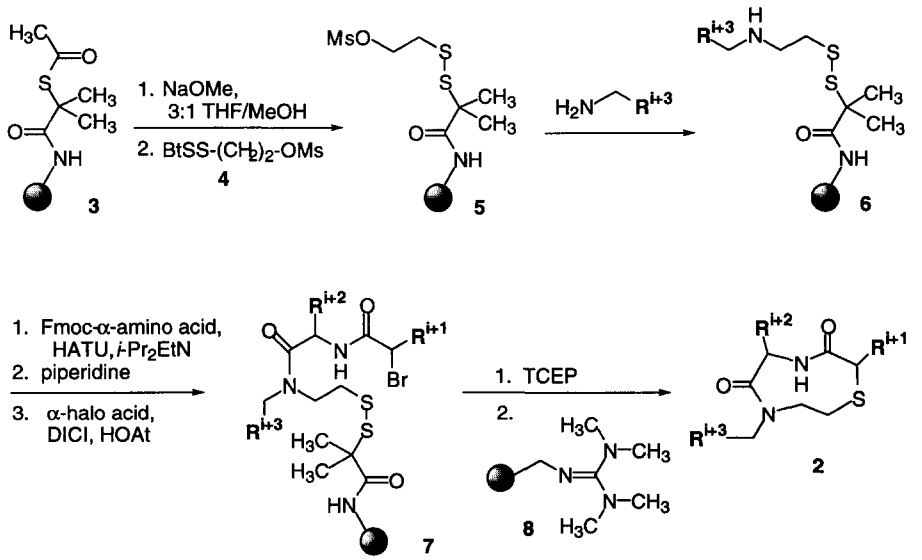


Figure 1. Heterocyclic β-turn mimetic.

Solid-phase synthesis is initiated by methanolysis of support-bound linker **3** followed by disulfide interchange with mixed disulfide **4** to afford the support bound mesylate **5**. This intermediate is then treated with a concentrated solution of the appropriate primary amine to introduce the i+3 side chain of **6**. The i+2 and i+1 side chains are then incorporated via HATU coupling, Fmoc deprotection, and standard DICI acylation, respectively, to afford the linear precursor **7**. The acyclic turn mimetic is then cleaved from the resin with tris-carboxyethyl phosphine, followed by treatment with the support-bound guanidine **8**. The latter reagent serves to remove all phosphine and phosphine oxide byproducts, as well as HBr, in addition to driving the cyclization. Filtration and concentration consistently affords the cyclized mimetics in good yields over eight steps on support (59% average overall yields), with small amounts (<5%) of the minor i+1 epimer being the only significant byproduct.[13]

Scheme I



Using this methodology, we constructed a library of over 5500 spatially separate β-turn mimetics (Figure 2),[14] the details of which will be described shortly. Both stereochemistries of 12 α-bromo acids and 14 Fmoc-α-amino acids were utilized at the i+1 and i+2 positions, respectively, to provide a highly representative cross-section of proteinogenic side chain functionality. Nine primary amines were employed at the i+3 position on the basis of diversity and compatibility with the overall sequence, and the spatially addressable format was layed out such that an exhaustive display of all side chain/stereochemistry combinations was present. From the available structures, 2304 compounds were selected based on the presence of the crucial aspartic acid residue at at least one site, with diversity emphasized at the remaining two positions.

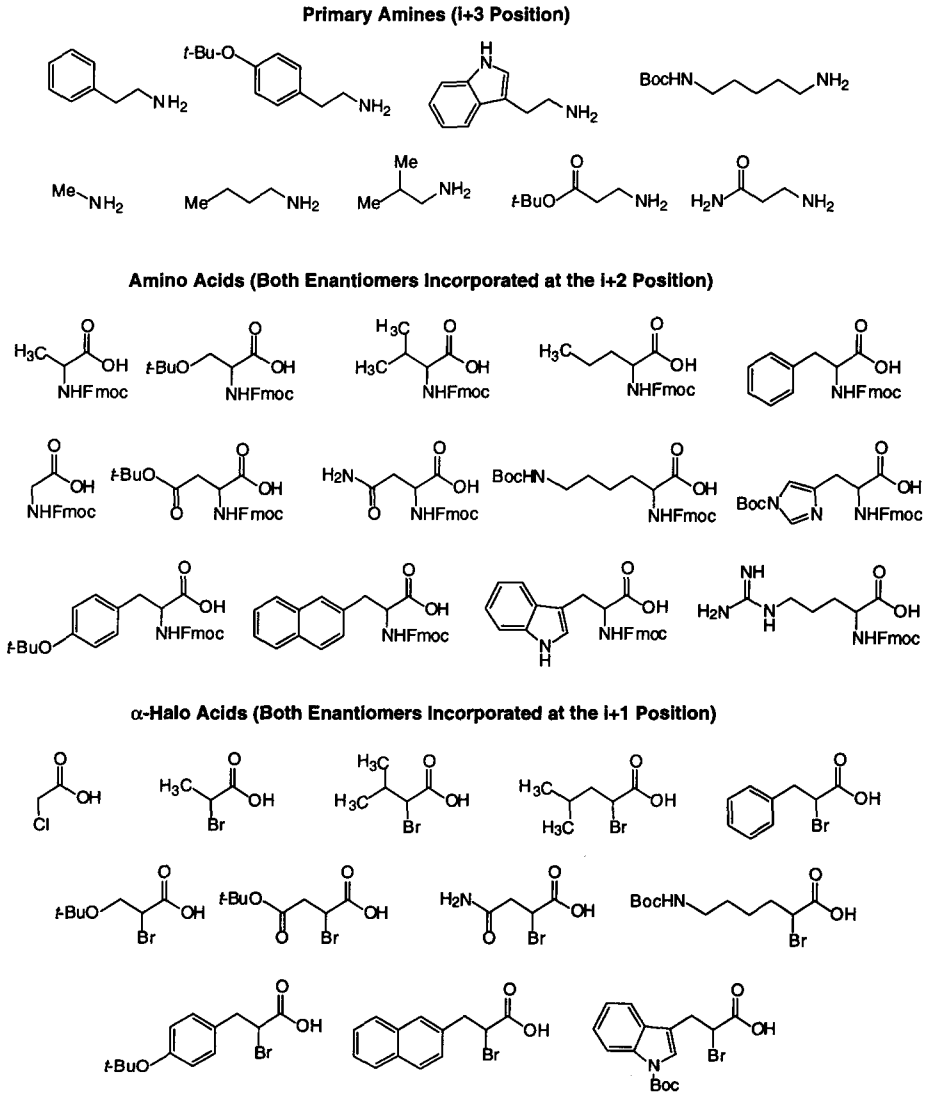


Figure 2. Building blocks used to prepare β-turn mimetic library.

The library of turn mimetics was evaluated for inhibitory effects on the binding of fluorescently labeled Ramos cells to immobilized CS1 peptide. The individual library compounds were assayed at a concentration of 1 μM.[11] Nearly all of the active compounds displayed an aspartic acid i+1 side chain, with both D and L stereochemistries being represented. Additionally, nearly all active compounds contained a hydrophobic residue at the i+2 site. Phenylalanine and tyrosine were present in over half the structures. Interestingly, the i+3 position displayed both hydrophilic and hydrophobic residues. The aspartic acid surrogate (β-alanine) at this position was present in half of the active compounds. A high preponderance of the diacid display at the i+1 and i+3 positions suggested that this motif is quite effective for inhibition. Notably, activity was not observed for mimetics with an aspartic acid side chain at the i+2 position juxtaposed by hydrophobic side chains at the i+1 and i+3 positions, as might be expected based upon the bioactive LDV sequence.

To validate these inhibitory effects, we carried out large scale syntheses of four of the most active compounds (Figure 3) employing the previously described conditions. β-turn mimetic **3**, with a display of Asp, Phe, and norvaline at the i+1, i+2, and i+3 positions, respectively, represented the closest similarity to the LDV sequence. To probe the structural requirements and at the i+3 position, a derivative (**4**) of compound **3** that contained a leucine surrogate was also synthesized.

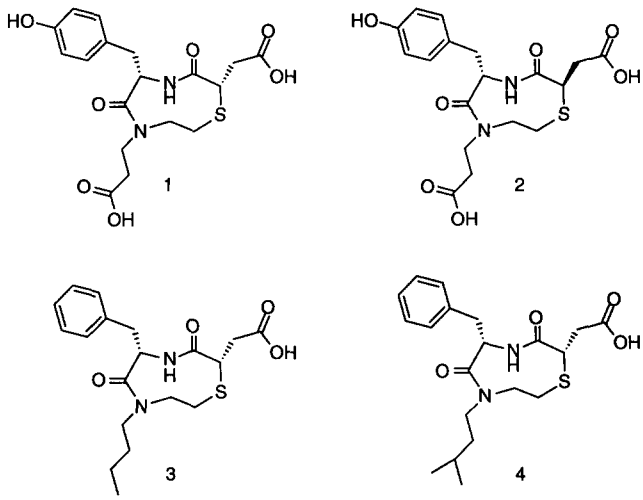


Figure 3. Four of the most potent ligands.

These four compounds were evaluated using the mentioned assay conditions, and IC_{50} values were determined. As seen in Table 1, compounds **1** and **2**, which differ only in their stereochemistry about the i+1 position, displayed validated activity.[15] The measured IC_{50} values for the two stereochemical displays of the i+1 side chain were 8 and 5 μM. The lack of activity observed for the Asp/Phe/norvaline mimetic suggests that the initially observed activity was a false positive, and consequently, the leucine analog was inactive as well. These

structures did serve as a control, however, and indicate that compounds **1** and **2** are not acting specifically as LDV mimetics.

Table 1. In vitro data of compounds 1–4.

Compound	IC_{50} (μM)	% Inhibition at 100 μM
1	8 ± 2	100 ± 0
2	5 ± 1	100 ± 0
3	>100	41 ± 3
4	>>100	0

Doyle and coworkers suggested that the activity of the LDV sequence is critically dependent on the two hydrophobic residues surrounding the key Asp functionality.[10] However, for mimetics **1** and **2**, it appears that one hydrophobic residue is sufficient for binding, while additional hydrophilics can increase potency. In particular, the observed data suggests that the diacid display is optimal in this scaffold for inhibitory activity.

In summary, compounds **1** and **2** represent some of the first nonpeptide ligands towards this class of integrins. The apparent lack of specificity for the i+1 aspartic residue stereochemistry implies that the scaffold is considerably flexible. Further rigidification may serve to enhance the activity of one or both of these compounds.

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15. (**1**) ^{1}H NMR (400 MHz, CD_3OD) δ 2.31 (m, 2H), 2.71–2.78 (m, 1H), 2.88–3.02 (m, 4H), 3.20–3.22 (m, 1H), 3.38–3.42 (m, 1H), 3.74–3.79 (m, 1H), 4.05–4.13 (m, 2H), 5.05 (m, 1H), 6.65 (d, J = 9.6 Hz, 2H), 7.12 (d, J = 9.6, 2H); HRMS (FAB) *m/z*, calcd for $C_{18}H_{22}N_2O_7S$ (MH^+) 411.4450; found: 411.1225. (**2**) ^{1}H NMR (400 MHz, CD_3OD) δ 2.33—2.42 (m, 1H), 2.51–2.62 (m, 2H), 2.68–2.82 (m, 1H), 2.79–2.85 (m, 2H), 3.08–3.21 (m, 2H), 3.32–3.43 (m, 3H), 3.63–3.81 (m, 2H), 4.88–4.92 (m, 1H), 6.64 (d, J = 9.6 Hz, 2H), 7.07 (d, J = 9.6 Hz, 2H); HRMS (FAB) *m/z*, calcd for $C_{18}H_{22}N_2O_7S$ (MH^+) 411.4450; found: 411.1230.