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R-Isomers of Arg-Gly-Asp (RGD) mimics as potent $\alpha_v\beta_3$ inhibitors

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Abstract—The integrin $\alpha_v\beta_3$, vitronectin receptor, is expressed in a number of cell types and has been shown to mediate adhesion of osteoclasts to bone matrix, vascular smooth muscle cell migration, and angiogenesis. We recently disclosed the discovery of a tripeptide Arg-Gly-Asp (RGD) mimic, which has been shown to be a potent inhibitor of the integrin $\alpha_v\beta_3$ and has excellent anti-angiogenic properties including its suppression of tumor growth in animal models. In other investigations involving RGD mimics, only compounds containing the *S*-isomers of the β-amino acids have been shown to be potent. We were surprised to find the potencies of analogs containing enantiomerically pure *S*-isomers of β-amino acids which were only marginally better than the corresponding racemic mixtures. We therefore synthesized RGD mimics containing *R*-isomers of β-amino acids and found them to be relatively potent inhibitors of $\alpha_v\beta_3$. One of the compounds was examined in tumor models in mice and has been shown to significantly reduce the rate of growth and the size of tumors.

1. Introduction

The cellular functions adhesion, migration, and survival are primarily mediated through the integrins, a family of cell surface adhesion molecules and receptors. Integrins are heterodimeric membrane receptors, having α and β subunits. They are classified broadly according to ligand specificity, forming four classes that recognize collagen, laminin, the tripeptide sequence Arg-Gly-Asp (RGD), and several specific leukocyte associated ligands.[1]

The tripeptide sequence RGD found in extracellular matrix and cell surface proteins such as vitronectin, fibronectin, fibrinogen, thrombospondin, von Willebrand factor, and osteopontin is recognized by the α_v integrins ($\beta_1, \beta_3, \beta_5, \beta_6, \beta_8$), the platelet glycoprotein receptor (GPIIb/IIIa, $\alpha_{IIb}\beta_3$), and the β_1 integrins, $\alpha_5\beta_1$, and $\alpha_8\beta_1$. These integrins function in bone remodeling, angiogenesis and the aggregation of activated platelets. In contrast to many other integrins, which have limited substrate selectivities, $\alpha_v\beta_3$ is promiscuous, recognizing several extracellular matrix proteins such as vitronectin and osteopontin that contain RGD. The integrin $\alpha_v\beta_3$ is expressed on several cell types, including osteoclasts, smooth muscle cells, and endothelial cells, and mediates several biological processes, for example, adherence to bone matrix, angiogenesis and the migration of endothelial cells, vascular smooth muscle cells, and tumor cells through the extracellular matrix.[2–9]

Several anti-integrin drugs are currently being evaluated clinically. Vitaxin (Abegrin) is a humanized monoclonal antibody (LM609) that reacts with $\alpha_v\beta_3$ and is reportedly

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in Phase III clinical trials for cancer. This antibody showed activity against solid tumors in a Phase I pharmacokinetic/tolerability trial.[10] Further, monoclonal antibody and small molecule antagonists of the platelet glycoprotein receptor, $\alpha_{IIb}\beta_3$, have been widely investigated. Abciximab, eptifibatide, and tirofiban have been evaluated in several major trials and have been shown to reduce thrombotic complications following percutaneous coronary interventions (PCI).[11]

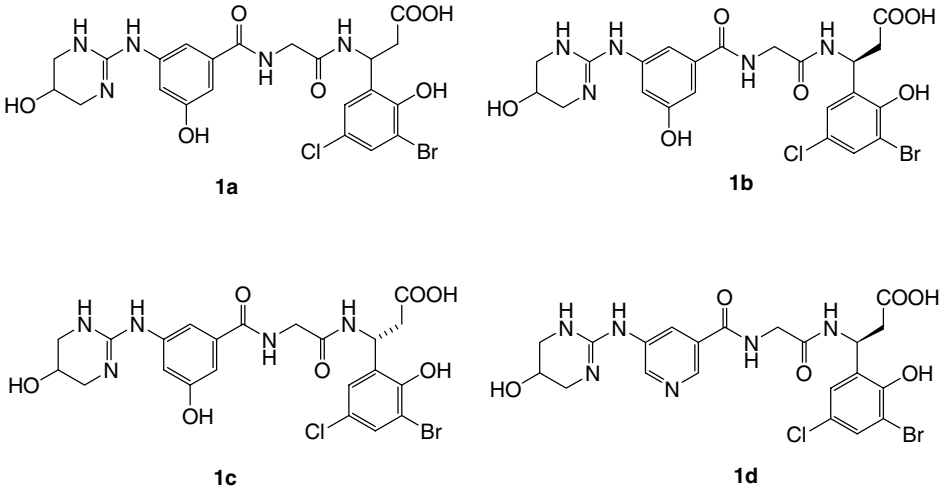
Integrins are thought to play a role in several pathological conditions, including cancer, arthritis, and pathologic thrombotic events. The role of integrins in cancer is complex as they are potentially involved in angiogenesis, cell migration, intravascular arrest, and metastasis. The RGD-selective integrins appear to play an important role in the angiogenic process. Monoclonal antibody and RGD peptidomimetic small molecule antagonists of α_v integrins inhibited angiogenesis and tumor growth in animal models.[12–17] We have demonstrated that the peptidomimetic S247 (**1d**), which inhibited α_v-dependent binding, significantly reduced the formation of colon cancer liver metastases and angiogenesis in a murine model of metastatic disease[18] (Scheme 1).

We had previously disclosed the discovery of the RGD mimetic **1a** and **1b** and their biological properties including the suppression of angiogenesis and tumor growth in animal models of human cancer.[19–22] We describe herein the extension of these studies to include *R*-enantiomers (e.g., **1c**).

The RGD mimics that have been synthesized in our laboratories have a chiral center in the β-amino acid moiety and we initially tested the racemic mixtures of our target compounds.[18–22] It was clear from other investigations involving RGD mimics that only analogs containing *S*-isomer of β-amino acids would be useful as inhibitors.[23–27] When the inhibitors incorporating enantiomerically pure *S*-isomers of β-amino acids were synthesized, the potencies of some of the chiral analogs were only marginally better than the racemic mixtures. We therefore synthesized inhibitors containing *R*-isomer of β-amino acids and found them also to be relatively potent (Racemic **1a**, 3.6 nM; *S*-isomer, **1b**, 2.6 nM; *R*-isomer, **1c**, 3 nM). We also ruled out the possibility that a minor contaminant containing the *S*-isomer of the β-amino acid was inhibiting $\alpha_v\beta_3$ binding. We therefore undertook a systematic study of the RGD mimics containing *R*-isomers of the β-amino acids and we were surprised to find that the SAR of these compounds is quite different from that of the corresponding *S*-isomers.

This paper describes our efforts in the synthesis of enantiomerically pure analogs of the RGD mimics containing β-amino acids with *R*-configuration and the identification of a compound that was a potent and selective $\alpha_v\beta_3$ inhibitor. Biological data for the compound including suppression of tumor growth in animal models are described. The SAR of three distinct regions of the molecule namely the β-amino acid moiety, guanidine, and the adjacent aromatic ring will be presented. The synthesis of the β-amino acids and the synthesis of different left hand portion of the molecules are also discussed.

The initial lead compound **1c** was divided into two parts, the β-amino acid and the guanidinobenzoic acid left hand side (LHS). None of the enantiomerically pure β-amino acids described are commercially available. They were synthesized using varieties of methods as shown below. The left hand side of the molecule was further divided into the *m*-amino substituted aromatic or heteroaromatic acid and the substituted tetrahydropyrimidine. The tetrahydropyrimidine part of the molecule is derived from 1,3-diaminopropane or substituted 1,3-diaminopropanes. Reaction of the isothiourea derived from the *meta*-amino aromatic acid with the diaminopropanes afforded a variety of LHS.



Scheme 1.

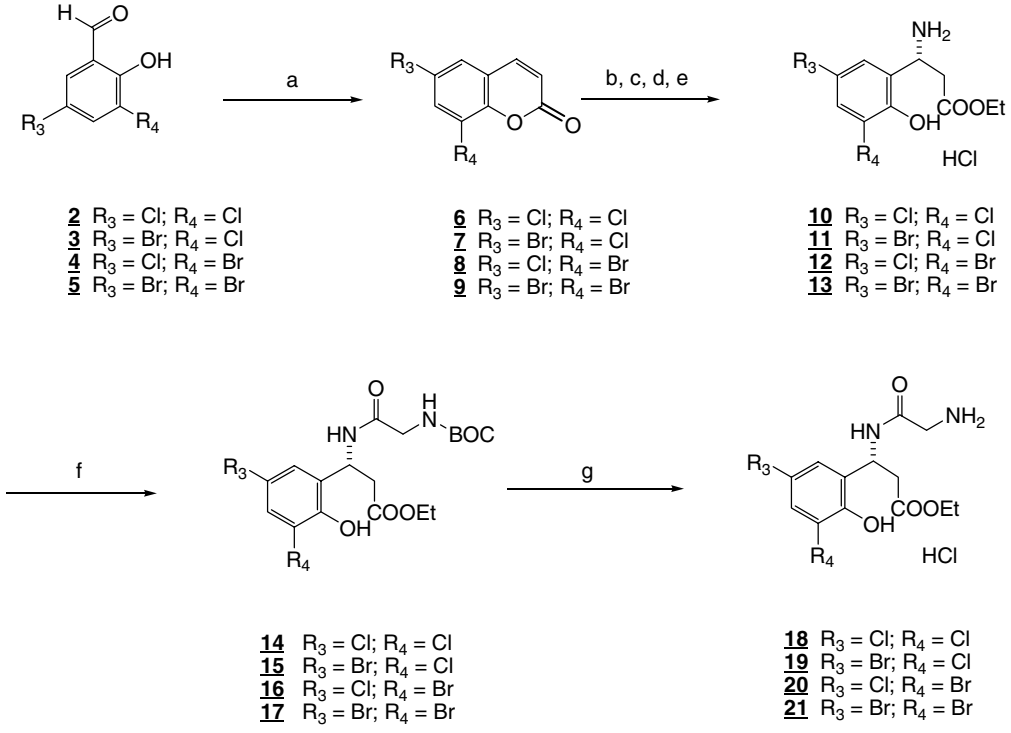
1.1. Synthesis of enantiomerically pure β-amino acids

1.1.1. Synthesis and resolution of racemic β-amino acids. Several methods are known for the synthesis of racemic β-amino acids[28–32] and we employed enzymatic resolution as well as chromatography (chiral packing) for the separation of enantiomers (Scheme 2). Structure–activity relationships established in our previous studies clearly indicated the 3,5-substitution pattern in the aromatic part of the molecule is essential for potency. 2-Hydroxy substitution is tolerated. Substitution in other positions resulted in loss of activity. For this study, we incorporated these limitations in choosing the starting salicylaldehydes. Substituted salicylaldehydes (**2**–**5**) were heated at reflux in acetic anhydride in the presence of an equivalent of triethylamine to give coumarins.[31] (**6**–**9**) Upon cooling of the reaction mixture, crystalline precipitates of the substituted coumarins were obtained. Conjugate addition of lithium hexamethyldisilazane to the coumarins **6**–**9** followed by quenching with acetic acid and treatment with HCl/dioxane gave hydrochloride salts of 4-aminodihydrocoumarins in very good yield.[31] Ring opening and esterification of the acids were accomplished by heating the aminodihydrocoumarins with ethanol/HCl to give the racemic β-amino acid esters in very high yield as their hydrochloride salts. The amino acid esters **10**–**13** were synthesized using this method.

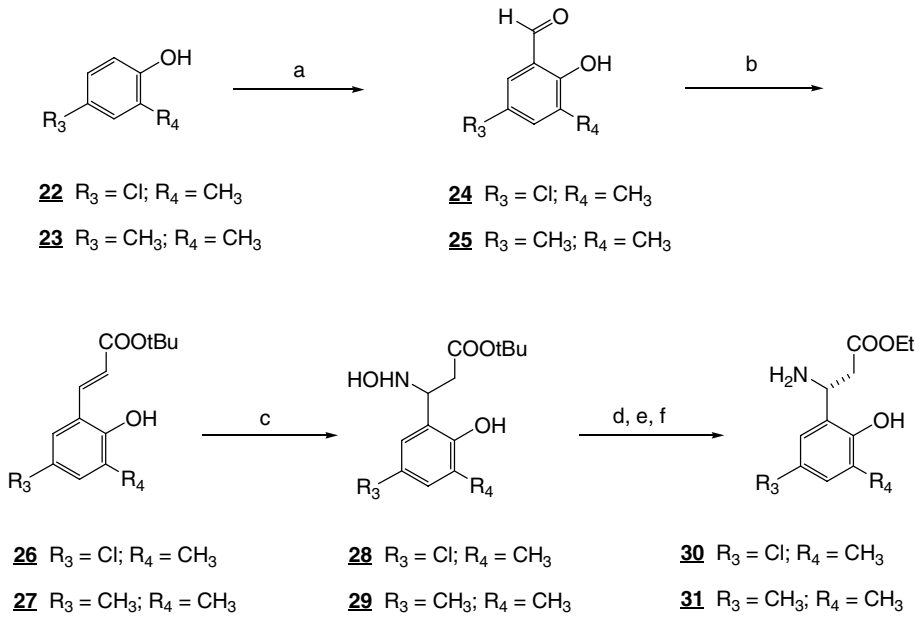
The coumarins derived from salicylaldehydes with alkyl substitutions in the aromatic ring do not undergo the conjugate addition with lithium hexamethyldisilazane as described in Scheme 2. A different approach was devised for these compounds (Scheme 3). The salicylaldehydes **24** and **25** were converted to the corresponding cinnamates **26** and **27** using the Wittig reaction. Michael addition of hydroxylamine to the resulting cinnamates afforded the *N*-hydroxylated β-amino acids **28** and **29** in good yield. Reduction of the *N*-hydroxy-β-amino acids was accomplished with Zn/acetic acid to give the esters of β-amino acids **30** and **31**.

The racemic esters **10**–**13**, **30**, and **31** were resolved using Amano PSC-I (immobilized *Pseudomonas cepacia* lipase) to give the pure *R*-isomers as the esters and *S*-isomers as the free acid.[33] Alternatively, the ethyl ester of the β-amino acid was converted to its CBZ derivative, which was separated on a reverse phase chiral column (Whelk-O, *R*,*R*, 10 micron) to give the pure enantiomers. Deprotection with TMSI afforded the *R*- and the *S*-isomers in pure form. The optical purity was established using analytical HPLC using a chiral column and employing both polarimeter and UV detectors. Each of the optical isomers was free of contamination from the other and the results are reported in Table 1. Ethyl *R*-3-(3,5-dichloro-2-hydroxyphenyl)-3-aminopropionate (**10**) was obtained using this method in addition to the enzymatic resolution.

1.1.2. Synthesis of β-amino acids using chiral auxiliary. The method has been previously described in several patent applications for the synthesis of *S*-isomers using *S*-phenylglycinol.[34,35] The method has been modified to protect the *o*-hydroxy group present in the β-amino acids of interest. *R*-Phenylglycinol was used as the chiral auxiliary as shown in Scheme 4. The substituted salicylaldehyde **32** was first treated with MEM chloride and potassium carbonate to afford the MEM protected salicylaldehyde **33**. The MEM ether protected salicylaldehyde

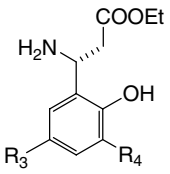


Scheme 2. Reagents: (a) Ac_2O/NEt_3; (b) LiHMDS; (c) HOAc; (d) EtOH/HCl; (e) resolve; (f) Boc-Gly-OSu/NEt_3; (g) EtOH/HCl.



Scheme 3. Reagents: (a) EtMgBr/$(HCHO)_n$; (b) $PH_3P{=}CHCOOtBu$; (c) NH_2OH; (d) Zn/HOAc; (e) EtOH/HCl; (f) resolve.

Table 1. Resolution data for *R*-isomers (yield, purity, and optical rotation)



Compound	R_4	R_3	Yield (%)	Purity (%)	Opt. rot *R*-isomer	Opt. rot *S*-isomer
10	Cl	Cl	32	99	−6.1	6.2
11	Cl	Br	47	99	−6.5	7
12	Br	Cl	ND	100	−5.7	5.6
13	Br	Br	33	100	−7.4	6.8
30	CH_3	Cl	41	100	−1.2	ND
31	CH_3	CH_3	41	100	−1.7	ND
37	I	Cl			−4.2	3.8

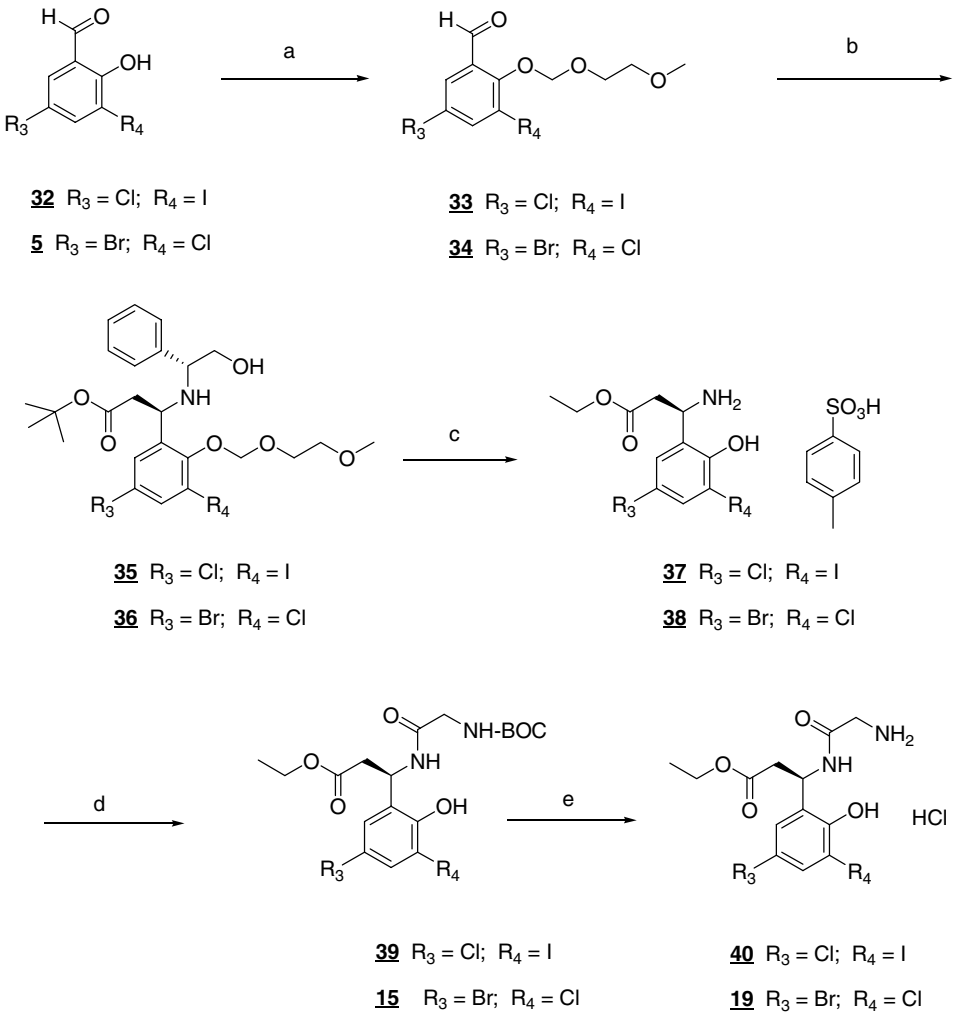
ND, not determined. The yield and purity data for **37** are not reported as it was synthesized using chiral auxiliary method.

was then reacted with *R*-phenylglycinol in the presence of magnesium sulfate in THF to afford the imine. The Reformatsky reagent, *tert*-butyl zincbromoacetate, was then added to the imine in *N*-methylpyrrolidine to give the β-amino acid derivative **35**. The chiral auxiliary was cleaved off by treatment with lead tetraacetate. Basic workup of the reaction mixture followed by heating it at reflux with *p*-toluenesulfonic acid in ethanol afforded the desired PTSA salt of the ethyl ester of the amino acid **37** as a crystalline powder. This method was not successful with aromatic ring containing alkyl substitutions. Ethyl *R*-3-(5-bromo-3-chloro-2-hydroxyphenyl)-3-aminopropionate (**15**) ethyl and *R*-3-(3-iodo-5-chloro-2-hydroxyphenyl)-3-aminopropionate (**37**) were synthesized using this methodology. The amino acid esters **10**, **11**, **12**, **13**, **30**, **31**, and **37** were treated with activated Boc-glycine and the protective group was removed by treatment with ethanol/HCl to afford the glycine amide of the corresponding β-amino acid esters.

1.1.3. Synthesis of 3-dihalophenyl-3-aminopropionic acids. The β-amino acids shown above have *o*-hydroxy group in the phenyl ring. We also synthesized β-amino acids that lack the hydroxyl group. This was accomplished as shown in Scheme 5 by heating at reflux the corresponding aldehyde with ammonium acetate and malonic acid in isopropyl alcohol.[30] Upon cooling, the amino acid precipitated and was isolated by filtration. The ethyl ester of the amino acid was resolved as described earlier.

1.2. Synthesis of the left hand side

1.2.1. Synthesis of thioureas. The thioureas have been synthesized by heating amino benzoic acid with ammonium isothiocyanate at reflux in concentrated hydrochloric acid for 24 h.[36] The vigorous conditions required for the reaction often resulted in difficult to purify products and we looked for milder and general conditions to generate the thioureas (Scheme 6). Substituted *meta*-amino benzoic acid (**51**) or 5-amino nicotinic acid (**52**) was treated with benzoylisothiocyanate to afford the benzoylthioureas **54** and **55** in quantitative yield. The removal of the benzoyl group in benzoylthiourea **54** was accomplished by treatment with two equivalents of sodium methoxide (or more if other acidic groups are present) in methanol. Upon concentration of the reaction mixture, a semisolid containing the sodium salt of the desired thiouridobenzoic acid and methyl benzoate byproduct was obtained. The semisolid obtained was then triturated with ether to remove the methyl benzoate and the solid was repeatedly washed with ether to give the sodium salt of the thiourea. The



32 R_3 = Cl; R_4 = I
5 R_3 = Br; R_4 = Cl

33 R_3 = Cl; R_4 = I
34 R_3 = Br; R_4 = Cl

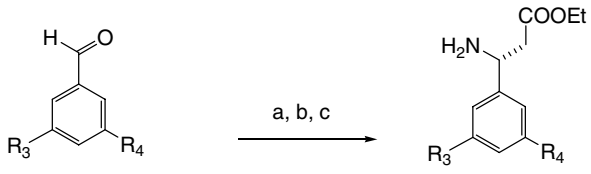
35 R_3 = Cl; R_4 = I
36 R_3 = Br; R_4 = Cl

37 R_3 = Cl; R_4 = I
38 R_3 = Br; R_4 = Cl

39 R_3 = Cl; R_4 = I
15 R_3 = Br; R_4 = Cl

40 R_3 = Cl; R_4 = I
19 R_3 = Br; R_4 = Cl

Scheme 4. Reagents: (a) MEM-Cl, K_2CO_3; (b) 1—*R*-phenylglycinol/$MgSO_4$; 2—$BrZnCH_2COOtBu$; (c) 1—$NaIO_4$; 2—PTSA/EtOH, reflux; (d) Boc-Gly-OSu; (e) EtOH/HCl.



41 R_3 = Br; R_4 = Br
42 R_3 = CH_3; R_4 = CH_3
43 R_3 =Cl; R_4 = Br
44 R_3 = Cl; R_4 = Cl
45 R_3 = Br; R_4 =I

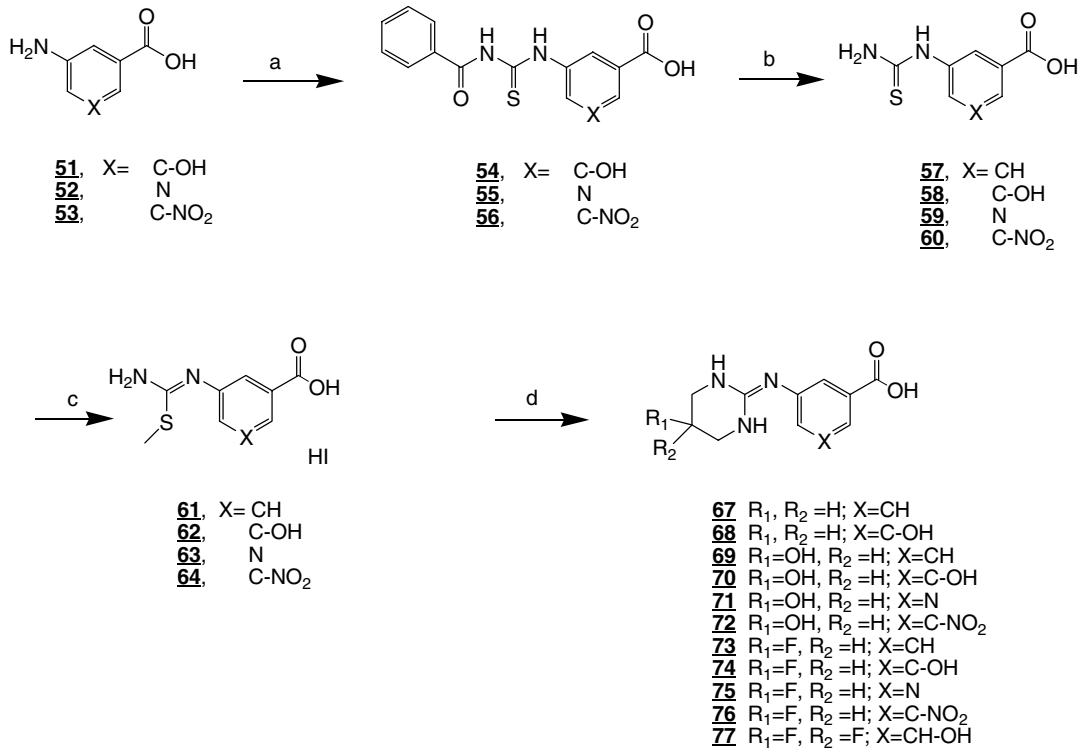
46 R_3 = Br; R_4 = Br
47 R_3 = CH_3; R_4 = CH_3
48 R_3 =Cl; R_4 = Br
49 R_3 = Cl; R_4 = Cl
50 R_3 = Br; R_4 =I

Scheme 5. Reagents: (a) malonic acid, ammonium acetate, isopropyl alcohol; (b) EtOH/HCl; (c) resolve.

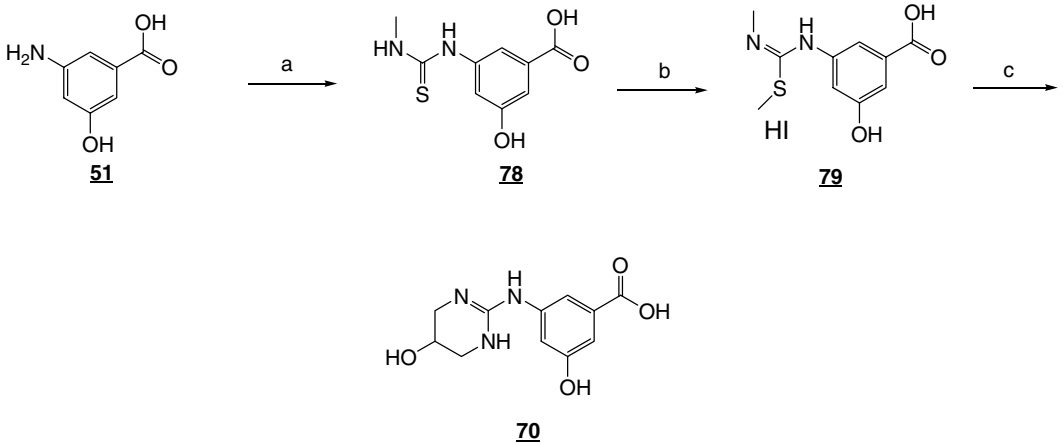
sodium salt, dissolved in minimum amount of water, was acidified to afford the thiourea **58** in very high yield. Treatment of the thiourea with methyl iodide gave the isothiourea **62** in very good yield.

For the pyridine containing substrates, better yield of the isothiourea **63** was obtained by skipping the isolation of the thiourea step. Iodomethane was added after the reaction with sodium methoxide had been completed. Careful acidification of the reaction mixture with acetic acid afforded the isothiourea as a solid. Reaction of the isothiourea with a diaminopropane gave the required guanidinobenzoic or nicotinic acid. The thiourea **57** is commercially available and it was converted to the *S*-methylisothiourea as described earlier. For the synthesis of the LHS on large scale, a modified approach was used (Scheme 7).

The substituted aminobenzoic acid **51** was reacted with methylisothiocyanate to give the *N*-methylthiourea **78**. Treatment of this with methyl iodide afforded *N*-methyl-*S*-methylisothiourea **79**. Upon reaction with 1,3-diamino-2-hydroxypropane, as in the previous example, the desired guanidine benzoic acid **70** was obtained. The byproducts, methylamine and methanethiol, are both volatile and are easily removed in a gas stream and scrubbed (with 2.5 N sodium hydroxide to remove the methyl mercaptan) leaving the product clean. We found this method to be quite useful for



Scheme 6. Reagents and condition: (a) PhCONCS, acetonitrile; (b) NaOMe/MeOH; (c) MeI/ethanol; (d) 1,3-diaminopropane, or 1,3-diamino-3-hydroxypropane, or 1,3-diamino-3-fluoropropane (**65**), or 1,3-diamino-3,3-difluoropropane (**66**), DMF, 100 °C.



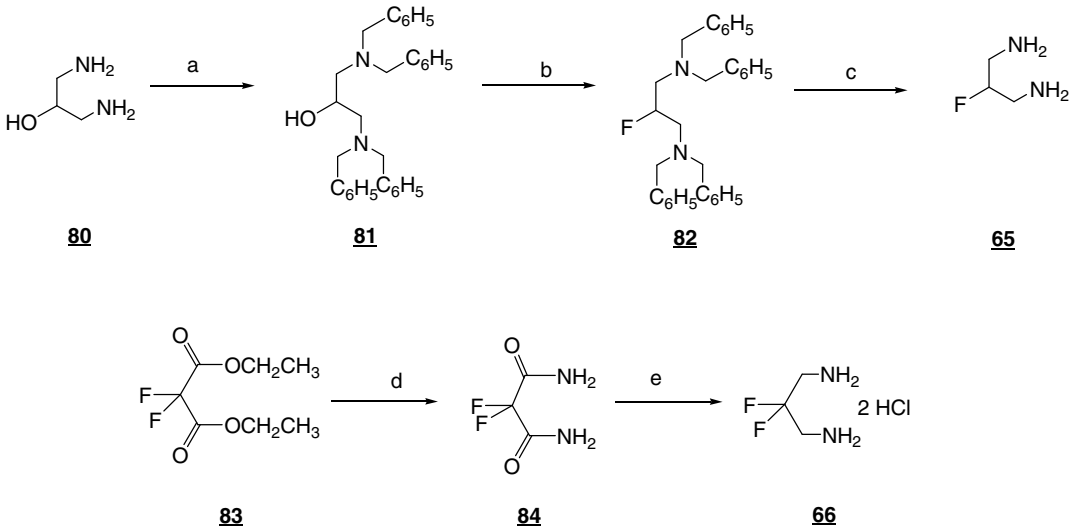
Scheme 7. Reagents and condition: (a) methylisothiocyanate, DMF; (b) iodomethane; (c) 1,3-diamino-3-hydroxypropane, DMF, 90 °C.

larger scale synthesis as it involves less number of manipulative steps.

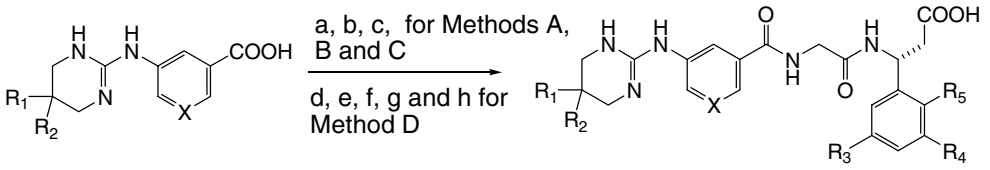
The syntheses of some of the substituted 1,3-diaminopropanes that are not commercially available are shown in Scheme 8. Treatment of 2-hydroxy-1,3-diaminopropane **80** with excess benzyl bromide afforded the tetra *N*-benzyl diamino-2-hydroxypropane **81**. Reaction of **81** with DAST followed by catalytic reduction gave the 2-fluoro-1,3-diaminopropane **65**. The synthesis of 1,3-diamino-2,2-difluoropropane (**66**) was accomplished by starting from diethyl difluoromalonate **83**.

The ester **83** was converted to the diamide **84** by reaction with methanolic ammonia. The diamide was reduced with diborane to afford the 2,2-difluorodiamino-propane (**66**).

1.2.2. Coupling of the LHS with the glycinamide of the β-amino acid ester (Scheme 9). The HCl salt of the carboxylic acid was treated with isobutyl chloroformate and *N*-methylmorpholine to give the mixed anhydride (Method A). Reaction of this with the glycinamide of the β-amino acid ester afforded the ethyl ester of the product. The ester was hydrolyzed with lithium hydroxide and the final purification was accomplished using reverse phase chromatography with a gradient of acetonitrile/water containing 0.1% TFA. The product was obtained as its TFA salt after lyophilization. This can be converted to the HCl salt using an ion exchange



Scheme 8. Reagents and conditions: (a) benzylbromide, EtOH/H_2O, 2 h, 60 °C; (b) DAST, CH_2Cl_2 −60 °C, rt, 16 h; (c) Pd $(OH)_2$ EtOH, EtOAc, 50 psi, 48 h; (d) MeOH/NH_3 (g); (e) BH_3/THF; EtOH/HCl (g).



Scheme 9. Reagents: (a) ClCOO*i*Bu/NMM (Method A) or HOBt/EDC (Method B) or HBTU/DIEA (Method C); (b) glycinamide of ethylester of β-amino acid/NMM; (c) NaOH (Method D); (d) DIICD/ethyl glycinate hydrochloride; (e) NaOH; (f) ClCOO*i*Bu/NMM; (g) β-amino acids **46**–**50**; (h) NaOH.

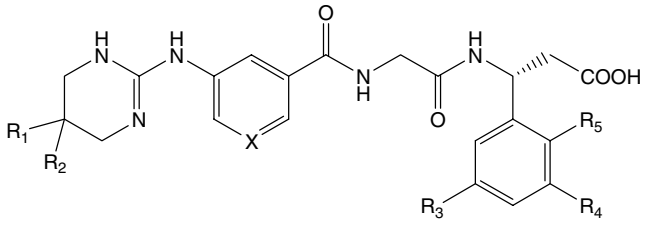
column. Alternatively, EDC/HOBt was also used as the coupling agent of the LHS to the β-amino acid derivative (Method B). A slightly different coupling agent HBTU was also used (Method C). For the amino acid lacking the *o*-hydroxyl moiety, a further modification was made (Method D). The left hand side was first coupled with glycine ethyl ester using diisopropylcarbodiimide and the ester was hydrolyzed to give the free acid. This was then coupled to the β-amino acids **46**–**50**. The final products were obtained by hydrolysis followed by purification by reverse phase HPLC.

2. Discussion

The coupling method used for each of the compounds synthesized is shown in Table 2. The table also shows cell data for the inhibition of the integrins $\alpha_v\beta_3$ (293-b3 cells) and $\alpha_v\beta_6$ (HT-29 cells). For certain compounds, $\alpha_v\beta_6$ data were determined with 293-b6 cells (reported in the table). The two assays produced the same rank order potency on a set of compounds, so either assay in isolation can be used to rank relative selectivity. However, the values should not be compared across the two assays because the IC_{50} values from the 293-b6 assay tend to be significantly lower than those from the HT-29. We thought this was probably due to the much higher expression of $\alpha_v\beta_6$ in the engineered 293 line than in the natural (untransfected) HT-29 line. The HT-29 assay has a greater dynamic range (i.e., the values were more spread apart making it easier to distinguish relative selectivity) than the 293-b6 assay, and therefore was employed for testing of most of the compounds.

The 293-b3 cell data for some of the *S*-isomers are also provided in the table for comparison. Data for other *S*-isomers will be published elsewhere. The data indicate that the *R*-isomers are in general less potent and the SAR follows a different pattern from that of the *S*-isomers. There are some apparent trends. The 2-hydroxyl substituent in the phenyl group in the β-amino acid moiety is critical for selectivity since lack of this substitution increases the potency against the integrin $\alpha_v\beta_6$ and decreases the potency against $\alpha_v\beta_3$ (**129**–**133**). The dibromo substitutions in the amino acid moiety also reduce the $\alpha_v\beta_3$ potency. With 5-chloro substituted β-amino acids, any halogen is tolerated at the 3-position. However, with 5-bromo substitutions, only chlorine is preferred at 3-position. Alkyl substitutions in the β-amino acid moiety reduce the potency significantly. For the left hand side, pyridine moiety as well as 5-hydroxy substitution on the phenyl group increases the potency. Introduction of a fluoro substitution in the tetrahydropyrimidine moiety considerably improves the potency of the molecule. A combination of 5-fluoro in the pyrimidine ring and a hydroxy group in the phenyl group provides the best combination for potency against the integrin $\alpha_v\beta_3$. Pyridyl compounds have been shown to

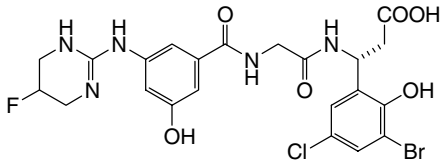
Table 2. Methods of synthesis, potency, and integrin selectivity of inhibitors



Compound	R_1	R_2	X	R_3	R_4	R_5	$\alpha_v\beta_3$	$\alpha_v\beta_6$	Method
85	H	H	C–OH	Cl	Cl	OH	3 (0.9)	3000	A
86	H	H	CH	Cl	Cl	OH	4	24	B
87	OH	H	C–OH	Cl	Cl	OH	3 (0.9)	64*	A
88	OH	H	CH	Cl	Cl	OH	0.7 (0.3)	20*	A
89	F	H	CH	Cl	Cl	OH	1	16	C
90	F	H	C–OH	Cl	Cl	OH	1	42	C
91	F	H	N	Cl	Cl	OH	1	14	C
92	H	H	C–OH	Cl	Br	OH	10 (2)	2740	B
93	H	H	CH	Cl	Br	OH	5 (2)	6543	A
1c	OH	H	C–OH	Cl	Br	OH	3 (2.6)	52*	A
94	OH	H	CH	Cl	Br	OH	5 (0.6)	3532	B
95	OH	H	N	Cl	Br	OH	1 (0.4)	615*	A
96	F	H	CH	Cl	Br	OH	1 (0.2)	1416	C
97	F	H	C–OH	Cl	Br	OH	1 (0.6)	366	A
98	F	F	C–OH	Cl	Br	OH	5 (5)	7258	A
99	F	H	N	Cl	Br	OH	0.7 (0.4)	1835	C
100	F	H	C–NH_2	Cl	Br	OH	3	3443	A
101	OH	H	C–NH_2	Cl	Br	OH	22	8624	B
102	H	H	C–OH	Br	Cl	OH	10	151	B
103	H	H	CH	Br	Cl	OH	8	88	B
104	OH	H	C–OH	Br	Cl	OH	7 (2.7)	588	A
105	OH	H	CH	Br	Cl	OH	8	48	B
106	OH	H	N	Br	Cl	OH	1 (0.3)	22,804	A
107	F	H	CH	Br	Cl	OH	2	25	B
108	F	H	C–OH	Br	Cl	OH	3	71	B
109	OH	H	C–NH_2	Br	Cl	OH	33	216	B
110	OH	H	C–OH	Br	Br	OH	21	96	B
111	H	H	C–OH	Br	Br	OH	20	204	B
112	H	H	CH	Br	Br	OH	4	3211	B
113	OH	H	CH	Br	Br	OH	10	25	B
114	OH	H	N	Br	Br	OH	2	1755	B
115	F	H	C–OH	Br	Br	OH	3	3420	B
116	OH	H	C–NH_2	Br	Br	OH	7	2790	B
117	H	H	C–OH	Cl	I	OH	9	1777*	A
118	H	H	CH	Cl	I	OH	7	10,000	B
119	OH	H	C–OH	Cl	I	OH	3 (6.4)	631	A
120	OH	H	CH	Cl	I	OH	1 (1.6)	11,475	A
121	OH	H	N	Cl	I	OH	1	622*	A
122	OH	H	C–NH_2	Cl	I	OH	0.3	12,253	A
123	F	H	C–OH	Cl	I	OH	3	164*	C
124	OH	H	C–OH	Cl	CH_3	OH	18 (0.2)	688	B
125	OH	H	C–OH	CH_3	CH_3	OH	54 (1)	118	B
126	F	H	C–NH_2	CH_3	CH_3	OH	10	65	B
129	F	H	C–OH	Br	Br	H	5	20	A
130	F	H	C–OH	CH_3	CH_3	H	56	49	A
131	F	H	C–OH	Cl	Br	H	5	57	A
132	F	H	C–OH	Cl	Cl	H	11	40	A
133	F	H	C–OH	Br	I	H	13	11	A

The values reported in the table are IC_{50}'s in nM. The values reported for the $\alpha_v\beta_6$ were determined with HT-29b6 cells. For some selected compounds (noted with an asterisk) 293-b6 cells were used. See Scheme 9 for the method of synthesis. The values reported in parentheses are for the corresponding *S*-isomers.

be potent inhibitors in the *S*-isomer series, but the PK properties were not acceptable. A similar trend has also been observed in the *R*-series. A combination of good potency versus $\alpha_v\beta_3$ and selectivity against other integrins was the criterion for compounds that were chosen for the studies in tumor models. Compound **97** (Scheme 10)



97

Scheme 10.

Table 3. Oral bioavailability and half-life data for selected compounds

Compound	Oral BA (%)		$t_{1/2}$ (h)	
	R-Isomer	*S*-Isomer	*R*-Isomer	*S*-Isomer
87	4.2	20	1.8	2.9
88	6.5	2.1	1.2	1.5
1c	7		17	
1b		18		4.7
97	5.9	13.6	2	5.1
104	3.7	13.3	2.1	2.1
119	7.4	19	2.8	6.3

The oral bioavailability and half-life were determined in mice.

has been shown to be one of the potent inhibitors of $\alpha_v\beta_3$ as well as selective against $\alpha_v\beta_6$. The compound **97** was further examined in tumor models (Table 3).

2.1. Inhibition of M21 melanoma tumor growth by 97

SCID mice were inoculated with M21 tumor cells and treated with **97**. Compound was administered intraperitoneally twice daily, beginning on the day of cell inoculation and continued through the end of the study. Tumors were measured with calipers over the course of the study to determine volume and then were resected for tumor weight determination at the end of the experiment. Human M21 melanoma cells implanted sc into the flank of female SCID mice formed palpable tumors within 14 days and reached 500–600 mm^3 by day 28. As shown in Figure 1, treatment of mice with **97** significantly inhibited the growth of M21 tumors in the mice in a dose-dependent manner. The experiment was terminated on day 28; tumor volumes were measured, and the tumors were resected and weighed. The average tumor volume in the 100 mg/kg **97** treated group was 227 mm^3 and the vehicle control treated group was 595 mm^3, representing a 62% reduction in tumor volume. Accordingly, the average tumor weight for the 100 mg/kg treated group showed a 53% decrease from the average tumor weight of the control treated mice (data not shown).

2.2. Inhibition of Colon 26 tumor growth and hypercalcemia by 97

Murine Colon 26 tumor cells were inoculated sc into syngeneic mice and treated with **97** intraperitoneally, twice daily through the end of the study. Tumors were measured with calipers to determine volume and then resected for tumor weight determination at the end of the experiment. Colon 26 cells implanted sc into the flank of Balb/c mice developed palpable tumors within 5 days and reached 2000–2500 mm^3 by day 21. As

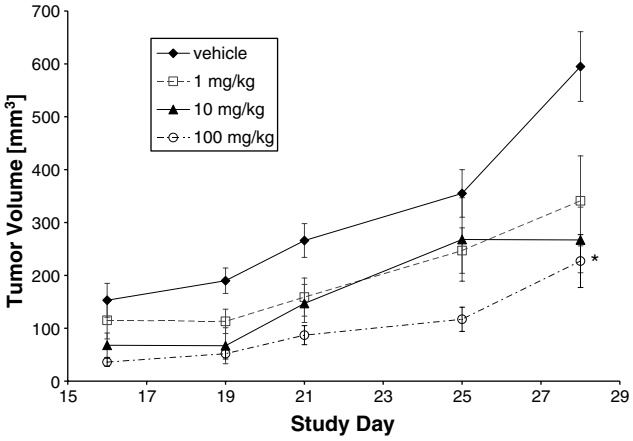


Figure 1. Efficacy of **97** on sc M21 xenograft growth in SCID mice. M21 tumor cells (5×10^6; $n = 10$ mice in each treatment group) were implanted sc into the hind flank of female SCID mice on day 0. Twice daily ip administration of **97** (1, 10, and 100 mg/kg in saline) or saline vehicle began ~6 h postimplantation. Tumor growth was measured using vernier calipers, and tumor volumes were calculated as the product of width$^2 \times$ length $\times$ 0.52. Values plotted are mean tumor volume ± SE. Statistical analysis was performed using unpaired Student's *t* test. $^*P = 0.0008$ versus vehicle control.

shown in Figure 2a, treatment with **97** significantly inhibited tumor growth. In the 100 mg/kg treatment group the average tumor volume was 947 mm^3 and in the control treated group the average tumor volume was 2351 mm^3, representing a 60% reduction in tumor volume. The average tumor weight for the 100 mg/kg treatment group showed a 55% reduction in tumor weight (data not shown).

The Colon 26 tumor induces hypercalcemia in Balb/c mice and the serum calcium correlates with tumor volume (data not shown). The compound **97** was shown to prevent the development of Colon 26 tumor-induced hypercalcemia in a dose-dependent manner. Balb/c mice with no palpable Colon 26 tumor showed a base line serum calcium of 8.99 mg/dL. As shown in Figure 2b, Colon 26 tumor bearing vehicle control treated mice showed an average serum calcium of 15.79 mg/dL on day 21. The 100 mg/kg **97** treatment group showed an average serum calcium of 11.43 mg/dL, indicative of a 64% reduction in tumor-induced hypercalcemia.

In both cases, the plasma levels of the drug **97** were measured at 6 h postdosing. The measured plasma levels were 3.3, 19.4, and 84.4 μg/mL for the dose of 1, 10, and 100 mpk, respectively. Significant efficacy was seen only at the highest plasma level.

3. Conclusions

We followed up on the unusual potency of the RGD mimics containing *R*-isomers of the β-amino acid. The SAR of the series is quite different from that of the *S*-isomers. In general, the mimics with *R*-isomers of β-amino acids are slightly less potent inhibitors, but have better selectivity for $\alpha_v\beta_3$ compared to the analogs

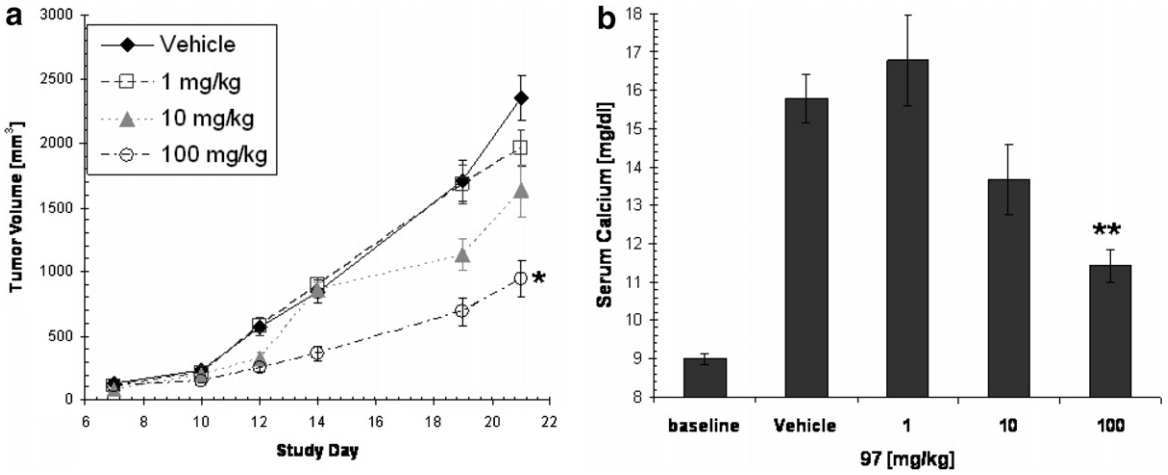


Figure 2. Efficacy of **97** on sc Colon 26 syngeneic tumor growth in mice and tumor-induced hypercalcemia. Colon 26 tumor cells (5×10^6; $n = 10$ mice in each treatment group) were implanted sc into the hind flank of female BALB/c mice on day 0. Twice daily ip administration of **97** (1, 10, and 100 mg/kg in saline) or saline vehicle began ~6 h postimplantation. Tumor growth was measured using vernier calipers, and tumor volumes were calculated as the product of width^2 × length × 0.52. Values plotted are mean tumor volume ± SE (a). Serum calcium was measured from serum collected on day 4 (baseline) and day 21 at the end of the study. Values plotted are mean serum calcium ± SE (b). Statistical analysis was performed using unpaired Student's t test. $^*P = 0.00001$ versus vehicle control. $^{**}P = 0.00002$ versus vehicle control.

with *S*-isomers of the β-amino acids. The bioavailability is also comparatively lower. One of the compounds was examined in mouse tumor models and was shown to significantly reduce the rate of growth and size of tumors. Further details of the biological properties of selected analogs will be published elsewhere.

4. Experimental

4.1. General: chemistry

Unless otherwise stated, starting materials were obtained from commercial sources and were used without further purification. All reactions were performed in anhydrous conditions in an atmosphere of nitrogen. Nuclear magnetic resonance spectra were recorded on a Varian VXR-400 spectrometer and chemical shifts are reported in ppm relative to TMS internal standard. Preparative HPLC was performed on a Waters Prep LC 2000 System using a UV detector (Waters 2487 Dual λ absorbance detector) on a Waters Delta pak C18-100A (5 cm × 30 cm column) at a flow rate of 80 mL/min using acetonitrile/water gradient containing 0.01% TFA. All final compounds were analyzed by analytical HPLC (gradient 5–100% acetonitrile in water containing 0.01% TFA) and peaks were monitored at 210 and 254 nM for purity. Mass spectral data were acquired on a Waters ZQ model single quadrupole instrument with an electrospray probe. High resolution data were acquired on a Perception Biosystems Mariner Time of Flight instrument with an electrospray probe. Optical rotations were determined on a Rudolph Autopol III Automatic polarimeter with 1 dm cell in methanol.

4.2. Biology

All animal studies were approved by the Pharmacia Institutional Animal Care and Use Committee. Animals were housed in Pharmacia facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Male Swiss-Webster mice were obtained at 6–8 weeks of age from Charles River Laboratories (Wilmington, MA). The test compound **97** was dissolved in sterile saline to the appropriate concentration and mice were dosed intraperitoneally with 100 μL/mouse/dose.

4.2.1. Ethyl *R*-3-amino-3-(5-bromo-3-chloro-2-hydroxyphenyl)propionate hydrochloride (11). Lithium hexamethyldisilazane (106 mL, 1 M, 106 mmol) was added to a solution of 6-bromo-8-chlorocoumarin[34] (**7**, 27.4 g, 105.8 mmol) in tetrahydrofuran (250 mL) at −78 °C. The reaction mixture was stirred at this temperature for 30 min, then at 0 °C for 1 h. Acetic acid (6.36 g, 106 mmol) was added to the reaction mixture. The reaction mixture was poured into ethyl acetate (300 mL) and saturated sodium carbonate (200 mL) solution. The organic layer was separated, washed with brine (200 mL), dried ($MgSO_4$), and concentrated to afford a residue. This was added anhydrous ether (200 mL) followed by dioxane/HCl (4 N, 30 mL) at 0 °C. The reaction mixture was stirred for 1 h at room temperature, filtered, and dried in vacuo to afford 25 g (76%) of the product as a powder. Saturated ethanolic HCl (250 mL) was added to the solid and the reaction mixture was heated at reflux for 6 h. Most of the solvent was removed by distillation. The cooled residue was added anhydrous ether and was stirred for 2 h. The gum that formed initially turned into a crystalline material. The crystalline product was filtered and was dried to afford 25 g (87%) of the product as off-white crystalline powder. ^{1}H NMR (CD_3OD) δ 7.57 (d, 1H, $J = 2.3$ Hz), 7.44 (d, 1H, $J = 2.3$ Hz), 4.8 (m, 1H), 4.15 (q, 2H, 7.1 Hz), 3.09 (m, 2H), 1.21 (t, 3H, $J = 7.1$ Hz). HRMS (ES, m/z) Calcd for $C_{11}H_{13}BrClNO_3$: 320.9846. Found: Mol. wt 321.9858 (M+H).

4.2.2. Enzymatic resolution of the racemic mixture. Ethyl 3-amino-(5-bromo-3-chloro-2-hydroxyphenyl)propano-

ate hydrochloride (4.0 g) was suspended in MTBE (100 mL) in a round-bottomed flask. While stirring this suspension at room temperature, 50 mL 1.3 M of $KHCO_3$ was slowly (over 30–60 min) added. After the addition of bicarbonate, the aqueous layer was removed. The organic layer was stirred and 6 g of Amano PSC-I (immobilized *P. cepacia* lipase) was added, and kept at room temperature. Extent of reaction was monitored by chiral HPLC. Once the reaction is complete (approximately 24 h), the suspension was filtered through Whatman #1 filter paper in a Buchner funnel (7.5 cm), postwashing the filter with 50 mL MTBE. The clear filtrate was transferred to a 250 mL round-bottomed flask, and 5 mL of 2.5 M HCl in ethanol was added and evaporated to dryness. Yield is approximately 90% of the starting (*R*)-ester. HPLC analysis of each of the isomers using a chiral column indicated them to be optically pure and free of the undesired isomer (UV detector). This method is general and the β-amino acids **10**–**13**, **30**, and **31** have been obtained using this procedure.

4.2.3. Ethyl 3-*R*-(*N*-Boc-gly)-amino-3-(5-bromo-3-chloro-2-hydroxyphenyl)propionate (15). A mixture of Boc-gly-OSu (6.29 g, 23.12 mmol), ethyl 3-amino-3-(5-bromo-3-chloro-2-hydroxyphenyl)propionate hydrochloride (8.30 g, 23.12 mmol), and triethylamine (3.3 mL) in DMF (100 mL) was stirred for 18 h at room temperature. DMF was removed in vacuo and the residue was partitioned between ethyl acetate (300 mL) and sodium bicarbonate (200 mL). The organic layer was washed with hydrochloric acid (1 N, 100 mL), brine (200 mL), dried ($MgSO_4$), and concentrated to afford 11.0 g (99%) of the product as a solid. ^{1}H NMR (CD_3OD) δ 7.38 (m, 1H), 7.29 (d, 1H, J = 2.4 Hz), 5.54 (m, 1H), 4.07 (q, 2H, 7.12 Hz), 3.69 (s, 2H), 2.84 (m, 2H), 1.44 (s, 9H), 1.21 (t, 3H, J = 7.1 Hz). HRMS (ES, *m*/*z*) Calcd for $C_{18}H_{24}BrClN_2O_6$: 478.0506. Found: 479.0610 (M+H).

4.2.4. Ethyl 3-*R*-(*N*-gly)-amino-3-(5-bromo-3-chloro-2-hydroxyphenyl)propionate hydrochloride (19). Ethanolic HCl (saturated, 250 mL) was added to ethyl 3-*R*-(*N*-Boc-gly)-amino-3-(5-bromo-3-chloro-2-hydroxyphenyl)propionate (10.8 g, 22.53 mmol) at room temperature and was stirred and heated at reflux for 6 h. The reaction mixture was concentrated, and concentrated once more after addition of toluene (100 mL). The residue obtained was suspended in ether and was filtered and dried to give 9.0 g (96%) of the product as a crystalline powder. ^{1}H NMR (CD_3OD) δ 7.41 (d, 1 H, J = 2.4 Hz), 7.30 (d, 1H, J = 2.4 Hz), 5.58 (m, 1H), 4.10 (q, 2H, 7.1 Hz), 3.69 (s, 2H), 2.88 (m, 2H), 1.19 (t, 3H, J = 7.1 Hz). HRMS (ES, *m*/*z*) Calcd for $C_{13}H_{16}BrClN_2O_4$: 377.9982. Found: 379.0067 (M+H).

4.2.5. Ethyl (3*R*)-3-amino-3-(3,5-dichloro-2-hydroxyphenyl)propanoate (10). To the racemic amino acid ester hydrochloride **10**, prepared as described above from 3,5-dichlorosalicylaldehyde (50.0 g, 158.9 mmol) and $NaHCO_3$ (38.2 g, 454.5 mmol) were added CH_2Cl_2 (500 mL) and water (380 mL). The mixture was stirred at room temperature for 10 min with vigorous gas evolution. A solution of benzyl chloroformate (43.4 g, 222.8 mmol) in CH_2Cl_2 (435 mL) was added over 20 min with rapid stirring. After 40 min, the reaction mixture was poured into a separatory funnel and the organic solution collected. The aqueous phase was washed with CH_2Cl_2 (170 mL). The combined organic solution was dried ($MgSO_4$) and concentrated in vacuo. The resulting gummy solid was triturated with hexane and collected by filtration. The tan solid was dried in vacuo to give the racemic product, 62.9 g (96%). This material was subjected to reverse phase HPLC on a chiral column Whelk-O (*R*,*R*) (10 micron) using a 90:10 heptane/ethanol mobile phase to give pure enantiomers. Optical purity was determined to be >98% using analytical HPLC with similar solvent and conditions. To a solution of the carbamate (38.5 g, 93.4 mmol) in CH_2Cl_2 (380 mL) was added trimethylsilyl iodide (25.0 g, 125.0 mmol) in CH_2Cl_2 (80 mL) via canula. The orange solution was stirred at room temperature for 1.5 h. Methanol (20.0 mL, 500 mmol) was added dropwise and the solution stirred for 20 min. The reaction solution was concentrated in vacuo to give orange oil. The residue was dissolved in methyl *tert*-butyl ether (450 mL) and extracted with 1 M HCl (320 mL) and water (1× 200 mL, 1× 100 mL). The aqueous extracts were backwashed with MTBE (130 mL). To the aqueous solution was added solid $NaHCO_3$ (40.1 g, 478 mmol) in small portions. The basified aqueous mixture was extracted with MTBE (1× 1.0 L, 2× 200 mL). The combined organic solution was washed with brine and concentrated in vacuo to give the product. Yield: 20.8 g (80%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.29 (d, 1 H, J = 2 Hz), 6.97 (d, 1H, J = 2 Hz), 4.42 (t, 1H, J = 6 Hz), 4.04 (q, 2H, J = 7 Hz), 2.71 (m, 2H), 1.13 (t, 3H, J = 7 Hz). Anal. Calcd for $C_{11}H_{13}Cl_2NO_3$: C, 47.50; H, 4.71; N, 5.04. Found: C, 47.11; H, 4.66; N, 4.93.

4.2.6. 5-Chloro-2-hydroxy-3-methylbenzaldehyde (24). The following was adapted from the published procedure.[40] To a solution of ethylmagnesium bromide (400 mL, 1.0 M in THF) was added 4-chloro-2-methylphenol (57 g, 0.4 mol) in toluene (75 mL) slowly at 4 °C followed by tetramethyl–ethylenediamine (45 g, 0.39 mol), paraformaldehyde (30 g), and HMPA (72.1 g, 0.4 mol) at room temperature. The reaction mixture was refluxed for 4 h, then stirred at room temperature for 48 h and was quenched with 50% HCl (450 mL). The aqueous solution was extracted with ethyl acetate (4× 250 mL). The combined organic extracts were washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by chromatography on silica gel (5% ethyl acetate in hexane) to give 40.8 g (60%) oil. ^{1}H NMR ($CDCl_3$) δ 11.16 (s, 1H), 9.8 (s, 1H), 7.36 (m, 2H), 2.25 (s, 3H). HRMS (ES, *m*/*z*) Calcd for $C_8H_7ClO_2$: 170.0135. Found: 170.0116.

4.2.7. *tert*-Butyl (2*E*)-3-(5-chloro-2-hydroxy-3-methylphenyl)prop-2-enoate (26). To a solution of 5-chloro-2-hydroxy-3-methylbenzaldehyde (22.6 g, 0.13 mol) in THF (200 mL) was added *tert*-butyl (triphenylphosphoranyl)acetate (50 g, 0.13 mol) followed by DBU (0.3 mL). The reaction mixture was stirred at room

temperature for 48 h and was concentrated in vacuo. The residue was added 10% ethyl acetate in hexane (100 mL) and the solid formed was filtered. The filtrate was concentrated and purified by chromatography on silica gel (10% ethyl acetate in hexane) to afford 33.4 g (88%) of white solid. ^{1}H NMR ($CDCl_3$) δ 8.01 (d, 1H, J = 16.11), 7.35 (m, 1H), 7.13 (m, 1H), 6.44 (d, 1H, J = 16.11 Hz), 6.20 (s, 1H), 2.32 (s, 3H), 1.56 (s, 9H). HRMS (ES, *m*/*z*) Calcd for $C_{14}H_{17}ClO_3$: 268.0866. Found: 268.0885.

4.2.8. Ethyl (3*R*)-3-amino-3-(5-chloro-2-hydroxy-3-methylphenyl)propanoate hydrochloride (30). To a solution of *tert*-butyl (2*E*)-3-(5-chloro-2-hydroxy-3-methylphenyl)prop-2-enoate (9 g, 33.5 mmol) in dioxane (21 mL) was added 50% hydroxylamine (8 mL) followed by tetrabutylammonium sulfate (0.1 g). The reaction mixture was stirred at room temperature for 48 h and was extracted with ethyl acetate (200 mL). The organic layer was separated, washed with water, brine, dried over $MgSO_4$, and concentrated to give 9.7 g of *tert*-butyl 3-(5-chloro-2-hydroxy-3-methylphenyl)-3-(hydroxyamino)propanoate (96%) as oil. ^{1}H NMR (DMSO) δ 7.2 (m, 2H), 5.1 (m, 1H), 3.06 (m, 2H), 2.19 (s, 3H), 1.24 (s, 9H). M+H = 302.1.

To a solution of *tert*-butyl 3-(5-chloro-2-hydroxy-3-methylphenyl)-3-(hydroxyamino)propanoate (9.5 g, 31.5 mmol) in acetic acid (65 mL) was added zinc dust (9 g) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min and for 6 h at room temperature. Zinc dust was filtered through Celite. The filtrate was concentrated and purified on reverse phase HPLC to afford 8 g (66%) of the product. ^{1}H NMR (400 MHz, CD_3OH) δ 7.17 (m, 1H), 7.11 (m, 1H), 4.76 (t, 1H, J = 7.25), 2.90–3.03 (m, 2H), 1.39 (s, 9H). HRMS (ES, *m*/*z*) Calcd for $C_{14}H_{20}ClNO_3$: 285.1132. Found: 286.1179 (M+H).

tert-Butyl 3-amino-3-(5-chloro-2-hydroxy-3-methylphenyl)propanoate trifluoroacetate (4 g, 10 mmol) was stirred in saturated HCl in ethanol (20 mL) under nitrogen for 4 h. The reaction mixture was concentrated in vacuo and the residue was added ether (100 mL) and the solution was stirred for 1 h. The solid formed was filtered. The *R*-isomer of ethyl 3-amino-3-(5-chloro-2-hydroxy-3-methylphenyl)propionate was enzymatically resolved as described earlier. The solid formed was dried to give 2.8 g (93%) of HCl salt of the title compound. ^{1}H NMR (400 MHz, CD_3OH) δ 7.17 (m, 1H), 7.13 (m, 1H), 4.16 (m, 1H), 2.98–3.15 (m, 2H), 2.24 (s, 3H), 1.21 (s, 3H). HRMS (ES, *m*/*z*) Calcd for $C_{12}H_{16}ClNO_3$: 257.0813. Found: 258.0896 (M+H).

4.2.9. 2-*O*-(MEM)-3-iodo-5-chlorosalicylaldehyde (33). Potassium carbonate (81.4 g, 5894 mol) was added to a solution of 3-iodo-5-chlorosalicylaldehyde (166.6 g, 0.5894 mol) in DMF (400 mL) at 20 °C. This resulted in yellow slurry and MEM-Cl (75.3 g, 0.589 mol) was added maintaining the reaction temperature. After 2 h, additional MEM-Cl (1.5 g) was added. After stirring for further 1 h, the reaction mixture was poured into ice-water mixture and was stirred. The precipitate formed was filtered and was dried in vacuo to afford the protected aldehyde. Yield: 212.7 g (98%). ^{1}H NMR ($CDCl_3$) δ 10.19 (s, 1H), 7.96 (d, 1H, J = 3.5 Hz), 7.75 (d, 1H, J = 3.5 Hz), 5.21 (s, 2H), 3.87 and 3.51 (m, 4H), 3.33 (s, 3H). HRMS (ES, *m*/*z*) Calcd for $C_{11}H_{12}ClIO_4$: 369.9469. Found: 387.9800 (M+NH_4).

4.2.10. *tert*-Butyl (3*R*)-3-{5-chloro-3-iodo-2-[(2-methoxyethoxy)methoxy]-phenyl}-3-{[(1*R*)-2-hydroxy-1-phenylethyl]amino}propanoate (35). (*R*)-Phenyl glycinol (78.68 g, 0.574 mol) was added to a solution of 2-*O*-(MEM)-3-iodo-5-chlorosalicyl-aldehyde (212.7 g, 0.574 mol) in THF (1 L) at room temperature. An endothermic reaction resulted. After 1 h of stirring $MgSO_4$ (100 g) was added and the stirring was continued for 2 h. The reaction mixture was filtered and the filtrate was concentrated and dried in vacuo for 2 h. A 2-neck round-bottomed flask was charged with the Reformatsky reagent (420 g, 1.7 mol) and *N*-methylpyrrolidone (1.7 L) and was stirred at −10 °C. A solution of the imine in *N*-methyl-pyrrolidone (100 mL) was slowly added maintaining the temperature at −10 °C. The mixture was maintained at this temperature for 2 h and for 1 h at −5 °C. After cooling the reaction mixture to −10 °C, a solution of concd HCl in saturated ammonium chloride (32 mL/400 mL) was added. Ethyl ether (900 mL) was added and was stirred for 2 h at room temperature. The ether layer was separated and the aqueous layer was further extracted with ether (800 mL). The combined ether layers were washed with saturated ammonium chloride (200 mL), water (200 mL), brine (200 mL), dried ($MgSO_4$) and was concentrated to give 332 g (95%) of an oil. ^{1}H NMR ($CDCl_3$) δ 7.60 (d, 1H, J = 3.2 Hz) 7.19–7.29 (m, 6H), 5.15 (s, 2H), 4.68 (m, 1H), 3.99 (m, 2H), 3.93 (m, 1H), 3.62 (m, 4H), 3.42 (s, 3H), 2.48–2.72 (m, 2H), 1.48 (s, 9H). HRMS (ES, *m*/*z*) Calcd for $C_{25}H_{33}ClINO_6$: 605.1041. Found: 606.1098 (M+H).

4.2.11. Ethyl 3-amino-3-(*R*)-(5-chloro-2-hydroxy-3-iodophenyl)propionate *p*-toluenesulfonic acid salt (37). A solution of the crude ester (332.0 g) was dissolved in ethanol (3.5 L) and was cooled to 0 °C. Lead tetraacetate (344.0 g, 0.776 mol) was added in one lot and the solution turned from orange to bright red orange before going back to orange. After 3 h, 15% solution of NaOH (800 mL) was added to the reaction mixture. Most of the ethanol was removed under reduced pressure. The residue was added 15% solution of NaOH (800 mL) and was extracted with ether (1600 mL). The ether layer was washed with water (500 mL), brine (500 mL), dried, and concentrated to afford orange oil. This was dissolved in ethanol (500 mL) and *p*-toluenesulfonic acid (192 g) was added and the solution was heated at reflux for 8 h and was concentrated under reduced pressure. The residue was diluted with THF (600 mL), heated at reflux, and cooled. The precipitate was filtered, washed with hexane/THF (300 mL, 1:1), and dried to yield 90.25 g of the product as the *p*-toluenesulfonic acid salt. ^{1}H NMR (CD_3OD) δ 7.8 (d, 1H, J = 3.2 Hz), 7.74 (d, 2H, J = 10.7 Hz), 7.66 (d, 1H, J = 3.2 Hz), 7.27 (d, 2H, J = 10.7 Hz), 5.17 (m, 1H), 4.17 (m, 2H), 3.30 (m, 2H), 2.43 (s, 3H), 1.25 (t, 3H, J = 9.4 Hz). HRMS

(ES, *m*/*z*) Calcd for $C_{11}H_{13}ClINO_3$: 368.9629. Found: 368.9629.

4.2.12. Ethyl 3-(*N*-Boc-gly)-amino-3-(*R*)-(5-chloro-2-hydroxy-3-iodophenyl)propionate (39). A mixture of Boc-gly-OSu (45.36 g, 166.6 mmol), ethyl 3-(*R*)-amino-3-(5-chloro-2-hydroxy-3-iodophenyl)propionate PTSA salt (90.25 g, 166.6 mmol) in DMF (500 mL) was added triethylamine (25 mL). The reaction mixture was stirred for 18 h at room temperature. DMF was removed in vacuo and the residue was partitioned between ethyl acetate (600 mL) and dil hydrochloric acid (100 mL). The organic layer was washed with sodium bicarbonate (200 mL), brine (200 mL), dried ($MgSO_4$), and were concentrated to afford 85 g (97%) of the product as a solid. ^{1}H NMR ($CDCl_3$) δ 7.62 (d, 1 H, J = 3.1 Hz), 7.15 (d, 1H, J = 3.1 Hz), 5.22 (m, 1H), 4.18 (m, 2H), 3.81 (m, 2H), 2.90 (m, 2H), 1.45 (s, 9H), 1.24 (t, 3H, J = 7.5 Hz). HRMS (ES, *m*/*z*) Calcd for $C_{18}H_{24}ClIN_2O_6$: 526.0368. Found: 527.0451 (M+H).

4.2.13. Ethyl 3-(*R*)-(*N*-gly)-amino-3-(5-chloro-2-hydroxy-3-iodophenyl)propionate hydrochloride (40). Ethanolic HCl (700 mL) was added to ethyl 3-(*R*)-(*N*-Boc-gly)-amino-3-(5-chloro-2-hydroxy-3-iodophenyl)propionate (84.5 g, 160.4 mmol) at 0 °C and was stirred at room temperature for 3 h. The reaction mixture was concentrated, and concentrated once more after addition of toluene (100 mL). The residue obtained was suspended in ether, filtered, and dried to yield 72.0 g (97%) of the product as a crystalline powder. ^{1}H NMR (CD_3OD) δ 7.67 (d, 1H, 3.5 Hz), 7.29 (d, 1H, J = 3.2 Hz), 5.61 (m, 1H), 4.14 (q, 2H, J = 9.7 Hz), 3.74 (s, 2H), 2.91 (m, 2H), 1.23 (t, 3H, J = 9.7 Hz). HRMS (ES, *m*/*z*) Calcd for $C_{13}H_{16}ClIN_2O_4$: 425.9843. Found: 426.9908 (M+H).

4.2.14. *N*-Benzoyl-*N*′-(5-hydroxy-3-carboxyphenyl)thiourea (54). A mixture of 3-amino-5-hydroxybenzoic acid (30.7 g, 200.7 mmol) and benzoyl isothiocyanate (26.57 g) in acetonitrile (450 mL) was stirred at room temperature for 1 h. The precipitate was filtered, washed with acetonitrile, and dried to afford 57.17 g (90%) of the product as a yellow powder. ^{1}H NMR (CD_3OD) δ 8.01–8.04 (m, 2H), 7.79 (m, 1H), 7.69 (m, 1H), 7.58–7.63 (m, 2H), 7.37 (m, 1H). HRMS (ES, *m*/*z*) Calcd for $C_{15}H_{12}N_2SO_4$: 316.0518. Found: 317.0593 (M+H).

4.2.15. *N*-(5-Hydroxy-3-carboxyphenyl)thiourea (58). Sodium methoxide (106 mL, 25%) was added slowly to a stirred mixture of *N*-(benzoyl)-*N*′-(5-hydroxy-3-carboxyphenyl)thiourea (51.77 g, 163.73 mmol) in anhydrous methanol (250 mL). A clear solution resulted in 10 min. After 1-h stirring at room temperature, methanol was removed in vacuo and the residue was dried in vacuo. The residue was triturated with ether (500 mL) to leave orange powder. The residue was dissolved in water (150 mL) and acidified to pH 6. The solid formed was filtered and dried. The solid was further washed with ether (100 mL). The residue obtained is the product. Yield: 34.6 g (99.5%). ^{1}H NMR (CD_3OD) δ 7.42 (m, 1 H), 7.28 (m, 1H), 7.11 (m, 1H). HRMS (ES, *m*/*z*) Calcd for $C_8H_8N_2SO_3$: 212.0256. Found: 213.0303 (M+H).

4.2.16. *N*-(5-Hydroxy-3-carboxyphenyl)-*S*-methylisothiourea (62). A mixture of *N*-(5-hydroxy-3-carboxyphenyl)thiourea (**58**, 32.22 g, 0.164 mol) and iodomethane (23.34 g) in ethanol (200 mL) was heated at reflux for 5 h, the solution turned homogeneous. The solution was concentrated. Yield 56.89 g (100%). ^{1}H NMR (CD_3OD) δ 7.26–7.32 (m, 2H), 6.93 (m, 1H), 2.67 (s, 3H). HRMS (ES, *m*/*z*) Calcd for $C_9H_{10}O_3N_2S$: 226.0412. Found: 227.0462 (M+H).

4.2.17. *N*-(3-Carboxy-5-pyridyl)-*S*-methylisothiourea (63). To the suspension of the thiourea, prepared from 5-aminonicotinic acid and benzoylisothiocyanate (11.1 g, 37.0 mmol) in anhydrous MeOH (230 mL), was added NaOMe (25 wt%) solution in methanol (21.1 mL, 92.0 mmol), at which point the reactant went into solution to give an orange-brown solution. This solution was stirred at room temperature for 3 h, cooled in an ice bath, and added methyliodide (3.45 mL, 0.055 mol). The resulting mixture was stirred at 10 °C for 30 min and 1.5 h at room temperature. The reaction mixture was then quenched with acetic acid (2 mL), cooled in an ice bath, and filtered. The solids were washed with cold MeOH and dried in vacuo to afford the product as beige solid (2.66 g, 37%). ^{1}H NMR (CD_3OD) δ 8.66 (s, 1H), 8.27 (s, 1H), 7.64 (s, 1H), 2.37 (s, 3H). HRMS (ES, *m*/*z*) Calcd for $C_8H_9O_2N_3S$: 211.0415. Found: 212.0490 (M+H).

4.2.18. *N*-(5-Hydroxytetrahydropyrimidinyl)-3-aminonicotinic acid (71). To a solution of the 1,3-diamino-2-hydroxypropane (11.2 g, 124 mmol) in anhydrous DMF (80 mL) was added the isothiourea **63** (8.7 g, 41.0 mmol). This mixture was heated at 85 °C under anhydrous conditions for 3 h. After 1–2 h of heating, the solution became turbid and turbidity increased during the course of heating. The reaction mixture was then cooled in an ice bath and filtered. The solids were washed with acetonitrile, water, acetonitrile and dried in vacuo to yield the product as beige solid (3.7 g, 38%). ^{1}H NMR (CD_3OD) δ 9.06 (s, 1H), 8.72 (s, 1H), 8.34 (d, 1H), 4.3 (d, 1H), 3.5 (m, 4H). HRMS (ES, *m*/*z*) Calcd for $C_{10}H_{12}O_3N_4$: 236.0909. Found: 237.0945 (M+H).

4.2.19. *N*,*N*,*N*,*N*-Tetrabenzyl-1,3-diamino-2-hydroxypropane (81). A mixture of 1,3-diamino-2-hydroxypropane (2.5 g) in ethanol (45 mL) and water (15 mL), containing potassium carbonate (11.5 g) and benzylbromide (14.6 mL), was heated at 60 °C for 2 h with vigorous stirring. Ethanol was removed under reduced pressure, and the residue was partitioned between water (100 mL) and EtOAc (200 mL). The organic phase was washed with water, dried (Na_2SO_4), and concentrated to dryness to give 12.4 g of the product as a colorless syrup. ^{1}H NMR ($CDCl_3$) δ 7.31 (m, 20 H), 3.83 (t, 1H), 3.67 (d, 4H, J = 13.5 Hz), 3.5 (d, 4H, J = 13.5 Hz), 3.4 (s, 1H), 2.45 (d, 4H, J = 6.0 Hz). HRMS (ES, *m*/*z*) Calcd for $C_{31}H_{34}N_2O$: 450.2669. Found: 451.2721 (M+H).

4.2.20. *N*,*N*,*N*,*N*-Tetrabenzyl-1,3-diamino-2-fluoropropane (82). To a solution of *N*,*N*,*N*,*N*-tetrabenzyl-1,3-diamino-2-hydroxypropane (25.0 g) in dichloromethane

(200 mL), at −65 °C, was added dropwise a solution of DAST (8.1 mL) in dichloromethane (25 mL) over a period of 15 min. with vigorous stirring under an atmosphere of argon. The reaction mixture was gradually allowed to warm to 15 °C overnight. It was cooled to −40 °C and poured in portions into saturated $NaHCO_3$ solution containing ice and the products were extracted with dichloromethane (2× 200 mL). The combined organic extracts were washed with water, dried (Na_2SO_4), and concentrated to dryness to give orange-colored syrup. This was dissolved in EtOAc (150 mL), added activated charcoal (5 g), stirred for 30 min, and filtered through Celite. The filtrate was concentrated to dryness and the residue was dried in vacuo to afford 23.5 g of a thick orange syrup. ^{1}H NMR ($CDCl_3$) δ 7.28 (m, 20 H), 4.92 and 4.75 (m, 1H), 3.67 (d, 4H, J = 13.8 Hz), 3.52 (d, 4H, J = 13.8 Hz), 2.6 (m, 4H). HRMS (ES, *m/z*) Calcd for $C_{31}H_{33}N_2F$: 452.2626. Found: 453.2709 (M+H).

4.2.21. 1,3-Diamino-2-fluoropropane (65). A solution of *N*,*N*,*N*,*N*-tetrabenzyl-1,3-diamino-2-fluoropropane (20.0 g) in EtOAc (50.0 mL) and MeOH (50.0 mL) was hydrogenated at 50 psi in the presence of 20% $Pd(OH)_2$ on carbon (10 g) for 16 h. The catalyst was removed by filtration and washed with ethanol. The combined filtrate and washings were again hydrogenated at 50 psi in the presence of 20% $Pd(OH)_2$ on carbon (10 g) for 24 h. The catalyst was removed by filtration, and it was washed with 10% water in ethanol (100 mL). The filtrate and the washings were combined and concentrated to dryness under reduced pressure to give 3.9 g of the diaminofluoropropane as a colorless syrup. ^{1}H NMR (CD_3OD) δ 4.62 and 4.45 (m, 1H), 2.77 (m, 2H) and 2.7 (m, 2H). HRMS (ES, *m/z*) Calcd for $C_3H_9N_2F$: 92.0743. Found: 93.0842 (M+H).

4.2.22. *N*-(5-Fluorotetrahydropyrimidinyl)-3-aminobenzoic acid (73). This was synthesized using the procedure for the synthesis of **69** by starting from 1,3-diamino-2-fluoropropane and *N*-(3-carboxyphenyl)-*S*-methylisothiourea (**61**). ^{1}H NMR (CD_3CD) δ 7.97 (m, 2H), 7.56 (m, 2H), 5.26 (m, 1H, J_H = 44 Hz), 3.65–3.5 (m, 4H). HRMS (ES, *m/z*) Calcd for $C_{11}H_{12}N_3FO_2$: 237.0912. Observed: 238.0996 (M+H).

4.2.23. 3-[(5-Fluoro-1,4,5,6-tetrahydro-2-pyrimidinyl)amino]-5-hydroxybenzoic acid hydrochloride (74). Prepared by using the procedure described for **69**, from 1,3-diamino-2-fluoropropane and *S*-methylisothiourea **62**. ^{1}H NMR (CD_3OD) δ 7.38 (m, 1H), 7.33 (m, 1H), 5.15 (m, 1H), 3.63–3.4 (m, 4H). HRMS (ES, *m/z*) Calcd for $C_{11}H_{12}N_3FO_3$: 253.0863. Found: 254.0944 (M+H).

4.2.24. *N*-(5-Fluorotetrahydropyrimidinyl)-3-aminonicotinic acid (75). This was prepared by starting from 1,3-diamino-2-fluoropropane and 3-aminonicotinic acid as described for the synthesis of **71**. ^{1}H NMR (CD_3OD) δ 9.0 (s, 1H), 8.7 (d, 1H), 8.4 (t, 1H), 5.2 (m, 1H, J_H = 46 Hz), 3.6 (m, 4H). HRMS (ES, *m/z*) Calcd for $C_{10}H_{11}O_2N_4F$: 238.0866. Found: 239.0984 (M+H).

4.2.25. 2,2-Difluoromalonamide (84). A solution of commercially available diethyl difluoromalonate (10.12 g, 51.5 mmol) in anhydrous methanol (110 mL) was saturated with ammonia (g) at 0 °C for 45 min. The resulting mixture was stirred in an ice bath for 3 h and monitored by MS (M+H, 139). The product was concentrated under reduced pressure to afford the amide as a white powder (7.1027 g, 99%). HRMS (ES, *m/z*) Calcd for $C_3H_3N_2F_2O_2$: 138.0234. Found: 139.0278 (M+H).

4.2.26. 1,3-Diamino-2,2-difluoropropane, dihydrochloride salt (66). 2,2-Difluoromalonamide (2.09 g, 15.14 mmol) was added to cold 1.0 M BH_3–THF (72 mL, 72.0 mmol) maintaining bath temperature at 0 to −4 °C. The ice bath was removed and mixture was allowed to warm up to room temperature at which time a clear solution was formed. The solution was then heated to reflux (75 °C) overnight. The reaction mixture was chilled in an ice bath and slowly quenched with methanol (25 mL). The solvents were removed under reduced pressure and the residue was co-evaporated with methanol again (3× 100 mL) to remove excess boric acid. The residue, white syrup, was dried overnight to remove excess solvent. Anhydrous ethanol (100 mL) was added to the residue and chilled in ice bath. The solution was then saturated with HCl (g) to give the product. The reaction mixture was allowed to stir at room temperature for 2 h. Filtered the white precipitate and rinsed with ethanol. The product was washed with fresh ethanol and filtered a second time. The desired product was isolated and dried as a white solid (0.4434 g, 35%). HRMS (ES, *m/z*) Calcd for $C_3H_8N_2F_2$: 110.0654. Found: 111.0702 (M+H).

4.2.27. 3-[(5,5-Difluoro-1,4,5,6-tetrahydro-2-pyrimidinyl)amino]-5-hydroxybenzoic acid, monohydrochloride salt (77). This was prepared from 1,3-diamino-2,2-difluoropropane hydrochloride salt and 3-hydroxy-5-[[imino-(methylthio)-methyl]amino]benzoic acid, monohydroiodide **62** to give the desired product as the hydroiodide salt which was converted to its HCl salt by treating with excess dioxane/HCl. ^{1}H NMR (CD_3OD) δ 7.4 (t, 1H), 7.35 (m, 1H), 6.8 (t, 1H), 3.75 (t, 4H). HRMS (ES, *m/z*) Calcd for $C_{11}H_{11}N_3F_2O_3$: 271.0767. Found: 272.0860 (M+H).

4.2.28. *N*-(3-Carboxyphenyl)-*S*-methylisothiourea (61). The thiourea **57** (28.0 g, 142.7 mmol) and iodomethane (20.25 g, 8.9 mL, 142.7 mmol) were dissolved in ethanol (280 mL) and heated to reflux under a drying tube overnight. The clear reaction mixture was concentrated to afford 48.2 g (94%) of the product. ^{1}H NMR (CD_3OD) δ 11.3 (br, 1H), 9.39 (br, 2H), 7.93 (d, 1H, J = 7.25 Hz), 7.85 (s, 1H), 7.54–7.62 (m, 2H), 2.66 (s, 3H). HRMS (ES, *m/z*) Calcd for $C_9H_{10}N_2O_2S$: Mol. wt 210.0463. Found: 211.0498 (M+H).

4.2.29. *N*-(Tetrahydropyrimidinyl)-3-aminobenzoic acid (67). *N*-(3-Carboxyphenyl)-*S*-methylisothiourea (11.09 g, 32.8 mmol) and 1,3-diaminopropane (7.3 g, 98 mmol) and DMF (25 mL) were added to 200 mL flask equipped with condenser and drying tube. The solution was heated at 80 °C for 18 h, cooled, and filtered. The solid was washed with ethyl acetate, then ether. Yield 5.3 g (74%). ^{1}H NMR (CD_3OD) δ 9.58 (s, 1H), 8.16

(s, 2H), 7.77 (d, 1H, J = 6.3 Hz), 7.72 (m, 1H), 7.47 (t, 1H, J = 7.9 Hz), 7.40–7.41 (m, 1H), 3.24–3.25 (m, 4H), 1.83–1.85 (m, 2H). HRMS (ES, *m/z*) Calcd for $C_{11}H_{13}O_2N_3$: 219.1008. Found: 220.1048 (M+H).

4.2.30. (β[1]*R*)-3,5-Dichloro-β-[[[3-[(5-hydroxy-1,4,5,6-tetrahydro-2-pyrimidinyl)-hydroxy]benzoyl]amino]acetyl] amino]-2-hydroxybenzenepropionic acid, trifluoroacetate (87). *Method A:* To a solution of the acid, 3-*N*-(5-hydroxytetrahydro-pyrimidinyl)-5-hydroxybenzoic acid (772 mg, 2.7 mmol) in DMF (10 mL) at 0 °C were added isobutylchloroformate (0.35 mL, 2.7 mmol) and NMM (0.58 mL, 5.4 mmol). The reaction solution was kept at 0 °C for 15 min. A solution of ethyl (3*R*)-3-(3-bromo-5-chloro-2-hydroxyphenyl)-3-(glycylamino)-propanoate, hydrochloride (1.0 g, 2.7 mmol), and NMM (0.29 mL, 2.7 mmol) in DMF (5 mL) was added. The reaction mixture was stirred at 0 °C for 15 min and then warmed to room temperature overnight. The mixture was concentrated in vacuo. The product was purified by reverse phase HPLC (90:10 H_2O/MeCN–50:50 H_2O/MeCN containing 0.1% TFA) to give the product (625 mg, 31%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.07 (br s, 1H), 9.92 (br s, 1H), 9.80 (s, 1H), 8.67 (t, 1H, J = 6 Hz), 8.54 (d, 1H, J = 6 Hz), 8.22 (br s, 2H), 7.41 (d, 1H, J = 2 Hz), 7.28 (d, 1H, J = 2 Hz), 7.12 (m, 2H), 6.75 (m, 1H), 5.50 (m, 1H), 4.05 (m, 4H), 3.90 (m, 2H), 3.34 (m, 2H), 3.17 (m, 2H), 2.72 (m, 2H), 1.15 (t, 3H, J = 7 Hz). HRMS (ES, *m/z*) Calcd for $C_{24}H_{27}Cl_2N_5O_7$ + 1.5TFA: C, 43.86; H, 3.88; N, 9.47. Found: C, 43.87; H, 4.08; N, 9.61. A solution of the ester (550 mg 0.74 mmol) in 1 M NaOH solution (7 mL) was kept at room temperature for 2.5 h. The reaction solution was acidified to pH 5 with TFA. The mixture was purified by reverse phase HPLC (95:5 H_2O/MeCN–60:40 H_2O/MeCN containing 0.1% TFA) to give the product (310 mg, 57%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.37 (br s, 1H), 10.02 (s, 1H), 9.90 (s, 1H), 9.58 (s, 1H), 8.65 (t, 1H, J = 6 Hz), 8.54 (d, 1H, J = 6 Hz), 8.10 (br s, 2H), 7.41 (d, 1H, J = 2 Hz), 7.23 (d, 1H, J = 2 Hz), 7.13 (m, 2H), 6.75 (m, 1H), 5.43 (m, 1H), 4.08 (m, 1H), 3.90 (m, 2H), 3.34 (m, 2H), 3.15 (m, 2H), 2.65 (m, 2H). Anal. Calcd for $C_{22}H_{23}Cl_2N_5O_7$ + 1.7TFA: C, 41.55; H, 3.39; N, 9.54. Found: C, 41.47; H, 3.36; N, 9.81.

4.2.31. (*R*)-3-Bromo-5-chloro-2-hydroxy-β-[[2-[[[3-hydroxy-5-[(1,4,5,6-tetrahydro-pyrimidin-2-yl)amino] phenyl] carbonyl]amino]acetyl]amino]benzene-propionic acid, tri fl uoroacetate salt (92). *Method B:* To a solution of 3-hydroxy-5-[(1,4,5,6-tetrahydro-2-pyrimidinyl)amino]benzoi c acid hydrochloride (0.3 g; 1.3 mmol) in DMF (7 mL), 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride (0.28 g; 1.5 mmol) and 1-hydroxybenzotriazole hydrate, HOBt (0.2 g; 1.5 mmol) were added. After stirring the reaction mixture at room temperature for 30 min, ethyl *R*-3-(*N*-gly)-amino-3-(3-bromo-5-chloro-2-hydroxyphenyl)-propionate hydrochloride (0.56 g; 1.3 mmol) and triethylamine (0.15 g; 1.5 mmol) were added and the resulting mixture was stirred at room temperature for 18 h. It was concentrated in vacuo and the residue was purified by reversed phase HPLC to afford the ethyl ester of the title compound (0.38 g, 40%) as white solid. This product was dissolved in acetonitrile:water/1:1 (8 mL), added lithium hydroxide (0.16 g), and stirred at room temperature for 3 h. The product was purified by HPLC to afford the title compound (0.3 g). ^{1}H NMR (CD_3OD) δ 7.41 (d, 1H, J = 2.42 Hz), 7.24 (d, 1H, J = 2.42 Hz), 7.18 (s, 1H), 7.14 (s, 1H), 6.78 (m, 1H), 5.5 (d, 1H, J = 5.5 Hz), 4.05 (s, 2H), 3.37 (t, 4H, J = 5.9 Hz), 2.90–2.77 (m, 2H), 2.01 (m, 2H). HRMS (ES, *m/z*) Calcd for $C_{22}H_{23}BrClN_5O_6$: 567.052. Found: 570.0534 (M+2+H, high resolution done on the second peak of the bromo).

4.2.32. (β[1]*R*)-3-Bromo-5-chloro-β-[[[[3-[(5-fluoro-1,4,5,6-tetrahydro-2-pyrimidinyl)-amino]-benzoyl]amino]acetyl]-amino]-2-hydroxybenzenepropionic acid, trifluoroacetate salt (96). *Method C:* To a solution of 3-[(5-fluoro-1,4,5,6-tetrahydro-2-pyrimidinyl)amino] benzoic acid, monohydrochloride salt (0.50 g, 1.61 mmol) in anhydrous DMF (5 mL) at −5 °C was added diisopropylethylamine (DIEA, 0.23 g, 1.81 mmol) followed by the addition of HBTU (0.64 g, 1.68 mmol). After stirring this mixture under argon atmosphere for 1 h at −5 °C, solution was allowed to warm up to room temperature and stirred for 1 h. After activating the ester, a cold solution of ethyl (3*R*)-3-(3-bromo-5-chloro-2-hydroxyphenyl)-3-(glycylamino)-propanoate, hydrochloride (0.655 g, 1.57 mmol) in anhydrous DMF (5 mL) and *N*-methylmorpholine (0.19 g, 0.0019 mol) was added. The pH of the resulting mixture was 7 at 1 h and was allowed to stir at room temperature overnight. Solvent was distilled in vacuo and the residue was purified by reverse phase HPLC to yield (after lyophilization) the ester as a white powder, 0.7 g (64% yield). ^{1}H NMR (CD_3OD) δ 7.6 (d, 1H), 7.73 (t, 1H), 7.55 (t, 1H), 7.41 (m, 2H), 7.24 (d, 1H), 5.57 (t, 1H), 5.25 (m, 1H, J_H = 48 Hz), 4.07 (m, 4H), 3.64 (m, 4H), 2.88 (m, 2H), 1.17 (t, 3H); HRMS (ES) *m/z* Calcd for C_{24} H_{26} N_5 $FClBrO_5$: 598.0868 (M+H). Found: 598.0850 (M+H). The ester (0.7 g, 0.98 mmol) was stirred with 1 M LiOH (4 mL) for 2 h at room temperature. The pH was adjusted to 2 with trifluoro-acetic acid and the product was purified by reverse phase HPLC to provide (after lyophilization) the acid as a white powder (0.4 g, 59%). ^{1}H NMR (CD_3OD) δ 7.79 (d, 1H), 7.73 (t, 1H), 7.55 (t, 1H), 7.42 (m, 2H), 7.24 (d, 1H), 5.55 (t, 1H), 5.26 (m, 1H, J_H = 46.4 Hz), 4.08 (s, 2H), 3.64–3.3 (m, 4H), 2.86 (m, 2H). HRMS (ES, *m/z*) Calcd for $C_{22}H_{22}N_5FO_5ClBr$: 569.0475. Found: 570.0550 (M+H).

4.2.33. (β[1]*R*)-3-Bromo-5-chloro-β-[[[[3-[(5-fluoro-1,4,5,6-tetrahydro-2-pyrimidinyl)-amino]-5-hydroxybenzoyl]amino]-acetyl]amino]-2-hydroxybenzenepropionic acid, trifluoroacetate salt (97). Coupling of **20** and **74** followed by saponification and purification by HPLC gave the desired product. ^{1}H NMR (CD_3OD) δ 7.41 (s, 1H), 7.24 (d, 1H, J = 1.8 Hz), 7.25 (s, 1H) 7.21 (s, 1H), 7.17 (s, 1H), 6.81 (s, 1H), 5.55 (t, 1H, J = 6.0 Hz), 5.20 (m, 1H, J_H = 46.4.0 Hz), 4.06 (s. 2H), 3.7–3.41 (m, 4H), 2.85 (ab q, 2H, J_1 = 5.6 Hz, J_2 = 9.6 Hz), 1.18 (t, 3H, J = 5.4 Hz). HRMS (ES, *m/z*) Calcd for $C_{22}H_{22}N_5FClBrO_6$: 585.0424. Found: 586.0495 (M+H).

The above three procedures described for the synthesis of compounds **87** (Method A), **92** (Method B), and **96** (Method C) have been utilized for the synthesis of the following compounds (**85**–**126**) starting from the appropriate left hand side and the glycine amide of the β-amino acid ester followed by saponification and purification. The method used for the individual compound is indicated in Table 2.

4.2.34. *N*-{3-[(5-Fluoro-1,4,5,6-tetrahydropyrimidin-2-yl) amino]-5-hydroxybenzoyl}glycine, trifluoroacetate (127). To a solution of 3-hydroxy-5-[(1,4,5,6-tetrahydro-5-fluoro-2-pyrimidyl)-amino]-benzoic acid hydrochloride prepared above (2.0 g, 6.9 mmol), 0.7 g (6.9 mmol) of *N*-methylmorpholine (NMM) and 0.96 g (6.9 mmol) of ethyl glycinate hydrochloride in 18 mL of anhydrous *N*,*N*-dimethylacetamide (DMA) were added, followed by the addition of 1.05 g (8.3 mmol) of diisopropylcarbodiimide (DIC) at ice bath temperature. The reaction mixture was stirred overnight at room temperature. The precipitate was filtered off, and DMA was removed in vacuo at 50 °C. 50–60 mL of water was added to the residue followed by 4.3 g (0.11 mol) NaOH. This mixture was stirred at room temperature for 3 h and filtered. The filtrate was neutralized with TFA, concentrated, and the residue was purified by reverse phase preparative HPLC to yield (after lyophilization) the title compound (0.85 g) as a white solid: ^{1}H NMR (D_2O) δ 7.08 (m, 2H), 6.83 (m, 1H), 5.19 (d m, J = 32 Hz, 1H), 4.03 (s, 2H), 3.33–3.64 (m, 4H). HRMS (ES, *m*/*z*) Calcd for $C_{13}H_{15}FN_4O_4$: 310.1076. Found: 311.1182 (M+H).

4.2.35. Ethyl-(*R*)-3-amino-3-(3,5-dibromo-phenyl)proprionate hydrochloride (46). 3,5-Dibromobenzaldehyde (20.0 g, 76 mmol), malonic acid (9.6 g, 90 mmol), ammonium acetate (7.0 g, 90 mmol), and 130 mL isopropyl alcohol were stirred at reflux for 5 h. After cooling to room temperature, the ppt. was filtered, washed with isopropyl alcohol, and dried to yield 10.8 g of racemic β-amino acid (zwitterion) product as a white solid. To 3.6 g of this β-amino acid in 40 mL of absolute ethanol is bubbled in excess HCl gas. The resulting solution is stirred at reflux for 45 min. The solvent is removed in vacuo and the residue is slurried twice in petroleum ether/isopropyl ether (~3:1) (solvent decanted off each time), and the resulting solid is dried in vacuo to yield 4.4 g of the racemic β-amino ester hydrochloride product as a white solid. The *R*-isomer was isolated by enzymatic resolution as described for **11**. ^{1}H NMR (DMSO-d_6) δ 8.94 (br s, 3H), 7.85 (m, 2H), 7.82 (m, 1H), 4.58 (m, 1H), 3.91–4.02 (m, 2H), 3.02–3.22 (m, 2H), 1.05 (t, 3H). HRMS (ES, *m*/*z*) Calcd for $C_{11}H_{13}Br_2NO_2$: 348.9313. Found: 351.9322 (M+H, high resolution for the second isotope peak of the bromo).

4.2.36. (β^1*R*)-3,5-Dibromo-β-[[[3-[(5-fluoro-1,4,5,6-tetrahydro-2-pyrimidinyl)amino]-5-hydroxybenzoyl]amino]-acetyl]amino]benzenepropionic acid, trifluoroacetate salt (129). *Method D:* To *N*-{3-[(5-fluoro-1,4,5,6-tetrahydropyrimidin-2-yl)amino]-5-hydroxybenzoyl}-glycine, trifluoroacetate (**127**) (0.25 g, 0.59 mmol), in 2 mL of anhydrous DMA in a flame-dried flask, was added (0.081 g, 0.59 mmol) of isobutyl chloroformate (IBCF) at ice bath temp, followed by of NMM (0.06 g, 0.59 mmol), and stirred at 5 °C for 5 min, under nitrogen atmosphere. After stirring at room temperature for 10 min, ethyl (*R*)-3-amino-3-(3,5-dibromo-phenyl)-proprionate hydrochloride (0.193 g, 0.5 mmol) was added, followed by the addition of NMM (0.05 g, 0.05 mmol). The reaction mixture was then stirred overnight at room temperature, and water (6.0 mL) and acetonitrile (2.0 mL) were added, followed by the addition of 600 mg of NaOH. This mixture was stirred at room temperature for 3 h, acidified with TFA, and the product was isolated by reverse phase preparative HPLC to yield (after lyophilization) the title compound (120 mg) as a white solid. ^{1}H NMR (D_2O) δ 7.56 (m, 1H), 7.39 (m, aromatic, 2H), 7.06 (m, 2H), 6.82 (m, 1H), 5.06–5.29 (m, 2H), 3.95 (s, 2H), 3.31–3.64 (m, 4H), 2.73–2.82 (m, 2H). HRMS (ES, *m*/*z*) Calcd for $C_{22}H_{22}Br_2FN_5O_5$: 612.9972. Found: 615.9999 (M+2+H, high resolution for the second isotope of the bromine).

4.2.37. Vitronectin adhesion assay: materials. Human vitronectin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were purified from human placenta[37] and from fresh frozen plasma[38] as previously described. Biotinylated human vitronectin was prepared by coupling NHS-biotin from Pierce Chemical Company (Rockford, IL) to purified vitronectin.[39] Assay buffer, OPD substrate tablets, and RIA grade BSA were obtained from Sigma (St. Louis, MO). Anti-biotin antibody was obtained from Sigma (St. Luois, MO). Nalge Nunc-Immuno microtiter plates were obtained from Nalge Company (Rochester, NY).

4.3. Methods: cell assays for potency and selectivity

While the β_3 subunit of $\alpha_v\beta_3$ is only known to complex with α_v or α_{IIb}, the α_v subunit complexes with multiple β subunits. The three α_v integrins most homologous with $\alpha_v\beta_3$ are $\alpha_v\beta_1$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$, with 43%, 56%, and 47% amino acid identity in the β subunits, respectively. To evaluate the selectivity of compounds between the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_6$, cell-based assays were established using the 293 human embryonic kidney cell line. 293 cells express $\alpha_v\beta_1$, but little to no detectable $\alpha_v\beta_3$ or $\alpha_v\beta_6$. cDNAs for β_3 and β_6 were transfected separately into 293 cells to generate 293-b3 and 293-b6 cells, respectively. High surface expression of $\alpha_v\beta_3$ and $\alpha_v\beta_6$ was confirmed by flow cytometry. Conditions were established for each cell line in which cell adhesion to immobilized human vitronectin was mediated by the appropriate integrin, as determined by a panel of integrin-specific, neutralizing monoclonal antibodies. Briefly, cells were incubated with inhibitor in the presence of 200 μM Mn^{2+}, allowed to adhere to immobilized vitronectin, washed, and adherent cells are detected by quantifying endogenous alkaline phosphatase and *para*-nitrophenyl phosphate. An 8-point dose–response curve using either 10- or 3-fold dilutions of compound was evaluated by fitting a four-parameter logistic, nonlinear model (using SAS).

To evaluate compound's potency for membrane-bound $\alpha_v\beta_6$ an additional cell-based adhesion assay was established using the HT-29 human colon carcinoma cell line. High surface expression of $\alpha_v\beta_6$ on HT-29 cells was confirmed by flow cytometry. Conditions were established in which cell adhesion to immobilized human latency associated peptide (LAP) was mediated by the $\alpha_v\beta_6$, as determined by a panel of integrin-specific, neutralizing monoclonal antibodies. Briefly, cells were incubated with inhibitor in the presence of 200 μM Mn^{2+}, allowed to adhere to immobilized LAP, washed, and adherent cells were detected by quantifying endogenous alkaline phosphatase using *para*-nitrophenyl phosphate. An 8-point dose–response curve using either 10- or 3-fold dilutions of compound was evaluated by fitting a four-parameter logistic, nonlinear model (using SAS).

4.4. Mouse pharmacokinetics

Test compounds were administered orally (po) at 20 mpk or intravenously (IV) at 10 mpk as solutions. For IV dosing the compounds were dissolved in normal saline. Compounds for po administration were suspended in 0.5% (w/v) methylcellulose and 0.025% (w/v) polyethylene sorbitan mono-oleate (Tween 80). Blood samples were collected by cardiac puncture following CO_2 euthanasia, citrated to prevent clotting, and the plasma immediately separated by centrifugation. Plasma samples were stored at −20 °C until assayed for $\alpha_v\beta_3$ inhibitory activity using a solid-phase competitive displacement assay. Pharmacokinetic parameters were calculated using WinNonLin software (Pharsight Corp., Mountain View, CA).

4.5. M21 tumor model

Human M21 melanoma cells were obtained from Dr. Jeff Smith (Burnham Institute, San Diego, CA). Cells were grown in RPMI with 10% FCS and 2 mM L-glutamine. Tumor cells were harvested from sub-confluent cultures, washed in medium, and resuspended in RPMI with no FCS at 5×10^7 cells/mL. Tumor cells were used below passage 40 for inoculation into mice.

Female SCID mice (Harlan, Indianapolis, IN), 4–6 weeks of age, were acclimated in a barrier care facility caged in groups of five. Mice were maintained on standard Purina mouse chow and had free access to water. Mice were injected subcutaneously in the flank with 5×10^6 tumor cells per mouse in the morning and dosing with **97** and vehicle was initiated the evening of the same day, and was continued twice daily through study day 27. Tumors were measured with a vernier caliper, and the volumes were determined using the formula: tumor volume = $\text{width}^2 \times \text{length} \times 0.52$. On day 28 of the study, the mice were sacrificed by CO_2 euthanasia, and the tumors were excised and weighed.

4.5.1. Colon 26 tumor model. Mouse Colon 26 tumor cells were obtained from National Cancer Institute, Frederick, MD. Cells were grown in RPMI with 10% FCS and 2 mM L-glutamine. Tumor cells were harvested from sub-confluent cultures, washed in HBSS, and resuspended in HBSS at 5×10^7 cells/mL. Tumor cells were kept in culture for 4 weeks or less prior to usage.

Female BALB/c mice (Charles River Laboratories, Wilmington, MA) were acclimated in a barrier care facility caged in groups of five. Mice were maintained on standard Purina mouse chow and had free access to water. Mice were injected subcutaneously in the flank with 5×10^6 tumor cells per mouse in the morning and dosing with **97** and vehicle was initiated the evening of the same day, and was continued twice daily through study day 20. Tumor volumes were measured at intervals over the course of the experiment. Tumors were measured with a vernier caliper, and the volumes were determined using the formula: tumor volume = $\text{width}^2 \times \text{length} \times 0.52$. On day 21 of the study, the mice were sacrificed by CO_2 euthanasia, and the tumors were excised and weighed. Blood was taken on study day 4 and day 21 to analyze the serum calcium concentration and was measured using a colorimetric kit (Sigma, St. Louis, MO).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.03.034.

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