Pergamon

Bioorganic & Medicinal Chemistry, Vol. 2, No. 9, pp. 881-895, 1994

0968-0896/94 $7.00 + .00

0968-0896(94)00108-1

Design of Orally Active, Non-Peptide Fibrinogen Receptor Antagonists. An Evolutionary Process from the RGD Sequence to Novel Anti-Platelet Aggregation Agents

P. R. Bovy,*† F. S. Tjoeng,† J. G. Rico,† T. E. Rogers,† R. J. Lindmark,† J. A. Zablocki, R. B. Garland, D. E. McMackins,† H. Dayringer,† M. V. Tóth,† M. E. Zupec,† S. Rao, S. G. Panzer-Knodle, N. S. Nicholson, A. Salyers, B. B. Taite, M. Herin, M. Miyano, L. P. Feigen and S. P. Adams†

Thrombosis Research, Searle, 4901 Searle Parkway, Skokie, IL 60077, U.S.A.
†Monsanto Corporate Research, Chemical Sciences, 700 Chesterfield Parkway North, St Louis, MO 63198, U.S.A.

Abstract—The evolutionary process from the Arg-Gly-Asp-Phe (RGDF) tetrapeptide to potent orally active anti-platelet agents is presented. The RGD sequence is an important component in the recognition of fibrinogen by its platelet receptor GP IIb–IIIa (integrin $\alpha_{IIb}\beta_3$). This work concentrates on the replacement of the Arg-Gly dipeptidyl fragment by an acylated aminobenzamidine. The C-terminal fragment has been replaced by a variety of β-amino acids, expanding on a previously reported paradigm. The lead compounds showed good potency in an *in vitro* platelet aggregation assay (dog PRP/ADP). The affinity for the fibrinogen receptor was confirmed in several cases by the ability to inhibit ^{125}I fibrinogen binding to activated human platelets. The ethyl ester prodrug form was tested by oral administration to dogs and monitoring of the anti-platelet effect on *ex vivo* collagen induced platelet aggregation. From the structural studies reported, the 4-[[(aminoiminomethyl)phenyl]amino]-4-oxobutanoic acid (**5**) was the best surrogate for the Arg-Gly dipeptide. Several conformationally restricted analogues are also reported which are compatible with the hypothesis of RGD binding to the $\alpha_{IIb}\beta_3$ in a turn-extended-turn conformation. The structure–activity relationships described also underline the importance of the β-amino acid substitution for potency. In particular, the absolute configuration at the β-carbon was crucial for high affinity. The best acid/ester pairs reported in this study had high potency (acid PRP/ADP $IC_{50} \simeq 50$ nM) and showed good oral activity in dogs at 5 mg/kg per os (ethyl ester).

Introduction of RGDX Peptidomimetic Concept

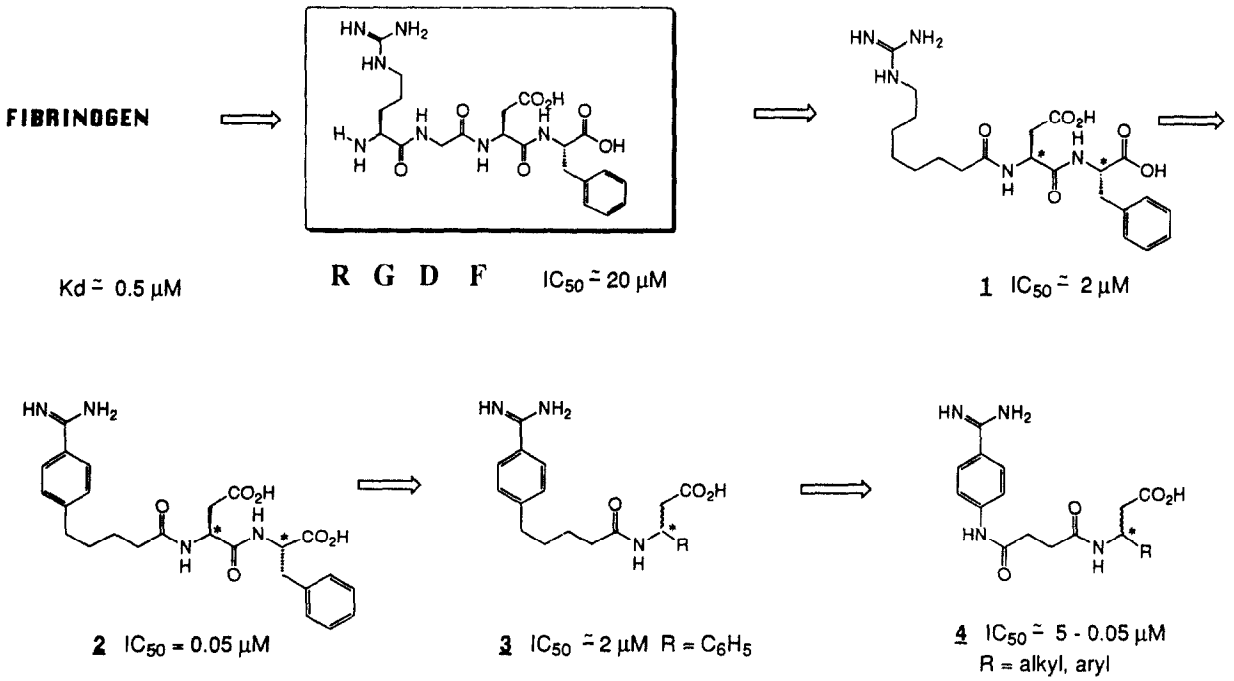
Platelet aggregation is an important component in the thrombotic process and is present in a wide variety of pathological circumstances. The binding of fibrinogen to its platelet receptor, glycoprotein IIb–IIIa (GPIIbIIIa, integrin $\alpha_{IIb}\beta_3$), was shown to be essential for thrombus growth.[1] Peptides and small proteins containing the sequence Arg-Gly-Asp (RGD) antagonize binding of fibrinogen to the GPIIbIIIa platelet receptor, thereby inhibiting platelet aggregation.[2,3] The tetrapeptides RGDS and RGDF, members of the ubiquitous integrin recognition sequence RGD, have an affinity for the GPIIbIIIa receptor which is about two to three orders of magnitude lower than that of fibrinogen. A number of structure–activity studies with peptides of the RGDX type converged to highlight the importance of the arginine guanidino function as well as the aspartic acid side chain.[4,6]

During the last five years, it has been a major goal among many pharmaceutical houses to identify compounds which (i) have higher affinity for the fibrinogen receptor while of small molecular size, (ii) have adequate plasma half-lives, (iii) display oral activity and, (iv) exhibit selectivity for the integrin $\alpha_{IIb}\beta_3$ versus other related integrin receptors, in particular the vitronectin receptor $\alpha_v\beta_3$.[5]

In Scheme I, we describe the evolution of the peptide RGDF, present in the α-chain of fibrinogen, toward a peptidomimetic entity endowed with the properties compatible with pharmaceutical development as a drug candidate.

Thus, compounds have been discovered that employ 8-guanidino-octanoic acid as a bioisosteric replacement for Arg-Gly (**1**, Scheme I).[6] Further structural modifications led to the introduction of benzamidine pentanoic acid as a replacement for the Arg-Gly dipeptide (**2**, Scheme I).[7] Compounds incorporating this moiety showed excellent affinity for $\alpha_{IIb}\beta_3$. Pioneering work has described the replacement of the N-terminal arginine by *p*-amidinophenylalanine and *p*-amidinobenzoyl derivatives.[8] More recently, a piperidine containing moiety has also been reported which appears to act as a surrogate for the guanidine.[9] The increased potency and selectivity obtained by the introduction of the benzamidine moiety as a surrogate for the arginine side chain is striking and has since been reported by many groups.[10,11] Further progress was made with the introduction of β-amino acids as surrogates for the Asp-Phe dipeptides (**3**, Scheme I). Compounds containing simple β-amino acids at the Asp position were found to display oral bioavailability when

*Author for correspondence: Affymax Research Institute, 4001 Miranda Av., Palo Alto, CA 94304, U.S.A.



Scheme I.

administered as ethyl ester prodrugs.[12] The novel modification that we report in this study is the introduction of an acylated aminobenzamidine at the Arg-Gly position.

Design and Synthesis of Aminobenzamidine Derivatives

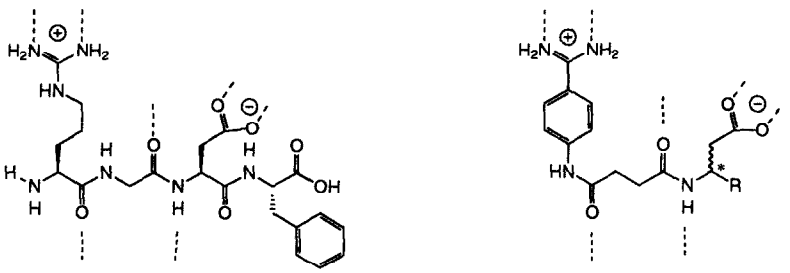
Compounds of structure **3** and **4** (Scheme I) differ by the introduction of an amide bond in the pentanoic acid chain. This structural modification was motivated by several considerations. Firstly, to probe a possible hydrogen bonding interaction with the receptor, secondly, to examine the effect of reducing the number of degrees of rotational freedom and modifying the spacer length and thirdly, to stabilize the benzamidine pentanoic linker (i.e. compound **3**) against a putative benzylic oxidation.

As indicated in Figure 1, an attractive spacer between the aminobenzamidine and the β-amino acid was a diacid linker based on structural features and synthetic accessibility. Thus we decided to examine the 4-[[4-(aminoiminomethyl)-phenyl]amino]-4-oxo-butanoic acid **5** fragment and homologues as a substitute for the Arg-Gly dipeptidyl fragment. We have explored the effect of several structural modifications of this linker on the biological activity. In particular, we have introduced conformational restriction in the succinic moiety by utilizing the unsaturated linkers fumarate and maleate, as well as the *trans*-cyclo-propyldicarboxylic derivatives.[13]

In the benzamidinopentanoic acid series (i.e. **3**) , the nature of the β-amino acid beta substituent has a profound effect on potency.[12] We have examined this structural parameter in the present series, including the influence of absolute configuration at the chiral center. We report here, in particular, the effect of a number of aromatic and heteroaromatic groups on the *in vitro* and *in vivo* potency.

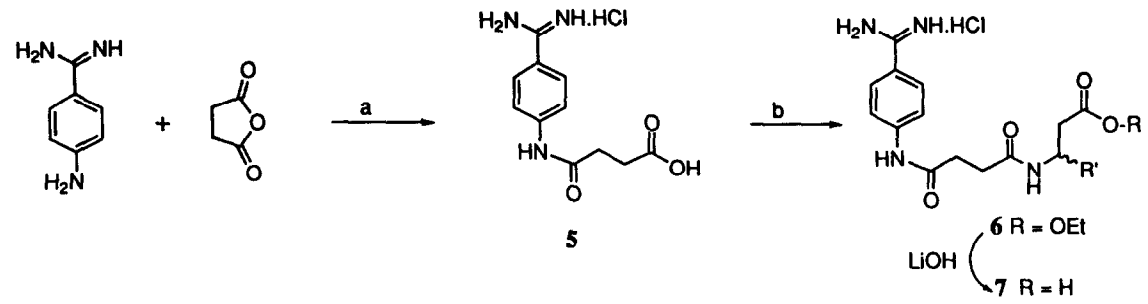
Synthesis of First Leads and Optimization

All compounds reported were prepared according to Schemes II–VII. The synthesis of the new linker 4-[[4-(aminoiminomethyl)phenyl]amino]-4-oxo-butanoic acid **5** has been previously reported.[14] Thus, *p*-aminobenzamidine



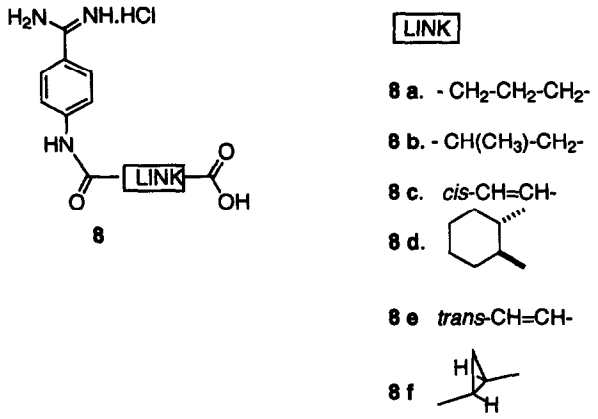
RGDF 4

Figure 1. Figure 1 schematically depicts a possible array of hydrogen bonds and ionic interactions between the RGD peptide. An aminobenzamidine derivative of general structure **4** may support the same critical interactions. When the β-amino acid is superposed to the aspartic acid residue, the succinic acid-derived linker allows the basic amidine and guanidine groups to occupy a similar position. Also, the carbonyl oxygens of the amide bonds assume similar relative positions.

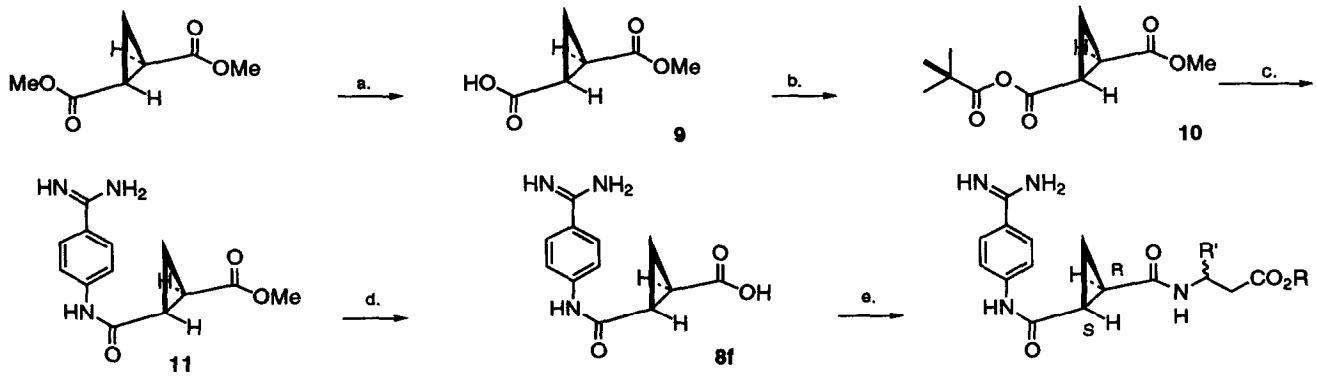


a. DMF, Pyr, DMAP; HCl; b. *i*-BuOCOCl, DMF, NMM, β-aminoester.

Scheme II.

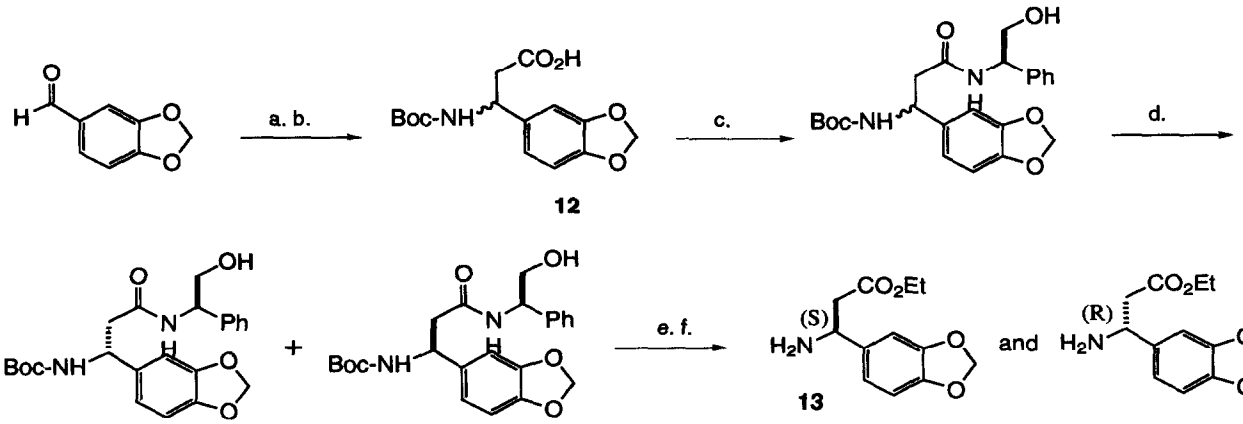


Scheme III.



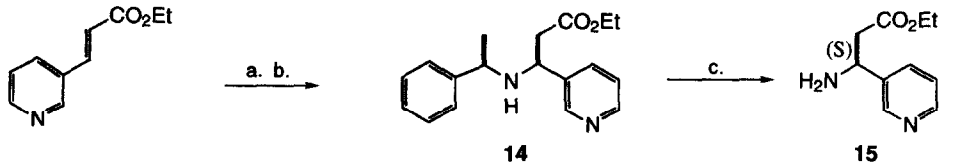
a. 1 eq. KOH; b. $(CH_3)_3COCl$, Pyr, DMF; c. NEt_3, aminobenzamidine; d. $LiOH/H_2O$; e. *i*-BuOCOCl, DMF, NMM, $NH_2CHR'CH_2CO_2R$.

Scheme IV.



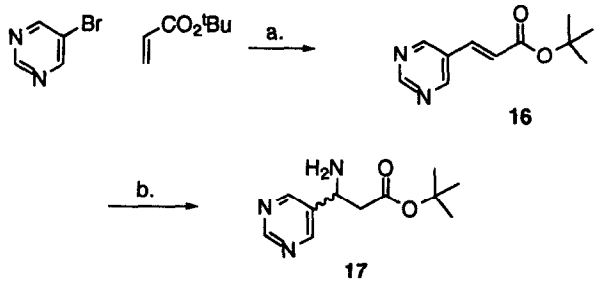
a. Malonic acid, NH_4OAc, EtOH, reflux, 16 h; b. Dioxane/water, NaOH, di-*t*-butyl dicarbonate; c. DMF/pyridine, disuccinimidylcarbonate, DMAP, (*R*)-2-amino-2-phenyl-ethanol 16 h; d. Resolution on C-18 silica gel with acetonitrile/water 35/75; e. H_2SO_4, water/dioxane, reflux, 16 h; f. EtOH, HClgaz.

Scheme V.



a. NEt_3, THF, (*R*)-(+)-α-Methylbenzylamine, TMSCl; b. Butyl lithium, THF, 0 °C; –78 °C to rt; c. $Pd/C/NH_4CO_2H$ or Pd/C/1,4-cyclo-hexadiene.

Scheme VI.



a. $Pd(OAc)_2$, NEt_3, 80 °C, 72 h; b. NH_3, t-BuOH, 80 °C, 72 h.

Scheme VII.

was reacted with succinic anhydride in a mixture of dimethylformamide and pyridine at 100 °C for 1 h to give 4- [[4- (aminoiminomethyl) phenyl] amino] -4-oxo-butanoic acid in isolated yields exceeding 80 % (Scheme II).

A similar approach was used to prepare some analogues of succinic acid (Scheme III), when the anhydride is available. Thus, glutaric anhydride, maleic anhydride, and methylsuccinic anhydride were used in the same way to provide the open adducts, respectively **8a**, **8c**, and **8b**. In the case of methyl succinic anhydride, no attempt was made to separate the expected two regioisomers. In that case, after coupling, two products could be isolated by HPLC, presumably the two regioisomers as a mixture of diastereoisomers (not resolved). In most cases, the hydrochloride salts of the benzamidine adduct were used in the subsequent coupling step in a manner similar to step b in Scheme II. Thus, compounds **8a–f** were activated by the mixed anhydride method and added to the desired β-amino acid, generally protected as esters. The best results were obtained when mixed anhydride formation was limited to 5 min at room temperature. This avoided the competitive cyclization to the succinimide when this process was anticipated. No protection was required for the amidine function when the carboxylic acid was activated by this method. Interestingly, the 3-amino-phenylpropanoic and 3-amino-methylpropanoic acids undergo coupling without protection of the carboxylic acid function. In all cases, purification of the crude mixture by RP-HPLC provided the desired compounds in 50–80 % yield. The corresponding acids could be easily obtained by hydrolysis of the ester with aqueous lithium or sodium hydroxide at room temperature.

When the anhydride could not be used, the aminobenzamidine had to be coupled to a carboxylic acid. For example, monoethyl fumarate and the monoethyl cyclopropanedicarboxylate **9** (Scheme IV) were coupled to aminobenzamidine using the mixed anhydride activation. Preparation of the pivaloyl mixed anhydride **10**[15] was found to give the best coupling in the case of the cyclopropane derivative. The desired acid **8f** was obtained by acid hydrolysis and coupled to the desired β-amino acid using the usual conditions.

Some of the simple β-amino acids were commercially available. Most of the more complex ones had to be prepared. In particular, when the homochiral material was necessary, practical preparative syntheses had to be devised.

The most common route to access the aromatic or heteroaromatic amino acids is the modified Knoevenagel condensation[16] as described in Scheme V for the preparation of the ethyl *N*-*t*-Boc-β-amino-1,3-benzodioxole-5-propanoate **12**. The racemic compound was resolved by separation of its diastereoisomeric adducts with (*R*)-2-amino-2-phenylethanol (Scheme V) and processed to the homochiral ester **13** and its (*R*)-enantiomer as outlined in steps e and f of Scheme V.

In another case, a stereoselective synthesis was preferred (Scheme VI).[14] The (*S*)-β-amino-3-pyridinepropionic acid, ethyl ester **15** was prepared by addition of a novel lithium amide derived from (*R*)-*N*-trimethylsilyl-1-phenyl-ethylamine to ethyl *trans*-3-pyridineacrylate in a highly stereoselective Michael addition (> 95 % *d.e.*) as described previously.[14] Debenzylation of the secondary amine intermediate proceeds under catalytic hydrogen transfer conditions to give the desired chiral β-aminoester **15** (95 % *e.e.*). The (*R*)-enantiomer could be obtained from the same procedure utilizing the (*S*)-1-phenylethylamine as chiral auxiliary. The enantiomeric purity was lower than in the latter case due to trace-contamination by the opposite enantiomer in the chiral auxiliary used.

To obtain non-homochiral material, other approaches have been devised. Thus, the ester (2*E*)-1,1-dimethylethyl-3-(5-pyrimidinyl)-2-propenoate **16**, obtained by Heck coupling between 5-bromopyrimidine and *t*-butyl acrylate undergoes nearly quantitative Michael addition in *t*-butanol saturated with ammonia to β-amino-5-pyrimidinepropanoic ester **17**.[17] This material was of suitable purity for coupling to the desired benzamidine derivative (Scheme VII). The free acid was obtained by deprotection of the final ester by trifluoroacetic acid treatment.

In some cases, a homochiral amino acid was coupled to a racemic linker (e.g. **8f**). For example, both enantiomers of the β-amino-3-pyridinepropionic acid were coupled to *trans*-cyclopropyl derivative **8d**. A total of four diastereoisomers were obtained, isolated and tested separately (Figure 2).

Biological Results and Basic SAR

The activity of the compounds reported in Tables 1–3 has been assessed by their ability to inhibit *in vitro* platelet

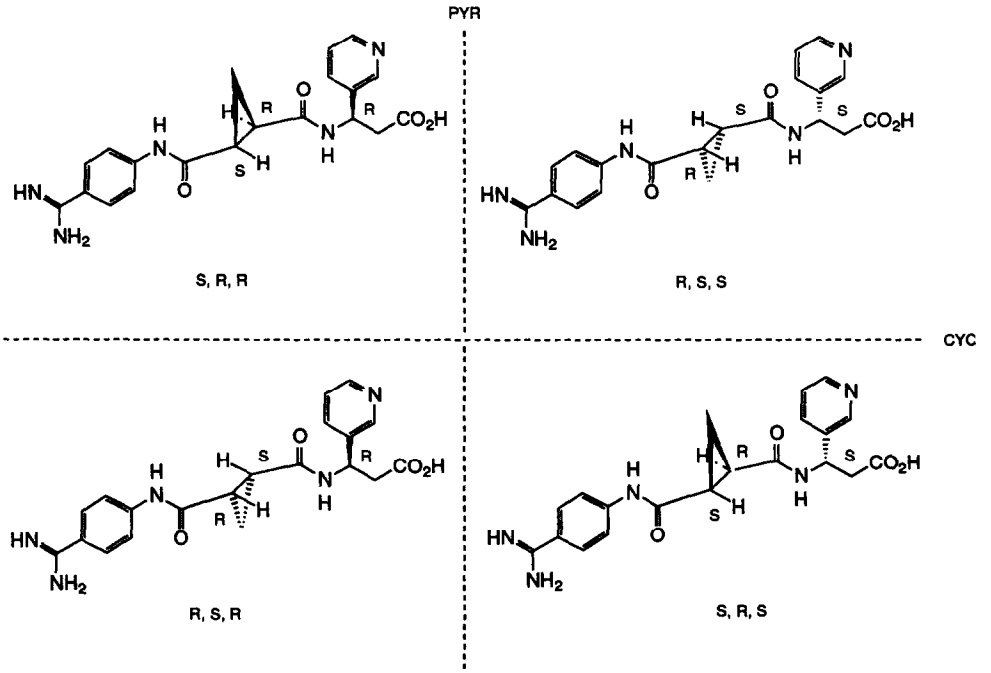
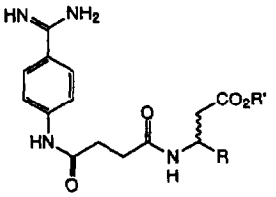


Figure 2. The four possible diastereoisomers (**57–60**) resulting from the coupling of (*R*)- and (*S*)-β-amino-3-pyridine propanoic acid with the cyclopropyl dicarboxylic derivative **8f** have been isolated and tested individually. The absolute configuration at the β-amino acid chiral center is firmly established by synthesis, while the absolute configuration at the cyclopropyl ring is unknown. One of the *S* diastereoisomers of the β-amino acid, **59**, is the most potent antagonist, enjoying a more than 50-fold increased potency over **60**.

Table 1. *In vitro* and *in vivo* evaluation of compounds containing the aminobenzamidine derivative **5**

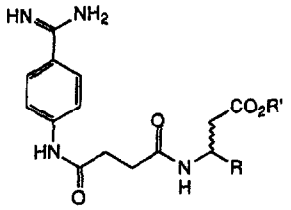


Comp. #	R	R'	Inhibn of Platelet[b] Aggregation IC_{50} (nM)	Fibrinogen Binding Assay[d] IC_{50} (nM)	Ex Vivo Inhibn (%) of Platelet Aggregation[c]
20/	H	H	700.0	NT[f]	(100/ 2mg/kg/iv)[e]
21/	H	Et	NA[a]	NT	100/ 20mg/kg
22/	Me	H	170.0	110	(100/ 10mg/kg/iv)[e]
23/	Me	Et	NA[a]	NA[a]	100/ 10mg/kg
24/	C_6H_5	H	200	NT	NT
25/	C_6H_5	Et	NA[a]	NT	100/ 20mg/kg
26/	$(CH_2)_2CO_2H$/	H	600	NT	NT
27/	CH_2CO_2Me/	H	400	NT	NT
28/	CO_2H	H	4800	NT	NT

[a]Not active when tested at 10 mM.
[b]Inhibition of platelet aggregation was determined in dog platelet rich plasma; the activation agent was ADP; all compounds were tested in duplicate. IC_{50}'s (dosage at which 50 % of platelet aggregation is inhibited) were calculated by linear regression of the dose–response curve.
[c]The inhibition of platelet aggregation (collagen) is measured *ex vivo* on blood from dogs which have been administered the test compound, intragastrically with the exception of the acids **20** and **22** (see e.).
[d]According to reference 19.
[e]The acids were administered intravenously as a bolus injection at the dose indicated and platelet aggregation was measured as under c.
[f]Not tested.

aggregation in platelet-rich plasma (PRP). PRP was prepared from dogs blood and platelet aggregation was initiated by adenosine-5'-diphosphate (ADP). All compounds were tested in duplicate for their ability to inhibit platelet aggregation and their median inhibitory concentrations (IC_{50}) are reported in Tables 1–3. IC_{50}'s were calculated by linear regression of the dose–response curve.[18] Several compounds have also been tested for their

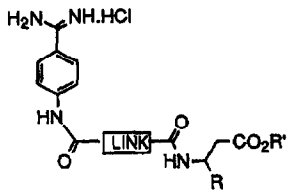
Table 2. *In vitro* and *in vivo* evaluation of aminobenzamidine derivatives with heteroaromatic and substituted aromatic β-amino acids



Comp. #	R	R'	Inhibn of Platelet[b] Aggregation IC_{50} (nM)	Fibrinogen Binding Assay [d] IC_{50} (nM)	Ex Vivo of Platelet Aggregatn[c]
29/	3-pyridyl	H	80	70	
30/	3-pyridyl	Et	NT[e]	NT	100/ 5mg/kg
31/	3-pyridyl	Me	NT	NT	100/ 5mg/kg
32/	(R)-3-pyridyl	H	2700	NT	
33/	(S)-3-pyridyl	H	48	50	100/ 8mg/kg
34/	2-furanyl	H	190	NT	
35/	2-furanyl	Et	NT	NT	100/ 10mg/kg
36/	2-thienyl	H	120	NT	
37/	2-thienyl	Et	>10000	NT	100/ 5mg/kg
38/	3-benzo-1,3-dioxole	H	68	NT	NT
39/	3-benzo-1,3-dioxole	Et	NA[a]	NT	100/ 5mg/kg
40/	(R)-3-benzo-1,3-dioxole	H	6800	NT	NT
41/	(S)-3-benzo-1,3-dioxole	H	46	45	100/ 3mg/kg
42/	2-pyridyl	H	290	NT	NT
43/	5-pyrimidinyl	H	59	NT	NT
44/	5-pyrimidinyl	Et	NA[a]	NT	100/ 5mg/kg
45/	4-pyridinyl	H	160	NT	NT
46/	3-thienyl	H	180	NT	NT
47/	C_6F_5	H	>10,000	NT	NT
48/	3,5-diF-C_6H_3	H	110	NT	NT
49/	3,4-diFC_6H_3	H	180	NT	NT

[a]Not active when tested at 10 mM
[b]Inhibition of platelet aggregation was determined in dog platelet rich plasma; the activation agent was ADP; all compounds were tested in duplicate. IC_{50}'s (dosage at which 50 % of platelet aggregation is inhibited) were calculated by linear regression of the dose–response curve.
[c]The inhibition of platelet aggregation (collagen) is measured *ex vivo* on blood from dogs which have been administered the test compound intragastrically.
[d]According to reference 19.
[e]Not tested.

Table 3. *In vitro* and *in vivo* evaluation of derivatives containing diverse linkers between the β-amino acid and the aminobenzamidine



Comp. #	R	R'	Link[c]	Inhibn of Platelet[b] Aggregation IC50 (nM)
51/	H	H	8e	>10000
52/	H	Et	8e	NA[a]
50/	C_6H_5	H	8a	3200
54/	C_6H_5	H	8c	>10000
55/	C_6H_5	H	8b[d]	5600/1100
53/	3-pyridyl	H	8e	110
56/	3-pyridyl	H	8d	>10000
57/	(R)-3-pyridyl	H	8f[e]	800
58/	(R)-3-pyridyl	H	8f[e]	>10000
59/	(S)-3-pyridyl	H	8f[e]	115
60/	(S)-3-pyridyl	H	8f[e]	6000
61/	3-pyridyl	H	8c	>10000

[a]Not active when tested at 10 mM
[b]Inhibition of platelet aggregation was determined in dog platelet rich plasma; the activation agent was ADP; all compounds were tested in duplicate. IC_{50}'s (dosage at which 50 % of platelet aggregation is inhibited) were calculated by linear regression of the dose response curve
[c]See Scheme III.
[d]Two regioisomers were isolated and tested individually; the regiochemistry was not established firmly.
[e]In this homochiral material the absolute configuration at the cyclopropyl is unknown.

ability to displace ^{125}I radiolabelled fibrinogen from its receptor on washed platelets.[19] For the evaluation of *in vivo* effects, compounds were administered to dogs orally (at doses varying between 5 and 20 mg/kg) and their antiplatelet effect was measured *ex vivo* on collagen-induced aggregation.[20]

Table 1 lists the *in vitro* and *in vivo* results for compounds **20–28**, a series of aminobenzamidine derivatives based on **5** in which a β-amino acid is used as a replacement for the Asp-Phe dipeptidyl fragment of RGDX. As can be seen from this table, the *in vitro* potency of the acid is modulated by the nature of the amino acid β-substituent. The phenyl and methyl substituted β-alanine derivatives produced analogues significantly more potent than the β-alanine itself. Aspartic acid is the least potent of the β-amino acids reported, indicating that a negative charge is not well tolerated at that position.

The acids **20** and **22** showed anti-aggregatory activity when injected iv in dogs but had no or a very low activity when administered orally. On the other hand, the corresponding ethyl esters showed no activity when tested *in vitro* at 10 μM but inhibited platelet aggregation when given orally to dogs at doses varying between 5 and 20 mg/kg. These observations are consistent with the ester being a prodrug form which releases the active acid by metabolic activation. Similar results have been previously observed and reported for the benzamidine pentanoic acid series.[21]

Since the nature of the β-amino acid substituent influences potency, efforts were devoted to determine the most favorable entities for *in vitro* affinity and *in vivo* activity. In Table 2 are reported the best aromatic and heteroaromatic substituents observed in this series. In several cases, the effect of the absolute configuration at the chiral β-carbon

was investigated. In all cases examined, the (*S*)-isomer, which has the same absolute configuration as L-aspartic acid, was found to be more active than the (*R*)-isomer by about 2 orders of magnitude. The heteroaromatic substituents at the β-position of the β-amino acid significantly increase the affinity for the receptor over the non-substituted β-alanine derivative **20**. The benzodioxole (**38**), the 3-pyridyl (**29**) and the 5-pyrimidinyl (**44**) derivatives were the most potent analogues with IC_{50}'s below 100 nM.

Table 3 reports binding data for a series of derivatives featuring various linkers between the β-amino acid and the aminobenzamidine fragments (see Scheme III). The nature of this 'link' sharply defines the accessible conformational space and is a determining factor for biological activity. The best compounds in the series are compounds **53** and **59** which are both frozen in a conformation forcing the amidine and carboxylic functions to remain away from each other, a feature which has been reported to be crucial for recognition of the fibrinogen receptor.[22,23] The distance between the β-carbon of the β-amino acid and the aniline nitrogen of the aminobenzamidine has been measured on two model linkers (Figure 3, Chem 3D Plus was used as a computer modeling software. Minimization of the 'links' was performed using the Chem 3D parameters). Thus, these linkers are capable of mimicking the distance between the Asp and Arg α-carbons which has been reported for a series of conformationally constrained RGD peptides presenting a β-turn or a turn-extended-turn conformation.[24,25]

The preferred chirality at the β-amino acids is (*S*), analogous to the L-Asp stereochemistry. The results obtained with the cyclopropyl-containing linker **8f** indicate that there is, in addition, a strict requirement on the absolute configuration of the linker between the β-amino acid and the aminobenzamidine moiety. The preferred absolute configuration for the cyclopropyl fragment in **59** has not been firmly established.

Conclusion

Design and synthesis of a novel class of orally active fibrinogen receptor antagonists are described.[26] This series features improved *in vitro* potency and *in vivo* activity over the previously reported benzamidine pentanoic acid series.[21] A large proportion of the new prodrug analogues had excellent anti-platelet activity when administered per os to beagles.[12b] The *in vivo* activity of most of these compounds lasted between 4 and 7 h or more. The 100 % inhibition level was usually reached in the first hour following administration and remained at that level for a variable period of time depending upon the potency, bioavailability and metabolic stability of the compound. Pharmacokinetic and pharmacodynamic properties as well as selectivity vs other integrin receptors will be reported in more detail elsewhere.

These results represent an exciting example in peptidomimetic design. Starting from a weakly active peptide lead, a combination of empirical observations and rational design led to compounds with high potency, long duration of action, and oral activity as practical drug candidates.

Experimental

The reagents and solvents were commercially available (Aldrich Chemical Co.) and of synthetic grade. The Analytical TLC plates and silica gel (230–400 mesh) were purchased from EM Reagents. Melting points were taken using a Mettler FP80/81 apparatus and are uncorrected.

^1H NMR spectra were routinely obtained on a Varian VXR-300 at 300 MHz in $CDCl_3$, DMSO-d_6 or CD_3OD. Mass spectra were obtained using a Finnigan MAT 90 or a VG model 250T spectrometer with either DCI or FAB ionization. Elemental analyses for C, H, N were obtained from Galbraith Laboratories, Inc. or Searle Physical Methods.

RP-HPLC column: Waters Delta Pak C, 100 Å, 15 m, 50 mm × 30 cm. Mobile phases: A—Water (0.05 % TFA), B—Acetonitrile (0.05 % TFA); gradient: 5 %–70 % B over 30 min; flow rate: 70 mL/min; detection at 215 nm. All compounds have been prepared according to the examples described below. The synthesis of intermediates **13**[14] and **17**[17] have been reported previously. The other non commercially available β-amino acids have been prepared according to Ref. 16 as illustrated for compound **12**.

4-[[4-(Aminoiminomethyl) phenyl] amino]-4-oxo-butanoic acid (5)

4-Aminobenzamidine dihydrochloride (25 g, 120 mmol) was added to dry dimethylformamide (100 mL). To this

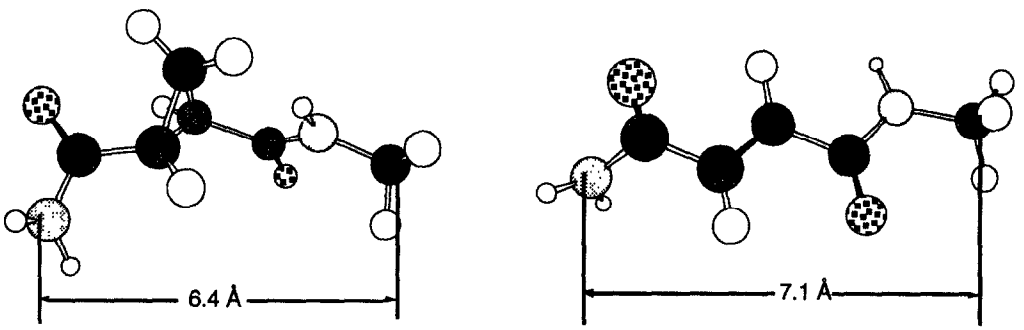


Figure 3. The distances between the β-carbon of the β-amino acid and the aniline nitrogen of the aminobenzamidine have been measured on models of linkers **5** and **8f** build and minimized with Chem3D Plus. These distances are similar to the distances between the similar α-carbon atoms of Asp and Arg in the cyclic RGD containing peptides presenting a β-turn or turn-extended-turn conformation (Ref. 24).

solution dry pyridine (100 mL) and succinic anhydride (12 g, 120 mmol) followed by dimethylaminopyridine (DMAP 1.5 g, 0.012 mmol) were added. The product precipitated after heating for 1/2 h at 100 °C. The product was filtered, washed with water, acetonitrile and ether. The white solid was suspended in ether, 4 N HCl in dioxane (100 mL) was added and the suspension was stirred for 1 h, filtered and dried in a desiccator to give 28 g, 88 % of 4-[[4-(aminoiminomethyl)phenyl]amino]-4-oxo-butanoic acid as a white yellow solid which decomposes between 270 and 290 °C.

4-[[4-(Aminoiminomethyl)phenyl]amino]-5-oxo-pentanoic acid (8a)

4-Aminobenzamidine dihydrochloride (1 g, 4.8 mmol) was added to dry dimethylformamide (20 mL). To this solution dry pyridine (5 mL) and glutaric anhydride (0.68 g, 5.3 mmol) followed by 10 mg dimethylaminopyridine (DMAP) were added. The product started to precipite after heating for 1/2 h at 100 °C. Heating was continued for 2 h and water (25 mL) was added after cooling to room temperature. An abundant precipitate was filtered, washed with water and ethyl acetate. The white solid was filtered and dried in a desiccator to give 0.8 g (50 %) of product as a white solid: ^{1}H NMR (DMSO) δ 1.95 (m, 2H), 2.4 (m, 2H), 2.5 (m, 2H), 7.85 (s, 4H), 9.05 (bs, 2H), 9.25 (bs, 2H), 10.4 (s, 1H); MS (FAB) m/z 250.1 (M + H$^+$).

4-[[4-(Aminoiminomethyl) phenyl] amino]-2(3)-methyl-4-oxo-butanoic acid (8b)

A procedure similar to that described for **8a** utilizing 5 g of 4-aminobenzamidine dihydrochloride and 2.85 g methyl succinic anhydride was used. The product was filtered, washed with water, acetonitrile and ether. The white solid (4.5 g) was suspended in dioxane, 4 N HCl in dioxane (100 mL) was added and the suspension was stirred for 1 h, filtered and dried. The resulting mixture of isomers was used without further purification.

trans-*2-[[[4-(Aminoiminoethyl) phenyl] amino] carbonyl]-cyclohexyl-carboxylic acid (8d)*

A mixture of 10 g *trans*-1,2-cyclohexane-dicarboxylic anhydride (0.065 mol), 13.7 g (0.065 mol) amino-benzamidine hydrochloride, 100 mL of pyridine and 100 mL of dimethylformamide was stirred at 100 °C for 3 h. The reaction mixture was concentrated *in vacuo*, brought to pH 7 with 0.5 N sodium hydroxide and water (total volume 200 mL). Upon cooling, a precipitate appeared that was filtered to give 14 g of desired product as a tan solid ^{1}H NMR (DMSO) δ 1.3 (bs, 4H), 1.75 (bs, 2H), 2.2 (bs, 2H), 2.5 (bs, 2H), 7.8 (s, 4H), 9.1 (bs, 2H), 9.2 (bs, 2H); 10.4 (s, 1H); MS (FAB) m/z 290.1 (M + H$^+$). The material was dissolved with aqueous HCl to obtain a solution of pH 2 which was lyophilized to a white powder.

4-[[4-(Aminoiminomethyl)phenyl]amino]-4-oxo-buten-(E)-oic acid (8e)

In a round bottomed flask under a static atmosphere of dry nitrogen were mixed 1.4 g of monoethyl fumarate, 1.36 g of isobutyl chloroformate and 1.01 g *N*-methylmorpholine in 100 mL dimethylformamide. 4-Aminobenzamidine dihydrochloride (2.06 g) and 2.02 g *N*-methylmorpholine were added at room temperature and the reaction mixture was stirred at 25 °C for 30 min. Water and sodium hydroxide were added to pH 10 and after 1 h stirring, neutralized to pH 7 to precipitate the zwitterion. Filtration provided 1 g of the desired compound as a white solid.

trans-*2-[[[4-(Aminoiminoethyl) phenyl] amino] carbonyl]-cyclopropyl-carboxylic acid (8f)*

To 6.0 g (0.038 mol) of *trans*-2-[ethoxycarbonyl]-cyclopropyl-carboxylic acid (**10**) dissolved in 100 mL anhydrous dimethylformamide and 10 mL anhydrous pyridine, were added 4.82 g (4.92 mL, 0.040 mol) trimethylacetyl chloride and a catalytic amount of DMAP. After about 1 h 9.49 g (0.046 mol) benzamidine hydrochloride was added and allowed to react under argon at room temperature overnight. The volatiles were removed under reduced pressure at 55 °C until a viscous oil was obtained. The residue was dissolved in water (100 mL) and the pH adjusted to 12 by addition of aqueous LiOH. After stirring overnight a precipitate formed. The pH was adjusted to 7 by addition of dilute aqueous HCl and the solids filtered and dried in a desiccator to give 5.0 g (53 %) of the desired zwitterion product. This material was converted to the hydrochloride salt by contacting with 4 N HCl in dioxane (100 mL) for several hours. The resulting solid was collected, washed with diethyl ether and dried. ^{1}H NMR (CD_3OD) δ 1.3 (m, 2H), 1.95 (m, 1H), 2.12 (m, 1H), 7.6 (m, 4H).

2-[Ethoxycarbonyl]cyclopropyl-carboxylic acid (10)

Diethylcyclopropylcarboxylate (50 g, 0268 mol; *trans* isomer from Aldrich) in 100 mL ethanol was added to a solution of 10 g LiOH (0.238 mole) in 100 mL water. After 5 min stirring, a yellow homogeneous mixture was observed and stirring continued for 24 h at 25 °C. The crude reaction mixture was partitioned between ethyl acetate and water (pH = 9). The aqueous layer was made acidic (pH 2 with conc. HCl) and extracted with ethyl acetate. The ethyl acetate extract was dried ($MgSO_4$) and concentrated to give 27 g of the desired mono acid as a solid (mp 46 °C).

β-[[N-(t-Butyloxycarbonyl) amino]-1,3-benzodioxole-5]-propanoic acid (12)

3,4-Methylenedioxybenzaldehyde (12.0 g; 80 mmol), malonic acid (10.5 g; 50 mmol) and ammonium acetate (8 g; 104 mmol) were gently refluxed in ethanol (600 mL) overnight. The reaction mixture was hot filtered and the solid was washed with ethanol/water (1:1; 3 × 100 mL). The product was air-dried to yield 8 g of white material. [FAB-MS: MH$^+$ = 210]. 3-Amino-[1,3-benzodioxole-5]propanoic acid (2.1 g; 10 mmol) was dissolved in 2.5 N NaOH (5 mL) and dioxane/water (2:1; 30 mL). To this mixture, di-*t*-butyldicarbonate (2.62 g; 12 mmol) was added with vigorous stirring. The reaction mixture was allowed to stir at room temperature overnight and taken down to dryness under reduced pressure. The residue was redissolved

in water (100 mL) and the solution was acidified with a dilute solution of $KHSO_4$. The white precipitate was then collected by filtration and dried *in vacuo* to yield 1.35 g of white solid. [FAB-MS: M+ Na = 332.]

Ethyl βS-amino-[1,3-benzodioxole-5]propanoate (13)

β-[[*N-t*-Butyloxycarbonylamido] -1,3-benzodioxole-5] propanoic acid (1.34 g; 4.34 mmol), disuccinimidylcarbonate (1.5 g; 6 mmol) and 4-dimethylaminopyridine (300 mg) were stirred in dimethylformamide/pyridine (2:1; 50 mL) at ambient temperature overnight. To this solution, (*R*)-2-amino-2-phenylethanol (1.15 g; 8 mmol) was added and the stirring was allowed to continue for another day. The mixture was taken down to dryness under reduced pressure and the residue was triturated with water. The solid was filtered and applied to a Waters Deltapak C-18 column (5 cm × 30 cm). The diastereomeric mixture was separated using an isocratic mixture of 35 % acetonitrile/water. Both 'early' and 'later' peaks were collected and lyophilized. Both compounds have the same mass ion of M+Li = 435.

2-[[β- *N-t*- Butyloxycarbonylamido -1,3- benzodioxole -5]-propanoylamido]-2-phenylethanol ('early peak', 0.62 g; 1 mmol) was suspended in conc. H_2SO_4 (2 mL) and dioxane/water (1:1; 20 mL). The mixture was refluxed for 16 h and taken down to dryness on rotavapor. The residue was redissolved in water (50 mL) and the mixture was titrated to pH 10 with 2.5 N NaOH and extracted with chloroform (3 × 75 mL). The aqueous phase was neutralized with 3 N HCl and taken down to dryness under reduced pressure. The solid was treated with ether and filtered to give 170 mg solid [FAB-MS: $M+H^+$ = 210]. This material was suspended in absolute ethanol (100 mL) and the suspension was cooled in an icebath and bubbled with HCl gas for 2 h. The mixture was stirred at room temperature overnight and filtered. The filtrate was taken down to dryness on rotavapor and the residue [240 mg; FAB-MS: $M+H^+$ = 238] was used without any further purification.

The other diastereoisomer ('early peak' of 2-[[β-*N-t*-butyloxycarbonylamido -1,3- benzodioxole-5] -propanoylamido]-2-phenylethanol, 0.42 g; 1.5 mmol) was suspended in conc. H_2SO_4 (2 mL) and dioxane/water (1:1; 20 mL). The mixture was refluxed for 16 h and worked up as described above. The acid, β*R*-amino-[1,3-benzodioxole-5]propanoic acid, [230 mg; FAB-MS: MH^+ = 210] was converted to its ethyl ester [165 mg; FAB-MS: $M+H^+$ = 238].

3-[[4-[[4-(Aminoiminomethyl) phenyl] amino]-1,4-dioxobutyl]amino]propionic acid (20)

A portion of ethyl-3-[[4-[[4-(aminoiminomethyl)phenyl]-amino]-1,4-dioxobutyl]amino]propanoate **21** (300 mg) was dissolved in 10 mL water. Sodium hydroxide (2 N) was added until the pH reached 10. The reaction mixture was allowed to stir at 25 °C for 30 min during which time a precipitate appeared. The mixture was acidified with HCl to pH 5. The precipitate was filtered, washed with water and diethyl ether and purified by RP-HPLC (acetonitrile/water) to give 50 mg of a white powder: ^1H NMR (DMSO) δ 2.4 (m, 4H), 2.6 (m, 2H), 3.25 (m, 2H), 7.8 (s, 4H), 8.0 (t, 1H, J = 7 Hz), 8.80 (br s, 2H), 9.18 (br s, 2H), 10.4 (s, 1H); MS (ES) m/z 307.1($M+H^+$).

Elemental analysis: required for $C_{19}H_{21}N_5O_4 \cdot F_3C_2O_2H \cdot 0.5H_2O$, C 44.86 H 4.47 N 13.08, found C 45.03 H 4.55 N 12.99.

Ethyl-3-[[4-[[4-(aminoiminomethyl) phenyl] amino]-1,4-dioxobutyl]amino]propanoate(21)

The hydrochloride **5** (1.36 g) was added to dry dimethylformamide (50 mL) followed by *N*-methyl morpholine (0.6 mL) and isobutyl chloroformate (0.65 mL) at 0 °C under nitrogen atmosphere. The mixture was stirred for 5 min, 0.45 g β-alanine ethyl ester hydrochloride was added, followed by 0.6 mL of *N*-methyl morpholine. After 4 h the solvent was removed under reduced pressure and the product was purified by reverse phase chromatography (water/acetonitrile) to give 4.4 g of white solid: ^1H NMR (DMSO) δ 1.2 (t, 3 H, J = 7 Hz), 2.45 (m, 4H), 2.6 (m, 2H), 3.25 (m, 2H), 4.05 (q, 2H, J = 7 Hz), 7.8 (s, 4H), 8.0 (m, 1H), 8.85 (br s, 2H), 9.18 (br s, 2H), 10.4 (s, 1H); MS (ES) m/z 335.1 ($M+H^+$).

Elemental analysis: required for $C_{16}H_{22}N_4O_4 \cdot 1.5F_3C_2O_2H \cdot 0.5H_2O$, C 43.21 H 4.53 N 11.20, found C 43.56 H 4.70 N 11.07.

D,L-3-[[4-[[4-(Aminoiminomethyl) phenyl] amino]-1,4-dioxobutyl]amino]-butanoic acid (22)

The hydrochloride **5** (2 g) was added to dry dimethylformamide (65 mL) followed by *N*-methyl morpholine (0.75 g, 1 eq.) and isobutyl chloroformate (1 g) at 25 °C. The mixture was stirred for 5 min. 3-Amino butyric acid (1.1 g, 1.1 eq.) was added followed by triethylamine (1.5 g; 1.3 eq.) and dimethylaminopyridine. After 1 h the solvent was removed under reduced pressure and the product was purified by reverse phase chromatography (water/acetonitrile) to give 750 mg of white solid: ^1H NMR (DMSO) δ 1.06 (d, 3H, J = 7 Hz), 2.2–2.6 (m, 6H), 4.05 (m, 1H), 7.8 (m, 4H), 7.85 (d, 1H, J = 8 Hz), 9.05 (br s, 2H), 9.15 (br s, 2H), 10.4 (s, 1H); MS (FAB) m/z 321.1($M+H^+$), 236.

Elemental analysis: required for $C_{15}H_{20}N_4O_4 \cdot F_3C_2O_2H \cdot 0.75H_2O$, C 45.66 H 4.90 N 12.52, found C 45.54 H 4.27 N 12.41.

D,L-Ethyl-3-[[4-[[4-(aminoiminomethyl)phenyl]amino]-1,4-dioxobutyl]amino]-butanoate (23)

The hydrochloride **5** (5 g, 18 mmol) was added to dry dimethylformamide (100 mL) followed by *N*-methyl morpholine (2.2 g, 22 mmol) and isobutyl chloroformate (2.8 g, 22 mmol) at 25 °C. The mixture was stirred for 5 min. Ethyl 3-amino butyrate (2.5 g, 22 mmol) was added followed by dimethylaminopyridine. After 1 h the solvent was removed under reduced pressure and the product was purified by reverse phase chromatography (water/acetonitrile) to give 4.4 g of white solid: ^1H NMR

(DMSO) δ 1.06 (d, 3H, J = 7 Hz), 1.2 (t, 3H, J = 7 Hz), 2.3–2.6 (m, 6H), 4.05 (m, 3H), 7.8 (s, 4H), 7.9 (d, 1H, J = 8 Hz), 9.1 (br s, 2H), 9.2 (br s, 2H), 10.4 (s, 1H); MS (FAB) m/z 349.2 ($M+H^+$), 321, 218.

Elemental analysis: required for $C_{17}H_{24}N_4O_4 \cdot F_3C_2O_2H$, C 49.35 H 5.44 N 12.11, found C. 49.18 H 5.44 N 11.98.

D,L-3-[[4-[[4-(Aminoiminomethyl) phenyl] amino]-1,4-dioxobutyl]amino]-3-phenylpropionic acid (24)

The hydrochloride (**5**) (1 g, 3.7 mmol) was added to dry dimethylformamide (35 mL) followed by *N*-methylmorpholine (0.39 g, 1 eq.) and isobutyl chloroformate (0.53 g, 3.9 mmol) at 25 °C. The mixture was stirred for 5 min. D,L-3-Amino-3-phenylpropionic acid (0.67 g, 4.05 mmol) was added followed by diisopropylethylamine (0.68 mL; 3.9 mmol) and a catalytic amount of dimethylaminopyridine. After 1 h the solvent was removed under reduced pressure and the product was purified by reverse phase chromatography (water/acetonitrile) and lyophilized to give 340 mg of white solid: ^{1}H NMR (DMSO) δ 2.45 (m, 2H), 2.6 (m, 2H), 2.7 (d, 2H, J = 7 Hz), 4.2 (dd, 1H, J = 7 Hz and 8 Hz), 7.3 (m, 4H), 7.8 (s, 4H), 8.45 (d, 1H, J = 8 Hz), 9.0 (br s, 2H), 9.2 (br s, 2H), 10.4 (s, 1H); MS (FAB) m/z 383.2 ($M+H^+$).

Elemental analysis: required for $C_{20}H_{22}N_4O_4 \cdot F_3C_2O_2H \cdot H_2O$, C 51.36 H 4.90 N 10.90, found C 51.67 H 4.74 N 10.72.

D,L-Ethyl-3-[[4-[[4-(aminoiminomethyl)phenyl]amino]-1,4-dioxobutyl]amino]-3-phenylpropanoate (25)

In a round bottomed flask under a static atmosphere of dry nitrogen were mixed 1.3 g of D,L-3-[[4-[[4-(aminoiminomethyl)phenyl]amino]-1,4-dioxobutyl]amino]-3-phenylpropionic acid (**24**) 200 mL absolute ethanol and 10 mL 4 N HCl in dioxane. The reaction mixture was stirred at 25 °C for 16 h. The volatiles were removed *in vacuo* and the remaining white solid was purified by RP-HPLC (water/acetonitrile gradient; 95:5 to 30:70 over 30 min) to provide 0.62 g of the desired ester as a white solid: ^{1}H NMR (DMSO) δ 1.1 (t, 3 H, J = 7 Hz), 2.45 (m, 2H), 2.6 (m, 2H), 2.75 (d, 2H, J = 7 Hz), 4.0 (q, 2H, J = 7 Hz), 4.2 (dd, 1H, J = 7 Hz and 8 Hz), 7.3 (m, 4H), 7.8 (s, 4H), 8.45 (d, 1H, J = 8 Hz), 9.05 (br s, 2H), 9.2 (br s, 2H), 10.4 (s, 1H); MS (FAB) m/z 411.2 ($M+H^+$), 135.2.

Elemental analysis: required for $C_{22}H_{26}N_4O_4 \cdot F_3C_2O_2H \cdot H_2O$, C 53.13 H 5.41 N 10.24, found C 53.13 H 5.39 N 10.33.

D,L-β-[[4-[[4-(Aminoiminomethyl) phenyl] amino]-1,4-dioxobutyl]amino]-3-pyridinepropanoic acid (29)

A portion of ethyl-β-[[4-[[4-(aminoiminomethyl)phenyl]-amino]-1,4-dioxobutyl]amino]-3-pyridinepropanoate **30** (550 mg of the trifluoroacetate salt) prepared as below was dissolved in 50 mL water and 300 μL of 50 % sodium hydroxide. The reaction mixture was allowed to stir at 25 °C for 2 h and was purified by RP-HPLC (acetonitrile/-water) to give 500 mg of a white powder: ^{1}H NMR (DMSO) δ 2.45 (m, 2H), 2.6 (m, 2H), 2.8 (m, 2H), 5.24 (m, 1H), 7.65 (m, 1H), 7.77 (m, 4H), 8.13 (m, 1H), 8.25 (m, 2H), 8.72 (br s, 1H), 9.18 (br s, 2H), 10.4 (s, 1H); MS (ES) m/z 384.1 ($M+H^+$).

Elemental analysis: required for $C_{14}H_{17}N_4O_4 \cdot 2\ F_3C_2O_2H \cdot 0.5H_2O$, C 44.52 H 3.90 N 11.28, found C 44.59 H 3.79 N 11.24.

D,L-Ethyl-β-[[4-[[4-(aminoiminomethyl)phenyl]amino]-1,4-dioxobutyl]amino]-3-pyridinepropanoate (30)

The hydrochloride **5** (2.35 g) was added to dry dimethylformamide (100 mL) followed by *N*-methyl morpholine (1.10 mL) and isobutyl chloroformate (1.30 mL) at 0 °C under nitrogen atmosphere. The mixture was stirred for 5 min and 3.56 g of β-amino-3-pyridinepropionic acid ethyl ester dihydrochloride (75 % pure) were added followed by 2.1 mL of *N*-methyl morpholine. After 1 h the solvent was removed under reduced pressure and the product was purified by reverse phase chromatography (water/acetonitrile) to give 4.4 g of white solid : ^{1}H NMR (DMSO) δ 1.12 (t, 3 H, J = 7 Hz), 2.45 (m, 2H), 2.6 (m, 2H), 2.85 (m, 2H), 4.05 (q, 2H, J = 7 Hz), 5.28 (m, 1H), 7.55 (m, 1H), 7.8 (s, 4H), 8.0 (m, 1H), 8.6 (m, 3H), 8.85 (br s, 2H), 9.18 (br s, 2H), 10.4 (s, 1H); MS (ES) m/z 412.1($M+H^+$), 277, 235, 218.

Elemental analysis: required for $C_{21}H_{25}N_5O_4 \cdot 2\ F_3C_2O_2H \cdot H_2O$, C 45.67 H 4.45 N 10.65, found C 45.83 H 4.31 N 10.63.

3-[[4-[[4-(Aminoiminomethyl) phenyl] amino]-1,4-dioxobuten-(Z)-yl]amino]-phenylpropanoic acid (54)

The compound was prepared from maleic anhydride, aminobenzamidine and 3-amino phenylpropionic acid in a manner similar to that described for **24**.

^{1}H NMR (DMSO) δ 2.45 (m, 2H), 3.6 (m, 2H), 7.05 (m, 2H), 7.85 (m, 4H), 8.6 (m, 1H), 8.9 (br s, 2H), 9.1 (d, 1H, J = 7 Hz), 9.25 (br s, 2H), 10.85 (s, 1H); MS (ES) m/z 381.2 ($M+H^+$), 216.

Elemental analysis: required for $C_{20}H_{20}N_4O_4.F_3C_2O_2H.1.5H_2O$, C 50.67 H 4.64 N 10.74, found C 50.28 H 4.15 N 10.63.

D,L-3-[[4-[[4-(aminoiminomethyl)phenyl]amino]-1,5-dioxopentyl]amino]-3-phenylpropionic acid (50)

The hydrochloride **8a** (1g, 3.5 mmol) was dissolved in dry dimethylformamide (35 mL) and *N*-methyl morpholine (0.39 g, 1 eq.) and isobutyl chloroformate (0.5 g) were added to the mixture cooled to 0 °C. The mixture was stirred for 5 min. D,L-3-Amino-3-phenyl propionic acid (0.58 g) was added followed by a catalytic amount of dimethylaminopyridine. After 1 h the solvent was removed under reduced pressure and the product was purified by reverse phase chromatography (water/acetonitrile) to give 440 mg of white solid: ^{1}H NMR (DMSO) δ 1.80 (m, 2H),

2.18 (t, 2H, J = 7 Hz), 2.4 (t, 2H, J = 7 Hz), 2.65 (d, 2H, J = 7 Hz), 4.2 (dd, 1H, J = 7 Hz and 8 Hz), 7.3 (m, 4H), 7.8 (s, 4H), 8.35 (d, 1H, J = 8 Hz), 8.95 (br s, 2H), 9.18 (br s, 2H), 10.34 (s, 1H); MS (FAB) m/z 397.2 ($M+H^+$), 351, 232.

Elemental analysis: required for $C_{21}H_{24}N_4O_4 \cdot F_3C_2O_2H \cdot H_2O$, C 52.27 H 5.15 N 10.60, found C 52.19 H 5.12 N 10.38.

Ethyl-3-[[4-[[4-(aminoiminomethyl) phenyl] amino]-1,4-dioxobuten-(E)-yl]amino]-propanoate (52)

The intermediate **8e** (1.35 g) was added to dry dimethylformamide (50 mL) followed by *N*-methyl morpholine (0.55 mL) and isobutyl chloroformate (0.65 mL) under nitrogen atmosphere. The mixture was stirred for 5 min and 0.75 g of β-alanine ethyl ester hydrochloride was added followed by 0.55 mL of *N*-methyl morpholine. After 2 h the solvents were removed under reduced pressure and the product was purified by reverse phase chromatography (water/acetonitrile) to give 700 mg of white solid.

3- [[4- [[4- (aminoiminomethyl) phenyl] amino] -1,4-dioxobuten-(E)-yl]amino]-propanoic acid (51)

A portion of ethyl 3-[[4-[[4-(aminoiminomethyl)phenyl]-amino]-1,4-dioxobuten-(Z)-yl]amino]-propanoate (**52**) (150 mg of the trifluoroacetate salt) was dissolved in 10 mL water and 10 mL acetonitrile and five drops of 50 % sodium hydroxide were added. The reaction mixture was stirred at 25 °C for 1 h and was acidified to pH 5 with TFA. The precipitate was collected by filtration, washed with acetonitrile, water, and diethylether to give 120 mg of a white powder which was lyophilized from HCl to give the hydrochloride salt: ^{1}H NMR (DMSO) δ 2.45 (m, 2H), 3.6 (m, 2H), 6.0 (m, 2H), 7.85 (m, 4H), 8.63 (m, 1H), 8.85 (br s, 2H), 9.20 (br s, 2H), 10.9 (s, 1H); MS (ES) m/z 333.1 ($M+H^+$).

Elemental analysis: required for $C_{14}H_{17}N_4O_4 \cdot 1.5\ F_3C_2O_2H \cdot 0.5H_2O$, C 41.39 H 3.98 N 11.36, found C 41.55 H 3.83 N11.72.

D,L-3-[[4-[[4-(aminoiminomethyl) phenyl] amino]-2(3)-methyl-1,4-dioxobutyl]amino]-3-phenylpropionic acid (55)

The intermediate **8b** (1.68 g, 5.8 mmol) was activated with isobutyl chloroformate (0.78 mL, 6 mmol) and coupled with D,L-3-amino-3-phenylpropionic acid (0.96 g, 5.8 mmol) in a manner similar to **24**. After usual work up, the reaction mixture was purified by reverse phase chromatography (water/acetonitrile). Two peaks (A and B) were isolated and lyophilized. Peak A gave 340 mg of yellow solid: ^{1}H NMR (DMSO) δ 1.0 (m, 3H); 2.4–2.6 (m, 4H), 2.8 (m, 2H), 4.1 (m, 1H), 7.15 (m, 4H), 7.7 (s, 4H), 8.45 (m, 1H), 9.0 (br s, 2H), 9.2 (br s, 2H), 10.4 (d, 1H, J = 8 Hz); MS (FAB) m/z 397.3 ($M+H^+$).

Elemental analysis: required for $C_{23}H_{27}N_4O_7 \cdot F_3C_2O_2H \cdot 1.5H_2O$, C 51.40 H 5.25 N 10.42, found C 51.69 H 4.86 N 10.38.

Peak B isolated from the crude reaction mixture gave 540 mg of white solid: ^{1}H NMR (DMSO) δ 1.05 (m, 3H); 2.2–2.6 (m, 5H), 2.85 (m, 1H), 5.15 (m, 1H), 7.2 (m, 1H), 7.25 (s, 4H), 7.8 (s, 4H), 8.45 (m, 1H), 8.75 (br s, 2H), 9.15 (br s, 2H), 10.4 (br s, 1H); MS (FAB) m/z 397.3 ($M+H^+$).

Elemental analysis: required for $C_{23}H_{27}N_4O_7 \cdot F_3C_2O_2H \cdot H_2O$, C 52.27 H 5.15 N 10.78, found C 52.08 H 4.84 N 10.45.

βR-[[4-[[4-(Aminoiminoethyl) phenyl] amino]-1,4-dioxobutyl]amino]-3-pyridinepropanoic acid (32)

The intermediate **5** (465 mg, 1.6 mmol) was added to dry dimethylformamide (50 mL) followed by *N*-methyl morpholine (0.18 mL) and isobutyl chloroformate (0.22 mL) at 25 °C. The mixture was stirred for 5 min. To β*R*-amino-3-pyridinepropionic acid ethyl ester ditrifluoroacetate (650 mg) in solution in a mixture of 50 mL dimethylformamide, 0.6 mL *N*-methyl morpholine was added at once. After 1 h at room temperature, water was added and the reaction concentrated *in vacuo*. The residue was purified by reverse phase chromatography (water/acetonitrile) to give 350 mg of white solid: ^{1}H NMR (DMSO) δ 1.1 (t, 3H, J = 7 Hz), 1.7 (s, 3H), 2.4 (m, 2H), 2.5 (m, 4H), 2.8 (d, 1H, J = 7 Hz), 3.95 (q, 2H, J = 7 Hz), 5.2 (m, 1H), 7.3 (m, 1H), 7.7 (m, 4H), 8.4 (dd, 1H, J_1 = 8 Hz and J_2 = 2.5 Hz), 8.5 (d, 1H, J = 2.5 Hz), 8.55 (d, 1H, J = 8 Hz). $[\alpha]_{25}^{Na}$: +1.4 ° (*c* 0.5; water).

A portion (100 mg) of the ester isolated above was dissolved in water and 2 N aqueous LiOH was added to pH 12. After stirring 1 h at 25 °C, the resulting mixture was purified by reverse phase high presure chromatography (R_t = 8 min on linear gradient 5–70 % acetonitrile in water over 30 min). After lyophilization, 90 mg of white solid was isolated: ^{1}H NMR (DMSO) δ 2.5 (m, 2H), 2.55 (m, 2H), 2.8 (m, 2H), 5.25 (m, 1H), 7.40 (m, 2H), 7.5 (br s, 1H), 7.75 (s, 4H), 7.95 (br s, 1H), 8.15 (m, 2H), 8.6 (m, 2H), 8.95 (br s, 2H), 9.15 (br s, 2H), 10.45 (s, 1H); MS (FAB) m/z 384 ($M+H^+$). $[\alpha]_{25}^{Na}$: +1.24 ° (*c* 1.0 , DMSO).

Elemental analysis: required for $C_{20}H_{23}N_5O_4 \cdot 2F_3C_2O_2H \cdot H_2O$, C 43.88 H 3.45 N 11.12, found C 43.53 H 3.99 N 10.82.

βS-[[4-[[4-(Aminoiminomethyl)phenyl]amino]-1,4-dioxobutyl]amino]-1,3-benzodioxole-5-propanoic acid (41)

The intermediate **5** (540 mg; 2 mmol) was dissolved in dimethylformamide (10 mL). Isobutylchloroformate (275 mg; 2 mmol) was added dropwise with stirring followed by *N*-methyl morpholine (200 mg; 2 mmol). In a separate flask, ethyl β*S*-amino-1,3-benzodioxole-5-propanoate. HCl (**13**, 165 mg, 1 mmol) and *N,N*-diisopropyl-*N*-ethylamine (130 mg; 1 mmol) were dissolved in dimethylformamide (10 mL). Both solutions were combined and stirred at room temperature for 2 h. Saturated sodium bicarbonate solution (5 mL) was added with stirring and the mixture was filtered. The filtrate was taken down to dryness on rotavapor. The

remaining residue was purified on a DeltaPak column (30 cm × 5 cm) using a linear gradient of 10–40 % acetonitrile/water/0.05 % TFA in 30 min. FAB-MS: $(M+H^+ = 455$. ^1H NMR (DMSO-d_6) δ 1.11 (t, 3H, $J = 7$ Hz), 2.45–2.57 (m, 4H), 2.69 (m, 2H), 4.0 (q, 2H, $J = 7$ Hz), 5.13 (m, 1H), 5.97 (s, 2H), 6.8–6.9 (m, 3H), 7.79 (s, 4H), 8.42 (d, 1H, $J = 8$ Hz), 9.0 (br s, 2H), 9.24 (br s, 2H).

Elemental analysis: required for $C_{23}H_{26}N_4O_6 \cdot HCl \cdot H_2O$, C 54.49 H 5.76 N 11.05, found C 54.39 H 5.49 N 11.01.

Ethyl β*S*-[[4-[[4-(aminoiminomethyl)phenyl]amino]-1,4-dioxobutyl]amino]-1,3-benzodioxole-5-propanoate (100 mg) was stirred in 2 N LiOH (5 mL) and methanol (5 mL) at room temperature for 20 min. The mixture was neutralized with 4 N HCl and diluted with water (20 mL). This material was then purified on a DeltaPak Column (30 cm × 5 cm) using a gradient of 10–40 % acetonitrile/water/0.05 % TFA in 30 min. FAB-MS: $(M+H)^+ = 427$. ^1H NMR (DMSO-d_6) δ 2.45 (m, 2H), 2.57 (m, 2H), 2.60 (m, 2H), 5.08 (m, 1H), 5.97 (s, 2H), 6.8–6.9 (br s, 3H), 7.77 (s, 4H), 8.4 (d, 1H, $J = 7$ Hz), 8.97 (br s, 2H), 9.15 (s, 2H).

Elemental analysis: required for $C_{21}H_{22}N_4O_6 \cdot HCl \cdot H_2O$, C 52.45 H 5.24 N 11.65, found C 51.62 H 4.89 N 11.35.

βR-[[4-[[4-(Aminoiminomethyl)phenyl]amino]-1,4-dioxobutyl]amino]-1,3-benzodioxole-5-propanoic acid [40]

The ester was prepared and purified according to the procedure described above from the enantiomer of **13** prepared from the 'later peak' [FAB-MS: $MH^+ = 455$). This ester (50 mg) was treated with 2 N LiOH (5 mL) and methanol (5 mL) at room temperature for 20 min. The mixture was neutralized with 4 N HCl and diluted with water (20 mL). This material was then purified on a DeltaPak Column (30 cm × 5 cm) using a gradient of 10–40 % acetonitrile/water/0.05 % TFA in 30 min. FAB-MS: $M+H^+ = 427$. ^1H NMR (DMSO-d6) δ 2.45 (m, 2H), 2.57 (m, 2H), 2.60 (m, 2H), 5.08 (m, 1H), 5.97 (s, 2H), 6.78–6.9 (m, 3H), 7.77 (s, 4H, Ar), 8.38 (d, 1H, $J = 8$ Hz), 8.88 (br s, 2H), and 9.42 (br s, 2H).

trans-β(S)-[[[2-[[[4-(Aminoiminoethyl) phenyl] amino]-carbonyl]cyclopropyl]carbonyl]amino]-3-pyridinepropanoic acid, bis(trifluoroacetate) (59)

The acid **8f** (210 mg; 0.7 mmol) was coupled to 420 mg of β-(*S*)-amino-3-pyridinepropionic acid ethyl ester ditrifluoroacetate using the mixed anhydride procedure. After concentration *in vacuo*, the residue (800 mg brown oil) was purified by HPLC (water/acetonitrile/0.05 %TFA). Two products, one eluting at 12 min and the second at 13.2 min (R_t on a linear water/acetonitrile 5:95–70:30 over 25 min), were collected which after lyophilization provided, respectively, 120 mg and 100 mg of white solids.

The ester (isomer 1, R_t 12 min, 120 mg) isolated above was dissolved in 10 mL water and LiOH was added to pH 12. The reaction was stirred for 2 h at 25 °C. After acidification to pH 4 with TFA, the reaction mixture was purified by HPLC. The main peak (R_t 10.3 min) was collected and lyophilized to 64 mg of white powder: MS (FAB) m/z 396.4$(M+H^+)$; ^1H NMR 500 MHz (DMSO) δ 1.16 (m, 2 H), 1.25 (m, 2H), 2.16 (m, 1H), 2.26 (m, 1H), 2.80 (m, 2H), 5.20 (m, 1H), 7.5 (m, 1H), 7.75 (m, 4H), 7.95 (m, 1H), 8.5 (m, 1H), 8.6 (s, 1H), 8.9 (br s, 2H), 8.96 (d, 1H, $J = 8$ Hz), 9.2 (br s, 2 H), 10.8 (s, 1H); $[\alpha]_{25}^{Na} = -89.2°$ (c 1.1; water).

trans-β(S)-[[[2-[[[4-(aminoiminoethyl) phenyl] amino]-carbonyl]cyclopropyl]carbonyl]amino]-3-pyridinepropanoic acid, bis(trifluoroacetate) isomer 2 (60)

The second ester (isomer 2, R_t 13.2 min, 100 mg) isolated above was dissolved in 10 mL water and LiOH was added to pH 12. The reaction was stirred for 2 h at 25 °C. After acidification to pH 4 with TFA, the reaction mixture was purified by HPLC. The main peak (R_t 10.4 min) was collected and lyophilized to 64 mg of white powder: MS (FAB) m/z 396.4$(M+H^+)$; ^1H NMR 500 MHz (DMSO) δ 1.16 (m, 2 H), 1.25 (m, 2H), 2.16 (m, 2H), 2.80 (m, 2H), 5.20 (m, 1H), 7.5 (m, 1H), 7.75 (m, 4H), 7.95 (m, 1H), 8.55 (m, 1H), 8.6 (s, 1H), 8.9 (br s, 2H), 8.96 (d, 1H, $J =$ 8 Hz), 9.12 (br s, 2 H), 10.78 (s, 1H); $[\alpha]_{25}^{Na} = 112.5°$ (c 0.9; water).

Elemental analysis: required for $C_{24}H_{31}N_5O_9F_6$, C 44.52 H 4.83 N 10.82, found C 44.66 H 4.59 N 10.77.

trans-β(R)-[[[2-[[[4-(Aminoiminoethyl) phenyl] amino]-carbonyl]cyclopropyl]carbonyl]amino]-3-pyridinepropanoic acid, bis(trifluoroacetate) isomer 1 (57)

The acid **8f** (283 mg; 1.1 mmol) was coupled to 420 mg of β-(*R*)-amino-3-pyridinepropionic acid ethyl ester ditrifluoroacetate using the mixed anhydride procedure. After concentration *in vacuo*, the residue (800 mg brownish oil) was purified by HPLC (water/acetonitrile/0.05 %TFA). Two products, one eluting at 12 min and the second at 13.2 min (R_t on a linear water/acetonitrile 5:95–70:30 over 25 min), were collected which after lyophilization provided, respectively, 300 mg and 100 mg of white solids.

The ester (isomer 1, R_t 12 min, 120 mg) was dissolved in 10 mL water and LiOH was added to pH 12. The reaction was stirred for 2 h at 25 °C. After acidification to pH 4 with TFA, the reaction mixture was purified by HPLC. The main peak (R_t 10.3 min) was collected and lyophilized to 40 mg of white powder: MS (FAB) m/z 396.4$(M+H^+)$; ^1H NMR 500 MHz (DMSO) δ 1.16 (m, 2 H); 1.25 (m, 2H), 2.16 (m, 1H), 2.26 (m, 1H), 2.80 (m, 2H), 5.20 (m, 1H), 7.5 (m, 1H), 7.75 (m, 4H), 7.95 (m, 1H), 8.5 (m, 1H), 8.6 (s, 1H), 8.9 (br s, 2H), 8.96 (d, 1H, $J = 8$ Hz), 9.2 (br s, 2 H), 10.8 (s, 1H).

β-(R)-[[[2-[[[4-(Aminoiminoethyl) phenyl]· amino]-carbonyl]cyclopropyl]carbonyl]amino]-3-pyridinepropanoic acid, bis(trifluoroacetate) isomer 2 (58).

The second ester (isomer 2, R_t 13.2 min, 100 mg) isolated above was dissolved in 10 mL water and LiOH was added

to pH 12. The reaction was stirred for 2 h at 25 °C. After acidification to pH 4 with TFA, the reaction mixture was purified by HPLC. The main peak (R_t 10.4 min) was collected and lyophilized to 55 mg of white powder: MS (FAB) m/z 396.4($M+H^+$); ^1H NMR 500 MHz (DMSO) δ 1.16 (m, 2 H), 1.25 (m, 2H), 2.16 (m, 2H), 2.80 (m, 2H), 5.20 (m, 1H), 7.5 (m, 1H), 7.75 (m, 4H), 7.95 (m, 1H), 8.55 (m, 1H), 8.6 (s, 1H), 8.9 (br s, 2H), 8.96 (d, 1H, J = 8 Hz), 9.12 (br s, 2 H), 10.78 (s, 1H); $[\alpha]_{25}^{Na} = -110.4°$ (*c* 1.0; water).

Biology: in vitro *platelet aggregation in PRP*

Healthy male or female dogs were fasted for 8 h and 30 mL whole blood was collected and mixed with 3 mL of 0.13 M buffered sodium citrate (3.8 %). Platelet-rich plasma (PRP) was prepared by centrifugation at 975 *g* for 3 min at room temperature. The PRP was collected and stored at room temperature. Platelet poor plasma (PPP) was prepared by centrifuging the remaining blood at 2000 *g* for 15 min at room temperature. The PRP was adjusted with PPP to a count of 2–3 × 108 platelets per mL. The PRP preparation (400 μL) and 50 μL of the test compounds solution or saline were preincubated for 1 min at 37 °C. Adenosine-5'-diphosphate (ADP, 50 μM final concentration) was added to the cuvettes and the aggregation monitored for 1 min. All compounds were tested in duplicate. IC_{50}'s (dosage at which 50 % of platelet aggregation is inhibited) were calculated by linear regression of the dose–response curve.

Inhibition of ex vivo *collagen induced aggregation*

Pretreatment (control) blood samples were drawn from either conscious or anesthetized dogs (beagles) and centrifuged to prepare platelet rich plasma (PRP). Aggregatory response to collagen was measured in an aggregometer and used as control. Compounds were administered, either intragastrically (by capsule or stomach tubes) or intravenously. Blood samples were drawn at predetermined intervals (every 15 min during the first hour, every 30 min during the next 2 h and every hour after that up to 7 h after compound administration), PRP prepared and aggregation to collagen determined. Compound inhibition of aggregation was determined by comparing the aggregation response after compound administration to the pretreatment response. The 100 % inhibition level was usually reached in the first hour following administration and remained at that level for a variable period of time depending upon the potency, bioavailability and metabolic stability of the compound.[27] The study was continued for a maximum of 7 h or until the platelet aggregation returned to control levels. If aggregation was still inhibited after 7 h, a blood sample was drawn the following morning and tested. Duration of activity was determined by the length of time platelet aggregation is inhibited after compound administration.

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(*Received* 20 *June* 1994)