Expedited Articles

Potent Noncovalent Thrombin Inhibitors That Utilize the Unique Amino Acid D-Dicyclohexylalanine in the P3 Position. Implications on Oral Bioavailability and Antithrombotic Efficacy

Thomas J. Tucker,*[,†] William C. Lumma,[†] S. Dale Lewis,[‡] Stephen J. Gardell,[‡] Bobby J. Lucas,[‡] Elizabeth P. Baskin,[§] Richard Woltmann,[§] Joseph J. Lynch,[§] Elizabeth A. Lyle,[§] Sandra D. Appleby,[§] I-Wu Chen,[∥] Kimberley B. Dancheck,[∥] and Joseph P. Vacca[†]

Departments of Medicinal Chemistry, Biological Chemistry, General Pharmacology, and Drug Metabolism, Merck Research Laboratories, West Point, Pennsylvania 19486

Received March 11, 1997[⊗]

In an effort to prepare orally bioavailable analogs of our previously reported thrombin inhibitor **1**, we have synthesized a series of compounds that utilize the unique amino acid D-dicyclohexylalanine as a P3 ligand. The resulting compounds are extremely potent and selective thrombin inhibitors, and the *N*-terminal Boc derivative **8** exhibited excellent oral bioavailability and pharmacokinetics in both rats and dogs. The des-Boc analog **6** was not orally bioavailable in rats. The high level of oral bioavailability observed with **8** appears to be a direct function of its increased lipophilicity versus other close analogs. Although increased lipophilicity may serve to increase the oral absorption of tripeptide thrombin inhibitors, it also appears to have detrimental effects on the antithrombotic properties observed with the compounds. Compound **6** performed extremely well in our in vivo antithrombotic assay, while the much more lipophilic but essentially equipotent analog **8** performed poorly. We have found that in general with this series of thrombin inhibitors as well as with other unreported series, increased lipophilicity and the associated increases in plasma protein binding have detrimental effects on 2X APTT values and subsequent performance in in vivo antithrombotic models.

Introduction

Thrombin is a trypsin-like serine protease that plays a pivotal role in the blood coagulation cascade. In an attempt to develop orally bioavailable antithrombotic agents, we have focused on the design and synthesis of novel thrombin inhibitors. Recently, we reported a series of potent thrombin inhibitors including compound **1**[1] (Figure 1). While these compounds were extremely potent and selective inhibitors of thrombin, they displayed poor oral bioavailability. Compound **1** is an extremely polar molecule, possessing a log *P* value of −0.539. The highly polar nature of **1** appears to be a major obstacle to absorption of the compound after oral dosing. In an effort to synthesize orally bioavailable derivatives of these earlier inhibitors by increasing their log *P* values, we have utilized the unique amino acid D-dicyclohexylalanine as a novel P3 group (Figure 1).[2] In this paper, we detail the results of these investigations and report a series of potent and orally bioavailable tripeptide thrombin inhibitors.

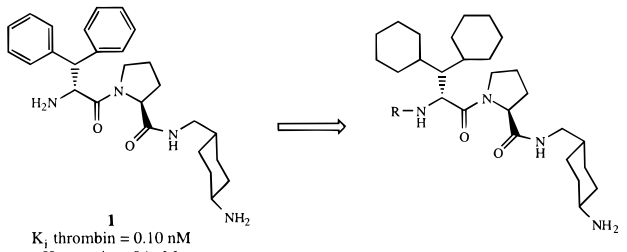


Figure 1.

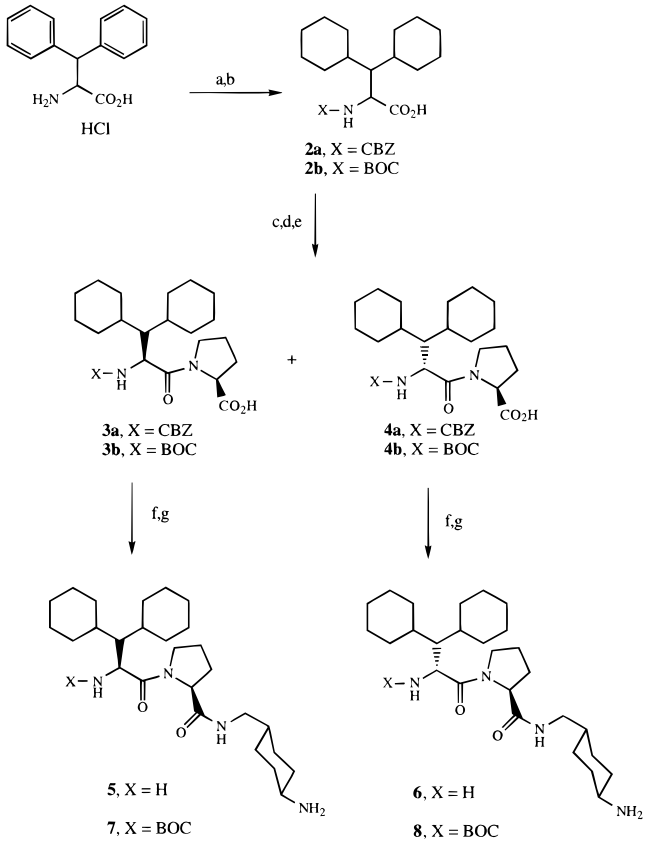
Chemistry

Since D-diphenylalanine is well tolerated by the S3 lipophilic binding pocket of the thrombin active site, we theorized that the fully reduced version of this amino acid should also be well tolerated. The increased lipophilicity of this compound could possibly enhance its absorption from the gut after oral dosing. The starting amino acid dicyclohexylalanine was prepared initially in its racemic form via catalytic reduction of racemic diphenylalanine[3] (Scheme 1). We found iridium black to be the most efficient catalyst for effecting this reduction at normal Parr apparatus pressures (approximately 60 psi). The CBZ-protected racemic amino acid **2a** was coupled to proline methyl ester using standard coupling conditions. Saponification, followed by reversed phase preparatory HPLC, provided the clean diastereomers **3a** and **4a**. Compounds **3a** and **4a** were each coupled to CBZ-protected *trans*-4-(aminomethyl)cyclohexylamine,[4] and subsequent deprotection provided the pure product diastereomers **5** and **6**. Stereochemical assignments were based on the results of the thrombin inhibition assay, in which compound **6** was shown to be the more potent inhibitor of the two and was thus assigned the (*R*) stereochemistry at the P3 asymmetric center. When D-diphenylalanine became commercially available,[3] the sequence was successfully

[†] Department of Medicinal Chemistry.
[‡] Department of Biological Chemistry.
[§] Department of General Pharmacology.
[∥] Department of Drug Metabolism.
[⊗] Abstract published in *Advance ACS Abstracts,* May 15, 1997.

S0022-2623(97)00140-4 CCC: $14.00

Scheme 1



[a] Reagents: (a) H_2 (60 PSI)/Ir black catalyst, AcOH/H_2O, 48 h; (b) CBZ-Cl, 2 N NaOH where X = CBZ or di-(*tert*-butyldicarbonate, 1 N NaOH/H_2O/dioxane, 0 °C where X = BOC; (c) EDC/HOBT/N $(Et)_3$/L-proline methyl ester hydrochloride, DMF; (d) 1 M LiOH/DME; (e) reversed phase (C_{18}) prep HPLC separation of diastereomers; (f) EDC/HOBT/N $(Et)_3$/*trans*-(4-*N*-CBZ-aminocyclohexyl)-methylamine, DMF; (g) H_2 (60 PSI)/Pd $(OH)_2$ catalyst, EtOH/H_2O/AcOH, 95:5:1.

Table 1. Thrombin Inhibition Activity[4] for Compounds and **1** and **5**–**8**

compd no.	K_i (nM)	compd no.	K_i (nM)
1	0.10	**7**	3000
5	440	**8**	0.10
6	0.056		

repeated on this material, with no racemization observed throughout the sequence. Results from this work were in agreement with the earlier assignments. The inhibitors which employed a Boc group at the *N*-terminus were prepared via an identical synthetic sequence, in which the racemic Boc-protected amino acid **2b** was used as the starting material. Stereochemical assignments were again based on the results of the thrombin inhibition assay, with compound **8** assigned the (*R*) stereochemistry at the P3 asymmetric center.

Results and Discussion

Compounds containing D-dicyclohexylalanine in the P3 position displayed inhibitory potency similar to that of their D-diphenylalanine analog, compound **1** (Table 1).[1] Compound **6** was evaluated for its selectivity against various human serine proteases and was shown to posess excellent selectivity versus all enzymes tested (Table 2).[5] log *P* values for these inhibitors were increased substantially as anticipated, rising above zero and into a region where reasonable oral absorption might be expected (Table 3). Compounds **6** and **8** were initially evaluated for oral bioavailabily in rats, and the results are summarized in Table 3. Compound **6** exhibited only 2% oral bioavailability in the rat, whereas the much more lipophilic Boc derivative **8** showed excellent oral bioavailabilty and good pharmacokinetic behavior. On the basis of these results, compound **8** was also evaluated for oral bioavailability in dogs, where it exhibited an excellent pharmacokinetic profile and oral bioavailability of 90% (Table 3).

Table 2. Inhibition of Various Human Serine Proteases[4] by Compound **6**

enzyme	K_i (μM)	selectivity ratio
thrombin	0.000056	
trypsin	0.12	2143
TPA	137	2.5×10^6
plasmin	165	3.0×10^6
plasma Kallekrein	5	89000
factor X_a	95	1.7×10^6
act protein C	417	7.5×10^6

Compounds **6** and **8** were also evaluated for their antithrombotic activity. We have extensively used 2X APTT (activated partial thromboplastin time) values[6] as a qualitative in vitro indicator of potential antithrombotic activity. The 2X APTT value is defined as the the inibitor concentration (in μM) required to double the APTT in plasma. The values determined for compounds **6** and **8** (Table 4) were high, which did not bode well for potential in vivo antithrombotic efficacy. Evaluation of compounds **6** and **8** in the rat carotid artery $FeCl_3$-induced thrombosis model[5] (Table 4) indicated that compound **6** possessed very good antithrombotic activity (1/5 occlusions dosed iv at 6 μg/kg/min), while the highly bioavailable compound **8** showed only marginal antithrombotic activity (4/6 occlusions, same dosing). Plasma concentrations for compounds **6** and **8** were 1.9 and 1.1 μM, respectively, at the end of the experiment. In the case of compound **6**, the plasma concentration was well in excess of the 2X APTT value determined for the compound in rat plasma. This data suggested that antithrombotic activity might be observed in vivo with this inhibitor. However, in the case of compound **8** the concentration of the inhibitor was well below the 2X APTT value determined for the compound, suggesting that the concentration of compound necessary to achieve good antithrombotic activity was never achieved. The rapid IV clearance of compound **8** from the rat, along with its high 2X APTT value in rat plasma appear to combine to compromise the antithrombotic activity observed in vivo.

The large difference in the 2X APTT values observed for compounds **6** and **8** is difficult to explain if one considers only the relative inhibitory potencies of these two compounds. Our results suggest that factors other than inherent potency play an important role in the antithrombotic properties of thrombin inhibitors. It appears that lipophilicity and the associated plasma protein binding (Table 4) are crucial determinants of potential antithrombotic activity. Despite the fact that compounds **6** and **8** are both extremely potent inhibitors of thrombin in vitro, compound **8** is far more lipophilic and is also more highly protein bound in rat plasma than **6**. We have found that in general with these as well as with other unreported series of compounds, increased lipophilicity and increased plasma protein

Table 3. Oral Bioavailability and Pharmacokinetic Parameters for Compounds **6** and **8** in Rat and Dog

compd no.	log *P*	dose (mg/kg)	route	AUC (μM h)	$T_{1/2}$ (min)	CI (mL/min/kg)	*F* (%)
			Rat				
6[a]	0.794	2	iv	3.30	12	6	
		10	po	0.36			2
8[b]	>3.088	2	iv	3.34	249	17.9	
		10	po	6.86			41
			Dog				
8[c]	>3.088	1	iv	90.0	196	6.06	-
		5	po	45.3	428		90

[a] Dosed po as 0.1% methocel suspension, iv in saline. [b] Dosed po as 0.1% methocel suspension, iv in 30% propylene glycol/saline. [c] Dosed po as 1% methocel suspension, iv in 20% PEG/saline. [d] log *P* values were determined by the standard partition method using octanol/pH 7.4 phosphate buffer.

Table 4. Antithrombotic Efficacy of Compounds **6** and **8**. Rat Carotid Artery Fe Cl_3-Induced Thrombosis Assay[4]

			2X APTT (μM)		plasma protein binding (% free)	
compd[a]	*n*	no. of occlusions	rat	human	rat	human
6	6	1/6	1.3	1.4	6	2
8	6	4/6	2.7	6.0	<1	ND
argatroban	6	0/6	ND	0.28	ND	ND

[a] Compounds **6** and **8** were infused at 6 μg/kg/min iv for 120 min prior to the $FeCl_3$ insult, and argatroban was infused at 10 μg/kg/min iv. The vehicle for administration of compound **6** and argatroban was saline; vehicle for compound **8** was 30% propylene glycol/saline. Vehicle control animals showed essentially complete occlusion. [b] The 2X APTT value is defined as the concentration of inhibitor in plasma (μM) required to double the activated partial thromboplastin time.

binding have detrimental effects on 2X APTT values and antithrombotic activity. While it is clearly possible to enhance oral bioavailability of tripeptide-type thrombin inhibitors by increasing the lipophilicity of these compounds, it appears that this approach also serves to simultaneously compromise the potential antithrombotic activity of the compounds. The in vitro properties and physical parameters of a molecule must be optimized simultaneously with the in vivo pharmacokinetic profile to obtain clinically viable thrombin inhibitors. It is likely that a much more careful and systematic approach will be necessary to develop clinically useful thrombin inhibitors that possess both good oral bioavailability and good antithrombotic efficacy, and understanding the requisite physical properties of the compounds will be crucial in this process. We are currently making use of these principles in an effort to develop clinically useful noncovalent thrombin inhibitors, and will detail our progress in future publications.

Experimental Section

Melting points were determined in open capillary tubes in a Thomas-Hoover apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian Unity 400 spectrometer. Chemical shifts are reported in δ (ppm) relative to tetramethylsilane. All reagents used were of commercial synthetic grade. Aldrich Sure-Seal dimethylformamide was used as solvent in all amino acid coupling reactions. All reversed phase preparatory HPLC purifications were performed on a Waters Prep 4000, using a Waters C18 Prep-Pac and various gradients.

***N*-CBZ-D,L-3,3-dicyclohexylalanine (2a).** A solution of 2.00 g (7.22 mmol) of D,L-3,3-diphenylalanine hydrochloride in 50 mL of acetic acid/10 mL of H_2O was hydrogenated at 62 psi on a Parr apparatus over 500 mg of Ir black catalyst. After 24 h, a second portion of catalyst was added and the reaction continued for a second 24 h interval. The reaction mixture was filtered through a Celite pad, and the filtrate was concentrated in vacuo to give a tan foam. The foam was diluted with Et_2O, scraped, and sonicated to give 1.38 g (66%) of D,L-3,3-dicyclohexylalanine hydrochloride as a tan solid, mp = 261−264 °C. The amino acid (4.76 mmol) was dissolved in 40 mL of 2 N NaOH, and the solution was cooled to 0 °C. The solution was treated dropwise with 1.06 g (6.19 mmol) of benzyl chloroformate with the temperature maintained at <5 °C. After completion of the addition, the reaction mixture was stirred at 0 °C for 15 min and then at room temperature for 1 h. The suspension was acidified to pH 2 with 2.75 M $KHSO_4$ solution, and the suspension extracted with 3 × 50 mL of EtOAc. The combined extracts were dried, decolorized with activated carbon, and filtered through a Celite pad. Concentration provided a peach-colored oil which partially crystallized on pumping. The residue was triturated with hexanes, which induced further crystallization. Filtration provided 1.00 g (55%) of desired product as a white crystalline solid: mp = 150−152 °C. 400 MHz ^{1}H NMR ($CDCl_3$) 0.91−1.38 (m, 10H), 1.48 (q, 1H), 1.54−1.89 (m, 12H), 4.69 (d, 1H), 5.06 (d, 1H), 5.20 (q, 2H), 7.41 (m, 5H); high-resolution FABMS M^+ theoretical 388.248 78, obsd 388.247 93.

***N*-CBZ-L-and D-3,3-dicyclohexylalanyl-L-proline (3a and 4a).** A solution of 850 mg (2.19 mmol) of **2a**, 363 mg (2.19 mmol) of proline methyl ester hydrochloride, 325 mg (2.41 mmol) of EDC, and 488 mg (4.82 mmol) of triethylamine in 12 mL of anhydrous DMF was treated with 462 mg (2.41 mmol) of EDC, and the resulting solution stirred at room temperature in an N_2 atmosphere for 18 h. The reaction mixture was diluted with 3 times its volume of 10% citric acid solution, and the suspension was extracted with 2 × 40 mL of EtOAc. The combined EtOAc extracts were washed with water and brine, and were dried and concentrated to provide the crude coupling product. The crude product was purified via column chromatography over silica gel with 2.5% MeOH/$CHCl_3$ to give the pure coupling product as a white foam. The foam was dissolved in 5 mL of 2 M LiOH/5 mL of DME, and the solution was stirred vigorously at room temperature for 18 h. The reaction mixture was acidified with 10% citric acid solution and extracted with EtOAc. The extract was washed with brine, dried, and concentrated to provide the crude *N*-CBZ-L- and -D-3,3-dicyclohexylalanine-L-proline as a mixture of diastereomers. The acid diastereomers were separated via reversed phase preparatory HPLC to provide 370 mg of the more polar diastereomer **3a** as a white foam: 400 MHz ^{1}H NMR ($CDCl_3$) 0.96−1.31 (m, 10H), 1.50−1.79 (m, 12H), 2.01 (m, 2H), 2.49 (m, 1H), 3.59 (m, 2H), 4.04 (t, 1H), 4.58 (m, 1H), 4.66 (t, 1H), 5.06 (d, J = 12Hz, 1H), 5.17 (d, J = 12Hz, 1H), 5.31 (d, J = 10 Hz, 1H), 7.32 (m, 5H); high-resolution FABMS M^+ theoretical 485.301 54, obsd 485.300 90. Also obtained was 363 mg of the less polar diastereomer **4a** as a glass (69% total yield for both diastereomers): 400 MHz ^{1}H NMR ($CDCl_3$) 0.98−1.24 (m, 10H), 1.39 (m, 1H), 1.51−1.78 (m, 11H), 2.04 (m, 2H), 2.39 (m, 2H), 3.61 (m, 1H), 3.99 (q, 1H), 4.64 (m, 2H), 5.04 (d, J = 12 Hz, 1H), 5.17 (d, J = 12 Hz, 1H), 5.29 (d, J = 10 Hz, 1H), 7.34 (m, 5H); high-resolution FABMS M^+ theoretical 485.301 54, obsd 485.301 62. Each diastereomer was completely free of the other diastereomer by analytical HPLC and NMR.

L- and D-3,3-Dicyclohexylalanyl-L-proline-*N*-[(*trans*-4-aminocyclohexyl)methyl]amide (5 and 6). A solution of 361 mg (0.75 mmol) of the more polar diastereomer of *N*-CBZ-3,3-dicyclohexylalanyl-L-proline, 197 mg (0.75 mmol) of *trans*-

(4-*N*-CBZ-aminocyclohexyl)methylamine, 112 mg (0.83 mmol) of HOBT, and 84 mg (0.83 mmol) of triethylamine in 5 mL of anhydrous DMF was treated with 159 mg (0.83 mmol) of EDC, and the resulting solution was stirred at room temperature in an N_2 atmosphere for 18 h. The reaction mixture was diluted with 3 times its volume of water, and the suspension was stirred vigorously at room temperature for 15 min. The suspension was filtered, and the white solid was washed with water and dried. A 528 mg (97%) yield of the bis-CBZ-protected coupling product was obtained, mp = 79−82 °C. A 500 mg sample of this material was dissolved in 40 mL of 4:1 EtOH/water and was hydrogenated on a Parr apparatus at 50 psi over 150 mg of Pd $(OH)_2$ catalyst for 18 h. The reaction mixture was filtered through a Celite pad, and the filtrate was concentrated to provide the crude product as a clear oil. The oil was purified via reverse phase prep LC to provide 330 mg (64%) of the desired product after lyophilization as an amorphous glass: 400 MHz ^{1}H NMR ($CDCl_3$) 0.98−1.57 (complex, 15H), 1.59−2.00 (complex, 16H), 2.00−2.19 (m, 4H), 2.40 (m, 1H), 3.02 (m, 2H), 3.14 (m, 1H), 3.55 (m, 1H), 3.64 (m, 1H), 4.36 (m, 1H), 4.44 (m, 1H), 7.37 (br s, 1H), 8.19 (br s, 4H). HR FABMS M^+ theoretical 461.385 55, obsd 461.386 46. Anal. ($C_{27}H_{48}N_4O_2$·2.40TFA·0.45 H_2O) C, H, N.

An identical procedure performed on 348 mg (0.72 mmol) of the less polar diastereomer of *N*-CBZ-dicyclohexylalanine-L-proline provided 275 mg (70%) of product as an amorphous tacky solid. 400 MHz ^{1}H NMR ($CDCl_3$) 0.90−1.05 (m, 4H), 1.05−1.43 (complex, 11H), 1.45−1.60 (m, 4H), 1.60−1.89 (complex, 12H), 1.95−2.18 (m, 4H), 2.12 (m, 1H), 2.86 (m, 1H), 2.94 (br s, 1H), 3.16 (m, 1H), 3.46 (m, 1H), 3.87 (m, 1H), 4.40 (m, 2H), 7.61 (t, 1H), 8.11 (br s, 2H), 8.41 (br s, 2H); HR FABMS M^+ theoretical 461.386 46, obsd 461.386 64. Anal. ($C_{27}H_{48}N_4O_2$·2.50TFA·0.50H_2O) C, H, N. This material was the more active diastereomer in a thrombin inhibition assay and was therefore assigned the (*R*)- configuration at the dicyclohexylalanine α-position.

***N*-boc-D,L-3,3-dicyclohexylalanine (2b).** To a solution of 2.00 g (6.90 mmol) of D,L-3,3-dicyclohexylalanine in in 17 mL 1 N NaOH/9 mL of water/17 mL of 1,4 dioxane cooled to 0 °C was added 1.66 g (7.59 mmol) of di-*tert*-butyl dicarbonate in portions over approximately 2 min. The solution was stirred in the cold for 5 min and then at room temperature for an additional 2 h. The reaction mixture was concentrated to remove most of the dioxane and was cooled in an ice bath. The cold reaction was acidified to pH 2 with 1 N $KHSO_4$ and was extracted 2 times with EtOAc. The combined extracts were washed with water and brine, dried, and concentrated to provide 2.43 g (90%) of the desired product as a white crystalline solid: mp = 188.0−189.5 °C; 400 MHz ^{1}H NMR ($CDCl_3$) 0.96−1.35 (m, 10 H), 1.46 (s, 9H), 1.51−1.83 (m, 13H), 4.56 (d, J = 8 Hz, 1H), 4.85 (d, J = 8 Hz, 1H); high-resolution FABMS M^+ theoretical 354.264 43, obsd 354.265 88.

***N*-Boc-L- and -D-3,3-dicyclohexylalanyl-L-proline (3b and 4b).** In a manner identical to the *N*-CBZ examples **3a** and **4a**, from 2.10 g (5.94 mmol) of *N*-BOC-D, L-3,3-dicyclohexylalanine was obtained 0.82 g of the more polar acid diastereomer **3b** as a clear oil/foam: 400 MHz ^{1}H NMR ($CDCl_3$) 1.04−1.32 (m, 10H), 1.43 (s, 9H), 1.51−1.76 (m, 11H), 2.10 (m, 3H), 2.34 (m, 1H), 3.62 (m, 1H), 4.01 (q, 1H), 4.62 (m, 2H), 5.17 (d, J = 9 Hz, 1H); HR FABMS M^+ theoretical 451.317 20, obsd 451.317 64. A 1.20 g quantity of the less polar diastereomer **4b** was also obtained HR FABMS M^+ theoretical 451.317 20, obsd 451.315 87; 400 MHz ^{1}H NMR ($CDCl_3$) 1.02−1.31 (m, 10H), 1.42 (s, 9H), 1.51−1.80 (m, 11H), 1.90−2.25 (m, 3H), 2.55 (m, 1H), 3.59 (q, 1H), 4.02 (t, 1H), 4.59 (m, 2H), 4.99 (d, J = 10 Hz, 1H). An 86% yield was obtained based on the recovery of both diastereomers. Each diastereomer was free of any of the other diastereomer by analytical HPLC and NMR.

***trans*-(4-Aminocyclohexyl)methyl]-*N*-boc-L- and -D-3,3-dicyclohexylalanyl-L-prolinamide (7 and 8).** In a manner identical to the above detailed preparation of compound **5**, from 264 mg (0.59 mmol) of compound **3b** (after coupling, CBZ removal, prep LC, and neutralization) was obtained 135 mg (41%) of the desired product **7** as a clear glass: 400 MHz ^{1}H NMR ($CDCl_3$) 0.84−1.39 (m, 16H), 1.46 (s, 9H), 1.50−1.99 (complex, 18H), 2.11 (m, 1H), 2.22 (m, 1H), 2.75 (m, 2H), 2.81 (m, 1H), 3.29 (m, 1H), 3.56 (br s, 1H), 3.88 (q, 1H), 4.59 (m, 2H), 4.99 (d, 1H), 7.09 (br s, 1H); FAB MS M^+ = 561. Anal. ($C_{32}H_{56}N_4O_4$·0.70EtOAc·0.70 H_2O) C, H, N.

In the same manner from 370 mg (0.82 mmol) of compound **4b** was obtained 155 mg (34%) of desired product **8** as a clear glass: 400 MHz ^{1}H NMR ($CDCl_3$) 0.94−1.35 (complex, 15H), 1.41 (s, 9H), 1.52−189 (complex, 20H), 1.86 (d, 1H), 1.99 (br s, 1H), 2.23 (m, 1H), 2.63 (br t, 1H), 3.02 (m, 2H), 3.54 (q, 1H), 3.95 (br t, 1H), 4.48 (t, 1H), 4.59 (d, 1H), 4.96 (m, 1H), 7.11 (br s, 1H); HRFAB MS M^+ theoretical 561.437 98, obsd 561.437 06. Anal. ($C_{32}H_{56}N_4O_4$·0.70EtOAc·0.15 H_2O) C, H, N. This material was the more active diastereomer in a thrombin inhibition assay and was therefore assigned the (*R*) configuration at the dicyclohexylalanine α-position.

Conscious Rat Bioavailability. Male Sprague−Dawley rats (Taconic Farms, 375−450 g) are anesthetized with Brevital (65 mg/kg). Under aseptic conditions, the carotid artery and in some animals (those to be dosed iv) the jugular vein are cannulated with PE 50 tubing (approximately 75 cm in length).

The tubing is advanced into the carotid artery for a distance of 3 cm and the jugular vein for 2 cm, and anchored securely. The tubing is exteriorized out the back of the neck and passed through a stainless steel spring having a button on one end. The neck incision is closed with autoclips, and the spring's button is sutured to the skin, thus protecting the tubing. The carotid catheter is loaded with approximately 0.3 mL of heparinized saline (5 units/mL) and sealed. Rats are kept in cages overnight where they can roam freely with access to food and water.

The following day a control blood sample (1 mL) is withdrawn from each rat. Collection of all blood samples are done as follows:

1. Approximately 0.3 mL is withdrawn from the carotid artery catheter to remove any traces of heparin.
2. A blood sample is withdrawn into a syringe containing 1 part 3.8% sodium citrate for each 9 parts of blood.
3. Once the sample is withdrawn the catheter is flushed with 0.3 mL of heparinized saline.
4. The blood sample is placed in a 1.5 mL microtube, mixed gently by inverting, and then centrifuged at maximum speed for 2.5 min. The plasma is removed to another microtube and placed on dry ice. (Any samples not centrifuged or plasma removed are kept on ice.)

Rats are now dosed with drug: 2 mg/kg intravenously or 10 mg/kg orally. Blood (0.5 mL) is collected at 1, 5, 15, 30, 60, 90, 120, 150, 180, 240, and 360 min posttreatment for rats dosed iv and (1 mL) at 15, 30, 60, 90, 120, 180, 240, and 360 min posttreatment for those dosed orally.

After the last sample is collected from the iv rats, anesthesia is administered through the iv catheter to prove that the iv dose was in fact received by the rat.

The collected plasma samples are analyzed using a thrombin enzyme activity assay. Plasma samples are extracted with acetonitrile prior to assay.

Conscious Dog Bioavailability. Two male beagle dogs weighing 10−12 kg were used for the absorption and kinetic studies. After an overnight fast, the dogs received oral doses of inhibitor at 5 mg/kg as a 1% methocal suspension in a crossover fashion. Blood samples were collected via the jugular vein at 0, 10, 20, 30, 40, 60, 90, 120, 180, 240, 300, 360, and 480 min after dosing. Plasma samples were kept frozen (−20 °C) until assayed by HPLC.

The same dogs used in the above study received iv doses of inhibitor at 1 mg/kg bolus in a 20% PEG/saline vehicle. Blood was collected at 0, 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, and 480 min after dosing. Plasma samples were kept frozen (−20 °C) until assayed by HPLC.

Determination of Percent Free. The fraction of free inhibitor in plasma is determined as follows: To 495 μL of platelet poor plasma (PPP) or HBSP buffer (50 mM HEPES, 150 mM NaCl, 0.1% PEG8000, pH 7.5) is added 5 μL of a concentrated inhibitor solution (water or DMSO) for a final concentration of 1 μM. After mixing, the solutions are passed through a Microcon 10 (Amicon No. 42406) by centrifugation (5000 rpm for 10 min at room temperature) and ∼50 μL is

collected from each. The eluent is mixed with a known concentration of thrombin ([thrombin] and [inhibitor] $\gg K_i$), and after a brief incubation chromogenic substrate is added. The inhibition generated from the eluent of the PPP sample is compared to a standard curve prepared from the inhibitor that has not been filtered. The nonspecific binding (generally <10%) of the inhibitor to the membrane is corrected for by using the eluent from the HSBP control. Eluent from filtered PPP with no added inhibitor was shown not to interfere with the thrombin chromogenic assay.

Acknowledgment. The authors thank Mr. Art Coddington and Dr. Harri Ramjet for Mass Spectra, Dr. Graham Smith, Mr. Matt Zrada, Mr. Ken Anderson, and Ms. Patrice Ciecko for log *P* determinations and elemental analyses, and Ms. Jean Kaysen for manuscript preparation. The authors also thank Prof. Barry Trost for helpful discussions.

References

(1) Tucker, T. J.; Lumma, W. C.; Naylor-Olsen, A. M.; Lewis, S. D.; Lucas, R.; Freidinger, R. M.; Mulichak, A. M.; Chen, Z.; Kuo, L. C. Design of Highly Potent Noncovalent Thrombin Inhibitors That Utilize a Novel Lipophilic Binding Pocket in the Thrombin Active Site. *J. Med. Chem.* **1997**, *40*, 830−832.

(2) There is a previous patent that generically describes the use of this amino acid as a P3 ligand in thrombin inhibitors: Teger-Nilsson, A. C. E.; Bylund, R. E. WO9311152-A1, 1995.

(3) Boc and Fmoc-D-diphenylalanine are commercially available from Synthetech, Inc., Albany, OR. Prior to this material becoming commercially available, racemic diphenylalanine was prepared according to Cheng et al.; Synthesis and Biological Activity of Ketomethylene Pseudopeotide Analogs as Thrombin Inhibitors. *J. Med. Chem.* **1992**, *35*, 3364−3370 and references cited therein.

(4) Full experimental details for the preparation of CBZ-protected *trans*-4- (aminomethyl)cyclohexylamine are provided in the patent claiming this series of compounds: Brady, S. F.; Feng, D. M.; Freidinger, R. M.; Lumma, W. C.; Lyle, T. A.; Sanderson, P. E. J.; Stauffer, K. J.; Tucker, T. J.; Vacca, J. P. US 5, 510, 369, 1996.

(5) Details of these assays are provided in several previous publications from our group: (a) Lewis, S. D.; Ng, A. S.; Baldwin, J. J.; Fusetani, N.; Naylor, A. M.; Shafer, J. S. Inhibition of Thrombin and Other Trypsin-like Serine Proteinases by Cyclotheonamide A. *Thromb. Res.* **1993**, *70*, 173−190. (b) Lewis, S. D.; Ng, A. S.; Lyle, E. A.; Mellott, M. J.; Appleby, S. D.; Brady, S. F.; Stauffer, K. J.; Sisko, J. T.; Mao, S.-S.; Veber, D. F.; Nutt, R. F.; Lynch, J. J.; Cook, J. J.; Gardell, S. J.; Shafer, J. A. Inhibition of Thrombin by Peptides Containing Lysyl-a Keto Carbonyl Derivatives. *Thromb. Haemostasis* **1995**, *74*, 1107−1112.

(6) Details of the experimental determination of this parameter are provided in the publications cited in ref 5.

JM970140S