Design and Synthesis of a Series of Potent and Orally Bioavailable Noncovalent Thrombin Inhibitors That Utilize Nonbasic Groups in the P1 Position

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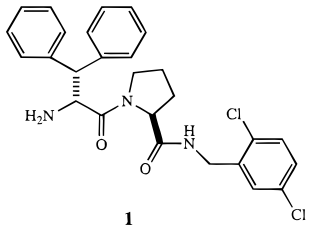
As part of an ongoing effort to prepare therapeutically useful orally active thrombin inhibitors, we have synthesized a series of compounds that utilize nonbasic groups in the P1 position. The work is based on our previously reported lead structure, compound **1**, which was discovered via a resin-based approach to varying P1. By minimizing the size and lipophilicity of the P3 group and by incorporating hydrogen-bonding groups on the N-terminus or on the 2-position of the P1 aromatic ring, we have prepared a number of derivatives in this series that exhibit subnanomolar enzyme potency combined with good in vivo antithrombotic and bioavailability profiles. The oxyacetic amide compound **14b** exhibited the best overall profile of in vitro and in vivo activity, and crystallographic studies indicate a unique mode of binding in the thrombin active site.

Introduction

The serine protease thrombin plays a critical role in the blood coagulation cascade. Thrombin is responsible for the conversion of fibrinogen to fibrin and is also the most potent known stimulator of platelet aggregation. In an attempt to develop novel, orally bioavailable antithrombotic agents with improved therapeutic potential over existing therapies, we have focused on the design and synthesis of selective thrombin inhibitors. A number of other research groups have also reported their efforts toward the design and synthesis of novel thrombin inhibitors.[1] We have detailed our strategies in a series of manuscripts[2−5] describing a number of potent, selective, and orally bioavailable tripeptide thrombin inhibitors. Most recently, we reported a series of potent thrombin inhibitors derived from a resin-based approach that utilized 2,5-disubstituted benzylamines as novel P1 ligands.[5] The key compound **1** (Chart 1) emerged as a novel lead structure from this work. Compound **1** is a potent and selective thrombin inhibitor and exhibited oral bioavailability of 45% in dogs with a half-life of 100 min. Unfortunately, compound **1** is extremely lipophilic (log P > 3.1), and the associated high level of plasma protein binding compromises the performance of the compound in the rat $FeCl_3$-induced thrombosis model. Despite its shortcomings, compound **1** became a crucial starting point for the design of a series of more potent and less lipophilic analogues. Crystallographic analysis[6] of **1** bound in the active site of thrombin indicated a novel binding interaction with the S1 pocket of thrombin (Figure 1). Most previously reported thrombin inhibitors rely on the interaction of a basic amino group in the P1 position with Asp 189 located at the bottom of the S1 pocket. However, in the case of **1** this key interaction with the S1 pocket is obviously lacking. Instead, the chlorine atom in the 5-position of the P1 aromatic ring makes a direct lipophilic contact with the aromatic ring of Tyr 228 located on the back wall of the S1 binding pocket. The binding energy derived from this key lipophilic contact appears to compensate for the lack of a direct interaction with Asp 189 to produce a novel thrombin inhibitor of nanomolar potency. The other key interactions of the D-diphenylAla-Pro series of thrombin inhibitors with the thrombin active site that we have detailed in our previous publications[2−5] are maintained by the P2 and P3 regions of this inhibitor.

Chart 1



1
K_i thrombin = 3 nM
K_i trypsin = 6.8 μM

Refinement of this lead compound **1** to increase its inhibitory potency, while simultaneously making the compound less lipophilic, became the goals of this study. In this manuscript, we describe our progress toward these goals and detail a series of structurally unique thrombin inhibitors that utilize nonbasic groups in the P1 position.

Synthetic Chemistry

The Boc-D-cyclohexylglycine-proline starting material (**2**) (Scheme 1) was prepared by standard amino acid

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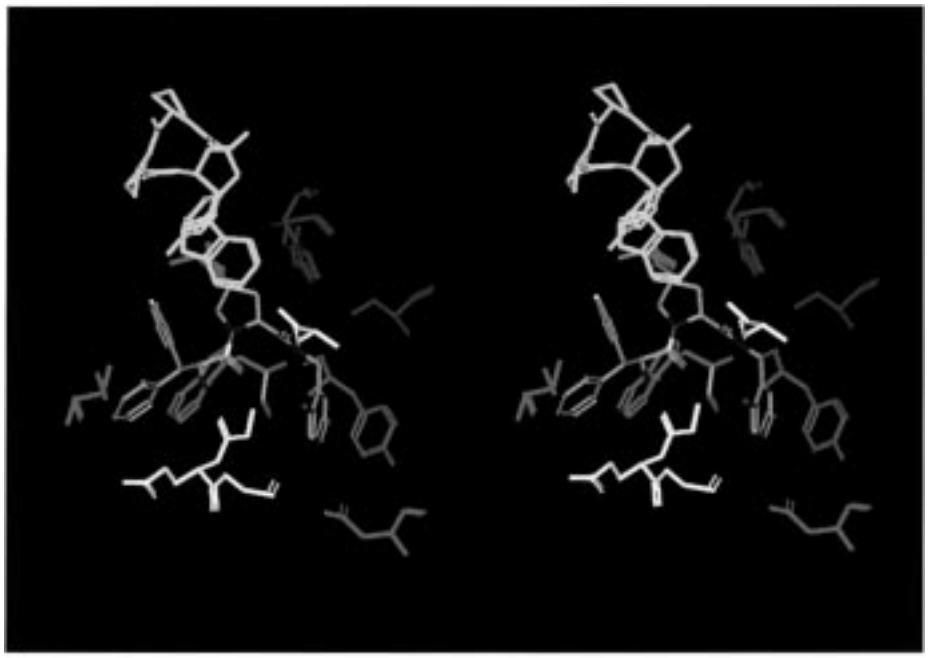


Figure 1. Stereoview of thrombin-bound inhibitor **1** (green with red oxygen and blue nitrogen atoms) with the inhibited thrombin active site. Active site residues are color-coded as follows: lipophilic P3 residues (Leu 99, Ile 174, Trp 215) are colored magneta; the thrombin β sheet is colored white (shown from residues Ser 214 to Gly 219); the catalytic triad is colored green; the S1 Asp 189 is colored red; the thrombin insertion loop is colored yellow; the lipophilic P1 residue Tyr 228 is colored blue. Some active site residues have been omitted for clarity.

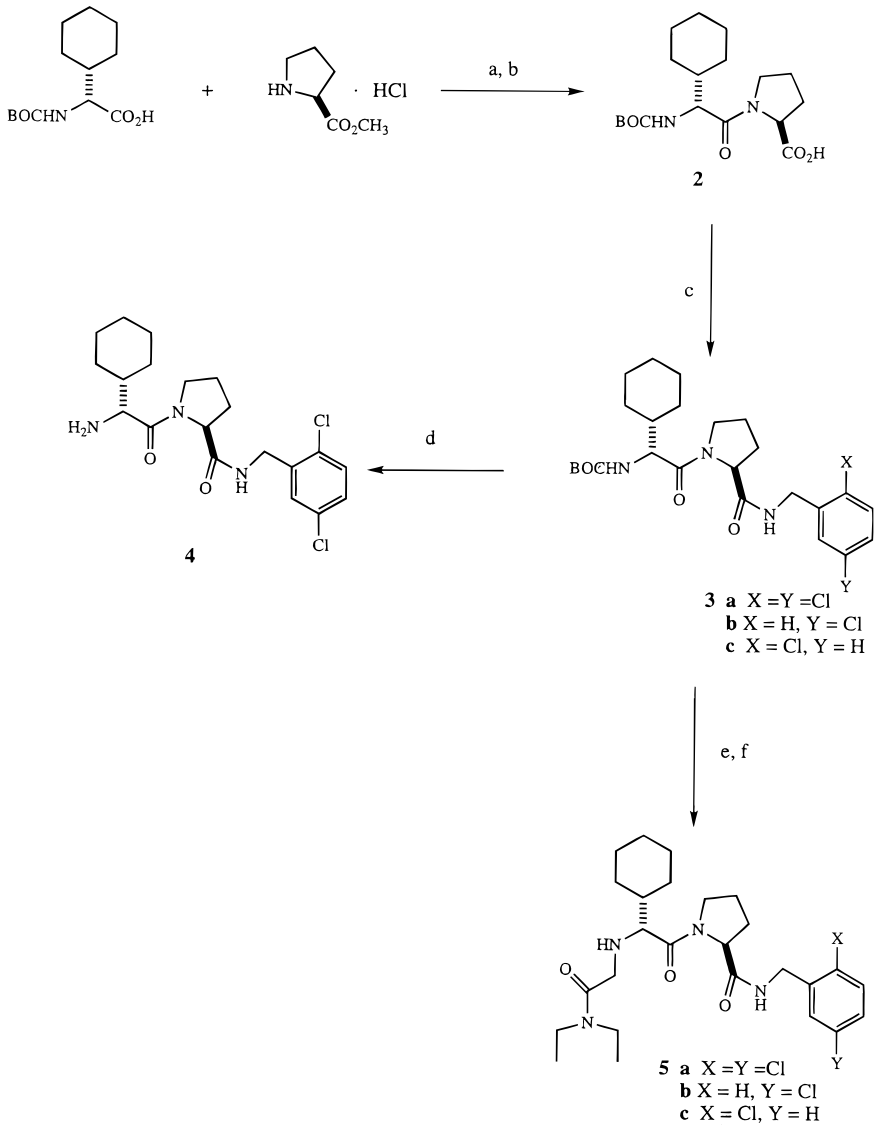
coupling of Boc-D-cyclohexylglycine with proline methyl ester, followed by ester hydrolysis with LiOH/DME. Standard amino acid coupling of **2** with the appropriate 2,5-disubstituted benzylamines provided **3a**−**c**. Treatment of **3a** with gaseous HCl in ethyl acetate at 0 °C provided a precipitate of compound **4**, which was found to be analytically pure after filtration and drying in vacuo. The N-terminal Boc group of **3a**−**c** was also efficiently removed by treatment with trifluoroacetic acid in methylene chloride (1:1). The crude amine salts were alkylated with α-bromo-*N*,*N*-diethylacetamide[7] and purified via reversed-phase preparatory LC to provide compounds **5a**−**c**.

The 2-hydroxy-5-chlorobenzylamine (**7**) was prepared in two steps from the aldehyde **6**[8] via reduction of the oxime (Scheme 2). Standard amino acid coupling of **7** with **2** provided the key tripeptide intermediate **8**. Treatment of **8** with trifluoroacetic acid in methylene chloride (1:1) followed by reversed-phase preparatory LC purification provided **9**. The phenol group of **8** was alkylated with ethyl bromoacetate/cesium carbonate in dioxane to give the ester **10** in good yield. Compound **10** was treated with TFA/CH_2Cl_2 as above to give the N-terminally deprotected ester compound **11**. The ester group of **10** was hydrolyzed with 1 M LiOH/DME to provide the acid intermediate **12**. As before, **12** was treated with TFA/CH_2Cl_2 and the crude product purified via reversed-phase preparatory LC to give compound **13**. Standard amino acid coupling of compound **12** with the appropriate amine, followed by TFA/CH_2Cl_2 deprotection and reversed-phase preparatory LC purification, provided the deprotected amides **14a**−**c**.

Results and Discussion

In an effort to reduce the lipophilicity of the lead structure **1**, the P3 diphenylalanine moiety became the focus of our initial efforts. Previous resin-based P3 modification work from our laboratories[9] had shown that, in a related series of thrombin inhibitors, the amino acid D-cyclohexylglycine could serve as a reasonable P3 group. Incorporation of this amino acid into the P3 position of our lead structure provided the essentially equipotent and selective compound **4** (Tables 1 and 2). Although **4** showed reasonable potency as a thrombin inhibitor, the compound exhibited only moderate oral bioavailability and poor performance in the rat $FeCl_3$ model of arterial thrombosis (Tables 3 and 4). We were convinced that additional inhibitory potency was required to obtain suitable compounds from this series. Molecular modeling studies[10] with **4** indicated that it might be possible to alkylate groups onto the N-terminus of the molecule that had the potential to make hydrogen-bonding interactions with the N−H of Gly 219 on the thrombin β sheet and also fill our previously reported lipophilic N-terminus binding site. We initially prepared benzylsulfonamide N-terminal versions of **4** based on our earlier reported work;[2] however, these compounds were much too insoluble and too lipophilic to pursue further. Instead, we chose to prepare compounds which were alkylated on the N-terminal nitrogen atom with the *N*,*N*-diethylacetamido group. Modeling indicated that the carbonyl functionality of this group could potentially interact with the N−H of Gly 219, while the *N*,*N*-diethyl group could serve to fill the lipophilic N-terminus binding site in a manner similar to our previously reported N-terminal benzylsulfonamides.[2] This group would have the clear advantage of adding far less lipophilicity to the compounds than an N-terminal benzylsulfonamide moiety. Synthesis of the prototype amide **5a** confirmed our assumptions, and this compound showed an approximately 12-fold increase in inhibitory potency (Table 1).

Scheme 1[a]

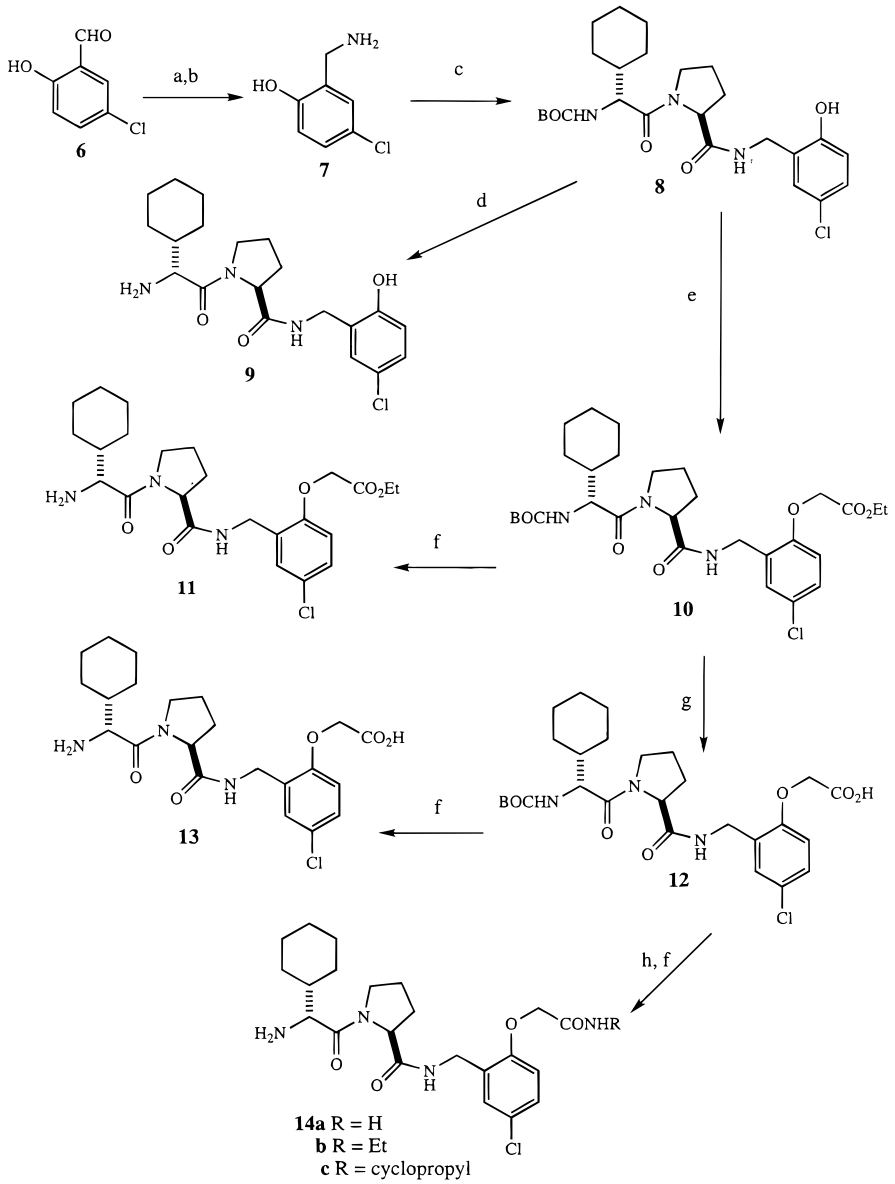


[a] Reagents: (a) EDC, HOBT, triethylamine/DMF, 18 h; (b) 1 M LiOH/DME; (c) EDC, HOBT, triethylamine, appropriate 2,5-disubstituted benzylamine/DMF, 18 h; (d) $HCl_{(g)}$, EtOAc, −10 °C; (e) 1:1 TFA/CH_2Cl_2; (f) α-bromo-*N*,*N*-diethylacetamide, DIEA/DMF, 50 °C, 18 h; purification by reversed-phase preparatory LC.

Removal of either the 2-chloro or the 5-chloro substituent from **5a** resulted in an 18-fold loss of inhibitory potency (**5b**,**c**), confirming the importance of the 2,5-disubstitution pattern. In addition to the critical interaction of the 5-chloro substituent with Tyr 228 in the S1 pocket of thrombin, it appears that the substituent in the 2-position of the aromatic ring also plays a crucial role in the unique binding mode exhibited by these inhibitors. The 2-position substituent may act as a conformational lock, helping to properly position the aromatic ring to allow maximum contact between the 5-chloro atom and the aromatic ring of Tyr 228. Unfortunately, **5a** exhibited only moderate performance in bioavailability (Table 4) and efficacy models (Table 3), forcing us to rethink our approach. This compound remained quite lipophilic (log $P = 2.67$), and on the basis of these data as well as our previously reported results, we concluded that further reductions in the lipophilicity of the molecules would be necessary to obtain good performance in our antithrombotic model. We therefore decided to explore replacement of the 2-chloro substituent on the P1 phenyl ring with more polar groups.

In considering potential substitutions at the 2-position of the P1 aromatic ring, an examination of the crystallographic results obtained with **1** indicated that the substituent on the 2-position of the aromatic ring fits in such a manner as to point directly at the N-terminal region around the amino acid residue Gly 219. We hypothesized that it might be possible to append potential hydrogen-bonding groups to this position of the P1 aromatic ring that could occupy the same region of space as the N-terminal diethylacetamide group of **5a**. Molecular modeling studies[10] (based on the crystal structure of **1**) on various derivatives indicated that a phenol moiety at the 2-position of the P1 ring alkylated with the appropriate hydrogen-bonding group might have the potential to achieve the desired interaction with Gly 219. The replacement of the 2-chloro substituent with a phenol or an appropriately alkylated phenol moiety would also address the key goal of making the compounds less lipophilic. On the basis of these hy-

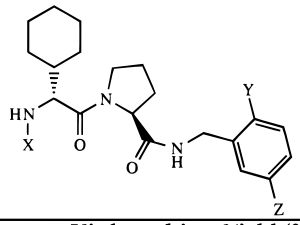
Scheme 2[a]

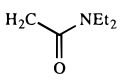
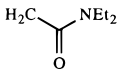
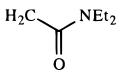
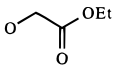
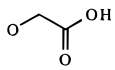
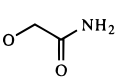
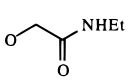
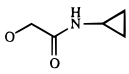


[a] Reagents: (a) $NH_2OH{\cdot}HCl$, NaOAc/EtOH−water, room temperature, 4 h; (b) 5% Rh on C/EtOH−H_2SO_4, Parr apparatus; (c) EDC, HOBT, triethylamine, **2**/DMF, 18 h; (d) $HCl_{(g)}$/EtOAc, −10 °C; (e) ethyl bromoacetate, Cs_2CO_3/1,4-dioxane, 36 h; (f) TFA/CH_2Cl_2, 30 min followed by preparatory LC purification; (g) 1 M LiOH/DME; (h) EDC, HOBT, NMM, appropriate amine/DMF, 18 h.

potheses, the 2-phenol derivative **9** was synthesized and was 2-fold more potent than its 2-chloro analogue **4** (Table 1). Compound **9** (log $P = 2.05$) was also one-half log less lipophilic than **4** (log $P = 2.63$). Most importantly, **9** provided a platform for continued synthetic efforts in this series. Guided by the previously described modeling studies on **9**, we hypothesized that an acetamide derivative similar to that used on the N-terminus of compound **5a**, but instead alkylated onto the phenolic oxygen of **9,** might achieve the desired hydrogen-bonding interaction with Gly 219. Preparation of the ester derivative **11** seemed to confirm the potential of this approach, as this compound exhibited an 8-fold increase in potency versus the phenol **9** and a 16-fold enhancement versus the 2-chloro derivative compound **4**. Unfortunately, the ester function of **11** was subject to rapid hydrolysis in vivo to the corresponding acid **13**, which exhibited a large loss in inhibitory potency. The reduced potency of **13** is consistent with its reduced capacity to make a hydrogen-bonding interaction with the thrombin β sheet. In an effort to produce stable compounds capable of making this key hydrogen-bonding interaction, we chose to focus on the preparation of a series of amide derivatives. We wanted to keep the alkyl groups on the amide of minimal size in an effort to keep the lipophilicity of the compounds in a reasonable range, so we chose to avoid bulky, disubstituted amides of the type used on the N-terminus of **5a**. Instead, we focused on a series of more polar monosubstituted amide derivatives. Preparation of the amide derivatives **14a**−**c** strongly supported our hydrogen-bonding hypothesis, as each of these derivatives retained in large measure the potency of the ester **11** (Table 1). Crystallographic analysis[11] of **14b** bound in the thrombin active site (Figure 2) confirmed the presence of the hydrogen-bonding interaction with the N−H of Gly 219 on the thrombin β sheet. The acetamide side chain on the phenolic oxygen bends toward

Table 1. Nonbasic P1-Containing Thrombin Inhibitors



Compd No.	X	Y	Z	Ki thrombin (nM)[a]	Yield (%)[b]	Anal.	Formula
4	H	Cl	Cl	5	97	CHN	$C_{20}H_{27}N_3O_2Cl_2 \cdot HCl$
5a		Cl	Cl	0.31	43	CHN	$C_{26}H_{38}N_4O_3Cl_2 \cdot 1.65\ TFA \cdot 0.65\ H_2O$
5b		H	Cl	18	41	CHN	$C_{26}H_{39}N_4O_3Cl \cdot 1.20\ TFA \cdot 0.15\ H_2O$
5c		Cl	H	20	29	CHN	$C_{26}H_{39}N_4O_3Cl \cdot 1.25\ TFA \cdot 0.35\ H_2O$
9	H	OH	Cl	2.5	63	CHN	$C_{20}H_{22}N_3O_3Cl \cdot 1.45\ TFA \cdot 0.50\ H_2O$
11	H		Cl	0.45	70	CHN	$C_{24}H_{34}N_3O_5Cl \cdot 0.45\ TFA \cdot 1.00\ H_2O$
13	H		Cl	60	82	CHN	$C_{22}H_{30}N_3O_5Cl \cdot 1.00\ TFA \cdot 0.45\ H_2O$
14a	H		Cl	0.50	17	CHN	$C_{22}H_{31}N_4O_4Cl \cdot 1.50\ TFA \cdot 0.35\ H_2O$
14b	H		Cl	0.74	63	CHN	$C_{24}H_{34}N_4O_4Cl \cdot 1.30\ TFA \cdot 0.15\ H_2O$
14c	H		Cl	0.61	62	CHN	$C_{25}H_{35}N_4O_4Cl \cdot 1.00\ TFA \cdot 0.10\ H_2O$

[a] K_i values for all serine proteases are the average of at least two determinations, where the variation from the mean value is ±10% or less. Experimental protocols are provided in ref 2 and references cited therein. [b] Yield refers to the final isolated and purified compound.

Table 2. Selectivity of Thrombin Inhibitors versus Various Serine Proteases[a]

compd no.	K_i thrombin (nM)	K_i (μM) trypsin	TPA	plasmin	plasma kallikrein	chymotrypsin
1	3	7	ND	ND	ND	>50
4	5	29	19	691	586	49
5a	0.31	27	29	244	399	174
14a	0.50	9	11	152	816	131
14b	0.74	23	ND	ND	ND	71
14c	0.61	18	36	113	79	67

[a] K_i values for all serine proteases are the average of at least two determinations, where the variation from the mean value is ±10% or less. ND = not determined.

the N-terminus of the inhibitor, with the carbonyl group making a hydrogen-bonding interaction with the N−H of Gly 219 and the ethyl group occupying the lipophilic N-terminus binding site region analogously to the benzyl group of our previously described sulfonamide inhibitors.[2] The hydrogen-bonding interaction of the amide carbonyl with the N−H of Gly 219 appears to be worth only approximately 5-fold in potency enhancement. While initially this appears to be a somewhat lower enhancement in potency than might be expected for a hydrogen-bonding interaction, it is consistent with the 10-fold potency enhancement observed for the incorporation of the benzylsulfonamide group onto the N-terminus in our previously reported work.[2] In this previously reported work,[2] crystallographic results indicated that one of the N-terminal sulfonamide oxygens was participating in a hydrogen-bonding interaction with the same N−H of Gly 219. These observations may be due to the fact that both of the aforementioned hydrogen-bonding interactions occur in a highly solvated region close to the water interface. As expected and consistent with our previously reported data,[2] the P3 cyclohexyl ring lies in the lipophilic S3 pocket, with the proline ring filling the S2 pocket. The usual three hydrogen-bonding interactions with amino acid residues Gly 216 and Ser 214 on the thrombin β sheet are maintained.

Table 3. Antithrombotic Efficacy of Thrombin Inhibitors: Rat Carotid Artery $FeCl_3$-Induced Thrombosis Model[a,b]

				2× APTT (μM)[c]		% free		
compd no.	log *P*	*r*	no. of occlusions	rat	human	rat	human	final plasma concn (nM)
4	2.63	6	6/6	ND	0.95	9 ± 2	4 ± 1	329
5a	2.67	6	3/6	ND	0.50	ND	ND	245
14a	1.96	10	8/10	0.31	0.25	7 ± 1	26 ± 4	726
14b	2.07	6	1/6	0.38	0.41	14 ± 2	15 ± 2	794
14c	2.04	6	1/6	0.42	0.35	11 ± 1	12 ± 2	709
argatroban	ND	6	0/6	ND	0.28	ND	ND	ND

[a] ND indicates that the parameter was not determined for that compound. [b] Compounds were infused at 10 μg/kg/min iv for 120 min prior to the $FeCl_3$ insult. Vehicle for compound administration was saline. Vehicle control animals showed complete occlusion. [c] The 2× APTT value is defined as the concentration of inhibitor in plasma required to double the activated partial thromboplastin time.

Table 4. Oral Bioavailability of Thrombin Inhibitors[a]

		F (%) (dose iv, mg/kg; dose po, mg/kg)		
compd no.	log *P*[b]	rat[c]	dog[d]	cynomolgous monkey[e]
1	>3.10	ND	45 ± 5 (1; 5)	ND
4	2.63	23 ± 4 (2; 10)	ND	ND
5a	2.67	10 ± 2 (2; 10)	ND	ND
14b	2.07	10 ± 4 (5; 20)	40 ± 10 (2; 5)	63 ± 9 (1; 5)
14c	2.04	20 ± 5 (5; 12)	ND	34 ± 8 (1; 5)

[a] Details of the experimental protocols are described in the Experimental Section. All values are the mean of at least three determinations. ND means not determined for that compound. [b] Log *P* values were determined experimentally using the standard octanol/water procedure.[2,3] [c] Compounds were dosed orally to rats as a suspension in 1% methocel. [d] Compounds were dosed orally to dogs as a 1% methocel suspension. [e] Compounds were dosed orally to monkeys as a 1% methocel suspension.

In general, members of this series exhibited excellent selectivity versus five other human serine proteases (Table 2). We were especially concerned about selectivity versus human chymotrypsin, as substrates of this enzyme do not contain charged groups in the P1 position. All compounds tested showed excellent selectivity versus this enzyme.

Key compounds in this series were evaluated for their in vivo antithrombotic efficacy in the rat carotid artery $FeCl_3$-induced thrombosis model (Table 3). The 2× APTT values determined in plasma for these compounds were in general quite low, suggesting that reasonable performance in an antithrombotic model could be expected from at least some members of this series. The more lipophilic compounds **4** and **5a** were poor performers in this model, while the less lipophilic amides **14b**,**c** performed much better. However, the unsubstituted amide **14a** showed results that were contrary to this trend, and these results clearly make any attempt at specific quantitative correlation of log *P* values and antithrombotic activity difficult. While **14a** is the least lipophilic compound tested, exhibits rat plasma protein binding essentially identical to the other compounds tested, and possesses a 2× APTT value suggesting that it should have antithrombotic activity, it performs poorly in the rat model. Further investigation of this behavior is ongoing.

Key compounds in this series were also evaluated for oral bioavailability in several species. The prototype compound **1** exhibited good oral bioavailability in dogs ($F = 45\%$), and the second-generation compound **4** also showed reasonable oral bioavailability in rats (Table 4). The N-terminal acetamide compound **5a** showed oral bioavailability of only 10% in rats. However, the phenoxyacetic amides **14b**,**c** both showed reasonable performance in several species. Compound **14b** exhibited the most consistent performance across species, showing oral bioavailabilities of 20%, 40%, and 63% in rats, dogs, and cynomolgous monkeys, respectively. Examination of the pharmacokinetic parameters for **14b**,**c** (Table 5) in all three species showed that the *N*-ethyl amide **14b** clearly exhibited a better overall performance than the *N*-cyclopropyl amide **14c**. Unfortunately, both compounds exhibited half-lives that were too short to be considered candidates for development as clinically useful oral thrombin inhibitors. Initial studies with these inhibitors indicate that the key issue contributing to the less than optimal in vivo half-life appears to be biotransformation.[12] Oxidative metabolism of the P3 cyclohexane ring appears to be one of the key biotransformations observed with these compounds.[12] Further investigation of the metabolic liabilities associated with this series of inhibitors is currently underway.

In summary, we have developed a series of potent and selective thrombin inhibitors based on the use of nonbasic groups in the P1 position. We have demonstrated that noncovalent thrombin inhibitors of subnanomolar potency can be designed that do not make hydrogen-bonding interactions with Asp 189 at the bottom of the S1 pocket of thrombin. The key interactions in the S1 pocket are instead lipophilic in nature, involving primarily a critical lipophilic interaction with the aromatic ring of Tyr 228 in the back wall of the S1 pocket. We have also demonstrated that additional binding energy can be achieved in this series by direct hydrogen-bonding interaction with the N−H of Gly 219, using groups appended to either the N-terminus of the molecules or the 2-position of the P1 aromatic ring. The properties of this series of molecules can be modified to produce thrombin inhibitors such as compound **14b** that exhibit excellent overall profiles of potency, selectivity, bioavailability, and antithrombotic efficacy. Lengthening the half-life of these compounds without compromising the overall profile has become the key challenge for this series of compounds, and efforts toward meeting these goals will be the subject of future publications from our laboratories.

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 instrument, using a Waters C18 Prep-Pac

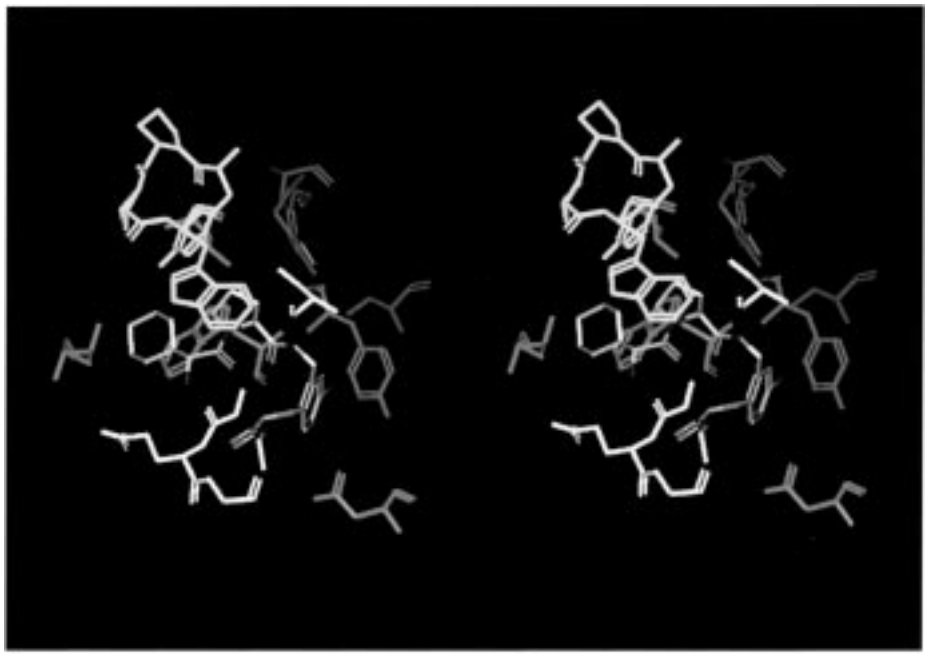


Figure 2. Stereoview of thrombin-bound inhibitor **14b** (green with red oxygen and blue nitrogen atoms) with the inhibited thrombin active site. Active site residues are color-coded as follows: lipophilic P3 residues (Leu 99, Ile 174, Trp 215) are colored magneta; the thrombin β sheet is colored white (shown from residues Ser 214 to Gly 219); the catalytic triad is colored green; the S1 Asp 189 is colored red; the thrombin insertion loop is colored yellow; the lipophilic P1 residue Tyr 228 is colored blue. Some active site residues have been omitted for clarity.

Table 5. Pharmacokinetic Parameters[a] for Compounds **14b**,**c** in Rat,[b] Dog,[c] and Monkey[d]

compd no.	log *P*	dose (mg/kg)	route	AUC (μM h)	$t_{1/2}$ (min)	Cl (mL/min/kg)	*F* (%)
				Rat			
14b	2.07	5	iv	3.88 ± 0.40	51 ± 6	35 ± 11	
		12	po	1.96 ± 0.20			10 ± 2
14c	2.04	5	iv	8.98 ± 0.94	68 ± 14	17 ± 6	
		20	po	3.20 ± 0.21			20 ± 5
				Dog			
14b	2.07	2	iv	4.76 ± 0.50	61 ± 6	14 ± 4	
		5	po	4.85 ± 0.51	73 ± 12		40 ± 10
				Cynomolgous Monkey			
14b	2.07	1	iv	2.23 ± 0.10	102 ± 10	29 ± 4	
		5	po	4.00 ± 0.40			63 ± 9
14c	2.04	0.5	iv	0.42 ± 0.05	35 ± 5	40 ± 10	
		5	po	1.46 ± 0.12			34 ± 8

[a] Details of the experimental protocols are described in the Experimental Section. Results are the mean of at least three determinations. [b] Dosed po as 1% methocel suspension, iv in saline. [c] Dosed po as 1% methocel suspension, iv in saline. [d] Dosed po as 1% methocel suspension, iv in 20% PEG 200.

and various gradients. Mass spectroscopy was performed on a VG HF mass spectrometer with resolving powers of 1000 for low resolution and 5000 for high resolution and FAB ionization using a glycerol matrix. Reagent abbreviations: HOBT, 1-hydroxybenzotriazole; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide HCl; DME, 1,2-dimethoxyethane; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid.

Boc-D-cyclohexylglycine-proline (2). A solution of 8.00 g (31 mmol) of Boc-D-cyclohexylglycine, 5.80 g (35 mmol) of proline methyl ester hydrochloride, 5.80 g (38 mmol) of HOBT, and 10.45 mL (75 mmol) of triethylamine in 100 mL of anhydrous DMF was treated with 7.90 g (40 mmol) of EDC, and the resulting solution stirred at room temperature under argon for 18 h. The reaction mixture was diluted with a 2-fold excess of 10% aqueous citric acid and the resulting mixture extracted with 2 × 150 mL of ethyl acetate. The combined extracts were washed with brine, dried ($MgSO_4$), filtered, and concentrated to give a clear oil. The crude oil was chromatographed over silica gel with 1:1 ethyl acetate/hexane to give the product ester as a clear oil. The oil was immediately dissolved in 100 mL of DME, and the solution was treated with 100 mL of 1 M LiOH. The mixture was stirred at room temperature for 18 h and was acidified with dilute aqueous $KHSO_4$ to pH 4. The mixture was extracted with 2 × 100 mL of ethyl acetate, and the combined extracts were washed with brine, dried ($MgSO_4$), filtered, and concentrated to give 6.45 g (72% for two steps) of pure title compound **2** as a foam. 400 MHz ^{1}H NMR ($CDCl_3$): 0.93−1.31 (complex, 6H), 1.44 (s, 9H), 1.56−1.89 (complex, 5H), 2.02 (complex, 3H), 2.52 (m, 1H), 3.56 (q, 1H), 3.94 (t, 1H), 4.25 (t, 1H), 4.59 (t, 1H), 5.09 (d, J = 9 Hz, 1H).

General Procedure for the Preparation of Compounds 3a−c and Compound 4. Boc-D-cyclohexylglycine-proline-*N*-(2,5-dichlorobenzyl)amide (3a) and D-Cyclohexylglycine-proline-*N*-(2,5-dichlorobenzyl)amide (4). A solution of 100 mg (0.28 mmol) of compound **2**, 50 mg (0.28 mmol) of 2,5-dichlorobenzylamine, 42 mg (0.31 mmol) of HOBT, and 43 mL (0.31 mmol) of triethylamine in 2 mL of anhydrous DMF was treated with 60 mg (0.31 mmol) of EDC, and the resulting solution stirred at room temperature under argon for 18 h. The reaction mixture was diluted with three times its volume of 10% citric acid solution, and the suspension stirred vigorously at room temperature for 2 h. The suspension was filtered to give 125 mg (87%) of compound **3a** as a tacky

amorphous white solid after drying. FAB MS: $MH^+ = 512$. 400 MHz ^{1}H NMR ($CDCl_3$): 0.90−1.21 (m, 5H), 1.25 (s, 9H), 1.58−1.83 (complex, 6H), 1.89 (m, 1H), 2.00 (m, 2H), 2.42 (m, 1H), 3.58 (q, 1H), 3.96 (t, 1H), 4.09 (t, 1H), 4.43 (dd, $J = 6$, 16 Hz, 1H), 4.58 (dd, $J = 6$, 16 Hz, 1H), 4.69 (d, $J = 6$ Hz, 1H), 5.02 (d, $J = 6$ Hz, 1H), 7.16 (d, $J = 3$ Hz, 1H), 7.24 (d, $J = 3$ Hz, 1H), 7.37 (s, 1H), 7.58 (br s, 1H, NH).

A 125-mg (0.24 mmol) sample of compound **3a** was dissolved in 2 mL of ethyl acetate, and the solution was cooled to −10 °C. The reaction mixture was bubbled with HCl gas for several minutes and was stirred in the cold for an additional 15 min. The reaction mixture was bubbled with argon to remove HCl, and the white precipitate was collected by filtration, washed with a little cold ethyl acetate, and dried in vacuo over P_2O_5; 105 mg (97%) of the desired product **4** was recovered after drying. HPLC purity = >98% at 214 nm. 400 MHz ^{1}H NMR (CD_3OD): 1.11−1.39 (complex, 5H), 1.64−1.96 (complex, 6H), 2.02 (m, 2H), 2.09 (m, 1H), 2.31 (m, 1H), 3.63 (m, 1H), 3.78 (m, 1H), 4.02 (d, 1H), 4.42 (q, 2H), 4.50 (m, 1H), 7.29 (d, 1H arom), 7.39 (d, 1H arom), 7.52 (s, 1H arom). Anal. for $C_{20}H_{27}N_3O_2Cl_2$·HCl: C (calcd 47.99, obsd 47.94); H (calcd 5.93, obsd 6.20); N (calcd 8.40, obsd 8.49).

General Procedure for the Preparation of Compounds 5a−c. *N*-(*N*′,*N*′-Diethylacetamido)-D-cyclohexylglycine-proline-*N*-(2,5-dichlorobenzyl)amide (5a). A 125-mg (0.24 mmol) sample of compound **3a** was dissolved in 1 mL of CH_2Cl_2/1 mL TFA, and the solution stirred at room temperature for 18 h. The reaction mixture was concentrated in vacuo and the oily residue placed on a high-vacuum pump for 3 h to remove any traces of solvent. A 50-mg (0.11 mmol) sample of the deprotected material was dissolved in 1 mL of anhydrous DMF and was treated with 38 mL (0.22 mmol) of DIEA, followed by 22 mg (0.11 mmol) of α-bromo-*N*,*N*-diethylacetamide. The resulting solution was stirred at room temperature for 18 h, at which time HPLC indicated that the reaction was only 50% complete. An additional 0.50 mol equiv of the bromide was added, and the reaction mixture was heated at 60 °C for 5 h. The reaction mixture was concentrated in vacuo and the tan oily residue purified via reversed-phase preparatory LC. Suspected product fractions were combined, concentrated to remove volatiles, and placed on a lyophilizer. Lyophilization gave 30 mg (43%) of the desired product **5a** as a tacky white amorphous powder. HR FAB MS: theor MH^+ 525.23992, obsd 525.23820. HPLC purity = >99% at 215, 254 nm. 400 MHz ^{1}H NMR ($CDCl_3$): 1.04−1.33 (complex, 5H), 1.12 (m, 6H), 1.56−1.77 (complex, 4H), 1.83−2.11 (complex, 4H), 2.21 (m, 2H), 3.20 (m, 2H), 3.38 (q, 2H), 3.52 (m, 1H), 3.88 (m, 1H), 4.02 (q, 2H), 4.09 (d, $J = 7$ Hz, 1H), 4.34 (dd, $J = 5$, 14 Hz, 1H), 4.58 (m, 2H), 4.61 (dd, $J = 5$, 14 Hz, 1H), 7.11 (d, 1H arom), 7.22 (m, 2H arom), 7.39 (s, 1H, NH). Anal. for $C_{26}H_{39}N_4O_3Cl$·1.20TFA·0.15H_2O: C (calcd 54.09, obsd 54.12); H (calcd 6.47, obsd 6.47); N (calcd 8.89, obsd 9.09).

***N*-(*N*′,*N*′-Diethylacetamido)-D-cyclohexylglycine-proline-*N*-(5-chlorobenzyl)amide (5b).** In a manner identical to that described above for compound **5a,** from 200 mg (0.48 mmol) of compound **3b** after Boc removal and treatment with 93 mg (0.50 mmol) of α-bromo-*N*,*N*-diethylacetamide and 167.00 mL (0.96 mmol) of diisopropylethylamine was obtained 119 mg (41%) of desired product **5b** as an amorphous fluffy white lyophilisate. FAB MS: $MH^+ = 491$. HPLC purity = >99% at 215, 254 nm. 400 MHz ^{1}H NMR ($CDCl_3$): 1.04−1.33 (complex, 5H), 1.05 (t, 3H), 1.22 (t, 3H), 1.65 (d, 2H), 1.81 (t, 2H), 1.96 (d, 2H), 2.01 (m, 1H), 2.11 (m, 1H), 2.19 (m, 2H), 3.19 (m, 2H), 3.35 (m, 2H), 3.51 (m, 1H), 3.88 (m, 1H), 4.14 (q, 2H), 4.08 (d, 2H), 4.31 (dd, $J = 6$, 15 Hz, 1H), 4.48 (dd, $J = 6$, 15 Hz, 1H), 4.52 (m, 1H), 7.12−7.30 (complex, 4H arom), 7.34 (br t, 1H NH). Anal. for $C_{26}H_{38}N_4O_3Cl_2$·1.65TFA·0.65H_2O: C (calcd 48.51, obsd 48.51); H (calcd 5.69, obsd 5.70); N (calcd 7.72, obsd 7.96).

***N*-(*N*′,*N*′-Diethylacetamido)-D-cyclohexylglycine-proline-*N*-(2-chlorobenzyl)amide (5c).** In a manner identical to that described above for compound **5a,** from 115 mg (0.24 mmol) of compound **3c** after Boc removal and treatment with 47 mg (0.24 mmol) of α-bromo-*N*,*N*-diethylacetamide and 84 mL (0.48 mmol) of DIEA was obtained 42 mg (29%) of desired product **5c** as an amorphous fluffy white lyophilisate. HR FAB MS: theor MH^+ 491.27889, obsd 491.27720. 400 MHz ^{1}H NMR ($CDCl_3$): 1.06 (m, 6H), 1.10−1.36 (complex, 5H), 1.73 (t, 2H), 2.79 (t, 2H), 1.99 (m, 4H), 3.17 (m, 2H), 3.26 (m, 2H), 3.36 (m, 2H), 3.56 (m, 1H), 3.83 (d, 1H), 3.88 (m, 1H), 4.08 (d, 1H), 4.29 (d, 1H), 4.49 (m, 1H and m, 2H), 6.85 (br t, 1H NH), 7.16−7.38 (complex, 4H arom). Anal. for $C_{26}H_{38}N_4O_3Cl_2$·1.25TFA·0.35H_2O: C (calcd 53.49, obsd 53.46); H (calcd 6.45, obsd 6.43); N (calcd 8.76, obsd 8.79).

2-Hydroxy-5-chlorobenzylamine Sulfate (7). A mixture of 4.70 g (30 mmol) of 5-chloro-2-hydroxybenzaldehyde (**6**), 2.10 g (30 mmol) of hydroxylamine hydrochloride, and 2.50 g (30 mmol) of sodium acetate in 50 mL of absolute ethanol/25 mL of water was stirred at room temperature for 4 h. The reaction mixture was diluted with 75 mL of water and the suspension filtered to give 4.50 g of the crude oxime which was used as recovered in the next step.

The crude oxime from above was dissolved in 45 mL of absolute ethanol by carefully adding 4.5 mL of concentrated H_2SO_4 without cooling. The resulting solution was treated with 450 mg of 5% Rh on C catalyst and was hydrogenated on a Parr apparatus for 36 h at 60 psi. The resulting solution was filtered through Celite and the filtrate diluted with 1 volume of water. The pH was adjusted to 7−8, at which point the product began to crystallize. Filtration and drying provided 4.00 g (67%) of the crude desired product as a tan solid. The material was 90−95% pure by HPLC analysis and was used crude in the next reaction. 400 MHz ^{1}H NMR (DMSO-d_6): 3.78 (s, 2H), 6.69 (dd, 1H), 7.05 (br d, 1H), 7.14 (br s, 1H).

Boc-D-cyclohexylglycine-proline-*N*-(2-hydroxy-5-chlorobenzyl)amide (8) and D-Cyclohexylglycine-proline-*N*-(2-hydroxy-5-chlorobenzyl)amide (9). A solution of 408 mg (1.15 mmol) of Boc-D-cyclohexylglycine-proline **(2)**, 151 mg (0.96 mmol) of 2-hydroxy-5-chlorobenzylamine sulfate (**7**), 191 mg (1.25 mmol) of HOBT, and 268 mL (1.92 mmol) of triethylamine in 4 mL of anhydrous DMF was treated with 258 mg (1.34 mmol) of EDC, and the resulting solution stirred at room temperature for 18 h. The reaction mixture was diluted with several volumes of water and the mixture extracted with ethyl acetate. The ethyl acetate extract was washed with dilute aqueous $KHSO_4$, water, dilute aqueous $NaHCO_3$, and brine and was dried ($MgSO_4$). Filtration and concentration provided a crude oily product which was purified via flash chromatography over silica gel with methylene chloride. Concentration of the product fractions gave 437 mg (92%) of desired product **8** as a foam.

A 200-mg (0.41 mmol) sample of **8** was dissolved in 2 mL of CH_2Cl_2/2 mL of TFA, and the resulting solution stirred under argon for 30 min. The reaction mixture was concentrated in vacuo and the crude oil product purified via reversed-phase preparatory LC. Suspected product fractions were concentrated to remove volatiles, and the residue was lyophilized. Lyophilization gave 100 mg (63%) of desired product **9** as an amorphous fluffy white powder. FAB MS: $MH^+ = 394$. HPLC purity = >99% at 214 nm. 400 MHz ^{1}H NMR (CD_3OD): 1.09−1.38 (complex, 5H), 1.64−1.95 (complex, 6H), 2.00 (m, 2H), 2.12 (m, 1H), 2.26 (m, 1H), 3.64 (m, 1H), 3.78 (m, 1H), 3.99 (d, 1H), 4.36 (s, 2H), 4.98 (m, 1H), 4.81 (m, 1H), 6.76 (d, 1H arom), 7.09 (d, 1H arom), 7.12 (s, 1H arom). Anal. for $C_{20}H_{28}N_3O_3Cl$·1.45TFA·0.45H_2O: C (calcd 48.40, found 48.41); H (calcd 5.40, found 5.35); N (calcd 7.40, found 7.50).

Boc-D-cyclohexylglycine-proline-*N*-[2-(*O*-carbethoxymethyl)-5-chlorobenzyl]amide (10) and D-Cyclohexylglycine-proline-*N*-[2-(*O*-carbethoxymethyl)-5-chlorobenzyl]-amide (11). A solution of 237 mg (0.48 mmol) of Boc-D-cyclohexylglycine-proline-*N*-(2-hydroxy-5-chlorobenzyl)amide (**8**) in 7 mL of anhydrous 1,4-dioxane was treated with 172 mg (0.53 mmol) of Cs_2CO_3 and 53 mg (0.53 mmol) of ethyl bromoacetate, and the mixture stirred at room temperature in an argon atmosphere overnight. An additional 0.50 mol equiv of base and bromide were added, and stirring was continued for an additional 18 h. The reaction mixture was partitioned between ethyl acetate and water and the organic

layer washed twice with brine and dried over anhydrous $MgSO_4$. Concentration provided the crude product, which was purified by flash chromatography over silica gel with ethyl acetate. Concentration of suspected product fractions gave 194 mg (78%) of Boc-protected product **10** as a foam.

A 139-mg (0.24 mmol) sample of **10** was dissolved in 2 mL of CH_2Cl_2/2 mL of TFA, and the solution was stirred under argon for 30 min. The reaction mixture was concentrated in vacuo and the oily residue purified via reversed-phase preparatory LC. Suspected product fractions were combined and concentrated to remove volatiles and placed on a lyophilizer for 18 h. Lyophilization provided 80 mg (70%) of the desired product **11** as an amorphous fluffy white powder. FAB MS: $MH^+ = 480$. HPLC purity = >98% at 214 nm. 400 MHz ^{1}H NMR (CD_3OD): 1.11−1.38 (complex, 5H), 1.29 (t, 3H), 1.64−1.96 (complex, 6H), 2.00 (m, 2H), 2.09 (m, 1H), 2.19 (m, 1H), 3.64 (m, 1H), 3.79 (m, 1H), 3.99 (d, 1H), 4.24 (q, 2H), 4.44 (d, 2H), 4.45 (m, 1H), 4.79 (m, 1H), 4.82 (m, 1H), 6.88 (d, 1H arom), 7.21 (d, 1H arom), 7.27 (s, 1H arom). Anal. for $C_{25}H_{32}N_3O_5Cl$·0.45TFA·1.00H_2O: C (calcd 48.78, found 48.80); H (calcd 5.41, found 5.42); N (calcd 6.35, found 6.39).

Boc-D-cyclohexylglycine-proline-*N*-[2-(*O*-carboxymethyl)-5-chlorobenzyl]amide (12) and D-Cyclohexylglycine-proline-*N*-[2-(*O*-carboxymethyl)-5-chlorobenzyl]amide (13). A 110-mg (0.24 mmol) portion of Boc-D-cyclohexylglycine-proline-*N*-[2-(*O*-carbethoxymethyl)-5-chlorobenzyl]amide **(10)** was dissolved in 3 mL of 1 M LiOH/3 mL of DME, and the solution stirred at room temperature for 18 h. The reaction mixture was concentrated to remove DME, and the pH was adjusted to approximately 6 with 10% aqueous citric acid. The material was extracted with ethyl acetate and the extract washed with brine, dried ($MgSO_4$), and concentrated in vacuo to 100 mg (96%) of the crude Boc acid product **12** as a foam.

A 50-mg (0.11 mmol) sample of compound **12** was dissolved in 1 mL of CH_2Cl_2/1 mL of TFA, and the resulting solution stirred at room temperature for 30 min. The reaction mixture was concentrated in vacuo and the oily residue purified via reversed-phase preparatory LC. Suspected product fractions were combined and concentrated to remove volatiles and placed on a lyophilizer for 18 h. Lyophilization gave 41 mg (82%) of the desired product **13** as a fluffy white amorphous powder. FAB MS: $MH^+ = 452$. 400 MHz ^{1}H NMR (CD_3OD): 1.09−1.38 (complex, 5H), 1.63−1.94 (complex, 6H), 1.99 (m, 2H), 2.09 (m, 1H), 2.26 (m, 1H), 3.63 (m, 1H), 3.78 (m, 1H), 3.99 (d, 1H), 4.45 (m, 2H), 4.48 (m, 1H), 4.82 (m, 1H), 6.92 (d, 1H arom), 7.23 (d, 1H arom), 7.32 (s, 1H arom). Anal. for $C_{22}H_{30}N_3O_5Cl$·TFA·0.45H_2O: C (calcd 45.79, obsd 45.81); H (calcd 4.89, obsd 4.89); N (calcd 6.21, obsd 6.41).

General Procedure for the Preparation of Compounds 14a−c. D-Cyclohexylglycine-proline-*N*-[2-(*O*-ethylacetamido)-5-chlorobenzyl]amide (14b). A solution of 91 mg (0.17 mmol) of Boc-D-cyclohexylglycine-proline-*N*-[2-(*O*-carboxymethyl)-5-chlorobenzyl]amide (**12**), 37 mg (0.20 mmol) of HOBT, 35 mg (0.20 mmol) of ethylamine hydrochloride, and 56 mL (0.51 mmol) of *N*-methylmorpholine in 5 mL of anhydrous DMF was treated with 58 mg (0.23 mmol) of EDC, and the resulting solution stirred at room temperature in an argon atmosphere for 18 h. The reaction mixture was concentrated in vacuo, and the residue was partitioned between ethyl acetate and water. The organic layer was washed with brine, dried ($MgSO_4$), filtered, and concentrated in vacuo to give a solid residue. The residue was dissolved in 2 mL of CH_2Cl_2/2 mL of TFA, and the solution stirred at room temperature for 30 min. The reaction mixture was concentrated in vacuo and the oily residue purified via reversed-phase preparatory LC. Suspected product fractions were combined and concentrated to remove volatiles and placed on a lyophilizer for 18 h. Lyophilization provided 64 mg (63%) of the desired product **14b** as an amorphous fluffy white powder. FAB MS: $MH^+ = 479$. 400 MHz ^{1}H NMR (CD_3OD): 1.11−1.39 (complex, 5H), 1.18 (t, 3H), 1.65−1.91 (complex, 6H), 1.98 (m, 2H), 2.08 (m, 1H), 2.25 (m, 1H), 3.64 (m, 1H), 3.77 (m, 1H), 4.02 (d, 1H), 4.43 (m, 1H), 4.45 (q, 2H), 4.56 (q, 2H), 6.91 (d, 1H arom), 7.25 (d, 1H arom), 7.37 (s, 1H arom). Anal. for $C_{24}H_{35}N_4O_4Cl$·1.30TFA·0.15H_2O: C (calcd 50.71, obsd 50.72); H (calcd 5.86, obsd 5.84); N (calcd 8.89, obsd 9.21).

D-Cyclohexylglycine-proline-*N*-[2-(*O*-acetamido)-5-chlorobenzyl]amide (14a). In a manner identical to that described above for compound **14b**, from 110 mg (0.20 mmol) of compound **10**, 12 mg (0.23 mmol) of ammonium chloride, 37 mg (0.24 mmol) of HOBT, 28 mL (0.26 mmol) of *N*-methylmorpholine, and 50 mg (0.26 mmol) of EDC was obtained 15 mg (17%) of the desired product **14a** as a fluffy white amorphous lyophilisate. HR FAB MS: MH^+ theor 451.21120, obsd 451.21195. 400 MHz ^{1}H NMR (CD_3OD): 1.09−1.39 (complex, 5H), 1.67−1.95 (complex, 6H), 1.98 (m, 2H), 2.11 (m, 1H), 2.25 (m, 1H), 3.64 (m, 1H), 3.78 (m, 1H), 4.00 (d, 1H), 4.42 (m, 1H), 4.43 (d, 2H), 4.60 (s, 2H), 6.95 (d, 1H arom), 7.28 (d, 1H arom), 7.36 (s, 1H arom). Anal. for $C_{22}H_{33}N_4O_4Cl$·1.50TFA·0.35H_2O: C (calcd 48.39, obsd 48.42); H (calcd 5.42, obsd 5.46); N (calcd 9.10, obsd, 8.93).

D-Cyclohexylglycine-proline-*N*-[2-(*O*-cyclopropylacetamido)-5-chlorobenzyl]amide (14c). In a manner identical to that described above for compound **14b**, from 140 mg (0.25 mmol) of compound **10**, 19 mL (0.28 mmol) of cyclopropylamine, 46 mg (0.30 mmol) of HOBT, 60 mL (0.60 mmol) of *N*-methylmorpholine, and 62 mg (0.33 mmol) of EDC was obtained 94 mg (62%) of the desired product **14c** as a fluffy white amorphous lyophilisate. FAB MS: $MH^+ = 491$. 400 MHz ^{1}H NMR (CD_3OD): 0.59 (m, 2H), 0.78 (m, 2H), 1.10−1.38 (complex, 5H), 1.64−1.91 (complex, 6H), 1.99 (m, 2H), 2.08 (m, 1H), 2.24 (m, 1H), 2.77 (m, 1H), 3.64 (m, 1H), 3.76 (m, 1H), 4.01 (d, 1H), 4.42 (m, 1H), 4.43 (m, 2H), 4.57 (s, 2H), 6.88 (d, 1H arom), 7.25 (d, 1H arom), 7.34 (s, 1H arom). Anal. for $C_{25}H_{35}N_4O_4Cl$·1.00TFA·0.10H_2O: C (calcd 51.98, obsd 51.92); H (calcd 5.76, obsd 5.77); N (calcd 8.72, obsd, 8.72).

Pharmacokinetic Studies in Rats, Dogs, and Monkeys. Pharmacokinetic studies in rats, dogs, and cynomolgus monkeys were conducted in the fully conscious state in animals which had been previously surgically prepared with indwelling arterial cannulae for repeated blood sampling. Also, pharmacokinetic studies in all species were conducted in the fasted state: i.e., animals were deprived of food for 24 h prior to test agent administration. Oral test agent administration in all species was by gavage; intravenous test agent administration was by indwelling jugular vein cannula in rat and by peripheral venipuncture in dogs and cynomolgus monkeys. Blood samples, collected on citrate (final concentration 0.38%), were obtained prior to treatment and at multiple time points for up to 24 h after treatment. Samples were analyzed by HPLC.

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Supporting Information Available: Crystallographic analysis of **1** and **14b** (2 pages). Ordering information is found on any current masthead page.

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(11) For the crystal structure of compound **14b**, a single crystal was used, diffracting to 1.9-Å resolution. The crystals were of space group *C*2. Full experimental details along with data collection and structure refinement parameters are provided as Supporting Information. The data will be deposited in the Protein Data Bank.

(12) Unpublished data, Department of Drug Metabolism, Merck Research Labs, West Point, PA.

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