J. Med. Chem. **1998,** *41,* 401−406

Notes

Discovery and Development of the Novel Potent Orally Active Thrombin Inhibitor *N*-(9-Hydroxy-9-fluorenecarboxy)prolyl *trans*-4-Aminocyclohexylmethyl Amide (L-372,460): Coapplication of Structure-Based Design and Rapid Multiple Analogue Synthesis on Solid Support

Stephen F. Brady,*,† Kenneth J. Stauffer,† William C. Lumma,† Graham M. Smith,† Harri G. Ramjit,† S. Dale Lewis,‡ Bobby J. Lucas,‡ Steven J. Gardell,‡ Elizabeth A. Lyle,§ Sandra D. Appleby,§ Jacquelynn J. Cook,§ Marie A. Holahan,§ Maria T. Stranieri,§ Joseph J. Lynch Jr.,§ Jiunn H. Lin,‖ I.-Wu Chen,‖ Kari Vastag,‖ Adel M. Naylor-Olsen,⊥ and Joseph P. Vacca†

Departments of Medicinal Chemistry, Biological Chemistry, Pharamacology, Drug Metabolism, and Molecular Design and Diversity, Merck Research Laboratories, West Point, Pennsylvania 19486

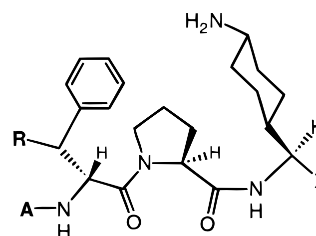
Received July 31, 1997

Early studies in these laboratories of peptidomimetic structures containing a basic P_1 moiety led to the highly potent and selective thrombin inhibitors **2** (K_i = 5.0 nM) and **3** (K_i = 0.1 nM). However, neither attains significant blood levels upon oral administration to rats and dogs. With the aim of improving pharmacokinetic properties via a more diverse database, we devised a resin-based route for the synthesis of analogues of these structures in which the P_3 residue is replaced with a range of lipophilic carboxylic amides. Assembly proceeds from the common P_2−P_1 template **7** linked via an acid-labile carbamate to a polystyrene support. Application of the methodology in a repetitive fashion afforded several interesting analogues out of a collection of some 200 compounds. Among the most potent of the group, *N*-(9-hydroxy-9-fluorenecarboxy)-prolyl *trans*-4-aminocyclohexylmethyl amide (L-372,460 **8**, K_i = 1.5 nM), in addition to being fully efficacious in a rat model of arterial thrombosis at an infusion rate of 10 μg/kg/min, exhibits oral bioavailability of 74% in dogs, and oral bioavailability of 39% in monkeys with a serum half-life of just under 4 h. On the basis of its favorable biological properties, inhibitor **8** has been subject to further evaluation as a possible treatment for thrombogenic disorders.

Introduction

Inhibitors of the enzyme thrombin, a key mediator in the blood coagulation system, have been found to be efficacious in animal models of thrombosis and offer the promise of improved treatment of a number of thrombogenic states.[1] Early studies in these laboratories of peptidomimetic structures containing a basic P_1 α-keto carbonyl moiety culminated in the highly potent, selective, and efficacious α-keto amide **1** (L-370,518).[2] In further work it was demonstrated that removal of the α-keto functionality from **1** afforded the still highly potent and selective *trans*-(4-aminocyclohexyl)methyl amide inhibitor **2** (L-371,912).[3] A key aim of researchers, however, has been to develop potent inhibitors effective upon oral administration, and neither of the above analogues meets this criterion.

X-ray crystallography demonstrated that binding of analogues **1** and **2** to thrombin places the basic aminocyclohexyl moiety in the S_1 subsite and the pyrrolidine ring of proline in the S_2 subsite (insertion loop), while the phenyl ring of the *N*-methyl-D-phenylalanyl residue fits into a unique hydrophobic binding pocket (S_3) on the enzyme.



1: **R** = H, **X** = CO-CONHCH3, **A** = CH3, K_i (thrombin) = **0.09 nM**

2: **R** = **X** = H, **A** = CH3 K_i (thrombin) = **4.9 nM**

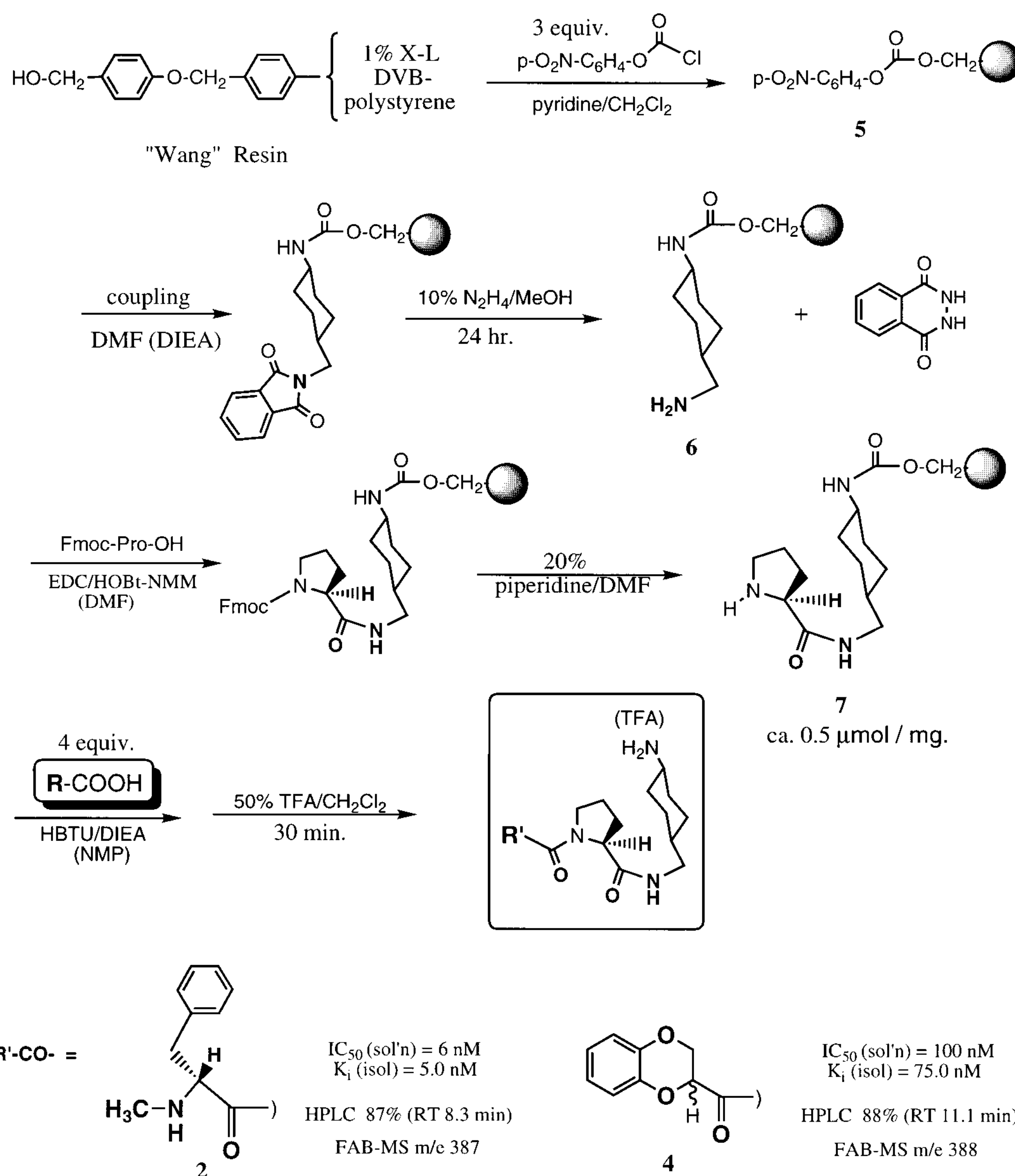
3: **R** = Ph, **X** = **A** = H K_i (thrombin) = **0.1 nM**

Initial results from SAR on inhibitor **2** led us to conclude that a variety of hydrophobic ligands might be accommodated within the S_3 region of thrombin. As an example, addition of a second phenyl group to the

† Department of Medicinal Chemistry.
‡ Department of Biological Chemistry.
§ Department of Pharmacology.
‖ Department of Drug Metabolism.
⊥ Department of Molecular Design and Diversity.

S0022-2623(97)00501-3 CCC: $15.00 © 1998 American Chemical Society
Published on Web 01/10/1998

Scheme 1. Validation of Methodology



β-carbon of the P_3 residue in **2**, via incorporation of D-β,β-diphenylalanine in place of *N*-Me-D-Phe, affords analogue **3**, which exhibits over an order of magnitude increase in inhibitory potency.[4] X-ray crystallographic studies, in conjunction with molecular modeling, revealed that whereas the one phenyl ring occupies essentially the same position as the phenyl of **2**, the second ring is situated at the front of the S_3 pocket close to the solvent interface, but near enough to the side chains of Ile-174 and Glu-217 for productive hydrophobic interaction. However, despite its increased lipophilicity relative to analogues **1** and **2**, significant blood levels of analogue **3** were not realized upon oral administration to rats and dogs.

In the belief that structures with improved pharmacokinetic properties might emerge from a more diverse database, we devised a resin-based approach to the rapid synthesis of multiple variants of structure **2** wherein the *N*-Me-D-Phe (P_3) residue is replaced with a range of carboxylic amides. While we sought to retain certain features at the S_3–P_3 interface that we felt were needed for reasonable potency, we also hoped to strike a balance of physicochemical parameters consistent with improved oral absorption and duration. At the same time, we included in our selection criteria a number of biases both delineating and broadening the scope of our coverage, namely substructures featuring (1) aromatic or hydrophobic moieties; (2) constraints or reinforcements affording "directional diversity"; (3) heteroatoms to access potential hydrogen bonding; and (4) chiral simplification, i.e., modifications to remove chirality.

Results

To evaluate the methodology, assembly proceeding from a common P_2–P_1 template synthesized on solid support was carried out in prototype runs to prepare samples of two analogues of known potency, the *N*-Me-D-Phe derivative **2** and the benzodioxane **4**, about 20-fold less potent, as depicted in Scheme 1. The template was linked to the support via an acid-labile carbamate, starting with commercially available 4-(hydroxymethyl)-phenoxybenzyl (Wang) resin,[5] which afforded resin-bound diamine **6** upon reaction of intermediate *p*-nitrophenyl carbonate **5**[6] with *trans*-4-(phthaloylamino-

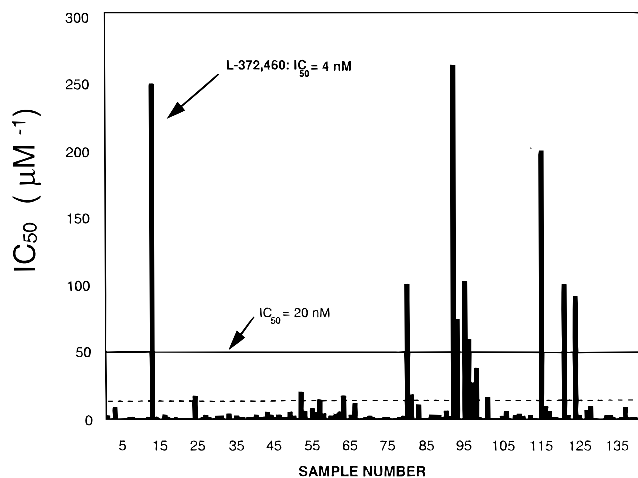


Figure 1. Plot of the reciprocal of the IC_{50} in μM^{-1} against sample number for 137 assay solutions prepared via multiple analogue synthesis, as described in the Experimental Section. Determinations were carried out as previously described[8] for fast-binding (reversible-noncovalent) thrombin inhibitors, with concentrations taken as equivalent to those of the reference compounds **2** and **4** (see Scheme 1).

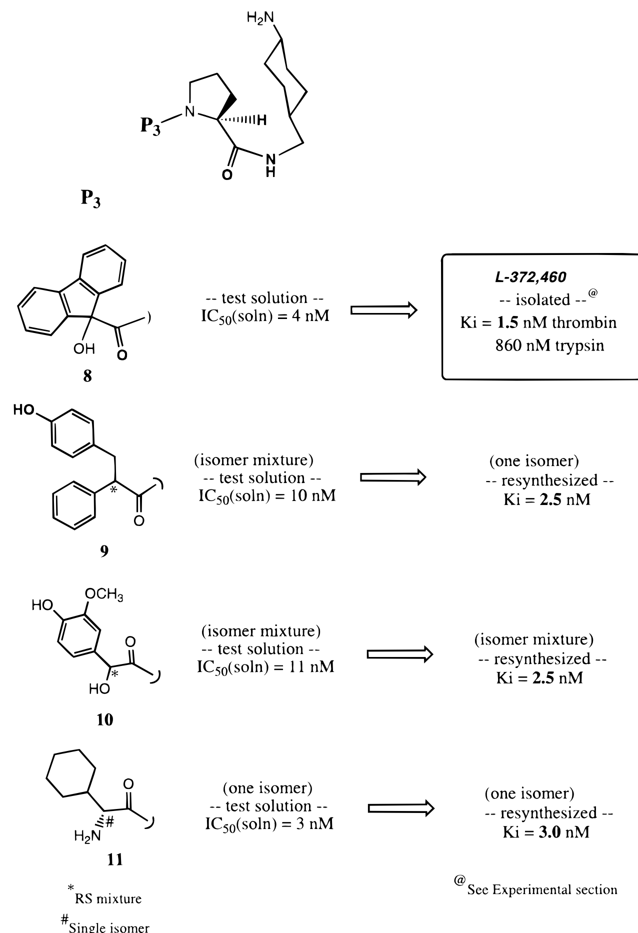


Figure 2. Results from rapid analogue synthesis.

methyl)cyclohexylamine.[7] Removal of the phthaloyl function was accomplished with 10% hydrazine in methanol, monitoring by HPLC to analyze for released phthalazinedione. Coupling with Fmoc-Pro-OH and subsequent deprotection was carried out using routine protocols to give resin-bound template **7**, ready for subdivision and reaction with chosen carboxylic

Table 1. Analogues from Figure 1

Example	HPLC (%)	P_3	IC_{50} (nM)
12	93		910
24	> 95		60
3	88		120
43	96		230
49	77		230
52	91		50
55	88		140
66	97 (doublet)		90
81	98		57
86	72		380
106	48/49 (doublet)		190
115	95		5
116	92		106

*RS isomer mixture

acids; the resin loading was established by amino acid analysis. Standard coupling conditions, followed by 30-min exposure to 50% TFA in methylene chloride, afforded reference analogues **2** and **4** in 87% and 89% purity, respectively, and nearly quantitative yields, as determined by HPLC comparison with authentic samples. Samples were prepared for enzyme assay in H_2O solution, and absolute potencies were gauged by comparison of IC_{50} with solutions of a known quantity of reference analogue.

With the process thus validated, we applied it to prepare P_3 variants generally meeting the criteria set forth above, splitting the resin-bound template **7** into 20-mg portions, each representing an end point of 3−5 mg of final product. Chemically diverse entities were

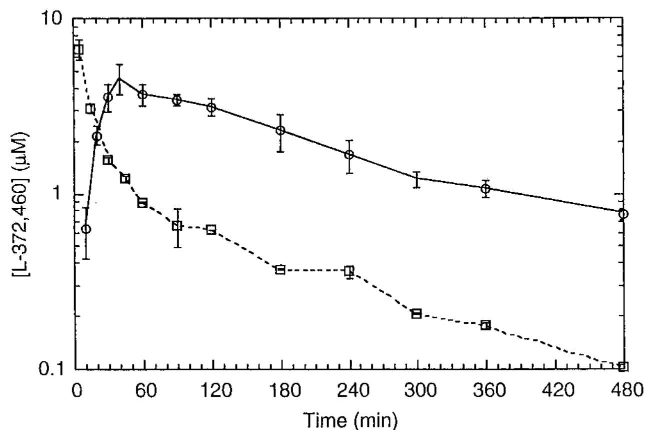


Figure 3. Oral activity of compound **8** over a period of 8 h after administration to dogs. Solid line measures the plasma concentration of drug after po dosing (5 mg/kg; $n = 4$); dashed line measures the plasma concentration after iv dosing (1 mg/kg; $n = 2$). Inhibitor levels were determined by HPLC analysis of acid-treated plasma.

selected from a database of 2200^+ carboxylic acids culled from commercial catalogs and our own in-house sample collection, and the synthesis of approximately 200 examples was carried out as depicted in Scheme 1, in groups of 25−40. The majority of crude products exhibited a major component (70−90%) on HPLC and the predicted molecular species on FAB-MS. The bioassay results for the first 137 compounds made are displayed graphically in Figure 1, which plots the reciprocals of the IC_{50} for each sample. The database in its entirety in fact comprises an evolving SAR, with selected later synthetic targets representing probes of structural classes based on an initial hit; hence the greater number of actives on the right side of the graph.

Some examples of structures that resulted from this effort are shown in Figure 2 and Table 1. One compound identified early on, **8** (example 13 in Figure 1), emerged as clearly worthy of follow-up; that is, resynthesis in pure form and in-depth biological evaluation (see below). The other three structures shown in Figure 2 represent "hits" prompting in turn further exploration of SAR based upon 2,3-diarylpropionate **9** (example 121 in Figure 1), mandelate **10** (example 124 in Figure 1), and α-cyclohexylglycine derivative **11**, respectively. Table 1 illustrates some examples representative of the range of structures made, with respective IC_{50} values and HPLC purity. Details of investigations on several structures of interest identified by this work will be the subject of a future publication.[9]

Biological and Structural Findings

Pharmacokinetic studies in rat, dog, and monkey were conducted in fully conscious 24 h fasted animals following surgical preparation with indwelling arterial cannula for repeated blood sampling. Analogue **8** was administered orally by gavage in all three species and by indwelling regular vein cannula in rat and peripheral veinpuncture in dog and monkey.

Briefly, **8** proved to be extremely potent ($K_i = 1.5$ nM), retaining good selectivity against trypsin ($K_i = 860$ nM) with no effect ($K_i \geq 20$ μM) on several other serine proteases (plasmin, tPA, activated protein C, plasma kallikrein, and chymotrypsin). The 2XAPTT value was 0.85 μM, and the compound was fully efficacious (0 out of 6 vessels occluded) in a rat model of $FeCl_3$-induced

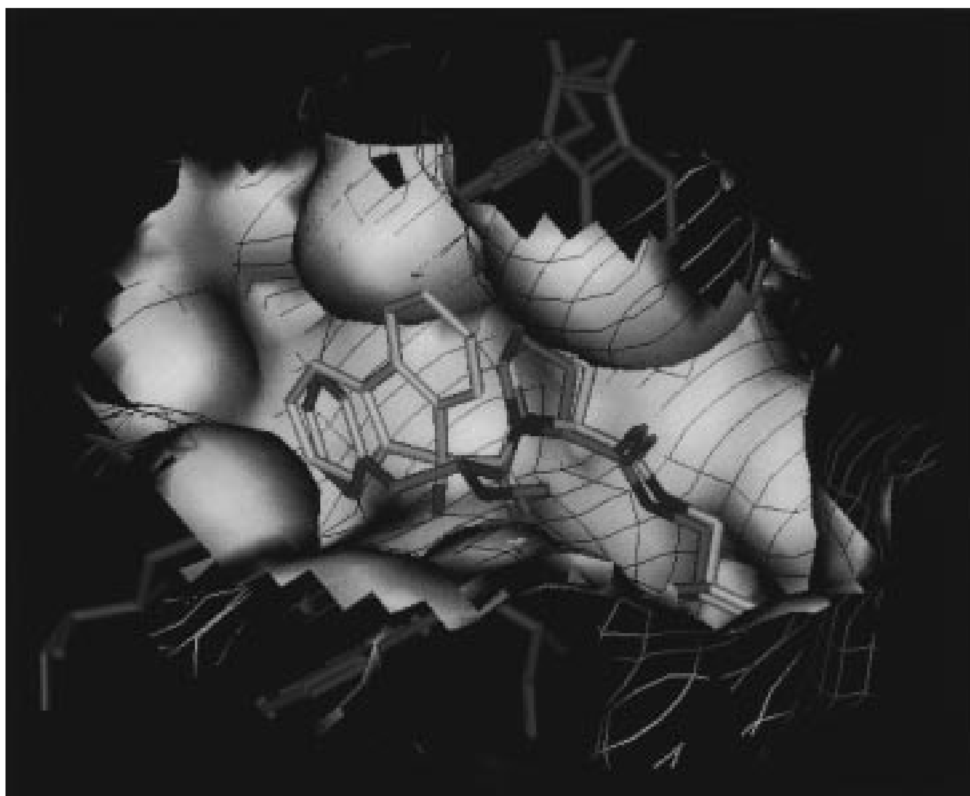


Figure 4. Molecular model of structure **8** created using the X-ray coordinates of analogue **2** to define the P_1-S_1 and P_2-S_2 subsite interactions. Models were constructed using AMF/C_View (AMF is proprietary Merck software). The ligands were energy-minimized within the context of the active site using OPTiMOL with the MMFF force field.

arterial thrombosis[10] at an infusion rate iv of 10 μg/kg/min, attaining a steady-state plasma concentration of 1.59 ± 0.27 μM. Although oral absorption was poor in the rat, the analogue exhibited oral bioavailability of 74% in the dog at a dosage of 5 mg/kg, with a C_{max} of 4.59 μM at 40 min and an iv plasma half-life of slightly over 2 h. The data are presented graphically in Figure 3. In cynomolgus monkeys under comparable conditions of administration, the oral bioavailability was 39%, with a C_{max} of 1.77 μM at 113 min and an iv plasma half-life of nearly 4 h (plot not shown).

Molecular modeling of inhibitor **8** provided key insights into several unique interactions arising from the constrained fluorenyl system. One of the benzene rings indeed occupies the same subsite at S_3 as does the phenyl of inhibitor **2**, but with less room to spare; the rigid tricyclic system places the other benzene ring squarely between the hydrophobic residues Tyr-60A and Trp-60D on the face of the insertion loop opposite that with which proline interacts; finally, this extremely compact structure positions the hydroxyl group to optimally hydrogen bond to the carbonyl of Gly-216, analogous to the NH in analogue **2**. Moreover, it is this replacement of the charged amino moiety with the neutral hydroxyl species that perhaps contributes to the improved oral absorption/plasma duration profile. It is also worth noting that the hydroxyl group very likely contributes significantly to the backbone binding of **8** to thrombin, as evidenced by the increase in IC_{50} of nearly 100-fold upon removal of this hydroxyl from **8** (Table 1, example 86). These various features are depicted in Figure 4, in which the model of structure **8** is superimposed on the X-ray crystal structure of **2**. Subsequent X-ray crystallographic data showed the model to be entirely consistent with the structure of the inhibitor bound to thrombin−hirudin in the crystal lattice.[11]

Conclusion

We have devised a route to the rapid synthesis of approximately 200 analogues of thrombin inhibitor **2** in which the P_3 residue has been replaced with a range of carboxylic acids. The process features (1) readily implemented template-based assembly on solid support; (2) rapid productivity and enzyme assay throughput; (3) access to analogues with a wide range of physicochemical properties. Application of the methodology in a repetitive fashion has afforded diverse potent inhibitors of thrombin, in particular the novel inhibitor **8**, which exhibits good efficacy and oral bioavailability in several animal models.

Experimental Section

General. Unless otherwise noted, all solvents and reagents were synthetic grade obtained from commercial sources and used without further purification. DMF was degassed before use. HPLC analyses were carried out on a Spectra-Physics SP8000 system having an SP4270 integrator with a Vydac Protein & Peptide C_{18} column, 300 A, 5 μm, 150 × 4.6 mm, using a 0.1% TFA /H_2O−acetonitrile gradient system over 30 min, with detection by UV at 210 or 280 nm. FAB mass spectra were obtained on a Fisons 7070E spectrometer.

***p*-Nitrophenyloxycarbonyl Resin 5.** A sample of 0.829 g of *p*-hydroxybenzyloxy alcohol 1% cross-linked polystyrene resin ("Wang" resin, available through several suppliers: Bachem, Inc., Advanced Chemtech, or Peptides International), approximately 0.6 mmol/g of hydroxymethyl substitution, was stirred in 5 mL of 9:1 CH_2Cl_2−pyridine with 0.25 mL of DIPEA, and 0.304 g (approximately 3 equiv) of 4-nitrophenyl chloroformate was added. After 24 h the resin was filtered through a sintered glass funnel, washed three times with DMF and three times with CH_2Cl_2, and dried in vacuo overnight to give 850 mg of orange−brown 4-nitrophenyloxycarbonyl resin **5**.

Resin-Bound *trans*-4-[(*N*-phthaloylamino)methyl]cyclohexylamine (6). A sample of 0.805 g of resin **5** was slurried in 7 mL of DMF, and 245 mg (2 equiv) of *trans*-4-(phthaloylmethyl)cyclohexylamine was added, followed by 0.174 mL (2 equiv) of DIPEA. The mixture was stirred slowly for 20 h and then filtered as above, and the solid was washed three times each with DMF, CH_3OH, and CH_2Cl_2 and then dried in vacuo to give 0.912 g of *N*-phthaloyl intermediate, 0.85 g of which was suspended in 20 mL of CH_3OH, followed by the addition of 2.0 mL of 98% hydrazine. After gentle stirring overnight, the resin was filtered as above, washed six times with CH_3OH, and dried in vacuo to give 700 mg of resin **6**. (Completeness of hydrazinolysis was determined by HPLC of the filtrate to determine release of the byproduct phthalazinedione (RT, 95→5%, 4.7 min.)

Resin-Bound Fmoc-prolyl-*trans*-4-(aminomethyl)cyclohexylamine (7). A sample of 0.686 g (calcd 0.45 mmol) of resin **6** suspended in 25 mL of DMF was treated in order with a solution of 674 mg (2.0 mmol) of Fmoc-L-proline in 4 mL of DMF; 305 mg (2.0 mmol) of *N*-hydroxybenztriazole (HOBt); DIPEA to pH 7−8 (moistened pH 5−10 paper); and 4 mL of 0.5 M DCC in CH_2Cl_2. The mixture was stirred, and the pH was maintained at 7−8 by the addition of DIPEA as needed. Completion of the coupling was determined by qualitative ninhydrin assay on an aliquot of the resin. After 20 h the resin was isolated by filtration as above, washed four times each with DMF, CH_3OH, and CH_2Cl_2, and dried in vacuo to give 0.750 g of Fmoc-protected **7**: amino acid analysis 0.44 mmol/g loading based on proline value.

The Fmoc group was removed by treatment of a 0.74-g sample with 22 mL of 4:1 (v/v) DMF−piperidine, stirring for 90 min. The filtered resin was washed three times each with DMF, CH_3OH, and CH_2Cl_2 and dried in vacuo to give 0.677 g of product **7**, ready for subdivision for multiple analogue synthesis.

Prototype Coupling/Cleavage of Resin 7 To Give Analogues 2 and 4. Samples of 19.9 and 21.2 mg (equivalent to 10.0 and 10.6 μmol based on loading of ca. 0.5 mmol/g) of resin **7** slurried in a 12 × 75 mm culture tube with 0.2 mL of *N*-methylpyrrolidone (NMP) were each treated with 12.0 mg (4.3 equiv) of Boc-*N*-methyl-D-phenylalanine (reaction 1) and 7.2 mg (3.8 equiv) of (±)-4-benzodioxane-2-carboxylic acid (reaction 2) and stirred to dissolve the acids. Then to each tube was added 0.075 mL (37.5 μmol) of a solution of 0.5 M 2-(1*H*-benzotriazolyl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in NMP, followed by 3 μL of DIPEA. After 24 h of agitation, each resin was washed, by centrifugation and pipet withdrawal, two times with DMF and six times with CH_3OH, and then dried in vacuo.

Each of the dried resins (R′-CO-**7**) was wetted with 0.1 mL of CH_2Cl_2 and then treated with 0.2 mL of 100% TFA. Each reaction was allowed stand for 30 min, then concentrated on a Speed-Vac, dried further in vacuo, and reconstituted in solution with 0.9 mL of H_2O. Quantitative analysis by HPLC gave the following results.

Reaction 1: major peak at t_R 8.4 min corresponding to authentic **2** at a concentration of 11.4 mM (purity 87% with no single contaminant >4%), equivalent to a yield of 8.9 μmol (89%) of analogue based on resin-bound precursor **7**.

Reaction 2: major peak at t_R 11.1 min corresponding to authentic **4** at a concentration of 8.8 mM (purity 89% with no single contaminant >4%), equivalent to a yield of 6.6 μmol (66%) of analogue based on resin-bound precursor **7**.

Assay for thrombin inhibition in vitro afforded the following IC_{50} values based on the above concentrations:[8] reaction 1, 6

nM (reference sample = 4.9 nM); reaction 2, 100 nM (reference sample = 71 nM).

Inhibitory potencies of all compounds in solution (see below) are reported as IC_{50} values, that is, the concentration of inhibitor at which the rate of thrombin-catalyzed hydrolysis of a standard substrate is reduced by 50%.[8] For most of the examples in Figure 1, the concentrations of which were not directly determined, values were set at 1 μM (equivalent to approximately 90% yield). In those instances where the compound was prepared independently and isolated with characterization, K_i values could be determined, based on known concentrations (see the Experimental Section). The closer the IC_{50} value to the K_i value, the better the yield of the resin-bound product.

Rapid Multiple Analogue Synthesis. In parallel fashion to the prototype runs, several rounds of rapid analogue synthesis were carried out by subdividing resin **7** into groups of 25−40 samples of ca. 20 mg (10 μmol) each, suspended in 0.2 mL of NMP, followed by addition of 4 equiv (ca. 40 μmol) of a selected carboxylic acid, then HBTU and DIPEA (ca. 3 equiv) for 48 h. Workup and processing as above yielded solutions for enzyme assay as above. Preliminary qualititative assessment was done by analytical HPLC and FAB-MS, and concentration was assumed as 10 μM by inference from the prototype runs.

Isolation of 8 (L-372,460). In the case of example 13, Figure 2, the crude product, after sampling for enzyme assay (IC_{50} thrombin = 4 nM), was purified by semipreparative HPLC on a 1.0-in. × 10-in. Vydac C_{18} column using a 100 → 50% 0.1% TFA/H_2O−CH_3CN gradient, to afford 1.5 mg of analogue **8** after lyophilization: FAB-MS *m/e* 434 (M + H); HPLC 95 → 5% gradient, >99% pure; NMR (400 MHz, D_2O) 2 amide bond rotamers at the proline N, ca. 1.5:1 ratio; δ 3.71/3.63 (2.45) 2H (Pro $^{\delta}$CH), 4.44 (3.47) 1H (Pro $^{\alpha}$CH), 2.48/2.05 (2.46/2.05) 2H (Pro $^{\beta}$CH), 1.67/1.55 (1.61) 2H (Pro $^{\gamma}$CH), 7.3−7.9 8H (fluorenyl CH), 3.20/3.04 3H (ring $CHNH_2$ and CH_2-NH), 0.91−2.08 9H (ring CH) (minor species in parentheses). K_i thrombin = 1.5 nM (see Figure 2).

Acknowledgment. The authors thank Dr. Michael J. Bogusky for determination and analysis of the NMR spectrum of analogue **8**. We also thank Ms. Jean F. Kaysen for preparation of the manuscript.

References

(1) Tapparelli, C.; Metternich, R.; Ehrhardt, C.; Cook, N. S. Synthetic Low-molecular Weight Thrombin Inhibitors: Molecular Design and Pharmacological Profile. *Trends Pharm. Sci.* **1993**, *14*, 366−373. See also: Lyle, T. A. Small-molecule Inhibitors of Thrombin. *Perspect. Drug Discovery Des.* **1994**, *1*, 453−460.

(2) Brady, S. F.; Lewis, S. D.; Colton, C. D.; Stauffer, K. J.; Sisko, J. T.; Ng, A. S.; Homnick, C. F.; Bogusky, M. J.; Shafer, J. A.; Veber, D. F.; Nutt, R. F. Development of Highly Potent and Selective α-Keto Carbonyl Thrombin Inhibitors with Novel P1 Side Chains: Synthesis and Biological Profile of L-370,518. In *Peptides: Chemistry, Structure and Biology* (Proceeding of the Fourteenth American Peptide Symposium); Kaumaya, P. T. P., Hodges, R. S., Eds.; Mayflower Scientific Ltd.: Kingswinford, England, 1996; pp 331−333.

(3) Lyle, T. A.; Chen, Z.; Appleby, S. D.; Freidinger, R. M.; Gardell, S. J.; Lewis, S. D.; Li, Y.; Lyle, E. A.; Lynch, J. J., Jr.; Mulichak, A. M.; Ng, A. S.; Naylor-Olsen, A. M.; Sanders, W. M. Synthesis, Evaluation, and Crystallographic Analysis of L-371,912: A Potent and Selective Active-site Thrombin Inhibitor. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 67−72.

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

(5) Wang, S. S. p-Alkoxybenzyl Alcohol Resin and p-Alkoxybenzyloxycarbonylhydrazide Resin for Solid Phase Synthesis of Protected Peptide Fragments. *J. Am. Chem. Soc.* **1973**, *95*, 1328−1335.

(6) Dixit, D. M.; Lesnoff, C. C. Insoluble Polymer Supports as Monoblocking Agents of Symmetrical Diamines *J. Chem. Soc., Chem. Commun.* **1977**, 798−799.

(7) Lumma, W. C., unpublished results, Merck Research Laboratories.

(8) 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 α-Keto Carbonyl Derivatives. *Thromb. Haemostasis* **1995**, *74* (4), 1107−1112.

(9) Stauffer, K. J., unpublished results, Merck Research Laboratories.

(10) Kurz, K. D.; Main, B. W.; Sandusky, G. E. Rat Model of Arterial Thrombosis Induced by Ferric Chloride. *Thrombosis Res.* **1990**, *60*, 269−274.

(11) Chen, Z.-G.; Kuo, L., unpublished results, Merck Research Laboratories.

JM9705014