Synthesis and Screening of Linear and Cyclic Oligocarbamate Libraries. Discovery of High Affinity Ligands for GPIIb/IIIa

Charles Y. Cho,[†,‡] R. Scott Youngquist,[#] Sari J. Paikoff,[†] Maureen H. Beresini,[¶] Andrea R. Hebert,[¶] Lea T. Berleau,[£] Corey W. Liu,[†] David E. Wemmer,[†,⊥] Thomas Keough,[#] and Peter G. Schultz*[,†,§]

Contribution from the Department of Chemistry and Howard Hughes Medical Institute, University of California, Berkeley, California 94720-1460, The Procter & Gamble Company, Miami Valley Laboratories, P.O. Box 538707, Cincinnati, Ohio 45253-8707, Departments of BioAnalytical Technology and Pharmakinetics and Metabolism, Genentech, Inc., One DNA Way, South San Francisco, California 94080-4990, and Physical Biosciences Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, California 94720-5230

Received October 2, 1997

Abstract: Synthetic methodology has been developed for the generation of large, diverse libraries of "unnatural" carbamate oligomers using the "one bead, one peptide" method. Using a pool of 27 structurally and functionally diverse monomers, one acyclic and two cyclic libraries were synthesized and screened for binding to the integrin GPIIb/IIIa. Several classes of oligocarbamate ligands for GPIIb/IIIa were discovered, and two cyclic ligands have activities that are within a factor of 3 of kistrin, a snake venom protein that effectively inhibits platelet aggregation. Preliminary pharmacokinetic characterization was performed on a linear oligocarbamate ligand, which was cleared from plasma with a half-life of 3.6 min.

Introduction

Peptides can be generated that bind many different proteins with both high affinity and high specificity, due in part to the chemical diversity resulting from permutations of the 20 natural amino acids as well as the unique structural properties of the amide backbone. Peptides, from a molecular recognition standpoint, are therefore a good starting point for the development of enzyme inhibitors or receptor agonists/antagonists for use as therapeutic agents. Typically, however, peptides are characterized by low bioavailability and poor transfer rates across cell membranes and are susceptible to proteolytic degradation.[1,2] It is thus not surprising that the vast majority of drugs are nonpeptidic small molecules, generally discovered through screening of natural products,[3] or more recently through structure-based design or the screening of synthetic combinatorial libraries.[4] The use of oligomeric molecules with unnatural backbones as drugs, however, remains largely unexplored. These "unnatural biopolymers" retain the advantages of the intrinsic diversity present in hetero-oligomers and the use of a single bond forming chemistry but might have improved pharmacokinetic properties compared to the peptide backbone. The syntheses of several unnatural biopolymers have been described, with chemical linkages that include amides,[5−11] carbamates,[12−15] ureas,[16−19] sulfonamides,[20,21] azatides,[22,23] pyrrolinones,[24] macrolactones (oligolides),[25] and acetals.[26]

The assessment of the pharmacological properties of an unnatural oligomer requires the discovery of high affinity ligands

* To whom correspondence should be addressed.
† Department of Chemistry, University of California, Berkeley.
‡ Present address: Department of Pharmacology, University of California, San Diego, La Jolla, California 92093-0647.
§ Howard Hughes Medical Institute.
# The Proctor & Gamble Company.
¶ Department of BioAnalytical Technology, Genentech, Inc.
£ Department of Pharmakinetics and Metabolism, Genentech, Inc.
⊥ Lawrence Berkeley National Laboratory.

(1) Humphrey, M. J.; Ringrose, P. S. *Drug. Metab. Rev.* **1986**, *17*, 283−310.
(2) Conradi, R. A.; Hilgers, A. R.; Ho, N. F. H.; Burton, P. S. *Pharm. Res.* **1992**, *9*, 435−439.
(3) Hirschmann, R. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1278−1301.
(4) Ellman, J. A. *Acc. Chem. Res.* **1996**, *29*, 132−143.
(5) Simon, R. J.; Kania, R. S.; Zuckermann, R. N.; Huebner, V. D.; Jewell, D. A.; Banville, S.; Ng, S.; Wang, L.; Rosenberg, S.; Marlowe, C. K.; Spellmeyer, D. C.; Tan, R.; Frankel, A. D.; Santi, D. V.; Cohen, F. E.; Bartlett, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 967−9371.
(6) Zuckermann, R. N.; Martin, E. J.; Spellmeyer, D. C.; Stauber, G. B.; Shoemaker, K. R.; Kerr, J. M.; Figliozzi, G. M.; Goff, D. A.; Siani, M. A.; Simon, R. J.; Banville, S. C.; Brown, E. G.; Wang, L.; Richter, L. S.; Moos, W. H. *J. Med. Chem.* **1994**, *37*, 2678−2685.
(7) Ostresh, J. M.; Husar, G. M.; Blondelle, S. E.; Dörner, B.; Weber, P. A.; Houghten, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11138−11142.
(8) Combs, A. P.; Kapoor, T. M.; Feng, S.; Chen, J. K.; Daudé-Snow, L. F.; Schreiber, S. L. *J. Am. Chem. Soc.* **1996**, *118*, 287−288.
(9) Hagihara, M.; Anthony, N. J.; Stout, T. J.; Clardy, J.; Schreiber, S. L. *J. Am. Chem. Soc.* **1992**, *114*, 6568−6570.
(10) Appella, D. H.; Christianson, L. A.; Klein, D. A.; Powell, D. R.; Huang, X.; Barchi, J. J.; Gellman, S. H. *Nature (London)* **1997**, *387*, 381−384.
(11) Matthews, J. L.; Overhand, M.; Kühnle, F. N. M.; Ciceri, P. E.; Seebach, D. *Liebigs Ann./Recueil* **1997**, 1371−1379.
(12) Cho, C. Y.; Moran, E. J.; Cherry, S. R.; Stephans, J. C.; Fodor, S. P. A.; Adams, C. L.; Sundaram, A.; Jacobs, J. W.; Schultz, P. G. *Science* **1993**, *261*, 1303−1305.
(13) Moran, E. J.; Wilson, T. E.; Cho, C. Y.; Cherry, S. R.; Schultz, P. G. *Biopolymers (Peptide Science)* **1995**, *37*, 213-219.
(14) Paikoff, S. J.; Wilson, T. E.; Cho, C. Y.; Schultz, P. G. *Tetrahedron Lett.* **1996**, *37*, 5653−5656.
(15) Wang, X.; Huq, I.; Rana, T. M. *J. Am. Chem. Soc.* **1997**, *119*, 6444−6445.
(16) Burgess, K.; Ibarzo, J.; Linthicum, D. S.; Russell, D. H.; Shin, H.; Shitangkoon, A.; Totani, R.; Zhang, A. J. *J. Am. Chem. Soc.* **1997**, *119*, 1556−1564.
(17) Kim, J.-M.; Bi, Y.; Paikoff, S. J.; Schultz, P. G. *Tetrahedron Lett.* **1996**, *37*, 5305−5308.
(18) Kim, J.-M.; Wilson, T. E.; Norman, T. C.; Schultz, P. G. *Tetrahedron Lett.* **1996**, *37*, 5309−5312.
(19) Nowick, J. S.; Powell, N. A.; Martinez, E. J.; Smith, E. M.; Noronha, G. *J. Org. Chem.* **1992**, *57*, 3763−3765.
(20) Gennari, C.; Salom, B.; Potenza, D.; Williams, A. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 2067−2069.

S0002-7863(97)03439-2 CCC: $15.00
Published on Web 07/24/1998

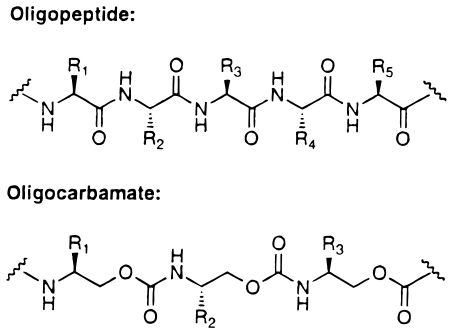


Figure 1. Structures of oligopeptides and oligocarbamates.

to a physiologically important receptor. Zuckermann and coworkers have described the synthesis and screening of a library of *N*-(substituted)glycines, or peptoids, that resulted in low nanomolar ligands for the α_1-adrenergic and μ-opiate receptors,[6] which are being assessed in vivo.[27] In addition, Rana and coworkers have synthesized a Tat-derived oligocarbamate sequence that binds TAR RNA with a K_d of 1.13 μM, which is within a factor of 1.5 of the natural peptide.[15] For our studies, we have chosen to pursue the identification of oligocarbamate ligands for the integrin GPIIb/IIIa. The association of GPIIb/IIIa with the plasma protein fibrinogen is a critical step in the aggregation of platelets, and blocking of this interaction can inhibit thrombus formation.[28] The binding of GPIIb/IIIa to fibrinogen, like that of other integrins to their target proteins, is mediated by an Arg-Gly-Asp (RGD) tripeptide sequence.[29] Several classes of molecules have been developed based on the RGD sequence that inhibit platelet aggregation.[30−32] No equivalent oligocarbamate pharmacophore, however, is known, thus necessitating the generation and screening of large libraries. Here we describe the synthesis and screening of one linear and two cyclic oligocarbamate libraries, with the subsequent identification and characterization of high affinity ligands for GPIIb/IIIa as well as the preliminary characterization of the pharmacokinetic properties of one of the linear carbamate ligands.

Results and Discussion

Monomer and Linear Oligomer Synthesis. The oligocarbamates described here (Figure 1) are synthesized by the stepwise coupling of *N*-protected amino-*p*-nitrophenyl carbonate monomers, which in turn are derived from the corresponding amino alcohols. Since GP IIb/IIIa is known to bind to molecules containing guanidinium and carboxylate moieties, the monomer set reflects this bias. Conformationally restricted monomers as well as both *R* and *S* enantiomers of several monomers were used. Oligocarbamates can be synthesized using virtually any amino alcohol building block, including hydroxyproline and aminoindanol derivatives. This allows rapid access to a wide variety of structurally diverse monomers, increasing both the chemical and conformational complexity of these libraries. Monomers were prepared by reaction of *N*-protected amino alcohols with *p*-nitrophenyl chloroformate.[12,13] The majority of the monomers were derived from the reduction of commercially available, Fmoc-protected amino acids used for peptide synthesis.[33] The monomeric units (in the context of the carbamate backbone) along with their three letter abbreviations used in this study are shown in Figure 2.

The efficient synthesis of oligocarbamates on insoluble polymeric supports was performed as previously described,[12,13] with some modifications. Briefly, stepwise oligomerization is accomplished by reacting *N*-protected amino *p*-nitrophenyl carbonate monomers with resin-bound amines in the presence of hydroxybenzotriazole (HOBt). Coupling yields are greater than 99% as monitored by the Kaiser ninhydrin test.[34] Main-chain amines are protected with the base-labile fluorenylmethoxycarbonyl (Fmoc) protecting group, while side-chain functional groups are generally protected with acid-labile protecting groups. For large-scale syntheses, ligands that contained the monomers **Hrg^c** and **Drg^c** (see Figure 2) were synthesized with side-chain allyloxycarbonyl-protected (Alloc) amine monomers in place of the 2,2,5,7,8-pentamethylchroman-6-sulfonyl-protected guanidinium monomers typically used in peptide synthesis. Deprotection of the Alloc group was accomplished with Pd^0,[35] and guanylation on support was performed as described.[36] Cleavage from support and deprotection of side-chain functional groups were accomplished by treatment of the resin with 5% triethylsilane in trifluoroacetic acid (TFA).[37] The crude oligocarbamates were precipitated in a mixture of cold *tert*-butyl methyl ether and hexanes. Sequences were purified by reverse-phase high-pressure liquid chromatography with monitoring at 205 nm and analyzed by MALDI mass spectrometry.

Cyclization of Oligocarbamates. Research from several labs directed toward the design of GPIIb/IIIa antagonists has shown that the conformational constraint afforded by cyclization of peptides results in increased binding affinity for the receptor when compared with their linear counterparts. Cyclic GPIIb/IIIa inhibitors have been synthesized by a number of means, including disulfide formation,[38−41] thioether formation,[42] ether formation,[43] amide formation,[44] and amide formation with an aromatic template.[45,46] In each of these cases, the conforma-

(21) Moree, W. J.; Marel, G. A. v. d.; Liskamp, R. J. *J. Org. Chem.* **1995**, *60*, 5157−5169.
(22) Han, H.; Janda, K. D. *J. Am. Chem. Soc.* **1996**, *118*, 2539−2544.
(23) Gante, J. *Synthesis* **1989**, 405−413.
(24) Smith, A. B.; Keenan, T. P.; Holcomb, R. C.; Sprengeler, P. A.; Guzman, M. C.; Wood, J. L.; Carroll, P. J.; Hirschmann, R. *J. Am. Chem. Soc.* **1992**, *114*, 10672−10674.
(25) Hoffmann, T.; Seebach, D. *Liebigs Ann. 1996*, 1277−1282.
(26) Hall, D. G.; Schultz, P. G. *Tetrahedron Lett.* **1997**, in press.
(27) Gibbons, J. A.; Hancock, A. A.; Vitt, C. R.; Knepper, S.; Buckner, S. A.; Brune, M. E.; Milicic, I.; J. F. Kerwin, J.; Richter, L. S.; Taylor, E. W.; MORE *J. Pharm. Exp. Ther.* **1996**, *277*, 885−899.
(28) Kieffer, N.; Phillips, D. R. *Annu. Rev. Cell Biol.* **1990**, *6*, 329−357.
(29) Pierschbacher, M. D.; Ruoslahti, E. *Nature (London)* **1984**, *309*, 30−33.
(30) Frishman, W. H.; Burns, B.; Atac, B.; Alturk, N.; Altajar, B.; Lerrick, K. *Am. Heart. J.* **1995**, *130*, 877−892.
(31) Verstraete, M.; Zoldhelyi, P. *Drugs* **1995**, *49*, 856−884.
(32) Ojima, I.; Chakravarty, S.; Dong, Q. *Bioorg. Med. Chem.* **1995**, *3*, 337−360.
(33) Rodriguez, M.; Llinares, M.; Doulut, S.; Heitz, A.; Martinez, J. *Tetrahedron Lett.* **1991**, *32*, 923−926.
(34) Sarin, V. K.; Kent, S. B.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147−157.
(35) Kates, S. A.; Solé, N. A.; Johnson, C. R.; Hudson, D.; Barany, G.; Albericio, F. *Tetrahedron Lett.* **1993**, *34*, 1549−1552.
(36) Bernatowicz, M. S.; Wu, Y. L.; Matsueda, G. R. *J. Org. Chem.* **1992**, *57*, 2497−2502.
(37) Mehta, A.; Jaouhari, R.; Benson, T. J.; Douglas, K. T. *Tetrahedron Lett.* **1992**, *33*, 5441−5444.
(38) Cheng, S.; Craig, W. S.; Mullen, D.; Tschopp, J. F.; Dixon, D.; Pierschbacher, M. D. *J. Med. Chem.* **1994**, *37*, 1−8.
(39) Scarborough, R. M.; Naughton, M. A.; Teng, W.; Rose, J. W.; Phillips, D. R.; Nannizzi, L.; Arfsten, A.; Campbell, A. M.; Charo, I. F. *J. Biol. Chem.* **1993**, *268*, 1066−1073.
(40) Samanen, J.; Ali, F.; Romoff, T.; Calvo, R.; Sorenson, E.; Vasko, J.; Storer, B.; Berry, D.; Bennett, D.; Strohsacker, M.; Powers, D.; Stadel, J.; Nichols, A. *J. Med. Chem.* **1991**, *34*, 3114−3125.
(41) Craig, W. S.; Cheng, S.; Mullen, D. G.; Blevitt, J.; Pierschbacher, M. D. *Biopolymers (Peptide Science)* **1995**, *37*, 157−175.

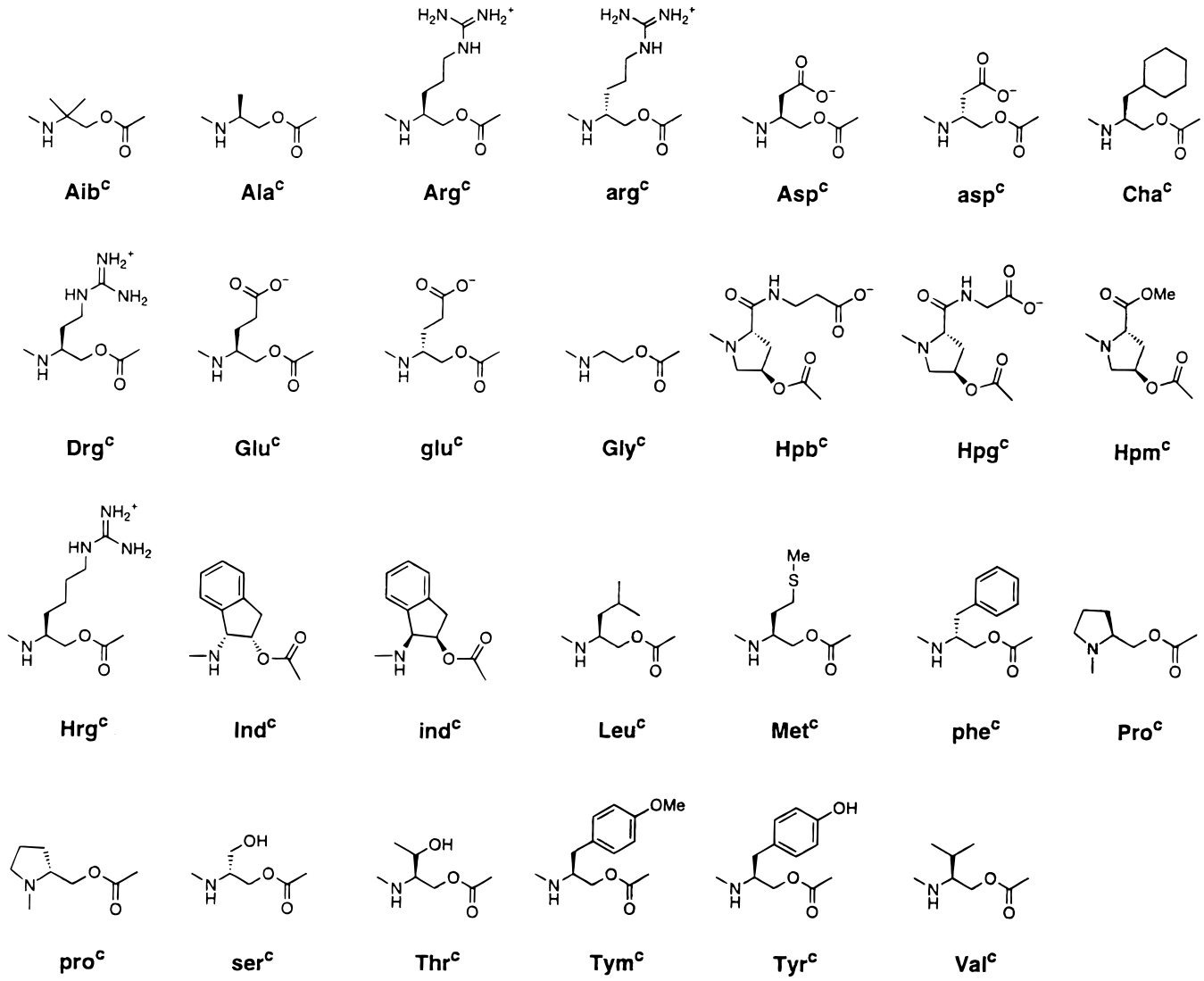


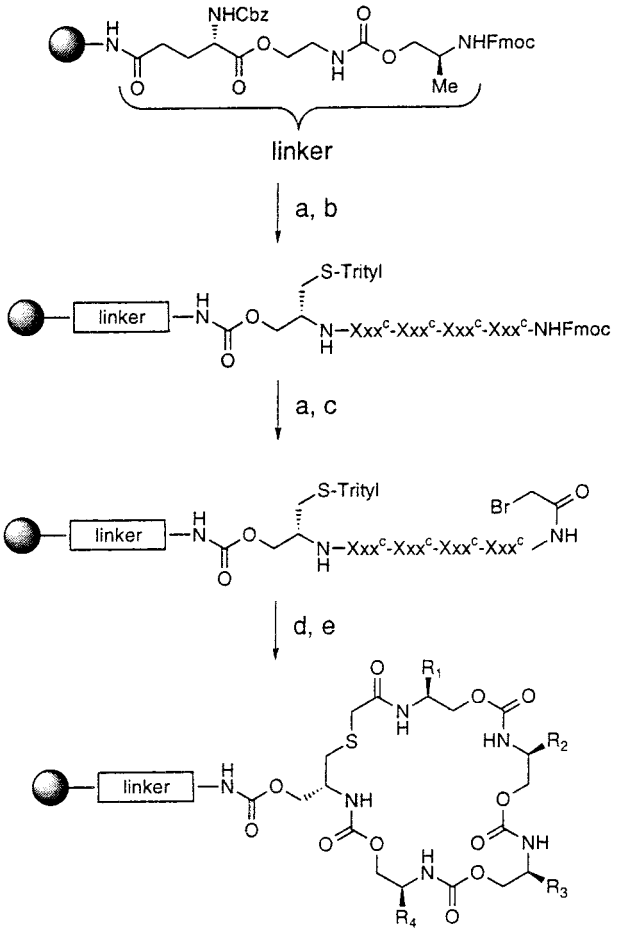
Figure 2. Monomers used in the synthesis of oligocarbamate libraries. Superscript "c" refers to the carbamate linkage.

tional constraint is built around an RGD or KGD tripeptide, a known pharmacophore. Cyclic, disulfide-linked peptides discovered through random screening of diverse libraries similarly contained RGD or KGD sequences.[47,48] In light of these studies with cyclic peptides, we decided to synthesize libraries of both cyclic and acyclic oligocarbamates.

A number of different strategies were considered for generating cyclic oligocarbamate libraries. In the past synthetic peptide libraries have been cyclized through disulfide bond formation[48−50] and thioether bond formation.[51] The efficient methods that have been developed for the resin bound cyclization of disulfide containing peptides[52] also proved to be effective for oligocarbamates (data not shown). However, use of disulfides as a means for conformational constraint may limit the range of receptors a combinatorial library can be screened against. Although GPIIb/IIIa is an extracellular receptor and therefore compatible with disulfide containing antagonists, intracellular targets in the reducing environment of the cytoplasm may not be compatible. Several methods and protecting group strategies have been developed for amide bond based cyclization of peptides.[53−57] One study, however, suggests that the efficiency

(42) Barker, P. L.; Bullens, S.; Bunting, S.; Burdick, D. J.; Chan, K. S.; Deisher, T.; Eigenbrot, C.; Gadek, T. R.; Gantzos, R.; Lipari, M. T.; Muir, C. D.; Napier, M. A.; Pitti, R. M.; Padua, A.; Quan, C.; Stanley, M.; Struble, M.; Tom, J. Y. K.; Burnier, J. P. *J. Med. Chem.* **1992**, *35*, 2040−2048.
(43) Stanley, M. S. *J. Org. Chem.* **1992**, *57*, 6421−6430.
(44) Lender, A.; Yao, W.; Sprengeler, P. A.; Spanevello, R. A.; Furst, G. T.; Hirschmann, R.; III, A. B. S. *Int. J. Pept. Prot. Res.* **1993**, *42*, 509−517.
(45) Jackson, S.; DeGrado, W.; Dwivedi, A.; Parthasarathy, A.; Higley, A.; Krywko, J.; Rockwell, A.; Markwalder, J.; Wells, G.; Wexler, R.; Mousa, S.; Harlow, R. *J. Am. Chem. Soc.* **1994**, *116*, 3220−3230.
(46) Bach, A. C.; Eyermann, C. J.; Gross, J. D.; Bower, M. J.; Harlow, R. L.; Weber, P. C.; DeGrado, W. F. *J. Am. Chem. Soc.* **1994**, *116*, 3207−3219.
(47) O'Neil, K. T.; Hoess, R. H.; Jackson, S. A.; Ramachandran, N. S.; Mousa, S. A.; DeGrado, W. F. *Proteins* **1992**, *14*, 509−515.
(48) Salmon, S. E.; Lam, K. S.; Lebl, M.; Kandola, A.; Khattri, P. S.; Wade, S.; Patek, M.; Kocis, P.; Krchnak, V.; Thorpe, D.; Felder, S. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11708−11712.
(49) McBride, J. D.; Freeman, N.; Domingo, G. J.; Leatherbarrow, R. J. *J. Mol. Biol.* **1996**, *259*, 819−827.
(50) Chen, J. K.; Lane, W. S.; Brauer, A. W.; Tanaka, A.; Schreiber, S. L. *J. Am. Chem. Soc.* **1993**, *115*, 12591−12592.
(51) Virgilio, A. A.; Bray, A. A.; Zhang, W.; Trinh, L.; Ellman, J. A. *Tetrahedron* **1997**, *53*, 6635−6644.
(52) Albericio, F.; Hammer, R. P.; Garcia-Echeverria, C.; Molins, M. A.; Chang, J. L.; Munson, M. C.; Pons, M.; Giralt, E.; Barany, G. *Int. J. Pept. Prot. Res.* **1991**, *37*, 402−413.
(53) Isied, S. S.; Kuehn, C. G.; Lyon, J. M.; Merrifield, R. B. *J. Am. Chem. Soc.* **1982**, *104*, 2632−2634.
(54) McMurray, J. S.; Lewis, C. A.; Obeyesekere, N. U. *Pept. Res.* **1994**, *7*, 195−206.

Scheme 1. Synthesis of Cyclic Oligocarbamates on Tentagel Resin[a]



[a] Reagents: (a) 20% piperidine/NMP; (b) Fmoc-amino-*p*-nitrophenyl carbonate/HOBt-H_2O/DIEA/NMP; (c) bromoacetic acid/DIPC/NMP; (d) 2% TFA/2% triethylsilane/CH_2Cl_2; (e) 5% *N*-methylmorpholine/NMP.

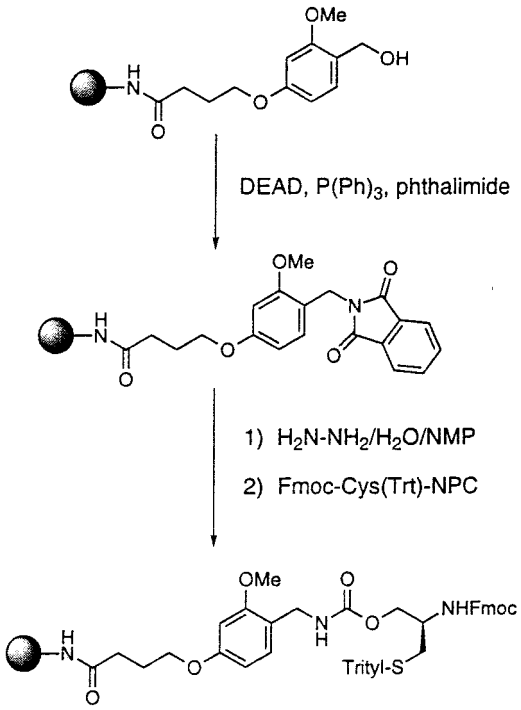
of amide-bond based cyclization is highly sequence dependent,[35] making the construction of a library problematic. We therefore chose to investigate cyclization of oligocarbamates through formation of a thioether bond.

Cyclization of peptides via a thioether bond can be performed through reaction of the side-chain thiol of a *C*-terminal cysteine residue with a bromoacetylated *N*-terminus, either in solution[42,58,59] or on solid support.[42,60] One method for cyclization on resin is accomplished by removing the trityl protecting group from Cys with 2% TFA/2% triethylsilane in dichloromethane (without cleaving the peptides from solid support[42]) followed by treatment of the resin with *N*-methylmorpholine in *N*,*N*-dimethylacetamide. We used a similar procedure for the solid-phase synthesis oligocarbamates, with some minor alterations (Scheme 1). Oligocarbamates were linked to solid support through a base cleavable alkyl ester linker **1** similar to the glutamate derivative used by Salmon et al.,[48] which is compatible with the synthesis and screening of oligocarbamate libraries. In addition, synthesis was carried out on a poly(ethylene glycol)-polystyrene graft copolymer, which has been used for the synthesis of one bead, one peptide libraries.[61,62] The efficiency of the cyclization procedure was monitored by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) on the product obtained from the cleavage of the ester linkage on single beads. MALDI-MS revealed no dimer formation or other side products. This synthetic scheme was then modified to make it compatible with sequencing by mass spectrometry (see below).

The preparative scale synthesis of soluble cyclic oligocarbamates with a *C*-terminal cysteine carbamate, however, proved to be more problematic. The solution-phase, basic aqueous conditions used for cyclization of peptides[42,58] could not be used for oligocarbamates since they are not soluble under these conditions, most likely due to the higher hydrophobicity of oligocarbamates in comparison to that of oligoamides. Resin derivatized with (4-((2′,4′-dimethoxyphenyl)aminomethyl)-phenoxy)acetic acid (Rink amide resin)[63] could not be used, since the oligocarbamate is cleaved from support under the conditions of trityl deprotection. A 5-(4′-aminomethyl-3′,5′-dimethoxyphenoxy)valeric acid handle (PAL) has been used under similar conditions in which a trimethoxybenzyl protecting group was removed from a cysteine while the peptide remained attached to solid support.[64] The *C*-terminal carbamate linkage, however, is considerably more acid labile than the corresponding amide. Upon treatment with 2% TFA/2% triethylsilane in methylene chloride, greater than 90% of the carbamate was cleaved from the PAL linker in minutes, as measured by quantitation of resin bound Fmoc groups.[65] We therefore synthesized cyclic carbamates using a 4-methyl benzhydrylamine resin (MBHA) derivatized with a 4-(4′-aminomethyl-3′-methoxyphenoxy)-butyric acid handle, which is less acid labile than the corresponding tris-alkoxy substituted system.[66] Rather than synthesizing the handle, we modified a commercially available 4-(4′-hydroxymethyl-3′-methoxyphenoxy)butyric acid (HMPB)-MBHA resin, originally developed for the synthesis of protected *C*-terminal acid fragments (Scheme 2).[67] The benzyl alcohol functionality was converted into an amine by formation of the phthalimide under Mitsunobu conditions[68,69] followed by deprotection with hydrazine-hydrate[16] and acylation with the *N*-Fmoc, *S*-trityl protected cysteine carbamate. The yield for three steps was 36% as determined by Fmoc quantitation,[65] based on the manufacturer's given loading of the HMPB-MBHA resin. Cleavage studies of this resin were conducted by monitoring for the presence of Fmoc groups. After 2 h treatment with 95% TFA/5% triethylsilane, greater than 90% of the carbamate was cleaved from the solid support, while less than 5% was cleaved

(55) Plaué, S. *Int. J. Pept. Prot. Res.* **1990**, *35*, 510−517.
(56) Schiller, P. W.; Nguyen, T. M. D.; Miller, J. *Int. J. Pept. Prot. Res.* **1985**, *25*, 171−177.
(57) Trzeciak, A.; Bannwarth, W. *Tetrahedron Lett.* **1992**, *33*, 4557−4560.
(58) Robey, F. A.; Fields, R. L. *Anal. Biochem.* **1989**, *177*, 373−377.
(59) Virgilio, A. A.; Schürer, S. C.; Ellman, J. A. *Tetrahedron Lett.* **1996**, *37*, 6961−6964.
(60) Virgilio, A. A.; Ellman, J. A. *J. Am. Chem. Soc.* **1994**, *116*, 11580−11581.
(61) Nikolaiev, V.; Stierandová, A.; Krchnák, V.; Seligmann, B.; Lam, K. S.; Salmon, S. E.; Lebl, M. *Pept. Res.* **1993**, *6*, 161−170.
(62) Youngquist, R. S.; Fuentes, G. R.; Lacey, M. P.; Keough, T. *Rapid Comm. Mass Spec.* **1994**, *8*, 77−81.
(63) Rink, H. *Tetrahedron Lett.* **1987**, *28*, 3787−3790.
(64) Munson, M. C.; Barany, G. *J. Am. Chem. Soc.* **1993**, *115*, 10203−10210.
(65) Fields, G. B.; Tian, Z.; Barany, G. In *Synthetic Peptides: A User's Guide*; Grant, G. A., Ed.; W. H. Freeman: New York, 1992; p 382.
(66) Albericio, F.; Barany, G. *Int. J. Pept. Prot. Res.* **1987**, *30*, 206−216.
(67) Flörsheimer, A.; Riniker, B. In *Peptides 1990: Proceedings of the Twenty-first European Peptide Symposium*; Giralt, E., Andreu, D., Eds.; Escom: Leiden, 1991.
(68) Krchnák, V.; Flegelová, Z.; Weichsel, A. S.; Lebl, M. *Tetrahedron Lett.* **1995**, *36*, 6193−6196.
(69) Mitsunobu, O. *Synthesis* **1981**, 1−28.

Scheme 2. Synthesis of 4-Aminomethyl-3-methoxyphenoxybutyric Acid-Linked Resin



after five 10 min treatments with 2%TFA/2% triethylsilane in methylene chloride, conditions sufficient for trityl group removal.

Cyclization on this resin, however, was far less efficient than on PEG-PS, both in terms of reaction time and purity of products. After overnight treatment with base, unreacted starting material was still observed, and unlike cyclization on PEG-PS, some dimer was formed. In addition, use of the bases *N*-methylmorpholine or tetramethylguanidine resulted in alkylation of the *N*-terminal bromoacetamide, based on MALDI mass spectral analysis of the crude products. The degree of alkylation was greatly reduced when the more hindered base diisopropylethylamine (DIEA) was used, although some unexplained side products were still present. The final conditions used for cyclization involved treatment of the trityl deprotected resin with 25% DIEA in *N*-methylpyrrolidone (NMP) for 24 h at 40 °C under nitrogen. The synthesis of cyclic oligocarbamate **2** (Cyclo(S)-Gly-Hpb^c-Tym^c-arg^c-Val^c-Cys^c-NH_2, Figure 3) is representative with an isolated overall yield of 26% for the purified product, based on the substitution level of the Fmoc-Cys^c(Trt)-derivatized resin. Figure 4 shows analytical HPLC traces of both the crude and purified products. This preparative scale synthesis may be improved by either attaching the HMPB linker to Tentagel resin or by using a *tert*-butyl disulfide protected thiol, as described by Virgilio and Ellman.[60] NMR spectroscopy was used to assign ^{1}H resonances for selected linear and cyclic oligocarbamates. Although the one-dimensional spectra were too complicated to interpret, most of the proton assignments could be made through analysis of DQF−COSY spectra,[70] and ambiguities in some of the sequences were resolved with the use of ROESY spectra. The proton assignments are given in the Experimental Section, and representative spectra are shown in the Supporting Information.

Library Synthesis. Three oligocarbamate libraries were synthesized for screening against GPIIb/IIIa. The first was a cyclic trimer library of the form Cyclo(S)-Gly-Xxx^c-Xxx^c-Xxx^c-Cys^c-NH_2 ("Xxx^c" referring to a randomized position) that used the 27 monomer units shown in Figure 2, resulting in 27^3 or 19 683 different compounds. The second library was a cyclic tetramer synthesized in the same fashion as the cyclic trimer library, except having an extra residue, resulting in 531 441 different compounds. The third library was a linear tetramer library of the form Ac^c-Xxx^c-Xxx^c-Xxx^c-Xxx^c-NH_2, which also used the 27 monomers in Figure 2. Although all of the libraries were constructed from the same building blocks, the structural frameworks of each are distinct and should sample nonoverlapping regions of conformational space.

Oligocarbamate libraries were synthesized on PEG-PS (Tentagel M, available from Advanced Chemtech) support to allow facile screening of ligands in aqueous buffers. The bead diameter was 35 μm compared with the more typical 88 μm, significantly simplifying screening procedures (1 g of 35 μm beads contains 45 million beads, 15-fold higher than if the diameter were 88 μm). The linker to the solid support should allow deprotection of the side-chain protecting groups without cleavage of the oligocarbamate from support yet also allow cleavage of ligands after screening for subsequent sequence identification by MALDI (see below). Earlier work with peptides involved a *C*-terminal methionine which can be cleaved with cyanogen bromide;[62] however, this deprotection procedure could potentially cleave a thioether bond in the cyclic oligocarbamates. We therefore used the alkyl ester linkage (Scheme 1) which is orthogonal to all steps of the synthesis and is cleaved in pH 11 aqueous ammonium hydroxide, leaving no salts upon removal of buffer in vacuo. After coupling of the linker to the resin, the amino acids Ser, Arg, Ser, and ϵ-aminohexanoic acid were coupled to support, providing both higher mass and an invariant positive charge, which simplified subsequent mass spectral analysis of the selected ligands.[62,71]

The libraries were generated using a divide and recombine approach as described by Furka *et al.*[72] In each coupling step, however, a small percentage of methyl-*p*-nitrophenyl carbonate was included along with the monomer, generating a small fraction of truncated oligomers for sequence determination (see below). For the synthesis of a linear tetramer carbamate library, the first randomized residue was coupled directly to the aminohexanoic amino acid, and the last randomized residue was capped with methyl-*p*-nitrophenyl carbonate. For the synthesis of the cyclic trimer and tetramer libraries, an invariant trityl-protected cysteine carbamate was coupled to the aminohexanoic acid linker, followed by randomized carbamate residues. The cyclic libraries were acylated with bromoacetic acid at the *N*-terminus, and cyclization was carried out as outlined in Scheme 1. Side-chain protecting groups were removed by treatment with 95% TFA/5% triethylsilane for 2 h, followed by extensive washes and neutralization with base.

Library Screening. A number of methods have been developed for the screening of large peptide libraries (for a review, see Gallop *et al.*[73]), involving either soluble ligands or ligands attached to solid support. Soluble libraries can be used in virtually any assay system but may be limited in terms of library size (ca. 10^4) or require lengthy deconvolution procedures.[74] Libraries tethered to insoluble supports often have the

(70) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; Wiley: New York, 1986.

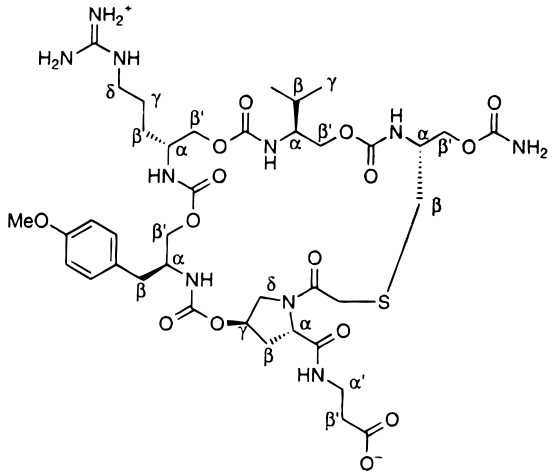
(71) Youngquist, R. S.; Fuentes, G. R.; Lacey, M. P.; Keough, T. *J. Am. Chem. Soc.* **1995**, *117*, 3900−3906.

(72) Furka, A.; Sebestyen, F.; Asgedom, M.; Dibo, G. *Int. J. Pept. Prot. Res.* **1991**, *37*, 487.

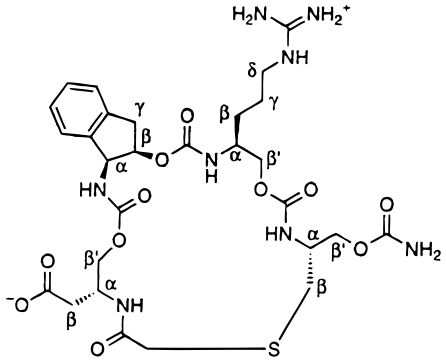
(73) Gallop, M. A.; Barrett, R. W.; Dower, W. J.; Fodor, S. P. A.; Gordon, E. M. *J. Med. Chem.* **1994**, *37*, 1233−1251.

(74) Houghten, R. A.; Pinilla, C.; Blondelle, S. E.; Appel, J. R.; Dooley, C. T.; Cuervo, J. H. *Nature* **1991**, *354*, 84−86.

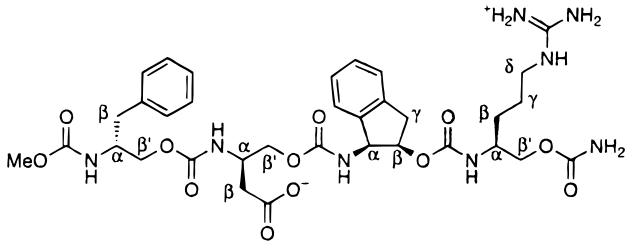
Carbamate **2**: Cyclo (S) Gly-Hpb^c-Tym^c-arg^c-Val^c-Cys^c-NH_2



Carbamate **3**: Cyclo (S) Gly-asp^c-ind^c-Arg^c-Cys^c-NH_2



Carbamate **21**: Ac^c-phe^c-asp^c-ind^c-Arg^c-NH_2



Carbamate **33**: Ac^c-Pro^c-Cha^c-Hrg^c-Gly^c-NH_2

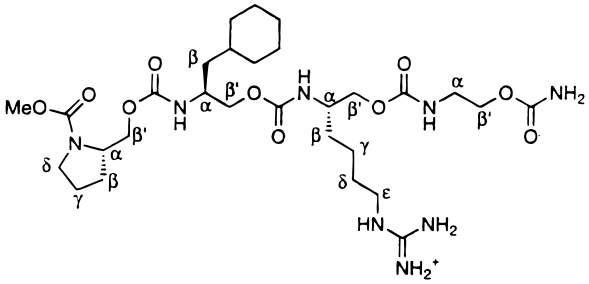


Figure 3. The structures of four oligocarbamates. The atoms are labeled as they are referred to in Tables 7−10 (^{1}H NMR assignments).

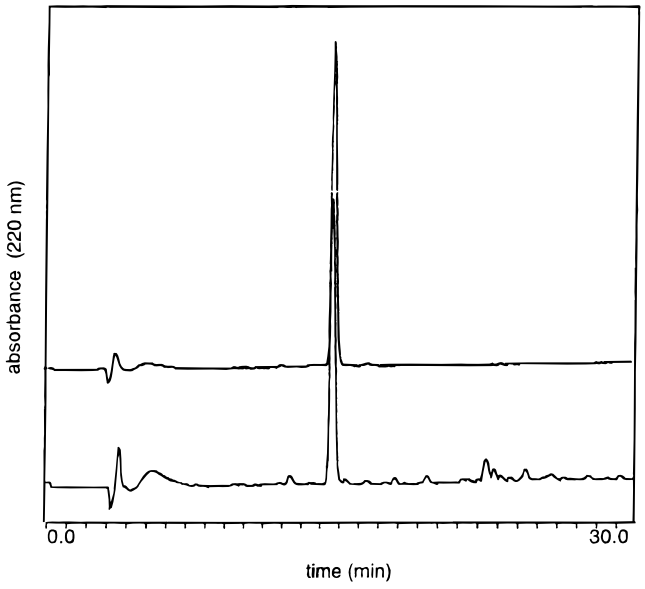


Figure 4. Analytical HPLC traces of carbamate **2** Cyclo(S)-Gly-Hpb^c-Tym^c-arg^c-Val^c-Cys^c-NH_2. The bottom trace is the crude product after precipitation; the top trace is the purified product. HPLC gradient: 25−45% B over 30 min (A: 0.1% TFA/H_2O, B: 0.08% TFA/CH_3CN), *l* = 220 nm.

advantage of more diversity (up to 10^7) but are generally screened for binding in ELISA type formats rather than directly for activity. Indeed, several peptide and non-peptide ligands for soluble proteins have been identified using this latter approach, including ligands for monoclonal antibodies, streptavidin, proteases, kinases, and SH3 domains. Because of the oligomeric nature of these carbamate constructs and the compatibility of GPIIb/IIIa with ELISA based assays, we chose to use a "one bead, one peptide" approach.[75]

To determine appropriate screening conditions for GPIIb/IIIa, the peptide ligand Cyclo(S)-Gly-D-Tyr-Arg-Gly-Asp-Cys- was synthesized on Tentagel resin as a positive control.[42] A mixture of beads containing either the control ligand or just the peptide linker itself was washed and treated with buffer containing 1% BSA, followed by incubation with GPIIb/IIIa. Binding of GPIIb/IIIa to beads was detected using the monoclonal antibody 4B12 that was conjugated with horseradish peroxidase. Although the peroxidase can be used as a marker for binding, better results were obtained by using a secondary polyclonal goat antibody (conjugated with alkaline phosphatase) specific for mouse antibody. Positive beads were stained upon incubation with standard alkaline phosphatase substrates as has been previously described.[71,75] Addition of soluble G4120, a high affinity ligand for GPIIb/IIIa,[42] during incubation of the beads with GPIIb/IIIa abolished binding, confirming that the observed interaction is specific. Buffers that contained detergent (Tween-20) and high concentrations of bovine serum albumin (10 mg/mL) were found to be essential to prevent nonspecific binding to the beads.

The three libraries were screened for GPIIb/IIIa binding at several receptor concentrations to vary the stringency of the assay. The cyclic trimer library consisted of approximately 2×10^4 distinct members, and approximately 6×10^4 beads were

(75) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *354*, 82−84.

screened at each of two receptor concentrations. At a receptor concentration of 720 pM, more than 100 beads were stained within 30 min, and 12 of these were picked for sequencing. To make the screen more stringent, the receptor concentration was reduced to 260 pM, and 10 beads were picked. Both tetramer libraries had approximately 5.3×10^5 members. The cyclic tetramer library was screened at 180, 360, and 720 pM receptor concentrations, with 1.5×10^5 beads screened at each concentration; seven positive beads were picked from each screen. Similarly, the linear tetramer library was screened at 180, 360, and 580 pM of GPIIb/IIIa, and 11, 13, and 14 positive beads were picked from the three screens, respectively. For each of the libraries, as the stringency of the screen increased, the extent of staining decreased to the point where the positive beads were barely discernible after a few hours of treatment with substrate.

MALDI Sequencing of Oligocarbamates. Since oligocarbamates cannot be sequenced via Edman degradation, a different means for determining the identity of positive ligands needed to be developed. Several sequencing procedures for combinatorial synthesis have been published, including use of "coding strands" of natural peptides,[61,76] parallel synthesis with oligonucleotides,[77,78] and indexing with molecular tags.[79] We instead chose to use a truncated synthesis approach involving sequence identification determined by matrix-assisted laser desorption ionization (MALDI) mass spectrometry, which was previously used in the screening of peptide libraries.[62,71] In this approach, a capping reagent is included with the monomer in each coupling step, resulting in a partial termination of the oligomeric chain. The products of the synthesis thus include the full length sequence and the ordered deletion sequences. The mixture of sequences is analyzed by MALDI mass spectrometry, and the sequence is determined from the differences in mass between the observed peaks, which correspond to the masses of the monomeric units.

Each of the beads from the screens was placed in a microcentrifuge tube, and the oligocarbamate was cleaved from support by treatment with ammonium hydroxide. After removal of buffer, the beads from the cyclic libraries were resuspended in 10 mM dithiothreitol to reduce any intermolecular cystine disulfides in the truncated sequences, while the beads from the linear library were suspended in aqueous TFA. One-third of the material from the beads was used for MALDI mass spectral analysis. Ninety-five percent of the beads could be sequenced, and if the most stringent linear tetramer screen is discounted, 99% of the beads were readable. The MALDI spectra of a representative cyclic trimer and a linear tetramer are shown in Figure 5(parts a and b, respectively). Some of the spectra, however, had peaks that could not be assigned to any monomer used in the library. For example, in the cyclic trimer series four of the spectra had a peak at 910, 112 mass units higher than the linker, which could not be assigned. In the linear series many spectra had "noise" in the mass range 860−885, or about 50−75 mass units higher than the linker. The sequences derived from each of the libraries are shown in Tables 1−3.

For several of the monomers (Arg, Asp, Glu, Ind, and Pro) both enantiomers were used in the synthesis of the library,

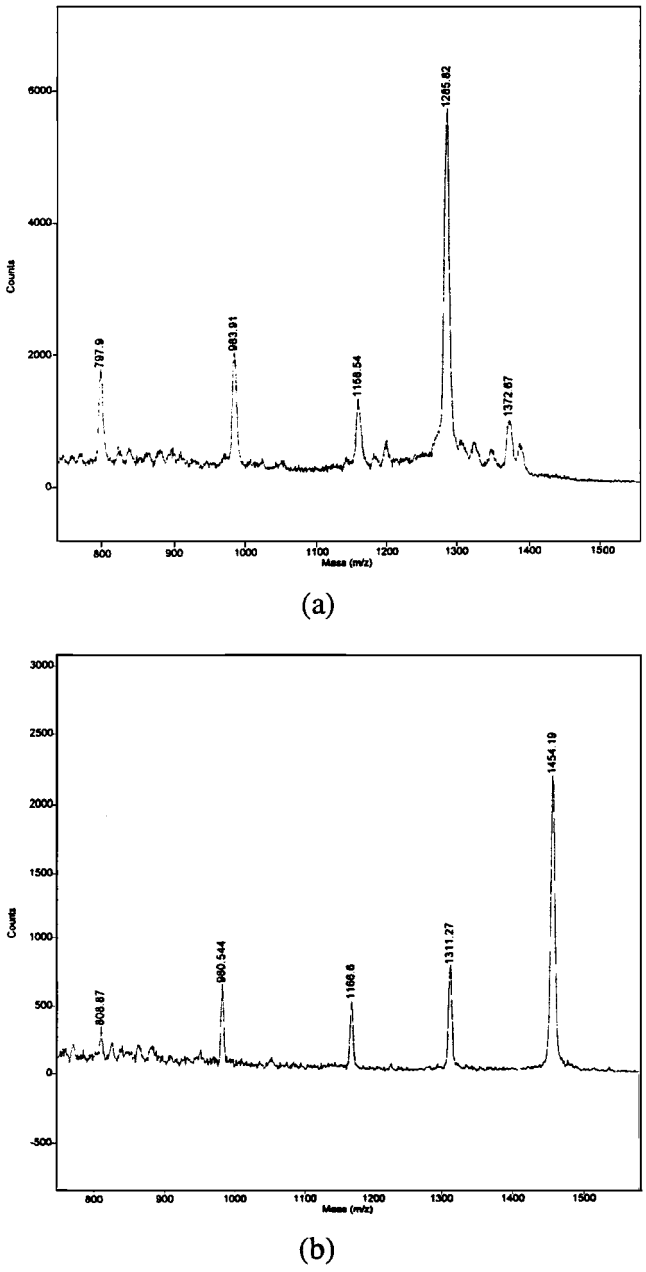


Figure 5. (a) MALDI mass spectrum of a carbamate from the cyclic trimer library from 33% of the total material. (b) MALDI mass spectrum of a carbamate from the linear tetramer library.

Table 1. Sequence Motifs from Oligocarbamate Cyclic Trimer Library

receptor concn (pM)	sequence			freq
Motif 1:	Cyclo(S)-Gly-asp^c-Xxx^c-Arg^c-Cys^c-NH_2			
720	asp^c	ind^c	Arg^c	3
260	asp^c	ind^c	Arg^c	4
720	asp^c	Arg^c	Arg^c	2
260	asp^c	Arg^c	Arg^c	2
720	Asp^c/asp^c	Hrg^c	Arg^c/arg^c	2
Motif 2:	Cyclo(S)-Gly-Hrg^c-Xxx^c-asp^c-Cys^c-NH_2			
720	Hrg^c	Arg^c	asp^c	3
260	Hrg^c	Hrg^c	asp^c	3
720	Hrg^c	Drg^c	Asp^c/asp^c	2

making it impossible to unambiguously determine the sequences of some members of the library by MALDI sequencing. Selective use of capping reagents allows one to differentiate

(76) Kerr, J. M.; Banville, S. C.; Zuckermann, R. N. *J. Am. Chem. Soc.* **1993**, *115*, 2529−2531.
(77) Nielsen, J.; Brenner, S.; Janda, K. D. *J. Am. Chem. Soc.* **1993**, *115*, 9812−9813.
(78) Needels, M. C.; Jones, D. G.; Tate, E. H.; Heinkel, G. L.; Kochersperger, L. M.; Dower, W. J.; Barrett, R. W.; Gallop, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10700−10704.
(79) Ohlmeyer, M. H. J.; Swanson, R. N.; Dillard, L. W.; Reader, J. C.; Asouline, G.; Kobayashi, R.; Wigler, M.; Still, W. C. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10922−10926.

Table 2. Sequence Motifs from Oligocarbamate Cyclic Tetramer Library

receptor concn (pM)	sequence			
Motif 3: Cyclo(S)-Gly-Xxx^c-asp^c-Xxx^c-Arg^c-Cys^c-NH_2				
720	Ala^c	asp^c	Drg^c	Arg^c
360[a]	Pro^c	asp^c	Arg^c	Arg^c
	pro^c	asp^c	Arg^c	Arg^c
180	Drg^c	asp^c	ind^c	Arg^c
720	Aib^c	Asp^c/asp^c	Arg^c/arg^c	Arg^c/arg^c
720	Pro^c/pro^c	Asp^c/asp^c	Hrg^c	Arg^c/arg^c
180	Val^c	Asp^c/asp^c	Drg^c	Arg^c/arg^c
180	Ala^c	Asp^c/asp^c	Arg^c/arg^c	Arg^c/arg^c
180	Pro^c/pro^c	Asp^c/asp^c	Hrg^c	Arg^c/arg^c
180	Gly^c	Asp^c/asp^c	Hrg^c	Arg^c/arg^c
Motif 4: Cyclo(S)-Gly-Xxx^c-Hrg^c-Xxx^c-asp^c-Cys^c-NH_2				
360	phe^c	Hrg^c	Hrg^c	asp^c
360	ser^c	Hrg^c	Hrg^c	Asp^c/asp^c
360	Arg^c/arg^c	Hrg^c	Hrg^c	Asp^c/asp^c
180	Hpm^c	Hrg^c	Arg^c/arg^c	Asp^c/asp^c
Motif 5: Cyclo(S)-Gly-Xxx^c-arg^c-asp^c-Hrg^c-Cys^c-NH_2				
720	Val^c	arg^c	asp^c	Hrg^c
720	Pro^c/pro^c	Arg^c/arg^c	Asp^c/asp^c	Hrg^c
720	Arg^c/arg^c	Arg^c/arg^c	Asp^c/asp^c	Hrg^c
Motif 6: Cyclo(S)-Gly-asp^c-Hrg^c-Xxx^c-Xxx^c-Cys^c-NH_2				
360	asp^c	Hrg^c	Arg^c	Gly^c
180	Asp^c/asp^c	Hrg^c	Hrg^c	Pro^c/pro^c
"Orphan" Sequences: Cyclo(S)-Gly-Xxx^c-Xxx^c-Xxx^c-Xxx^c-Cys^c-NH_2				
720	Arg^c	Ala^c	Hrg^c	asp^c
360	Pro^c/pro^c	Asp^c/asp^c	Arg^c/arg^c	Drg^c

[a] Sequence occurred twice, and both diastereomers found to be active.

Table 3. Sequence Motifs from Oligocarbamate Linear Tetramer Library

receptor concn (pM)	sequence			
Motif 7: Ac^c-(Xxx^c)-asp^c-Xxx^c-Arg^c-(Xxx^c)-NH_2				
580		asp^c	Arg^c	Arg^c pro^c
580	phe^c	asp^c	ind^c	Arg^c
360[a]		asp^c	Pro^c	Arg^c Arg^c
360	Ala^c	asp^c	Hrg^c	Arg^c
180		asp^c	Val^c	Arg^c Hrg^c
580	Pro^c/pro^c	Asp^c/asp^c	Drg^c	Arg^c/arg^c
580		Asp^c/asp^c	Aib^c	Arg^c/arg^c Hrg^c
360	Thr^c	Asp^c/asp^c	Hrg^c	Arg^c/arg^c
360	Hrg^c	Asp^c/asp^c	Ind^c/ind^c	Arg^c/arg^c
360		Asp^c/asp^c	Thr^c	Arg^c/arg^c Hrg^c
360	phe^c	Asp^c/asp^c	Hrg^c	Arg^c/arg^c
180	Arg^c/arg^c	Asp^c/asp^c	Drg^c	Arg^c/arg^c
Motif 8: Ac^c-(Xxx^c)-asp^c-Xxx^c-Hrg^c-(Xxx^c)-NH_2				
580		asp^c	Pro^c	Hrg^c Pro^c
580	arg^c	asp^c	ind^c	Hrg^c
580	Tyr^c	Asp^c/asp^c	Hrg^c	Hrg^c Arg^c/arg^c
580		Asp^c/asp^c	Aib^c	Hrg^c
580		Asp^c/asp^c	Arg^c/arg^c	Hrg^c Arg^c/arg^c
580	Arg^c/arg^c	Asp^c/asp^c	Hrg^c	Hrg^c
360	Hrg^c	Asp^c/asp^c	Arg^c/arg^c	Hrg^c
360	Hrg^c	Asp^c/asp^c	Ala^c	Hrg^c
Motif 9: Ac^c-(Xxx^c)-asp^c-Xxx^c-Drg^c-(Xxx^c)-NH_2				
580[a]		asp^c	Pro^c	Drg^c Hrg^c
580		Asp^c/asp^c	Ind^c/ind^c	Drg^c Arg^c/arg^c
360	Leu^c	Asp^c/asp^c	Arg^c/arg^c	Drg^c
Motif 10: Ac^c-Xxx^c-Xxx^c-asp^c-Hrg^c-NH_2				
180[a,b]	Arg^c	Arg^c	asp^c	Hrg^c
	Arg^c	arg^c	asp^c	Hrg^c
360	Hrg^c	Leu^c	Asp^c/asp^c	Hrg^c
180	Arg^c/arg^c	Hrg^c	Asp^c/asp^c	Hrg^c
180	Hrg^c	Arg^c/arg^c	Asp^c/asp^c	Hrg^c
Motif 11: Ac^c-Arg^c-Arg^c-Hrg^c-asp^c-NH_2				
360[a,b]	Arg^c	Arg^c	Hrg^c	asp^c
	arg^c	Arg^c	Hrg^c	asp^c
180[a,b]	Arg^c	Arg^c	Hrg^c	asp^c
	arg^c	Arg^c	Hrg^c	asp^c
Motif 12: Ac^c-Hpg^c-Xxx^c-Xxx^c-Xxx^c-NH_2				
180	Hpg^c	ind^c	Arg^c	Drg^c
360	Hpg^c	Tym^c	Drg^c	Arg^c/arg^c
180	Hpg^c	Ind^c/ind^c	Drg^c	Drg^c

[a] Sequence occurred twice. [b] Both diastereomers found to be active.

isomers;[71] however, only one capping reagent was used in these libraries to simplify the mass spectra. To determine the chirality of monomers in sequences where it was ambiguous, each diastereomer of the parent sequence was resynthesized on Tentagel resin. For each diastereomer, a different and characteristic linker of amino acids was used to attach the sequence to resin, so that cleavage of different diastereomers of the same sequence would give a different mass. The beads containing the different diastereomers with different mass linkers were recombined, and the pool was screened for GPIIb/IIIa binding using the same procedure as used for the original libraries. The stained beads were picked, and the mass of the product was determined by MALDI-MS, allowing assignment of the active diastereomer. Thus, in Tables 1−3, sequences for which the correct diastereomer was determined show a single diastereomer; in a few instances, however, the deconvolution screen revealed two active diastereomers and both are shown. For the remaining sequences where the active diastereomer was not determined, both enantiomers of the monomer are shown.

Sequence Motifs of Oligocarbamate GPIIb/IIIa Ligands. The ligands identified from the GPIIb/IIIa screens are organized into several well-defined consensus sequences or motifs which contain identical residues at two or more sites in the sequence (Tables 1−3). The number of distinct consensus sequences for each framework (linear tetramer, cyclic trimer, and cyclic tetramer) increases with increasing conformational flexibility. Whereas the cyclic trimer sequences fall into two motifs, the cyclic tetramer sequences have four and the linear tetramer sequences have six motifs. The motifs overlap between frameworks, with the cyclic trimer motifs present in all three libraries, while the cyclic tetramer motifs are mostly present in the linear tetramer library. These results suggest that the more flexible acyclic molecules can access the binding conformations of the conformationally constrained molecules in addition to conformations that cannot be accessed by the cyclic molecules.

All of the ligands possess both guanidinium and carboxylate groups, as would be expected. Except for a few of the linear tetramer sequences which contained the **Hpg^c** monomer (motif 12), all sequences contained **asp^c** as the carboxylate functionality, in contrast to peptides, which have L-Asp in active sequences. In addition, many of the sequences contain multiple guanidinium-containing monomers, suggesting a second guanidinium binding site, consistent with a model proposed by Cheng *et al.*[38] It is unlikely that the presence of multiple guanidium-containing monomers is an artifact of ligand presentation on the bead during the screening process, since hydrophilic, carboxylate-containing monomers aside from **asp^c** were absent.

Binding Properties of Oligocarbamate Ligands. Several of the ligands from the three libraries were synthesized and assayed for their ability to inhibit the binding of solubilized GPIIb/IIIa to fibrinogen in an ELISA format. The assays were performed as described[42] using G4120 (Cyclo(S)-Gly-D-Tyr-Asp-Gly-Arg-Cys-OH) as a positive control, a peptide benchmark for comparison to oligocarbamates. The IC_{50} values for

Table 4. Inhibition of GPIIb/IIIa-Fibrinogen Binding by Cyclic Trimer Oligocarbamates

carbamate	sequence	ELISA IC_{50}, nM
	G4120	1.5
	kistrin	2.0
3	Cyclo(S)-Gly-asp^c-ind^c-Arg^c-Cys^c-NH_2	3.9 ± 1.3
4	Cyclo(S)-Gly-asp^c-ind^c-Arg^c-Cys-NH_2	27 ± 8
5	Cyclo(S)-Gly-Hrg^c-Hrg^c-asp^c-Cys^c-NH_2	33 ± 4
6	Cyclo(S)-Gly-asp^c-Arg^c-Arg^c-Cys^c-NH_2	41 ± 3
7	Cyclo(S)-Gly-asp^c-Leu^c-Arg^c-Cys^c-NH_2	64 ± 10
8	Cyclo(S)-Gly-Hrg^c-Arg^c-asp^c-Cys^c-NH_2	120 ± 20
9	Cyclo(S)-Gly-asp^c-Ala^c-Arg^c-Cys^c-NH_2	170 ± 40
10	Cyclo(S)-Gly-asp^c-Leu^c-Arg^c-Cys-OH	700 ± 210
11	Cyclo(S)-Gly-Asp^c-Arg^c-Arg^c-Cys^c-NH_2	$>10^5$
12	Cyclo(S)-Gly-Asp^c-Leu^c-Arg^c-Cys^c-NH_2	$>10^5$
13	Cyclo(S)-Gly-Asp^c-Ala^c-Arg^c-Cys^c-NH_2	$>10^5$

Table 5. Inhibition of GPIIb/IIIa-Fibrinogen Binding by Cyclic Tetramer Oligocarbamates

carbamate	sequence	ELISA IC_{50}, nM
	G4120	1.5
	kistrin	2.0
14	Cyclo(S)-Gly-phe^c-Hrg^c-Hrg^c-asp^c-Cys^c-NH_2	4.9 ± 0.6
15	Cyclo(S)-Gly-Arg^c-Ala^c-Hrg^c-asp^c-Cys^c-NH_2	31 ± 5
16	Cyclo(S)-Gly-Val^c-arg^c-asp^c-Hrg^c-Cys^c-NH_2	100 ± 20
17	Cyclo(S)-Gly-Ala^c-asp^c-Drg^c-Arg^c-Cys^c-NH_2	240 ± 50
18	Cyclo(S)-Gly-pro^c-asp^c-Arg^c-Arg^c-Cys^c-NH_2	260 ± 40
19	Cyclo(S)-Gly-asp^c-Hrg^c-Arg^c-Gly^c-Cys^c-NH_2	330 ± 30
20	Cyclo(S)-Gly-Drg^c-asp^c-ind^c-Arg^c-Cys^c-NH_2	350 ± 10

Table 6. Inhibition of GPIIb/IIIa-Fibrinogen Binding by Linear Tetramer Oligocarbamates

carbamate	sequence	ELISA IC_{50}, nM
	G4120	1.5
	kistrin	2.0
21	Ac^c-phe^c-asp^c-ind^c-Arg^c-NH_2	13 ± 1
22	Ac^c-asp^c-Pro^c-Arg^c-Arg^c-NH_2	25 ± 6
23	Ac^c-arg^c-asp^c-ind^c-Hrg^c-NH_2	120 ± 20
24	Ac^c-asp^c-Val^c-Arg^c-Hrg^c-NH_2	140 ± 40
25	Ac^c-Ala^c-asp^c-Hrg^c-Arg^c-NH_2	230 ± 100
26	Ac^c-asp^c-Pro^c-Hrg^c-Pro^c-NH_2	240 ± 30
27	Ac^c-Hpg^c-ind^c-Arg^c-Drg^c-NH_2	260 ± 60
28	Ac^c-Arg^c-Arg^c-Hrg^c-asp^c-NH_2	430 ± 90
29	Ac^c-asp^c-Pro^c-Drg^c-Hrg^c-NH_2	460 ± 40
30	Ac^c-asp^c-Arg^c-Arg^c-Pro^c-NH_2	470 ± 160
31	Ac^c-Arg^c-arg^c-asp^c-Hrg^c-NH_2	1300 ± 400
32	Ac^c-Arg^c-Arg^c-asp^c-Hrg^c-NH_2	1800 ± 500

each independent determination were normalized against that of G4120, which has been previously determined to be 1.5 nM. The results are shown in Tables 4−6.

The cyclic trimer library contained only two consensus motifs, Cyclo(S)-Gly-asp^c-Xxx^c-Arg^c-Cys^c-NH_2 and Cyclo(S)-Gly-Hrg^c-Xxx^c-asp^c-Cys^c-NH_2. The ligand Cyclo(S)-Gly-asp^c-ind^c-Arg^c-Cys^c-NH_2 **3** (Figure 3) from motif 1 bound the receptor with high affinity ($IC_{50} = 3.9$ nM), approximately 3-fold weaker than G4120 and within a factor of 2 of kistrin, a naturally occurring snake venom protein that is a potent inhibitor of platelet aggregation.[80] Carbamate **3** contains the rigid aminoindanol based monomer, which may account for its high binding affinity. Consistent with this notion, replacement of the **ind^c** residue with **Arg^c** (Cyclo(S)-Gly-asp^c-Arg^c-Arg^c-Cys^c-NH_2, carbamate **6**) reduces binding affinity by approximately 10-fold. To further examine the importance of this central residue, we synthesized and assayed ligands **7** (Cyclo(S)-Gly-asp^c-Leu^c-Arg^c-Cys^c-NH_2) and **9** (Cyclo(S)-Gly-asp^c-Ala^c-Arg^c-Cys^c-NH_2), sequences that were not found among the positive beads in the library. Both ligands bound the receptor with lower affinity (15 and 45-fold, respectively) than **3**; however, **7** was bound only 1.5-fold less tightly than **6**. The second motif, Cyclo(S)-Gly-Hrg^c-Xxx^c-asp^c-Cys^c-NH_2, was present in fewer numbers among selected sequences than the first motif, and carbamates **5** (Cyclo(S)-Gly-Hrg^c-Hrg^c-asp^c-Cys^c-NH_2, IC_{50} 33 nM) and **8** (Cyclo(S)-Gly-Hrg^c-Arg^c-asp^c-Cys^c-NH_2, IC_{50} 120 nM) bound less tightly to receptor than did carbamate **3**. Under more stringent screening conditions, only carbamate **5** was found for motif 2.

The binding determinants of the Cyclo(S)-Gly-asp^c-Xxx^c-Arg^c-Cys^c-NH_2 motif were investigated further by examining the importance of the *C*-terminus and the stereochemistry of the D-aspartate carbamate monomer. Replacement of the *C*-terminal carbamate with an amide resulted in a 6.9-fold loss in activity (**3** vs **4**), while replacement with the less conservative carboxylate resulted in an 11-fold decrease in activity (**7** vs **10**). The preference for the *C*-terminal carbamate is not surprising, since the library had been synthesized with a carbamate linkage to solid support. This preference is in contrast to cyclic thioether peptides, where the highest affinity ligands have a *C*-terminal carboxylate.[42] In addition, as suggested by the sequence data, the **asp^c** is essential for activity; replacement with **Asp^c** results in at least a 500-fold reduction in binding affinity (carbamates **11**−**13**). A similar degree of specificity is seen in RGD peptides, where Cyclo(S,S)-CRGDC has a 100-fold higher affinity than Cyclo(S,S)-CRGdC.[48]

While motif 1 possesses both guanidinium and carboxylate moieties, as expected for a ligand for GPIIb/IIIa, the particular sequence could not have been predicted. In peptides containing the RGD tripeptide sequence, the guanidinium and carboxylate groups are separated by 11 atoms. In the -asp^c-Xxx^c-Arg^c-carbamate trimer, however, the two groups are separated by 15 atoms; in addition, the number of atoms between the backbone and the side-chain functional group is the same for the carbamate as it is for the peptide, and thus the increased distance between the functional groups (four atoms) is along the backbone. The conformational properties of the carbamate backbone are therefore distinct, perhaps allowing a "bulge" in the backbone region connecting the recognition monomers compared to the peptide backbone in RGD peptides. NMR studies of cyclic peptides that have high affinity for GPIIb/IIIa have shown that the Gly residue in RGD peptides is in an extended conformation.[46] In addition, the Arg C_β-Asp C_β distance in another cyclic peptide has been measured as being between 7.5 and 8.5 Å.[81] Thus GPIIb/IIIa recognizes peptides that are in a relatively extended conformation, providing an explanation for the comparatively large distance between the guanidinium and carboxylate groups in the carbamate ligands.

In the case of the cyclic tetramer library (Table 2), motif 3 (Cyclo(S)-Gly-Xxx^c-asp^c-Xxx^c-Arg^c-Cys^c-NH_2) has the most members and is analogous to motif 1 (Cyclo(S)-Gly-asp^c-Xxx^c-Arg^c-Cys^c-NH_2) of the cyclic trimers. The three ligands that were synthesized and assayed (carbamates Cyclo(S)-Gly-Ala^c-asp^c-Drg^c-Arg^c-Cys^c-NH_2 **17**, Cyclo(S)-Gly pro^c-asp^c-Arg^c-Arg^c-Cys^c-NH_2 **18**, and Cyclo(S)-Gly-Drg^c-asp^c-ind^c-Arg^c-Cys^c-NH_2 **20**) from this class, however, had only moderate binding affinity, with IC_{50}'s ranging from 240 to 350 nM (Table 5). Motif 4 (Cyclo(S)-Gly-Xxx^c-Hrg^c-Xxx^c-asp^c-Cys^c-NH_2) of the cyclic

(80) Dennis, M. S.; Henzel, W. J.; Pitti, R. M.; Lipari, M. T.; Napier, M. A.; Deisher, T. A.; Bunting, S.; Lazarus, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *87*, 2471−2475.

(81) Pfaff, M.; Tangemann, K.; Müller, B.; Gurrath, M.; Müller, G.; Kessler, H.; Timple, R.; Engel, J. *J. Biol. Chem.* **1994**, *269*, 20233−20238.

tetramers is analogous to motif 2 (Cyclo(S)-Gly-Hrg^c-Xxx^c-asp^c-Cys^c-NH_2), whose ligands had moderate affinity. However, in this case the cyclic tetramer **14** (Cyclo(S)-Gly phe^c-Hrg^c-Hrg^c-asp^c-Cys^c-NH_2, IC_{50} 4.9 nM) binds the receptor with 6.7-fold higher affinity than its cyclic trimer counterpart, carbamate **5** (Cyclo(S)-Gly-Hrg^c-Hrg^c-asp^c-Cys^c-NH_2), and within a factor of 4 of G4120. Thus, although the cyclic trimer is presumably more constrained than the cyclic tetramer (21 vs 26 atoms in the ring), the cyclic tetramer version of the -Hrg^c-Xxx^c-asp^c- has higher affinity. In contrast, the smaller ring size resulted in higher affinity for the members of motif 1 (Cyclo(S)-Gly-asp^c-Xxx^c-Arg^c-Cys^c-NH_2) compared to motif 3 (Cyclo(S)-Gly-Xxx^c-asp^c-Xxx^c-Arg^c-Cys^c-NH_2). The conformational framework of the cyclic trimer is apparently best suited for the -asp^c-Xxx^c-Arg^c- motif, while the cyclic tetramer framework seems best suited for the Hrg^c-Xxx^c-asp^c- motif. The nature of the respective cyclic frameworks that would cause the cyclic trimer to favor a $C \rightarrow N$ orientation of guanidinium to carboxylate compared to $N \rightarrow C$ orientation for the cyclic tetramer is unclear. Such variation in orientation of peptide binding to GPIIb/IIIa has not been observed, although most studies of cyclic peptide ligands have varied frameworks and monomers around a constant -Arg-Gly-Asp-tripeptide.

The other motifs from the cyclic tetramer library do not have cyclic trimer analogues and have adjacent **Hrg^c** and **asp^c** monomers in their sequences. This pair of monomers have the same number of atoms between the guanidinium and carboxylate functionality as the RGD tripeptide, and earlier work has shown simple molecules having the appropriate number of atoms in a spacer can have modest affinity for GPIIb/IIIa.[82,83] When the -asp^c-Hrg^c- dimer is in the first two positions of the cyclic tetramer, as in motif 6 (Cyclo(S)-Gly-asp^c-Hrg^c-Xxx^c-Xxx^c-Cys^c-NH_2), the IC_{50} is 330 nM (carbamate **19**, Cyclo(S)-Gly-asp^c-Hrg^c-Arg^c-Gly^c-Cys^c-NH_2); the affinity increases by a factor of 3 when the dimer is in the last two positions (carbamate **16**, motif 5, Cyclo(S)-Gly-Val^c-arg^c-asp^c-Hrg^c-Cys^c-NH_2). In carbamate **15** (Cyclo(S)-Gly-Arg^c-Ala^c-Hrg^c-asp^c-Cys^c-NH_2), which is an "orphan" sequence that does not fall into any motif, -Hrg^c-asp^c is in the middle two positions. The affinity of carbamate **15** is surprisingly high (IC_{50} 31 nM), given that it is unique among the sequences. Thus, the large range in binding affinities observed in these ligands suggests that the neighboring residues and the cyclic carbamate framework play a significant role in binding to the receptor.

The range of binding affinities, and the number of sequence motifs, is considerably larger for the ligands from the linear tetramer library. The two highest affinity ligands belong to motif 7 (Ac^c-(Xxx^c)-asp^c-Xxx^c-Arg^c-(Xxx^c)-NH_2), which has the most members, and corresponds to the analogous motifs 1 and 3 in the cases of the cyclic trimer and tetramer libraries, respectively. Carbamate **21** (Ac^c-phe^c-asp^c-ind^c-Arg^c-NH_2, IC_{50} 13 nM) and carbamate **22** (Ac^c-asp^c-Pro^c-Arg^c-Arg^c-NH_2, IC_{50} 25 nM) which have conformationally constrained monomers between **asp^c** and **Arg^c** have high affinities for GPIIb/IIIa. The other members of motif 7 (carbamates **24**, **25**, and **30**) which lacked conformationally constrained monomers bind considerably less well with IC_{50} values in the range of 140−470 nM. Motifs 8 (Ac^c-(Xxx^c)-asp^c-Xxx^c-Hrg^c-(Xxx^c)-NH_2) and 9 (Ac^c-(Xxx^c)-asp^c-Xxx^c-Drg^c-(Xxx^c)-NH_2) are similar in form to motif 7 but have different distances between the side-chain guanidinium group and the carbamate backbone. Carbamate **23** (Ac^c-arg^c-asp^c-ind^c-Hrg^c-NH_2, IC_{50} 120 nM) binds 9-fold less tightly than the analogous sequence **21**, and carbamates **26** (Ac^c-asp^c-Pro^c-Hrg^c-Pro^c-NH_2, IC_{50} 240 nM) and **29** (Ac^c-asp^c-Pro^c-Drg^c-Hrg^c-NH_2, IC_{50} 460 nM) bind less tightly than the analogous sequence **22**. These examples indicate that the optimal spacing between the backbone and the guanidinium group is three methylene groups in the context of the motifs 7−9. Motifs 10−12 are represented by carbamates **27**, **28**, **31**, and **32**, which all bind receptor with moderate or low affinity.

Comparison of Cyclic and Linear Oligocarbamates. Several groups have reported that cyclization of peptides improves their binding affinity to GPIIb/IIIa compared to their linear counterparts, presumably due to entropic effects associated with binding of ligand to the receptor. A similar overall trend is observed when comparing cyclic and linear oligocarbamates, although the magnitude of the effect varies widely and depends on sequence and whether the framework is a cyclic trimer or tetramer. In addition, the comparison of cyclic and linear forms is of course indirect, since flanking residues in the linear form not present in the cyclic trimers or altered in the cyclic tetramer may have an important influence on binding.

The highest affinity linear ligand has the -asp^c-ind^c-Arg^c- sequence (Ac^c-phe^c-asp^c-ind^c-Arg^c-NH_2, **21**, IC_{50} 13 nM). Cyclization of the -asp^c-ind^c-Arg^c- sequence in the context of the cyclic trimer results in a ligand that binds 3.4-fold more tightly (Cyclo(S)-Gly-asp^c-ind^c-Arg^c-Cys^c-NH_2, **3**, IC_{50} 3.9 nM). When the -asp^c-ind^c-Arg^c- sequence is placed in the context of the cyclic tetramer, however, the opposite effect is seen: carbamate **20** (Cyclo(S)-Gly-Drg^c-asp^c-ind^c-Arg^c-Cys^c-NH_2, IC_{50} 350 nM) binds 27-fold less tightly than the linear sequence **21**. Thus cyclization in itself does not improve affinity; ring size and sequence play a key role. In other cases, the effect of cyclization may be sufficiently large that the corresponding linear analogue was not identified in the screen, since the sequence motif -Hrg^c-Xxx^c-asp^c- was not found among the linear ligands. These results underscore the importance of synthesizing diverse combinatorial libraries with several distinct frameworks to find high affinity ligands. Knowledge of a linear, oligomeric pharmacophore cannot necessarily be translated into a higher affinity cyclic molecule.

Interpretation of the bound conformation of the high affinity cyclic oligocarbamates is difficult, since the solution structures of ligands **3** and **4** have not been determined, and no structure of GPIIb/IIIa is known. A model for the ligand binding domain of β_3, however, has been built,[84] and the site of interaction in α_{IIb} has been determined.[85] Furthermore, the solution-phase structure of several peptides have been solved by NMR. NMR studies on G4120 (a cyclic thioether based molecule)[86] and the cyclic peptide Cyclo[GRGDfV] (formed through a head-to-tail amide cyclization)[81] show that both classes of high affinity cyclic peptides adopt a type-II′ β turn in solution, although with different registers in sequence. The cyclic oligocarbamates **3** and **4** may thus be mimicking a type-II′ β turn. Further comparisons between the cyclic oligocarbamates and cyclic peptides can also be made based on sequence. The structure of G4120 shows a "hydrophobic moment" in the molecule, which McDowell and Gadek suggest may enhance binding by

(82) McDowell, R. S.; Gadek, T. R.; Barker, P. L.; Burdick, D. J.; Chan, K. S.; Quan, C. L.; Skelton, N.; Struble, M.; Thorsett, E. D.; Tischler, M.; Tom, J. Y. K.; Webb, T. R.; Burnier, J. P. *J. Am. Chem. Soc.* **1994**, *116*, 5069−5076.

(83) Greenspoon, N.; Hershkoviz, R.; Alon, T.; Varon, D.; Shenkman, B.; Marz, G.; Federman, S.; Kapustina, G.; Lider, O. *Biochemistry* **1993**, *32*, 1001−1008.

(84) Tozzer, E. C.; Liddington, R. C.; Sutcliffe, M. J.; Smeeton, A. H.; Loftus, J. C. *J. Biol. Chem.* **1996**, *271*, 21978−21984.

(85) Kamata, T.; Irie, A.; Tokuhira, M.; Takada, Y. *J. Biol. Chem.* **1996**, *271*, 18610−18615.

(86) McDowell, R. S.; Gadek, T. R. *J. Am. Chem. Soc.* **1992**, *114*, 9245−9253.

shielding the guanidinium and carboxylate groups from bulk solvent, strengthening the ionic interaction with GPIIb/IIIa.[86] Moreover, Cheng *et al*. postulate a hydrophobic binding pocket in the receptor based on the high affinity of their cyclic disulfide peptide, Cyclo(S,S)-Ac−(D-Pen)-NPRGD(Y−OMe)RC−NH_2.[38] These models are consistent with both carbamate **3** (Cyclo(S)-Gly-asp^c-ind^c-Arg^c-Cys^c-NH_2) and carbamate **4** (Cyclo(S)-Gly phe^c-Hrg^c-Hrg^c-asp^c-Cys^c-NH_2) having aromatic groups. Furthermore, mutagenesis data of α_{IIb} are also suggestive that there may be a hydrophobic binding site. Mutation of the residues Gly-184, Tyr-189, Tyr-190, Phe-191, and Gly-193 to Ala in α_{IIb} reduces the affinity of GPIIb/IIIa for fibrinogen significantly. A more comprehensive understanding of the binding modes of peptides or carbamates, however, requires more detailed structural information for GPIIb/IIIa.

Pharmacokinetic Characterization of a Linear Oligocarbamate. To begin to evaluate the utility of oligocarbamates as therapeutic agents, the serum half-life (as measured by the fibrinogen/GPIIb/IIIa ELISA assay) of linear carbamate **21** (Ac^c-phe^c-asp^c-ind^c-Arg^c-NH_2) was determined by intravenous administration in rats. The concentration of carbamate **21** in the 2-min plasma sample was 43 μM and was below the detectable limit (39 nM) by 180 min. The initial half-life was 3.6 min. The mechanism of clearance of carbamate **21** is uncertain, and whether the degradation is of side-chain functionality or the carbamate backbone itself has not been determined. However, we have previously shown[12] that carbamates are poor substrates for serine proteases, suggesting that an alternative clearance mechanism is involved.

Conclusions

In summary, we have described the synthesis and screening of linear and cyclic oligocarbamate libraries on solid support using the one bead, one peptide approach. The libraries were sequenced using the termination synthesis method previously described for linear peptides. This work demonstrates that the method is effective for unnatural oligomers as well as for cyclic compounds. It is simpler to carry out than other mass spectrometric methods for sequencing combinatorial libraries[87] and straightforward, requiring no alternative chemistries (as with oligonucleotide encoded libraries)[88] or further synthesis (as with small molecule tags).

Screening of these libraries identified unrelated high affinity oligocarbamate ligands for GPIIb/IIIa that could not have been predicted by rational design. Two of the cyclic ligands have affinities that are approaching those of natural protein ligands for GPIIb/IIIa, and the linear oligocarbamates have affinities comparable to those of linear peptides. Screening of both cyclic and acyclic libraries also allowed us to begin to explore the effects of conformational constraint in an unnatural polymeric backbone. Initial pharmacokinetic characterization of a linear oligocarbamate ligand suggests that this class of compounds is rapidly cleared from serum; however, the mechanism of degradation is unknown, and cyclic ligands were not tested.

Experimental Section

General Synthetic Materials and Methods. Protected amino acids, Rink amide resin, and 4-hydroxymethyl-3-methoxyphenoxybutyric acid 4-methylbenzhydramine resin (HMPB-MBHA) were from Novabiochem (San Diego, CA). Tentagel M resin was from Advanced ChemTech (Louisville, KY). *N*-Methylpyrrolidone was OmniSolv grade from E. Merck. Other chemicals were from Aldrich, and all chemicals were used without further purification. Dry dichloromethane was distilled from CaH_2, and dry tetrahydrofuran was distilled from Na/benzophenone.

Semipreparative HPLC was performed on a Rainin system on a Vydac C18 reverse phase column (10 μm, 10 × 250 mm) with monitoring at 205 nm. Solvent A was 0.1% TFA in water, and solvent B was 0.08% TFA in acetonitrile, with varying gradients depending on the ligand and a flow rate of 4 mL/min. Analytical HPLC was performed on a Rainin C-18 Microsorb column (5 μm, 4.6 × 250 mm) with gradients as specified and monitoring at 220 nm. Solid-phase steps were carried out in peptide flasks (B&H Scientific Glassware, Santee, CA) with agitation by mixing with N_2, unless otherwise noted.

Synthesis of Linear Oligocarbamates. Linear oligocarbamates were synthesized as described[12,13] on Rink amide resin. The *N*-terminus was acylated with 10 equiv of methyl-*p*-nitrophenyl carbonate and 20 equiv of HOBt-H_2O, followed by extensive washes with NMP and dichloromethane and drying of the resin. Sequences that contained the monomers **Hrg^c** or **Drg^c** were synthesized with Fmoc-Lys(Alloc)-NPC or Fmoc-Dab(Alloc)-NPC in their place. After *N*-terminal acylation, the allyloxycarbonyl groups were removed as described.[35] The side-chain amines were then guanylated with 1*H*-pyrazole-1-carboxamidine-HCl[36] (50 equiv, 5% DIEA/NMP, 0.15 M) followed by washes with NMP and CH_2Cl_2 and drying of the resin. Sequences were cleaved from support and side chains deprotected by treatment of resin with 95% TFA/5% triethylsilane for 2 h. The TFA and triethylsilane were removed in vacuo, and the crude oil was resuspended in 250 μL of TFA (for a 30 μmol scale synthesis). The TFA mixture was added to two 1.3 mL portions of an ice-cold 50/50 mixture of *tert*-butylmethyl ether and hexanes in microcentrifuge tubes. The precipitated oligocarbamate was vortexed vigorously and then pelleted. This washing procedure was repeated several times, and the crude carbamate was dried in vacuo. Sequences were purified by HPLC and lyophilized. Selected sequences were characterized by ^{1}H NMR, and the remaining sequences were characterized by high-resolution mass spectrometry (Supporting Information).

Preparation of 4-(4′-Aminomethyl-3′-methoxyphenoxy)butyric Acid-MBHA Resin. HMPB-MBHA resin (2.01 g, 1 mmol) and triphenylphosphine (1.31 g, 5 mmol) were added to a silanized round-bottom flask equipped with a stir bar. Phthalimide (0.75 g, 5 mmol) suspended in 20 mL of 50/50 dry CH_2Cl_2/THF was added to the flask, followed by the addition of an additional 40 mL of dry 50/50 CH_2Cl_2/THF. The suspension was cooled in an ice bath under N_2. Diethylazodicarboxylate (0.88 g, 5 mmol) dissolved in 20 mL of 50/50 CH_2Cl_2/THF was added over approximately 1 min, followed by an additional 20 mL of solvent. The reaction was removed from ice and stirred at room temperature for 18 h. The resin was transferred to a silanized peptide flask and washed with CH_2Cl_2 (3 × 2 min) and NMP (7 × 2 min). Hydrazine monohydrate (10 mL) mixed with NMP (6 mL) was added to the resin, and the resin was mechanically agitated for 12 h at room temperature. After the hydrazine/NMP mixture was drained, the resin was washed with NMP (7 × 2 min) and CH_2Cl_2 (5 × 2 min) and dried in vacuo.

Synthesis of Cyclic Oligocarbamates. The bis-alkoxybenzylamine resin was solvated in NMP for 1 h and then acylated with Fmoc-Cys-(Trt)-NPC under standard conditions. Substitution of the resin was 0.14 mmol/g as determined by quantitation of the Fmoc groups on support, which corresponds to a 36% yield for the three steps based on the manufacturer's given loading of the HMPB resin. The ninhydrin test for free amines was negative. The Cys^c(Trt) loaded resin was dried, and cyclic carbamates were synthesized at 20 μmol scale. Carbamates were coupled under standard conditions, and the *N*-terminal Fmoc group was removed. The *N*-terminus was acylated with 40 resin equiv of bromoacetic acid mixed with 20 resin equiv of diisopropylcarbodiimide (DIPC) in NMP for 2 h. Following acylation, the resin was washed with NMP (5 × 1 min) and CH_2Cl_2 (3 × 1 min), which was followed immediately with treatment with 2% TFA/2% triethylsilane/CH_2Cl_2 (5 × 10 min) for trityl deprotection. Washes with CH_2Cl_2 (3 × 1 min) and NMP (2 × 1 min) followed. Cyclization was initiated by addition of 25% diisopropylethylamine/NMP with N_2 agitation for 30 min. The

(87) Brummel, C. L.; Lee, I. N. W.; Zhou, Y.; Benkovic, S. J.; Winograd, N. *Science* **1994**, *264*, 399−402.

(88) *Antibodies: A Laboratory Manual*; Harlow, E., Lane, D., Eds.; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1988; p 726.

Table 7. Chemical Shifts for Protons in Carbamate **21** (Ac^c-phe^c-asp^c-ind^c-Arg^c-NH_2)

residue	NH	α-CH	β′-CH	others
Ac^c				CH_3 3.53
phe^c	6.88	4.03	4.20	β-CH_2 2.81, 2.91
				aromatic CH 7.19−7.36
asp^c	7.07	4.28	4.11, 4.31	β-CH_2 2.65
ind^c	6.94	5.35	5.44	γ-CH_2 3.03, 3.25
Arg^c	6.72	3.77	3.99	β-CH_2 1.50, 1.62
				γ-CH_2 1.64, 1.71
				δ-CH_2 3.29
				guan NH 8.20
				guan NH_2 7.6
				C-term NH_2 6.35

Table 8. Chemical Shifts for Protons in Carbamate **33** (Ac^c-Pro^c-Cha^c-Hrg^c-Gly^c-NH_2)

residue	NH	α-CH	β′-CH	others
Ac^c				CH_3 3.67
Pro^c		3.97	4.10	β-CH_2 1.87, 1.98
				γ-CH_2 1.84, 1.97
				δ-CH_2 3.37, 3.38
Cha^c	6.79	3.89	3.96, 3.99	β-CH_2 1.40
				Cyclohexyl 0.88−1.83
Hrg^c	6.78	3.76	3.99,4.05	β-CH_2 1.52, 1.59
				γ-CH_2 1.42, 1.50
				δ-CH_2 1.61, 1.66
				ϵ-CH_2 3.29
				guan NH 8.16
				guan NH_2 7.60
Gly^c	6.91	3.33	4.04	*C*-term NH_2 6.30

resin and solvent was then transferred to a 25 mL silanized pear shaped flask equipped with a stir bar. The suspension was heated to 40 °C in an oil bath under N_2 for 24 h. The resin was transferred back to a peptide flask, washed extensively with NMP (5 × 1 min) and CH_2Cl_2 (5 × 1 min), and dried. Cleavage, purification, and characterization were identical to linear oligocarbamates.

^{1}H NMR Characterization of Oligocarbamates. Oligocarbamates **2** (Cyclo(S)-Gly-Hpb^c-Tym^c-arg^c-Val^c-Cys^c-NH_2), **3** (Cyclo(S)-Gly-asp^c-ind^c-Arg^c-Cys^c-NH_2), **21** (Ac^c-phe^c-asp^c-ind^c-Arg^c-NH_2), and **33** (Ac^c-Pro^c-Cha^c-Hrg^c-Gly^c-NH_2) were dissolved in *N*,*N*-dimethylformamide (DMF-d_7) to final concentrations of 10−15 mM. A series of 1D ^{1}H NMR spectra were acquired on the oligocarbamates at varying temperatures to determine conditions favorable for acquiring assignment spectra. The temperature series experiments were performed on the Bruker AM-400 spectrometer with the temperature controlled by a variable temperature unit. Spectra were acquired with 16 384 complex points and 4237.9 Hz spectral width for 24−64 scans. Optimal temperatures for the carbamates **2**, **3**, **21**, and **33** were 80, 45, 50, and 60 °C, respectively.

^{1}H NMR assignment spectra were acquired on a GE Omega500 spectrometer operating at a frequency of 500.13 MHz, using a dedicated 5 mm ^{1}H probe. Chemical shift assignments are referenced to the residual aldehyde proton of DMF (8.03 ppm) present in the solvent. Spectra were processed and analyzed using the Felix 95.0 (Molecular Simulations, Inc.) program on Silicon Graphics workstations.

1D ^{1}H NMR spectra were acquired with 32 768 complex points and 5000 Hz spectral width for 16 scans. DQF−COSY spectra were acquired with a minimum of 2048 × 512 complex points and 5000 Hz spectra width for 16 scans per t1 increment. ROESY spectra, utilizing a 300 ms spinlock, were acquired with a minimum of 2048 × 1024 complex points and 5000 Hz spectral width for 16 scans per t1 increment.

Residue assignments for the oligocarbamates were made primarily through the DQF−COSY spectra, with the ROESY aiding in certain through-space connectivities. The ^{1}H assignments for carbamates **21**, **33**, **2**, and **3** are given in Tables 7−10, respectively. Complete ^{1}H assignments were made for these carbamates with the exception of some degenerate methylene resonances in **2** and **33**. Assignments for **2** were

Table 9. Chemical Shifts for Protons in Carbamate **2** (Cyclo(S)-Gly-Hpb^c-Tym^c-arg^c-Val^c-Cys^c-NH_2)

residue	NH	α-CH	β′-CH	others
Gly		deg[a]		β-CH_2 2.18, 2.26
$Hpb^{c\,\dagger}$		4.49		γ-CH 5.18
				δ-CH_2 3.83, 3.92
				NH 7.75
				α′-CH_2 3.43
				β′-CH_2 2.51
$Hpb^{c\,\dagger\dagger}$		4.79		β-CH_2 2.28, 2.49
				γ-CH 5.11
				δ-CH_2 3.60, 3.98
				NH 8.14
				α′-CH_2 3.49
				β′-CH_2 2.56
Tym^c	6.75	4.01	deg*	β-CH_2 2.78
				aromatic CH 6.90, 7.22
				CH_3 3.81
arg^c	6.63	3.77	4.13	β-CH_2 1.58, 1.68
				γ-CH_2 1.70, 1.79
				δ-CH_2 3.33
				guan NH 8.02
				guan NH_2 7.49
Val^c	6.44	3.62	3.95, 4.14	β-CH 1.88
				γ-CH_3 0.96
Cys^c	6.59	4.03	deg*	β-CH_2 2.75, 2.81
				C-term NH_2 6.25

* Unassigned due to degenerate nature. † Primary confomation. †† Secondary conformation.

Table 10. Chemical Shifts for Protons in Carbamate **3** (Cyclo(S)-Gly-asp^c-ind^c-Arg^c-Cys^c-NH_2)

residue	NH	α-CH	β′-CH	others
Gly		3.32		
asp^c	7.92	4.55	4.27, 4.54	β-CH_2 2.63
ind^c	7.02	5.33	5.53	γ-CH_2 2.97, 3.26
Arg^c	6.75	3.77	3.89, 4.33	β-CH_2 1.51, 1.59
				γ-CH_2 1.64, 1.70
				δ-CH_2 3.30
				guan NH 8.21
				guan NH_2 7.64
Cys^c	7.13	3.93	4.03, 4.42	β-CH_2 2.82
				C-term NH_2 6.43

further complicated by the presence of two conformations in solution (see Table 9), likely a result of cis/trans isomerization of the **Hpb^c** residue. Figures of the 1D spectra, DQF−COSY spectra illustrating the residue assignments, and a representative ROESY spectra are included in the Supporting Information.

Linear Oligocarbamate Library Synthesis. All libraries were synthesized using a divide-and-recombine strategy.[72] Tentagel M resin (35 μm bead diameter, 0.26 mmol/g loading) was solvated in NMP for several hours, and clumps of resin were physically dispersed. The resin was acylated with linker **1** through an HBTU/HOBt mediated peptide coupling in DMF. The resin was washed with NMP (5 × 1 min), and the Fmoc group was deprotected by treatment with 20% piperidine in NMP (1 × 5 min/1 × 10 min) followed by extensive washes with NMP (10 × 1 min). The tetrapeptide linker ϵ-aminohexanoic acid-Ser-Arg-Ser was then synthesized on resin. After removal of the Fmoc group and washes with NMP, the resin was split into 27 equal portions (by mass) in 1.5 mL microcentrifuge tubes and coupled to a 10-fold excess of Fmoc-amino-*p*-nitrophenyl carbonate monomer. To generate termination sequences, a small percentage of methyl-*p*-nitrophenyl carbonate was included in each coupling; the capping reagent was 7.5%, 5.2%, 4.6%, and 3.9% of the coupling mixture for the successive couplings. After the fourth carbamate coupling, the resin was recombined, and the Fmoc group was removed followed by final capping with methyl-*p*-nitrophenyl carbonate. The side-chain protecting groups were removed by treatment with 95% TFA/5% triethylsilane for 2 h. The resin was then washed with CH_2Cl_2 (1 × 1 min), NMP (2 × 1 min), 10% DIEA/NMP (1 × 1 min, 1 × 10 min), NMP (7 ×

1 min), and CH_2Cl_2 (5 × 1 min). The resin was dried in vacuo and stored at 4 °C.

Cyclic Oligocarbamate Library Synthesis. The cyclic libraries were synthesized with the same linker as the linear library. The linker was acylated with Fmoc-Cys(Trt)-NPC, followed by randomization of the following three or four residues. Following the last randomized residue, the Fmoc group was removed, and the *N*-terminal amine was acylated with bromoacetic acid/DIPC/NMP. The trityl group was removed as described above, and cyclization was initiated by suspension of the resin in 5% *N*-methylmorpholine in NMP. The resin was agitated with N_2 overnight. The side-chain protecting groups were removed as described for the linear library.

Screening of Oligocarbamate Libraries. Small portions of resin (2−5 mg) in siliconized, 0.5 mL microcentrifuge tubes were washed (3 × 0.3 mL × 2 min) with blocking buffer (274 mM NaCl, 5.5 mM KCl, 20 mM Na_2HPO_4, 3.5 mM KH_2PO_4, 1% BSA, 0.1% Tween-20, pH 7.2). The beads were blocked with blocking buffer for 1 h with gentle rotation at room temperature. After removal of blocking buffer, GPIIb/IIIa in TACTS.1 (20 mM Tris, 150 mM NaCl, 2 mM $CaCl_2$, 0.02% NaN_3, 0.1% Tween-20, 1% BSA, pH 7.4) was added to the resin and incubated for 1 h. The GPIIb/IIIa was removed, and the resin was washed with wash buffer (274 mM NaCl, 5.5 mM KCl, 20 mM Na_2HPO_4, 3.5 mM KH_2PO_4, 0.1% Tween-20, pH 7.2) four times, 1 min each wash. The beads were treated with monoclonal antibody 4B12 (0.3 mL, concentrated 0.3 μg/mL) in blocking buffer for 1 h, followed by standard washes. Secondary antibody (goat-anti-mouse-alkaline phosphatase conjugate) was added in blocking buffer (0.3 mL, 0.3 nM) and incubated for 1 h, followed by washes. The beads were stained with bromochloroindolyl phosphate and nitro blue tetrazolium as described.[88] Beads were distributed into ELISA wells, and positive beads were picked with a 500 μL syringe and placed into 500 μL siliconized microcentrifuge tubes. Carbamates were cleaved from the beads by addition of aqueous NH_4OH (pH 11, 3 μL) for 3 h, followed by removal of solvent in vacuo.

MALDI Mass Spectroscopy of Library Sequences. MALDI spectra were obtained on a PerSeptive Biosystems Voyager Biospectrometry Workstation. Samples from cyclic libraries were taken up in 3 μL of 10 mM DTT for 2 h before sample preparation. Samples from the linear library were taken up in 3 μL solvent A. Equal volumes of reduced oligocarbamate and matrix (5 mg α-cyano-4-hydroxycinnamic acid dissolved in 350 μL solvent A/250 μL ethanol/150 μL solvent B) were mixed, and 1 μL of this mixture was used for MALDI analysis. Generally 50−100 laser pulses were used to obtain spectra.

Competition ELISA. The relative affinities of carbamate compounds for GPIIbIIIa were evaluated with a receptor binding ELISA. Nunc 96-well Maxisorp plates were coated overnight at 4 °C with 100 μL of 10 μg/mL fibrinogen in 50 mM sodium carbonate, pH 9.6. The liquid was decanted, and the plates were blocked for 1 h with 0.5% BSA in PBS. The plates were washed with 0.05% Tween-20 in PBS, followed by the addition of 50 μL of sample (serial dilutions of oligocarbamate inhibitors) and 50 μL of 17.5 μg/mL GPIIbIIIa in 20 mM Tris-HCl, 0.5% BSA, 0.05% Tween-20, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4. The plates were incubated for 2 h at room temperature with gentle agitation. After the unbound GPIIbIIIa was removed by washing with 0.05% Tween-20 in PBS, 100 μL of horseradish peroxidase conjugated anti-GPIIbIIIa antibody was added. The plates were again incubated for 2 h at room temperature with gentle agitation. After washing to remove the unbound antibody, 100 μL of tetramethylbenzidine substrate was added. The enzymatic reaction was allowed to proceed for approximately 10 min, after which it was stopped by the addition of 100 μL of 1 M H_3PO_4. The absorbance in the wells was read at 450 nm. The data were graphed as absorbance value vs log[oligocarbamate], and curves were fit using a four-parameter equation. IC_{50} values were normalized against G4120, which was included in each run, and the given values are the average of three independent determinations.

Serum Stability of a Linear Oligocarbamate. Jugular vein catheters were surgically implanted in two adult male Sprague−Dawley rats (0.26 kg average weight) (Charles River Laboratories, Hollister, CA) while under ketamine/xylazine anesthesia. Upon recovery, the animals received 10 mg/kg carbamate **21** (Ac^c-phe^c-asp^c-ind^c-Arg^c-NH_2) (approximately 0.5 mL) via bolus tail vein injection. The test material was formulated in 10% DMSO/H_2O (5 mg/mL). Blood samples (0.2 mL) were collected via the catheters at the following times: predose, and 2, 7, 15, 30, 45, 60, 90, 120, 180, and 240 min postdose. Catheters were flushed with normal saline before and after each collection. Plasma samples (EDTA) were assayed for drug concentration by ELISA (limit of quantitation, 0.039 μM). The plasma drug concentration vs time data for each rat were fitted to a two-compartment model with a weighting scheme of $1/y^2$ (WinNonLin, Scientific Consulting Inc., Cary, NC).

Acknowledgment. The authors wish to thank J. K. Chen and H. D. Ulrich for helpful discussions concerning the assay, B. Blackburn and J. P. Burnier for insights into GPIIb/IIIa inhibitor structure and function, and J. A. Ellman, E. K. Kick and the Ellman group for the use of their MALDI-MS. C.Y.C. thanks Glaxo-Wellcome and Eli Lilly for predoctoral fellowships. P.G.S. is a Howard Hughes Medical Institute Investigator. This work was supported by the Director, Office of Energy Research, Office of Basic Energy Sciences, Division of Material Sciences, and also by the Division of Energy Biosciences of the U.S. Department of Energy under Contract No. DE-AC03-76SF00098. D.E.W. and C.W.L. have been supported by the Director, Office of Energy Research, Office of Biological and Environmental Research, General Life Science Division of the U.S. Department of Energy under contract No. DE-AC03-76F00098. Instrumentation grants to D.E.W. were provided by the U.S. Department of Energy, DE-FG05-86ER75281, and NSF, DMB86-09305.

Supporting Information Available: Text giving experimental details, tables giving masses of oligocarbamates, and ^1H NMR, DQF-COSY, and ROESY spectra (30 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA9734399