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Identification of Novel Macrocyclic Peptidase Substrates via On-Bead Enzymatic Cyclization

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Received June 16, 2003

Peptidase-catalyzed formation of macrocyclic lactams on solid phase identifies ring systems that are favorably bound in the enzyme active site. We evaluated several cyclic peptide motifs linked by ester bonds between the P2 and P1′ or the P1 and P2′ side chains. The depsipeptide represented by structure **5** was readily generated by a variety of peptidases from precursor ω-amino acids or ω-amino esters. This strategy for identifying ring systems for potential macrocyclic transition state analogues was demonstrated with the serine peptidases trypsin and chymotrypsin, with the aspartic peptidase pepsin, and with the zinc peptidase thermolysin.

Introduction

Peptidases are important targets for drug discovery because of the key roles they play in physiological processes.[1] These enzymes have occupied a prominent position in the modern era of "rational" drug design, since they were among the first target classes whose chemical mechanisms and 3-dimensional structures were elucidated. As a consequence, many schemes for both irreversible and reversible inhibition of peptidases have been devised. Rational strategies for peptidase inhibition generally lead to molecules with peptide-like traits but with altered functionality in place of the scissile linkage. Peptidic character is difficult to avoid in an inhibitor because of the requirement of the peptidase active site, which usually interacts with an extended substrate at multiple points. Carbonyl oxygens and amide NH groups in the vicinity of the cleavage point are stitched in with hydrogen bonds, and the side chains fit into complementary binding pockets in the active site for both affinity and selectivity.

A variety of inhibitor motifs has been developed to replace the scissile peptide bond. Electrophilic replacements may alkylate an active site nucleophile, and mimics of the high-energy, tetrahedral intermediate along the reaction pathway bind as transition state analogues.[2] These replacements typically enhance the affinity of the inhibitor over that of the parent peptide substrate, in addition to conveying resistance to the activity of the enzyme. However, they do not often bring selectivity among related peptidases, which is determined by the neighboring amino acid side chains.

An additional strategy for enhancing the affinity of a peptidase inhibitor is to reduce its conformational flexibility. If the inhibitor is prevented from adopting conformations that do not fit into the active site, fewer degrees of freedom are lost on association, resulting in a more favorable free energy of binding. One of the most straightforward approaches for constraining a conformationally mobile, acyclic chain is to embed it in a cyclic structure by bridging from one end to the other.[3,4] In principle, cyclization does not perturb any of the direct interactions between the inhibitor and enzyme or solvent except for those due to the bridge itself.[5] However, the entropy penalty for binding a cyclic compound to an active site can be dramatically less than that for an acyclic compound. In the absence of other torsional constraints, a linear chain loses five degrees of freedom on cyclization as the bond rotations become dependent on each other.[6,7] Additional advantages of a macrocyclic structure are enhanced metabolic stability and bioavailability.[8]

The binding advantage of a cyclic compound over the acyclic analogue does not come automatically. Where the bridge should go and what low-energy conformations it induces in the inhibitor need to be considered carefully. Highly flexible bridges may introduce more conformational mobility than they restrict, and a poorly designed

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§ The Center for New Directions in Organic Synthesis is supported by Bristol-Myers Squibb as a sponsoring member and Novartis Pharma as supporting member.

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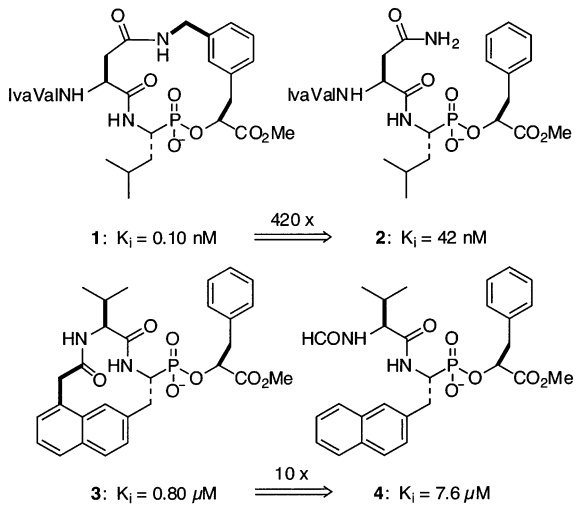
10.1021/jo0348367 CCC: $25.00
Published on Web 10/08/2003

bridge may distort the binding region away from the bound conformation. Several examples of naturally occurring, macrocyclic inhibitors of serine peptidases are known, including cyclotheonamide A[9,10] and the cyanopeptolides.[11−13] Interesting as these molecules are, in many respects they are more complex than traditional inhibitors and they provide limited insight into how to design simpler macrocycles.

Structure-based design approaches are high-risk and imprecise, and they often lead to challenging targets for synthesis.[3,14−19] The modest success we have had in enhancing the affinity of peptidase inhibitors by designing macrocyclic derivatives based on the bound structures of acyclic analogues has not only underscored the potential payoff but also illustrated the design challenge. For example, the phosphonates **1**and **2** bind to the aspartic peptidase penicillopepsin almost identically,[5] yet the macrocycle has 420-fold greater affinity.[19] In contrast, the cyclic and acyclic naphthalene analogues **3** and **4** differ by only a factor of 10 in affinity[18] and they do not bind to the enzyme in quite the same way.[20]



The idea of screening a library of diverse macrocyclic structures offers complementary challenges, not the least of which would be the difficulty in preparing these structures in an automated fashion. For this reason, we have developed an inverse approach: screening *acyclic* product-like molecules to identify analogues that are readily *cyclized* by the peptidase.[21] The acyclic substrates are readily synthesized with terminal amine and carboxylic acid or carboxylate ester groups, as required for different peptidases. If the bridging moiety allows the peptidic part of the structure to interact favorably with the enzyme, under appropriate conditions, the enzyme will catalyze peptide bond formation via a macrocyclic transition state. Since both the forward (hydrolysis) and reverse (amide synthesis) reactions catalyzed by a peptidase involve the same enzyme intermediate,[22] linear derivatives that are readily cyclized by the enzyme should point to bridging units that facilitate formation of this transition state and, thus, would be good candidates for incorporation into a transition state analogue inhibitor.

A variety of strategies is available for inducing peptide bond formation by a peptidase, including using activated precursors, lowering the pH, and reducing the water content of the medium.[22] In addition to our preliminary work,[21] there are two earlier reports of the use of enzymes to synthesize cyclic peptides (thermolysin[23] and subtiligase[24]), although not in the context of library screening. More recently, TycC thioesterase has been used to create a library of cyclic peptide antibiotic products.[25]

A colorimetric, on-bead assay is used to identify analogues that are cyclized by the enzyme (Figure 1). The substrates are designed with an ester moiety as a cleavable linker between the amine and carboxyl ends of the acyclic substrate. A dye molecule and the point of attachment to the resin particle are also located on opposite sides of the ester linkage. Thus, after treatment with the enzyme and subsequent saponification of the ester linkage, the dye molecule remains associated with the resin only if the lactam amide bond has been formed, i.e., if the substrate has been cyclized. By simple visual inspection of the beads, competent substrates can be distinguished from poor substrates. This method facilitates the rapid identification of optimal scaffolds for the construction of transition state analogue inhibitors. This screening strategy was first tested using trypsin on a derivative of the cyanopeptolin A90720A, a potent inhibitor of trypsin.[21] In this report, we describe the design and synthesis of smaller, novel macrocycles that incorporate the features of the screening strategy and are synthesized with serine, aspartic, and metallopeptidases.

Design of Macrocyclic Targets

Peptidases bind their substrates in a relatively extended conformation. As a consequence, modeling suggests that at least seven atoms are required to bridge the Cα positions of the P2 and P1′ residues in order to preserve the desired conformation of the peptide back-

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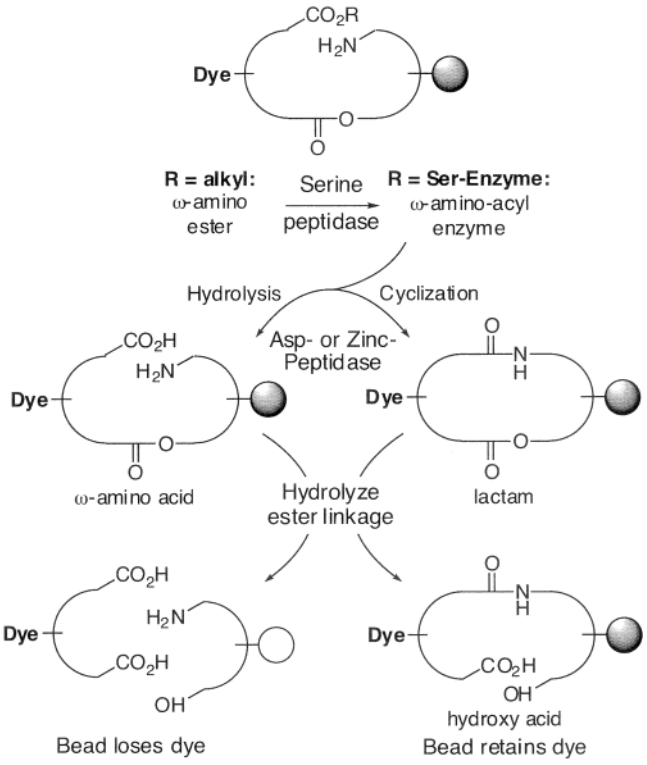


FIGURE 1. Screening strategy to identify good cyclization substrates.

bone in the macrocyclic analogues. To avoid intramolecular transacylation, the ester linkage in the bridge was carefully located away from the free amine in the acyclic precursor. The positions of the dye molecule and the linkage to resin were also incorporated on opposite sides of the bridging ester unit in locations that did not interfere with binding to the enzyme. Some of the bridging motifs that fit these criteria are illustrated in Figure 2. Modeling suggested that the extended conformation of the peptide backbone between the P2 and P1′ residues was best conserved in the low-energy conformations of macrocycle **5**.

Interestingly, the conformation adopted by the macrocyclic framework of **5** appeared to be appropriate for the active sites of three very different classes of peptidases, represented by α-chymotrypsin, trypsin, pepsin, and thermolysin. The peptide backbone of this macrocycle consists of Asp-Xaa-Dap, where Xaa represents a variable amino acid and Dap represents 2,3-diaminopropanoic acid. The ring is completed by a hydroxy acid bridging the carboxyl and amino groups of the Asp and Dap side chains, respectively. Within this common motif, some differences could be anticipated with respect to the substituents on the hydroxy acid bridge that would be tolerated in the enzyme active sites. For example, while *N*-Cbz-L-threonine served well as the hydroxy acid unit for the serine peptidases, modeling suggested that the Cbz-NH group would not fit into the active site of the aspartic peptidase. Thus, glycolic acid (R′ = H, n = 0) and 3-hydroxypropanoic acid (R′ = H, n = 1) were employed for the latter enzyme. Cbz-L-threonine, 3-hydroxypropanoic acid, and hydroxyproline were all shown to be effective in substrates for the metallopeptidase thermolysin.

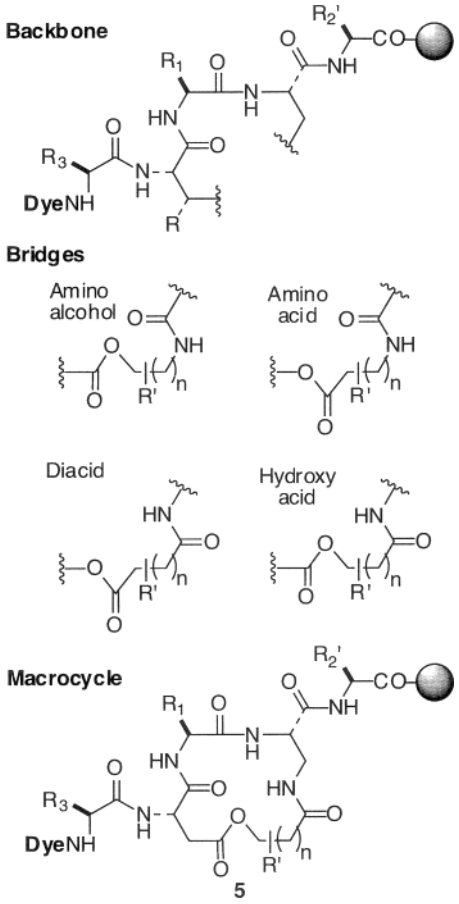


FIGURE 2. Possible resin-bound macrocycles for library.

Another significant difference among the designs of the acyclic substrates for the various enzymes is the amide bond formed on cyclization. For the serine and aspartic peptidases, this linkage is between the Xaa and Dap residues; for the metallopeptidase thermolysin, the cyclization reaction occurs between Asp and Xaa. This difference in the active site of thermolysin also requires the (*R*)-enantiomer of the Dap residue. Finally, different functional groups are required for cyclization by the serine peptidases than for the aspartic and metallopeptidases. The mechanisms of the latter enzymes involve direct formation of the amine−acid products on collapse of the tetrahedral intermediate; therefore, the substrates for the reverse reaction of amide bond formation must likewise have free amine and carboxylic acid groups. In the case of the serine peptidases, a carboxylate ester can be used as substrate to facilitate formation of the acyl enzyme intermediate; cyclization then occurs in competition with hydrolysis of the acyl enzyme.

Practical Considerations in Implementing the Screening Strategy

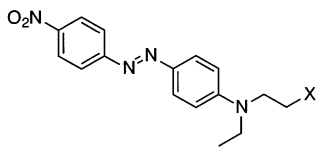
Solid Support. The resin used in this on-bead screening strategy needs to be compatible with organic solvents for synthesis of the substrates and with water for the enzymatic reaction. In addition, the resin beads must swell in aqueous solvents so that the enzymes can penetrate to the interior. In our initial experiments,

PEGA800[26−28] was found to work well for cyclization reactions catalyzed by trypsin, but it proved to be unsuitable for α-chymotrypsin, even for substrates that were readily cyclized by that enzyme in solution. Although both of these enzymes have a molecular weight below the 32 kDa threshold for this resin, the tendency of α-chymotrypsin to dimerize at high concentrations and ionic strength[29] may have pushed the effective molecular weight beyond this limit.

PEGA1900 has a longer poly(ethylene glycol) chain, which makes the core of the beads accessible to enzymes with molecular weights up to 60 kDa.[30] Indeed, this resin proved to be compatible with trypsin, α-chymotrypsin, pepsin, and thermolysin for the cyclization reactions. Three other solid supports were evaluated (Argopore, Novagel, and Tentagel), but none allowed sufficient enzyme permeability for our enzymatic assay.

Linker. The substrates were attached to the resin by the photocleavable linker 4-(4-(1-(9-Fmoc-amino)ethyl)-2-methoxy-5-nitrophenoxy)butanoic acid.[31] This linker is stable to both the acidic and basic conditions required for their synthesis and the subsequent assay conditions, while facilitating analysis of the intermediates and products by photolytic cleavage at 365 nm.

Dye. The dyed glycine derivative **6** was prepared from Disperse Red 1 via oxidation to the aldehyde, reductive amination of glycine methyl ester, acylation, and hydrolysis.[32] This component is readily incorporated in peptide synthesis and in assembly of the acyclic substrates.



Disperse Red 1: X = OH
6: X = N(Fmoc)CH_2CO_2H

Spacer. In preliminary experiments with pepsin, we evaluated the coupling of Cbz-Ala-Phe to a series of Phe-$(Gly)_n$-[linker] peptides on PEGA1900 in 10% DMF/buffer. The products, Cbz-Ala-Phe-Phe-$(Gly)_n$-NH_2, were observed for $n = 2$ or higher. A spacer of two glycines was therefore included between the photolabile linker and the R_2' amino acid residue for all of the cyclization substrates.

Conditions for Amide Bond Formation. The conditions used for cyclization with the serine peptidases were based on literature precedents for enzymatic synthesis on solid support. Both 3:3:4 DMF/EtOH/Tris pH 6.5[21] and 3:2 MeCN/Tris pH 8[33] catalyzed the cyclization reaction efficiently with trypsin and chymotrypsin.

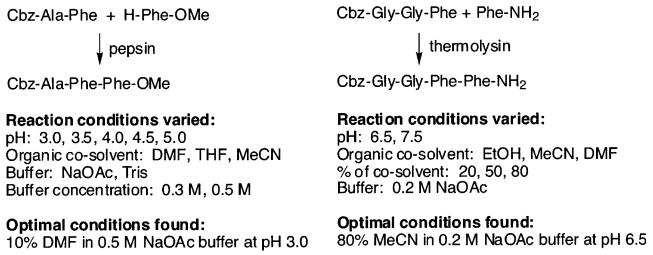


FIGURE 3. Reaction development for pepsin- and thermolysin-catalyzed peptide synthesis.

The conditions for cyclization with pepsin were optimized by a more systematic approach. The enzymatic coupling of Cbz-Ala-Phe to Phe-OMe with pepsin was evaluated in solution with variation in buffer, organic cosolvent, and pH in a parallel format (Figure 3). A slight excess of the acid component was used, so the reaction was judged to be near completion when the signal for the amine disappeared by LC/MS or RP-HPLC analysis. The best conditions for pepsin-catalyzed synthesis of this peptide in solution were found to be 10% DMF in 0.5 M NaOAc buffer, pH 3.0. On translation to the cyclization substrate on solid phase, an increase in the proportion of the organic cosolvent to 40% proved to be necessary for optimal reaction, presumably for solvation of the larger resin-bound peptide.

A similar series of experiments was performed to identify the best conditions for enzymatic cyclization with thermolysin (Figure 3). These experiments also revealed that the coupling conditions optimized from the straightforward solution assay translated well to the resin-bound reaction.

Synthesis of the Acyclic Substrates

The synthesis of the cyclization substrates for trypsin, α-chymotrypsin, and pepsin is depicted in Scheme 1. Standard Fmoc-amino acid coupling procedures were employed to add the linker, two glycine residues, the P2′ and P1′ residues, and the hydroxy acid portion of the bridging unit. The alcohol was acylated with the side chain carboxyl of suitably protected Asp or Glu, followed by addition of the P1, P3, and P4 residues and the dye **6**. The acyclic substrates for thermolysin were assembled in slightly different fashion because of the different position for ring closure and the incorporation of different components as described above (Scheme 2). Table 1 summarizes all of the substrates prepared.

Results of On-Bead Cyclizations

Serine Peptidases: Trypsin and Chymotrypsin. The acyclic substrate **7a**, with lysine at the P1 position, was treated with trypsin (4 mg/mL) in MeCN/tris buffer (3:2) at pH 6.5 at room temperature. Monitoring the reaction by reverse-phase HPLC showed that cyclization was 68% complete after 2 min and 100% complete after 30 min. Similarly, the phenylalanine analogue **7b** was readily lactamized on treatment with α-chymotrypsin (4 mg/mL) in MeCN/tris buffer (3:2) at pH 8 at room temperature, albeit at a slower rate (25% complete after 5 min, and only 60% complete after 40 h). (As noted above, chymotrypsin is less effective as a catalyst for

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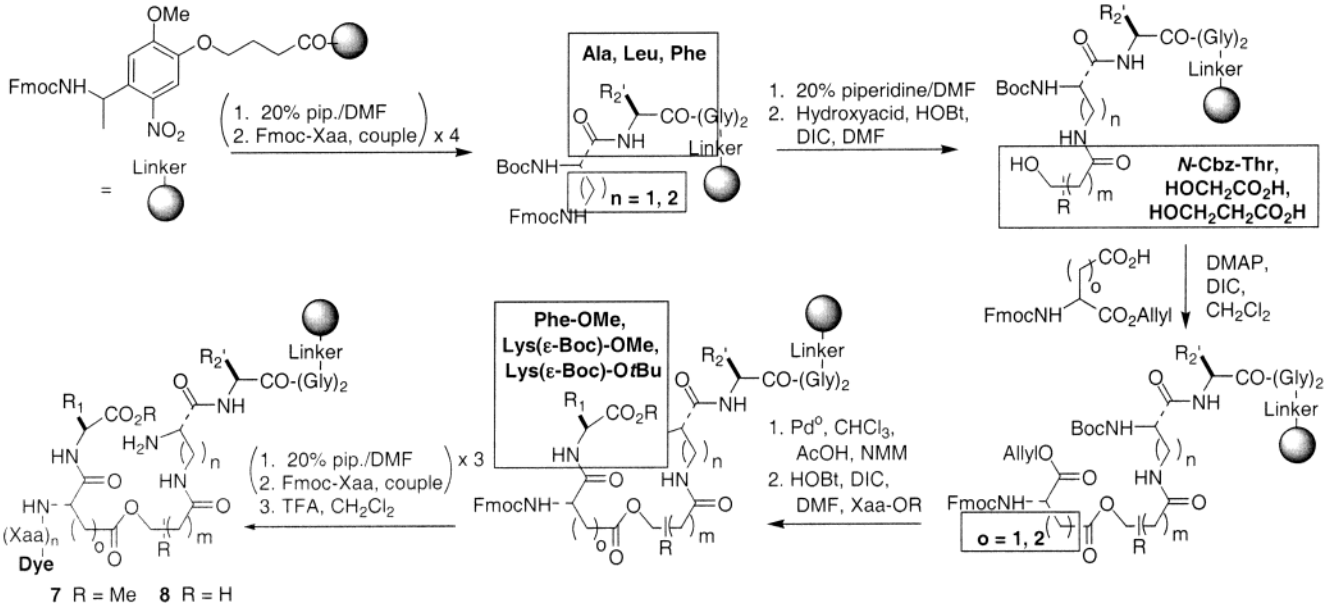
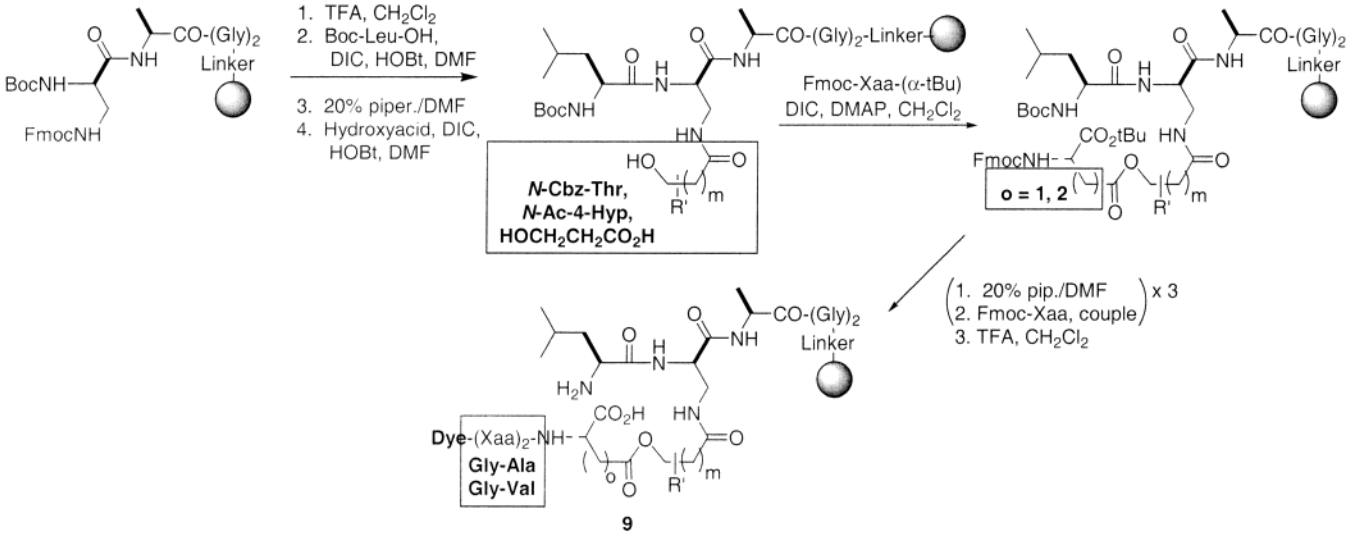
SCHEME 1. Synthesis of Cyclization Substrates for Trypsin, α-Chymotrypsin, and Pepsin



SCHEME 2. Synthesis of Cyclization Substrates for Thermolysin



polymeric substrates than is trypsin, even with the PEGA1900 resin as solid support.) For these two serine peptidases, it is apparent that cyclization of the acyl enzyme intermediate competes effectively with attack by water to give the hydrolysis product.

Aspartic Peptidase: Pepsin. In contrast to the serine peptidase substrates, in which the carboxyl group is activated as an ester, the substrates required by the aspartic and zinc peptidases are the free *ω*-amino acids. The carboxyl groups of the pepsin substrates are protonated under the low pH conditions that favor peptide bond formation by this enzyme. Treatment of **7c** with pepsin in 40% DMF in 0.5 M NaOAc at pH 3 afforded the cyclic product, demonstrating that enzyme-catalyzed lactam formation from the free *ω*-amino acids is indeed feasible. The utility of the dye retention/release assay was explored with the substrates **8a**−**f**, which were treated with pepsin (8 mg/mL) in 40% DMF in 0.5 M NaOAc at pH 3 for 6 h and subsequently with K_2CO_3 (0.5 M in MeOH/H_2O, 1:1) for 24 h. The color intensity of beads in all the samples was higher and clearly distinct from controls that had not been exposed to the peptidase (and thus did not contain cyclic product). After photochemical release of substrate from the beads, the presence of the ring-opened hydroxy acids was confirmed for derivatives **8a, 8c**, **8e**, and **8f**.[34]

Zinc Peptidase: Thermolysin. In a similar fashion, substrates **9a**−**j** were exposed to thermolysin (5 mg/mL) in 80% MeCN/20% NaOAc buffer (0.2 M, pH 6.5) for 6 h. After treatment of the beads with K_2CO_3 (0.5 M in MeOH/H_2O, 1:1) for 24 h, mass spectral analysis of the supernatant confirmed that all substrates except **9a** and **9e** had been cyclized by thermolysin. It appears that the Ala-Asp combination is disfavored as a substrate except when

(34) Since we showed conclusively by mass spectral analysis that analogues of substrates **8b** and **8d** with an Fmoc group in place of the dye molecule could be converted to the cyclic product, we attribute our failure to observe the hydroxy acids from **8b** and **8d** to poor yields or sample decomposition in these particular cleavage experiments.

TABLE 1. Summary of Cyclization Substrates Prepared on Solid Phase[a]

compound	dye	$(Xaa)_n$	diacid	R_1 amino acid	hydroxy acid	diamino acid	R_2' amino acid	enzyme
7a	Fmoc	Val	Asp	Lys	*N*-Cbz-Thr	(*S*)-Dap	Leu	TRP
7b	Fmoc	Val	Asp	Phe	*N*-Cbz-Thr	(*S*)-Dap	Leu	CHY
7c	Fmoc	$(Val)_2$	Asp	Leu	Hpa	(*S*)-Dap	Phe	PEP
8a	dye	$(Val)_2$	Asp	Leu	Ga	(*S*)-Dab	Ala	PEP
8b	dye	$(Val)_2$	Glu	Leu	Ga	(*S*)-Dab	Ala	PEP
8c	dye	$(Val)_2$	Asp	Leu	Hpa	(*S*)-Dap	Ala	PEP
8d	dye	$(Val)_2$	Asp	Leu	Hpa	(*S*)-Dab	Ala	PEP
8e	dye	$(Val)_2$	Glu	Leu	Hpa	(*S*)-Dap	Ala	PEP
8f	dye	$(Val)_2$	Glu	Leu	Hpa	(*S*)-Dab	Ala	PEP
9a	dye	Gly-Ala	Asp	Leu	*N*-Cbz-Thr	(*R*)-Dap	Ala	TLN
9b	dye	Gly-Val	Asp	Leu	*N*-Cbz-Thr	(*R*)-Dap	Ala	TLN
9c	dye	Gly-Ala	Glu	Leu	*N*-Cbz-Thr	(*R*)-Dap	Ala	TLN
9d	dye	Gly-Val	Glu	Leu	*N*-Cbz-Thr	(*R*)-Dap	Ala	TLN
9e	dye	Gly-Ala	Asp	Leu	Hpa	(*R*)-Dap	Ala	TLN
9f	dye	Gly-Val	Asp	Leu	Hpa	(*R*)-Dap	Ala	TLN
9g	dye	Gly-Ala	Glu	Leu	Hpa	(*R*)-Dap	Ala	TLN
9h	dye	Gly-Val	Glu	Leu	Hpa	(*R*)-Dap	Ala	TLN
9i	dye	Gly-Ala	Asp	Leu	*N*-Ac-Hyp	(*R*)-Dap	Ala	TLN
9j	dye	Gly-Val	Asp	Leu	*N*-Ac-Hyp	(*R*)-Dap	Ala	TLN
9k	dye	Gly-Ala	Glu	Leu	*N*-Ac-Hyp	(*R*)-Dap	Ala	TLN
9l	dye	Gly-Val	Glu	Leu	*N*-Ac-Hyp	(*R*)-Dap	Ala	TLN

[a] Abbreviations used: Dap = 2,3-diaminopropanoic acid, Dab = 2,4-diaminobutanoic acid, Ga = glycolic acid, Hpa = 3-hydroxypropanoic acid, *N*-Ac-Hyp = *N*-acetyl-4-hydroxy-L-proline, TRP = trypsin, CHY = α-chymotrypsin, PEP = pepsin, TLN = thermolysin.

combined with the more rigid *N*-acetyl-4-hydroxyproline moiety.

Limitations. While the degree of cyclization can be assessed qualitatively by this assay, a number of practical limitations frustrate attempts to discern a quantitative relationship between bead color and rate of cyclization. Variation in color intensity due to bead size could be reduced with uniform beads, but variations in the rate-limiting step for different substrates and enzymes are less readily controlled. For example, diffusion of the enzyme into and within the polymer may take place on a time scale that is comparable to that of cyclization and/or hydrolysis of the best substrates. As a consequence, it may not be possible to distinguish kinetically from thermodynamically favored ring systems. Nevertheless, even a qualitative strategy for identification of macrocyclic structures that are easily formed and whose conformations are compatible with the enzyme active site is of value in the design of peptidase inhibitors.

Conclusion

The on-bead screening method for enzymatic cyclization of depsipeptides was first demonstrated with trypsin on an analogue of a naturally occurring inhibitor.[21] We have now shown the generality of this strategy with much simpler derivatives and its application to an additional serine peptidase (chymotrypsin) and to enzymes from the aspartic peptidase (pepsin) and zinc peptidase (thermolysin) classes. The formation of cyclic products was easily detected qualitatively by retention of the dye moiety on resin or by mass spectrometric identification of the ring-opened hydroxy acid after photocleavage. The following report describes the synthesis and evaluation of a macrocyclic inhibitor based on a scaffold identified from this cyclization strategy.[35]

Acknowledgment. This work was supported by a grant from the National Institutes of Health (Grant No. GM30759), a Carlsberg Foundation postdoctoral fellowship to H.C.H., and a UC LEADS summer fellowship to R.C.C.

Supporting Information Available: Experimental procedures for synthesis and characterization of the cyclization substrates and for enzyme-catalyzed cyclization. This material is available free of charge via the Internet at http://pubs.acs.org.

JO0348367

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