Enzymatic, Polymer-Supported Formation of an Analog of the Trypsin Inhibitor A90720A: A Screening Strategy for Macrocyclic Peptidase Inhibitors

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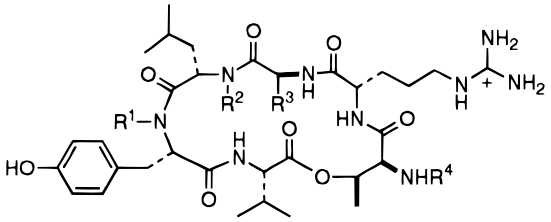
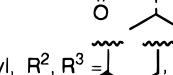
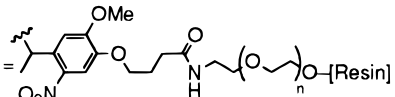
Macrocyclic constraint is effective in reducing conformational flexibility and thereby enhancing binding affinity and metabolic stability in enzyme inhibitors and other protein ligands.[1] Such derivatives present challenges both in design and synthesis: ring systems must be identified that induce the correct conformation in the binding region while avoiding unfavorable contacts with the receptor, and a significant investment in effort is required to prepare the analogs. A rapid and convenient method for identifying favorable ring systems prior to synthesis would be useful, especially if the method could be applied as a combinatorial approach. In this paper, we propose such a strategy for the discovery of macrocyclic peptidase inhibitors, demonstrating the key features of the method with the synthesis of an analog (**2**) of A90720A (**1**), a naturally-occurring inhibitor of trypsin.[2]



1 R^1 = methyl, R^2, R^3 =  , R^4 = N-(β-sulfo-D-glyceryl)-D-Leu

2 R^1 = 2-(N-(4-(4-nitrophenylazo)phenyl)-N-ethylamino)ethyl (**Dye**), R^2 = H, R^3 = CH_2OH, R^4 = $CO(CH_2)_3CONH$–**L**

a: **L** = H, **b**: **L** = 

= [Photolinker][PEGA resin]

We reasoned that a ring system that is favorably bound and turned over as a substrate by the enzyme should be similarly effective in the form of an inhibitor (for example, if the scissile linkage were replaced with a transition state analog). Since both the forward (hydrolysis) and reverse (amide synthesis) reactions catalyzed by a peptidase involve the same intermediate, discovering which *linear* derivatives are readily *cyclized* by the enzyme should also point to structures that would make good macrocyclic inhibitors.[3] Linear molecules are inherently easier to synthesize than macrocycles, and a variety of strategies are available for inducing peptide bond formation by a peptidase (activated precursors, lower pH, low-water content, etc.[4]).

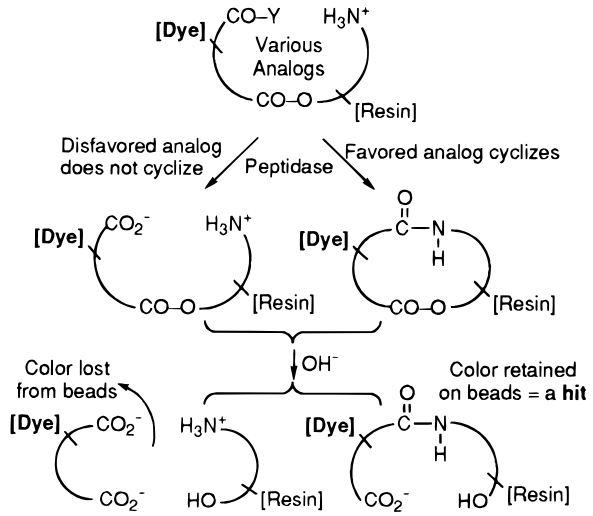


Figure 1. Strategy for distinguishing, from a library of analogs, molecules that cyclize from those that do not.

Key for the success of this strategy is a simple assay for cyclization that can identify individual beads from an encoded library and thus take full advantage of the combinatorial approach.[5] The scheme depicted in Figure 1 relies on the cyclization reaction to establish a connection between the polymeric synthesis support and a dye that is stable to conditions which cleave the molecule at a different point. Cleavage removes the dye from beads carrying derivatives that did not cyclize, while dye is retained on those that have the desired analogs. We have reduced to practice the major elements in this strategy by synthesizing macrocycle **2** on a solid phase by enzymatic cyclization and demonstrating that beads with cyclized and uncyclized molecules can be differentiated by hydrolysis of the ester linkage.

A90720A (**1**) furnished an ideal test system for this approach; it binds to trypsin with the Thr-Arg-Ahp [Ahp = 3-amino-6-hydroxy-2-piperidone] tripeptide in the S2-S1-S1′ subsites, with the tyrosine *N*-methyl projecting away from the enzyme, and the sulfoglyceryl-Leu residues in an open region on the protein surface.[2] These positions on A90720A were thus logical places to locate the dye molecule (a derivative of Disperse Red 1) and the tether point, respectively, since they are on opposite sides of the cleavable ester linkage. Design of the synthetic target **2** was completed by substituting Ser for the Ahp subunit for ease of synthesis, and replacing the sulfoglyceryl-Leu moiety with a simple acid- and base-stable attachment to a photocleavable tether to the resin. The acyclic precursor was assembled in solution in protected form (see the Supporting Information),

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(2) Bonjouklian, R.; Smitka, T. A.; Hunt, A. H.; Occolowitz, J. L.; Perun, T. J., Jr.; Doolin, L.; Stevenson, S.; Knauss, L.; Wijayaratne, R.; Szewczyk, S.; Patterson, G. M. L. *Tetrahedron* **1996**, *52*, 395. Lee, A. Y.; Smitka, T. A.; Bonjouklian, R.; Clardy, J. *Chem. Biol.* **1994**, *1*, 113.

(3) Because some ring systems are inherently easier to form, no direct relationship between the rate of enzymatic cyclization and transition state binding affinity is expected (Radzicka, A.; Wolfenden, R. *Methods Enzymol.* **1995**, *249*, 284); however, the fact that ring systems which are easily formed would be favored in this screen may have practical benefits in identifying macrocyclic inhibitors that are easier to synthesize.

(4) Heiduschka, P.; Dittrich, J.; Barth, A. *Pharmazie* **1990**, *45*, 164.

(5) Joyce, G. F.; Still, W. C.; Chapman, K. T. *Curr. Opin. Chem. Biol.* **1997**, *1*, 3 and companion articles in that issue.

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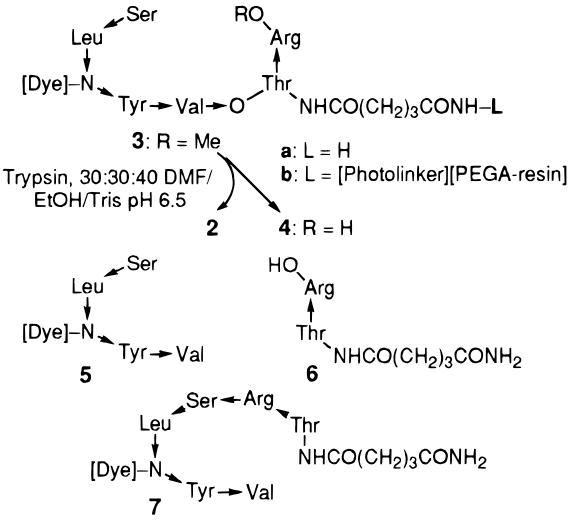
attached to PEGA (polyethyleneglycol-polyacrylamide)[6] beads bearing the photolinker,[7] and deprotected to the methyl ester **3b**.[8] Photolytic detachment of this material in methanol at 366 nM yielded **3a** in solution as a single compound, as characterized by ^{1}H NMR, HRMS, and RPLC.



Cyclization of soluble amino ester **3a** by trypsin was readily achieved in 30:30:40 DMF/ethanol/pH 6.5 Tris buffer, affording a 2:1 ratio of cyclic/hydrolyzed products **2a** and **4a**. Conditions for on-bead cyclization of **3b** were explored by incubation with trypsin, photolysis, and analysis for precursor **3a**, macrocycle **2a**, and the hydrolysis product **4a** by HPLC. In a preparative experiment, treatment of 2 mg of **3b** (ca. 2000 beads, 0.6 μmol) with 40 mg (ca. 3 mol equiv) of trypsin in the mixed solvent system for 26 h, followed by washing and photolysis, gave a 1:1 mixture of **2a** and **4a**.[9] The formation of both products presumably proceeds via the acyl-enzyme as a common intermediate, which partitions to the hydrolyzed product **4b** by attack of water and to the macrocycle **2b** by intramolecular attack of the terminal amine.[10] Only a small amount of precursor **3a** was present in the eluant, indicating that most of the material on the resin is accessible to the enzyme; a control experiment without the enzyme afforded only the unaltered amino ester **3a** after photolysis.

Treatment of the resin-bound precursor **3b** with 1:1 methanol/1 M NaOH for 7 h cleaves the Val-Thr ester linkage, and the beads turn colorless after filtration and washing. MS analysis of the filtrate confirms the presence of **5**, and after photolysis of the remaining resin, the expected dipeptide derivative **6** is released. In contrast, from beads that have been treated with trypsin prior to ester cleavage, the red color is not removed by the saponification step, and the hydrolyzed product **7** can be identified in the eluant after photolysis. The difference in coloration is illustrated in Figure 2, which shows a mixture of beads that were taken before (**3b**) and after (**2b** and **4b**) trypsin treatment, combined, and saponified together.

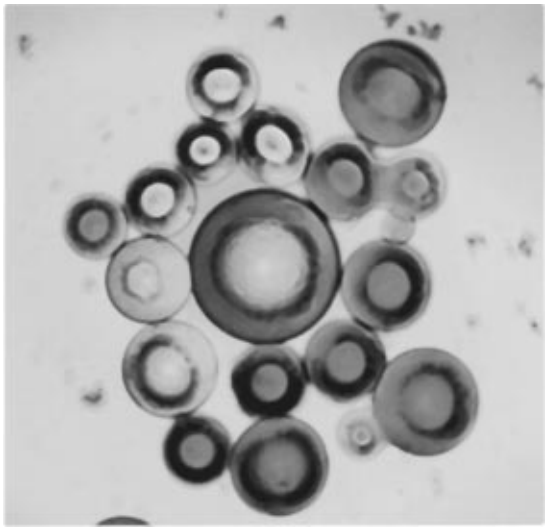


Figure 2. A mixture of beads gathered before and after treatment with trypsin, combined, and saponified.

Not surprisingly, analog **2a** is a substrate for trypsin in aqueous solution ($k_{cat} \approx 0.1$ s^{-1} in 5% DMSO, pH 8.0), but it persists long enough at low enzyme concentrations (22 nM) that an inhibition constant of 230 ± 30 nM can be determined.[11] The binding affinity of **2a** is only 25-fold less than that of A90720A itself ($K_i = 9$ nM),[2] reflecting loss of the active site contacts of the sulfoglyceryl-Leu moiety of the natural product and the rigidification induced by the Ahp residue. The acyclic, unconstrained hydrolysis product **4a** is a weak inhibitor: $K_i = 15\ \mu$M. Although modest alterations in substrate selectivity may be expected for enzymatic reactions in organic versus aqueous solutions,[12] it is clear that the ability of **3a** to cyclize in the mixed solvent translates into the ability of **2a** to inhibit the enzyme in water.

An eventual goal of this project is to determine whether the information needed for discovery of potent, macrocyclic peptidase inhibitors can be obtained more easily than through iterative design and synthesis or by the preparation and assay of libraries of macrocyclic structures. The results presented here demonstrate the essential elements that make screening a library of linear analogs for enzymatic cyclization a viable approach. This method should be general for a variety of proteases, macrocyclic motifs, and cleavage sites, requiring only that the enzyme be able to function in the direction of amide synthesis.

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Supporting Information Available: Experimental procedures for preparation of intermediates, solution synthesis, characterization, and enzymatic evaluation of macrocycle **2a** (17 pages). See any current masthead page for ordering and Internet access instructions.

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(8) We first explored Tentagel (PEG-polystyrene) as the support; however, no products from enzymatic transformation, either cyclization or hydrolysis, were detected under a variety of conditions (Lowe, G.; Quarrell, R. *METHODS: A Companion to Methods in Enzymology* **1994**, *6*, 411. Vágner, J.; Barany, G.; Lam, K. S.; Krchnák, V.; Sepetov, N. F.; Ostrem, J. A.; Strop, P.; Lebl, M. *Proc. Nat. Acad. Sci. U.S.A.* **1996**, *93*, 8194).

(9) It should be noted that 2 mg of resin beads may represent more than 1000 compounds in the context of a combinatorial library; hence, even without optimization, the conditions of the preparative experiment could be applied efficiently as a screen.

(10) The ratio of **4a**:**2a** did not appear to increase during the course of the reaction, indicating that **4b** did not arise from hydrolysis of **2a**.

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