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Amplification of Screening Sensitivity through Selective Destruction: Theory and Screening of a Library of Carbonic Anhydrase Inhibitors

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Abstract: A new method for identifying enzyme inhibitors is to conduct their synthesis in the presence of the targeted enzyme. Good inhibitors form in larger amounts than poorer ones because the binding either speeds up synthesis (target-accelerated synthesis) or shifts the synthesis equilibrium (dynamic combinatorial libraries). Several groups have successfully demonstrated this approach with simple systems, but application to larger libraries is challenging because of the need to accurately measure the amount of each inhibitor. In this report, we dramatically simplify this analysis by adding a reaction that destroys the unbound inhibitors. This works similar to a kinetic resolution, with the best inhibitor being the last one remaining. We demonstrate this method for a static library of several sulfonamide inhibitors of carbonic anhydrase. Four sulfonamide-containing dipeptides, EtOC-Phe$_{sa}$-Phe (**4a**), EtOC-Phe$_{sa}$-Gly (**4b**), EtOC-Phe$_{sa}$-Leu (**4c**) and EtOC-Phe$_{sa}$-Pro (**4d**), were prepared and their inhibition constants measured. These inhibitors migrated to the carbonic anhydrase compartment of a two-compartment vessel. Although higher concentrations of the better inhibitors were observed in the carbonic anhydrase compartment, the concentration differences were small (1.83:1.71:1.54:1.46:1 for **4a:4b:4c:4d:5**, where **5** is a noninhibiting dipeptide EtOC-Phe-Phe). Addition of a protease rapidly cleaved the weaker inhibitors (**4d** and **5**). Intermediate inhibitor **4c** was cleaved at a slower rate, and at the end of the reaction, only **4a** and **4b** remained. In a separate experiment, the ratio of **4a** to **4b** was found to increase over time to a final ratio of nearly 4:1. This is greater than the ratio of their inhibition constants (approximately 2:1). The theoretical model predicts that these ratios would increase even further as the destruction proceeds. This removal of poorer inhibitors simplifies identification of the best inhibitor in a complex mixture.

Introduction

Synthesis using combinatorial chemistry allows testing of hundreds of thousands of drug candidates using high throughput screening techniques. Although this rapid pace has revolutionized drug development, the search for faster and more efficient testing methods continues. One promising method is in situ screening of mixtures such as in dynamic combinatorial libraries.[1] Dynamic combinatorial libraries are equilibrating mixtures of organic molecules. Equilibration in the presence of a therapeutic target increases the equilibrium amounts of those library members that bind tightly to that target. The difference in library composition with and without a stoichiometric amount of target identifies the best inhibitors.

Dynamic libraries are still in the developmental stage, and only a few examples have been reported.[2] For example, Ramström and Lehn[3] created a dynamic library of 10 disaccharides by disulfide exchange starting from a mixture of monosaccharide thiols. The library was screened against concanavalin A, which binds mannose-rich oligosaccharides. A mannoside homodimer was the strongest binder in the library. When the disulfide exchange was carried out in the presence of concanavalin A, the amount of mannoside homodimer present increased by 40%. This increase in the amount of mannoside homodimer identifies it as the best-binding disaccharide. Analysis of a larger 21-member library was more difficult because HPLC did not resolve each member. Nevertheless, the mannoside homodimer was clearly favored in this library as well.

To make a real impact on drug discovery, methods must be developed to screen dynamic combinatorial libraries with thousands of members. This screening is complicated because

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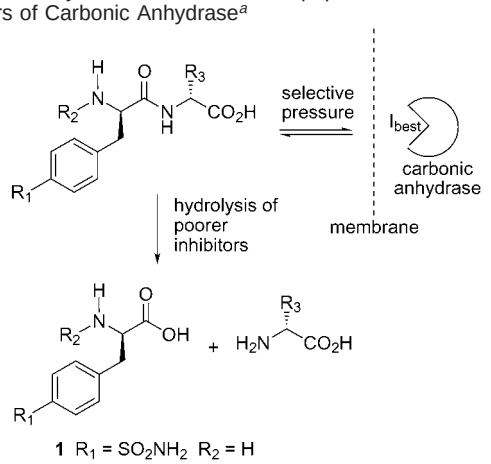
(1) Reviews: Ganesan, A. *Angew. Chem., Int. Ed.* **1998**, *37*, 2828−2831; Lehn, J.-M. *Chem. Eur. J.* **1999**, *5*, 2455−2463; Cousins, G. R. L.; Poulsen, S.-A.; Sanders, J. K. M. *Curr. Opin. Chem. Biol.* **2000**, *4*, 270−279; Huc, I.; Nguyen, R. *Comb. Chem. High Throughput Screening* **2001**, *4*, 53−74.

(2) For examples aimed towards biological targets, see: (a) Huc, I.; Lehn, J.-M. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2106−2110. (b) Nicolaou, K. C.; Hughes, R.; Cho, S. Y.; Winssinger, N.; Smethurst, C.; Labischinski, H.; Endermann, R. *Angew. Chem., Int. Ed.* **2000**, *39*, 3823−3828. (c) Karan, C.; Miller, B. L. *J. Am. Chem. Soc.* **2001**, *123*, 7455−7456. (d) Bunyapaiboonsri, T.; Ramström, O.; Lohmann, S.; Lehn, J.-M.; Peng, L.; Goeldner, M. *ChemBioChem* **2001**, *2*, 438−444. (e) Nguyen, R.; Huc, I. *Angew. Chem., Int. Ed.* **2001**, *40*, 1774−1776.

(3) Ramström, O.; Lehn, J.-M. *ChemBioChem* **2000**, *1*, 41−48.

10.1021/ja017099+ CCC: $22.00

Scheme 1. Aryl Sulfonamide-Based Dipeptide Libraries as Inhibitors of Carbonic Anhydrase[a]



1 $R_1 = SO_2NH_2$ $R_2 = H$

[a] Strong binding inhibitors will be bound to carbonic anhydrase and protected. Weaker inhibitors will be hydrolyzed by a protease.

it is often difficult to measure the concentration of each library member in the absence and presence of a target. Further, the libraries will likely contain not one but many good inhibitors because many library members have similar structures and thus similar binding constants. In these cases, adding the target increases the concentration of many library members, rather than a single member, and makes analysis very difficult or impossible. Eliseev and Nelen[4] estimated that a dynamic library combined with an affinity column containing the target would yield one major compound (>50%) only if K_{strong}/K_{weak} was at least *n*, where *n* is the number of members of the library. Thus, for one member to predominate in a library of 1000 members, that member would have to bind >1000 times stronger than the others, an unlikely possibility. This inability to distinguish between inhibitors of similar binding constants is a major limitation of the current dynamic combinatorial libraries.

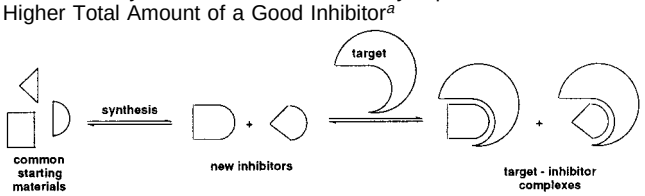
In this report, we propose a screening method that enhances the ability to detect the best inhibitor in a mixture of similar inhibitors. The key to the method is an irreversible destruction reaction that destroys the unbound and weakly bound inhibitors, similar to a kinetic resolution. The best inhibitor is the last one remaining. We demonstrate that this method works for a static library and discuss its potential application to a dynamic system.

Our library targets carbonic anhydrase and consists of dipeptides with an N-terminal 4′-sulfonamidophenylalanine (**1**, Phe_{sa}).[5] These dipeptides can either bind to carbonic anhydrase or be cleaved by a protease, Scheme 1. This cleavage increases the ratio of the strongest binder relative to weaker binders. Importantly, the ratio may increase to values significantly greater than the ratio of the binding constants, thus overcoming the limitation identified by Eliseev and Nelen and making it easy to identify the best inhibitor in the mixture.

Theory

Finding the Best Inhibitor by Shifting the Equilibrium To Make More of the Better Inhibitors. Most dynamic combinatorial library experiments contain two equilibria: an equilibrium for the synthesis of inhibitors and an equilibrium for binding of the inhibitors to the target, Scheme 2. The binding equilibrium removes the good inhibitors from the synthesis equilibrium, and to reestablish equilibrium, the synthesis produces more of the good inhibitors than it would in the absence of target. First, we show that the ratio of good inhibitor to a poor inhibitor depends linearly on the ratio of the binding constants.

Scheme 2. Dynamic Combinatorial Library Equilibria Yield a Higher Total Amount of a Good Inhibitor[a]



[a] Binding of the inhibitor to a target removes it from the synthesis equilibrium so that the synthesis produces more of the good inhibitor.

Consider a common starting material, SM, in equilibrium with two inhibitors, A and B, which can each bind reversibly to a target, T, to form complexes T·A and T·B

$$\text{SM} \overset{K_{sA}}{\rightleftharpoons} A + T \overset{K_{aA}}{\rightleftharpoons} T\cdot A \qquad (1)$$

$$\text{SM} \overset{K_{sB}}{\rightleftharpoons} B + T \overset{K_{aB}}{\rightleftharpoons} T\cdot B \qquad (2)$$

If $[A_T]$ is the total of bound and unbound forms of A ($[A_T] = [A] + [T\cdot A]$), it can be shown that under typical conditions (e.g., tight binding and an excess of target at a high concentration), the equilibrium ratio of the total amounts of the two inhibitors, $[A_T]$ and $[B_T]$, depends linearly on their relative association constants (eq 3, see Supporting Information for a derivation).[6]

$$\frac{[A_T]}{[B_T]} = \frac{K_{sA} \times K_{aA}}{K_{sB} \times K_{aB}} \qquad (3)$$

For the ideal case where there is one good inhibitor in a pool of noninhibitors, these equilibria indeed will yield the good inhibitor. For example, assuming the rates of synthesis are equal, a mixture of two inhibitors differing in their inhibition constants by a factor of 10 will give a 1:1 mixture in the absence of target (50 mol % of better inhibitor) but gives a 1:10 mixture in the presence of target (91 mol % of better inhibitor). Similarly, a mixture of 1000 inhibitors would yield 0.1 mol % of each inhibitor in the absence of target, but in the presence of target, the poorer inhibitors would decrease to 0.09 mol % each, while the one good inhibitor would increase to 0.9 mol %. This very simple example is already a difficult analysis problem. The more common situation where many inhibitors with similar binding constants are present may become difficult or impossible to analyze.

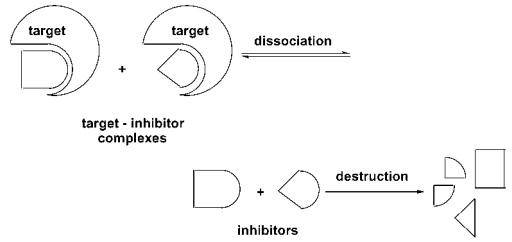
Finding the Best Inhibitor by Destruction of Poorer Inhibitors. One way to enhance the concentration differences between inhibitors with similar binding constants is to add an irreversible reaction that removes the unbound poorer inhibitor.

(4) (a) Eliseev, A. V.; Nelen, M. I. *J. Am. Chem. Soc.* **1997**, *119*, 1147−1148. (b) Eliseev, A. V.; Nelen, M. I. *Chem. Eur. J.***1998**, *4*, 825−834.

(5) Glaucoma patients often take carbonic anhydrase inhibitors to reduce the pressure in the eye. All commercial inhibitors contain a sulfonamide moiety. We chose carbonic anhydrase as a test case for inhibitor design and screening methods.

(6) If the binding is not tight or the target is not in excess at high concentration, then the concentrations of the inhibitors will be more similar than those discussed in the text above, and it will be even harder to distinguish which is the better inhibitor.

Scheme 3. Destruction of Inhibitors[a]



[a] The free concentration of the poorer inhibitor is higher, thus it is destroyed more readily. This destruction reaction exponentially increases the relative concentration of the good inhibitor similar to a kinetic resolution.

This situation is similar to a kinetic resolution of enantiomers.[7] As the destruction reaction winnows away the poorer inhibitors, the relative concentration of the best inhibitor increases exponentially (Scheme 3). The analysis below is similar to that for kinetic resolutions.[7]

Consider two inhibitors, A and B, that compete for a target, T, and are also destroyed by an irreversible reaction to yield P and Q with rates of k_{2A} and k_{2B}.

$$\mathrm{T{\cdot}A} \overset{K_{dA}}{\rightleftharpoons} \mathrm{T} + \mathrm{A} \xrightarrow{k_{2A}} \mathrm{P} \tag{4}$$

$$\mathrm{T{\cdot}B} \overset{K_{dB}}{\rightleftharpoons} \mathrm{T} + \mathrm{B} \xrightarrow{k_{2B}} \mathrm{Q} \tag{5}$$

The rate of disappearance of inhibitor A is

$$\frac{\mathrm{d[A]}}{\mathrm{d}t} = -k_{2A}[\mathrm{A}] \tag{6}$$

If $[A_T]$ is the total concentration of bound and unbound forms of inhibitor A, it can be shown that

$$[\mathrm{A_T}] = [\mathrm{A}]\left(1 + \frac{[\mathrm{T}]}{K_{dA}}\right) \tag{7}$$

Upon solving for [A] and substituting into eq 6, the rate of disappearance of A is given by

$$\frac{\mathrm{d[A]}}{\mathrm{d}t} = -\frac{k_{2A}K_{dA}[\mathrm{A_T}]}{K_{dA} + [\mathrm{T}]} \tag{8}$$

When the target is in excess of the inhibitor, the concentration of the free target, T, will be much larger than the dissociation constant, K_{dA}, so $[T] \gg K_{dA}$, therefore, eq 8 simplifies to

$$\frac{\mathrm{d[A]}}{\mathrm{d}t} = -\frac{k_{2A}K_{dA}[\mathrm{A_T}]}{[\mathrm{T}]} \tag{9}$$

A similar equation is obtained for inhibitor B. The ratio of their partial reaction rates is

$$\frac{\mathrm{d[A]}}{\mathrm{d[B]}} = \frac{K_{dA}}{K_{dB}} \times \frac{k_{2A}}{k_{2B}} \times \frac{[\mathrm{A_T}]}{[\mathrm{B_T}]} = \frac{1}{S} \times \frac{[\mathrm{A_T}]}{[\mathrm{B_T}]},$$

$$\text{where } S = \frac{K_{dB}}{K_{dA}} \times \frac{k_{2B}}{k_{2A}} \tag{10}$$

This equation shows that the relative rate of disappearance of the two inhibitors depends linearly on their total concentration, their relative binding ability, and their relative rate of destruction. For simplicity, we define *S* as the product of the relative binding abilities and relative rates of destruction of the two inhibitors. If the rates of destruction of the two inhibitors are equal, then *S* is the ratio of the dissociation constants and will be greater than one if A is a stronger inhibitor of the target than B.

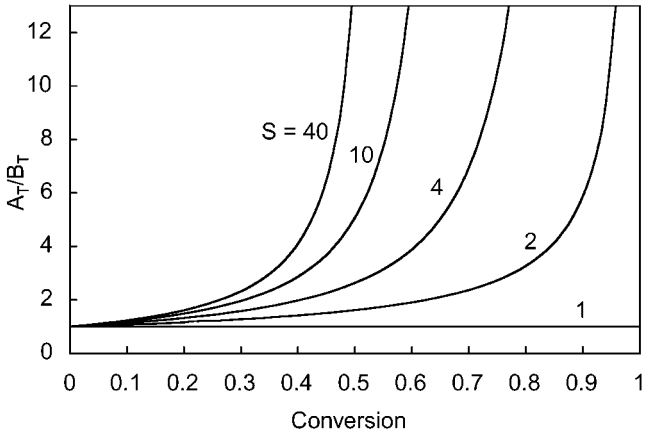


Figure 1. Predicted ratio of the total (bound and unbound) concentrations of two hypothetical inhibitors, A and B, as a function of the degree of conversion for given values of S. The degree of conversion is the fraction of the total amount of inhibitors that have been destroyed. The calculated lines follow eqs 11 and 12 where the initial total concentration of the inhibitors is one. This graph shows that the ratio of the two inhibitors can be much larger than the value of S, even for values of *S* as low as 2.

Upon integration of eq 10, one finds that the ratio of the total amounts of the two inhibitors varies exponentially with *S* (eq 11), where $[A_T]_0$ represents the initial total concentration of inhibitor A. This exponential relationship enhances the ability to detect small differences as the destruction reaction progresses.

$$\frac{\ln([\mathrm{B_T}]/[\mathrm{B_T}]_0)}{\ln([\mathrm{A_T}]/[\mathrm{A_T}]_0)} = S \tag{11}$$

By measuring the relative concentration of the two inhibitors during the destruction reaction, the value of *S* can be determined using eq 11. Alternatively, eq 12 below, which expresses $[A_T]$, $[B_T]$, $[A_T]_0$, and $[B_T]_0$ in terms of the conversion, *C*, and the ratio of the total concentrations of the two inhibitors, can be used to determine *S*.

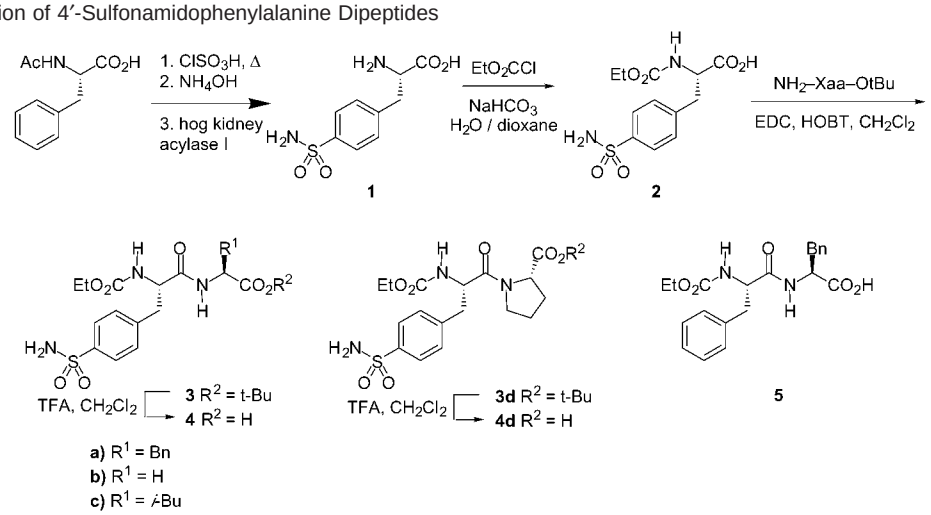
$$\frac{\ln[(1-C)(2/(1+R))]}{\ln[(1-C)(2R/(1+R))]} = S$$

$$\text{where } C = 1 - \frac{[\mathrm{A_T}] + [\mathrm{B_T}]}{[\mathrm{A_T}]_0 + [\mathrm{B_T}]_0} \quad \text{and } R = \frac{[\mathrm{A_T}]}{[\mathrm{B_T}]} \tag{12}$$

These predictions are shown graphically for several values of *S* in Figure 1. If the rates of destruction of the two inhibitors are equal, then *S* is the ratio of the dissociation constants. As the destruction reaction proceeds (conversion increases from 0 to 1), the ratio of the total amounts of the two inhibitors, $[A_T]/[B_T]$, varies when $S \neq 1$. When *S* is large (e.g., 40), the relative concentration of the good inhibitor increases steeply near 50% conversion. When *S* is small (e.g., 2), the relative concentration of the good inhibitor increases steeply near 90% conversion.

(7) The analysis below follows closely the mathematical treatment for kinetic resolutions. For examples, see: Martin, V. S.; Woodard, S. S.; Katsuki, T.; Yamada, Y.; Ikeda, M.; Sharpless, K. B. *J. Am. Chem. Soc.* **1981**, *103*, 6237−6240; Chen, C. S., Fujimoto, Y., Girdaukas, G., Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294−7299; Kagan, H. B., Fiaud, J. C. *Top. Stereochem.* **1988**, *18*, 249−330.

Scheme 4. Preparation of 4′-Sulfonamidophenylalanine Dipeptides



In either case, the ratio of the total amounts of the two inhibitors, $[A_T]/[B_T]$, can be much larger than the value of S.

Results

Preparation of 4′-Sulfonamidophenylalanine Dipeptides. (*S*)-4′-Sulfonamidophenylalanine (**1** or Phe_{sa}) was prepared from (*S*)-*N*-acetylphenylalanine by a modification of the procedure described by Escher et al.[8] Thus, chlorosulfonylation of *N*-acetylphenylalanine in chlorosulfonic acid at 60 °C followed by ammonolysis afforded *N*-acetyl-4′-sulfonamidophenylalanine. Direct purification of this intermediate proved difficult. Therefore, it was deacetylated using hog kidney acylase I,[9] and the resulting free amino acid **1** was purified by ion-exchange chromatography and recrystallization. Using this procedure, **1** was prepared as an analytically pure solid in 40% yield from *N*-acetyl-Phe. The α-amino group was selectively blocked using ethyl chloroformate under standard Schotten−Baumann conditions. The requisite dipeptides were then prepared by coupling **2** with *tert*-butyl amino acid esters using EDC/HOBT,[10] followed by trifluoroacetic acid-mediated deprotection of the ester function to afford dipeptides **4a**−**d** (Scheme 4). No acylation of the sulfonamide nitrogen was observed under either the Schotten−Baumann or peptide-coupling conditions. Dipeptide EtOC-Phe-Phe (**5**), which does not contain a sulfonamide group and serves as a control, was prepared by standard methods.

Inhibition of Carbonic Anhydrase. Sulfonamides **1** and **2** as well as sulfonamide dipeptides **4a**−**d** all inhibited the carbonic anhydrase-catalyzed hydrolysis of 4-nitrophenyl acetate (pNPA). The inhibition was competitive and Lineweaver−Burk plots revealed similar inhibition constants, which varied by only a factor of 10 (Table 1). The parent amino acid **1** (Phe_{sa}) was the poorest sulfonamide inhibitor ($K_I = 13\ \mu M$), while dipeptides **4a** (EtOC-Phe_{sa}-Phe) and **4b** (EtOC-Phe_{sa}-Gly) were the best sulfonamide inhibitors ($K_I = 1.2$ and $2.5\ \mu M$, respectively). Dipeptides **4c** (EtOC-Phe_{sa}-Leu) and **4d** (EtOC-Phe_{sa}-Pro) showed slightly higher inhibition constants (4.4 and 9.4 μM, respectively). Other simple sulfonamides also inhibit carbonic anhydrase with similar inhibition constants.[11] As expected, the dipeptide lacking a sulfonamide group, **5**, did not inhibit carbonic anhydrase.

Table 1. Inhibition of Carbonic Anhydrase by Sulfonamides **1** and **2**, Sulfonamide Dipeptides **4a**−**d**, and Dipeptide **5**

compound	K_I (μM)[a]
Phe_{sa} (**1**)	13 ± 1.6
EtOC-Phe_{sa} (**2**)	12 ± 1.4
EtOC-Phe_{sa}-Phe (**4a**)	1.2 ± 0.2
EtOC-Phe_{sa}-Gly (**4b**)	2.5 ± 0.5
EtOC-Phe_{sa}-Leu (**4c**)	4.4 ± 0.7
EtOC-Phe_{sa}-Pro (**4d**)	9.4 ± 1.6
EtOC-Phe-Phe (**5**)	≫1000[b]

[a] Competetive inhibition constants for the carbonic anhydrase-catalyzed hydrolysis of *p*-nitrophenyl actetate (*p*NPA) at 25 °C in phosphate buffer (10 mM pH 7.5). A typical procedure was to add carbonic anhydrase solution (100 μL, 0.05 mg/mL) containing inhibitor (0.0−100 μM in most cases) to an acetonitrile solution of *p*NPA (5.0 μL, 2.0−32 mM) and follow the release of *p*-nitrophenoxide spectrophotometrically at 404 nM. [b] No inhibition detected at an inhibitor concentration of 1 mM.

Selective Extraction of Inhibitors by Carbonic Anhydrase. First, we demonstrated that a strongly binding inhibitor concentrates into the carbonic anhydrase-containing compartment of a two-compartment vessel (cf. Figure 4 without Pronase). The two compartments were created by suspending a dialysis bag containing a solution of bovine carbonic anhydrase[12] (0.34 mM) in a solution of phosphate buffer. The dialysis membrane (12-kDa cutoff) separated the two compartments so that small molecules such as the sulfonamide dipeptides could diffuse freely across the membrane, while carbonic anhydrase (30 kDa) could not. Both compartments initially contained a mixture of 0.16 mM sulfonamide dipeptide **4a** and 0.19 mM noninhibitor dipeptide **5**. Over several hours the total sulfonamide concentration increased in the inside compartment containing carbonic

(8) Escher, E.; Bernier, M.; Parent, P. *Helv. Chim. Acta* **1983**, *66*, 1355−1365.
(9) Researchers often use hog kidney acylase to resolve enantiomers of *N*-acetyl amino acids. Chenault, H. K.; Dahmer, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1989**, *111*, 6354−6364; Roberts, S. M., Ed. *Preparative Biotransformations*; Wiley: Chichester 1992−1998; Module 1:14. In our case, this intermediate was already enantiomerically pure. We used hog kidney acylase to cleave the acetyl group under milder conditions than those required by chemical cleavage methods.
(10) EDC = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; HOBT = 1-hydroxybenzotriazole.
(11) For example, Nguyen and Huc investigated a simple sulfonamides with inhibition constants of ∼0.1−1 μM (Nguyen, R.; Huc, I. *Angew. Chem., Int. Ed.* **2001**, *40*, 1774−1776), while Doyon et al. investigated other simple sulfonamides with inhibition constants of ∼0.001 μM (Doyon, J. B.; Hansen, E. A. M.; Kim, C.-Y.; Chang, J. S.; Christianson, D. W.; Madder, R. D.; Voet, J. G.; Baird, T. A., Jr.; Fierke, C. A.; Jain, A. *Org. Lett.* **2000**, *2*, 1189−1192).
(12) These experiments required stoichiometric amounts of carbonic anhydrase. We used an inexpensive mixture of isozymes from bovine sources. Although material was not pure carbonic anhydrase, we calculated the concentrations assuming it was pure. Thus, the true concentration will be less than the number given.

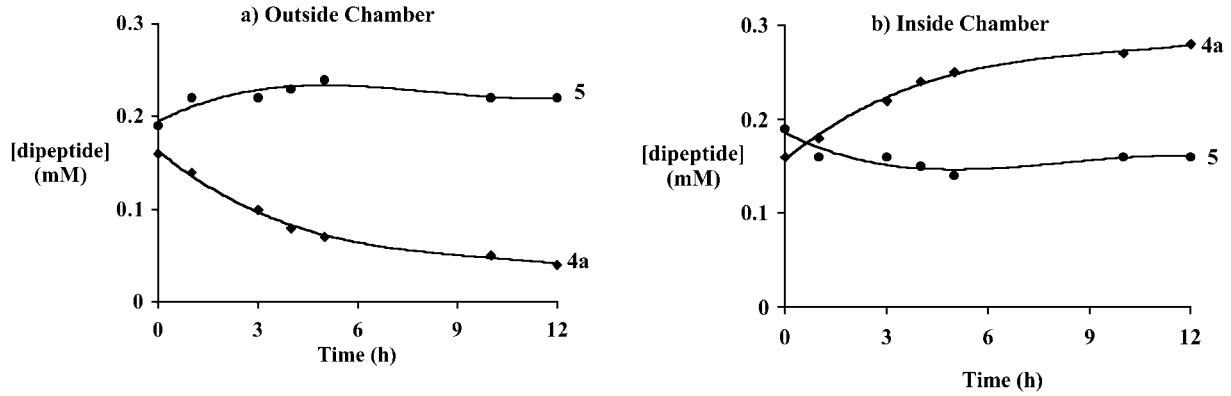


Figure 2.[13] Selective concentration of the sulfonamide **4a** over noninhibitor **5** into the carbonic-anhydrase-containing compartment of a two-compartment vessel. One compartment contained carbonic anhydrase (0.34 mM), while both compartments (20 mL each) initially contained equal concentrations of sulfonamide **4a** (0.16 mM) and noninhibitor **5** (0.19 mM). The sulfonamide diffused freely across the dialysis membrane and concentrated in the carbonic-anhydrase-containing compartment as shown. In contrast, the concentrations of noninhibitor **5** remained similar in both compartments. After 12 h, the concentration of sulfonamide **4a** in the outside compartment decreased to 0.04 mM and increased in the inside compartment to 0.28 mM (total of free and carbonic anhydrase-bound). The final ratio of **4a** to **5** in the carbonic anhydrase chamber was 1.75:1.

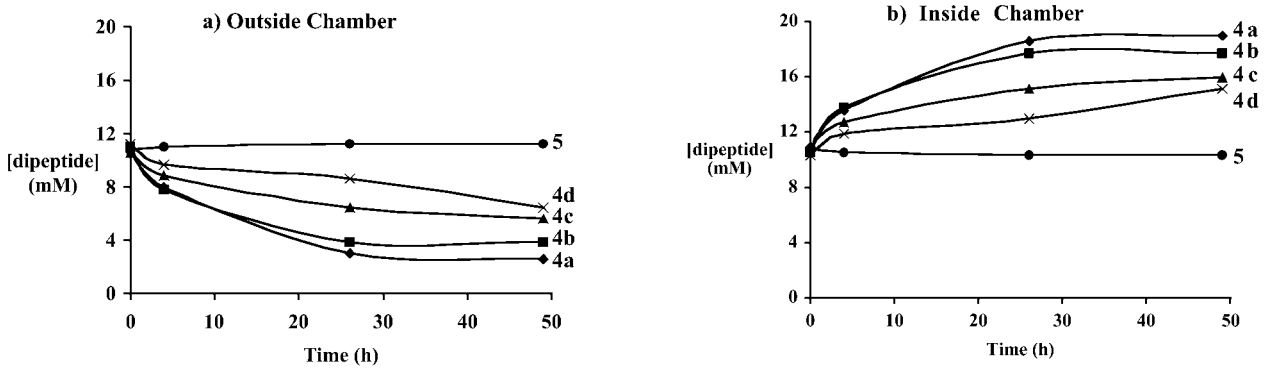


Figure 3.[13] Selective concentration of the sulfonamides **4a-4d** over noninhibitor **5** into the carbonic-anhydrase-containing compartment of a two-compartment vessel separated by a dialysis membrane. One compartment contained carbonic anhydrase (0.485 mM), while both compartments (20 mL each) initially contained equal concentrations of sulfonamides **4a-4d** and noninhibitor **5** (~0.11 mM each). The sulfonamides diffused freely across the dialysis membrane and concentrated in the carbonic-anhydrase-containing compartment. In contrast, the concentration of noninhibitor **5** increased slightly in the outer compartment.

anhydrase and decreased in the outside compartment (Figure 2). Alternatively, the concentrations of the noninhibitor **5** remained similar in both compartments. This result showed that tight binding to a target could concentrate a good inhibitor into one compartment of a two-compartment reaction vessel.

In a similar experiment using a mixture of inhibitors, we could further detect differences in relative inhibition constants. A more tightly binding inhibitor concentrated in the carbonic anhydrase compartment to a greater extent than a less tightly binding inhibitor. Starting with an equimolar mixture of sulfonamide dipeptides **4a**−**d** and the noninhibitor **5** in both compartments, the sulfonamide dipeptides concentrated into the carbonic anhydrase compartment, Figure 3. The fraction of dipeptide in the carbonic anhydrase compartment varied: 88, 82, 74, 70, and 48% for **4a**, **4b**, **4c**, **4d**, and the noninhibitor **5**, respectively (or a ratio of 1.83:1.71:1.54:1.46:1 for **4a:4b:4c:4d:5**). The order of highest to lowest concentration in the carbonic anhydrase chamber reflects the order of the binding constants of the inhibitors.

These results show that is possible to distinguish between inhibitors, but the differences in concentration are small, especially among inhibitors of similar strength. Even comparing the best inhibitor (**4a**) with a noninhibitor (**5**) gives a concentration differing by less than a factor of 2. To enhance this difference in concentration, we explored the use of proteases to destroy the poorer inhibitors.

Screening of Proteases. We screened 22 commercially available proteases to identify those that could hydrolyze the dipeptide EtOC-Phe$_{sa}$-Phe (**4a**). All proteases showed some activity. Using 0.1 mg of protease and 2 μmol (2 mM) dipeptide **4a**, the five most active proteases (α-chymotypsin, protease from *Streptomyces casepitosus*, proteinase K, Pronase from *Streptomyces griseus*, and protease from *Bacillus thermoproteolyticus rokko*) cleaved all of the dipeptide within 24 h, while two moderately active proteases (protease N "Amano", protease from *Bacillus polymyxa*) cleaved all of the dipeptide within 48 h. The remaining proteases cleaved less than half of the dipeptide after 72 h. We chose one of the most active yet inexpensive enzymes, Pronase from *S. griseus*, for subsequent experiments. Pronase was found to cleave all five dipeptides (**4a**−**d** and **5**), although the glycine and proline dipeptides (**4b** and **4d**) were cleaved more slowly (80−90% hydrolysis within 24 h). To ensure high cleavage rates, larger amounts of Pronase were used in the competitive degradation experiments described below.

Selective Protection of Inhibitors by Carbonic Anhydrase from Hydrolysis. We compared the ability of carbonic anhydrase to protect sulfonamide inhibitor **4a** from hydrolysis while allowing a noninhibitor, **5**, to be hydrolyzed. An experiment

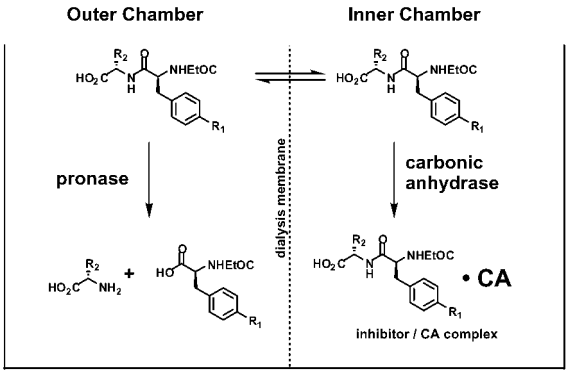


Figure 4. Reaction design for the selective destruction experiments. The dipeptides can diffuse across the dialysis membrane into either chamber. One chamber contains carbonic anhydrase, the other contains Pronase. Dipeptides in the Pronase chamber are rapidly cleaved to their constituent pieces. Carbonic anhydrase prevents strong binding dipeptides from diffusing across the membrane and thus slows their hydrolysis.

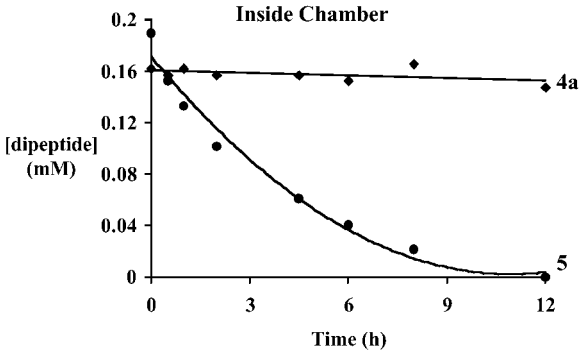


Figure 5.[13] Selective protection from hydrolysis of sulfonamide **4a** over noninhibitor **5** by carbonic anhydrase. A vessel containing two compartments of equal volume (20 mL each) separated by a dialysis membrane was filled with a solution of. sulfonamide **4a** (0.16 mM) and noninhibitor **5** (0.19 mM). The inside compartment contained carbonic anhydrase (0.34 mM), while the outside compartment contained Pronase. The protease rapidly cleaved the dipeptides in the outside compartment to the corresponding amino acids (data not shown). The noninhibitor **5** diffused freely across the dialysis membrane and was cleaved by the protease. In contrast, the inhibitor **4a** bound to carbonic anhydrase in the inside compartment and was not consumed at a significant rate. After 6 h, the concentration of sulfonamide **4a** in the inside compartment decreased by only 6% (0.15 mM), while the concentration of noninhibitor **5** decreased to 0.041 mM during the same time period (ratio = 3.7:1).

similar to that described above, except with Pronase added to the outer chamber was set up (Figure 4). On the one hand, in the Pronase-containing chamber, both dipeptides were rapidly cleaved to the constituent pieces within 15 min. On the other hand, the inside compartment showed a steady decrease in the concentration of noninhibitor **5** over 12 h (Figure 5), while the concentration of sulfonamide **4a** remained nearly constant (a decrease of 9% over 12 h).[14] After even just 6 h, the ratio, **4a**:**5**, in the inside compartment was 3.7:1 and continued to increase to greater than 20:1 after 12 h. By comparison, the experiment which does not contain Pronase had a final ratio of **4a** to **5** of 1.75:1.

In a simlar experiment, dipeptides **4a** and **4b**, which have very similar binding constants, were exposed to carbonic anhydrase and Pronase. In this experiment, the dipeptides were

(13) Lines drawn in all figures (except Figure 1 and Figure 9) are for illustration purposes only. They do not represent theoretical lines of any sort.
(14) Both **4a** and **5** diffused through the membrane at identical rates with a half-life of about 3 h. (Data not shown.)

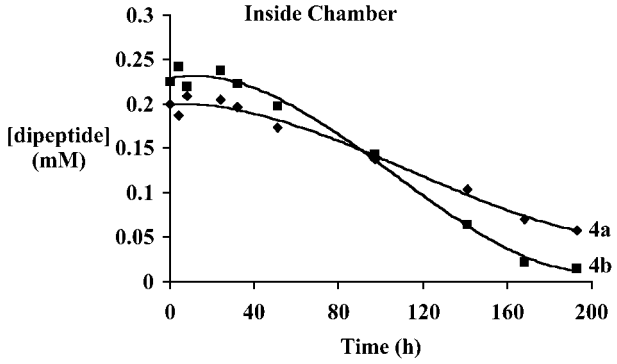


Figure 6.[13] Selective protection from hydrolysis of dipeptide **4a** over **4b** by carbonic anhydrase. A reaction vessel was separated into two compartments (20 mL each) by a dialysis membrane. The inside compartment contained carbonic anhydrase (13.6 mol), dipeptide **4a** (4.3 mol) and dipeptide **4b** (4.3 mol) in 20 mL of buffer. The outer compartment contained Pronase (5 mg) dissolved in 20 mL of buffer. The time course of the reaction in the carbonic anhydrase chamber is shown in the figure. At 83% conversion (193 h) the ratio of **4a** to **4b** was 3.8:1.

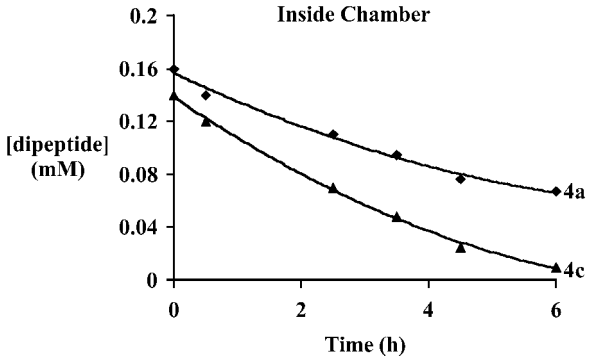


Figure 7.[13] Selective protection from hydrolysis of dipeptide **4a** over **4c** by carbonic anhydrase. A reaction vessel was separated into two compartments (20 mL each) by a dialysis membrane. The inside compartment contained carbonic anhydrase (0.34 mM), while the outside compartment contained Pronase (4 mg). Both compartments initially contained similar concentrations of dipeptide **4a** (0.16 mM) and dipeptide **4c** (0.14 mM). The protease rapidly cleaved the dipeptides in the outside compartment to the corresponding amino acids (data not shown). The time course of the reaction in the carbonic anhydrase chamber is shown in the figure. After 6 h, 93% of **4c** inside the CA chamber had been hydrolyzed, while only 58% of **4a** had hydrolyzed. A control experiment which did not contain carbonic anhydrase showed an equal rate of hydrolysis for the two dipeptides in the chamber not containing Pronase.

placed only in the carbonic anhydrase chamber, and an excess of carbonic anhydrase was used (1.6:1 ratio of CA to dipeptides) so that the conditions adhered rigorously to those of the theory described above. As expected, due to the excess of target and tight binding of both dipeptides, the hydrolysis of **4a** and **4b** was slow. However, as in the first reaction, the weaker binder, **4b**, was consumed at a higher rate (Figure 6). After 193 h, 83% of the total dipeptides had been hydrolyzed, and the ratio of **4a** to **4b** was 3.8:1. This final ratio is in excess of the ratio of the independently determined binding constants of the dipeptides (2.1:1).

In a related experiment, we compared two sulfonamide dipeptides **4a** and **4c** which also have similar inhibition constants (Figure 7). In this experiment, both the inside and outside chambers initially contained equal concentrations of the dipeptides, and the total concentration of dipeptides was in excess (2.1:1 ratio of dipeptides to CA). The result was a much faster hydrolysis of both dipeptides in the carbonic anhydrase chamber. This faster rate reflects the rapid release of 1 equiv of Phe_{SA}

(**2**) from the Pronase chamber. Although **2** is a weaker binder than either **4a** or **4c**, enough of it was produced such that it could displace a small amount of **4a** and **4c** from the carbonic anhydrase binding pocket, thus accelerating their hydrolysis by Pronase. However, the net result was still the same. After 6 h, 93% of **4c** was hydrolyzed after 6 h, but only 58% of **4a** was hydrolyzed. Thus, the ratio of concentrations was 6:1, which is much larger than the 1.6:1 ratio observed in a control experiment which did not contain Pronase and larger than the 3.7:1 ratio of their binding constants.

Finally, an experiment containing all five dipeptides (**4a**−**d** and **5**) was conducted using an excess of carbonic anhydrase (ratio of CA to dipeptides is 1.2:1). The experiment was consistent both with the theory and with the prior results. Dipeptide **5** was cleaved rapidly while dipeptides **4a**−**d** disappeared at rates which corresponded to their binding constants (Figure 8).

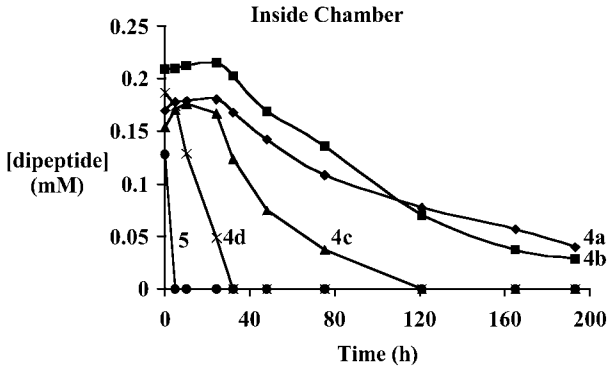


Figure 8.[13] Selective protection from hydrolysis of dipeptides by carbonic anhydrase. A reaction vessel separated into two compartments (20 mL each) by a dialysis membrane was set up. The inside compartment contained carbonic anhydrase (25.6 mol) and dipeptides **4a**−**d** and **5** (4.3 mol each) in 20 mL of buffer. The outer compartment contained Pronase (5 mg) dissolved in 20 mL of buffer. The time course of the reaction in the carbonic anhydrase chamber is shown in the figure.

Discussion

As expected, the four sulfonamide dipeptides **4a**−**d** all inhibit carbonic anhydrase competitively with similar inhibition constants (within a factor of 10 of each other). Classical kinetics using initial rates easily identified these differences, but these classical methods are slow and require the separate measurement of each inhibitor. This becomes laborious for libraries containing thousands of members.

To rapidly identify the best inhibitor, we used competitive binding to carbonic anhydrase in one compartment of a two-compartment cell. The inhibitors concentrated into the carbonic anhydrase compartment of a two-compartment cell. Higher concentrations of the better inhibitors were observed in the carbonic anhydrase compartment, but the concentration differences were small (1.83:1.71:1.54:1.46:1 for **4a:4b:4c:4d:5**). If the mixture contained 1000 dipeptides, this competitive experiment would not identify the best inhibitor because it would be difficult to separate all the dipeptides, and the differences in concentration with and without target would be small.

Although this experiment does not include a dipeptide-synthesis step and thus is not a dynamic library, the diffusion across the membrane mimics a synthesis step in a dynamic library in that both are equilibrium processes. For the diffusion process, in the absence of a target, each compartment should contain equal amounts of each inhibitor. In the presence of the target, the carbonic anhydrase chamber contains more of the tight-binding inhibitors. Thus, the equilibrium for the diffusion process has shifted.

A nonselective destruction of the library members should enhance differences in the relative concentrations of the members bound to the target. The poor binding members are destroyed at a rate higher than that for the strong-binding members, and as the degradation progresses, the ratio improves exponentially in favor of the latter. This was observed in our library, where dipeptide hydrolysis by Pronase was used as the destruction process. In a competition experiment between a strong and weak binder (**4a** vs **5**), the ratio of **4a** to **5** in the carbonic anhydrase chamber increased from 1.75:1 in the absence of Pronase to 3.7:1 in the presence of Pronase (at 45% conversion). Furthermore, this ratio continued to increase to >20:1 as the reaction progressed. A second experiment with two species with very similar K_i's (**4a** vs **4b**) had a final ratio of 3.8:1 when the ratio of the binding constants was 2.1:1. As shown in Figure 9a, these results follow the theoretical model closely. Similar results were obtained for an experiment containing two inhibitors (**4a** and **4c**) where an excess of a weaker binder, Phe_{SA} (**2**), was generated in the reaction mixture. The presence of **2** accelerated the rate of cleavage of **4a** and **4c**, but as can be seen in Figure 9b, the ratio of dipeptides during the course of the reaction still followed the theoretical model. At 70% conversion, the ratio of **4a** to **4c** was 6:1, which is much larger than the 1.6:1 ratio observed in a reaction not containing Pronase. In all cases, the model indicates that the ratios should continue to increase if the reactions are carried out for even longer periods. In experiments with a large number of library members, this increase will be critical in allowing the tightest binding species to be easily identified.[15]

One potential limitation of this screening method is selectivity in the destruction reaction. For example, dipeptide **4c** is cleaved by Pronase at a much slower rate than that for dipeptide **4a**. In such a case, *S* from eqs 10−12 will not be equal to the ratio of the binding constants, and thus the degradation reaction will not follow the theoretical curves of Figure 1. To accommodate this situation, we used a large amount of protease, and more importantly, we employed a dialysis membrane to separate the target−inhibitor complexes from the protease. In this setup, the rate-limiting step in the destruction reaction is not the protease-catalyzed cleavage but diffusion across the dialysis membrane. Unlike the protease-catalyzed cleavage, the rate of diffusion does not vary significantly with the structure of the inhibitor, and the result is that the destruction reaction follows the theoretical curve. Although Pronase accepts a wide variety of peptides, substrate specificity of the enzyme may become problematic if highly diverse libraries are studied. A dipeptide which is not cleaved by Pronase would be retained in the reaction mixture even if it did not bind to carbonic anhydrase. One way to alleviate this problem would be to use a mixture of enzymes with a wide range of specificities. Alternatively, it is important to note that the degradation reaction is not limited to enzymic processes. Other chemical degradation methods can be envisioned, depending upon the type of library being studied. For

(15) The reaction mixture will contain the products of the degradation reactions. However, in most cases, this method will be applied to combinatorial libraries, and as such, the degradation products will often be the common starting materials used to make the library members. Thus, only a limited number of degradation products will be produced.

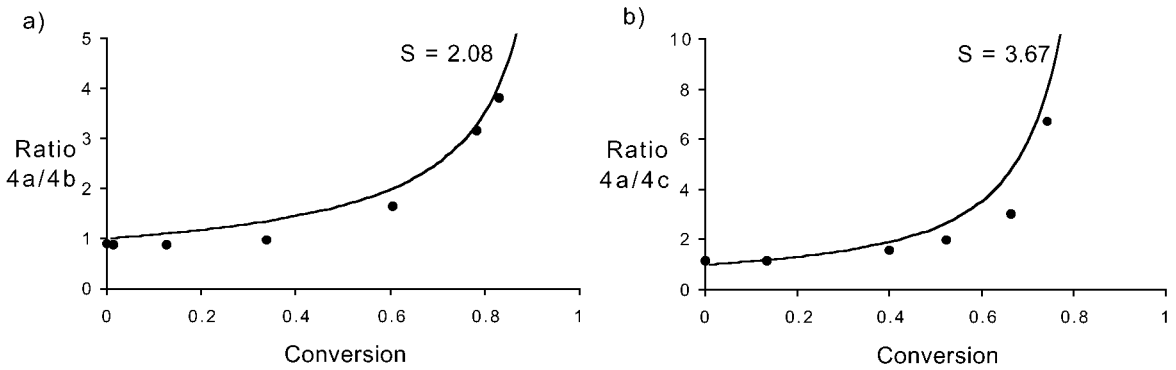


Figure 9. The graph shows theoretical and experimental ratios for the screening experiments. Theoretical lines are shown as smooth lines. The *S* values correspond to the ratios of the experimentally determined binding constants. The data points show the experimentally determined ratios at different conversions for a) **4a**/**4b** (cf. Figure 6) and b) **4a**/**4c** (cf. Figure 7).

example, a library based on disulfide exchange could be degraded by adding a reducing agent (e.g., a phosphine) to cleave any unbound disulfides. Alternatively, physical methods for removal of unbound inhibitors (e.g., adsorption to a solid phase, extraction) should accomplish the same effect as a chemical degradation.

Another potential limitation of this screening method, and indeed for methods based on the dynamic combinatorial library technique, is the need for stoichiometric amounts of the target. The initial experiments reported here used large amounts of carbonic anhydrase (100−500 mg/experiment) as we expect to apply it to a dynamic library process where the best inhibitor will actually be isolated and characterized. However, for purely analytical screening purposes, the methods can easily be scaled down using smaller compartments, assuming that more sensitive analytical tools are used (e.g., mass spectroscopy). These modifications could reduce the amount of target needed to <0.1 mg/experiment, an amount that is easily accessible for targets that have been cloned and overexpressed.

In conclusion, we have developed a method for screening mixtures of compounds against a therapeutic target which readily identifies the best binder in a library. The method works by selectively degrading the poorer inhibitors with an enzyme. This results in a significant improvement in the ability to distinguish between inhibitors which have very close binding constants. We plan to extend this method to dynamic libraries with the goal of improving the enhancement observed in synthesis of good inhibitors in the presence of a therapeutic target.

Experimental Section

General Experimental. *p*-Nitrophenyl acetate (*p*NPA), carbonic anhydrase (CA, from bovine erythrocytes, a mixture of isozymes, C-3934) and proteases were purchased from Sigma unless otherwise noted and used without further purification. HPLC analyses were conducted using a Phenomenex-C_8 reversed phase HPLC column (10 mm × 250 mm) with detection at 220 nm, unless otherwise noted. Elemental analyses were obtained from Quantitative Technologies Inc., Whitehouse, NJ. High-resolution mass spectra were obtained from Université de Sherbrooke, Sherbrooke, QC.

4′-Sulfonamidophenylalanine (1). *N*-Acetylphenylalanine (37.7 g, 178 mmol, 1 equiv) was added in portions over a 1-h period to neat chlorosulfonic acid (110 mL, 1.65 mol, 9.5 equiv) at −10 °C. The resulting yellow solution was stirred at −10 °C for 2.5 h, at 25 °C for 2.5 h, and then heated to 60 °C until gas evolution had ceased (approximately 12 h). The resulting orange solution was cooled to 0 °C and poured carefully onto 750 mL of ice (Caution: exotherm!). The resulting mixture was extracted with ethyl acetate (3 × 1 L), and the combined organic layers were dried over Na_2SO_4, filtered, and concentrated in vacuo to afford the sulfonyl chloride (45.1 g, 83%) as an orange solid which was used immediately without further purification. ^{1}H NMR ($(CD_3)_2SO$) δ 8.26−8.21 (d, 1H, J = 8.5 Hz), 7.55 (d, 2H, J = 6.9 Hz), 7.22 (d, 2H, J = 6.8 Hz), 4.49−4.34 (m, 1H), 3.13−3.00 (dd, 1H, J = 14.4 and 6.8 Hz), 2.92−2.79 (dd, 1H, J = 11.0 and 10.2 Hz), 1.80 (s, 3H).

The sulfonyl chloride was dissolved in 28% NH_4OH (240 mL), and the resulting solution was heated at reflux for 3 h. After cooling to 0 °C, the solution was acidified to pH 1 by addition of 3 M H_2SO_4 (ca. 200 mL) and extracted with ethyl acetate (3 × 500 mL). The combined organic extracts were dried over Na_2SO_4, filtered, and concentrated in vacuo to afford the sulfonamide (29.9 g, 71%) as a white solid. The *N*-acetyl sulfonamide could not be purified to homogeneity by either chromatography or recrystallization. ^{1}H NMR ($(CD_3)_2SO$) δ 8.29−8.24 (d, 1H, J = 8.5 Hz), 7.77 (d, 2H, J = 3.9 Hz), 7.45 (d, 2H, J = 6.9 Hz), 7.33 (s, 2H), 4.53−4.41 (m, 1H), 3.20−3.09 (dd, 1H, J = 14.2 and 6.8 Hz), 3.01−2.87 (dd, 1H, J = 11.2 and 10.1 Hz), 1.80 (s, 3H).

A suspension of the sulfonamide (20.0 g, 69.9 mmol, 1 equiv) in distilled water (300 mL) was adjusted to pH 5.00 with LiOH (900 mg). A 0.25 M solution of Na_2HPO_4 (85 mL) was used to raise the pH to 7.50. Acylase I from hog kidney (200 mg, 17.8 U/mg, 3560 U) was added as an aqueous solution (12 mL), and the resulting solution was stirred at 21 °C for 70 h. The solution was then acidified to pH 1.0 with 3 M H_2SO_4 and extracted with ethyl acetate (3 × 500 mL); the organic layer was then dried with anhydrous sodium sulfate and concentrated in vacuo to afford 2.28 g (11%) of the sulfonamide starting material. The aqueous layer was neutralized with 2 M NaOH and concentrated. The solution was then applied to an Amberlite 120(plus) acidic ion-exchange column. The column was rinsed with water until the eluent was at pH 6.0, and then it was rinsed with 0.50 M NH_4OH solution until the eluent became basic. The basic wash was concentrated in vacuo and recrystallized from water to afford the provided 4′-sulfonamidophenylalanine as a white solid (11.60 g, 68%).^{1}H NMR (D_2O/DCl) δ 7.62 (d, 2H, J = 8.1 Hz), 7.26 (d, 2H, J = 8.1 Hz), 4.14 (t, 1H, J = 6.8 Hz), 3.19−3.12 (dd, 1H, J = 14.6 and 5.7 Hz),3.08−3.01 (dd, 1H, J = 14.4 and 6.9 Hz). ^{13}C NMR (D_2O/DCl) δ 170.73, 140.451, 139.49, 130.38, 126.55, 53.49, 35.19. FABMS in saturated NaCl *m*/*z* 267 (M + Na, $C_9H_{12}N_2O_4SNa$ requires 267.)

***N*-Ethoxycarbonyl-4′-sulfonamidophenylalanine (2).** Ethyl chloroformate (398 μL, 4.17 mmol, 1.10 equiv) was added to a two-phase mixture of 4′-sulfonamidophenylalanine (925 mg, 3.73 mmol, 1 equiv) in 1,4-dioxane (25 mL) and saturated $NaHCO_3$ solution (25 mL) at 0 °C, and the resulting solution was stirred for 6 h at 0 °C. The mixture was extracted with ethyl acetate (100 mL), and the aqueous layer was acidified to pH 1 by addition of 2 M HCl (ca. 20 mL) and then extracted with ethyl acetate (3 × 50 mL). Latter organic extracts were combined, dried over Na_2SO_4, filtered, and concentrated in vacuo to afford the

ethyl carbamate (879 mg, 83%) as an analytically pure oil. ^{1}H NMR ($(CD_3)_2CO$) δ 7.85 (d, 2H, J = 7.1 Hz), 7.51 (d, 2H, J = 6.9 Hz), 6.54 (s, 2H), 6.45 (d, 1H, J = 6.7 Hz), 4.62−4.45 (m, 1H), 4.01−3.97 (q, 2H, J = 2.4 Hz), 3.41−3.28 (dd, 1H, J = 11.3 and 4.0 Hz), 3.16−3.05 (dd, 1H, J = 10.4 and 7.8 Hz), 1.13 (t, 3H, J = 6.0 Hz). HR-CIMS (m/z): [MH^+] calculated for $C_{12}H_{17}N_2O_6S$, 317.0807; found, 317.0817.

EtO$_2$C-(4′-SO$_2$NH$_2$)Phe-Gly-*O*-*tert*-butyl (3b). EDC·HCl (136 mg, 0.711 mmol, 1.10 equiv), HOBT (87.3 mg, 0.646 mmol, 1.00 equiv), and triethylamine (269 μL, 1.94 mmol, 3.00 equiv) were added to a solution of **2** (204 mg, 0.646 mmol, 1 equiv) in THF (3 mL) at 0 °C. Glycine *tert*-butyl ester·HCl (119 mg, 0.711 mmol, 1.10 equiv) was added, and the resulting solution was allowed to warm to 21 °C while stirring for 13 h, at which point the bulk of the THF was removed by concentration in vacuo. The residue was dissolved in ethyl acetate (45 mL) and extracted with 0.1 M HCl (3 × 25 mL) and saturated $NaHCO_3$ solution (3 × 25 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated in vacuo. The solid residue was purified by mixed solvent recrystallization (ethyl acetate/hexanes) to afford 193 mg (70%) of **3b**. ^{1}H NMR ($(CD_3)_2CO$) δ 7.82 (d, 2H, J = 7.5 Hz), 7.63 (s, 1H), 7.50 (d, 2H, J = 7.5 Hz), 6.52 (s, 2H), 6.40 (d, 1H, J = 7.5 Hz), 4.50 (m, 1H), 4.00−3.88 (m, 4H), 3.40 (dd, 1H, J = 14.1 and 4.2 Hz), 3.02 (dd, 1H, J = 13.5 and 9.9 Hz), 1.45 (s, 9H), 1.12 (t, 3H, J = 6.9 Hz). ^{13}C NMR ($(CD_3)_2CO$) δ 171.48, 168.93, 156.33, 142.70, 130.06, 126.18, 81.04, 60.48, 55.92, 41.79, 37.83, 27.52, 14.22. Analysis calculated for $C_{18}H_{27}N_3O_7S$ C, 50.34; H, 6.34; N, 9.78. Found: C, 50.33; H, 6.35; N, 9.73.

EtO$_2$C-(4′-SO$_2$NH$_2$)Phe-Gly-OH (4b). TFA (7 mL) was added to a solution of **3b** (175 mg, 0.409 mmol, 1 equiv) in CH_2Cl_2 (8 mL), and the solution was stirred for 25 min at 21 °C under an atmosphere of argon. The solvents were removed in vacuo, and the residue was purified by recrystallization from acetone to afford 121 mg (79%) of **4b.** ^{1}H NMR (CD_3OD) δ 8.55 (s, 1H), 7.83 (d, 2H, J = 7.2 Hz), 7.46 (d, 2H, J = 7.2 Hz), 4.45−4.42 (m, 1H), 4.02−3.98 (q, 2H, J = 6.8), 3.95−3.92 (m, 1H), 3.32−3.25 (m, 2H), 2.97−2.89 (dd, 1H, J = 13.5 and 9.9 Hz), 1.18−1.14 (t, 3H, J = 6.8). ^{13}C NMR (CD_3CD) δ 173.0, 171.8, 157.3, 148.7, 142.4, 137.6, 129.8, 126.0, 60.9, 56.0, 37.7, 13.7. HR−CIMS (m/z): [MH^+] calculated for $C_{14}H_{20}N_3O_7S$, 374.1022; found, 374.1030.

Measurement of Inhibition Constants. Kinetic constants for carbonic anhydrase (CA) were measured according to Pocker and Stone using *p*-nitrophenyl acetate (*p*NPA) as the substrate.[16] The CA-catalyzed hydrolysis of *p*NPA was followed spectrophotometrically at 25 °C in a 96-well microplate spectrophotometer by monitoring the appearance of *p*-nitrophenolate at 404 nm. The values of K_m and V_{max} were determined by measuring the hydrolysis rate as a function of the *p*NPA concentration. To determine the inhibition constants, the values of K_m and V_{max} were redetermined in the presence of varying amounts of inhibitor. Since the values of K_m for pNPA increased in the presence of the inhibitor, but the values of V_{max} remained unchanged, we concluded that the inhibition is competitive. The concentration of inhibitor that increased the K_m for pNPA by a factor of 2 is the inhibition constant. A typical procedure was to add CA solution (100.0 μL) with inhibitor to acetonitrile solution of *p*NPA (5.0 μL). In the assay solution, the concentration of inhibitor ranges from 0.0 to 6.0 μM, while the concentration of *p*NPA ranged from 0.2 to 2.5 mM. The microplate was shaken for 5 s before the first reading and for 3 s between readings.

Selective Concentration of EtOC-Phe$_{sa}$-Phe (4a) over EtOC-Phe-Phe, (5) into a Compartment Containing Carbonic Anhydrase. A solution of **4a** (2.9 mg, 6.3 μmol) and **5** (2.9 mg, 7.5 μmol) in 0.01 M aqueous phosphate buffer (pH 7.5, 40 mL) was divided into two equal portions. Carbonic anhydrase (0.20 g, approx. 6.7 μmol) was dissolved in the first portion, and the resulting solution (20.0 mL) was transferred to a dialysis bag (12 000-MW cutoff, Sigma D-0655). This dialysis bag was suspended in the second portion, and the reaction vessel was shaken gently (200 rpm) at 30 °C. Aliquots were removed periodically from each compartment, heated to 80 °C until a white precipitate formed (~5 min), and centrifuged, and the supernatant was filtered through a 0.22-μm pore filter. The amount of dipeptides was measured by HPLC using a Zorbax C8 column and 40/60/0.1 water/methanol/trifluoroacetic acid at 0.40 mL/min. After 12 h 88% of **4a** (retention time 11.4 min) had accumulated inside the dialysis bag while only 42% of **5** (retention time 25.5 min) was found inside the bag.

Selective Concentration Of EtOC-Phe$_{sa}$-Phe (4a) from a Mixture of EtOC-Phe$_{sa}$-Leu (4c), EtOC-Phe$_{sa}$-Gly (4b), and EtOC-Phe-Phe (5) by Carbonic Anhydrase. Dipeptides **4a** (2.0 mg, 4.3 μmol), **4b** (1.6 mg, 4.3 μmol), **4c** (1.9 mg, 4. μmol) **4d** (1.8 mg, 4.3 μmol), and **5** (1.7 mg, 4.3 μmol) were dissolved in 40 mL of 10 mM KH_2PO_4 buffer, pH 7.5 containing 0.1 mg/mL penicillin G (to avoid bacterial growth). Carbonic anhydrase (CA) (0.29 g, 9.7 μmol, 0.45 equiv) was dissolved in 20 mL of this solution and placed in a dialysis bag (the bag was washed in ddH_2O for 1 h, rinsed in EtOH once, and then washed again with ddH_2O). The bag was suspended in the remaining 20 mL of inhibitor solution in a 100-mL container and shaken at 60 rpm on a three-dimensional orbital shaker at room temperature for 49 h. Samples (1 mL) were taken periodically from inside and outside the dialysis bag, heated in an 80 °C water bath for 5 min, and then centrifuged for 10 min. The supernatant was filtered through a 0.22-μM sterile filter. The supernatant (700 μL) was added to MeOH (300 μL) to form the HPLC sample (30% MeOH, 70% aqueous). The sample was run on a Phenomenex C8 reverse phase column under the following conditions: 0−15 min 30% MeOH, 70% H_2O, 15−60 min 37% MeOH 63% H_2O, 60−90 min 62% MeOH, 38% H_2O. The peak areas were monitored: Phe$_{sa}$Gly: 7.9 min, PhesaPro: 17.6 min, Phe$_{sa}$Leu: 54.5 min, Phe$_{sa}$Phe: 60.0 min, PhePhe: 69.5 min. The percentages are accurate to ±2%. All nonsterile apparatus used was autoclaved prior to use to avoid bacterial growth.

Screening of Proteases for the Hydrolysis of EtOC-Phe-Phe Dipeptide (4a). The protease to be screened (0.1 mg) was added to a solution of **4a** (1.0 mg, 2.2 mol) in 0.01 M aqueous phosphate buffer (pH 7.5). The solution was kept at 30 °C, and aliquots were removed periodically, worked up as above, and analyzed by HPLC.

Selective Protection of Inhibitors from Hydrolysis by Carbonic Anhydrase. A solution of **4a** (3.0 mg, 6.5 μmol) and **5** (2.8 mg, 7.3 μmol) in 0.01 M aqueous phosphate buffer (pH 7.5, 40 mL) was divided into two equal portions. Carbonic anhydrase (0.20 g, approx 6.7 μmol) was dissolved in the first portion, and the resulting solution (20.0 mL) was transferred to a dialysis bag (12 000-MW cutoff, Sigma D-0655). Pronase from *S. griseus* (Sigma P-5147, 4 mg) was dissolved in the second portion, and the dialysis bag was then suspended in the resulting solution. The reaction vessel was then shaken gently (200 rpm) at 30 °C, and aliquots were removed periodically from each compartment, worked up as above, and analyzed by HPLC. After 30 min, neither substrate was detectable in the solution outside the dialysis bag. Inside the dialysis bag, 78% of **5** had hydrolyzed, while only 6% of **4a** had hydrolyzed after 6 h. In a control experiment containing no carbonic anhydrase, inside the dialysis bag, 76% of **4a** and 80% of **5** had hydrolyzed after 6 h.

Selective Binding of EtOC-Phe$_{sa}$-Phe (4a) over EtOC-Phe$_{sa}$-Leu (4c). A solution of **4a** (3.3 mg 7.1 mol) and **4c** (3.6 mg, 8.4 mol) in 0.01 M aqueous phosphate buffer (pH 7.5, 40 mL) was divided into two equal portions. Carbonic anhydrase (0.20 g, approx 6.7 μmol) was dissolved in the first portion, and the resulting solution (20.0 mL) was transferred to a dialysis bag (12 000-MW cutoff, Sigma D-0655). This dialysis bag was suspended in the second portion, and the reaction vessel was shaken gently (200 rpm) at 30 °C. Aliquots were removed periodically from each compartment, worked up as above, and analyzed by HPLC using a Zorbax C8 column. After 12 h 98% of **4a** had accumulated inside the dialysis bag, while only 60% of **4c** was found inside the bag.

Hydrolysis of EtOC-Phe$_{sa}$-Gly (4b) and EtOC-Phe$_{sa}$-Phe (4a) in the Presence of Carbonic Anhydrase. Phe$_{sa}$Phe **4a** (2.0 mg, 4.3 μmol)

(16) Pocker, Y.; Stone, J. T. *Biochemistry* **1968**, *7*, 3021−3031.

and Phe_{sa}Gly **4b** (1.6 mg, 4.3 μmol) were dissolved in 20 mL of 10 mM KH_2PO_4 buffer, pH 7.5. Carbonic anhydrase (CA) (0.4090 g, 13.6 μmol, 1.60 equiv) was dissolved in this solution and placed in a dialysis bag (the bag was washed in ddH_2O for 1 h, rinsed in EtOH once, and then washed again with ddH_2O). The bag was suspended in 20 mL of the phosphate buffer containing Pronase from *S. griseus* (5.0 mg, 0.01 equiv) in a 150-mL beaker and shaken at 150 rpm at 30 °C for 313 h. Samples (1 mL) were taken periodically from inside, worked up as above, and analyzed by HPLC. After 193 h, only 71% of **4a** had hydrolyzed, while 93% of **4b** had hydrolyzed.

Hydrolysis of EtOC-Phe_{sa}-Leu (4c) and EtOC-Phe_{sa}-Phe (4a) in the Absence of Carbonic Anhydrase. A solution of **4a** (2.9 mg 6.3 mol) and **4c** (2.4 mg, 5.6 mol) in 0.01 M aqueous phosphate buffer (pH 7.5, 40 mL) was divided into two equal portions. The first portion was transferred to a dialysis bag (12 000-MW cutoff, Sigma D-0655). Pronase from *S. griseus* (Sigma P-5147, 4 mg) was dissolved in the second portion, and the dialysis bag was then suspended in the resulting solution. The reaction vessel was then shaken gently (200 rpm) at 30 °C, and aliquots were removed periodically from each compartment, worked up as above, and analyzed by HPLC using a Zorbax C8 column. After 30 min, neither substrate was detectable in the solution outside the dialysis bag. After 8 h, 86% of **4a** and 88% of **4c** inside the dialysis bag had hydrolyzed.

Hydrolysis of EtOC-Phe_{sa}-Leu (4c) and EtOC-Phe_{sa}-Phe (4a) in the Presence of Carbonic Anhydrase. A solution of **4a** (2.9 mg, 6.3 mol) and **4c** (2.4 mg, 5.6 mol) in 0.01 M aqueous phosphate buffer (pH 7.5, 40 mL) was divided into two equal portions. Carbonic anhydrase (0.14 g, approx 4.7 μmol) was dissolved in the first portion, and the resulting solution (20.0 mL) was transferred to a dialysis bag (12 000-MW cutoff, Sigma D-0655). Pronase from *S. griseus* (Sigma P-5147, 4 mg) was dissolved in the second portion, and the dialysis bag was then suspended in the resulting solution. The reaction vessel was then shaken gently (200 rpm) at 30 °C, and aliquots were removed periodically from each compartment, worked up as above, and analyzed by HPLC. After 6 h, 93% of **4c** had hydrolyzed, while only 58% of **4a** was hydrolyzed.

Hydrolysis of EtOC-Phe_{sa}-Phe (4a), EtOC-Phe_{sa}-Gly (4b) EtOC-Phe_{sa}-Leu (4c), EtOC-Phe_{sa}-Pro (4d) and EtOC-Phe-Phe (5), in the Presence of Carbonic Anhydrase. Phe_{sa}Phe **4a** (2.0 mg, 4.3 μmol), Phe_{sa}Gly **4b** (1.6 mg, 4.3 μmol), Phe_{sa}Leu **4c** (1.9 mg, 4.3 μmol), Phe_{sa}-Pro **4d** (1.8 mg, 4.3 μmol), and PhePhe **5** (1.7 mg, 4.3 μmol) were dissolved in 20 mL of 10 mM KH_2PO_4 buffer, pH 7.5. Carbonic anhydrase (CA) (0.7670 g, 25.6 μmol, 1.20 equiv) was dissolved in this solution and placed in a dialysis bag (the bag was washed in ddH_2O for 1 h, rinsed in EtOH once, and then washed again with ddH_2O). The bag was suspended in 20 mL of the phosphate buffer containing Pronase from *S. griseus* (4.9 mg, 0.01 equiv) in a 150-mL beaker and shaken at 150 rpm at 30 °C for 193 h. Samples (1 mL) were taken, worked up as above, and analyzed by HPLC. Data for this experiment is shown in Figure 8.

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Supporting Information Available: A derivation of eq 3 and characterization data for **3a,c,d** and **4a,c,d** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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