Selective Binding

Pseudodynamic Combinatorial Libraries: A Receptor-Assisted Approach for Drug Discovery**

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Emerging methods of combinatorial chemistry incorporate receptor assistance to combine synthesis and screening.[1] Stoichiometric binding to a receptor alters either the thermo-

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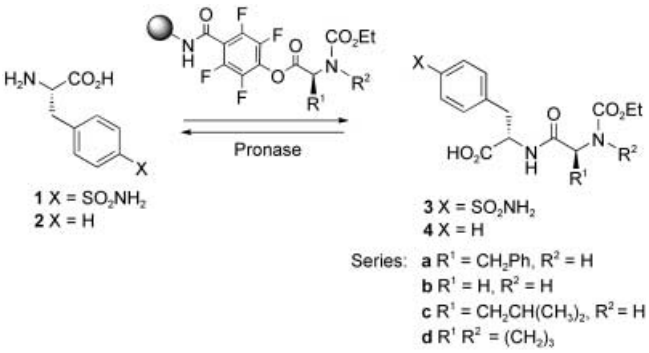
dynamics or kinetics of library synthesis. Dynamic combinatorial libraries[2] use a thermodynamic approach where binding shifts a synthetic equilibrium to increase the amounts of the best-binding compounds, in accordance with LeChâtelier's principle. These libraries usually identify the library members that bind the tightest, but some experimental conditions can give small or misleading changes in concentration.[3] An alternative method, receptor-accelerated synthesis, uses a kinetic approach.[4] Starting components that bind to the receptor can couple to create a new, tight-binding compound. The receptor accelerates the coupling of the better-fitting starting components as a result of their proximity, but requires that both components bind tightly to the receptor. Here we demonstrate a new method, a pseudodynamic library, which adds a kinetic contribution to traditional dynamic libraries to dramatically increase the selectivity.

A pseudodynamic combinatorial library combines an irreversible synthesis of library members with an irreversible destruction step. Those library members that bind to the receptor are protected from destruction. Subsequent synthesis reuses fragments from destroyed library members, thus amplifying the amounts of the better binders at the expense of the lesser ones. The separate irreversible synthesis and destruction steps allow adjustment to optimize both the amplification and selectivity.

We developed a pseudodynamic library of eight dipeptides to identify the best inhibitor of carbonic anhydrase (CA). Carbonic anhydrase, a zinc metalloenzyme, is a therapeutic target for glaucoma and is inhibited by aromatic sulfonamides, which coordinate to the zinc ion. Four of the eight dipeptides in our library contain 4′-sulfonamidophenylalanine (Phe_{sa}, **1**), and thus should bind to CA, while the remaining four contain only Phe and serve as negative controls. The irreversible synthesis of dipeptides used a solid-supported coupling of activated esters with an amino acid in aqueous solution (Scheme 1). TentaGel-supported tetrafluorophenyl active esters react cleanly with free amino



Scheme 1. Creation of a pseudodynamic library of dipeptides.

acids in water under alkaline (pH 8–10) conditions to form dipeptides.[5] A nonselective protease from *Streptomyces griseus* (Pronase) destroyed these dipeptides by catalyzing their irreversible hydrolysis.[6]

The pseudodynamic library was prepared in a three-chambered reaction vessel formed by suspending two dialysis bags in a surrounding solution (Figure 1). One dialysis bag (the synthesis chamber) contained the active esters and the other dialysis bag (hydrolysis chamber) contained the protease, while the surrounding solution (screening chamber) contained the carbonic anhydrase. Adding nucleophiles **1** and **2** to the synthesis chamber generated the dipeptide library. These dipeptides diffused into the surrounding solution where they could bind to carbonic anhydrase and then diffuse into the hydrolysis chamber where Pronase cleaved them. This

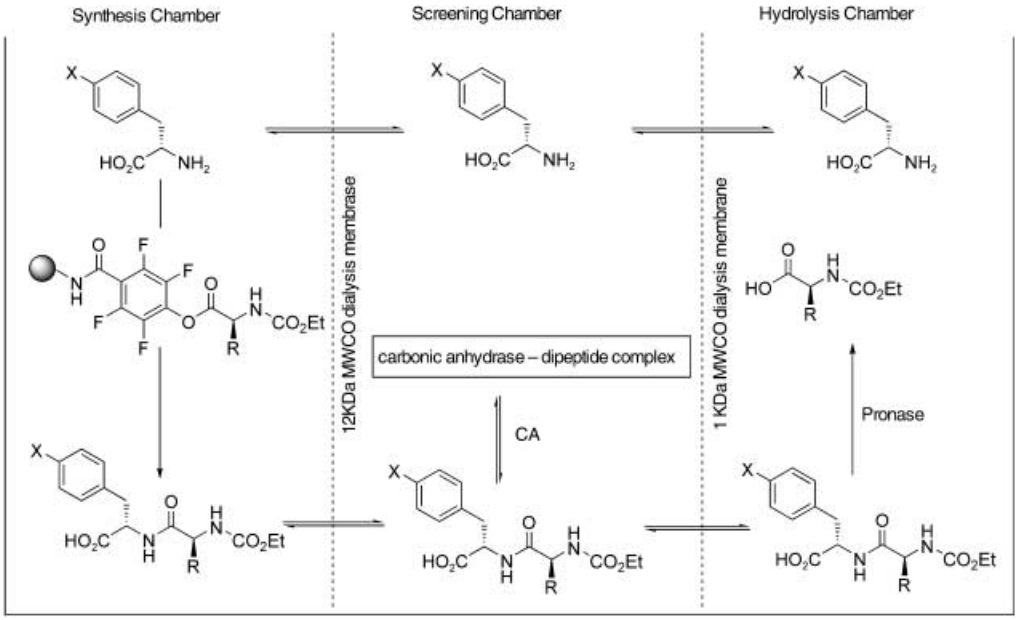


Figure 1. Schematic representation of the pseudodynamic combinatorial library experiment. Reaction of two free amino acids (Phe_{sa} (**1**) and Phe (**2**)) with four solid-supported active esters (*N*-EtO_2C-Phe, *N*-EtO_2C-Gly, *N*-EtO_2C-Leu, and *N*-EtO_2C-Pro) creates an eight-member library. MWCO = molecular-weight cut off.

cleavage regenerated **1** and **2**, which could diffuse back into the synthesis chamber to repeat the cycle. This arrangement prevented Pronase-catalyzed destruction and active-ester-mediated modification of the receptor (CA) and also permitted periodic replenishment of the activated ester to regulate the rate of synthesis.

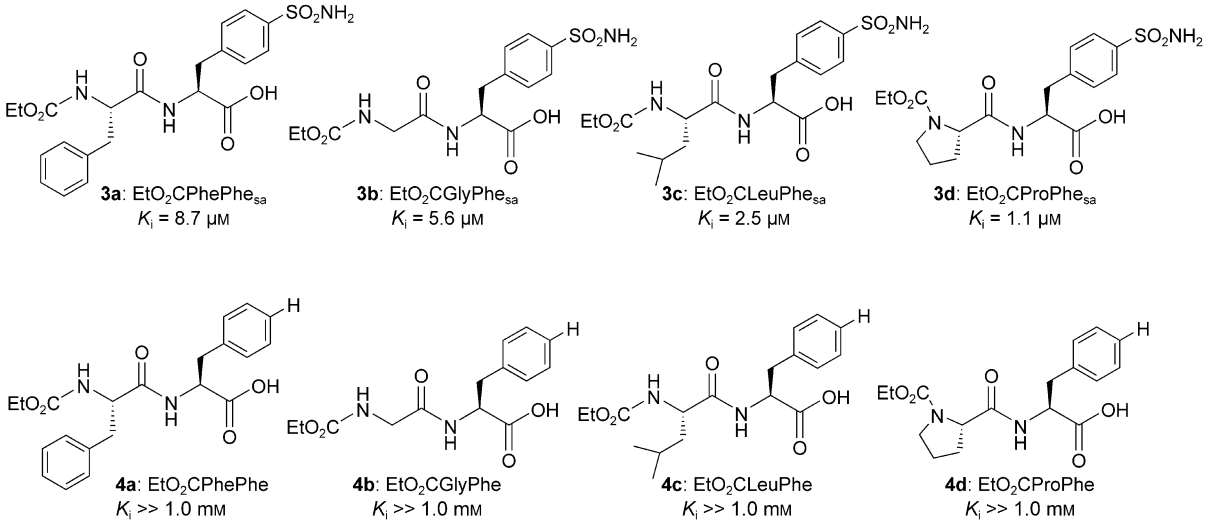
The experiments used four active ester resins derived from *N*-EtO_2C-Phe, *N*-EtO_2C-Gly, *N*-EtO_2C-Leu, and *N*-EtO_2C-Pro (0.8 equiv each), nucleophiles **1** and **2** (6.4 equiv each), carbonic anhydrase (28 μmol, 1 equiv), and Pronase (25 mg mL^{-1}). The large amount of Pronase made diffusion across the dialysis membrane the rate-limiting step for hydrolysis; hence, all the dipeptides were cleaved at similar rates in spite of the substrate selectivity of Pronase. Periodic addition of fresh portions of active ester resin (defined as the cycle time) regulated the overall rate of library synthesis. We conducted three experiments with this system using cycle times of 8, 12, and 16 h. HPLC analysis of aliquots from the screening chamber showed the progress of the experiments (Figure 2).

Two control experiments established, first, that the synthetic process afforded all the expected dipeptides and, second, that the sulfonamide-containing dipeptides inhibited carbonic anhydrase. Combining equal amounts of the four active esters with Phe_{sa} (**1**) as the nucleophile produced four dipeptides **3a**–**d** in a ratio of 18:44:15:23. Not surprisingly, the coupling of **1** with the less-hindered glycine ester to produce **3b** was more efficient than with the more-hindered phenylalanine, leucine, or proline esters. In spite of these differences, all four dipeptides formed in significant amounts. The use of phenylalanine as the nucleophile gave similar results. For the second control experiment, all eight dipeptides were prepared individually and their ability to inhibit the CA-catalyzed hydrolysis of *p*-nitrophenyl acetate (Scheme 2) was measured. As expected, the sulfonamide-containing dipeptide competitively inhibited this hydrolysis, with inhibition constants of 1.1–8.7 μM, while the non-sulfonamide dipeptides showed no detectable inhibition. Dipeptide **3d** was the best inhibitor, with an inhibition constant of 1.1 μM, and dipeptide **3c** the next best, with an inhibition constant of 2.5 μM. Compound **1** also inhibits CA ($K_i = 13$ μM), but approximately tenfold less effectively than the tightest binding dipeptide (**3d**).

In the first pseudo-dynamic experiment (8-hour cycle, Figure 2a), the cycle time was too short for the destruction reaction to remove the less-effective inhibitors. During the first four hours of each cycle, the screening chamber contained all eight dipeptides, thus indicating that all eight had formed as expected. At the end of each 8 h cycle, prior to the next addition of active ester, the hydrolysis had removed the four non-sulfonamide dipeptides, thus leaving only the four sulfonamide dipeptides. At the end of six cycles of active ester addition, dipeptide **3b** was present in the highest amount (58% yield, relative to CA), followed by **3d** (33%), **3c** (27%), and **3a** (8%). These relative amounts differ from their relative binding constants. The higher yield of **3b** instead reflects its more favorable rate of synthesis. In addition, the sum of all the sulfonamide dipeptides at 48 h was greater than the amount of target (126% yield). This high yield shows that unbound dipeptides remained and that the destruction reaction had not had enough time to distinguish between the different sulfonamide inhibitors.

Lengthening the cycle time from 8 h to 12 h yielded the best three inhibitors, in relative amounts in the order of their inhibition constants (Figure 2b). Although sulfonamides **3b**–**d** were present in high concentrations early in the experiment, the concentration of these weaker binding dipeptides had diminished substantially at the end of four cycles. The tightest binding dipeptide (**3d**) was present in the highest amount (15% yield relative to CA), followed by **3c** (5%) and **3b** (1.5%). Notably, the ratio at the end of the experiment (10.1:3.5:1) exceeded the ratio of their binding constants (5.1:2.2:1). None of the weaker binding **3a** or of the non-sulfonamide dipeptides remained at the end of the experiment.

The selectivity of the dynamic process improved even further upon extending the cycle time to 16 h (Figure 2c). The



Scheme 2. Competitive inhibition constants of the library members for the CA-catalyzed hydrolysis of *p*-nitrophenyl acetate. The non-sulfonamide compounds showed no detectable inhibition at 1 mM.

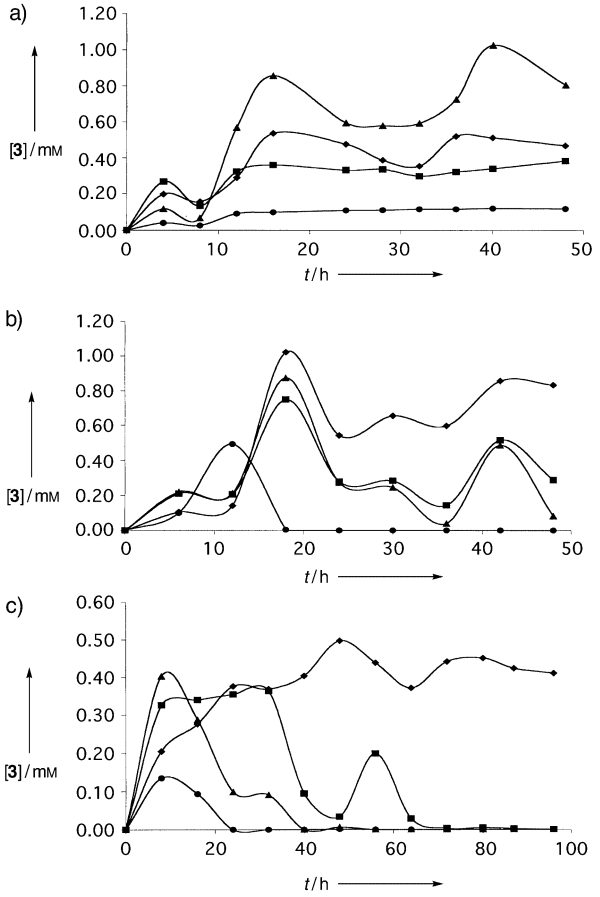


Figure 2. Pseudodynamic library experiments. Concentrations of sulfonamide containing dipeptides **3a** (●), **3b** (▲), **3c** (■), and **3d** (◆) over the course of experiments: a) 8 h per cycle, b) 12 h per cycle, and c) 16 h per cycle.

initial synthesis during the first cycle favored dipeptide **3b**, the most rapidly synthesized dipeptide, but this dipeptide disappeared in later cycles where the main competition was between **3d** and **3c**, the tightest binding dipeptides. After four cycles (64 h), only these two remained and the ratio of their concentrations (13:1) was significantly higher than the ratio of their binding constants (2.3:1). The selectivity increased to >100:1 in favor of the strongest binding dipeptide **3d** after three more cycles. The yield was 29% relative to the amount of CA and corresponded to 4 mg of dipeptide. Thus, adjusting the relative rate of the library synthesis and destruction optimized the selectivity so that only the best-binding dipeptide remained and was present in a good overall yield.

The selectivity in the pseudodynamic library is significantly greater than that in many traditional dynamic libraries. The optimum conditions produced only the single, tightest-binding dipeptide (>100:1 selectivity), while a traditional approach would yield a mixture because the binding constants for the two tightest-binding dipeptides differed by only 2.3-fold. This higher selectivity greatly simplifies the analysis, as only one compound need be identified and characterized. The optimization of a pseudodynamic library arises through control of the relative rates of synthesis and destruction. We previously showed that a destruction reaction operating on a static library in the presence of a receptor distinguishes between library members with very similar binding constants, selectively removing the weaker-binding species.[6] However, when selectivity arises from destruction alone, significant amounts of the best-binding library member must be destroyed to achieve high ratios of good binder to slightly poorer binder. This situation leaves only a small amount of the best binder for analysis. The high selectivity in pseudodynamic libraries also stems from the competition between binding to the receptor and destruction.

The iterative nature of the experiment also contributes to the high selectivity. Cleavage by Pronase has reduced the amounts of weak-binding dipeptides toward the end of each cycle, which leaves dipeptide **3d** as the major species bound to CA. The subsequent burst of synthesis produces a mixture of all dipeptides which compete for the smaller amount of free target. Pronase then rapidly cleaves all unbound species, which would consist of a higher proportion of weak binders. Following our static model, continued action of Pronase further increases the ratio in favor of the bound species, which results ultimately in high selectivity for the tightest-binding speices.

Our static model of pseudodynamic combinatorial libraries[6] indicates that selectivity stems from the relative binding constants of the inhibitors, not their absolute affinity for the target. Thus, we expect that pseudodynamic combinatorial libraries will also work with even tighter-binding inhibitors, but would require longer cycle times to distinguish between these more tightly binding inhibitors. Indeed, we are currently expanding our studies to larger pseudodynamic libraries to discover such tighter-binding inhibitors.

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