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ARTICLE *in* JOURNAL OF MEDICINAL CHEMISTRY · FEBRUARY 2014

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Off-Rate Screening (ORS) By Surface Plasmon Resonance. An Efficient Method to Kinetically Sample Hit to Lead Chemical Space from Unpurified Reaction Products

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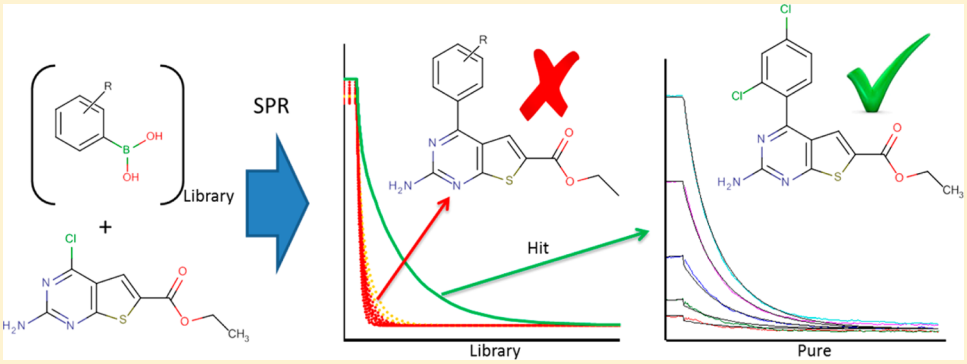
Supporting Information



ABSTRACT: The dissociation rate constant k_d (off-rate) is the component of ligand−protein binding with the most significant potential to enhance compound potency. Here we provide theoretical and empirical data to show that this parameter can be determined accurately from unpurified reaction products containing designed test compounds. This screening protocol is amenable to parallel chemistry, provides efficiencies of time and materials, and complements existing methodologies for the hit-to-lead phase in fragment-based drug discovery.

INTRODUCTION

The pressure on the pharmaceutical industry to develop new drugs has led to a search for efficiency gains within research organizations globally. Since the widespread adoption of parallel chemistry techniques, the purification of synthesized products has become a significant bottleneck within medicinal chemistry. The use of automated HPLC systems has improved this situation. However, such systems are expensive to operate, use large volumes of solvents, and suffer from poor material recovery. Alternative approaches, such as solid-supported scavenger reagents or ion-exchange chromatography, are only applicable to certain reaction types with limited substrate scope. These methods still require individual weighing and dilution prior to biological screening.

Fragment-based drug discovery (FBDD)[1,2] has become prevalent within the pharmaceutical industry as an effective method to identify hits for existing and novel targets. However, fragments hits are typically of a lower affinity than HTS hits and often generate many tens, if not hundreds, of potential starting points. The problem can perhaps be best described as not "Do I have anything to work on?" but more "What should I work on?"

The affinity of a compound for the target is described by the steady state affinity equilibrium dissociation constant K_D (or its surrogates IC_{50}/EC_{50}). Affinity (K_D) can determined from the bound and unbound concentrations of ligand and target or as a simple ratio of dissociation/association rate constants as described in eq 1:

$$K_D = \frac{[P_F][C_F]}{[PC]} = \frac{k_d}{k_a} \quad (1)$$

where P is target, C is compound, F is free, and k_d and k_a are the dissociation and association rate constants, respectively.

Measuring affinity kinetically throughout a drug discovery program is more informative. For example, a compound with a 10-fold slower on- and off-rate would not be recognized as different if evaluated by equilibrium measures of affinity. Such an observation may indicate a novel protein conformation or significant internal strain in the bound ligand. Either way, the bound conformation is a productive one that may yield to the significant progress toward the drug candidate if exploited further. This is exemplified with the concept of kinetic efficiency.[3]

A number of papers have been devoted to the analysis and consequences of varying residence times for drug candidates. The dissociation rate can vary from the immeasurably fast to the immeasurably slow (i.e., stable covalent complex). Thus, the off-rate (defined by Copeland et al)[4] is the component of

Received: November 29, 2013
Published: February 12, 2014

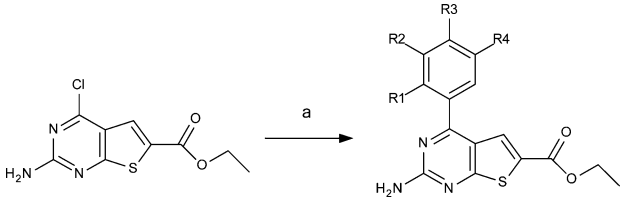
binding that has the greatest potential to improve potency. Early papers in this field were devoted to biological consequences of prolonged target inhibition, leading to lower toxicity due to more rapid off-target dissociation.[4,5] For in vivo systems, the maximal contribution of the dissociation rate to efficacy will be limited by the rate of target resynthesis. More recently, there has been an effort to understand the molecular determinants leading to increased residence times.[6,7] While progress is being made, this remains a largely unsolved problem. We reason here that one could exploit the dissociation rate empirically and remove significant bottlenecks in drug discovery, in particular within fragment-to-lead efforts.

We provide both the theoretical and experimental support for this approach. We demonstrate this by using surface plasmon resonance (SPR) assessment of the dissociation rate constants from a library of crude (*unpurified*) reaction mixtures which contain designed target compounds.

RESULTS

We have compared the k_d observed for crude reaction mixtures with the k_d of the pure compound. First, this was done by recapitulating early medicinal chemistry efforts targeted at HSP90 that led to the preclinical candidate NVP-BEP800.[8] A small library of 13 compounds was generated for off-rate screening (ORS) experiments using the same Suzuki chemistry employed in the original work, Scheme 1. Workup was kept to a minimum, and upon completion the reactions were evaporated and dissolved in DMSO to nominally match the stock concentration of the pure compound (assuming 100% yield). These samples were diluted (1:99) with the running buffer and injected over HSP90 immobilized on the SPR chip. Injection of blank reaction sample served as the reference control.

Scheme 1[a]



[a]Reagents and conditions: (a) $ArB(OH)_2$, $NaHCO_3$, $PdCl_2(PPh_3)_2$, DMF, H_2O, 100 °C microwave, 10 min.

Dissociation phase of the resulting double referenced sensorgrams were evaluated using BIAevaluation 1.1 (BIAcore GE Healthcare Bio-SciencesCorp) or Scrubber2 (BioLogic) software by fitting with a one-phase exponential decay model, eq 2:

$$R = R_0 \cdot e^{-k_d \cdot (t - t_0)} + R_\infty \quad (2)$$

where the R_0 and t_0 are the response and the time at the start of the dissociation phase, respectively, and R_∞ is a residual response after complete dissociation.

Initial SPR experiments were carried out using a BIAcore T100. We have stored all the samples at −18 °C and retested these 3.5 years later using a BIAcore T200. This permits the evaluation of the crude library stability over a considerable time and the responses of differing equipment (T200 *cf* T100). The dissociation rate constants determined for the pure samples and those generated from the ORS library are shown in Table 1. The T200 data shows and average difference in the k_ds between crude and pure samples of 19%, similarly, the older T100 data had a 15% difference. Satisfyingly, we observe that the k_ds vary by an average of only 30% when compared across instruments and across time. This demonstrates that carryover contamination, long-term storage, and differing equipment has a modest effect on the observed k_ds. Indeed, these small deviations in the k_ds closely reflect the differences observed in multilaboratory studies where variability observed has been reported to be from 14% to 40% depending on the system.[9]

Potter et al. have previously described a series of inhibitors of the prolyl-isomerase PIN1 based on the *β*-(benzimidazol-2-yl)alanine scaffold (*R*)-**7**.[10] We resynthesized a set of these compounds according to Scheme 2 and analyzed the crude reaction products by ORS. Furthermore, we investigated whether the more readily available racemates (*rac*-**7a**−**g**)

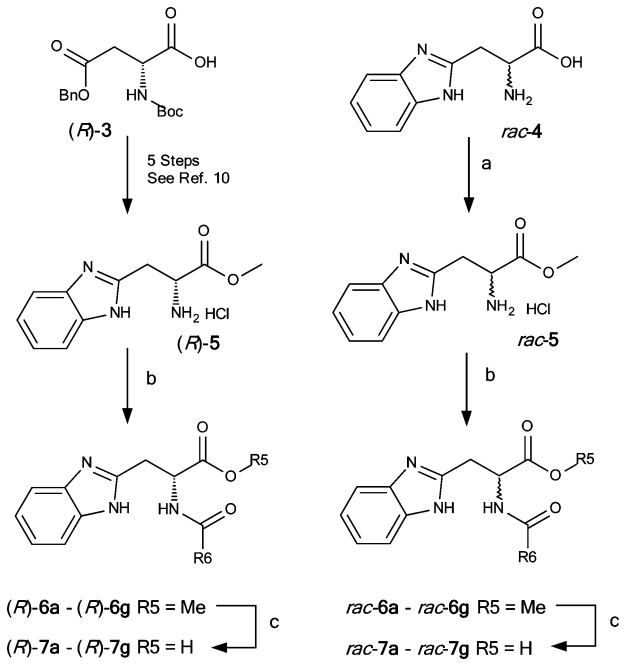
Table 1. Results of the Hsp90 Screen: Dissociation Rate Constants Determined by ORS for the Crude Reaction Mixtures and for the Respective Pure Compounds

					T200 k_d (s^{-1})		T100 k_d (s^{-1})	
compd	R1	R2	R3	R4	pure	crude	pure	crude
1					fast[a]		fast	
2a	H	H	H	H	1.05	1.08	fast	fast
2b	Me	H	H	H	0.16	0.19	0.10	0.10
2c	H	H	Cl	H	0.26	0.29	0.09	0.09
2d	Me	Me	H	H	nt	fast	nt	fast
2e	Me	H	H	Me	0.15	0.09	0.12	0.10
2f	Me	H	Me	H	0.03	0.03	0.02	0.02
2g	H	H	CN	H	0.16	0.20	0.12	0.15
2h	Cl	H	Cl	H	0.02	0.02	0.01	0.02
2i	Me	H	H	CO_2Me	0.03	0.03	0.03	0.02
2j	Me	H	CN	H	nt	0.02	nt	0.02
2k	H	F	CN	H	0.63	nt	fast	fast
2l	Et	H	H	H	fast	fast	fast	fast
2m	F	H	F	H	fast	0.78	fast	fast
2n	OMe	H	H	H	nt	0.31	nt	0.37

[a]Fast = k_d > 1.2 s^{-1}. nt is not tested

dx.doi.org/10.1021/jm401848a | J. Med. Chem. 2014, 57, 2845−2850

Scheme 2[a]



[a]Reagents and conditions: (a) $SOCl_2$, MeOH, 80%; (b) RCO_2H, COMU, Et_3N, DMF; (c) (1) LiOH (5 equiv), MeOH-H_2O, (2) AcOH (5 equiv).

would also demonstrate the expected SAR from ORS. Additionally, we chose to conduct the repeat syntheses in disposable plastic tubes with rubber septa. The tubes were tested for solvent compatibility by loading with deuterated solvents and assessed for solvent loss (by weighing), tube condition (by visual inspection), and leaching of contaminants by the solvent (by ^{1}H NMR analysis).

At ambient temperature (18−22 °C), only tubes containing DCM and $CDCl_3$ showed appreciable solvent loss. At 55 °C, $CDCl_3$ also showed significant losses (Figure 1). In all cases,

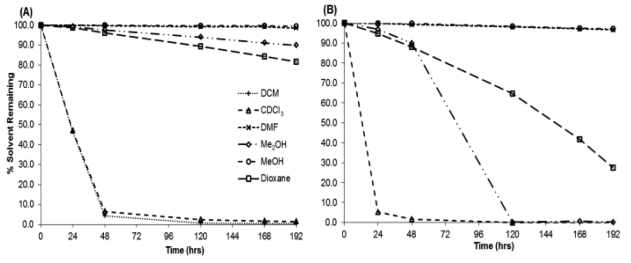
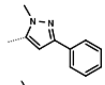
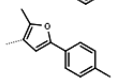
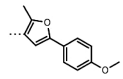


Figure 1. Solvent loss from 96-well Matrix polypropylene tubes: (A) at ambient temperature 18−22 °C, (B) at 55 °C.

there was leaching of unidentified aliphatic hydrocarbon contaminants into the solvents, especially for acetone and chlorinated solvents. If this material interferes (unlikely) with the SPR binding signal, it is compensated for by inclusion of an appropriate "blank" control. However, the tubes maintained their integrity throughout the experiment. Because of evaporation, the more volatile solvents such as DCM or $CDCl_3$ were unsuitable for high temperature or long reaction times.

Within the set of PIN1 compounds, all of the reaction mixtures containing the most active compounds were easily identified (Table 2). Closer inspection showed that, as above, the k_d determined for the crude reactions was in very good

Table 2. Measured Dissociation Rate Constants of 7a−7g Binding to PIN1

Cpd	R6	k_d (R)	k_d (rac)	k_d (R) (Pure)	Conversion (5 : 6 : 7)[a]
7a		1.54	1.23	1.93	0 : 19 : 81
7b	Et	Fast*	Fast	Fast	0 : 0 : 100
7c		Fast	Fast	Fast	0 : 0 : 100
7d		Fast	Fast	Fast	0 : 0 : 100
7e		0.93	0.99	0.99	0 : 1 : 99
7f		0.36	0.48	0.47	0 : 9 : 91
7g		0.25	0.20	0.30	0 : 9 : 91

[a]Ratio of starting material (**5**) to products as defined in Scheme 2. *Fast refers to k_d > 2 s^{-1}.

agreement with those for the pure compound. We can see that the dissociation rates differ by less than 15% on average for either the racemic or enantiomerically pure reagents. This clearly demonstrates that, even with complex crude mixtures, we are able to recapitulate the observed k_ds of pure samples and to readily identify the most active final compound with minimal effort.

Finally, to further explore if any of the most commonly deployed chemistries within the drug discovery industry[11] possess any inherent carryover liability, we conducted a series of Faux reactions by combining simple example reagents and catalysts to generate simple compounds (see Supporting Information) that were unlikely to bind to the target but able to replicate reagent carryover and related degradation products. The resultant mixtures were then subjected to the normal heating/cooling or irradiation procedures (See Supporting Information) and worked up by evaporation. A 20 mM stock solution of pure compound (**2i** for HSP90; **7g** for PIN1) was added to the dried residue and mixed with gentle heating for 30 min. The final samples were evaluated as per the standard ORS protocol (Table 3).

It can be seen that the dissociation rate constant varied on average by 5.8% and 7.8%, with maximal variations of 12% and 18% for HSP90 and PIN1, respectively. This clearly demonstrates that generic carryover from more than a half of the most commonly utilized drug discovery chemistries has no significant effect on the observed off-rates.

DISCUSSION

Our approach relies on two independent components. The first one is the fractional ligand binding at equilibrium. It relates to the extent to which a compound ("product" compound or starting material (SM)) in the crude reaction mixture is bound to the protein immobilized on the surface of the SPR chip. The second one is the rate of decay of the chip-bound complexes. It relates to the dissociation rates of each individual complex present in the mixture.

dx.doi.org/10.1021/jm401848a | J. Med. Chem. 2014, 57, 2845−2850

Table 3. Measured Dissociation Rate Constants of 2i Binding to HSP90 in the Faux Reaction Mixtures[a]

Faux reaction	HSP90 k_d (s^{-1})	HSP90 variation (%)	PIN1 k_d (s^{-1})	PIN1 variation (%)
amide formation	0.042	4.0	0.41	13.6
Boc deprotection	0.042	3.7	0.42	18.4
N-alkylation with R-X	0.043	0.2	0.37	4.4
N-arylation (SNAr)	0.046	5.6	0.38	6.0
O-alkylation (Mitsunobu)	0.042	3.8	0.37	4.0
O-alkylation with R-X	0.042	4.6	0.37	3.3
reductive amination	0.041	5.1	0.40	12.6
Sonogashira coupling	0.040	8.8	0.36	1.7
sulphonamide formation	0.040	8.6	0.40	11.5
Suzuki reaction	0.042	3.5	0.37	4.4
urea formation	0.038	11.7	0.38	6.2
control	0.044		0.36	

[a]Percentage variation is calculated relative to the Blank well (reagent **2i**).

Active site occupancy: in a two-component system, relative site occupancy at equilibrium is simply a ratio of the potency of the two components and the concentration at which they are present in the solution. The relative response at equilibrium for the components can be given by eq 3:

$$RU^1_{eq} = \frac{K_{D2}}{K_{D1}} \times M_{w_1} \times \frac{F_1/(1-F_1)}{1 + \left(\frac{K_{D2}}{K_{D1}} \times \frac{F_1}{1-F_1}\right)} \quad (3)$$

where RU^1_{eq} is the relative response at equilibrium and is assumed to be directly proportional to the mass. K_D is the equilibrium affinity constant of the relevant component. F is the fractional purity of the relevant component.

Thus, when two components with masses of 230 and 270 Da are present in equal concentrations, and one is 10-fold more potent than another, 93% of the binding signal will be due to the more potent component. Indeed, even at concentration ratio of 19:1, 40% of the binding signal will be due to the more potent component. Screening is conducted at high nominal concentration to ensure that equilibrium on the surface of the SPR chip is reached rapidly and bound signal is high.

Ligand efficiency[12] (LE; kcal/mol/HA) gains will be apparent even at relatively low yields and few heavy (HA) atom additions. For example, at 5% yield with the addition of three heavy atoms (17 to 20 HA) and maintenance of a LE of 0.4 kcal/mol, 31% of the occupancy (initial response units in SPR) will be due to the product. Interestingly, for the greater heavy atom additions, significant losses in LE are more tolerated. For example, if we have added seven heavy atoms and the product LE was reduced to 0.35 kcal/mol (17 to 24 HA), over 50% of the initial response will be due to the product, despite the poor group efficiency[13] of 0.22 kcal/mol. This is exemplified more fully in Table 4 for a 5% reaction yield.

Here the assumptions are that the product LE is maintained (0.4) or degraded (0.35 and 0.3) compared with the SM LE. This demonstrates that as more diverse chemical space is explored, the higher the initial product signal is likely to be, even with poor group efficiencies.

Table 4. Initial Response As Function of LE/HA Addition with 5% Yield

	product LE		
added HA	0.4	0.35	0.3
1	0.098	0.024	0.005
3	0.317	0.080	0.016
5	0.661	0.236	0.046
7	0.891	0.521	0.127
9	0.971	0.792	0.301

Protein−ligand dissociation: once sample is no longer applied to the protein surface, the bound components dissociate in a zero-order process. The sensorgram signal is proportional to the relative fraction bound at the end of the injection and the k_d of each bound component. For a simple single-step mechanism with two binding components, the dissociation sensorgram can be described by eq 4.

$$RU_{obs} = RU^1_{eq}\, e^{-k^1_d(t-t_0)} + RU^2_{eq}\, e^{-k^2_d(t-t_0)} \quad (4)$$

where RU^1_{eq} and RU^2_{eq} are the weighted responses at the start of the dissociation phase, see eq 3. t_0 is time at the start of dissociation.

Starting materials of low affinity ($K_D > 10^{-5}$ M) and molecular weight (app 190) will typically have a fast dissociation rate. The exemplified compounds will typically be of greater mass in average of higher than 270. If the extra mass contributes to affinity in an efficient way, the newly synthesized compound should be correspondingly 20−50-fold more potent, much of which will be exhibited through the slower off-rate. Figure 2 demonstrates the effect on the observed dissociation rate for such a mixture.

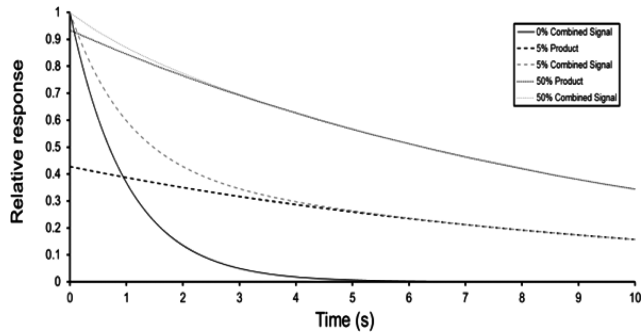


Figure 2. Theoretical dissociations phase for sensorgrams with varying reaction yield using eq 4. The SM (mw 190) with K_D 1E^{-5} M and k_d 1 s^{-1} and the putative product (mw 270) with K_D of 1E^{-6} M and k_d 0.1 s^{-1}.

For a 1:1 ratio of starting material to product at 3 s into the dissociation, more than 99% of the signal observed is due to the product. Furthermore, at 19:1 ratio and 3 s of dissociation, 92% of the observed signal is due to the more potent compound. Thus, even at low yields the more substantial the gain in off-rate, the more readily the active component will be detected.

Each reaction mixture is designed to generate a single product (with starting material plus remaining unreacted reagents and by products). LCMS analysis was used to provide

dx.doi.org/10.1021/jm401848a | J. Med. Chem. 2014, 57, 2845−2850

evidence that the desired target compound was present in each crude reaction mixture.

We have tested this concept in two sets of experiments on the drug targets HSP90 (classically drugable) and PIN1 (hard to drug). These tests were to determine: (a) if we could reliably detect the most active substituents from crude reaction mixtures and, (b) if the most commonly employed reactions were amenable to this approach. As shown in the Results section and Supporting Information, we could easily identify the active substituents for both HSP90 and PIN1, with the k_d values correlating to the known SAR derived from biochemical assays. Gratifyingly, the measured k_ds are typically within 25% of the pure k_d, which is as expected due to the slowly dissociating component dominating the dissociation phase.

Using a series of Faux reactions (Table 3), we sought to determine the effect on the observed off-rate of any carryover contaminants from 62% of the most commonly employed reactions.[11] We observed a maximal difference of 12% in the observed k_d, clearly indicating that there is no appreciable effect caused by the reaction components.

An important consideration is the dissociation rate of the SM, if this is slow, then in low yielding reactions it will be difficult to identify the product with slower k_d. This can be ameliorated by conducting the screening at higher temperatures where the k_d is increased, thereby contributing less to the observed dissociation sensorgram. Conversely, for rapidly dissociating systems, the screening temperature can be lowered, e.g., PIN1.

Considerable resources are expended to evaluate compounds in concentration sensitive assays. The likely outcome for any one compound would be a poor response in the relevant bioassay, particularly during the early stages of fragment-to-lead chemistry. These resources are primarily: large scale synthesis (typically >5 mgs), time-consuming analytical chemistry, purification (high solvent use), accurate weighing (time-consuming), storage of both liquid and solid samples (extensive compound management), and bioassays (multiple dilutions/data analysis). These all add significantly to the cost of the early stage drug development. The ORS approach described here mitigates the use of such expensive and time-consuming resources, although it is important to note that hits identified from ORS are generally reprepared and purified for confirmation and further characterization

Mild "near-ambient" conditions exist for many of the most commonly deployed reactions, enabling large libraries to be generated without special equipment to handle difficult high throughput chemistries (e.g., benzamide and amide synthesis).[14] Furthermore, as poor conversion can be tolerated in our approach, more challenging chemistries requiring forcing conditions to obtain good conversion, which have previously been difficult to apply to library synthesis may also become accessible using this approach.

We anticipate that further efficiency increases can be realized by the elimination of protecting groups (which account for around 1 in 5 of all medicinal chemistry transformations) prior to library synthesis, in situations where derivatization of second reactive functionalities involved directly in binding will result in a noninterfering nonbinder, for example, N−H functions responsible for hinge-binding interactions in kinase ligands.

We have demonstrated that the screening of crude unpurified reaction mixtures of elaborated fragments allows the rapid identification of compounds with increased residence times without need for significant reaction workup and purification, thus eliminating a significant bottleneck in the early stages of medicinal chemistry efforts.[15] Application of this approach will shorten fragment to lead times, reduce solvent and reagent use, leading to "greener" more productive fragment-based lead generation.[16]

EXPERIMENTAL SECTION

Libraries were made and stored as 20 mM solutions in DMSO or DMSO-d_6 at −20 °C.

General Procedure A: Preparation of 4-Arylthieno[2,3-*d*]pyrimidines 2a−2n by Suzuki Cross-Coupling. To each of 14 microwave vials was added 2-amino-4-chloro-thieno[2,3-*d*]pyrimidine-6-carboxylic acid ethyl ester **1** (50 mg, 0.194 mmol),[8] DMF (3 mL), $NaHCO_3$ (1N aq; 0.5 mL), the appropriate boronic acid (0.291 mmol, 1.5 equiv), and bis(triphenylphosphine)palladium(II) dichloride (14 mg, 10 mol %). Each vial was sealed and heated in microwave synthesizer at 100 °C for 10 min. The reaction mixtures were transferred to 50 mL boiling tubes and solvents evaporated in vacuo (Genevac). Each crude product was partitioned between satd NaCl (aq) solution (3 mL) and EtOAc (3 mL), stirred for 2 min, and the EtOAc layer pipetted off and filtered through a small plug of anhydrous Na_2SO_4 in a SPE cartridge. The filtrates were evaporated in vacuo to generate the crude products which were analyzed by LCMS and ca. 1 mg of each submitted for SPR testing.

General Procedure B: Synthesis of Acids 7a−7g for Crude Screening. To a Matrix tube was added the acid ("RCO_2H" in Scheme 2; 1 M in DMF; 5.0 μL. 5.0 μmol), the amine 5 (1 M in DMF; 5.0 μL, 5.0 μmol), and triethylamine (3.5 uL, 25.0 μmol). A solution of COMU (1.2 M in DMF; 5.0 μL, 6.0 μmol) was added and the tubes capped and agitated briefly to ensure mixing. After 21 h, the solvents were evaporated (Genevac EZ2; medium bp solvent; T_{max} 45 °C). The crude esters were redissolved in MeOH (25 μL) and LiOH (1.0 M aq; 25 μL, 25.0 μmol) added. The tubes were capped, agitated briefly, and allowed to stand at rt for 4 h. Acetic acid (1.72 μL; 30 μmol) was added and the mixtures evaporated to dryness (Genevac EZ2; medium−low bp mixture; T_{max} 45 °C). The crude products were redissolved in DMSO (250 μL) to a nominal concentration of 20 mM. Libraries were made and stored as 20 mM solutions in DMSO or DMSO-d_6 at −20 °C.

ASSOCIATED CONTENT

Supporting Information

Additional experimental for chemical syntheses, SPR data, and equation derivations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

k_d, dissociation rate constant; k_a, association rate constant; K_D, dissociation constant; ORS, off-rate screening; SM, starting material; MeOH, methanol; COMU, 1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholinomethylene)]methanaminium hexafluorophosphate

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