

A Novel Method for the Determination of Isokinetic Ratios and Its Application in the Synthesis of Two New Positional Scanning Libraries

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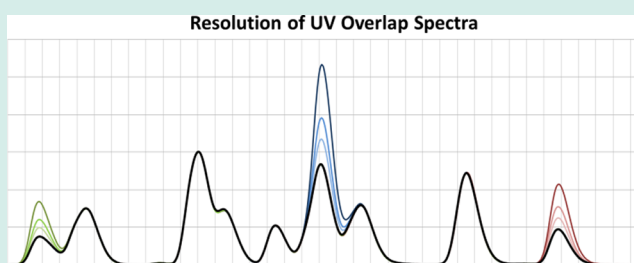
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Supporting Information

ABSTRACT: A novel method for the direct evaluation of the equimolarity of the compounds contained in a mixture is presented. We applied the method toward calculating isokinetic ratios for the reaction between the amine termini of a resin bound peptide fragment and a sulfonyl chloride to produce equal molar mixtures of sulfonamides. The results of this study and the application of the method to the synthesis of two new positional scanning synthetic combinatorial libraries (PS-SCL) are discussed.

KEYWORDS: positional scanning library, isokinetic ratios, combinatorial libraries, harmonic mean, piperazine, sulfonamide, guanidine



INTRODUCTION

Combinatorial chemistry is a valuable tool in the rapid production of compound libraries for biological evaluation.^{1,2} Specifically, positional scanning synthetic combinatorial libraries (PS-SCL) enable the systematic screening of thousands to trillions of compounds through the use of only hundreds of samples.^{2,3} This exponential reduction in sample size enables many endeavors, such as the evaluation of nearly 4 million small molecules through a direct in vivo screening to identify two novel potent, low-liability antinociceptive compounds⁴ and the multiclonal evaluation of trillions of 10-mer peptides to identify immunoprevalent antigens recognized by human CD4+ T cells.⁵ None of these efforts could have been currently conducted without the use of PS-SCL.

A key component of PS-SCL involves the ability to produce near equimolar amounts of each individual compound in each mixture sample. Two methods for accomplishing equimolar mixtures are the use of physical mixtures (“split and mix”)^{6–8} or chemical mixtures.⁹ While the use of physical mixtures has the advantage of assuring near equimolar incorporation of each individual compound in the mixture samples, it is limited in that as the number of R groups increases around the scaffold the corresponding number of split and mixes required increases exponentially, quickly rendering the technique less valuable. The use of chemical mixtures where “isokinetic mixtures” are applied to produce equimolar mixtures does not have the same limitations of the split and mix method; however, to utilize chemical mixtures in the production of PS-SCL, one needs to determine the isokinetic ratios for the reagents used.

Several methods have been reported for determining the isokinetic ratio for a variety of different reagent classes, such as

amino acids, carboxylic acids, and aldehydes.^{9–13} These methods include the use of amino acid analysis, as well as binary and iterative HPLC analysis, and MALDI-TOF. In this publication, we introduce a novel method for the comparison of LCMS UV spectra, which allows direct relative evaluation of the equimolarity of the compounds contained in that mixture; further, this method is applicable in cases more complex than would be feasible by simply using a reference compound. We applied it to the reaction between the amine termini of a resin bound peptide fragment and a sulfonyl chloride to produce a sulfonamide. The isokinetic ratio was determined and the effect of the on-resin peptide fragment was evaluated. Two novel PS-SCLs were synthesized based on the calculated ratios. Furthermore the two PS-SCLs were each screened in two opioid (mu and kappa) binding assays in part to determine the synthetic integrity.

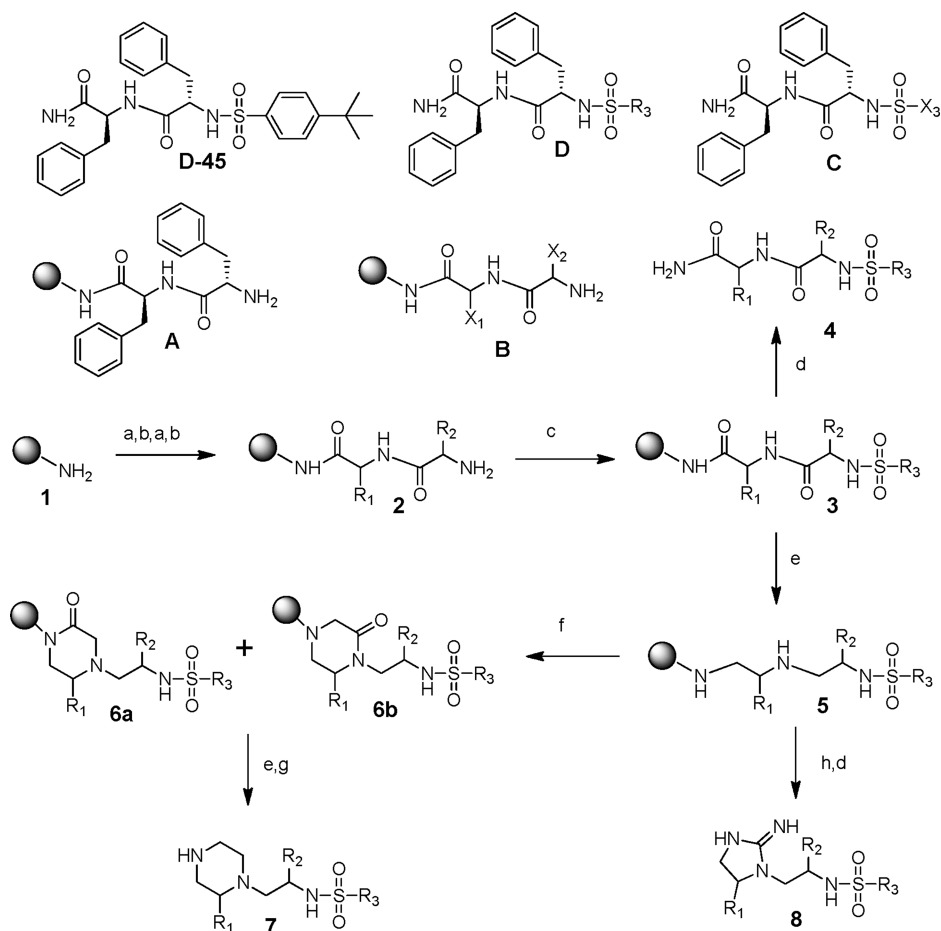
The two novel PS-SCLs were designed around two separate scaffolds (1) a piperazine linked to a sulfonamide (Scheme 1 Structure 7) and (2) a cyclic guanidine linked to a sulfonamide (Scheme 1 Structure 8). A number of approved pharmaceuticals contain piperazines, such as imatinib (cancer treatment), quetiapine (antipsychotic), and eszopiclone (treatment of insomnia), sulfonamides, such as sulfamethoxazole (antibiotic), xipamide (hypertension and edema), and darunavir (HIV), and guanidines, such as guanadrel (antihypertensive) and guanethidine (antihypertensive). The synthesis (Scheme 1) utilizes our libraries from libraries approach,¹⁴ where multiple libraries are

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Scheme 1. (a) Boc-AA/DIC/HOBt (6ex) in DMF, (b) (1) 55%TFA/DCM, (2) 5%DIEA/DCM, (c) Sulfonyl Chloride/DIEA (8ex) in DMF, (d) HF 0 °C 1.5 h, (e) (1) Borane–THF 60 °C 96 h, (2) Piperidine 60 °C 24 h, (f) Bromoacetic Acid/DIC/DIEA (5ex/5ex/2.5ex), (g) HF 0 °C 7 h, (h) Cyanogen Bromide/DMF (5ex); (A) mBHA Resin-(L-Phenylalanine)-(L-Phenylalanine), (B) mBHA Resin-(Equal Molar Mixture of Amino Acids; Ls, Ds, and Unusals)-(Equal Molar Mixture of Amino Acids; Ls, Ds, and Unusals), (C) Mixture Sample Defined with L-Phenylalanine (R₁), L-Phenylalanine (R₂), and a Mixture of Different Sulfonyl Chlorides at R₃, (D) Compound Derived from Using L-Phenylalanine (R₁), L-Phenylalanine (R₂), and Any One Sulfonyl Chloride (R₃), (D-45) Compound Derived from Using L-Phenylalanine (R₁), L-Phenylalanine (R₂), and 4-*tert*-Butylbenzene-1-sulfonyl Chloride (R₃)



synthesized from the same core in this case the peptide linked sulfonamide (Scheme 1 Structure 3). In fact, we could have made separate libraries or libraries from libraries of both 3 and 4. Additionally we could expand the diversity of our collection by utilizing different cyclization conditions on 5 to produce additional “libraries from libraries”, such as cyclic urea linked sulfonamides and cyclic thiourea linked sulfonamides utilizing carbonyldiimidazole and thiocarbonyldiimidazole to name just a couple¹⁵.

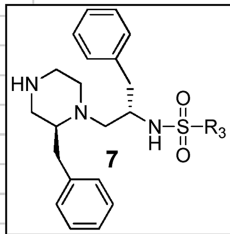
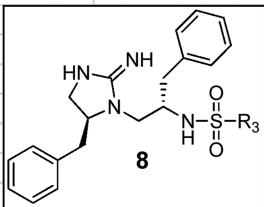
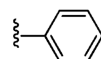


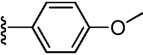
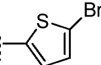
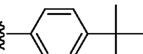
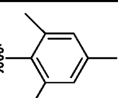

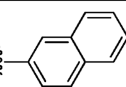


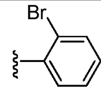
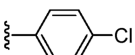
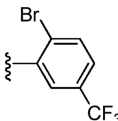
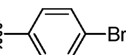
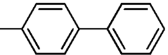
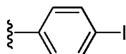

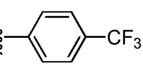
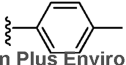
Determination of Proper Reagent Ratio. We have previously calculated, utilized, and reported the reagent ratios for amino acids competitively coupled to solid supported free amines⁹. These ratios are utilized in the generation of Library 7 and 8. However, until now we had not determined the proper reagent ratios for sulfonyl chlorides reacting with solid supported free amines. To synthesize an equimolar mixture of sulfonamides, ratios must be determined to adjust for the fact that steric and electronic hindrance will vary among the different sulfonyl chlorides used in the mixture.

Initially, to test the robustness of different amino acids and sulfonyl chlorides to the reaction conditions described in Scheme 1, 104 individual compounds (52 of 7 and 52 of 8)

were synthesized and analyzed by LCMS (see Table S1 in Supporting Information). Of the 104 compounds, 40 tested the robustness of the R₃ position, 20 for 7, and 20 for 8 (see Table 1). Of these 40, 30 (15 for 7 and 15 for 8) were shown to have final UV purities greater than >75%. Therefore the 15 sulfonyl chlorides that provided these 30 compounds (with >75% purity) were selected to determine isokinetic ratios. It may be noted that compounds in the R₃ position that contained fluorine substitution yielded low product purity. This may be due to fluorine reacting with the piperazine during the reduction step (Scheme 1 step e2); however, this side reaction was not explored, and these sulfonyl chlorides were eliminated from further study.

Having tested the robustness of the reaction conditions to different reagents, it is then necessary to determine a correction factor for each compound to account for differences in UV absorbance. Applying the tea bag method¹⁶, 15 individual compounds where Boc-L-Phenylalanine was utilized in the R₁ and R₂ position were synthesized according to scheme 1. The 15 sulfonyl chlorides of interest were coupled to this sequence separately (Scheme 1 compound 3) providing 15 separate compounds that differ at only the R₃ position (Scheme 1

Table 1. 40 Different Individual Compounds (20 of Type 7 and 20 of Type 8) Were Made to Test the Robustness of the R₃ Position^a

 7		 8	
ID	R ₃	ID	R ₃
-33		-43	
-34		-44	
-35		-45	
-36		-46	
-37		-47	
-38		-48	
-39		-49	
-40		-50	
-41		-51	
-42		-52	

^aThe boxed functionalities are the functionalities that provided the desired compounds in purities sufficient enough to be included in the final library syntheses.

compound D). L-Phenylalanine in the R₁ and R₂ position was chosen because of its strong UV absorbance properties at 214 nm and nonreactive side chain. All individual compounds were analyzed using reverse phase HPLC-MS (See Table 2). The UV component of the HPLC-MS is used in the study in order to quantify the relative ratios while the MS component is used to aid in the identification of the compounds. A compound,

obtained from using 4-tert-butylbenzene-1-sulfonyl chloride at the R₃ position (Scheme 1 compound D-45), with a sufficiently distinct retention time was selected as the control to which all other compounds were compared. Each compound was then physically mixed with the control to create an equal molar mixture. These samples were analyzed using HPLC-MS. The integration parameters were set to allow the best integration possible for the UV peaks 214 nm, and all data sets were analyzed using these same integration parameters. Since the concentration of each compound is known to be equal, any differences in peak area can be attributed to differences in molar absorbance. A correction factor is therefore calculated for each compound as the ratio of the control compound's peak area to the peak area of that compound:

$$CF_n = \frac{\text{Area}_{\text{control}}}{\text{Area}_n}$$

This definition insures that by multiplying the peak area of the *n*th compound by its corresponding control factor, an area equal to peak area of the control is obtained. This process was performed in duplicate to confirm the correction factors and to minimize experimental error. See Table 2.

The rate at which each sulfonyl chloride reacts will vary based on steric and electronic hindrance. To synthesize an equimolar mixture, a ratio of each component of the sulfonyl chloride mixture must be calculated in order to compensate for the reaction rate differences. Each test mixture C was synthesized using a 1:1 mixture of the test sulfonyl chloride and the control sulfonyl chloride (Scheme 1 step c), each at a 4-fold excess over the resin (for a combined 8-fold excess total). The resulting on-resin synthetic mixtures were then cleaved from the solid support and analyzed by HPLC-MS using the same method and integration parameters as were used to calculate the correction factor. The UV 214 nm peak area under the curve is used to calculate the reaction rate ratio:

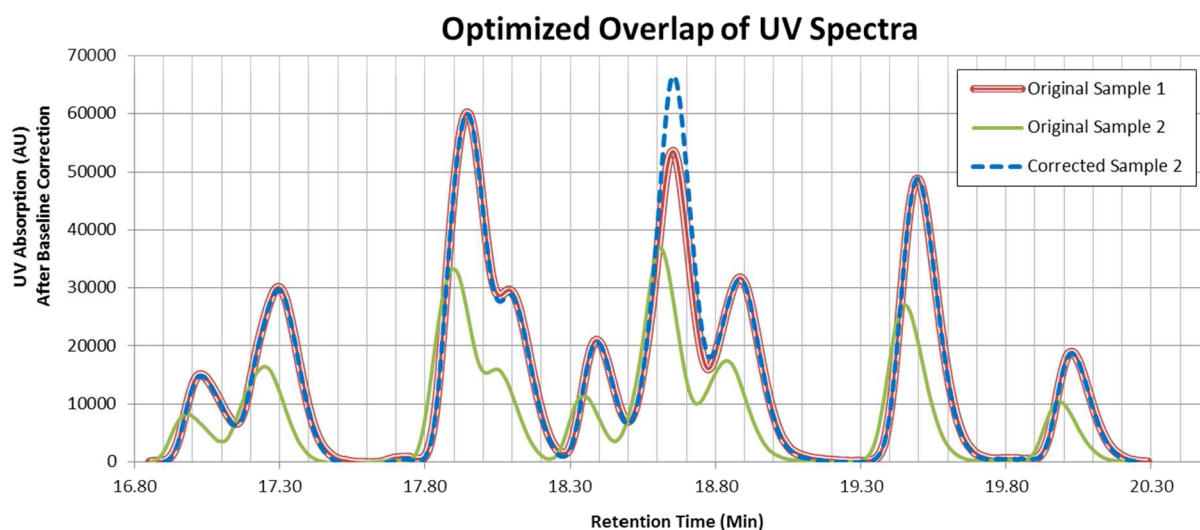
$$BR_n = \frac{\text{Area}_{\text{control}}}{CF_n \text{Area}_n}$$

The ratios obtained are thus the rate at which the *n*th sulfonyl chloride reacts relative to the control compound, with the differences in absorptivity accounted for via multiplication by the control factor. See Table 2.

Differences in Reaction Rate Caused by Multiple Simultaneous Reactions. The above calculations were done as individual comparisons between a given sulfonyl chloride and the control sulfonyl chloride. To determine if the presence of additional (more than two at a time) sulfonyl chlorides simultaneously would change the reaction rate of each sulfonyl chloride, these ratios were then tested in the presence of multiple sulfonyl chlorides including the control. A mixture of all the sulfonyl chlorides would yield a final mixture that would not allow for the analysis of individual peaks due to some final compounds D (i.e., D-52 rt 17.89 min and D-47 rt 17.91 min) having similar retention times and/or same molecular weights, and therefore individual changes in reaction rates would not be determinable. Because of this, multiple sample sets were set up to maximize the amount of compounds present that would still be distinguishable by HPLC-MS analysis. The control compound, D-45, was present in every sample set. Each designed mixture of sulfonyl chlorides was competitively coupled to L-phenylalanine, L-phenylalanine-MBHA resin (Scheme 1 Compound A) using an 8-fold excess (meaning

Table 2. Calculated Values for the Various Synthesized Compounds with L-Phenylalanine in the First Two Positions and a Sulfonyl Chloride in the Third Position

sulfonyl chloride	compound	molecular weight	retention time	UV absorptivity correction factor relative to control	ratio relative to control	final reagent ratio relative to control
thiophene-2-sulfonyl chloride	D-34	458.10	17.00	1.30	4.08	3.15
benzenesulfonyl chloride	D-35	451.90	17.23	0.89	0.60	0.67
2,4,6-trimethylbenzene-1-sulfonyl chloride	D-36	494.05	19.50	0.81	0.37	0.45
naphthalene-2-sulfonyl chloride	D-37	502.00	18.66	0.51	0.26	0.52
4-chlorobenzene-1-sulfonyl chloride	D-39	486.10	18.38	0.97	0.49	0.51
4-bromobenzene-1-sulfonyl chloride	D-40	531.90	18.58	1.03	0.51	0.50
4-iodobenzene-1-sulfonyl chloride	D-41	578.00	18.83	1.10	0.48	0.44
4-(trifluoromethyl)benzene-1-sulfonyl chloride	D-42	519.90	18.91	1.02	0.41	0.40
4-methoxybenzene-1-sulfonyl chloride	D-44	481.95	17.31	1.06	1.11	1.04
4-tert-butylbenzene-1-sulfonyl chloride (control)	D-45	508.15	20.01	1.00	1.00	1.00
2-chlorobenzene-1-sulfonyl chloride	D-47	485.80	17.91	0.88	0.77	0.87
2-bromobenzene-1-sulfonyl chloride	D-48	532.00	18.09	0.87	0.69	0.79
Biphenyl-4-sulfonyl chloride	D-50	528.15	19.46	0.84	0.72	0.85
3-methylbenzene-1-sulfonyl chloride	D-51	466.20	17.96	0.78	0.63	0.81
4-methylbenzene-1-sulfonyl chloride	D-52	465.95	17.89	0.96	0.70	0.73

**Figure 1.** Example of two UV spectra (red and green), which are not directly comparable because of differences in attenuation and concentration, and the result (blue) of finding the optimal overlap between them. The difference in the spectra is now apparent and quantifiable.

the total excess of the sulfonyl chlorides) over the resin. The experiment was done so that every compound was analyzed in duplicate. The HPLC-MS 214 nm UV peaks were analyzed using the same integration parameters that were used to obtain the ratios in the previous experiments. The correction factor for each compound was used to compare the amount of that compound to the amount of the control compound. No compound deviates in amount by more than 20% from that of the control (see Figure 1). This is well within experimental error and shows that there is no substantial effect on the reaction rates of the individual sulfonyl chlorides from the presence of other sulfonyl chlorides.

Differences in Reaction Rate Caused by Different On-Resin Amino Acids. It has been demonstrated that differences in the on-resin amino acid onto which the reagents couple will cause differences in the reaction rates of the individual reagents.^{9,17} To explore the extent to which this is the case, we have developed a novel technique to quantify the overall

difference between two mixtures containing multiple compounds. In general, direct comparison between two different HPLC-MS 214 nm UV spectra is complicated by differences in concentration and attenuation from run to run. Further, use of a reference compound cannot quantify differences in peak shape that may result when multiple compounds with similar retention times are present, and can only be used to adjust relative peak heights. However, working under the assumption that two identical samples will have geometrically similar 214 nm UV spectra, it is posited that through appropriate shifting and scaling two 214 nm UV spectra may be compared directly for similarity. In particular, given two samples, first the HPLC-MS was used to determine the initial retention time of the first peak of interest, t_i , and the final retention time of the final peak of interest, t_f , of both samples. Then, the baseline was assumed to be linear and was removed from both samples via the equation:

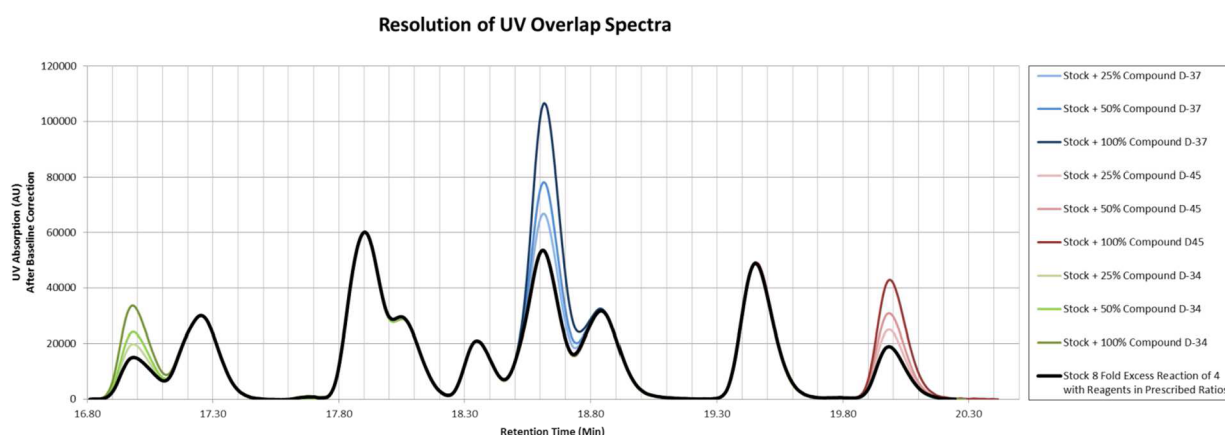


Figure 2. 214 nm UV spectra of stock solution spiked with specific compounds, as compared to the stock solution alone, after optimal overlap correction.

Linear Relationships Between Optimal Overlap Area & Amount of Compound Added

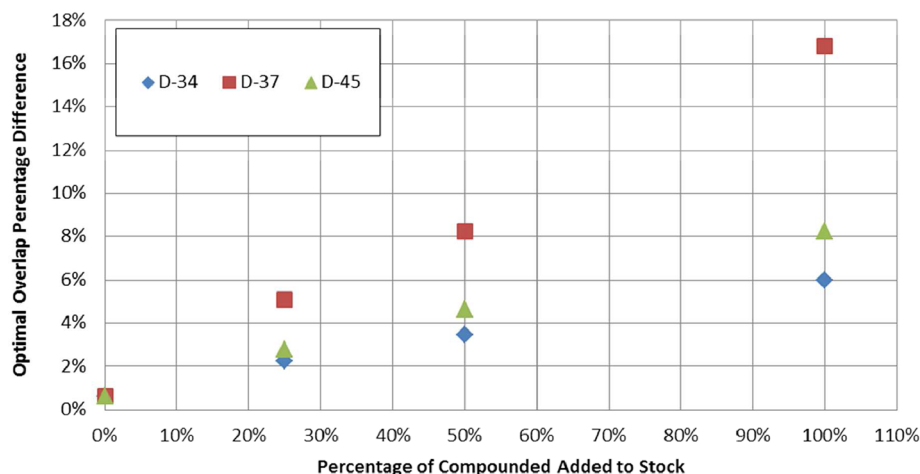


Figure 3. Errors are between 214 nm UV spectra of stock solution spiked with specific compounds, as compared to the stock solution alone, after optimal overlap correction. The relationship between the percentage errors and amount of compounded added is clearly linear, with all R^2 values exceeding 0.99. Steeper slopes correspond to higher UV absorptivity.

$$UV_{\text{withoutbaseline}}(t) = U(t) - \frac{UV(t_f) - UV(t_i)}{t_f - t_i}(t - t_i) - UV(t_i)$$

It was assumed that for two identical samples there exist parameters A , b , h , and k such that

$$UV_{\text{withoutbaseline}}^{\text{sample1}}(t) = A \cdot UV_{\text{withoutbaseline}}^{\text{sample2}}(b \cdot (t - h)) + k$$

In other words, it was assumed that there exists appropriate vertical and horizontal scaling parameters (A and b , respectively) and vertical and horizontal shifting parameters (k and h , respectively) so that the UV spectra of identical samples can be made to “overlap” one another. Furthermore, for two samples that are not identical it may be thought to have the relationship

$$UV_{\text{withoutbaseline}}^{\text{sample1}}(t) = A \cdot UV_{\text{withoutbaseline}}^{\text{sample2}}(b \cdot (t - h)) + k + D(t)$$

where integration of the difference term $D(t)$ corresponds to the area between the two spectra after overlapping has occurred, and therefore quantifies differences in the relative makeups of the two samples. To this end, given two 214 nm UV spectra, a variant of the Accelerated Random Search Algorithm¹⁸ can be used to find the optimal overlap parameters A , b , h , and k which minimize the area between the two spectra; this minimum area serves as a measure of the “difference” between the two samples (See Figure 1). The Accelerated Random Search Algorithm is an optimization algorithm shown in ref 18 to have excellent rapid convergence properties while maintaining consistency and requiring minimal parameter manipulation to achieve optimal results.

To test the resolution of the above method a synthetic mixture of 15 compounds (type C scheme 1) (termed “stock solution” from here on out) was made using *L*-phenylalanine (R_1), *L*-phenylalanine (R_2), and X_3 (where X_3 represents a mixture of the 15 sulfonyl chlorides of interest using the prescribed ratios calculated as in Table 2). Multiple aliquots of this stock solution were made and spiked with one of three

different individual compounds (D-34, D-37, D-45) at one of three different percentage increases, 25%, 50%, and 100%. The samples were then analyzed using the HPLC-MS 214 nm UV spectra, with each sample compared to the unspiked stock solution. Optimal overlapping was performed as described above, and the area between the resulting curves was calculated (see Figures 2 and 3, and Table 3). The spectra after

Table 3. Percentage Error between 214 nm UV Spectra of Stock Solution Spiked with Specific Compounds, As Compared to the Stock Solution Alone

test sample	comparison to stock solution (%)
stock solution	0.61
stock solution +25% D-34	2.22
stock solution +50% D-34	3.48
stock solution +100% D-34	5.97
stock solution +25% D-37	5.09
stock solution +50% D-37	8.24
stock solution +100% D-37	16.79
stock solution +25% D-45	2.77
stock solution +50% D-45	4.62
stock solution +100% D-45	8.22

overlapping clearly show differences even when only 25% additional compound is added, and the area difference between the curves scales linearly with the amount of compound added. Unsurprisingly, the compound with the lowest correction factor (and therefore highest UV absorptivity), D-37 (correction factor of 0.51, Table 2), also had the greatest slope; the compound with the highest correction factor (and therefore lowest UV absorptivity), D-34, had the lowest slope.

With the ability to compare UV spectra directly, the following experiment was devised to determine the impact of differing on-resin amino acids to the reaction rates of the sulfonyl chlorides. Ideally, in order for the above ratios to produce as equimolar a mixture as possible, the group of all on-resin functionalities should react at approximately the same average rate as the specific functionality used to calculate the ratios (L-phenylalanine). Two different on-resin starting materials were prepared; one with L-phenylalanine used in the R₁ and R₂ positions (Scheme 1 Compound A) and one with an equal molar mixture of 66 amino acids (including L, D,

and unusuals) in the R₁ and R₂ positions (Scheme 1 Compound B). The two reaction vessels were set up. In the first reaction vessel two “tea bags”¹⁶ were added, one containing compound A and the other containing compound B. In the second reaction vessel, two tea bags both containing compound A were added. To each vessel an equal molar mixture of the 15 sulfonyl chlorides was added. It was added so as to provide only a 1 equivalent amount of sulfonyl chloride to available free amine (note this differs from the 8-fold excess used above). The assumption is that in the presence of Compound B, if Compound B has an average reaction rate to any of the sulfonyl chlorides that differs drastically from that of Compound A, it will disrupt the equimolarity of the reagents reacting with Compound A and cause an unequal distribution of products. The final mixture products (of the form Compound C Scheme 1) from Compound A reacted in the presence of Compound B and in the absence of Compound B were analyzed using HPLC-MS 214 nm UV, and their spectra were compared both to each other and to the aforementioned stock solution (done using the corrected ratios with an 8-fold excess) (see Figure 4).

The largest difference between spectra for the stock solution (using the 8-fold excess) and the spectra from the mixture products derived from compound A using the 1-fold excess in the presence and absence of compound B occurs at an approximate retention time of 17.0 min. This corresponds to compound D-34, L-phenylalanine (R₁), L-phenylalanine (R₂), thiophene-2-sulfonyl chloride (R₃); this difference is unsurprising since D-34 has such a slow reaction rate (Table 2). Most importantly, the spectra for both the 1-fold excess compound C samples do not differ substantially from one another, with an overall difference of 7.01%. There are slight differences between the spectra at all retention times, but the largest difference occurs at an approximate retention time of 18.85 min. This peak corresponds to compound D-41, L-phenylalanine (R₁), L-phenylalanine (R₂), 4-iodobenzene-1-sulfonyl chloride (R₃), and compound D-42, L-phenylalanine (R₁), L-phenylalanine (R₂), 4-(trifluoromethyl)benzene-1-sulfonyl chloride (R₃), both of which have similar correction factors to the control D-45, L-phenylalanine (R₁), L-phenylalanine (R₂), 4-*tert*-butylbenzene-1-sulfonyl chloride (R₃) (see Table 2). In the spiking experiment described early, it was determined that a mixture which contained 2-fold the amount of D-45 corresponded to a

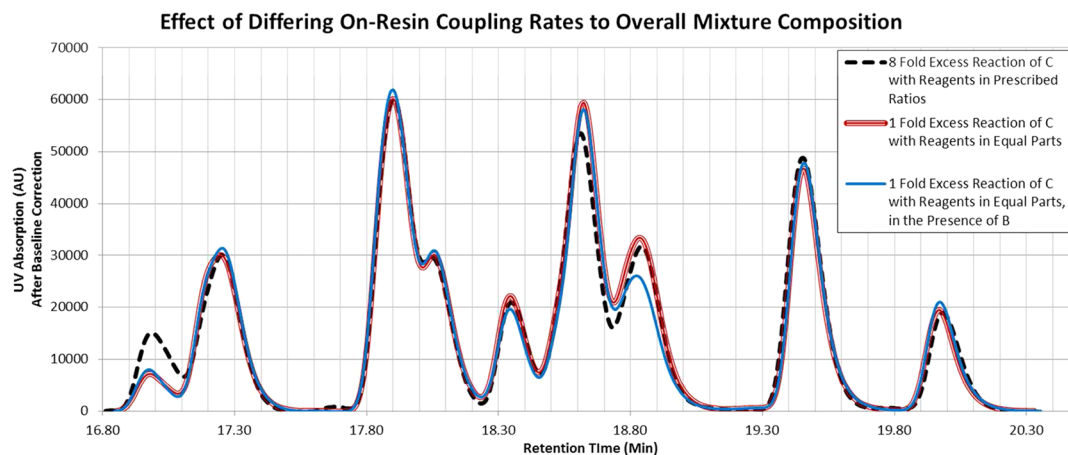


Figure 4. 214 nm UV spectra after optimal overlap correction of 8-fold stock solution and 1-fold reactions. The differences between the samples are not substantial.

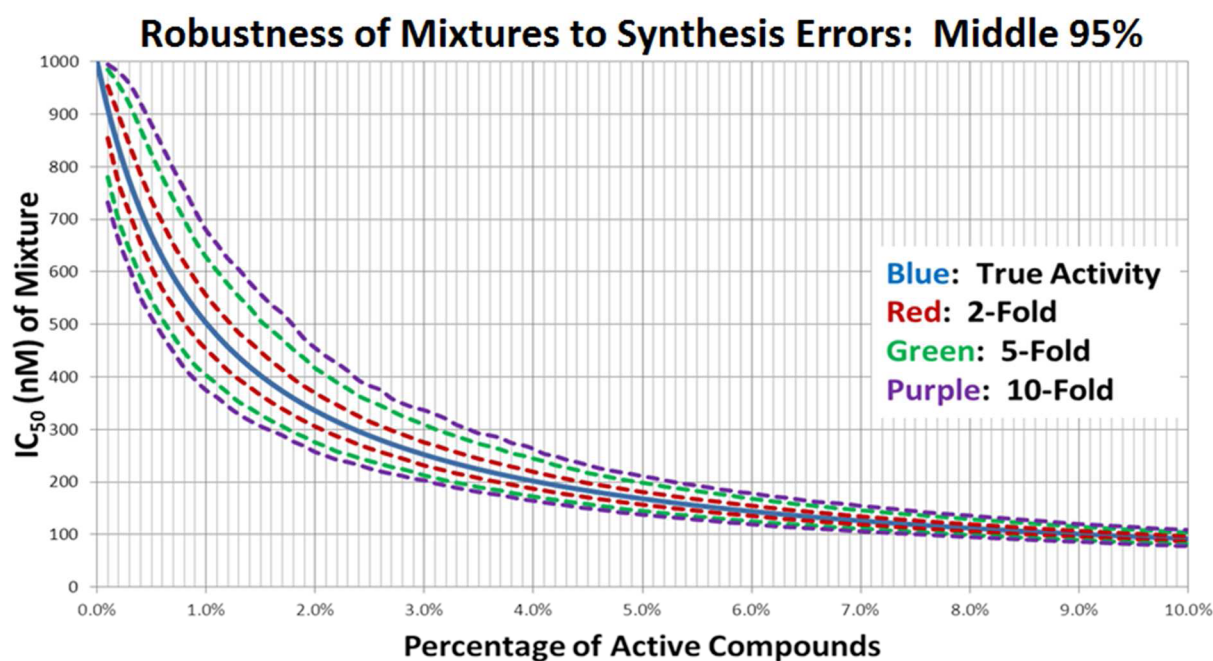


Figure 5. Results of a Monte Carlo simulation demonstrating the robustness of mixtures to errors in equimolarity when tested in simple competitive binding assays.

difference in spectra of 8.22%, therefore, we posit that no single compound molarity in the mixture obtained from reacting compound A in the presence of compound B differs more than 2-fold from that of the mixture obtained from reacting compound A in the absence of compound B. In fact, since the 7.01% error is distributed among multiple peaks, and multiple compounds are present in certain peaks, it is likely that the difference in relative constituency is far less than 2-fold. This means that the average reaction rate of all on-resin functionalities is only marginally different from the reaction rates used to calculate the ratios for this specific reaction.

Robustness of Combinatorial Libraries to Errors in Equimolarity. Although the possibility of differing reaction rates resulting in a 2-fold error in equimolarity may seem to preclude usefulness for this library for meaningful screening data, this is in fact not the case. To demonstrate this, hypothetical mixtures containing various percentages of 10 nM IC_{50} active compounds and 1000 nM IC_{50} inactive compounds were considered in association with a simple competitive binding assay. The IC_{50} of such a mixture as a function of its percentage of active compounds may be calculated directly using the harmonic mean¹⁹. To determine the effect of n -fold error in the equimolarity of compounds in this setting, for each percentage of active compounds a Monte Carlo simulation was performed, generating a uniform error in relative abundance of at most n -fold, for $n = 2, 5,$ and 10 . The upper and lower bounds of the middle 95% of simulations is plotted in Figure 5 as a function of the percentage of active compounds; as is evident, even a 10-fold maximal error in equimolarity did not result in even a 2-fold error in mixture IC_{50} at any percentage of active compounds. The maximal errors in equimolarity presented herein are therefore well within the acceptable range for use in screening.

The values 10 nM and 1,000 nM are representative of the harmonic mean of the “active” individual compounds versus the “inactive” individual compounds **within the mixture**. This does not mean to imply that the mixture will always contain a

compound 100-fold better than the current best, but rather that the mixture contains some compounds that bind well, and others that do not bind at all. In our experience, a 100-fold difference in activity among the thousands of compounds in the library is a reasonable assumption.

Application to the Design of Two New Positional Scanning Libraries. The correction factors obtained for the sulfonyl chlorides were then applied to synthesize two heterocyclic positional scanning libraries. A linear sulfonamide precursor is synthesized using the “tea bag” method. Two different cyclizations are applied to form either a 2-piperazine sulfonamide library or a 2-guanidine sulfonamide library (See scheme 1 structure 7 and 8). As mentioned earlier and reported in Table S1 in the Supporting Information, 104 individual compounds were synthesized and analyzed to test the robustness of different functionalities at the three diversity positions. The results of the sulfonyl chlorides analyzed and the resulting 15 chosen for ratio determination are shown in Table 1. These 15 sulfonyl chlorides were all used in the synthesis of the two new positional scanning libraries (7 and 8). In Table S1 in the Supporting Information we also report the results obtained from testing different functionalities at the R_1 and R_2 positions. From these results 34 amino acids (L, D, and unusual) were selected for the libraries. Each library therefore contains 17 340 compounds ($34 \times 34 \times 15$). A list of the amino acids used in the libraries as well as their associated ratios can be found in the Supporting Information (Table S2).

Evaluation of Two New Positional Scanning Libraries in Mu and Kappa Binding Assays. The two new libraries 7 and 8 were both screened in two different opioid binding assays, a mu binding assay and a kappa binding assay (Complete results can be found in the Supporting Information, See Table S3 Supporting Information). The screening was done in part to confirm the integrity of the synthesis of the libraries. The harmonic mean offers an excellent method of using such positional scan data to assess the integrity of the synthesis process¹⁹. In particular, since each position

subdivides the entire library, if the individual compounds are equimolar in all mixtures then the harmonic mean of the IC_{50} values of the mixtures in each position should be the same, and that value should also be the IC_{50} of the all X mixture. On the other hand, if there is substantial difference in the constituents of the mixtures by position, then the harmonic means at each position may be drastically different. In Table 4, we see the

Table 4. Harmonic Mean Values for Library 7 and 8 in Mu and Kappa Binding Assays

	experimental IC_{50} , untested extrapolated from %Inh (nM)			
	library 7		library 8	
	mu	kappa	mu	kappa
R1	11147	10971	4444	15357
R2	8864	9019	4992	15792
R3	9508	9345	5249	13343
all X	8134	7416	6884	14337

harmonic means of the IC_{50} values of the mixtures at each position, for both targets and both libraries. For mixtures that did not show substantial activity at high doses, IC_{50} values were extrapolated using single-point percent inhibition. The All X was also treated in this manner. As is evident, the resulting harmonic means do not vary substantially, with a maximal deviation of 1.55-fold and most values quite a bit more similar. This is well within the resolution of the assay, and we can therefore be further confident that there is not substantial difference in the constituencies of the mixtures at the different positions.

Discussion Regarding Use of Detection Methods in Determining Isokinetic Ratios. It should be noted that we initially assessed the validity of utilizing the MS portion of the HPLC-MS in determining the ratios for the reaction studied in this manuscript. However it quickly became apparent that correction factors would be needed for MS as equal molar mixtures of two compounds did not produce equal ratios of ions and the MS signal does not scale linearly with molarity. Additionally we determined that the MS was less consistent than the UV component at providing reproducible data. For example if we injected the same equal molar mixture of two components into the HPLC-MS over multiple runs the run to run variation in UV absorption was significantly lower than the variation in the MS ionization. Therefore the MS did not provide any advantage over the UV component in determining our isokinetic ratios.

At the time of this study, we did not have reasonable access to an NMR for use in determining the ratios of this reaction. This in of itself is one advantage of the method we report in our manuscript as a researcher would theoretically only need use of an HPLC to determine relative ratios. The MS component is only used as confirmation of compound identity. Since an HPLC is more affordable and likely more available to a wider number of researchers than either HPLC-MS or NMR we felt our reported approach would be more applicable. It should be noted that we now do have reasonable access to an NMR and are currently working out a method for calculating ratios with the NMR. However at this time we are finding that one limiting factor is the nonconsistent confounding influence of the solvent peak which strongly biases attempts for direct overlap. We are hoping to resolve this problem and soon report our findings on the use of NMR for the determination of isokinetic ratios.

CONCLUSION

We have demonstrated the utility of a new method for determining the isokinetic ratios of reactant species. We then applied it in order to calculate the ratios for mixtures of sulfonyl chlorides reacted with on-resin amines. In doing so, we also demonstrated a new method to access the effect of the on-resin amines toward the relative reaction rates of mixtures of sulfonyl chlorides and shown that our calculated ratios are not substantially effected by the on-resin amines. These new methodologies can be applied to study a wide variety of isokinetic ratios and the only instrument of analysis needed is an HPLC.

Additionally the determination of ratios for the formation of a sulfonamide bond enables us to expand the diversity of current positional scanning libraries. The two specific libraries described in this publication have been added to our current collection of positional scanning libraries that are available for use through collaborative efforts.

EXPERIMENTAL PROCEDURES

General Procedure for Individual Compounds 5 (As Outlined in Scheme 1). A tea bag filled with mBHA resin is neutralized with 3 washes of 5% DIEA in DCM. The tea bag filled with neutralized resin is added to a reaction vessel that has been allowed to preactivate for 15 min with a Boc-AA [6 equiv, 0.1 M dimethylformamide (DMF)], DIC (6 equiv, 0.1 M DMF), and HOBt (6 equiv, 0.1 M DMF) for 2 h at room temperature, followed by washes DMF (2 \times) and dichloromethane, DCM (2 \times). The Boc was removed using 55% trifluoroacetic acid in DCM for 30 min, followed by washes with DCM (2 \times), IPA (2 \times), and DCM (3 \times). Then the resin was neutralized using 5% DIEA in DCM (3 \times) followed by washes of DCM (3 \times). This was repeated so that a compound 2 was obtained. Sulfonyl chloride (8 equiv, 0.1 M anhydrous DCM) was coupled to 2 using DIEA (8 equiv) overnight producing compound 3. Each coupling reaction was monitored by the use of the ninhydrin test to ensure the coupling was complete. The tea bag containing 3 was added to a reaction vessel containing a 40-fold excess of BH_3 -THF and heated at 60 °C for 96 h after which the solution was poured off and the resin filled tea bag was treated with piperidine at 60 °C for 24 h.

General Procedure for Individual Compounds 7. A tea bag containing compound 5 is added to a reaction vessel containing bromoacetic acid (5 equiv, 0.1 M anhydrous DCM), DIC (5 equiv), and DIEA (2.5 equiv) and shaken overnight. The solution is poured off and excess reagents are washed off by successive washes with DMF and DCM. The resulting products 6a and 6b are then added to a reaction vessel containing a 40 fold excess of BH_3 -THF and heated at 60 °C for 96 h, after which the solution was poured off and the resin filled tea bag was treated with piperidine at 60 °C for 24 h. The resin is allowed to dry prior to cleavage with HF/anisole at 0 °C for 7hrs. The final product 7 is extracted from the resin using 95% acetic acid frozen and lyophilized. After which it is dissolved in a solution of 50% acetonitrile and 50% water, frozen, and lyophilized before the dry powder is then analyzed. (Note: It should be noted that several other methods for transforming compounds of type 5 to compounds of type 7 were attempted including treatment with oxalylidimidazole followed by reduction, as well as several different conditions involving the use of 1,2-dibromoethane, to circumvent the need for the second reduction. However none of the tested conditions produced the desired test

compounds in the yields and quality that the method described here did. The results of the 1,2-dibromoethane experiments are the subject matter of previous publication²⁰ and will also be addressed in future publications.) ¹H-NMR of 7–33 *N*-((S)-1-((S)-2-benzylpiperazin-1-yl)-3-(4-hydroxyphenyl)propan-2-yl)-benzenesulfonamide (500 MHz, DMSO-*d*₆): δ 8.30 (1H, s), 7.67 (2H, d, *J* = 5.0 Hz), 7.54 (1H, t), 7.45 (2H, t), 7.27–7.02 (10H, m), 3.33 (2H, s), 2.78 (1H, q), 2.64–2.53 (6H, m), 2.39 (2H, s), 2.31 (2H, q), 2.24 (1H, q), 1.23 (1H, s).

General Procedure for Individual Compounds 8. A tea bag containing compound 5 is added to a reaction vessel containing cyanogen bromide (5 equiv, 0.1 M anhydrous DMF) and shaken overnight. The solution is poured off and excess reagents are washed off by successive washes with DMF and DCM. The resin is allowed to dry prior to cleavage with HF/anisole at 0 °C for 1.5 h. The final product 8 is extracted from the resin using 95% acetic acid frozen and lyophilized. After which it is dissolved in a solution of 50% acetonitrile and 50% water, frozen, and lyophilized before the dry powder is then analyzed. ¹H-NMR of 8–40 *N*-((S)-1-((S)-5-benzyl-2-iminoimidazolidin-1-yl)-3-phenylpropan-2-yl)-4-bromobenzenesulfonamide (500 MHz, DMSO-*d*₆): δ 8.45 (1H, s), 7.50 (1H, d, *J* = 1.7 Hz), 7.49 (4H, d, *J* = 1.8 Hz), 7.37–7.22 (5H, m), 7.09–7.01 (6H, m), 3.68 (2H, quin), 3.32 (1H, t), 3.22 (1H, t), 3.22 (1H, q), 2.96–2.93 (2H, m), 2.67–2.62 (3H, m).

General Procedure for μ Receptor Binding Assay. Rat cortices were homogenized using 50 mM Tris, pH 7.4, and centrifuged at 16 500 rpm for 10 min. The pellets were resuspended in fresh buffer and incubated at 37 °C for 30 min. Following incubation, the suspensions were centrifuged as before, the resulting pellets resuspended in 100 volumes of buffer A plus 2 mg/mL bovine serum albumin (membrane buffer) and the suspensions combined. Each assay tube contained 0.5 mL of membrane suspension, 2 nM [³H]-DAMGO, and 0.02 mg/mL mixture in a total volume of 0.65 mL. Assay tubes were incubated for 1 h at 25 °C. The reaction was terminated by filtration through GF/B filters, soaked in 5 mg/mL bovine serum albumin, 50 mM Tris, pH 7.4, on a Tomtec Mach II Harvester 96. The filters were subsequently washed with 6 mL of assay buffer. Unlabeled DAMGO was used as a competitor to generate a standard curve and determine nonspecific binding. Bound radioactivity was counted on a Wallac Betaplate Liquid Scintillation Counter.

General Procedure for κ Receptor Binding Assay. Guinea pig cortices and cerebella were homogenized using 50 mM Tris, pH 7.4, 10 mM MgCl₂–6H₂O, 200 μM PMSF (assay buffer) and centrifuged at 16 500 rpm for 10 min. The pellets were resuspended in fresh buffer and incubated at 37 °C for 30 min. Following incubation, the suspensions were centrifuged as before, the resulting pellets resuspended in 100 volumes of buffer A plus 2 mg/mL bovine serum albumin (membrane buffer) and the suspensions combined. Each assay tube contained 0.5 mL of membrane suspension, 2 nM [³H]U69 593, and 0.02 mg/mL mixture in a total volume of 0.65 mL. Assay tubes were incubated for 2 h at 25 °C. The reaction was terminated by filtration through GF/B filters, soaked in 5 mg/mL bovine serum albumin, 50 mM Tris, pH 7.4, on a Tomtec Mach II Harvester 96. The filters were subsequently washed with 6 mL of assay buffer. Unlabeled U50,488 was used as a competitor to generate a standard curve and determine nonspecific binding. Bound radioactivity was counted on a Wallac Betaplate Liquid Scintillation Counter.

■ ASSOCIATED CONTENT

■ Supporting Information

Data for library members. 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.

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