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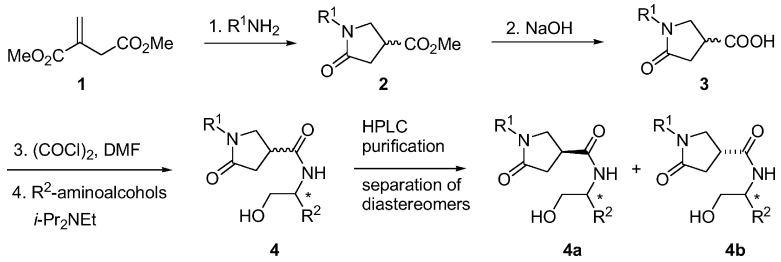

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High-Throughput Purification of Combinatorial Libraries II: Automated Separation of Single Diastereomers from a 4-Amido-pyrrolidone Library Containing Intentional Diastereomer Pairs

Mark Irving,[‡] Clinton A. Krueger,[‡] Janice V. Wade, John C. Hodges,[†] Kyle Leopold, Nathan Collins, Cecilia Chan, Suzan Shaqair, Alex Shornikov, and Bing Yan*

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A 4-amido-pyrrolidone library that was intentionally synthesized as pairs of diastereomers was produced by solution-phase parallel syntheses and purified by an automated high-throughput purification system. A total of 2592 4-amido-pyrrolidinones were ultimately isolated as single diastereomers from a matrix of 1920 syntheses. After the four-step synthesis and HPLC purification, the average yield of a single diastereomer was 36.6%. The average chemical purity was >90%, and the average diastereomeric purity was >87%. The choice of chiral amines used to make amides with heterocyclic acid chlorides had a dramatic effect on success. Analysis of the relationship between amines used for synthesis and the diastereomeric separation showed that amides made from chiral 1,2-amino alcohols gave superior separation to amides from chiral morpholines. The presence of a hydrogen bond donor on the amide side chain seems to be required for a better diastereomeric separation.

Introduction

Biological targets such as receptors and enzymes possess well-defined three-dimensional structures. They recognize and bind the ligand or drug in a stereochemically specific binding site. The three-dimensional structure of the protein or a drug target also determines the binding affinity of the drug. For these reasons, the chirality of a drug molecule has become the focus of pharmaceutical research. Recently, there has been a noticeable increase in the sale of chiral drugs and the effort to develop chiral drugs.[1] However, the development of chiral drugs relies on more effective lead discovery, especially our ability to prepare optically pure compound libraries and screen them.

Combinatorial chemistry has provided a vast supply of compounds for lead discovery.[2] The early practice to make and screen compound mixtures has been replaced by making and screening individual compounds. By "individual compound", one normally means compounds with the same chemical formula, not necessarily the same stereochemistry. Racemic mixtures are normally considered as "pure" compound and used in high-throughput screening without chiral separation. In lead discovery research, the optical purity of compounds made from combinatorial chemistry and parallel synthesis determines the effort required to fully understand biological screening results. Results from screening racemic mixtures are ambiguous as to the correct stereochemistry for the hit, necessitating further work to resolve and rescreen each enantiomer before further development. Preparation of the combinatorial libraries without chiral ambiguity is a critical step toward a more effective and rapid drug discovery process.

For molecules that are enantiomers, purification by high performance liquid chromatography (HPLC) requires stationary and mobile phases that have chiral modifications. For nonsuperimposable molecules (diastereomers), separation is frequently possible without the need for such chiral modifications, given sufficient development of a suitable preparative HPLC method. However, pursuit of a high-throughput separation protocol for pairs of diastereomers in combinatorial libraries remains a formidable undertaking, since the diversity of functional groups may vary the retention time difference for each pair.

Several automated high-throughput purification methods have been reported in addition to our purification system.[3−8] Reported here are our results on diastereomer separation of a 4-amido-pyrrolidone library that contains intentional pairs of diastereomers that were made by solution-phase parallel synthesis. The library was purified using an automated high-throughput purification system.[8] This system offers short cycle times, affordable operation cost, and individualized separation conditions for each compound.

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Scheme 1

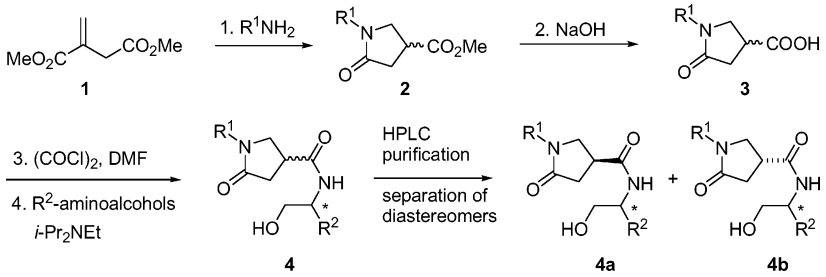


Table 1. Comparison of Separation Parameters from Validation Libraries

sample set	column size, mm	sample load, mmol	gradient range (ACN%)	av peak width (min)	av resolution
A	30 × 50	0.6	29.8	0.42 (±0.06)	1.62 (±0.46)
B	30 × 50	0.3	33.8	0.33 (±0.07)	1.83 (±0.51)
C	30 × 50	0.3	29.8	0.26 (±0.02)	2.01 (±0.47)
D	21.2 × 50	0.6	29.8	0.44 (±0.11)	1.04 (±0.23)
E	21.2 × 100	0.25	29.8	0.38 (±0.14)	1.46 (±0.39)

Results and Discussion

Purification Overview. Our separation method is a modified version of our high-throughput purification process, called the accelerated retention window (ARW) method.[8] First, crude reaction mixtures are rapidly analyzed by LC/MS/UV using an eight-channel MUX-LCT LC/MS system. The retention time of each desired compound is then used to predict the initial and ending solvent strength to elute a compound at a fixed time using the same gradient steepness for both analytical and preparative separations. A collecting window is then calculated around this target time. Within this time window, UV or ELSD signals are used to trigger the fraction collection.

Separation Considerations. We developed and optimized a high-throughput diastereomer separation method by studying a test library. Our goal was to separate diastereomers effectively with automated operation to achieve high recovery in terms of both compound number and mass.

A measure of resolution is defined as

$$\mathrm{Rs} = 1.18(t_2 - t_1)/[W_{1(50)} + W_{2(50)}] \qquad (1)$$

where t_1 and t_2 are the retention times of isomer peaks, $W_{1(50)}$ and $W_{2(50)}$, are the peak widths at half-height. Figure 1 shows chromatograms of two closely eluting components at different separation resolution. It shows that an Rs of 1.0 is required to give a satisfactory separation.

Purification Method Development Using the Reagent Validation Library for 4-Amido-pyrrolidinones. To develop high-throughput diastereomer purification methods, we synthesized and purified a reagent validation library as described in Scheme 1. In the validation library, some of the syntheses held R_1 constant while varying R_2 and the remainder held R_2 constant while varying R_1. The cutoff for validated reagents was set at >60% product purity (**4a** + **4b**) by HPLC analysis (AUC at 214 nM) of the crude reaction product. The effects of column size, loading level, gradient time, and gradient slope were examined on products from the validation library.

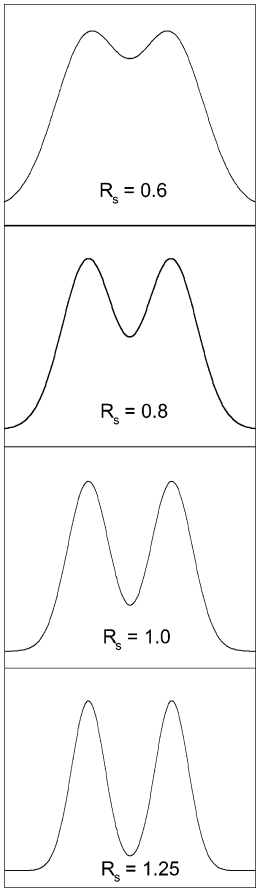
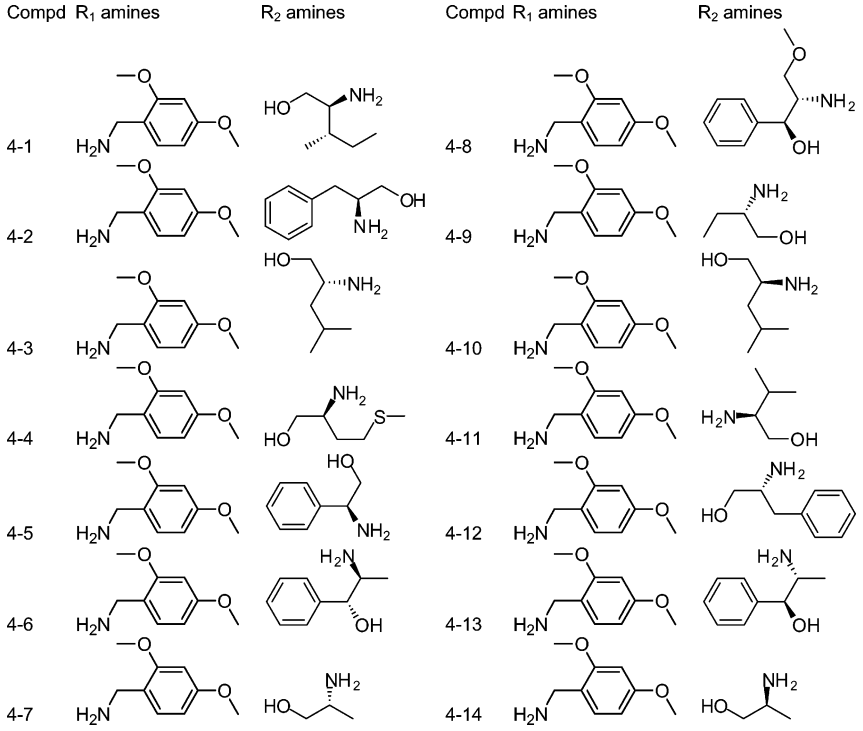


Figure 1. The rating of peak resolution for two adjacent chromatographic components according to eq 1.

Ninety-six amidopyrrolidonones were divided into five sample sets and purified by preparative HPLC using the variety of conditions shown in Table 1. The method used for sample set C (containing 19 samples) gave the narrowest peaks and best resolution on average. Figure 2 illustrates the chromatographic behavior of 14 representative diastereomer pairs, **4-1** to **4-14** (Table 2), from sample set C. From the 19 injections of this sample set, 4 pairs failed to show any separation (not shown), 3 pairs had near baseline separation (**4-2**,**4-8**,**4-12**), and the remaining 12 pairs exhibited complete baseline separation. On the basis of these studies, a column size of 30 × 50 mm and a gradiant range of 29% change of ACN percent were used. The starting ACN percent was individualized on the basis of a calculation using the analytical retention time of each compound.[8]

Synthesis and Purification of 4-Amido-pyrrolidone Production Library. The 4-amido-pyrrolidinone production library (Scheme 1) consisted of 64 proprietary methyl esters (**2**), which were converted to acid chlorides in the same

Table 2. 4-Amido-pyrrolidinones: Reagent Validation Library Examples



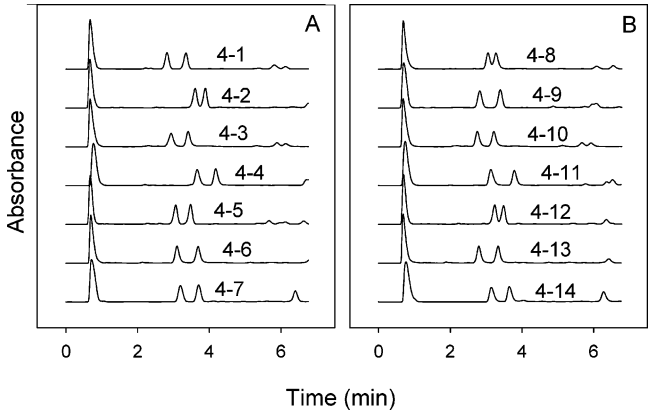


Figure 2. Chromatograms of preparative HPLC for 14 compounds (Table 2) in the reagent validation library.

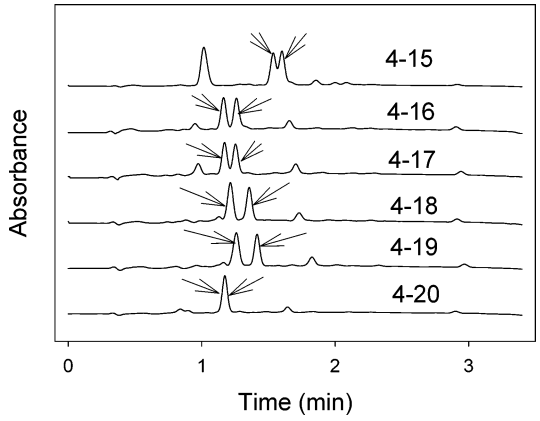
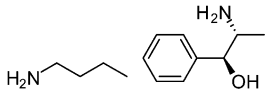
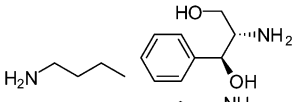
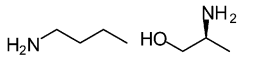
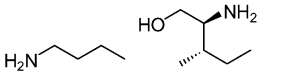
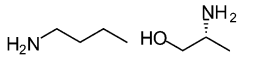
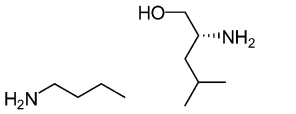
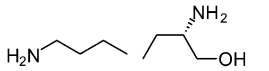
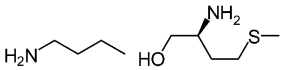
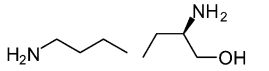
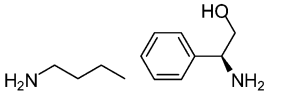
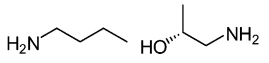
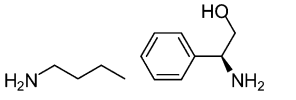


Figure 3. Examples for LC/MS analysis of the production library before purification. LC chromatograms are shown for samples **4-15** through **4-20**. Diastereomer pairs of desired products are labeled by arrows.

manner as the test library, whereby they were reacted with 30 different chiral amino alcohols. A total of 80 plates (24-well) were used to produce 1920 diastereomeric pairs **4**. The individual diastereomers **4a** and **4b** were then separated using the optimized method described above.

Two batches of 960 compounds each were synthesized, as shown in Scheme 1, at a 0.6 mmol scale. Mainly aliphatic R_1 diversity was used in synthesizing batch A (960 compounds) and aromatic R_1 diversity for batch B (960 compounds). Compounds were first analyzed by parallel LC/MS analysis to assess the initial purity. As examples, Figure 3 shows prepurification analysis of **4-15** to **4-20**. The crude purity distributions for batches A, B, and C (see below) are shown in Figure 4. Compounds with a purity $\geq 10\%$ were purified. The analytical retention time of each compound was used to calculate a preparative solvent gradient segment. This process individualizes the starting and ending ACN percent in order to elute two diastereomers with their midpoint at 4 min in a 6.5-min run. As examples, the preparative purification chromatograms for diastereomer pairs **4-15** to **4-26** from batch A and **4-27** to **4-38** from batch B are shown in Figures 5 and 6. Structures of these compounds are shown in Tables 3 and 4. Sufficient separation was observed for all compounds shown in Figure 5, except for diastereomer pair **4-20**, which was expected to be difficult to separate from the analytical LC/MS analysis (Figure 3). Figure 6 shows some examples of compound separations in batch B. Nearly one-half of the diastereomer pairs in Figure 6 did not give sufficient separation. Fraction collection control by avoiding the overlapping portion of the peak made the diastereomer separation possible, such as in **4-29** and **4-30**. Collected fractions were again analyzed by parallel LC/MS to determine their purity. As examples, the postpurification LC/MS data for diastereomers from samples **4-15** to **4-20** are shown in Figure 7. Note that diastereomer pair **4-20** was not separated. Most of the diastereomer pairs in the production

Table 3. 4-Amido-pyrrolidinones: Batch A Examples

Compound	R_1 amines	R_2 amines	Compound	R_1 amines	R_2 amines
4-15			4-21		
4-16			4-22		
4-17			4-23		
4-18			4-24		
4-19			4-25		
4-20			4-26		

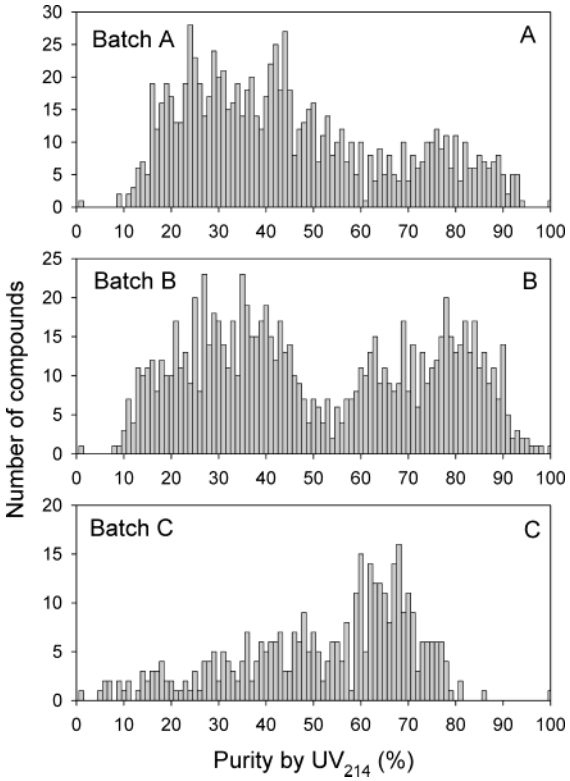


Figure 4. Purity distribution of batches A, B, and C of the 4-amido-pyrrolidinone library before purification.

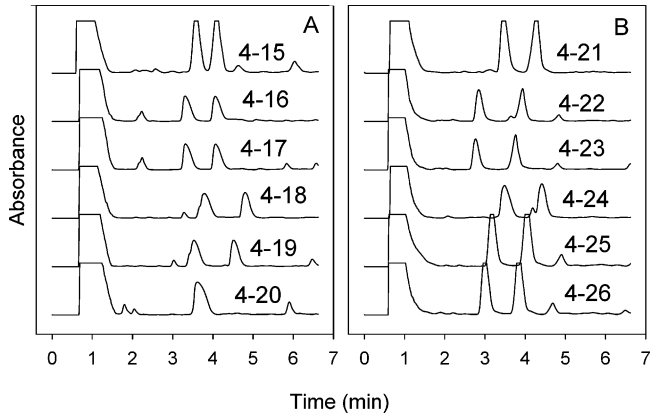


Figure 5. Examples for HPLC purification of diastereomer pairs from batch A. Examples are diastereomer pairs **4-15** through **4-26**.

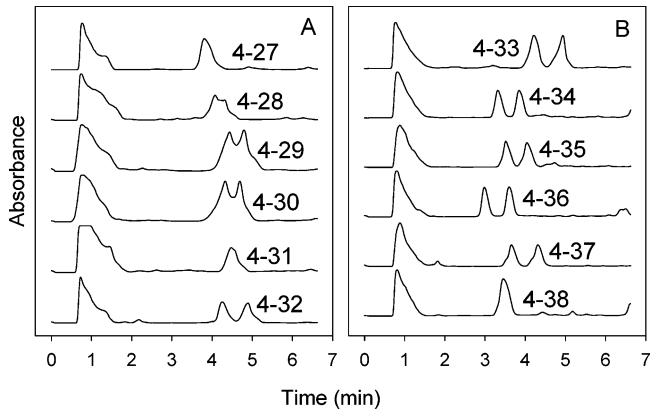


Figure 6. Examples for HPLC purification of diastereomer pairs from batch B. Examples are diastereomer pairs **4-27** through **4-38**.

library showed good separation. Although some did not show a baseline separation, careful control of the fraction collection cutoff was used to achieve optimal separation. A total of 206 pairs from batch A and 285 pairs from batch B did not show any separation. Separated diastereomers were named D1 and D2 according to their elution order.

Since not all samples were baseline-separated, some of the purified compounds contained small amounts of the other diastereomer. We therefore define the "chemical purity" to be the UV purity at 214 nm of both isomers relative to the other impurities in the final sample and the "diastereomeric purity" to be the AUC for one diastereomer divided by the sum of the AUCs for both diastereomers. Figures 8−11 summarize the purification results, including the chemical purity, diastereomeric purity, and the weight for all successfully purified compounds. Figure 8 (diastereomer 1 data) and Figure 9 (diastereomer 2 data) summarize the data from batch A (960 aliphatic R_1 compounds). Figure 10 (diastereomer 1 data) and Figure 11 (diastereomer 2 data) summarize the data

Table 4. 4-Amido-pyrrolidinones: Batch B Examples

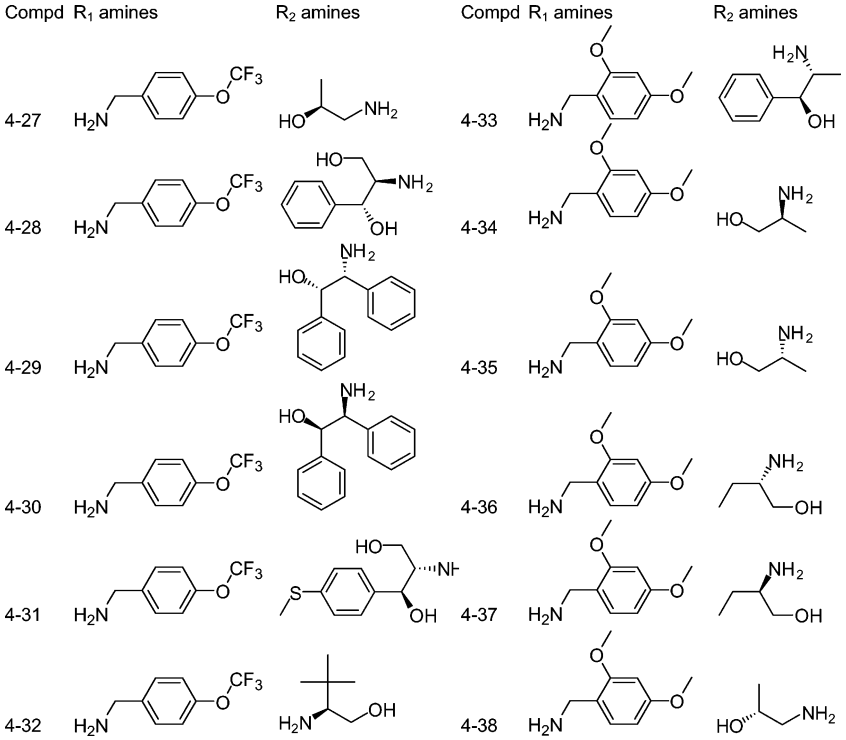
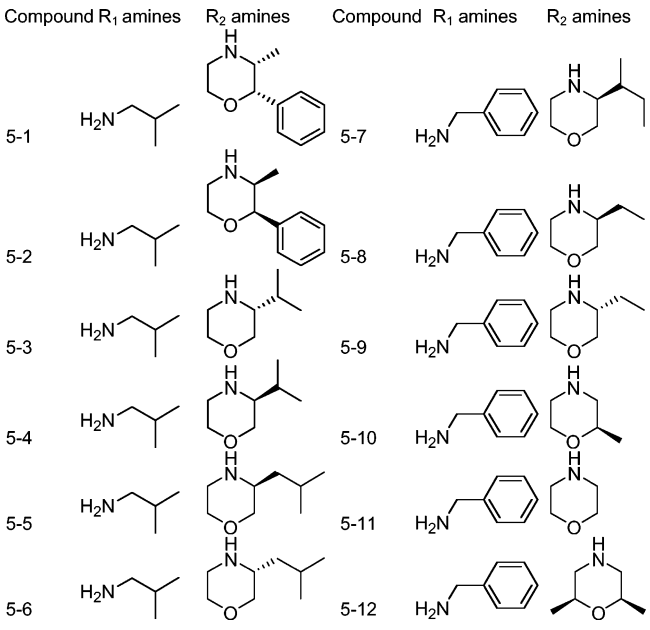


Table 5. 4-Amido-pyrrolidinones: Batch C Examples



from batch B (960 aromatic R_1 compounds). Using 0.03 mmol and 90% chemical and diastereomeric purities as a combined cutoff, 2592 4-amido-pyrrolidinone single diastereomers were obtained. The purification success rates for diastereomers D1 and D2 of batch A are 76 and 69%, and for diastereomer D1 and D2 of batch B are 72 and 53%. The slightly lower success rate for batch B is due to a poorer separation for diastereomer pairs in this batch, as compared to batch A (see Figure 6).

The average weight for each diastereomer in batch A was 0.116 mmol, with an average chemical purity of 95.2% and

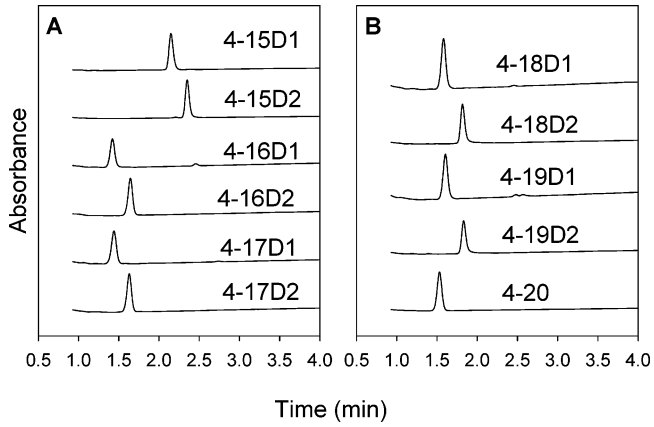


Figure 7. Examples for LC/MS analysis of the production library after purification. LC chromatograms (UV_{214} detection) are shown for purified diastereomer D1 and D2 for samples **4-15** through **4-20**. Among these samples, only the diastereomer pair **4-20** was not separated.

an average diastereomeric purity of 89.6%. The average weight for each diastereomer in batch B was 0.102 mmol, with an average chemical purity of 90.9% and an average diastereomeric purity of 87.7%. The initial synthesis scale was 0.6, or 0.3 mmol per diastereomer. After a four-step synthesis and purification, 39 and 34% average yields were achieved for batches A and B, respectively, for single diastereomers.

An additional 384-member library (batch C) was also synthesized using chiral morpholines as the R_2 component instead of the chiral amino alcohols used in batches A and B (Scheme 2). Batch C diastereomer pairs were subjected to purification under conditions identical to those used for **4**. As examples, Figure 12 shows the purification chromatograms of 12 compounds, **5-1** to **5-12**, from batch C (Table

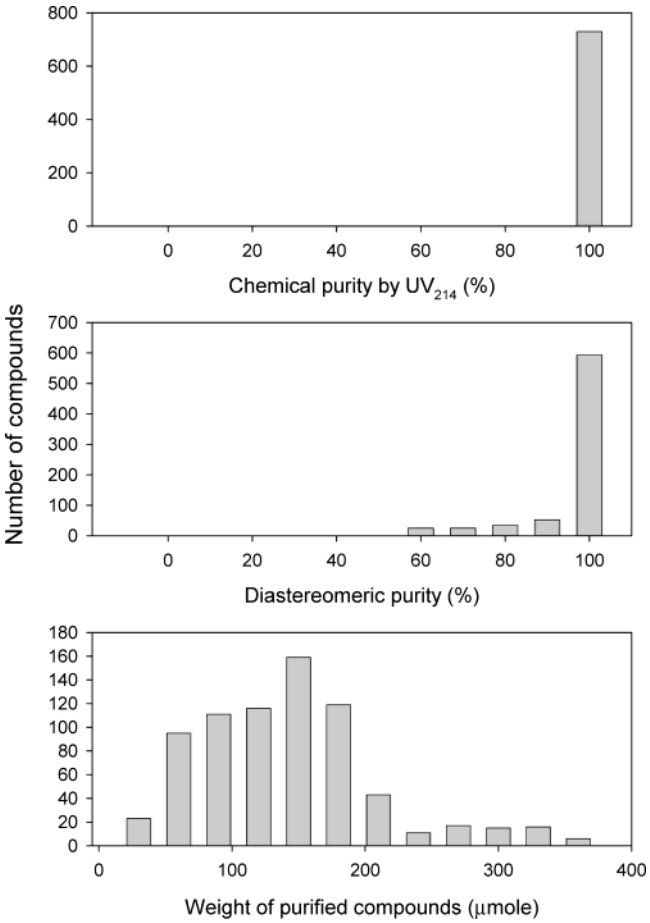


Figure 8. Distributions of the chemical purity, diastereomeric purity, and the weight of diastereomer 1 (D1) in batch A after purification.

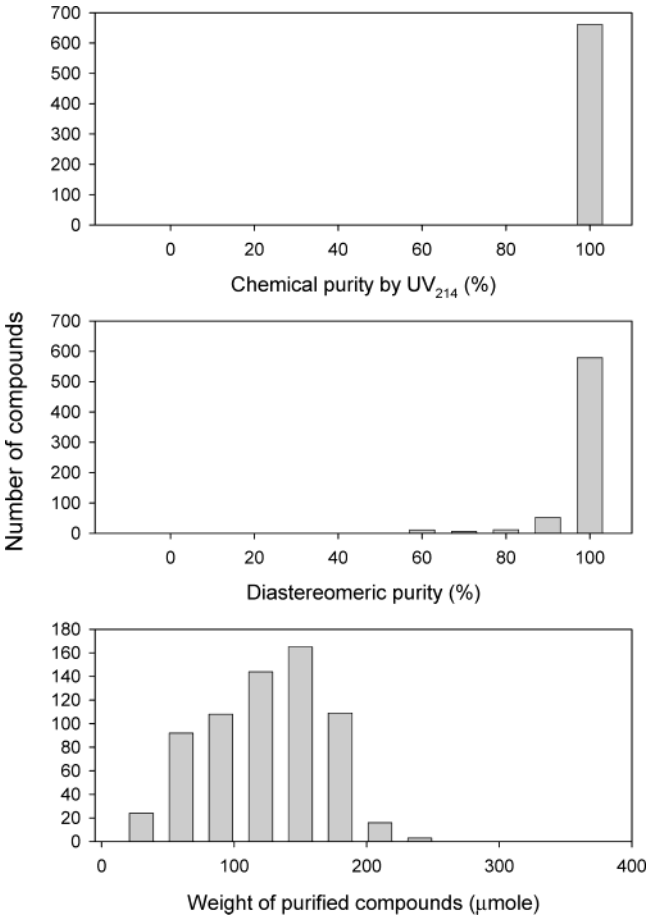


Figure 9. Distributions of the chemical purity, diastereomeric purity, and the weight of diastereomer 2 (D2) in batch A after purification.

5). No diastereomer separation was observed for **5** in all cases. The replacement of a hydrogen bond donor with a lipophilic ethylene bridge in compound **5** in batch C has a remarkably detrimental effect on diastereomer separation.

In summary, batch A, in which R_1 was primarily an aliphatic side chain, showed a better average peak resolution than batch B, in which R_1 was an aromatic side chain. However, it was possible to achieve high chemical and diastereomeric purity for both batches by controlling the collection cutoff. Finally, we found that when chiral morpholines were used as the R_2 diversity instead of the chiral amino alcohols (batch C), no separation was observed. We speculate that hydrogen bond donors on the R_2 containing alcohols have an important role in effecting chromatographic separation. The factors affecting diastereomer separation are now under investigation and will be reported in the future.

Experimental Section

Materials. All reagents and solvents were reagent grade and were used without further purification.

Step 1: Synthesis of Pyrrolidinone-4-carboxylic Acid Methyl Esters (2). Dimethyl itaconate **1** (10.1 g, 63.8 mmol, 1.11 equiv) was added to a 250-mL Nalgene bottle, followed by 130 mL of methanol and the amine (R^1) (57.6 mmol, 1.00 equiv). After shaking, the bottle was placed in an oven at 60 °C for 16−20 h. The excess dimethyl itaconate **1** was removed with 14.2 g of AP-Trisamine resin (2.71 mmol/g, 38.4 mmol, Argonaut Technologies Catalog No. 800399). The resin was added to the reaction solution, placed in an oven at 60 °C, and allowed to react for 16−20 h. The resin was removed by filtration using a medium fritted funnel, and the solution (**2**) was collected in a 2-L round-bottom flask. The resin was rinsed three times with 60-mL aliquots of methanol, and the rinses were collected in the same flask.

Step 2: Preparation of Pyrrolidinone-4-carboxylic Acid (3). Aqueous sodium hydroxide (1 M, 75 mL, 75 mmol, 1.3 equiv) was added to the solution (**2**). The reaction was stirred for 4 h, and the solution was concentrated in vacuo until only the water remained. AG MP-50 cation-exchange resin (28 g, 98 mmol, 3.5 mmol/g, 1.7 equiv), purchased from BioRad Laboratories (Catalog No. 143-0841), was rinsed with 75 mL each of methanol, 1 N HCl, ether, methanol, and 1:1 THF/H_2O (repeating the 1:1 THF/H_2O wash once more). A mixture of 1:1 THF/H_2O was prepared, and 105 mL was added to the 2-L round-bottom flask containing the concentrated scaffold. The solution was transferred to a 250-mL Nalgene bottle. The flask was then washed twice with 20 mL off 1:1 THF/H_2O, and the rinses were added to the bottle, followed by the AG MP-50 resin. The bottle was placed on a shaker for 1.5 h. The resin was filtered off, and the solution (**3**) was collected in a 2-L round-bottom flask. The resin was then rinsed twice with 75 mL of THF,

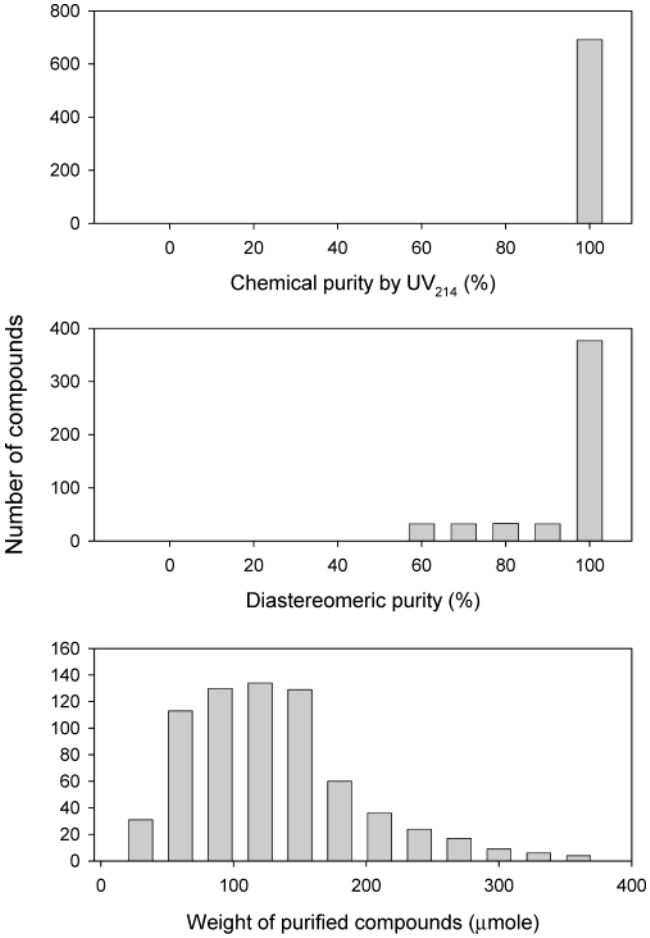


Figure 10. Distributions of the chemical purity, diastereomeric purity, and the weight of diastereomer 1 (D1) in batch B after purification.

collecting the filtrate in the same flask. The pH of the solute **3** was tested to ensure a pH of <5. The solution was concentrated to remove all of the THF and most of the water. Crude **3** was dissolved in 140 mL of 1,4-dioxane, and the solution was transferred directly into a clean, tared 1-L lyophilizing flask. The original flask was then rinsed twice with 50 mL of 1,4-dioxane, and the rinses were combined in the lyophilizer flask. The solution was frozen at −80 °C and then lyophilized for at least 48 h, yielding the pyrrolidinone-4-carboxylic acid (**3**). The overall yield for steps 1 and 2 for all 64 precursors were between 80 and 95% with purities between 90 and 100% at 214 nm.

Steps 3 and 4: Preparation of 4-amido-pyrrolidinone (4 and 5). (Note: These steps were performed in a glovebox and were completed on the same day.) A 0.25 M solution of pyrrolidinone-4-carboxylic acid (**3**) (32 mmol, 130 mL) was made using anhydrous THF. *N*,*N*-Dimethylformamide (250 μL, 3.2 mmol, 0.10 equiv) was added to the solution. Oxalyl chloride (4.2 mL, 49 mmol, 1.5 equiv) was added slowly to the solution. *Note: The reaction is exothermic and generates CO_2 and CO gases.* The solution turned yellow upon formation of the acid chloride. The reaction was allowed to sit for 15 min or until gases ceased to evolve after the solution was swirled. When complete, the solution was concentrated in vacuo. Crude acid chlorides were used as below.

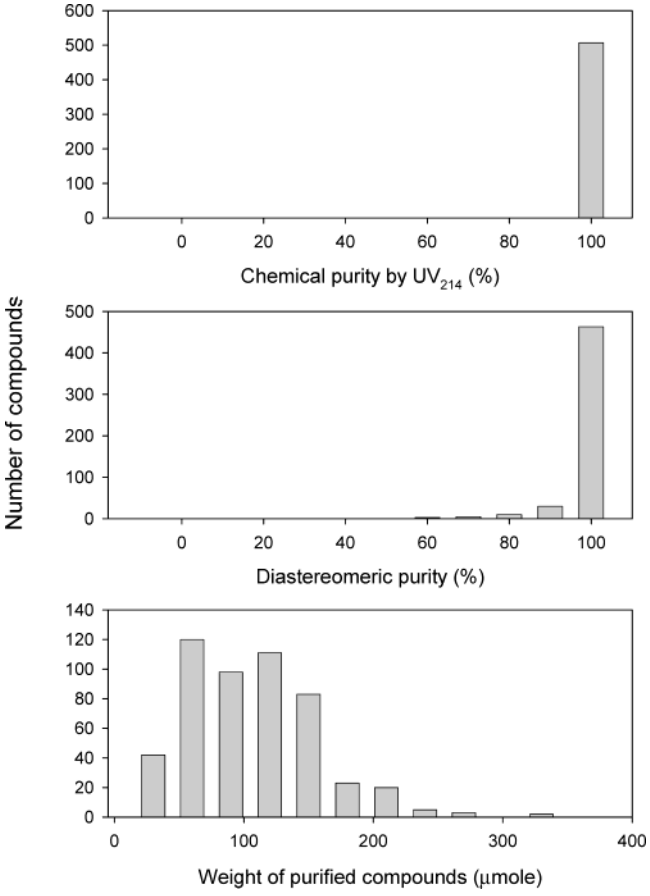


Figure 11. Distributions of the chemical purity, diastereomeric purity, and the weight of diastereomer 2 (D2) in batch B after purification.

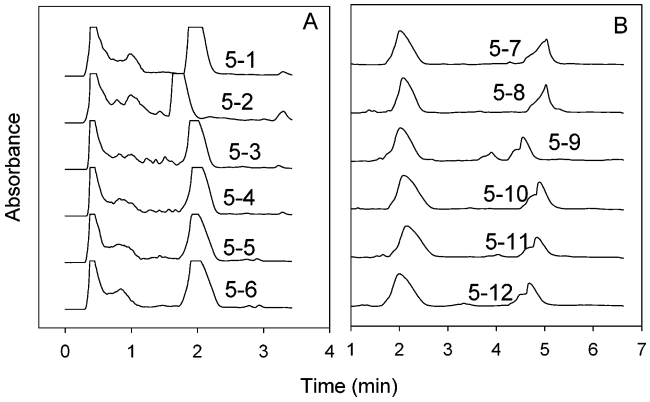
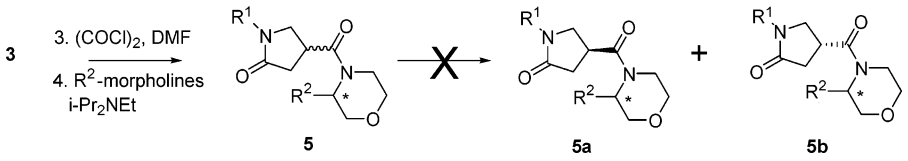


Figure 12. Examples for HPLC purification of diastereomer pairs from batch C. Examples are diastereomer pairs **5-1** through **5-12**.

The R_2 amino alcohols or morpholines (1.2 mmol, 2.0 equiv) were diluted in 2.8 mL of anhydrous THF. *N*,*N*-Diisopropylethylamine (210 μL, 1.2 mmol, 2.0 equiv) was added to each amino alcohol or morpholine. All morpholines were soluble. Insoluble amino alcohols were removed from the glovebox and sonicated to dissolve or to make a suspension. The solutions were delivered to the appropriate well in a 24-well Multichem plate according to the plate layout. Anhydrous THF was added to the flask containing the crude pyrrolidinone-4-carboxylic acid chloride. Using the Robbins Hydra 96-well dispenser, acid chloride/THF solution was delivered 10 times to each well of the 24-well plate in aliquots of 400 μL per well, waiting for at least 15 s between

Scheme 2



each delivery. The final quantity of acid chloride in each well was 0.6 mmol. The plates were clamped and put on a shaker for 16 h. The contents of the plates were then concentrated on the Savant Speed-Vac rotary evaporator.

Analysis. Six representative compounds were characterized by ^{1}H NMR, ^{13}C NMR, and elemental analysis. ^{1}H NMR and ^{13}C NMR spectra were measured on a JEOL Eclipse 270 spectrometer at 296 K, respectively. Chemical shifts are reported in parts per million relative to TMS ($\delta = 0$). HPLC data were obtained on an Agilent HPLC 1100 using a Phenomenex C18 (3.0 × 100 mm) column. The following conditions were used: mobile A, water/ACN (99:1) and 0.05% TFA; mobile B, ACN/water (99:1) and 0.05% TFA; flow rate, 0.5 mL/min; sample volume, 10.0 μL; temperature, 40 °C; gradient, 0−100% B in 6.5 min, 100% B for 3.0 min, 0% B for 2.5 min; detection, UV at 214 and 254 nm.

LC/MS analysis was carried out on a MUX-LCT system (Waters, Beverly, MA). The LC/UV system consisted of a Gilson pump system (two 306 pumps, an 811C dynamic mixer, and an 805 manometric module), a Gilson 215 autosampler with a 889 eight-injection module, and eight Gilson 115 UV detectors. (Gilson, Inc., Middleton, WI). The solvent delivered by the pump at 16 mL/min was equally split into eight LC columns (each had a precolumn filter) to carry out reversed-phase HPLC separation. Eight samples (from a column on a 96-well microtiter plate) were simultaneously injected into eight columns, separated by the same gradient, and detected by individual UV detectors at selected wavelengths. After the UV detectors, eluent from eight channels, each with a flow rate of 50 μL/min, was introduced into an eight-channel multiplexed electrospray ion source (MUX) while the remaining flow was directed to waste.

A Micromass LCT orthogonal acceleration time-of-flight mass spectrometer (Micromass U.K. Limited, Manchester, England) equipped with an eight-channel MUX was used as a mass detector. The MUX interface consists of eight electrospray probes and a sampling aperture positioned coaxial with the sampling cone. Each of the probes within the MUX source was indexed using an optical position sensor and selected using a programmable stepper motor, controlled by the MassLynx software. The position of the sampling aperture in MUX was controlled by the stepper motor, which only allows ions from one probe at a time into the sampling cone of the mass spectrometer. Acquisition times per spray were set to 0.1 s, with the interspray time of 0.05 s. This produced a data point for each spray every 1.2 s.

The instrument was operated in positive mode. The following settings were used: capillary voltage, 3.5 kV; sample cone, 30 V; RF lens, 250 V; extraction cone, 5 V; RF DC offset 1, 4 V; RF DC offset 2, 7 V; aperture, 10 V; acceleration, 200 V; steering, 0 V; and ion energy, 34 V. Desolvation and source temperature were set at 350 and 100 °C. The nitrogen desolvation and nebulizer gas flows were set at 900 and 300 L/h. For negative ion mode, the capillary voltage was 3.3 kV.

C-18 columns (4.6 × 50 mm packed with 5-μm particles from Phenomenex,Torrance, CA) were used for LC/MS analysis. Mobile phase A contained 99% water, 1% acetonitrile, and 0.1% acetic acid. Mobile phase B contained 1% water, 99% acetonitrile, and 0.1% acetic acid. The gradient was programmed to go from 0 to 100% B in 3.0 min, stay at 100% B for 0.5 min, and reequilibrate to initial condition for 0.5 min. An injection volume of 10 μL was used. For postpurification LC/MS analysis, a gradient was programmed to go from 0 to 100% B in 7.0 min, stay at 100% B for 0.5 min, and reequilibrate to initial condition for 0.5 min.

Diastereomer Purification. Separation of library samples was achieved using Gilson (Gilson Inc., Middleton, WI) liquid handlers and HPLC equipment. Instruments were controlled by Unipoint, version 3.2. Initial HPLC gradient conditions and peak levels for triggering fraction collection were set according to ARW calculations from analytical LC/MS data. Four pumps (three 306 piston pumps and one 305 piston pump) were used to control mobile phase flow through two matched, 30 × 50 mm Phenomenex Hydro-RP (C-18 based), 10-μm columns (Phenomenex, Torrance, CA). Liquid streams were mixed with Gilson 811C dynamic mixers, and pressure spikes were moderated using Gilson 806 manometric modules. Two Gilson switching valves were also used: an 819 unit controlled the injection port, and a Valvemate controlled the column flow. Aqueous mobile phase consisted of HPLC purity water with 0.1% acetic acid (HAc) as additive, organic mobile phase was HPLC purity acetonitrile with 0.1% (HAc) as modifier; flow rate was 35 mL/min. The starting and ending solvent compositions were calculated on the basis of the analytical retention time to allow the first detectable isomer to elute at 4 min. The total gradient time was 7 min.

Sample injection and fraction collection were automated using two separate Gilson 215 liquid handlers. An alternative configuration was also used so that one liquid handler operated in both inject and collect modes. Samples were dissolved in DMSO and then drained through filter plates (Thomson Inc., Oceanside, CA). The total sample volume was kept below 1200 μL. Fraction collection was triggered by a UV detector set at 412 nm or a Sedex 55 evaporative light scattering detector (S.E.D.E.R.E., France). Sample fractions were collected into 13 × 100 Pyrex test tubes.

After each purification run, the prep chromatograms were processed by an in-house "compound-tracking file" to cull the desired fractions. The culled fractions were then consolidated using a Bohdan BA-200 (Bohdan Automation Inc. Mundelein, IL) and sampled for postpurification LC/MS analysis using a Tecan Genesis (Tecan AG, Hombrechtikon,

Switzerland). These samples were then frozen at −80 °C in a Revco Ultima II freezer (Kendro Laboratory Products Inc., Ashville, NC) for 12−24 h and then freeze-dried in a Virtis Ultra EL tray lyophilizer (Virtis Inc., Gardiner, NY). Culled samples that passed internal MUX analysis criteria were then dissolved in $CHCl_3$/MeOH (2:1). The tubes were vortexed to ensure sample dissolution and transferred (via Tecan Genesis) to Bohdan tared test tubes. These tubes were then dried at 50 °C for 24 h for final weighing by Bohdan.[8]

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