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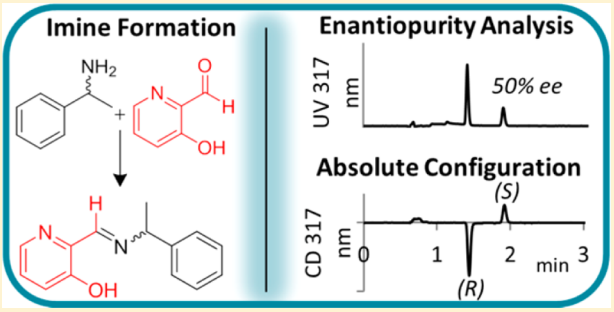
Hydroxypyridyl Imines: Enhancing Chromatographic Separation and Stereochemical Analysis of Chiral Amines via Circular Dichroism

Leo A. Joyce,* Erik L. Regalado, and Christopher J. Welch

Analytical Research & Development, Merck Research Laboratories, Rahway, New Jersey 07065, United States

Supporting Information

ABSTRACT: Imine-bond formation between chiral amines and commercially available 3-hydroxypyridine-2-carboxaldehyde (HCA) was exploited for rapid determination of stereochemical composition. Chiral supercritical fluid chromatography (SFC) screening of the derivatized imine compounds led to the elucidation of multiple combinations of mobile and stationary phases that gave resolution of all members of a series of chiral amines. The first eluting enantiomer was generally the derivative of the (*R*)-amine enantiomer across the series that was studied, indicating that the imine formed from the (*S*)-amine has more favorable interaction with the chiral stationary phase of the column. These conditions were then applied to more challenging compounds, namely amino alcohols and diastereomers possessing more than one stereocenter. The approach was utilized to monitor stereoselective biocatalytic transamination and assign the absolute configuration of the enantiomeric products. Finally, hydrolysis of the imine bond of the derivative was shown to generate enantiopure amine starting materials without racemization. This further highlights the value of this approach for creating readily reversed derivatives that enhance chromatographic separation and aid in the determination of absolute configuration.



INTRODUCTION

The development and optimization of asymmetric reactions that selectively produce a single stereoisomeric product has been an intense area of research focus for several years.[1−3] The chromatographic separation of enantiomers on chiral stationary phases is a cornerstone capability that facilitates this area of modern chemical research.[4−8] Today, chromatographic separation is almost always carried out directly on unmodified compounds of interest. Indirect methods requiring chemical derivatization were widespread in the past,[9,10] and are still necessary in many cases where the compounds of interest have poorly absorbing chromophores or their enantioseparation proves challenging.[11−13] Chiral primary amines, important to the synthesis of active pharmaceutical ingredients,[14,15] make up one such class of compounds that can be difficult to separate. The ideal derivatization reagents for chromatographic enantioseparation will allow fast and convenient reaction, while enhancing detectability or separability. In addition, the convenient removal of the derivatizing group is important, should access to the original underivatized analyte be desired.

We recently reported the development of a method for the study of chiral amines by circular dichroism (CD) spectroscopy based around derivatization with 3-hydroxy pyridine-2-carboxaldehyde (HCA).[16] Imine-bond formation between the HCA and the amine to generate a CD-active species occurs rapidly and in high yield. The resultant imine has strong UV and CD signals at 317 nm, avoiding interference with most organic compounds. We also found that these imines were reasonably stable to high pH liquid chromatography conditions, affording well shaped peaks in these studies. This allowed us to couple the CD detector with an HPLC instrument to facilitate high-throughput analysis of the enantiopurity of synthetic chiral amines. Additionally, the sign of the CD signal could be related to the absolute configuration of the original amine within the suite of compounds that were tested. In this study, we investigate the potential for the use of HCA derivatization to provide an "easy on/easy off" method for improving chromatographic enantioseparation of amine analytes on chiral stationary phases while enhancing UV absorption and providing a detectable CD signal that can be useful for stereochemical analysis.

Supercritical fluid chromatography (SFC), based on the application of supercritical carbon dioxide as the main mobile phase component, was selected as a separation technique for this work. Chiral SFC has recently emerged as the preferred tool for analytical and preparative separation of chiral molecules,[17−20] because it affords fast separations, tolerates a large range of analyte polarities, and has an economical and green carbon dioxide mobile phase.

RESULTS AND DISCUSSION

The first step of this study was to identify possible general SFC conditions for enantioseparation of the imine derivatives. In

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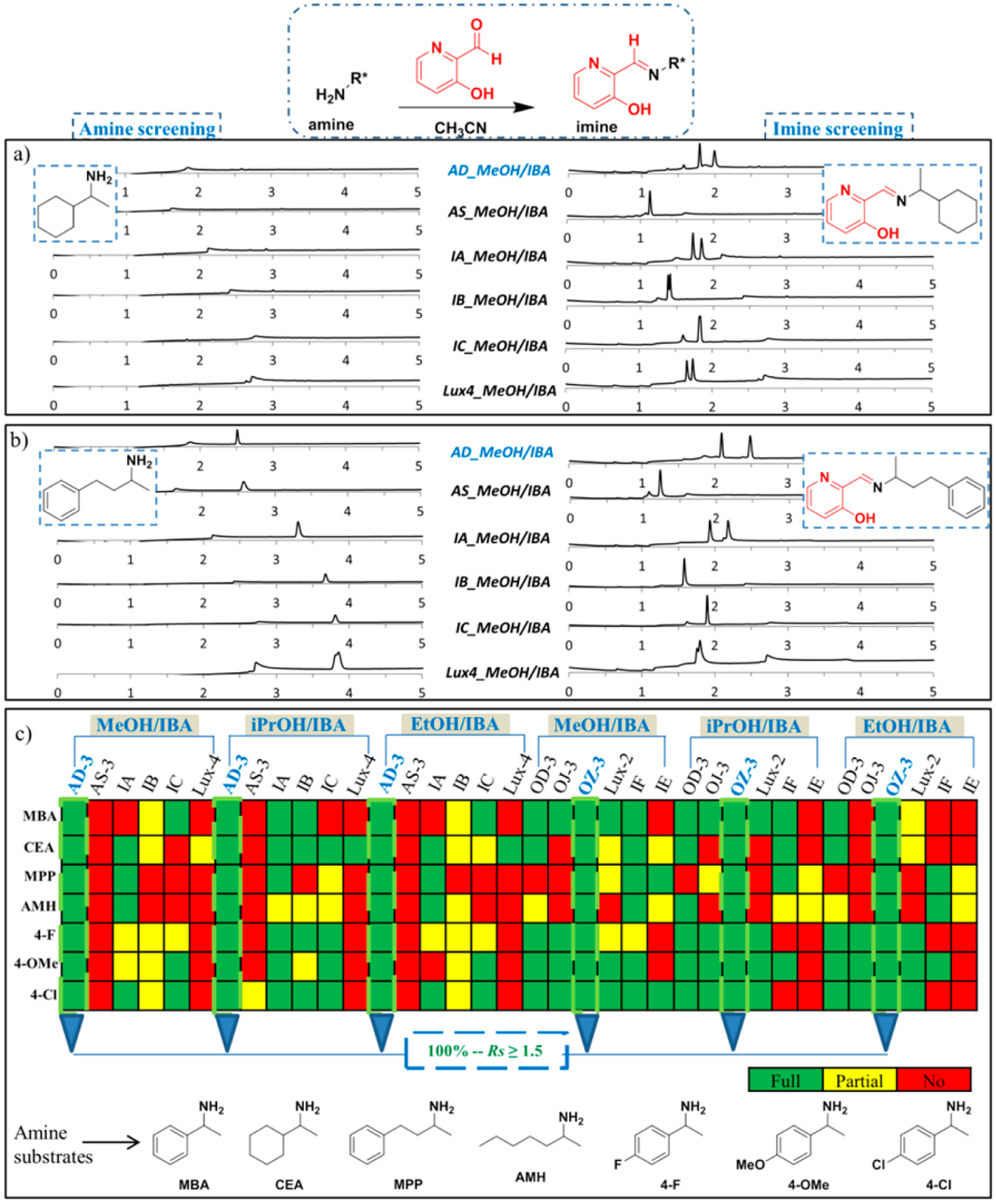

Figure 1. Chiral SFC-UV separation of amine and imine derivatives using standard screening conditions on six different CSPs, as detailed in the Experimental Section. a,b) Enantiomers of CEA, MPP, and respective imine derivative on the same columns using 25 mM $iBuNH_2$ in MeOH as polar modifier. The y scale was kept from imine to amine experiment to allow comparison. c) Map of best chiral SFC conditions for separation of imine-derived products of seven different amines. Three different mobile phases (25 mM $iBuNH_2$ in MeOH, iPrOH, and EtOH/CO_2) were screened. The scoring color system was developed based on baseline (green), partial (yellow), or no separation (red) as illustrated.

order to do this, a diverse set of imines was generated from the reaction of the corresponding chiral amines and HCA. The resulting chiral imine mixtures were subjected to standard SFC screening using systems routinely used for developing analytical methods in support of drug discovery and development.[21,22] A total of 12 chiral stationary phases (CSPs) in combination with several polar modifiers were used in this study (see Supporting Information, SI, for full screening chromatograms).

Selected examples from this evaluation are shown in Figure 1. Cyclohexylethylamine (CEA) does not have any chromophoric elements, and thus is invisible to UV detection even at 210 nm. As a result, no chiral separation methods can be identified during direct screening of this amine with UV detection (Figure 1a). However, when the imine derivative formed between CEA and HCA was screened, baseline enantioseparation was found for multiple combinations of column and modifier. In another example shown in Figure 1b, the low wavelength UV signal for 1-methyl-3-phenylpropylamine (MPP) is sufficient for detection, but the poor chromatographic performance (peak shape and incomplete resolution) on every CSP is not suitable to directly develop a chiral separation method. Similar to the CEA-imine example, multiple chromatographic screening conditions delivered baseline separation of the MPP-imine enantiomers with no need for subsequent optimization experiments.

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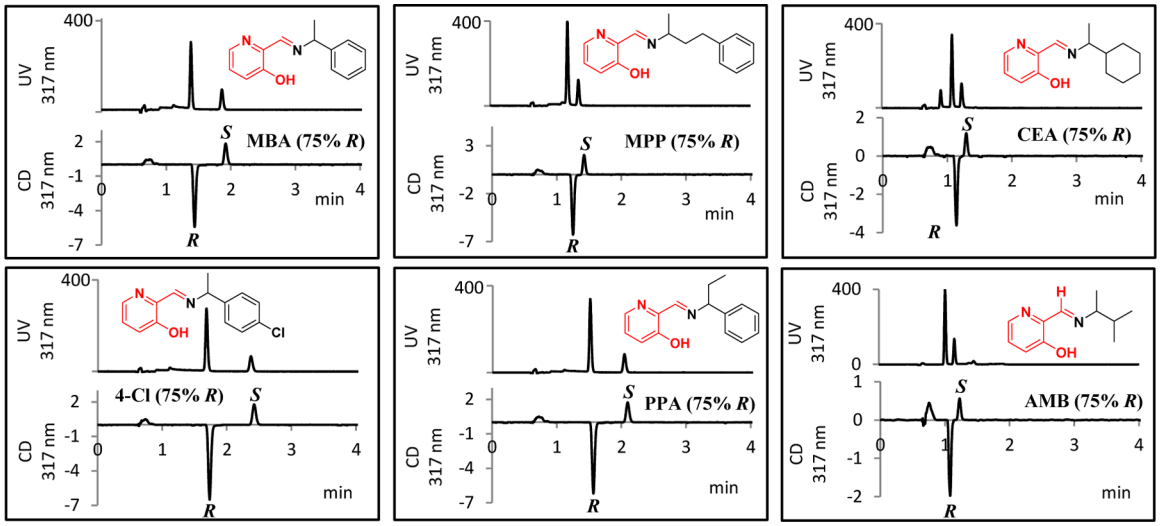

Figure 2. Chiral SFC-UV-CD analysis of six different imine-derivatives. Optimized chiral SFC method from best screening conditions in Figure 1: Samples: 5 μL injection of each mixture (75% (*R*)-enantiomer and 25% (*S*)-enantiomer) in ACN; column: Chiralpak AD-3 (4.6 × 150 mm, 3 μm); temperature: 40 °C; flow rate: 3 mL/min; isocratic mobile phase: 15% polar modifier (25 mM $iBuNH_2$ in iPrOH)/85% CO_2, except for AMB: 5%/95%. Backpressure regulator (BPR): 150 bar. Detection: UV and CD at 317 nm.

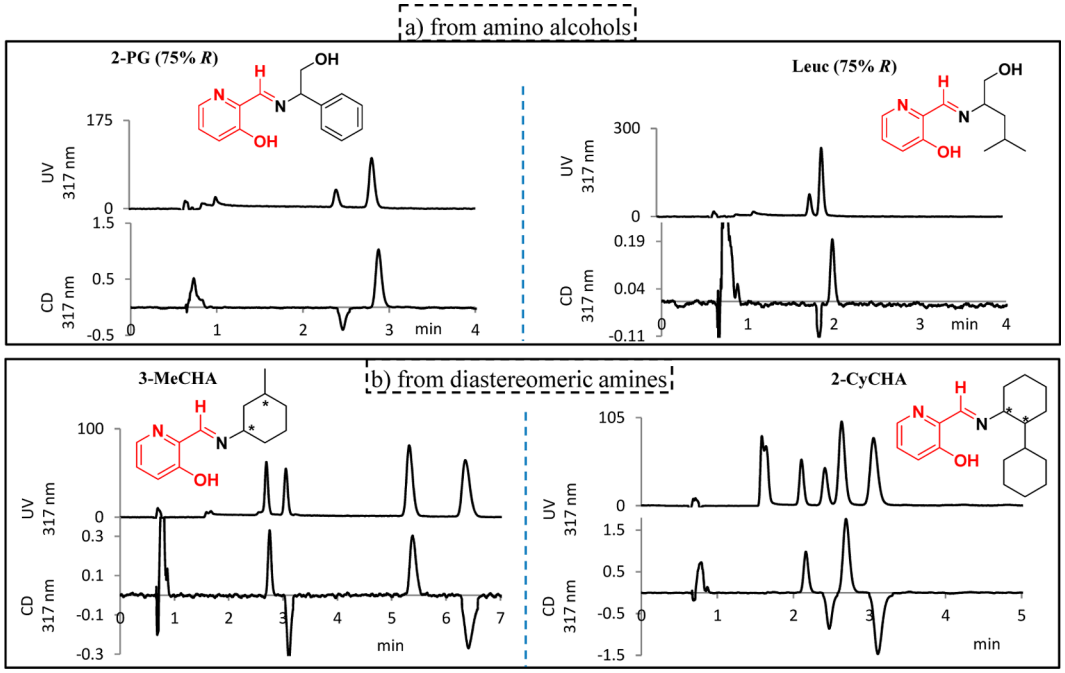

Figure 3. a) Chiral SFC-UV-CD analysis of imine-derived amine alcohols using the method described in Figure 2 at 40% modifier/60% CO_2. Samples: 5 μL injection of each mixture (75% (*R*)-enantiomers) in ACN. b) Chiral SFC-UV-CD analysis of two different diastereomeric imine-derived amines. Chromatographic conditions as described in Figure 2 at 5% modifier/95% CO_2.

To fully test the generality of this approach, imine derivatives of seven commercially available amines were prepared and subjected to the same screening protocol as described for CEA and MPP (Figure 1c). This set of compounds was composed of both aliphatic and aryl amines, and this representative set was used to help identify general trends in enantioselectivity of the CSPs for the imine species. The results were qualitatively sorted into three categories based on how well the enantiomers were separated: full baseline resolution (green), partial resolution that could likely be optimized (yellow), and no separation (red). This approach allowed us to identify two CSPs (Chiralpak AD-3 and Chiralcel OZ-3) that were general toward the chiral imine derivatives, providing baseline enantioseparation for all of the compound mixtures with any of the polar modifiers. Having identified suitable conditions for the enantioseparation of chiral imine-derived amines, we next probed the elution order of the imine enantiomers.

In order to track the absolute configuration of the eluting imine enantiomer, we made use of a commercially available SFC-CD detector (Jasco, Inc.). This is the same detector used for HPLC-CD analysis, but makes use of a high pressure flow cell designed to handle the high back pressure (~200 bar) required for SFC analysis.[23] A single isocratic method that gave the best resolution across the series was developed using a

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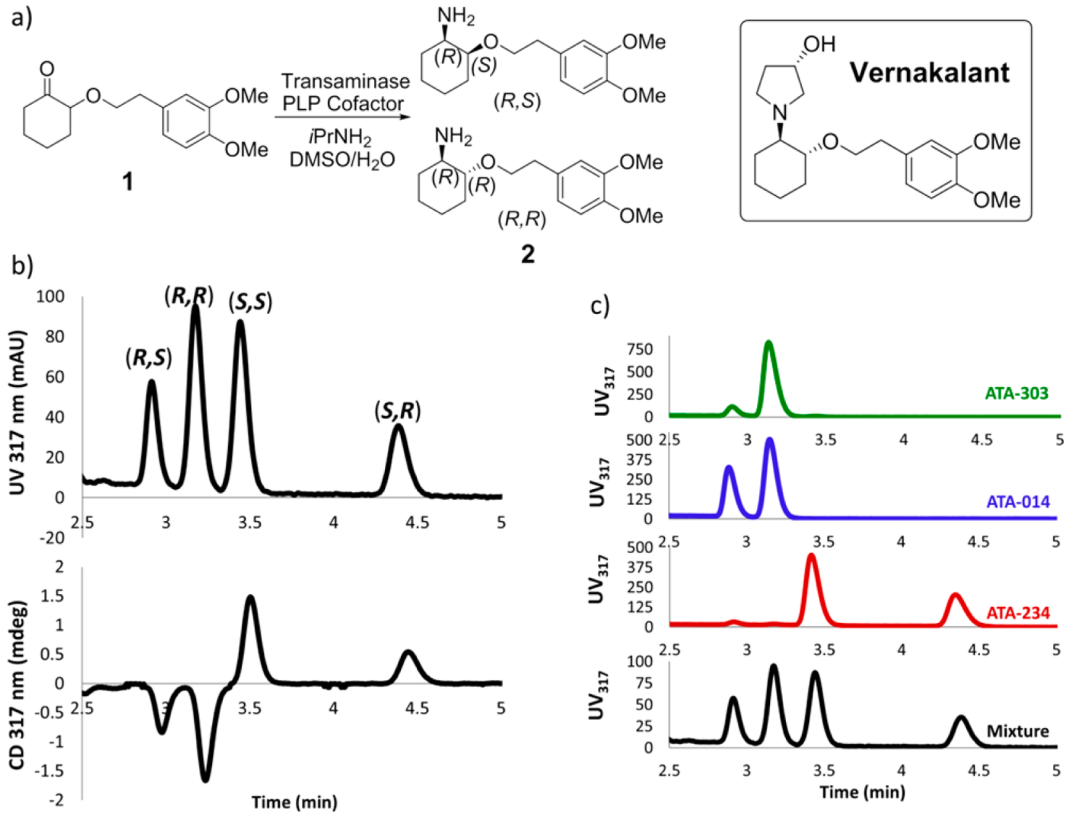

Figure 4. a) Stereoselective enzymatic transaminaton to afford Vernakalant intermediate **2**. b) Chiral SFC-UV-CD analysis for the vernakalant amine as its imine derivative. c) Results of the transamination and subsequent imine-formation, showing formation for an (*S*)-selective (ATA-234), or (*R*)-selective (ATA-014) enzymes, as well as a variant evolved to give selectively (*R*,*R*) for this substrate (ATA-303). Chromatographic conditions; column: Chiralpak AD-3 (4.6 × 150 mm, 3 μm); temperature: 40 °C; flow rate: 3 mL/min; isocratic mobile phase: 15% polar modifier (25 mM $iBuNH_2$ in 50% MeOH, 50% iPrOH)/85% CO_2. Backpressure regulator (BPR): 150 bar. Detection: UV and CD at 317 nm.

Chiralpak AD-3 column and iPrOH with $iBuNH_2$ (25 mM). Although $iBuNH_2$ itself is a primary amine, virtually no reversibility of the desired imines were observed on the experimental time scale. Each sample was made up of 75% (*R*)-amine to ensure that the absolute configuration could accurately be assigned to the eluting peak. Two additional amines that had not been screened were included in this set, namely 1-phenylpropylamine (PPA) and 2-amino-3-methylbutane (AMB), to demonstrate the applicability of these chromatography conditions beyond the set of compounds that was originally screened. The UV and the CD signals at 317 nm were recorded for these separations. As illustrated in Figure 2, the first eluting peak in each case is the larger 75% area peak, representing the (*R*)-enantiomer. This first peak has a negative CD signal at 317 nm, which is consistent with what we reported previously for imines derived from a series of related (*R*)-amines.[16] The enantiomeric elution order observed here is expected to apply only among closely related compounds under study, and is not likely to be general across all amines. These conditions delivered baseline resolution of both of the imines that had not been screened previously, further demonstrating the generality of this approach.

In order to expand the utility of this method, we broadened the scope of our analysis to incorporate amino alcohols (Figure 3a). The resultant imino alcohols formed from the amino alcohols leucinol (Leuc) and 2-phenylglycinol (2-PG) were more polar than those formed with the alkyl and aliphatic amines, and required an isocratic method with a higher amount of iPrOH to elute the compounds from the column. These imino alcohols also appeared to be more prone to hydrolysis back to the amino alcohol and HCA, and required a lower column temperature (20 °C) to prevent on-column degradation. After making the required adjustments, baseline resolution was observed for these two analytes. In both of these cases, the 25% area peak corresponding to the imino alcohol formed from the (*S*)-enantiomer of the amino alcohol is the first to elute, and has a negative CD signal at 317 nm. These seemingly contradictory results are consistent with what has been observed previously for this type of substrate.[15,24] In this case, a change in Cahn-Ingold-Prelog priority leads to a change in absolute configuration, while the largest and smallest groups are consistent with the previous model. Hence, the imines formed from the (*S*)-amino alcohols give rise to a negative CD signal at 317 nm, and elutes first under these chromatographic conditions.

To further demonstrate the utility of this method, we turned our attention to amines with two stereocenters (Figure 3b). Developing a method for separating four stereoisomers is more challenging than that for a pair of enantiomers. In this instance, additional screening and optimization using the same column but with a different mobile phase afforded superior resolution of the stereoisomeric products. It is clear from the UV and CD signals above that the two enantiomers of a single diastereomer elute consecutively, followed by both enantiomers of the other diastereomer. For example, the first two imine peaks derived from 3-methylcyclohexylamine (MCHA) in Figure 3b, between 2.5 and 3.5 min, have the same UV signal and CD signals with equal and opposite magnitude. Similarly, the last set of peaks

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have the same UV signals, with equal and opposite CD signals at 317 nm. The first eluting peak for each diastereomer, the first and third overall eluting stereoisomeric peaks, have a positive CD signal at 317 nm. The positive CD signal indicates that these peaks likely have the (*S*)-configuration at the carbon bearing the nitrogen. This switch in elution order may be due to the change in solvent from iPrOH to MeOH. These compounds were only commercially available as racemic mixtures of stereoisomers, and the relative configuration of each diastereomer could not be assigned using this methodology. Caution must be taken in using this method for determining the diastereomeric ratio (dr), since the diastereomeric imines likely form to different extents and are prone to potential hydrolysis at different rates while passing through the chromatographic stationary phase.

To demonstrate the practical application of this system, we turned our attention to the synthesis of Vernakalant. Vernakalant was discovered by Cardiome Pharma Corp, and was developed to treat recent-onset atrial fibrillation through selective inhibition of Na- and K-channels.[25] Merck recently reported an efficient asymmetric synthesis of this compound, proceeding through chiral amine intermediate **2** shown in Figure 4a.[26,27] The stereochemistry for the two contiguous stereocenters on the cyclohexane ring was set in a highly stereoselective fashion through an enzyme-catalyzed dynamic asymmetric transamination from the corresponding α-chiral ketone substrate **1**. Three rounds of evolution gave an enzyme that was selective in delivering the desired stereoisomer of amine **2** with very high enantio- and diastereoselectivity. The imine derivatization and subsequent SFC-CD analysis was performed on amine intermediate **2**. A mixture of all four stereoisomers was obtained by reductive amination in the absence of any enantioenriched influence. The crude reaction material could be used directly after filtration through diatomaceous earth without further purification, since the absorbance of the HCA-imine is at a sufficiently long wavelength to avoid interference from any reaction components.

Baseline resolution of the four HCA-imines was not achieved using either pure MeOH or iPrOH as a polar modifier, but rather a 1:1 mixture of the two solvents was used to give the best resolution (Figure 4). The first two eluting peaks showed negative CD signals at 317 nm, indicating that they likely have the (*R*)-configuration at the carbon bearing the nitrogen. This is different from what we observed previously, where the two enantiomers of each diastereomer eluted together as a pair. This could be due to the mixture of the solvents, or possibly because of the size and additional interactions available to the aromatic side chain attached to the other stereocenter on the cyclohexane ring. The second eluting peak was confirmed to be the desired (*R*,*R*)-stereoisomer by injection of the authentic sample, confirming the tentative assignment made by the sign of the CD signal.[28] On the basis of this information, the assignment of each stereoisomer is made according to the label in Figure 4a. After optimizing the separation method for the four stereoisomers, we focused on analyzing the formation of the product by enzyme-mediated transamination. Three enzymes were selected for the synthesis: ATA-234, ATA-014, and ATA-303. This panel of enzymes was selected to contain diversity in selectivity, with one general S-selective (ATA-234) component, one general R-selective (ATA-014) enzyme, and one that had been evolved for ketone substrate **1** (ATA-303). The reactions were allowed to stir for 1 day, and the products were subsequently analyzed after extraction into ethyl acetate and subsequent imine formation. The results of these reactions are shown in Figure 4. The product generated by ATA-234 was composed of only two stereoisomers, each with approximately equal area by UV. These stereoisomers gave positive CD signals at 317 nm, which supports formation of the (*S*)-configuration at the carbon attached to nitrogen. In contrast, the other two stereoisomers were formed in approximately the same concentration with ATA-014. These stereoisomers had negative signals at 317 nm, indicating that the stereoisomeric products formed both have the (*R*)-configuration at the carbon bearing nitrogen. Finally, ATA-303 gave rise to the same two stereoisomers as ATA-014. As anticipated, this enzyme afforded selective formation of the desired Vernakalant intermediate stereoisomer.

This approach allows relatively straightforward differentiation of enantiomeric imines formed from corresponding chiral amines. The imine formation occurs rapidly, and is stable to high pH chromatographic conditions. A large CD signal is generated in a portion of the spectrum that previously had no discernible signals, and the sign of the output signal allows the assignment of absolute configuration to be made with some degree of confidence. The ability to remove the HCA derivatizing reagent to afford the pure enantiomer, desirable in preparative enantioseparations or enantiopurity upgrades, was studied further. In order to test that HCA met this criterion as a derivatizing reagent, we studied the hydrolysis of the imine formed between HCA and MBA. Imines are known to be susceptible to acid-catalyzed hydrolysis, and hence we began our study focusing on hydrochloric acid (1 N HCl). Addition of five equivalents of HCl relative to the imine gave complete hydrolysis of the imine bond and regeneration of the starting amine and HCA (Figure 5). There was concern that the

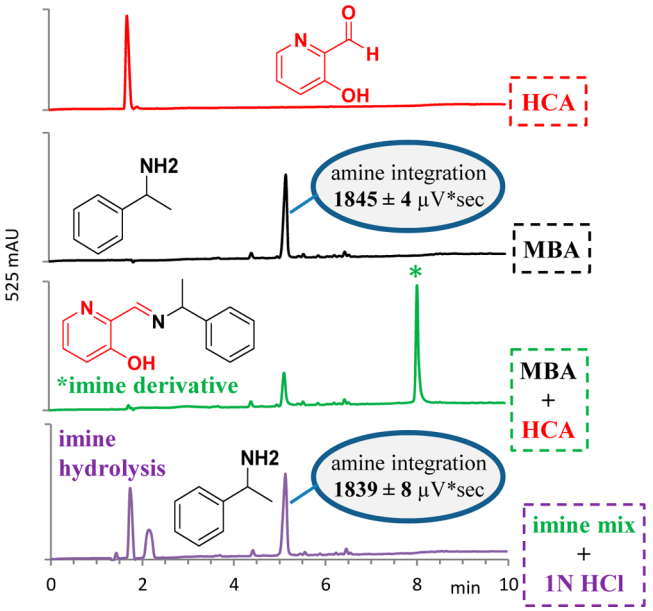

Figure 5. Monitoring amine recovery by comparison of achiral reversed phase HPLC profiles before and after imine hydrolysis. Two mM 3-hydroxypyridine-2-carboxaldehyde (HCA) + 2 mM methylbenzylamine (MBA). Ten μL 1 N HCl (5 equiv) was added to the respective imine derivative mixture and then analyzed. All experiments were performed in triplicate (n = 3). Detailed chromatographic conditions are described in the Experimental Section.

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addition of acid could lead to undesired racemization of the stereocenter during hydroloysis. A chiral SFC method was developed for the MBA analyte alone (Figure 1c), and this method showed that no observable racemization occurred for either enantiomer during the process of hydrolysis (see the Supporting Information for additional details). Hence, this group indeed performs as an "easy on easy off" protecting group while maintaining stereochemical integrity during its incorporation and removal.

CONCLUSIONS

We have demonstrated that 3-hydroxypyridine-2-carboxaldehyde (HCA) can function as an efficient derivatizing agent for subsequent chiral SFC analysis. Optimal conditions were found to give good resolution of a large suite of imine-derived chiral amines, with the (*R*)-enantiomer generally eluting first for this suite of compounds under the optimized conditions. These conditions were then expanded to encompass a wider substrate scope, including amino alcohols and compounds with multiple stereocenters. Separation across all of the substrates was achieved using the same chromatographic stationary phase, namely Chiralpak AD-3. To demonstrate the practical application of this sensing ensemble, a biocatalytic transamination forming a chiral amine intermediate in the synthesis of Vernakalant was monitored. Conditions were identified that formed predominantly the (*R*)- or (*S*)-enantiomer, with respect to the stereocenter bound to nitrogen. Finally, the imine bond was hydrolyzed to generate enantiopure amine starting material and HCA with no corresponding racemization seen.

EXPERIMENTAL SECTION

Chiral SFC Screening Conditions. Chiral SFC screening experiments were carried out on 4.6 × 150 mm, 3 μm columns by gradient elution at a flow rate of 3 mL/min, as described previously in the literature.[21] The SFC eluents were solvent A (CO_2) and solvent B (25 mM $iBuNH_2$ in MeOH/iPrOH/EtOH or ACN). The mobile phases were programmed as follows: linear gradient from 1% to 40% B in 5 min, hold at 40% B for 1 min. The column and samples were maintained at a temperature of 40 and 20 °C, respectively.

Derivatization of Chiral Amine Analytes. Derivatization of chiral amines was carried out using the protocol described previously.[16] Briefly, 10 mM acetonitrile stock solutions were made of HCA and the desired amine analyte. Next, equimolar amounts of the amine analytes and HCA (200 uL) were mixed to afford the respective imine products, followed by dilution with 600 uL acetonitrile This analysis was done in situ, with no need to purify the product or remove water from the mixture. The formation of chiral imine-derived amines leads to a strong CD signal at 317 nm which has been used in this study for the analysis.

Vernakalant Amine Synthesis. Synthesis of Vernakalant ketone intermediate **1**, as well as subsequent transamination reactions, were carried out as described in the literature.[26] To form the mixture of stereoisomers, ketone intermediate **1** (0.2 mmol) was stirred in methanol (5 mL). Ammonium hydroxide (10 mmol) and sodium cyanoborohydride (10 mmol) were then added, and the mixture was allowed to stir overnight at room temperature. The mixture was filtered through diatomaceous earth, concentrated in vacuo, and used without further purification.

Imine Hydrolysis. The imine formation was carried out as described previously, through equimolar addition of the desired amine and HCA. To this solution was added 1 N HCl (10 uL), and the product was briefly mixed before HPLC analysis to determine extent of imine hydrolysis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b01162.

SFC chromatograms of chiral screening; monitoring racemization in imine hydrolysis (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: leo.joyce@merck.com (L.A.J.)

Notes

The authors declare no competing financial interest.

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