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Use of Achiral/Chiral SFC/MS for the Profiling of Isomeric Cinnamonitrile/Hydrocinnamonitrile Products in Chiral Drug Synthesis

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A directly coupled achiral/chiral SFC/MS method has been developed for the profiling of a three-step stereoselective synthesis of cinnamonitrile and hydrocinnamonitrile intermediates. Semipurified reaction mixtures were screened in *one* step to determine the diastereomeric/enantiomeric composition of the final product as well as to identify any remaining *E/Z* isomers present from the starting material. The coupled achiral/chiral column combination was found to significantly enhance the separation of both enantiomers and diastereomers, without adding significantly to the overall analysis time. This analytical technique should prove to be generally useful for the profiling of isomeric reaction products in chiral drug synthesis.

Due, in part, to more stringent FDA guidelines for the marketing of chiral drugs,[1] stereoselective drug syntheses are rapidly becoming the norm in the development of new drug candidates. However, in the scale-up of synthetic methods, many other products (such as structural isomers, or diastereoisomers) are often formed, either in addition to or instead of the expected enantiomer of interest. This, in-turn, requires the development of analytical methods that can both separate and characterize potentially unknown isomeric side products in crude or semipurified reaction mixtures.

Both reversed-phase chiral liquid chromatography−mass spectrometry (LC/MS)[2] and chiral supercritical fluid chromatography−mass spectrometry (SFC/MS)[3] have recently been shown to be powerful techniques for the characterization of chiral mixtures. Due to the low efficiency and poor achiral selectivity of most chiral columns, MS detection is essential to distinguish the enantiomers of interest from other achiral impurities,[4] but importantly, molecular ion data alone cannot be used to distinguish between the presence of enantiomers, diastereomers, or structural isomers of the same compound. Thus, the focus of many of the studies in this area has been on the chiral separation of relatively simple mixtures,[5,6] or more specifically, in the case of SFC/MS, on the rapid chiral screening of lead compounds in the drug discovery environment.[7] Indirectly coupled achiral and chiral columns are commonly employed for the chiral analysis of drugs in complex biological matrixes.[5] This form of multidimensional chromatography is typically implemented on-line via the use of integrated switching valves and uses the first column (achiral) to remove interferences from endogenous compounds and to deliver primarily the drug of interest to the chiral column.[8] This is a very powerful technique as the sample cleanup and analysis steps are isolated, thus allowing a wide range of columns/mobile phases to be explored to achieve the desired enantiomeric separation, but it does require compatible mobile phases for the two separations.[8] Although multidimensional SFC has been described,[9] it does not appear to have been widely adopted for complex mixture analysis. A simpler technique, which potentially allows for the simultaneous achiral/chiral separation of a range of components in a complex mixture is the direct *serial* coupling of achiral and chiral columns.[10] In LC, this approach is limited by the increased total system backpressure and by the fact that the mobile phase requirements for the achiral and chiral separations are generally different.[11] In contrast, direct serial coupling in SFC is easily accomplished because the mobile phase fluid (CO_2 plus modifier) has a significantly lower viscosity than a liquid and is an effective eluent for both chiral and achiral stationary phases.[12] Surprisingly, despite these advantages, no recent applications using directly coupled achiral/chiral stationary phases in SFC have been reported (as far as the authors are aware). In this work, the utility of this technique combined with MS detection is initially demonstrated for the screening of a three-step synthesis of

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cinnamonitrile and hydrocinnamonitrile intermediates, which generates both enantiomeric and diastereomeric products.

EXPERIMENTAL SECTION

Reagents and Chemicals. Carbon dioxide (SFC grade) was obtained from AirGas (Cheshire, CT). Methanol (HPLC grade) was purchased from EMD Chemicals, Inc. (Gibbstown, NJ). Trifluoroacetic acid (TFA), isopropylamine, 1,2,4-triazole, 2-chloro-2′,4′-difluoroacetophenone, and diethyl(1-cyanoethyl)phosphonate were purchased from Sigma-Aldrich (St. Louis, MO).

Equipment and Instrumentation. Separations were performed using a Berger Analytical SFC system supplied by Mettler-Toledo AutoChem, Inc. (Columbia, MD), equipped with an SFC pump (pump A), a modifier pump (pump B), a column oven, and a dual-wavelength UV detector. SFC grade liquid CO_2 and HPLC grade methanol were used as mobile phases A and B, respectively. Mass spectrometric detection was performed using a Sciex (Concord, Ontario, Canada) model API 4000 triple quadrupole mass spectrometer utilizing a heated nebulizer (APCI) probe. The chiral columns, a Diacel Chiralcel OD-H and a Diacel Chiralpak AD-H (both 2.0 × 150 mm, 5 μm), were purchased from Chiral Technologies, Inc. (West Chester, PA). The achiral column, a Phenomenex Luna Silica 2 (4.6 × 150 mm, 5 μm), was purchased from Phenomenex (Torrance, CA). The SFC was connected directly to the mass spectrometer via a stainless steel T-piece with a bore of 150 μm (VICI Valco Instruments, Houston, TX), which was inserted between the UV detector and the back-pressure regulator (BPR). The existing UV-to-BPR connection (1/16 o.d. × 0.020-in. i.d. tubing) was removed, and the T-piece was connected using two appropriate lengths of 1/16 i.d. × 0.005-in. i.d. stainless steel tubing and fittings (VICI Valco Instruments). The final connection to the APCI probe was made with a 5-ft length of 65-μm i.d. × 1/16 o.d. PEEK tubing (Upchurch Scientific, WA) using standard finger-tight PEEK fittings. The final split ratio was not determined.

Experimental Conditions. The SFC system was operated isocratically under the following conditions: oven temperature 30 °C, UV detection wavelength 220 nm, and set outlet pressure 120 bar. Flow rates of 0.8 mL/min were used for chiral-only chromatography and 1.0 mL/min for coupled achiral/chiral separations. Methanol, 5−15 vol %, was used as the modifier for all separations. The injection volume was 5 μL, and the total run time was 10 min. The mass spectrometer was operated in positive ion mode under the following conditions: probe temperature 400 °C, nebulizing gas pressure 50 psi, curtain gas flow rate 0.9 L/min, discharge current 2 μA, and declustering potential (DP) 70 V. Due to the low flow rates required for narrow-bore columns, no nebulizer makeup gas flow or additional heating of the transfer line was necessary. Data acquisition, 150−500 Da. scan range in 0.5 min, was initiated by a contact closure out signal from the SFC controller.

Synthetic Chemistry. A three-step synthesis was used to transform 1,2,4-triazole into a final mixture of hydrocinnamonitrile diastereomers and enantiomers, as outlined in Figures 1 and 2. In reaction 1, 1,2,4 -triazole (compound **1**) was N-alkylated with 2-chloro-2′,4′-difluoroacetophenone (compound **2**) under basic conditions to yield a mixture of triazoloketone positional isomers (compounds **3** and **4**). Isomer **3**, which was produced in ~85% yield, was isolated using radial chromatography over silica

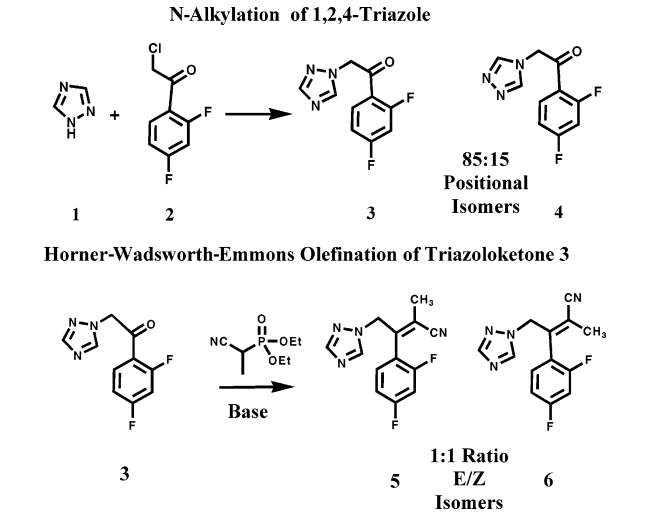

Figure 1. Synthetic chemistry steps 1 and 2. N-Alkylation of 1,2,4-triazole. Horner−Wadsworth−Emmons olefination of triazoloketone **3**.

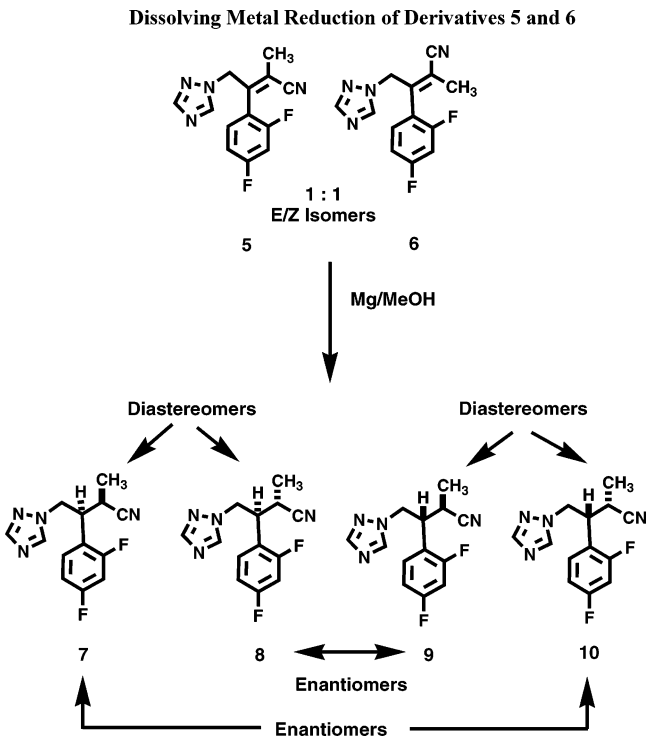

Figure 2. Synthetic chemistry step 3. Dissolving metal reduction of derivatives **5** and **6**.

gel. In reaction 2, ketone **3** was reacted with diethyl(1-cyanoethyl)phosphonate under basic conditions (Horner−Wadsworth−Emmons olefination) to generate a roughly 1:1 mixture of the *E*/*Z* isomers of cinnamonitrile derivatives **5** and **6**. In reaction 3, derivatives **5** and **6** were subjected to a dissolving metal reduction using a mixture of magnesium in methanol to yield the expected final products (diastereomers/enantiomers **7**−**10**). The solution containing the final products was semipurified by filtration to remove the excess magnesium, followed by aqueous workup to remove inorganic magnesium salts. Aliquots were typically diluted 250:1 with methanol prior to chromatographic analysis.

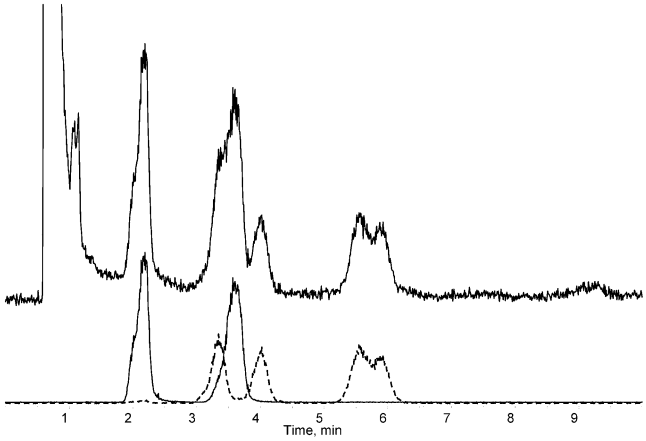

Figure 3. Chiral SFC/MS separation of semipurified final product. Upper trace: total ion chromatogram (TIC). Lower trace (dashed): product $[M + H]^+$ EIC (*m*/*z* 263) diastermers/enantiomers **7**−**10**. Lower trace (solid): starting material $[M + H]^+$ EIC (*m*/*z* 261) *E*/*Z* isomers **5**, **6**.

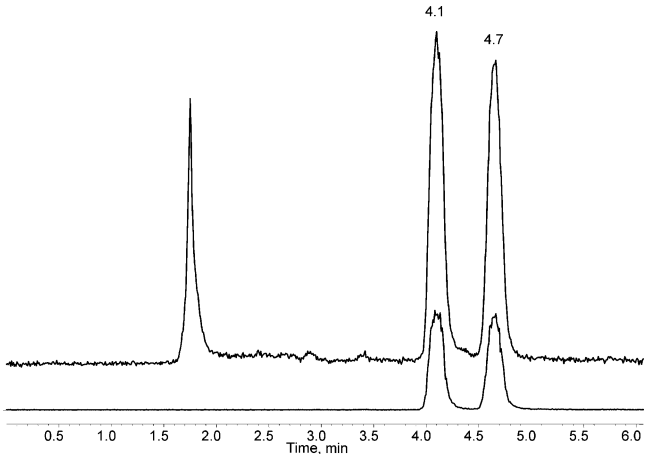

Figure 4. Achiral SFC/MS separation of semipurified product. Upper trace: TIC. Lower trace: product $[M + H]^+$ EIC (*m*/*z* 263).

RESULTS AND DISCUSSION

On the basis of previous experience, two small-bore (2.0 × 150 mm, 5 μm) polysaccharide chiral columns, Chiralcel OD-H and Chiralpak AD-H, were evaluated for this study (data not shown). The most effective SFC separation of the semipurified final product mixture (from reaction 3 after ~2 h) is shown in Figure 3. This was obtained using the Chiralcel OD-H column at a flow rate of 0.8 mL/min and with 5% methanol as additive. The addition of small amounts (≤0.1% v/v) of either acid (TFA), or base (isopropylamine), were not found to improve the peak symmetry. Note that, although the resolution is poor, the extracted ion chromatograms (EIC) for starting material (*E*/*Z* isomers **5** and **6**, $[M + H]^+$ *m*/*z* 261) and product (species **7**−**10**, $[M + H]^+$ *m*/*z* 263) can be used to distinguish the partially resolved components. Interestingly, the four expected product peaks are present, indicating that this column does exhibit some selectivity for both the separation of stereoisomers, as well as enantiomers. The isocratic achiral SFC separation of the same final product mixture, obtained using an analytical scale silica column (4.6 × 150 mm), is shown in Figure 4. This was obtained at a flow rate of 1 mL/min and with 15% methanol, conditions that were not too different from those used for the chiral separation. The

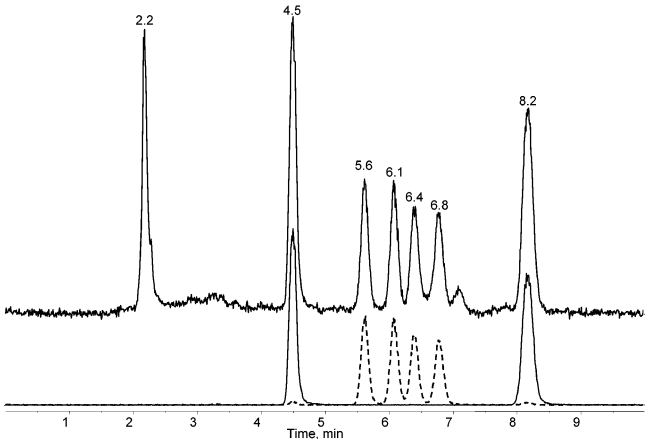

Figure 5. Coupled achiral and chiral SFC/MS separation of semipurified final product. Upper trace: TIC. Lower trace (dashed): product $[M + H]^+$ EIC (*m*/*z* 263) diastereomers/enantiomers **7**−**10**. Lower trace (solid): starting material $[M + H]^+$ EIC (*m*/*z* 261) *E*/*Z* isomers **5**, **6**.

chromatogram clearly shows two well-resolved components of equal abundance with the expected mass of the product ($[M + H]^+$ *m*/*z* 263), which is consistent with the presence of the two expected diastereomers **7**(**10**) and **8**(**9**). The achiral/chiral SFC separation of the same mixture, achieved simply by the serial coupling of the two columns, is shown in Figure 5. This was obtained under the conditions optimized for the achiral separation, that is, at a flow rate of 1 mL/min and with 15% methanol. As can be seen, a dramatic improvement in resolution has been achieved, and all components are now baseline-separated. Note also that this has been achieved without a significant increase in analysis time (9 min versus the original 7 min). In addition, the relative intensities of the four products (two enantiomeric and two diastereomeric species) are now close to being equal, as would be expected from the mechanism of the dissolving metal reduction reaction. With regard to peak identity, the combined achiral/chiral separation would be expected to yield one set of enantiomers, followed by the other, that is, either **7**(**10**) followed by **8**(**9**), or **8**(**9**) followed by **7**(**10**). However, because the four peaks are all of nearly equal intensity, this is difficult to confirm without the use of a chiral detector. To investigate this, an artificial final product mixture was generated by isolating the two diastereomers (using achiral normal phase silica gel chromatography) and recombining them in a ratio 1:4 instead of the natural ratio of 1:1. This artificial mixture was then subjected to the same achiral/chiral separation as shown in Figure 5 (data not shown). In this case, the four previously observed chromatographic peaks were replaced by two consecutive sets of two peaks (doublets). Each peak within a given doublet was of equal intensity, and the intensity ratio between the first and second sets of doublets was ~1:4. This confirmed the expected elution order of consecutive sets of enantiomeric pairs.

CONCLUSION

Direct serial coupling of achiral and chiral columns in SFC has previously been shown to improve the limited achiral selectivity of chiral stationary phases for the separation and measurement of enantiomers in drug mixtures.[10] It was also expected to be useful for the analysis of compounds having multiple chiral

centers, when both enantiomers and diastereomers may be present in a complex sample. We have shown this to be the case if directly coupled achiral/chiral SFC is further combined with the specificity of mass spectrometric detection. Specifically, in this study, we have demonstrated that semipurified reaction mixtures of cinnamonitrile and hydrocinnamonitrile intermediates can be profiled in *one* step to determine the diastereomeric/enantiomeric composition of the final product, as well as to identify any remaining *E*/*Z* isomers present from the starting material. The coupled achiral/chiral column combination was found to significantly enhance the separation of both enantiomers and diastereomers without adding significantly to the overall analysis time.

Note Added after ASAP Publication. Due to a production error, this paper was posted on the Web on May 5, 2006, before a correction to the first sentence of the Equipment and Instrumentation paragraph had been made. The correction was made, and the paper was reposted on May 5, 2006.

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