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An Alternative Procedure To Screen Mixture Combinatorial Libraries for Selectors for Chiral Chromatography

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An alternative process for the analysis of mixture library components for their potential as selectors for chiral chromatography is described. The procedure involves the immobilization of each enantiomer of the target racemic analyte to silica gel, followed by incubation of each resulting stationary phase with a mixture library. The adsorbed library components on the two stationary phases are then analyzed by reversed-phase liquid chromatography. A comparison of the resulting two chromatograms is made. Any peak of identical retention time but with a significant difference in intensity in the two chromatograms indicates that this component is most likely a chiral selector. Its chemical structure is then determined by LC−MS or LC−MS−MS. This new screening method significantly increases the efficiency of chiral selector determination by eliminating the need for multilibrary syntheses, as opposed to our previous method. This technique should also allow for the screening of much larger libraries as compared to our previous work.

Combinatorial libraries have become very powerful methods for the development of selective binders for a given target molecule.[2] In a short period of time, they have found widespread applications in both pharmaceutical research and material sciences.[3] Potential applications of combinatorial libraries in chiral separation have also been demonstrated.[4] In fact, some of the early works in chromatography, such as the rapid solution screening of chiral selectors by NMR or by CE[5] and the reciprocal development of chiral selectors,[6] are combinatorial in nature.

Among the more recent examples of the application of combinatorial libraries in chiral separation, some use mixture libraries,[7] while others use libraries of pure components (parallel libraries).[8] Although the screening process can be more straightforward in a parallel library method, a larger number of compounds can be synthesized and analyzed more readily in a mixture library approach. Of the reported mixture library examples, one is based on a one bead−one selector visual assay.[7b] In this method, the enantiomers labeled with tags of different color are allowed to interact with potential chiral selectors on a solid bead. The resulting chiral selectors are identified on the basis of the color of the bead. Another method involves the selective precipitation of racemates from solution by using mixtures of potentially selective precipitants.[7c] The third method is the library-on-beads approach.[7a] In this method, all the library members are immobilized onto the same solid support, and the ability of the resulting mixed stationary phase to resolve the target racemic analyte is tested. Two other examples exist in CE that use the direct addition of mixture libraries to the buffer solution of the CE system.[7d,e]

We have also published a procedure to screen mixture combinatorial libraries for chiral selectors.[9] In that method, one enantiomer of the racemic analyte is immobilized onto silica gel, and the resulting stationary phase is packed into a typical HPLC column. Two libraries are then prepared: one library consists of

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(1) Unless otherwise noted, all the amino acids adopt the L configuration. NMM, *N*-methylmorpholine; PyBop, benzotriazolyloxytris(pyrrolidino)phosphonium hexafluorophosphate; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; Abu, 4-aminobutyric acid; Fmoc, 9-fluorenylmethoxycarbonyl; TFA, trifluoroacetic acid; DIPEA, *N*,*N*-diisopropylethylamine; DCM, dichloromethane; IPA, 2-propanol; ACN, acetonitrile.

(2) For some examples, see: (a) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *354*, 82−84. (b) Houghten, R. A.; Pinilla, C.; Blondelle, S. E.; Appel, J. R.; Dooley, C. T.; Cuervo, J. H. *Nature* **1991**, *354*, 84−86. (c) Janda, K. D. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 10779−10785. (d) Bunin, B. A. *The Combinatorial Index*; Academic Press: New York, 1998; pp 5−8. See also: Thompson, L. A.; Ellman, J. A. *Chem. Rev.* **1996**, *96*, 555−600.

(3) For a general review, see: Borman, S. *Chem. Eng. News* **1998**, *76* (14), 47−67.

(4) Examples in chiral separation are cited in refs 7 and 8. For examples in protein purification, see: (a) Huang, P. Y.; Carbonell, R. G. *Biotechnol. Bioeng.* **1999**, *63*, 633−641. (b) Palombo, G.; De Falco, S.; Tortora, M.; Cassani, G.; Fassina, G. *J. Mol. Recognit.* **1998**, *11*, 243−246.

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(8) (a) Welch, C. J.; Protopopova, M. N.; Bhat, G. *Enantiomer* **1998**, *3*, 471−476. (b) Tobler, E.; Lammerhofer, M.; Oberleitner, W. R.; Maier, N. M.; Lindner, W. *Chromatographia* **2000**, *51*, 65−70. (c) Lewandowski, K.; Murer, P.; Svec, F.; Frechet, J. M. J. *Chem. Commun.* **1998**, 2237. (d) Wang, Y.; Li, T. *Anal. Chem.* **1999**, *71*, 4178−4182.

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a mixture of enantiomerically pure components, and the other contains a mixture of the opposite enantiomers of the first library. By using the column mentioned above, chromatograms of these two libraries are obtained. Since the two libraries are enantiomeric to each other, any difference in the appearance of the two chromatograms most likely indicates the presence of at least one chiral selector in the library. The components responsible for the differing chromatograms (thus, the potential chiral selector) are then identified by sublibrary synthesis. Compared with these other published methods, our mixture library screening method appears to be applicable to larger, more general classes of compounds.

Although the feasibility of this library screening method is demonstrated, the technique is not without its drawbacks. First, the method requires the synthesis of two enantiomerically pure libraries, which can be rather laborious. Moreover, identification of the active component by sublibrary synthesis is cumbersome. Furthermore, due to the relatively poor column efficiency of a typical chiral column, the size of the library amenable to such a screening method may be limited. Therefore, we investigated an alternative library screening method and the results are reported in this article.

Our new procedure involves the use of two stationary phases to eliminate the need for two enantiomeric libraries and the application of LC−MS or LC−MS−MS to simplify active component identification. Specifically, two stationary phases, one immobilized with one enantiomer of the racemic analyte of interest and the other immobilized with its opposite enantiomer, need to be prepared. One library containing a mixture of enantiomerically pure potential chiral selectors must also be prepared. Equal amounts of the mixture library dissolved in a suitable solvent are then added and allowed to equilibrate with an equal amount of these two stationary phases separately but under identical conditions. After equilibration, the library members adsorbed onto the stationary phases are analyzed with reversed-phase chromatography. Subsequently, a comparison of the resulting two chromatograms is made. Any peak of identical retention time but with a significant difference in intensity would indicate that this component is most likely a chiral selector. Its chemical structure could then be determined by LC−MS or LC−MS−MS.

EXPERIMENTAL SECTION[1]

Chemicals and Instrumentation. All protected amino acids, coupling reagents, aromatic carboxylic acids, and solid-phase synthesis resin involved in the synthesis of the library were obtained from Aldrich (Milwaukee, WI) or Novabiochem (San Diego, CA) with the exception of HATU, which was from Perseptive Biosystems (Warrington, England). Selecto silica gel (32−63 μm) from Fisher Scientific (Pittsburgh, PA) was used for purification of the library. All chemicals needed for making the *R* and *S* enantiomers of the *N*-(1-naphthyl)leucine ester **1** were from Aldrich or Fluka (Ronkonkoma, NY). The *R* and *S* esters were attached to HPLC grade Allsphere silica gel (particle size 5 μm, pore size 80 Å, and surface area 220 m^2/g) obtained from Alltech (Deerfield, IL). All organic solvents were HPLC grade and were obtained from Fisher or E. Merck (Darmstadt, Germany). Water for chromatographic purposes was first purified with a MilliQ filtration apparatus from Millipore (Bedford, MA). Centrifugal filtration tubes used to complete library equilibration experiments were from Millipore. Elemental analyses were conducted by Atlantic Microlab, Inc. (Norcross, GA).

HPLC−MS Measurement. All HPLC analyses of the library involved an Adsorbosphere octadecylsilica (ODS) column from Beckman Scientific (Fullerton, CA) with 5-μm particle size and dimensions of 250 mm × 4.6 mm i.d.. Chromatographic method development was completed on a Beckman analytical gradient system with UV detection at 254 nm using System Gold software. All mass spectral analyses were completed on a LC−UV−MS system consisting of an Alliance 2690 liquid handler from Waters (Milford, MA), a 785A programmable absorbance UV detector from PE Biosystems (Foster City, CA), and a Finnegan TSQ-7000 triple quadrupole mass spectrometer equipped with electrospray ionization (San Jose, CA). The chromatographic elution conditions employed throughout the study consisted of a binary mixture of 0.1% HCOOH in H_2O and ACN with gradient elution (15% ACN isocratic for 3 min; to 55% ACN in 61 min; return to 15% ACN in 1 min). To facilitate MS analysis, the eluant flow from the column was reduced from 1.0 to 0.2 mL/min by means of a flow-splitting apparatus. The electrospray ionization source was outfitted with a 100-μm deactivated fused-silica capillary. Nitrogen was used for both the sheath gas (70 psi) and auxiliary gas (10 psi). The mass spectrometer was operated in the positive ion mode and the electrospray needle was maintained at 4 kV. The heated capillary was operated at 16 V and 220 °C, and the tube lens voltage was set to 75 V. Full-scan spectra were acquired from m/z 100 to 1000.

To obtain further structural information for specific library members, LC−UV−MS−MS was used. After ascertaining which chromatographic peaks contained which selectors by LC−MS as described above, selective ion monitoring was used to obtain fragmentation data for compounds of interest. During the course of a chromatographic run, Q1 was maintained at $[M + H]^+$ for the compound of interest as it eluted from the column, Q2 served as the CID chamber (2.5 mTorr Ar gas), and Q3 served as the detector for any resulting fragments. The mass range was m/z 10−500, and the scan rate was ∼1.5 scans/s. Once the compound had eluted, the molecular weight monitored at Q1 was changed in anticipation of the next compound to elute from the column. In this manner, MS fragmentation data were obtained for the chiral selectors of interest.

Preparation of L-[4 × 4]-Abu-CONH$_2$ Library. This library was made using well-established solid-phase synthesis techniques with Rink amide AM resin (0.66 mmol/g loading). After swelling 250 mg of resin (0.165 mmol) in DMF for 30 min, the Fmoc (see ref 1 for a list of reagent abbreviations) protecting group attached to the resin was removed using 20% piperidine/DMF for 30 min. After rinsing with DMF, DCM, IPA, and DCM (1 mL × 5 times), Fmoc-Abu-OH (2 equiv) was combined with the resin, along with HATU (2 equiv) and DIPEA (2 equiv) in DMF (5 mL). This mixture was shaken for 2 h, after which the resin was washed as described previously. After removal of the Fmoc protecting group with another 30-min treatment with 20% piperidine/DMF, module 1, which consisted of Fmoc-protected L-amino acids, was attached to the resin. This was done by combining Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, and Fmoc-Pro-OH (0.25 equiv each), PyBOP (1 equiv), and DIPEA (2 equiv) in DMF (5 mL) with the resin and shaking for 2 h. This step was repeated to ensure complete modification of the resin surface. After rinsing and

removing the Fmoc protecting group from this module, module 2, which consisted of aromatic acids, was added to the resin. This was accomplished by combining Anth-OH (0.15 equiv), Bz-OH (0.30 equiv), DNB-OH (0.30 equiv), Naph-OH (0.15 equiv), PyBOP (1 equiv), and DIPEA (2 equiv) with DMF (5 mL) and shaking for 2 h. As with module 1, this step was repeated to ensure complete modification of the resin surface. To collect the library, the resin was treated with 95% TFA for 30 min, after which the solution was collected by filtration. The filtrate was dissolved in DCM and passed through a plug of silica gel using DCM followed by EtOH as eluting solvents. After the sample was concentrated, the library was kept refrigerated as an ethanolic stock solution and was reconstituted in the appropriate solvent as needed for all subsequent experiments.

Preparation of (*R*)- and (*S*)-*N*-(1-Naphthyl)leucine Stationary Phases. Both the synthesis and attachment to silica gel of *N*-(1-naphthyl)leucine ester **1** have been reported previously.[9]

Incubation and Analysis Procedure. The (*R*)- and (*S*)-*N*-(1-naphthyl)leucine silica gel stationary phases were placed in separate centrifugal filtration modules (60 mg), which had been sealed with Teflon tape to prevent solvent leakage. The library mixture (0.66 μmol in 200 μL of 1:4 EtOH/hexanes) was then added to each filtration tube. After the appropriate equilibration period, the Teflon tape was removed and the filtrate removed by centrifugation. Then, 500 μL of 1:1 MeOH/DCM was placed in each filtration tube and immediately centrifuged again in order to collect the library members that had adsorbed to the silica gel samples. These rinsings were concentrated and then reconstituted in equal amounts of 1:1 MeOH/DCM (0.2 mL) and analyzed by reversed-phase HPLC.

Preparation of Dnb-Ala-Abu-Silica Gel Stationary Phase 3, Dnb-Leu-Abu-Silica Gel Stationary Phase 4, and Dnb-Pro-Abu-Silica Gel Stationary Phase 5. All stationary phases were prepared following procedures similar to that reported for the preparation of DNB-Ala-Gly-silica gel.[9] The ligand surface concentrations were estimated to be 0.24 mmol/g of silica gel for stationary phase 3, 0.11 mmol/g of silica gel for stationary phase 4**,** and 0.20 mmol/g of silica gel for stationary phase 5**,** as determined by elemental analysis of nitrogen.

RESULTS AND DISCUSSION

To demonstrate the general procedure outlined in the introduction, we studied the chiral resolution of *N*-(1-naphthyl)leucine ester **1**[10] (Figure 1). Following this screening protocol, two stationary phases ((*R*)-**2**, (*S*)-**2**), one immobilized with the *R* enantiomer of **1** and the other immobilized with the *S* enantiomer of **1**, were prepared according to procedures published previously.[9] To be consistent with our previous study, a small (4 × 4) peptide library, which is essentially identical to the previous library,[9] was employed in this work (Figure 2). The Gly used as a component of module 2 served as a negative internal control for the screening procedure.

In this study, the library was synthesized using Rink amide resin instead of the oxime resin used previously (Scheme 1). Synthesis on oxime resin is prone to premature peptide cleavage and thus requires careful control of reaction conditions. Moreover, even with carefully controlled reaction conditions, the library synthesized with oxime resin often contains some impurities. In comparison, Rink amide resin is more robust and the resulting library usually contains fewer impurities. With the exception of the cleavage of final product from the resin, the chemistry needed to prepare this library on Rink amide resin is identical to that described previously.

The resulting library was analyzed using reversed-phase HPLC interfaced with both a UV and a MS detector. By using a gradient elution system (15% ACN to 55% ACN in 0.1% HCOOH in water), all except one library member are resolved (Figure 3, the

(10) For the synthesis of this compound and its related chiral studies, see: (a) Pirkle, W. H.; Pochapsky, T. C. *J. Am. Chem. Soc.* **1986**, *108*, 352−354. (b) Pirkle, W. H.; Deming, K. C.; Burke, J. A. *Chirality* **1991**, *3*, 183−187.

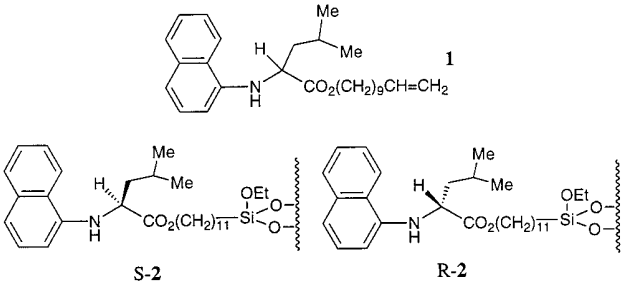


Figure 1. Racemic analyte *N*-(1-naphthyl)leucine ester **1** and silica gel-based stationary phases immobilized with its enantiomers.

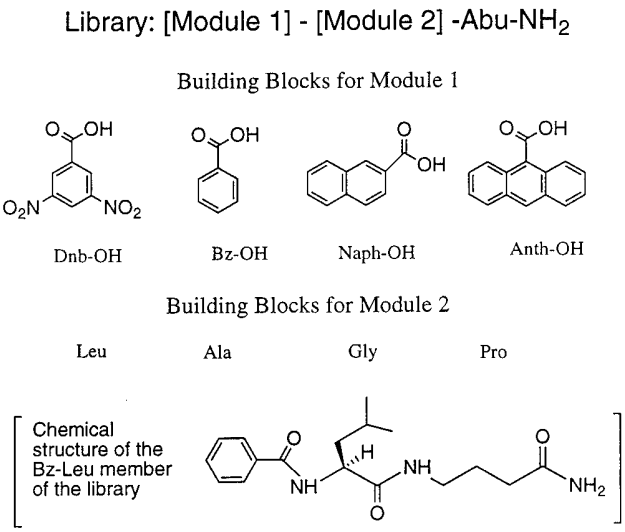
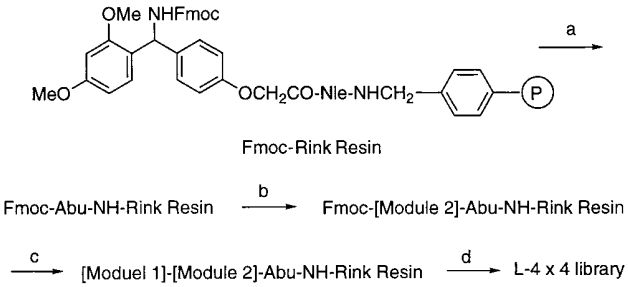


Figure 2. The L-4 × 4-[Anth, Bz, Dnb, Naph]-[Ala, Gly, Leu, Pro] library. Abu, 4-aminobutyric acid. Abu is a linker and the Gly in module 2 serves as a negative internal control for screening.

Scheme 1. Preparation of the L 4 × 4 Library[a]



[a] (a) (1) Piperidine; (2) Fmoc-Abu-OH, PyBop. (b) (1) Piperidine; (2) Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Pro-OH, PyBop. (c) (1) Piperidine; (2) Anth-OH, Bz-OH, Dnb-OH, Naph-OH, PyBop. (d) TFA.

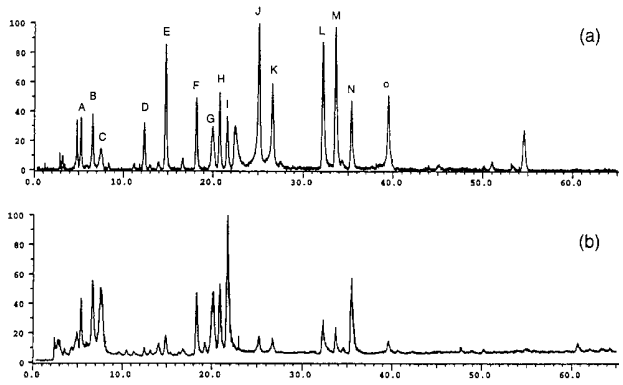


Figure 3. Chromatograms of the library: (a) recorded with a UV detector, (b) recorded with a MS detector. Peak assignments: A, Bz-Gly; B, Bz-Ala; C, Bz-Pro, D, Dnb-Gly; E, Dnb-Ala and Dnb-Pro; F, Naph-Gly; G, Naph-Pro; H, Naph-Ala; I, Bz-Leu; J, Anth-Gly; K, Anth-Ala; L, Dnb-Leu; M, Anth-Pro; N, Naph-Leu; O, Anth-Leu. See Experimental Section for chromatographic conditions.

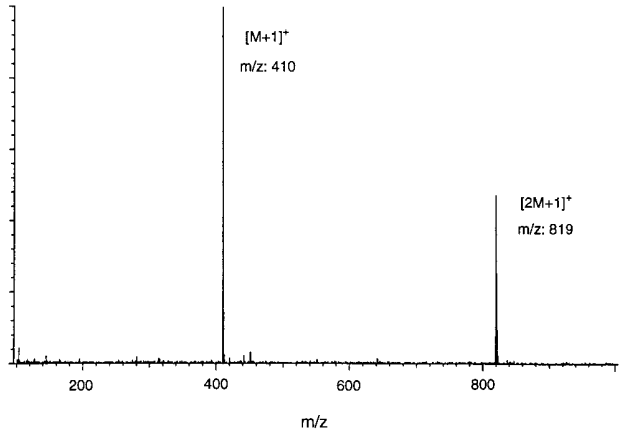


Figure 4. LC−ESI-MS of Dnb-Leu selector. See Experimental Section for instrument operating conditions.

unresolved peak E is composed of two library members, Dnb-Ala and Dnb-Pro). The chemical identities of the chromatographic peaks were determined by the molecular weights as measured with the LC−MS. Under electrospray ionization conditions, all the peptide library members yielded clean, easily identifiable mass spectra (Figure 4). Judging from the chromatogram, few other impurities existed, thereby demonstrating the reliable synthesis of the library afforded by Rink amide resin.

An equal amount of the library in 20% EtOH/hexane was then allowed to equilibrate with equal amounts of the two stationary phases separately. After equilibration for 30 min,[11] the supernatants were removed from the stationary phases by centrifugal filtration. The stationary phases were then washed with 1:1 MeOH/DCM to elute these adsorbed library members. The collected eluents from both stationary phases were concentrated and redissolved in an equal amount of 1:1 MeOH/DCM. These reconstituted samples were analyzed using reversed-phase HPLC equipped with a UV detector, under the same chromatographic conditions as described earlier for the LC−MS analysis of the library. The chromatograms obtained are shown in Figure 5.

(11) The chromatograms obtained at a longer equilibration time (1 h) are virtually identical to these obtained at 30-min equilibration time, indicating that the latter is sufficient for the system to reach equilibrium.

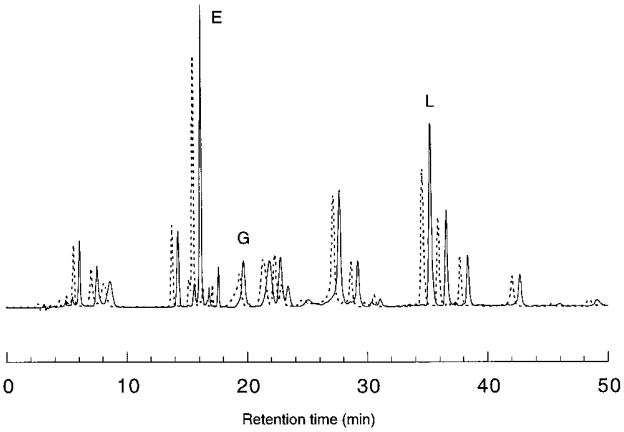
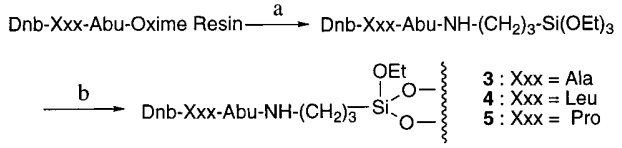


Figure 5. Chromatograms of library members adsorbed onto the stationary phases. Solid line: from (*S*)-**2** stationary phase. Dotted line: from (*R*)-**2** stationary phase. Chromatogram from (*S*)-*2* is shifted 0.5 min to the right to provide a clear comparison.

Scheme 2. Immobilization of Potential Chiral Selectors onto Silica Gel[a]



[a] (a) 3-Aminopropyl)triethoxysilane. (b) Silica gel, 120 °C.

As seen from these chromatograms in Figure 5, two peaks (E and L) have clearly different intensities in these chromatograms corresponding to the two different stationary phases. For peak G, although the peak height in one chromatogram is higher than that in the other one, its peak width is narrower than the corresponding peak. Therefore, peaks E and L mostly likely represent chiral selectors. As determined by LC−MS and shown in Figure 3, peak L results from a single compound, Dnb-Leu. On the other hand, peak E corresponds to a mixture of Dnb-Pro and Dnb-Ala. Therefore, Dnb-Leu is most likely a chiral selector, and either one or both Dnb-Pro and Dnb-Ala may offer chiral discrimination for the target analyte (**1**).

The three potential chiral selectors were then immobilized onto silica gel according to procedures reported previously (Scheme 2), and the resulting stationary phases were packed into columns using a standard slurry packing method.[12] Both Dnb-Ala and Dnb-Leu columns were found to resolve racemic naphthyl leucine ester **1** well; the separation factor[13] with the Dnb-Ala column 3 was 4.7, while that of the Dnb-Leu column 4 was 12 (Figure 6). However, Dnb-Pro 5 failed to resolve the racemic analyte under the same chromatographic conditions.

For this small library, LC−MS proved sufficient to identify all the library components, as they all have different molecular weights. For other libraries, LC−MS−MS may prove essential as such libraries may contain components that have the same

(12) Poole, C. F.; Poole, S. K. *Chromatography today*; Elsevier: New York, 1991; pp 350−353.

(13) Retention factor (k') equals $(t_r - t_0)/t_0$, in which t_r is the retention time and t_0 is the dead time. The separation factor (α) equals k'_2/k'_1. Dead time t_0 was measured with 1,3,5-tri-*tert*-butylbenzene as the void volume marker according to: Pirkle, W. H.; Welch, C. J. *J. Liq. Chromatogr., A* **1991**, *14*, 1−8.

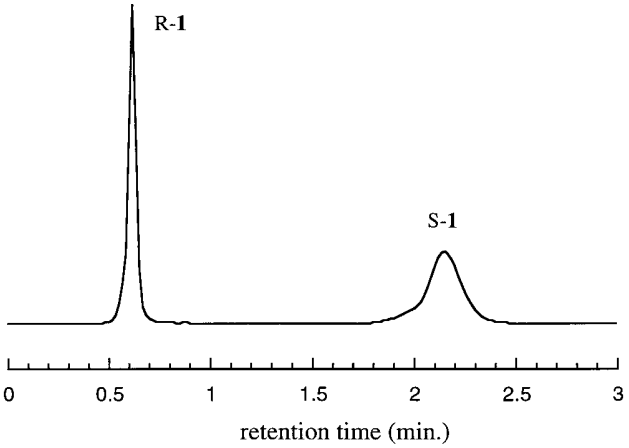


Figure 6. Chromatogram of racemic *N*-(1-Naphthyl)leucine ester **1** on Dnb-Leu stationary phase 4. Column size, 50 × 4.6 mm; mobile phase, 20% IPA in hexanes; flow rate, 1.2 mL/min; UV detector (254 nm); t_0 = 0.48 min.

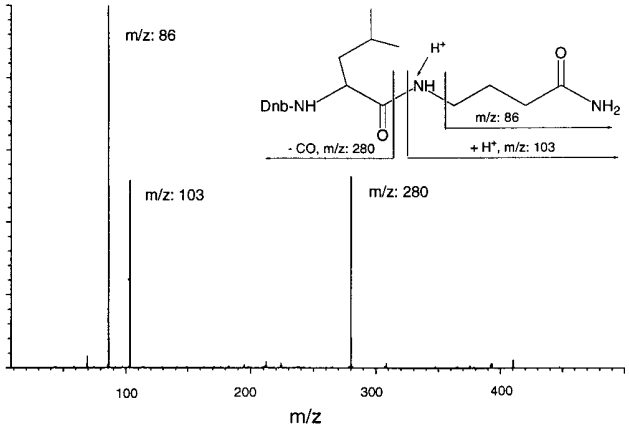


Figure 7. LC−ESI-MS−MS spectrum of Dnb-Leu selector. The spectrum was obtained by monitoring the fragmentation of the $[M + 1]^+$ peak of Dnb-Leu selector. See Experimental Section for operating conditions.

molecular weights. Even when identifying compounds with the same molecular weight is not an issue, MS−MS could still provide additional valuable structural information for active component identification. For these reasons, the LC−MS−MS spectra of chiral selectors Dnb-Ala and Dnb-Leu were studied. Fragmentation of the molecular peak $[M + 1]^+$ yielded mainly three mass signals: For Dnb-Ala, at m/z 86.2, 103.2, and 238.1; and for Dnb-Leu, at m/z 86.2, 103.2, and 280.1 (Figure 7). The peak at m/z 86.2 in both compounds is assigned to $(CH_2CH_2CH_2CONH_2)^+$, and the peak at m/z 103.2 is assigned to $(NH_3CH_2CH_2CH_2CONH_2)^+$. Both are derived from the Abu unit common to the two selectors. The peak at m/z 280 is unique for Dnb-Leu, while the peak at m/z 238 is specific for Dnb-Ala.

It should be noted that obtaining mass spectra of the entire library is not necessary, unless one is assessing the quality of the library synthesis, as in our case. In a routine screening experiment, one could identify chromatographic peaks corresponding to chiral selectors using HPLC coupled with a UV or other detectors capable of providing reliable peak intensity measurement. One could then repeat the chromatographic experiments using HPLC−MS or HPLC−MS−MS to obtain structural information of only those identified compounds.

One potential source for complications for this technique, as in the previous method, involves peak overlapping. It is possible that one peak with identical intensity in both chromatograms could actually be the summation of two peaks, one with enhanced intensity and the other with reduced intensity. Therefore, it is possible to overlook a chiral selector in this library screening method. However, the possibility of two compounds having exactly the same retention time and exact opposing chiral selectivity (not only in direction but also in magnitude) is rather slim. Moreover, one can minimize complications arising from peak overlapping by repeating the chromatographic screening experiments using different mobile phases, thereby possibly altering selectivity. It should also be pointed out that, in many combinatorial library experiments, missing a potential hit is not detrimental as long as other chiral selectors are identified. Peak overlapping could also lead to the identification of false chiral selectors, as the Dnb-Pro example demonstrated in this study. False chiral selectors introduce an additional amount of work but no other serious consequences. One can also minimize such a possibility by repeating the chromatographic screening experiments using different mobile phases.

CONCLUSIONS

Several advantages to this method exist as compared to our previous screening method. Unlike the previous method, this method requires the synthesis of only one enantiomerically pure library and it does not require the syntheses of sublibraries. Moreover, the size of the library that can be screened with this approach could be increased significantly for two reasons. First, in this new screening procedure, the library was analyzed with a reversed-phase column, whose column efficiency and thus peak capacity is typically much higher than an unoptimized chiral column. As a result, more compounds can be resolved in a reversed-phase chromatographic experiment.[14] Second, the number of mixture library components that need to be analyzed for any library screening experiment could be reduced dramatically, since only those adsorbed library members, not the entire library, need to be analyzed.

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