Tetrahedron: *Asymmetry* 20 (2009) 1476–1486

Contents lists available at ScienceDirect

Tetrahedron: *Asymmetry*

journal homepage: www.elsevier.com/locate/tetasy

Development of a practical mass spectrometry based assay for determining enantiomeric excess. A fast and convenient method for the optimization of PLE-catalyzed hydrolysis of prochiral disubstituted malonates

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ARTICLE INFO

Article history:
Received 20 April 2009
Accepted 29 May 2009

ABSTRACT

A practical mass spectrometry-based enantioselectivity assay is presented which makes use of enantiomerically enriched, but not enantiomerically pure, probe molecules readily obtained from esterase hydrolysis of prochiral malonates. The technique presented here allows us to recycle materials obtained from esterase hydrolysis which give substantial, but synthetically insufficient, enantiomeric excess as probe molecules in an enantioselectivity assay. The enantiomerically enriched products are esterified using deuterium-labelled alcohol. The enantiomeric excess is measured using mass spectrometry (LC–MS and LDI) by measuring the D_5/H_5 ratio in the resulting products obtained from an enzymatic hydrolysis. The D_5/H_5 ratio is corrected to account for the enantiomeric purity of the probe. Herein we report the results obtained from Pig Liver Esterase hydrolyses of prochiral malonate esters and outline the strengths and limitations of this approach to enantioselectivity determinations. This assay strategy was able to identify reaction conditions that led to an improvement in ee from 70% ee to >97% ee in the PLE-catalyzed hydrolysis of a prochiral malonate used to prepare unnatural serine analogues.

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1. Introduction

Synthetic chemists have made significant use of enzymatic reactions in the recent years to provide high value synthetic intermediates. These high value synthetic intermediates are usually enantioenriched molecules where the enantioselectivity is provided by the enzymatic process being utilized. It is arguably the case that the hydrolysis of esters is by far the most widely used enzymatic reaction for the preparation of high value synthetic intermediates.[1–3] Enzymatic hydrolysis has been utilized in the preparation of biologically active compounds containing stereogenic quaternary centers. The synthetic value of hydrolytic enzymes is unquestioned and has been effectively demonstrated in the preparation of various quaternary carbon containing unnatural amino acids,[4–6] anti-cancer agents,[7] enzyme inhibitors,[8,9] and analgesics.[10] It is anticipated that the use of enzymes in organic synthesis will continue to increase now that the most versatile of the hydrolytic enzymes, Pig Liver Esterase (PLE), has been cloned and expressed in yeast.[11,12]

It is well documented that hydrolytic enzymes (e.g., PLE) are capable of hydrolyzing a wide variety of substrates with high levels of enantiocontrol and chemical yield.[13–20] However, there are instances where the enantioselectivity of the enzymatic hydrolysis is good but at unacceptable levels for synthetic purposes.[5,16,21,22,15] There are numerous reports in the literature of using various co-solvents and altered reaction conditions, which drastically improve the enantioselectivity of the enzymatic hydrolysis.[23,21,24–26] However, it appears that there is no general model which can be used to predict which co-solvents or altered reaction conditions will provide high levels of enantioselectivity for a particular substrate/enzyme combination.[21] At present, synthetic chemists must use a combinatorial approach for the identification of optimal reaction conditions for each substrate under consideration. This has led to a significant research effort in the recent years toward the development of methods which can rapidly determine enantioselectivity from such reactions.[27]

Traditionally accepted methods of determining enantiomeric excesses include polarimetry,[28] chiral chromatography (both GC and HPLC),[29–32] and NMR (derivatives with chiral auxiliaries and chiral shift reagents).[33–35] Although these appear to be the most straightforward methods for determining enantiomeric excess, there are significant drawbacks to each of these which make their use as a convenient and rapid technique questionable. For example, polarimetry requires compounds of high purity as the measurement of [α] is susceptible to the presence of both chiral and achiral contaminates. Chiral chromatographic methods overcome many of the issues associated with polarimetry, but in many cases the chromatographic methods are too time consuming to be practical in a combinatorial approach. The use of chiral shift reagents in NMR also

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doi:10.1016/j.tetasy.2009.05.037

has considerable drawbacks such as insufficient resolution of the diastereomeric NMR signals to obtain accurate signal integrations. Chiral compounds can also be derivatized with enantiomerically pure auxiliaries to generate diastereomeric pairs, which can be analyzed by NMR. However, this method requires additional manipulation prior to analysis, resulting in reduced sample throughput.

Significant advances have been made with NMR allowing for high throughput screening for enantioselectivity. Reetz has developed an NMR technique based on the use of ^{13}C-labelled *pseudo* enantiomers and *pseudo meso* compounds.[36,37] The technique makes use of a *pseudo* racemate comprising one enantiomer that is ^{13}C-enriched while the other enantiomer is ^{12}C-enriched. The reporting moiety in each case is a methyl group. The products of the reaction are analyzed using ^{1}H NMR by integration of the singlet (^{12}C-enriched methyl group) and the doublet (^{13}C-enriched methyl group) giving the enantioselectivity of the reaction under investigation. The NMR technique developed by Reetz et al. utilizes recent advances in NMR flow cell technology and an auto-sampler resulting in a throughput of approximately 1400 determinations per day.

Several groups have recently developed methods to determine the enantioselectivity using various spectrophotometric reporter systems. Anslyn et al. have developed UV–vis and fluorescence systems capable of determining the enantiomeric excess of various compound classes.[38–41] In one embodiment, Anslyn and co-workers made use of amino acid coordination to a preformed chiral copper complex containing an appropriate spectroscopic reporter.[38] The reporter is displaced by coordination of the amino acid resulting in an absorbance change. The change in absorbance was found to be proportional to the enantiomeric purity of the amino acid under consideration. Anslyn has recently reported on an attractive assay using CD spectroscopy to determine both enantiomeric excess and concentration.[42] Berkowitz has developed an ISES (in situ enzymatic screening) assay[43,44] that is capable of readily determining enantiomeric excess and kinetic information by making use of a dual cuvette and two reporter enzymes.[45,46] The dual-cuvette ISES assay was used successfully to screen catalysts for their efficiency in the hydrolytic kinetic resolution of various racemic epoxides.[45,46] The ISES assay was able to identify, in a combinatorial fashion, several catalysts capable of resolving epoxides with acceptable levels of enantiomeric purity. Reetz has reported on the use of FTIR to screen for enantioselectivity.[47] The FTIR technique exploits the fact that a ^{13}C-labelled carbonyl is more significantly shifted to a lower wave number ($\sim$40–50 cm^{-1}) in the carbonyl region of the IR spectrum than is its ^{12}C-enriched counterpart. The FTIR screening method makes use of a *pseudo* racemic mixture where one enantiomer has a ^{13}C-labelled carbonyl while the other enantiomer is a ^{12}C-carbonyl. Applying the Beer–Lambert law then allows for the calculation of enantiomeric excess. Unfortunately, in order to apply the Beer–Lambert law, one must know the molar extinction coefficient for each species under investigation. However, the FTIR method allows for up to 10,000 samples per day to be processed using commercially available FTIR equipment coupled to auto-sampler/plate reader devices.

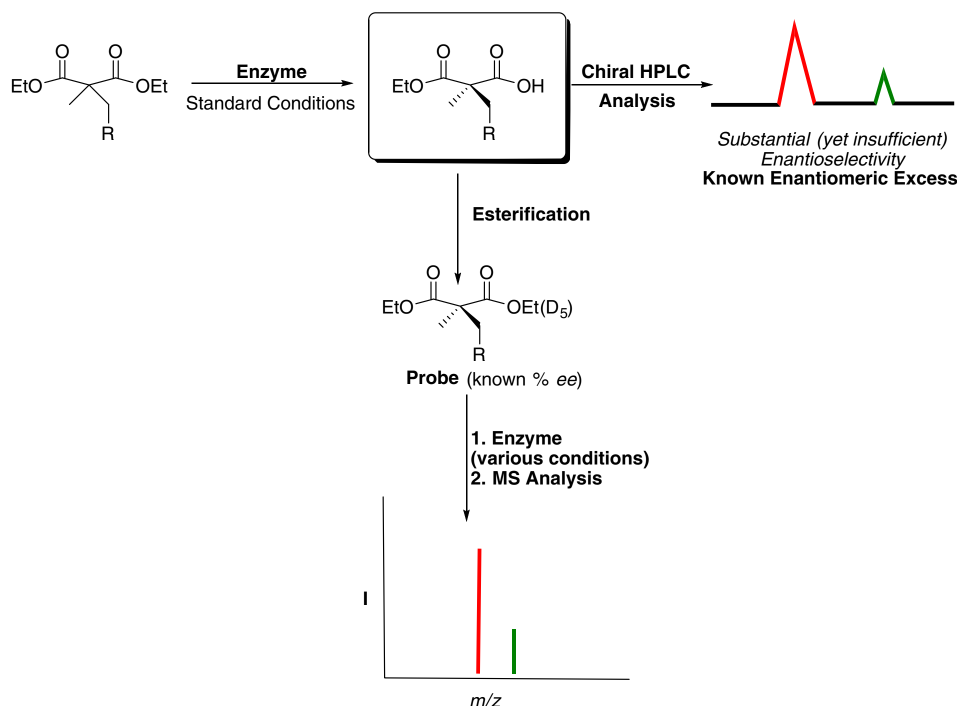
Mass spectrometry has gained interest in the recent years as a technique for the determination of enantiomeric excess. Several techniques have been reported that make use of *pseudo* racemates in which the *pseudo* enantiomers differ from one another by mass.[48–52] Pfaltz et al. have developed an MS-based enantioselectivity assay which was used to screen a library of ligands in palladium-promoted allylic substitution reactions.[53] In this embodiment one of the *pseudo* enantiomers contains a methyl substituent while the other *pseudo* enantiomer contains an ethyl substituent sufficiently removed from the reaction center. This mass tagging strategy allows for the rapid determination of enantiomeric excess by simply measuring the ion intensities using electrospray ionization. Reetz has exploited mass spectrometry in enantioselectivity assays using the *pseudo* racemate and *pseudo meso* compound strategy.[54,55] Reetz successfully used this strategy to assay enzymes for their ability to perform kinetic resolutions. Reetz further refined the technique using a specialized eight-channel multiplexed sprayer system coupled to a mass spectrometer increasing the sample throughput to approximately 10,000 samples per day.

Herein we report on our efforts to develop and implement a convenient assay for the determination of enantiomeric excess, which is practical for the synthetic chemists with specific target compounds under consideration. We are interested in exploiting hydrolase enzymes, particularly PLE, to provide half-ester intermediates from prochiral malonate esters for the preparation of various amino acid classes.[4] We have frequently encountered situations where the PLE hydrolysis provides the half-ester intermediates in significant but insufficient enantioselectivities (approximately 50–70% ee). Scheme 1 illustrates our assay strategy for enantioselectivity which allows for the recycling of the half-ester products obtained from a significant yet insufficient hydrolysis. The probes obtained from these recycled products are then utilized, using the classical isotope dilution method,[56] to screen for reaction conditions which provide acceptable levels of enantioselectivity. The enantiomeric excess calculated from the MS analysis can then be corrected to account for the enantiomeric purity of the probe.[35] To the best of our knowledge there are no reports of recycling reaction products for assay purposes as illustrated in Scheme 1. We believe that the methodology described below will find widespread use by synthetic chemists given that the technique is straightforward and utilizes readily available instruments and synthetic methods.

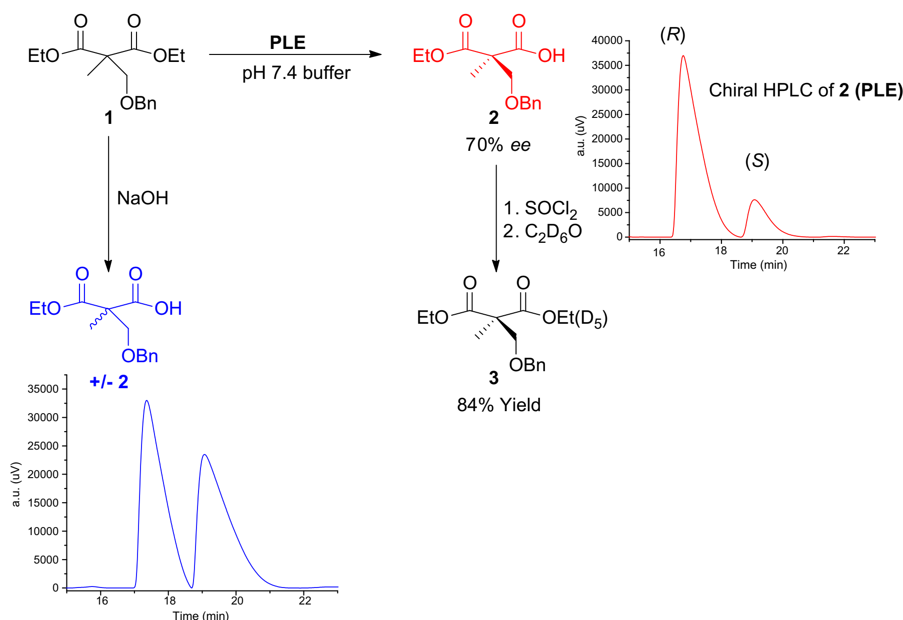
2. Results and discussion

We initially attempted a PLE hydrolysis of malonate **1** as shown in Scheme 2 using the standard PLE hydrolysis protocol to ultimately prepare a series of unnatural serine analogues.[4] Chiral HPLC analysis of the half-ester product **2** obtained revealed that the hydrolysis proceeded with only 70% ee. Although this is considerable enantioselectivity, it is of little synthetic value for our purposes as we require nearly enantiomerically pure material. Half-ester **2** was converted into the *pseudo* prochiral probe **3** by preparing the acid chloride of **2** followed by treatment with ethanol-d_6. We had attempted the DCC coupling protocol to prepare probe **3**, but found that the acid chloride method required less extensive workup. The resulting compound **3** was isolated by chromatography and completely characterized by NMR (^{1}H and ^{13}C) and high resolution MS analysis.

We first wanted to determine which MS technique would be the most advantageous (MALDI or ESI). However, given the low molecular weight of our samples we could not use traditional MALDI due to matrix interferences in the needed *m*/*z* range. Fortunately, our samples contain an aromatic ring and we were able to observe strong signal intensities of our half-ester products as potassium adducts using laser desorption ionization (LDI). Unfortunately, obtaining useful LDI data required extensive workup of the enzymatic reactions prior to analysis. The workup involved an extraction of the half-ester products into methylene chloride to separate the products from the buffer in the reaction medium. Attempts to directly analyze the reaction medium proved difficult due to salt-promoted ion suppression. The extractions required relatively large aliquots be taken from the enzymatic hydrolysis reactions and resulted in dramatically decreased sample throughput. We also observed varying signal intensities from sample to sample using LDI. We attributed the variability in signal intensity to the varying thickness of the sample on the MALDI plate from sample to sample. Using LC–MS proved to be more convenient; the LC conditions were optimized such that the samples required little preparation and analysis could be performed on small aliquots of reaction medium. It was determined that useful data could be obtained on samples in the low micromolar concentration range



Scheme 1. Mass spectrometry enantioselectivity assay using optically impure probes.



Scheme 2. Preparation and analysis of probe **3** (Chiralcel OJ-H column).

using select ion monitoring (SIM) of our half-ester products. Figures 1 and 2 show selected spectra obtained by LDI and LC–MS in the SIM mode of the half-esters obtained by PLE hydrolysis of probe **3**. The LDI provided strong signals for potassium adducts as the buffer system utilized in the PLE hydrolysis is potassium phosphate while the LC–MS provided either intense proton or sodium adducts.

Our initial experiments were to test the hypothesis that enantiomerically impure probe molecules could be used in an MS-based assay for enantioselectivity. The initial data were used to compare the corrected ee values obtained by MS to the ee values obtained by the traditional chiral HPLC method (without any correction of the data). In order to accomplish this we needed half-ester products whose ee values spanned a reasonable range of enantiomeric purities. We obtained the necessary half-ester products in a variety of ee values as shown in Scheme 3. Starting with probe **3**, we performed a typical PLE hydrolysis to obtain half-ester **4**. We performed both MS analysis and chiral HPLC analysis on **4** and found the values to be in close agreement with one another. Aliquots of **4** were then spiked with varying amounts of racemic D_5/H_5-labeled mixture **5** (50% H_5/50% D_5). The ee of the spiked samples was determined by chiral HPLC analysis and each sample was analyzed by LC–MS in the SIM mode.

Figure 3 shows that the data obtained by chiral HPLC analysis and the corrected MS values are in reasonable agreement with

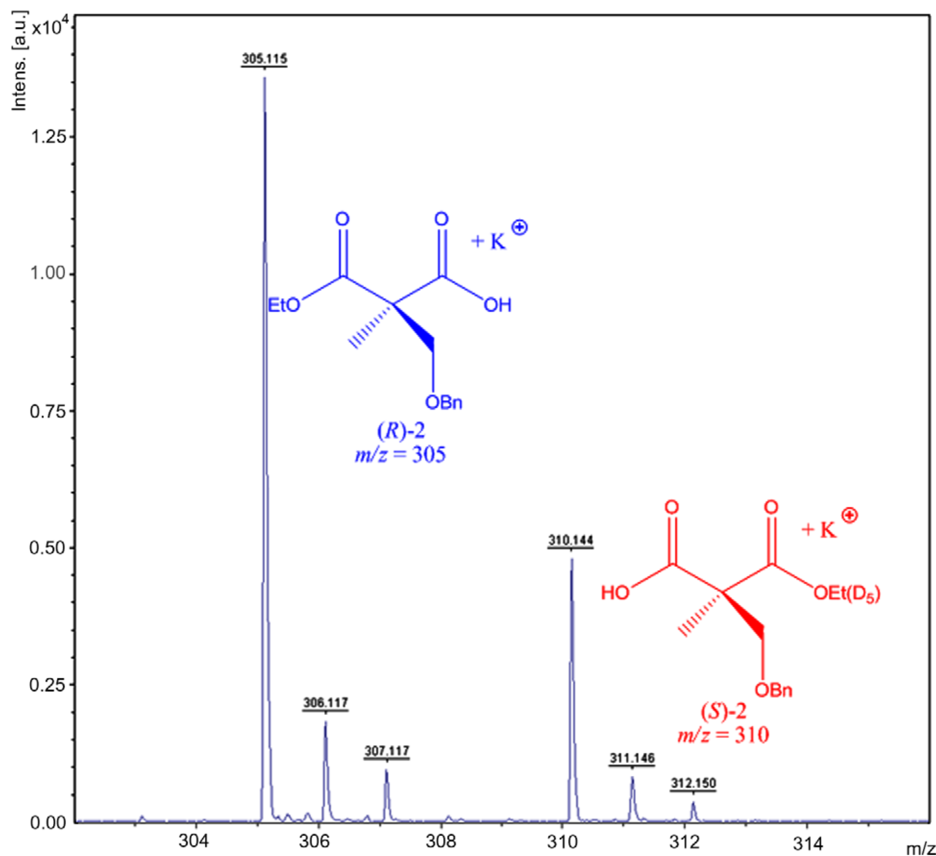


Figure 1. Laser desorption ionization (LDI) analysis of half-esters obtained by PLE hydrolysis of **3**.

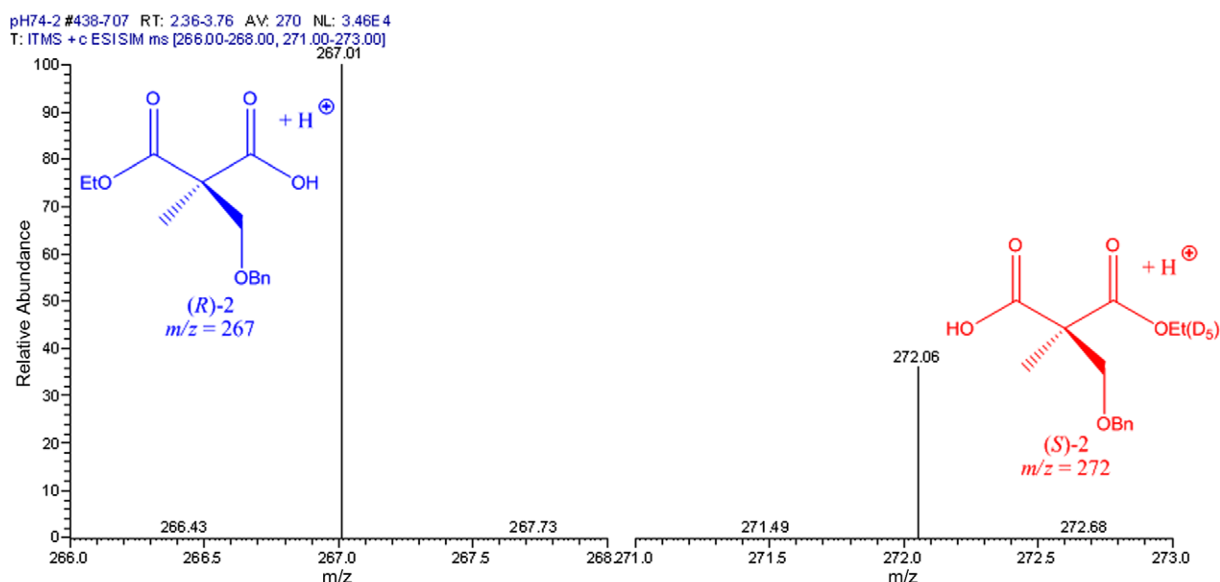
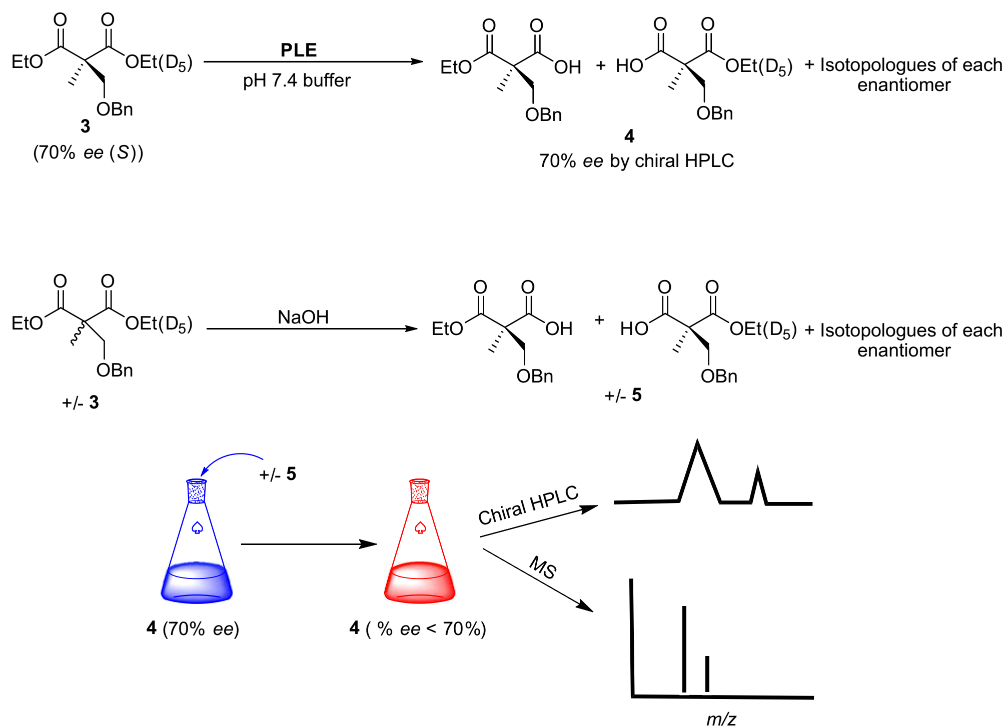


Figure 2. LC–MS analysis in SIM mode of half-ester products obtained by PLE hydrolysis of **3**.

one another. Figure 3 illustrates a slope of near unity and an acceptable fit to a straight line with a *Y*-intercept zero as expected. This limited data set suggests that it is feasible to utilize enantiomerically impure probes in a mass spectrometry-based enantioselectivity assay by correcting for the enantiomeric purity of the probe. Secondary kinetic isotope effects are expected to be insignificant and within the limit of error for this method. The work published by Reetz et al. shows an excellent correlation of MS ee values with ee values obtained by chiral GC demonstrating that the secondary isotope effects are insignificant.[55]

It has been demonstrated in several studies that hydrolytic enzymes can be tuned to provide better enantioselectivities by altering the reaction medium, a process known as reaction medium engineering.[2] Although the nature of the effect is uncertain, it is clear that the effect can be exploited in the preparation of advanced synthetic intermediates.[57,21] We sought to explore various conditions such as



Scheme 3. Preparation of **4** with various % ee.

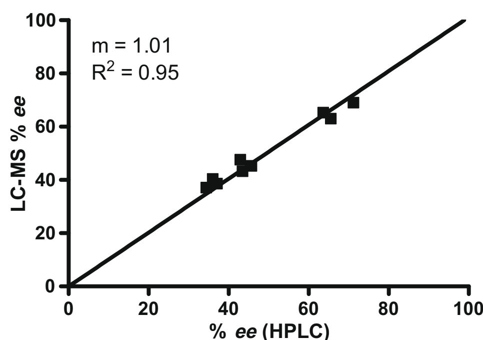


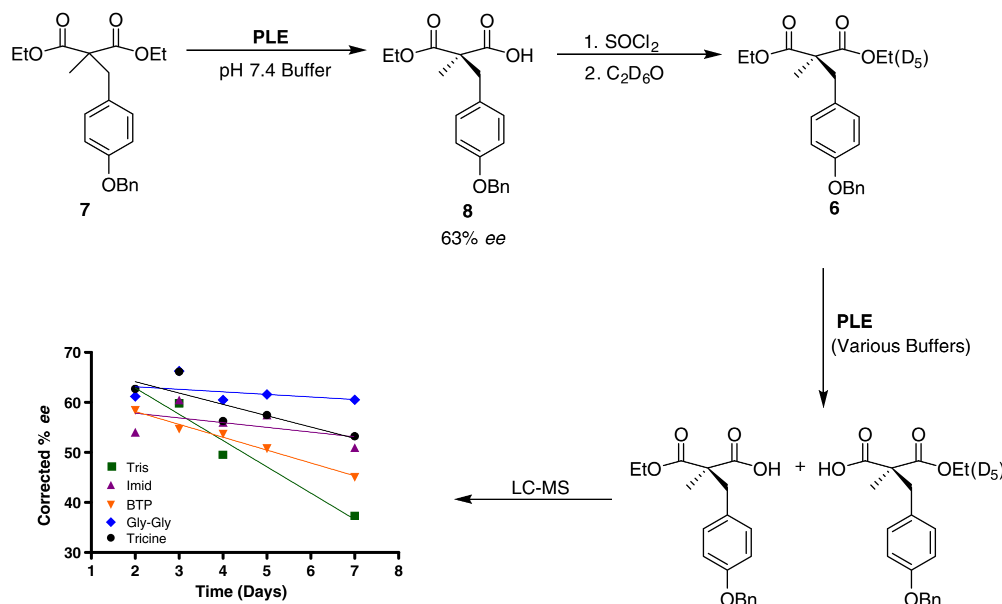
Figure 3. Correlation of HPLC% ee with corrected% ee values from LC–MS analysis.

pH, buffer type, and co-solvent composition on the PLE-catalyzed hydrolysis of **3** and **6** in the hope of obtaining conditions capable of providing **4** and **8** with synthetically useful levels of enantioselectivity (Scheme 4). Compound **8** was chosen due to the fact that **8** can be readily transformed into tyrosine analogues. Initially we decided to determine if it would be possible to simply alter the pH of the phosphate buffer and observe significant changes in enantioselectivity. We conducted our assays in small conical vials containing approximately 10 mg of either probe **3** or **6**, 1.5 mL of phosphate buffer at the desired pH, and 3 μL of PLE suspension (4 units/assay). The assay samples were shaken vigorously at 25 °C for a specified time and then small aliquots were removed for analysis by LC–MS, LDI, and chiral HPLC. The data presented in Figure 4 clearly indicate that changes in pH, over the narrow range studied here, have at best a minimal influence on the enantioselectivity of the PLE-catalyzed hydrolysis of probes **3** and **6**. Figure 4 also illustrates that this general trend is observed regardless of the analytical technique used (HPLC, LDI, or LC–MS). This suggests that small changes in pH around the pH optimum for PLE do not alter the dimensions of the chiral pocket to any useful extent.[16]

We turned our attention to the buffering species to determine if the choice of buffer could have an effect on the outcome of the PLE-catalyzed hydrolysis. We chose a variety of commonly used buffer systems, such as phosphate (control), glycine–glycine, TRIS, imidazole, tricine, and bis–tris propane. This set of assays was performed in a similar manner as described above for the assay varying pH. We noticed that most of the buffer systems we chose initially resulted in similar enantioselectivities to those obtained using the simple phosphate buffer system. This suggests that the choice of buffer is relatively unimportant. However, we did observe significantly lower enantioselectivity compared to the control for several of the buffer systems over time using probe **6** (Scheme 4). This observation suggests that extended reaction times in the presence of nucleophilic buffering species can significantly degrade the enantioselectivity of the final product. We suspect these result in lower enantiomeric excesses over time due to racemic background hydrolysis.[58,59]

We next turned our attention to the addition of various co-solvents to the PLE reaction medium. We decided to use the phosphate buffer system for the co-solvent study based on our results from the buffer assay outlined above. We chose to study the following miscible co-solvents: methanol, ethanol, isopropanol, diglyme, triglyme, acetonitrile, DMF, dioxane, and DMSO. We found that varying the co-solvent composition between 5% and 30% was tolerated by the phosphate buffer and all co-solvent compositions were completely homogenous. Attempts to make compositions greater than 30% co-solvent frequently resulted in the crystallization of phosphate and/or two phase systems and were not pursued further in this study. The assays were conducted as described above substituting the co-solvent/phosphate buffer mixtures for the phosphate buffer. The pH of the resulting co-solvent/buffer solutions was not adjusted. The assay samples were shaken vigorously at 25 °C and were analyzed by LC–MS.

We analyzed the primary alcohol co-solvent compositions first as we were concerned about the possibility of transesterification with the alcohol co-solvent. The chromatogram in Figure 5 shows



Scheme 4. Preparation of probe **6** and buffer assay results.

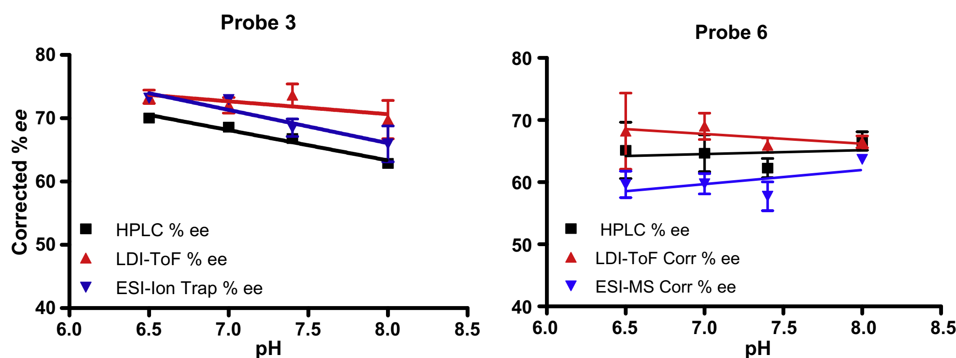


Figure 4. Effect of pH on the enantioselectivity of PLE hydrolysis of **3** and **6**.

the total ion current chromatogram (TIC) and the reconstructed ion chromatograms (RICs) for the assay performed in 10% methanol co-solvent. It is apparent that several chromatographic peaks are present indicating significant transesterification with the primary alcohols. The identity of each of the chromatographic peaks was determined using MS/MS analysis and comparison of the retention times to those of authentic samples. MS analyses of the half-ester products obtained using methanol and ethanol co-solvents produce flawed results due to the loss of the deuterium label by equilibration with the co-solvent. This finding, although not entirely unexpected, does place a limit on the assay in that highly nucleophilic co-solvents capable of forming transesterification products with isobaric species must be avoided. A similar analysis with both *tert*-butanol and isopropanol did not result in significant production of transesterification products. Table 1 shows the results of the co-solvent assays performed with probes **3** and **6**. The non-nucleophilic co-solvents studied demonstrate their ability to alter the enantioselectivity of the PLE-catalyzed hydrolysis of **3**. However, most of the co-solvents studied resulted in only marginal improvements in enantioselectivity. The data in Table 1 show that 10% isopropanol co-solvent resulted in essentially enantiomerically pure material [~103% ee, (*R*)-isomer] for probe **3**. The authors realized that 103% ee is impossible.[60] The value is obtained following the correction for the probe purity. We chose to scale up the PLE hydrolysis using 10% isopropanol co-solvent and diester **1**. The resulting half-ester was isolated in 60% yield and was analyzed by chiral HPLC. Chiral HPLC indicated that the half-ester **2** was produced in >97% ee and that the major isomer was of the (*R*) absolute configuration (Scheme 5). This finding suggests that isopropanol should be examined as a co-solvent in the PLE-catalyzed hydrolysis of other prochiral malonates.[24,61]

Diester **7**, a precursor to unnatural tyrosine analogues, is hydrolyzed by PLE in reasonable yield and moderate enantioselectivity (63% ee). The half-ester **8** was recycled into probe **6** using the acid chloride esterification method described above to produce **3** (Scheme 4). Probe **6** was subjected to PLE hydrolysis in various co-solvents and was analyzed by LC–MS in the SIM mode. The data in Table 1 identify 30% isopropanol co-solvent as the best reaction medium for the hydrolysis of **8** with respect to enantioselectivity. Diester **7** was hydrolyzed by PLE in 30% isopropanol co-solvent in a scaled up reaction to produce **8** in <10% yield. The chiral HPLC analysis of **8** obtained from the scaled up reaction confirmed the production of the half-ester in 85% ee with the (*R*)-absolute stereochemistry. The stereochemistry was confirmed by transforming acid ester **8** into the α-tyrosine analogue **10** via a Curtius rearrangement and comparison of the specific rotation with that of authentic material (Scheme 6). Although the use of such high co-solvent composition has a deleterious effect on the isolated yield

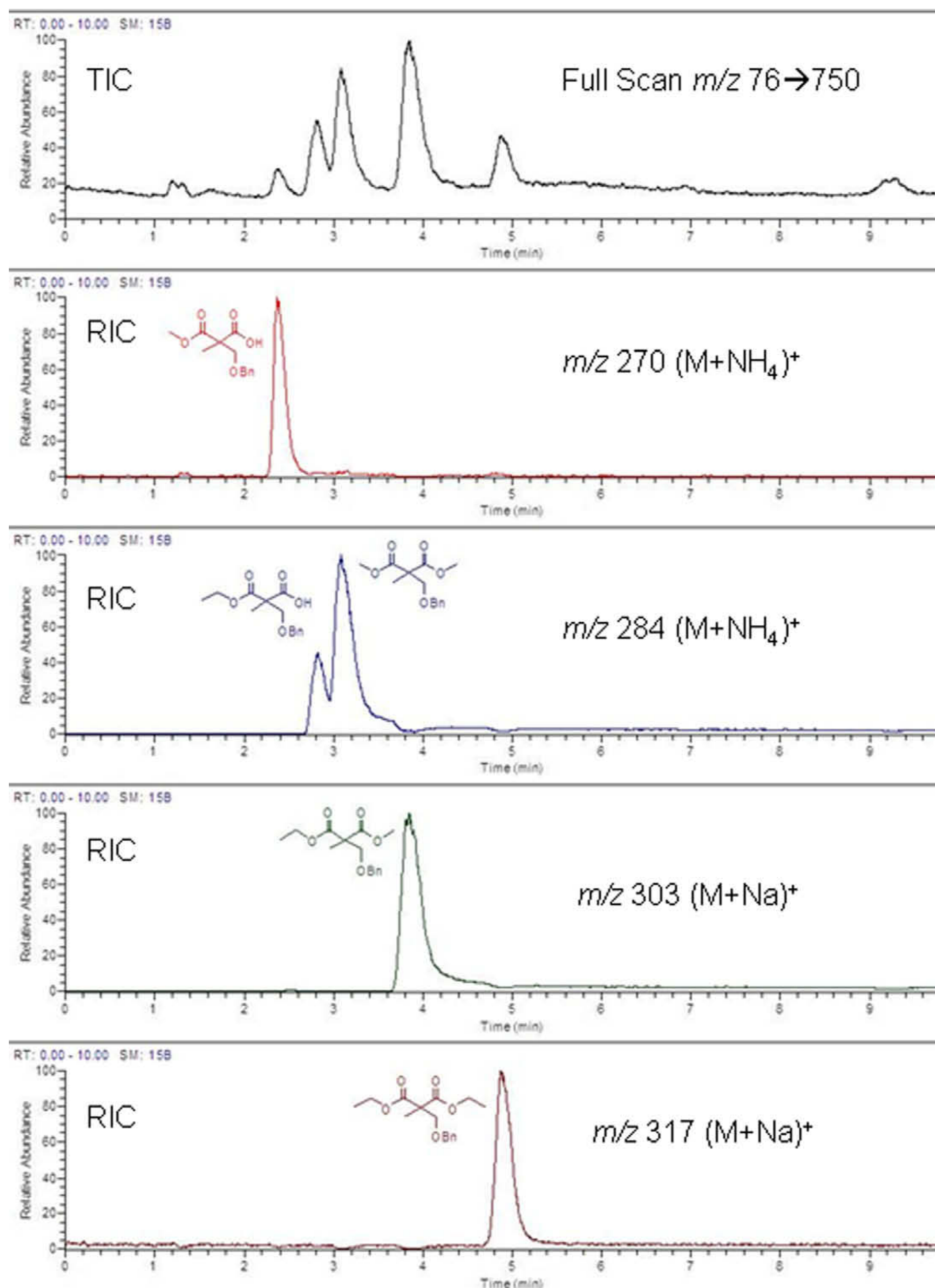


Figure 5. LC–MS chromatogram of the PLE hydrolysis of **3** in methanol co-solvent.

of **8**, the improvement in enantioselectivity is significant. We are currently performing additional co-solvent assays on probe **6** to identify reaction conditions that can provide the high levels of enantioselectivity observed with the 30% isopropanol co-solvent without detrimental effects on the yield.

3. Conclusion

Much of the data obtained in this study are plotted in Figure 6 to illustrate the correlation of the corrected % ee values obtained by LC–MS analysis with the values obtained by chiral HPLC analysis. The linear regression of the data shows a line of excellent fit with a slope of near unity and a *Y*-intercept near zero. The excellent fit is noteworthy that the corrected MS-values contain the error of two independent measurements (the observed % ee by MS and the % ee of the probe as determined by chiral HPLC). This study demonstrates the viability of a 'green' methodology by recycling chiral compounds as probe molecules, which can be used in assays designed to optimize reaction conditions. The assay also allows us to determine the absolute

Table 1
Selected co-solvent assay data for probes **3** and **6**

Co-solvent	Probe **3** corrected % ee			Probe **6** corrected % ee		
	10%	20%	30%	10%	20%	30%
i-PrOH	103	n.r.	n.r.	71	79	90
t-BuOH	85	n.r.	n.r.	71	77	85
DMSO	72	73	75	72	73	n.a.
MeCN	46	68	72	46	45	n.a.
DMF	70	60	66	67	72	n.a.
Dioxane	68	74	75	75	77	n.a.
Diglyme	66	72	74	71	71	n.a.
Triglyme	68	71	66	72	75	n.a.

n.r. = not reported due to weak signal intensity.
n.a = not available.

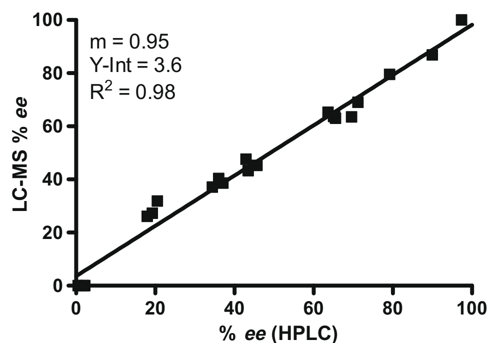


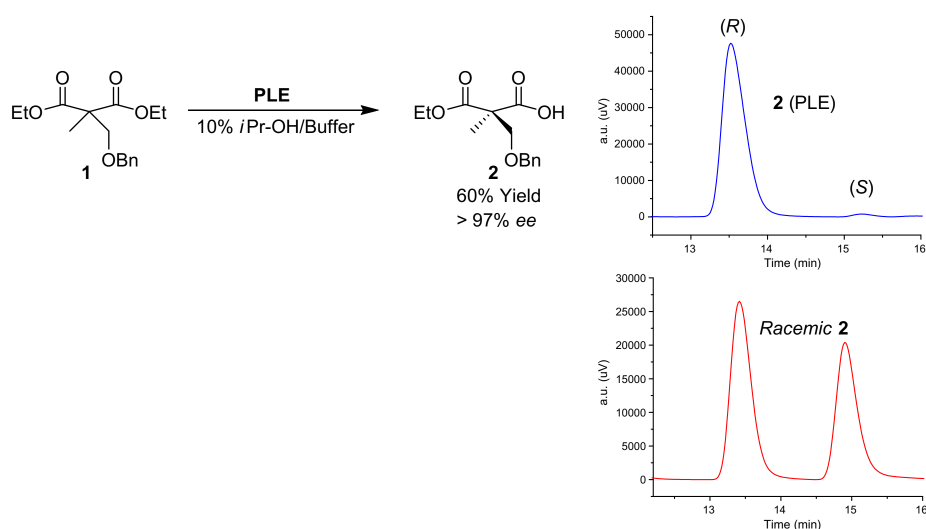
Figure 6. Correlation of HPLC% ee with corrected% ee by LC–MS.

stereochemistry of the hydrolysis reaction if the starting probe configuration is known by simple inspection of the H_5/D_5 half-ester ratio. We are currently using the MS assay described herein to find optimum reaction conditions for enzymatic hydrolysis of other prochiral malonates of interest in our laboratories and we will report on those efforts in due course.

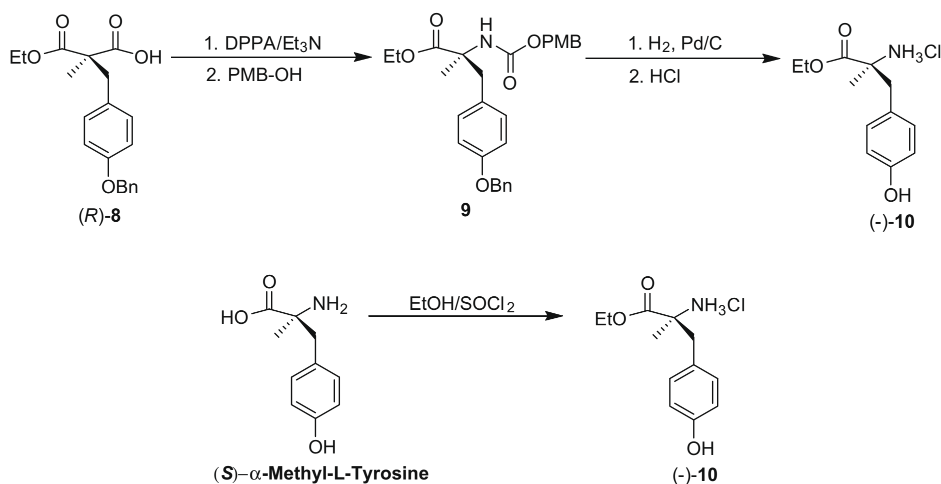
4. Experimental

4.1. General

THF was distilled from sodium under a nitrogen atmosphere prior to use. Methylene chloride and 1,2-dichloroethane were distilled from CaH_2 under a nitrogen atmosphere prior to use. Triethylamine



Scheme 5. Scaled-up synthesis of **2** by PLE hydrolysis in 10% *i*Pr-OH co-solvent (Chiralcel AD-H column).



Scheme 6. Assignment of absolute stereochemistry as (*S*) for **11**.

was distilled from NaOH pellets under a nitrogen atmosphere prior to use. Diphenylphosphorylazide (DPPA) was prepared using a literature procedure.[62] All other chemicals and enzymes were obtained from Aldrich Chemical and were used as received unless otherwise noted. Diethyl 2-(benzyloxymethyl)-2-methylmalonate **1** was synthesized according to a literature procedure.[4]

Mass spectrometry grade methanol was purchased from Aldrich. HPLC grade water was purchased from Fisher. Acetic acid was purchased from Aldrich. Auto sampler vials and caps were purchased from VWR. Samples were analyzed using either a ThermoFisher LXQ ESI-Ion trap mass spectrometer coupled to a ThermoFisher Accela HPLC system or a Bruker Microflex MALDI mass spectrometer in the LDI mode. All NMR spectra were acquired with a Varian Mercury 300 MHz spectrometer and referenced to either residual solvent protons or to TMS. IR spectra were acquired with a Thermo-Nicolet Nexus 470-FT-IR using a diamond anvil ATR accessory. UV–vis spectra were acquired with an HP 8452 spectrophotometer. Optical rotation measurements were acquired with a Rudolph Research Autopol III autopolarimeter using a 1 dm cell at ambient temperature. TLC analysis was performed on EMD science silica coated aluminum plates and was visualized using UV or phosphomolybdic acid stain. Flash chromatography was performed using Silicycle silica gel (Silia-P). Chiral HPLC was performed using a LabAlliance Series III isocratic pump coupled to a LabAlliance Model 500 UV–vis detector. HPLC chromatograms were recorded using the Peaksimple® data acquisition system and software. Chiral HPLC was performed using a Chiralcel OJ-H analytical column or a Chiralcel AD-H column from Chiral Technologies, Inc. HRMS analysis was performed at Old Dominion University on an Apex FT-MS using a 1:1 THF/MeOH solvent system with added NaCl to observe sodium adducts of the compounds of interest. Melting points were determined in an open capillary tube using a Hoover melting point apparatus and are uncorrected.

4.2. Enzyme assays (general procedure)

Approximately 10 mg of the probe under study **3** or **6** was placed in a 2.0 mL microcentrifuge tube along with 1.5 mL of reaction media (buffer or buffer/co-solvent mixtures). A 3 μL aliquot of a 50 mg/mL PLE solution (in 0.1 M phosphate buffer) was added (4.1 units total). The samples were loaded into an Eppendorf™ Thermomixer at 25 °C and 1400 RPM mixing rate. The samples were incubated and mixed continuously for three to five days for **3** and **6**, respectively. Aliquots were taken at the desired time intervals and were analyzed by either LC–MS or LDI-ToF MS. The observed ee values were corrected for the enantiomeric purity of the probe by dividing the observed ee by the enantiomeric purity of the probe as determined by chiral HPLC analysis.[35]

4.3. LDI-ToF MS

A 200 μL aliquot of reaction media was placed in a glass vial and acidified with 30 μL of 10% HCl solution. The acidified media were then extracted with 200 μL of CH_2Cl_2. A 2 μL aliquot of the organic phase was spotted directly onto a polished steel Microscout® chip (Bruker Daltonics). The sample was allowed to dry at ambient temperature by gently passing a stream of air over the Microscout® chip. An average of 800 laser pulses were collected for each sample and the intensities of the peaks were used for the calculation of enantiomeric excess.

4.4. Esi-ion trap ms

A 200 μL aliquot of reaction media was placed in a glass autosampler vial and was diluted with 200 μL of methanol. The Accela autosampler was programmed to inject 1 μL onto a Hypersil Gold RP HPLC column (50 × 2.1 mm, ThermoFisher). The mobile phase was 70:30 methanol/water at a flow rate of 100 μL/min. The LC–MS system was programmed to divert the initial 2 min of flow to waste to desalt the sample. The data were collected for the chromatographic peak in SIM mode and the intensities were used to calculate enantiomeric excess.

4.4.1. (*R*)-2-(4-(Benzyloxymethyl)-3-ethoxy-2-methyl-3-oxopropanoic acid 2

Synthesized using a literature procedure.[4] The characterization data match those of authentic material. The % ee was determined by analytical chiral HPLC Chriacel OJ-H (257 nm, flow rate = 1 mL/min, 4% *i*Pr-OH/96% hexane) $t_{R_{(R)}}$ = 16.90 min, $t_{R_{(S)}}$ = 19.20 min or Chiracel AD-H (45 °C, 257 nm, flow rate = 1 mL/min, 4% *i*Pr-OH/96% hexane) $t_{R_{(R)}}$ = 13.65 min, $t_{R_{(S)}}$ = 15.20 min.

4.4.2. D_5-Diethyl 2-(benzyloxymethyl)-2-methylmalonate 3

Compound **2** (0.45 g, 1.7 mMol) was dissolved in 25 mL of CH_2Cl_2 in a 50 mL round-bottomed flask with a magnetic stirbar. Thionyl chloride (1.8 mL, 25.2 mMol) was added and the solution was heated to reflux solvent overnight. The solution was concentrated in vacuo to remove excess thionyl chloride and solvent. The acyl chloride was dissolved in 10 mL of dry CH_2Cl_2. A solution of 220 μL of Et_3N in 1 mL of ethanol-d_6 was added dropwise to the stirring acyl chloride under a dry nitrogen atmosphere. The reaction mixture was stirred overnight at ambient temperature. The reaction mixture was diluted with 20 mL of ether and was washed three times each with 10% HCl and 1.0 M NaOH. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo to give **3** as a clear, viscous oil 0.43 g (1.41 mMol 84% yield) TLC (30% ether/hexane) R_f = 0.28. IR (cm^{-1}) 1727, ^1H NMR (300 MHz, $CDCl_3$) 1.21 (3H, t, *J* = 7 Hz), 1.52 (3H, s), 3.82 (2H, s), 4.16 (2H, q, *J* = 7 Hz), 4.53 (3H, s), 7.28 (5H, m). ^{13}C NMR (75 MHz, $CDCl_3$) 14.2, 18.6, 55.0, 61.5, 72.8, 73.6, 81.8, 127.6, 127.8, 128.5, 138.2, 170.8. HRMS: [$C_{16}H_{17}D_5O_5Na^+$] calcd = 322.1679 obsd = 322.1670.

4.4.3. (±)-D_5/H_5-2-(4-(Benzyloxymethyl)-3-ethoxy-2-methyl-3-oxopropanoic acid 5

Compound **1** (4.00 g, (13.6 mMol) was dissolved in 11 mL of ethanol after which 0.790 g of KOH was added in a 50 mL round-bottomed flask with a magnetic stirbar. After 48 h, the reaction mixture was diluted with 20 mL of water and extracted three times with 20 mL of ether. The aqueous layer was then acidified to pH 2 and extracted three times with 20 mL of CH_2Cl_2, the combined organic layers were dried over $MgSO_4$, filtered and concentrated in vacuo to give the racemic half ester of **2** (2.21 g, 8.30 mMol, 61% yield). The racemic half ester of **2** (0.446 g, 1.68 mMol) was then esterified by the same procedure reported to prepare **3** to give 0.422 g (1.41 mmol, 84% yield) of the racemic D_5/H_5 diester. The racemic D_5/H_5 diester (0.357 g) was dissolved in a 4:1 *i*Pr-OH/H_2O solution containing 95.5 μL of a 50% w/v aqueous NaOH solution. The reaction was monitored by TLC and was complete after 48 h. The reaction mixture was diluted with 20 mL of 1.0 M NaOH and was extracted three times with ether. The aqueous layer was acidified to pH 2 and extracted three times with CH_2Cl_2, dried over $MgSO_4$, filtered, and concentrated in vacuo to give racemic D_5/H_5 half ester **5** [0.209 g, 0.775 mMol (based on average molecular weight), 65% yield]. TLC (5% MeOH/CH_2Cl_2) R_f = 0.317. ^1H NMR (300 MHz, $CDCl_3$) 1.23 (1.5H, t, *J* = 7 Hz), 1.54 (3H, s), 3.81 (2H, s), 4.20 (1H, q, *J* = 7 Hz), 4.55 (2H, s), 7.28 (5H, m). The % ee was determined by analytical chiral HPLC (Chiracel AD-H, 257 nm, flow rate = 1 mL/min, 4% *i*Pr-OH/96% hexane) $t_{R_{(R)}}$ = 14.97 min, $t_{R_{(S)}}$ = 17.62 min.

4.4.4. Diethyl 2-(4-(benzyloxy)benzyl)-2-methylmalonate 7

Prepared in a similar fashion as reported in the literature.[59]A 250 mL three-necked round-bottomed flask was charged with

100 mL of dry THF, a stirbar, and 1.70 g (41.9 mMol) of NaH (60% dispersion in mineral oil). The flask was fitted with a rubber septum, a glass stopper, and a reflux condenser to which a nitrogen inlet was attached. The flask was placed in an ice bath and allowed to stir for 15 min. A solution of diethyl methylmalonate (6.07 g, 34.9 mMol in 5 mL dry THF) was added dropwise to the NaH suspension at 0 °C. The solution was allowed to stir at 0 °C until no further gas evolution was observed. The reaction mixture was removed from the ice bath and allowed to stir at ambient temperature for 90 min. A solution of 1-(benzyloxy)benzyl)-4-(chloromethyl)benzene (6.00 g, 38.3 mMol, in 30 mL dry THF) was added dropwise and the rubber septum was rapidly replaced with a glass stopper. The resulting solution was heated at reflux overnight. The solution was then allowed to cool to ambient temperature and 1 mL of water was added to quench any remaining NaH. The resulting suspension was diluted with 100 mL of ether and placed in a separatory funnel. The suspension was then washed three times with brine and three times with 10% HCl. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude material was dissolved in warm pentane and cooled to −25 °C for 5 h. The resulting white solid was isolated by vacuum filtration and washed with three 20 mL portions of cold pentane. 11.73 g (31.7 mMol, 91% yield) of **9** as a white amorphous solid. TLC (20% EtOAc/hexane) R_f = 0.3. Mp = 49 °C, IR (cm^{-1}) 1727, ^{1}H NMR (300 MHz, $CDCl_3$) 1.25 (6H, t, J = 7 Hz), 1.33 (3H, s), 3.17 (2H, s), 4.19 (4H, q, J = 7 Hz), 5.02 (s, 2H), 6.92 (2H, d, J = 9 Hz), 7.04 (2H, d, J = 9 Hz) 7.38 (5H, m). ^{13}C NMR (75 MHz, $CDCl_3$) 14.3, 19.9, 40.5, 55.1, 61.5, 70.2, 114.7, 127.7, 128.1, 128.7, 128.8, 131.4, 137.2, 158.0, 172.2. HRMS: [$C_{22}H_{26}O_5Na^+$] calcd = 393.1678 obsd = 393.1672.

4.4.5. (*R*)-2-(4-(Benzyloxy)benzyl)-3-ethoxy-2-methyl-3-oxopropanoic acid 8

Compound **7** (4.00 g, 10.8 mMol) was dispersed in 600 mL of rapidly stirring phosphate buffer (0.1 M, pH 7.4). Hundred milligrams of PLE (2100 units) was suspended in 1.0 mL of 3 M $(NH_4)_2SO_4$ and added to the buffer solution. The pH of the reaction mixture was maintained using a 798 MPT Titrino in the pH stat mode. The Titrino was set to titrate to a volume of 10.2 mL (1 equiv of 1.06 M NaOH). The hydrolysis proceeded for 20 h, after which time 40 mL of 1.06 M NaOH was added to make the solution sufficiently basic. The aqueous solution was then extracted three times with 300 mL portions of ether. The aqueous layer was acidified using concentrated HCl to a pH of 2. The aqueous layer was then extracted three times with CH_2Cl_2. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo to give 2.81 g (8.21 mMol, 76% yield, 63% ee) of a white, amorphous solid. TLC (20% EtOAc/hexane) R_f = 0.25. $[\alpha]_D^{22} = -1.0$ (c 0.066, CH_2Cl_2). Mp = 99.0 °C ^{1}H NMR (300 MHz, $CDCl_3$) 1.22 (3.0 H, t, J = 7 Hz), 1.40 (3H, s), 3.15 (1H, d, J = 14 Hz), 3.25 (1H, d, J = 14 Hz), 4.21 (2H, q, J = 7 Hz), 5.02 (2H, s), 6.88 (2H, d, J = 8 Hz), 7.08 (2H, d, J = 8 Hz)7.36 (5H, m), 11.50 (1H, br s). ^{13}C NMR (75 MHz, $CDCl_3$) 14.2, 20.1, 40.1, 55.2, 62.0, 70.1, 114.8, 127.7, 128.1, 128.2, 128.8, 131.4, 137.1, 158.1, 172.8, 178.0. HRMS: [$C_{20}H_{22}O_5Na^+$] calcd = 365.1365 obsd = 365.1359. The % ee was determined by analytical chiral HPLC (Chiracel AD-H, 282 nm, flow rate = 1 mL/min, 4% *i*Pr-OH/96% hexane) $t_{R_{(R)}}$ = 20.87 min, $t_{R_{(S)}}$ = 30.18 min.

4.4.6. (±)-D_5/H_5-2-(4-(Benzyloxy)benzyl)-3-ethoxy-2-methyl-3-oxopropanoic acid 8b

Compound **7** (3.24 g, 8.75 mMol) was dissolved in 20 mL of ethanol and 701 μL of a 50% w/v aqueous NaOH solution was added in a 50 mL round-bottomed flask with a magnetic stirbar. After 24 h, the reaction mixture was diluted with 40 mL of water and washed three times with Et_2O. The aqueous layer was then acidified to pH 2 and extracted three times with CH_2Cl_2, dried over $MgSO_4$, filtered, and concentrated in vacuo to give the racemic (2.27 g, 6.62 mmol, 76% yield) half ester (±)-**8**. Compound (±)-**8** was then esterified by the same procedure reported for **3** to give 0.391 g (1.043 mMol, 71% yield) of the racemic D_5/H_5 diester. The racemic D_5/H_5 diester (0.618 g, 1.646 mMol) was then dissolved in 13 mL of a 4:1 *i*Pr-OH/water solution containing 132 μL of 50% w/v aqueous NaOH solution was added. The reaction was monitored by TLC (5% MeOH/CH_2Cl_2) and was completed after 48 h. The reaction mixture was diluted with 20 mL of 1.0 M NaOH and washed three times with Et_2O. The aqueous layer was then acidified to pH 2 and extracted three times with CH_2Cl_2, dried over $MgSO_4$, filtered, and concentrated in vacuo to give 0.49 g (0.078 Mol, 87% yield) of the racemic D_5/H_5 half ester **8b**. TLC (5% MeOH /CH_2Cl_2) R_f = 0.5. ^{1}H NMR (300 MHz, $CDCl_3$) 1.26 (1.5H, t, J = 7 Hz), 1.40 (3H, s), 3.13 (1H, d, J = 14 Hz), 3.25 (1H, d, J = 14 Hz), 4.20 (1H, q, J = 7 Hz), 5.01 (2H, s), 6.88 (2H, d, J = 8 Hz), 7.08 (2H, d, J = 8 Hz)7.38 (5H, m). Compound **8b** was used to prepare samples of **8** whose % ee was less than 63% to provide additional data points for Figure 6 in a similar fashion to that shown in Scheme 3.

4.4.7. D_5-Diethyl 2-(4-(benzyloxy)benzyl)-2-methylmalonate 6

Compound **10** (0.50 g, 1.46 mMol) (from PLE hydrolysis) was dissolved in 15 mL of CH_2Cl_2 in a 25 mL round-bottomed flask with a magnetic stirbar. Thionyl chloride (2 mL, 27.8 mMol) was added and the solution was brought to reflux for 15 h. The solution was concentrated in vacuo and the crude acyl chloride was dissolved in 10 mL of dry CH_2Cl_2.Then, 220 μL of Et_3N was dissolved in 1 mL of ethanol-d_6 and added dropwise to the stirring acyl chloride. The reaction mixture was stirred for 15 h. at ambient temperature under an N_2 blanket. The reaction mixture was diluted with 20 mL of Et_2O and washed three times each with 10% HCl and 1.0 M NaOH. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo to give **6** as a white amorphous solid.10% EtOAc/hexane (R_f = 0.2). This gave 0.39 g (1.04 mMol, 71% yield) of a white amorphous solid. Mp = 49 °C, IR (cm^{-1}) 1726, ^{1}H NMR (300 MHz, $CDCl_3$) 1.24 (3H, t, J = 7 Hz), 1.33 (3H, s), 3.17 (2H, s), 4.18 (2H, q, J = 7 Hz), 6.86 (2H, d, J = 9 Hz), 7.04 (2H, d, J = 9 Hz) 7.37 (5H, m). ^{13}C NMR (75 MHz, $CDCl_3$) 13.1 (sept, J = 20 Hz), 14.2, 19.8, 40.4, 55.0, 60.6 (quint, J = 22 Hz), 61.5, 70.1, 114.6, 127.6, 128.0, 128.5, 128.7, 131.3, 137.1, 157.9, 172.1. HRMS: [$C_{22}H_{21}D_5O_5Na^+$] calcd = 398.1992 obsd = 398.1983.

4.4.8. (*S*)-Ethyl-3–(benzyloxy)-2-((4-methoxybenzyloxy)carbonylamino)-2-methylpropanoate 9

Compound **8** (0.310 g, 0.90 mMol) was dissolved in 10 mL of dichloroethane in a 50 mL round-bottomed flask with a magnetic stirbar. Then 215 μL (0.996 mMol, 1.1 equiv) of DPPA and 409 μL (2.72 mMol, 3.0 equiv) of Et_3N was added and the solution was heated at reflux for 1.5 h, at which time 169 μL (1.36 mMol, 1.5 equiv) of *para*-methoxybenzyl alcohol (PMB-OH) was added and the solution was allowed to reflux for 15 h. The mixture was then cooled and diluted with 20 mL of chloroform and washed with 10% HCl. The organic layer was dried and concentrated in vacuo. The residue was purified by flash chromatography (SiO_2, 50% Et_2O/50% hexane) to give 0.29 g (0.61 mMol, 67% yield, 63% ee) of a clear viscous oil. TLC (50% Et_2O/hexane) R_f = 0.35. $[\alpha]_D^{17.8} = +24.2$ (c 0.07, CH_2Cl_2). IR 3426, 3354, 1716. ^{1}H NMR (300 MHz, $CDCl_3$) 1.27 (3H, t, J = 7 Hz), 1.61 (3H, s), 3.09 (1H, d, J = 13 Hz), 3.36 (1H, d, J = 13 Hz), 3.80 (3H, s), 4.18 (1H, m), 5.00 (2H, s), 5.00 (1H, d, J = 12 Hz), 5.10 (1H, d, J = 12 Hz), 5.44 (1H, br s), 6.78 (2H, d, J = 9 Hz), 6.89 (4H, m), 7.37 (7H, m). ^{13}C NMR (75 MHz, $CDCl_3$) 14.3, 23.8, 41.0, 55.5, 60.9, 61.9, 66.4, 70.1, 114.0, 114.7, 127.7, 128.1, 128.6, 128.8, 128.9, 130.3, 131.1, 137.2, 154.9, 157.9, 159.7, 173.8. HRMS: [$C_{28}H_{31}NO_6Na^+$] calcd = 500.2049 obsd = 500.2044. The ee was determined by analytical chiral HPLC (Chiralcel AD-H, 282 nm, 4% *i*Pr-OH/96% hexane) $t_{R_{(S)}}$ = 34.38, $t_{R_{(R)}}$ = 39.35.

4.4.9. (*S*)-α-Methyl tyrosine ethyl ester 10

At first, 0.5 g of 10% Pd/C was placed in a 50 mL round-bottomed flask and was carefully wetted with 10 mL of ethanol. Then, 0.650 g of **9** was dissolved in 10 mL of ethanol and was slowly added to the flask. The solution was sparged with hydrogen gas for 15 min and then placed under a hydrogen blanket for 24 h with rapid stirring. The reaction mixture was filtered through a Celite pad to remove the Pd/C and concentrated in vacuo. The residue was dissolved in 10% HCl and washed three times with CH_2Cl_2, pH adjusted to 9 with NaOH, and extracted with CH_2Cl_2. The resulting residue was concentrated in vacuo to give a white hygroscopic amorphous solid. Polarimetry was performed in 1.21 M HCl. Due to the hygroscopic nature of **10**, an accurate mass of the sample was difficult to obtain. The levorotary direction of rotation $[\alpha]_{obs}^{21.7} = -0.1$ (*c* 1.2 M, HCl)) of **10** matched that of an authentic sample. ^{1}H NMR (300 MHz, CD_3OD) 1.26 (3H, t, *J* = 7 Hz), 1.54 (3H, s), 2.95 (1H, d, *J* = 14 Hz), 3.15 (1H, d, *J* = 14 Hz), 4.23 (2H, m), 6.73 (2H, d, *J* = 9 Hz), 6.97 (2H, d, *J* = 9 Hz).

Acknowledgments

We would like to thank the National Science Foundation (MCB0639817) for support of this work and (CHE 0639208 and DBI 0619455) for funds to acquire the MS facilities used in this study. We would also like to thank the Department of Chemistry and Biochemistry at USM for start-up funds. DAR would like to thank the Department of Education for a GAANN Fellowship (#P200A060323). We would also like to thank Mrs. Tina Masterson for reviewing the manuscript prior to submission.

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