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Synthesis of The Stereoismomers of DEHP and MEHP from Kinetically Resolved 2-ethyl-1-hexanol

Lonnie Alexander Harris

SYNTHESIS OF THE STEREOISMOMERS OF DEHP AND MEHP FROM KINETICALLY RESOLVED 2-ETHYL-1-HEXANOL

An honors paper submitted to the Department of Chemistry
of the University of Mary Washington
in partial fulfillment of the requirements for Departmental Honors

Lonnie Alexander Harris
April 2016

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Lonnie Harris
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Title: Synthesis of the Stereoisomers of DEHP and MEHP from Kinetically Resolved 2-ethyl-1-hexanol

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Synthesis of the Stereoisomers of DEHP and MEHP from Kinetically Resolved 2-ethyl-1-hexanol

By

Lonnie Harris

Thesis submitted to the faculty of the University of Mary Washington in partial fulfillment of the requirements for graduation with Honors in Chemistry.

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Abstract

Di(2-ethylhexyl) phthalate (DEHP) is a known chiral persistent organic pollutant found in many different consumer products, and the toxicities of its stereoisomers and their metabolites are not well known. To determine their adverse health effects, each enantiomer has to be synthesized. Using lipase PS from *Burkholderia cepacia* in dichloromethane at 0˚C for 48 hours, 2-ethyl-1-hexyl acetate was obtained in 55% yield (75:25 e.r.), while (R)-2-ethyl-1-hexanol (**1**) was obtained in 29% yield (96:4 e.r.). The acetate was then hydrolyzed to recover the (S) enriched alcohol (82% yield). The enriched alcohol was acylated a second time to yield (S)-(**1**) in 39% yield (91:9 e.r.). R-(**1**) and phthalic anhydride were reacted in 1:3 pyridine/toluene at 100˚C for 2 hours to synthesize (R)-mono(2-ethylhexyl) phthalate ((R)-MEHP) in 50% yield. Difficulty was encountered in separating MEHP from (**1**) in the reaction mixture, with numerous solvent systems, vacuum distillation, and chemically active extraction attempted without success. The resulting impure (R)-MEHP was reacted with (R)-(**1**) in the presence of N,N'-diisopropylcarbodiimide and 4-dimethylaminopyridine in dichloromethane for 19 hours at room temperature to synthesize(R,R)- DEHP in 36% yield. Future work will synthesize the other isomers of DEHP, further purify (**1**), and successfully separate MEHP from (**1**).

Acknowledgements

I would first and foremost like to thank Dr. Oldham for his guidance over the last three years. I have learned a lot from him, and will miss his mentorship in the future. Secondly, I would like to thank Kelly McDaniel for being a great lab partner, even when I made the lab a total disaster. I would also like to thank my parent for always believing in me, even when I did not. Finally, I would like to thank Abigail Milone for her constant emotional support. I could not have done it without you.

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Introduction

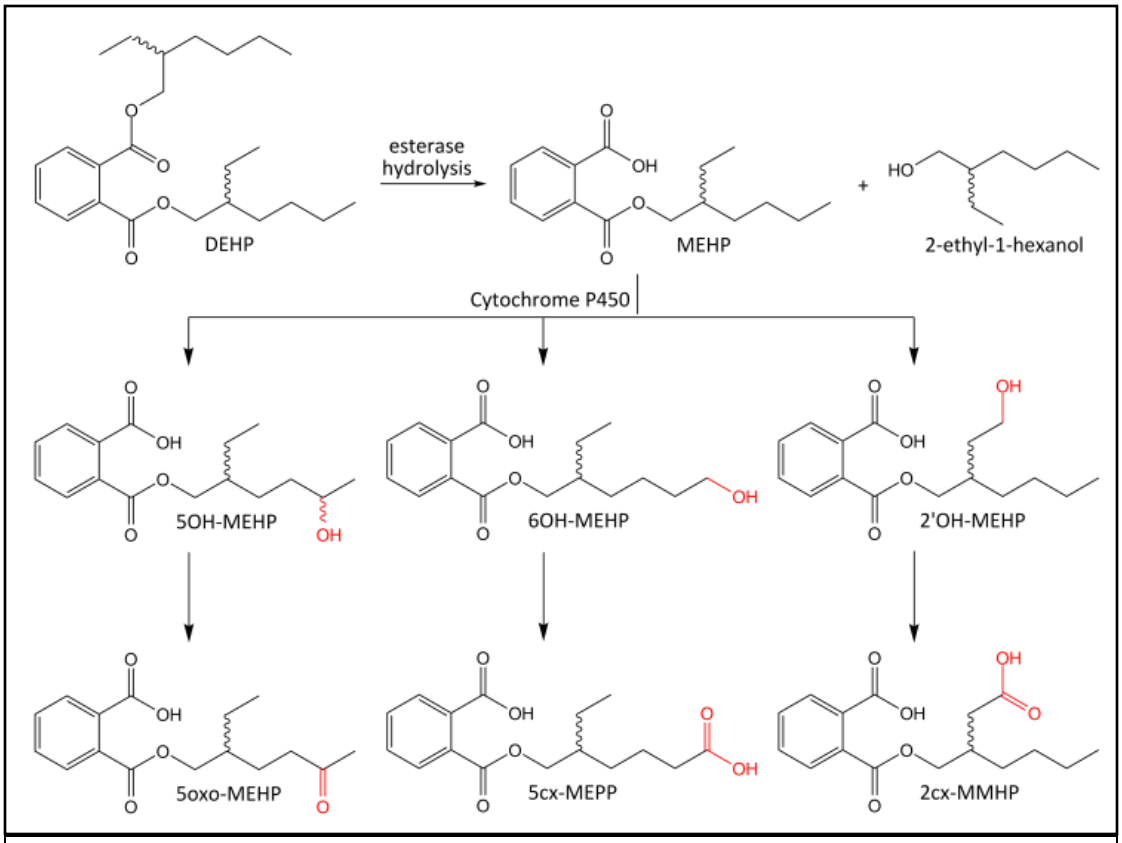
Di(2-ethylhexyl) phthalate (or DEHP) is a compound used industrially as a plasticizer for polyvinyl chloride (PVC) (Scheme 1). In this application, it can be found in many common products, such as blood bags and other medical equipment, toys, and vinyl flooring.[1] In addition, DEHP and other phthalates are a common additive to products ranging from oils and lubricants to cosmetics. Industrial production of DEHP for these applications exceeds 2 million tons per year.[2] Due to this widespread usage and production, DEHP is a common environmental contaminant. Human exposure to DEHP is mainly thought to occur through ingestion, with leaching from plastic containers, food wrappers and inhalation of contaminated dust being the predominant pathways. Other exposure routes include intravenous exposure through blood and other medical bags, dermal contact with contaminated surfaces, and transfer between mother and fetus during pregnancy.[3,4]

Previous research has shown that DEHP and other phthalates are endocrine disruptors,[5] with exposure to phthalates causing developmental and reproductive abnormalities in laboratory animals. Some of these effects include reduced sperm production in zebra fish,[6] decreased estradiol levels and growth of antral follicles cultured from mice,[7] reduced fertility and birth defects in rats,[8] and testicular abnormalities in rodents[1]. Studies in human populations have also correlated high levels of phthalates with abnormal reproduction and development. These include decreased masculine behavior in male children,[9] reduced anogenital distance in male newborns,[10] and reduced fertility.[11]

Upon ingestion, DEHP is hydrolyzed to form mono(2-ethylhexyl) phthalate (MEHP), which is subsequently oxidized to form several metabolites (Scheme 1). The oxidation of MEHP is accomplished in the liver by the cytochrome P450 (CYP) family of enzymes. These heme containing enzymes use molecular oxygen to oxidize a wide range of compounds, including

fatty acids, sterols, and therapeutic drugs. Indeed, it is estimated that CYP enzymes are responsible for metabolizing 75% of the drugs on the market.[12] The purpose of this oxidation is to increase the polarity of these molecules so that they are more soluble in water, allowing them to be excreted in urine. The effect of this oxidation is highly variable. For many compounds, metabolism by CYP enzymes renders them inactive. On the other hand, some are rendered more potent. Notable examples of this are prodrugs, which are ingested in an inactive form but are metabolized into their therapeutic form.[13] This dichotomy is not just limited to therapeutic drugs but is also seen in toxic compounds.

Some of the oxidative metabolites produced by CYP enzymes that have been detected in urine and blood serum are mono(2-ethyl-6-hydroxyhexyl) phthalate (6-OH –MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (5-OH-MEHP), mono(2-ethyl-5-oxyethyl) phthalate (5-oxo-MEHP), and mono(2-ethyl-5-carboxypentyl) phthalate (5-carboxy-MEPP) (Scheme 1).[5,14] Some of these metabolic products are thought to be more toxic than DEHP itself, with MEHP causing higher fetal mortality and abnormalities in mice than DEHP.[8] The toxicity of DEHP has been attributed to both estrogen receptor and peroxisomal proliferator-activated receptor (PPAR) activation. Considerable research has been devoted to determining the relative contributions of each pathway to the toxicity of DEHP.[15,16] Both PPAR and estrogen receptors are nuclear receptors, meaning that they are associated with nuclear DNA and regulate gene expression at the transcriptional level. The estrogen receptor mainly regulates genes associated with sexual development and reproduction, but also regulates such disparate processes as lipid metabolism and glucose uptake.[17] PPARs as a class are mainly involved in lipid metabolism, but like estrogen have disparate roles in inflammation and fertility.[18]

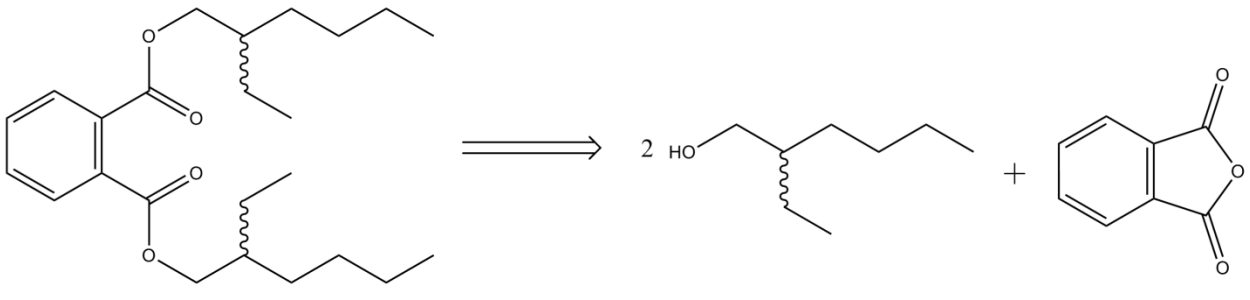


Scheme 1: Metabolism of DEHP and the resulting oxidative metabolites (Reproduced courtesy of Kelly McDaniel)

In recent years, the chirality of environmental pollutants has become an important avenue of research. Biological systems are inherently sensitive to changes in three dimensional configuration, and the change of a single chiral center can affect the biological activity of a compound. A classic example of this phenomenon is the drug thalidomide, a sedative and antiemetic sold from 1957 to 1961. Due to its effectiveness in treating nausea, the drug was widely prescribed to treat morning sickness in pregnant women. It was discovered that taking thalidomide during pregnancy caused abnormal limb development in fetuses. Thalidomide contains a single chiral center and was sold as a racemic mixture. The R enantiomer was an effective morning sickness treatment, while the S enantiomer caused birth defects.[19] Other examples include pyrethroid insecticides, organophosphate pesticides, and some polychlorinated biphenyls (PCB's). These three classes of compounds are chiral and have very different environmental fates and biological activity that are dependent upon stereochemistry.[20]

Industrially synthesized DEHP contains three different stereoisomers, (R,R)-DEHP, (R,S)-DEHP, and (S,S)-DEHP. Both (R,R)-DEHP and (S,S)-DEHP are chiral molecules, while (R,S)-DEHP is a meso compound. In addition, each DEHP metabolite mentioned above has at least two stereoisomers. Due to the different spatial configuration of these isomers, it is likely that they have different binding affinities for PPAR and estrogen receptors and thus vary in their toxicity. Moreover, the relative rates of metabolism between the different stereoisomers of MEHP and its metabolites by cytochrome P450 enzymes are also unknown, meaning that their retention in the body could vary. However, measuring receptor binding and enzyme metabolism would be difficult using the racemic mixtures of DEHP and its metabolites. In an achiral environment, enantiomers have the same physical and chemical properties under most conditions. Thus, a method for synthesizing enantiomerically pure samples of these compounds is needed.

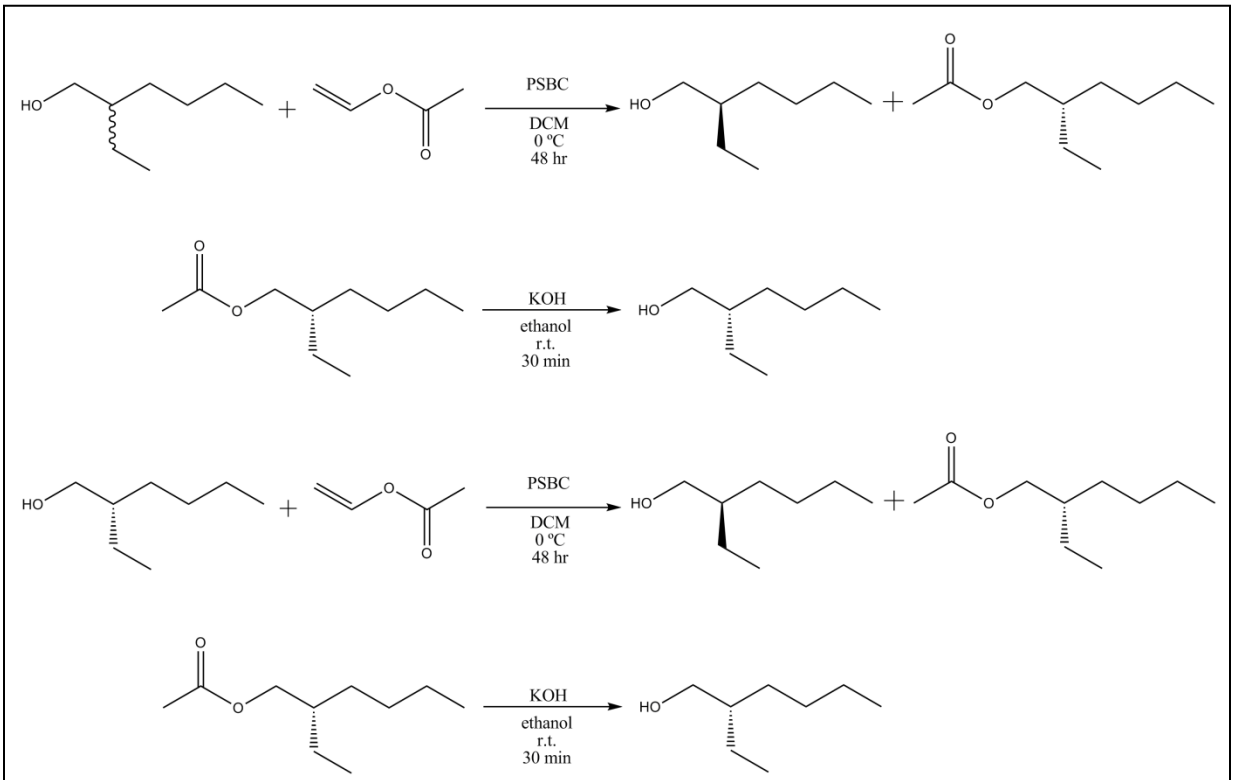
DEHP is industrially produced via an esterification reaction between one equivalent of phthalic anhydride and two equivalents of 2-ethyl-1-hexanol (Scheme 2). Since the two chiral centers of DEHP are introduced via the two 2-ethyl-1-hexyl chains, resolution of 2-ethyl-1-hexanol and then a stepwise reaction of two equivalents of enantiomerically pure 2-ethyl-1-hexanol would produce a single stereoisomer of DEHP with a known configuration.



Scheme 2: Synthesis of DEHP from 2-ethyl-1-hexanol and phthalic anhydride. The two chiral centers present in DEHP are introduced through the 2-ethylhexyl moieties, meaning that the stereoisomers of DEHP can be synthesized using enantiomerically pure samples of 2-ethyl1-hexanol.

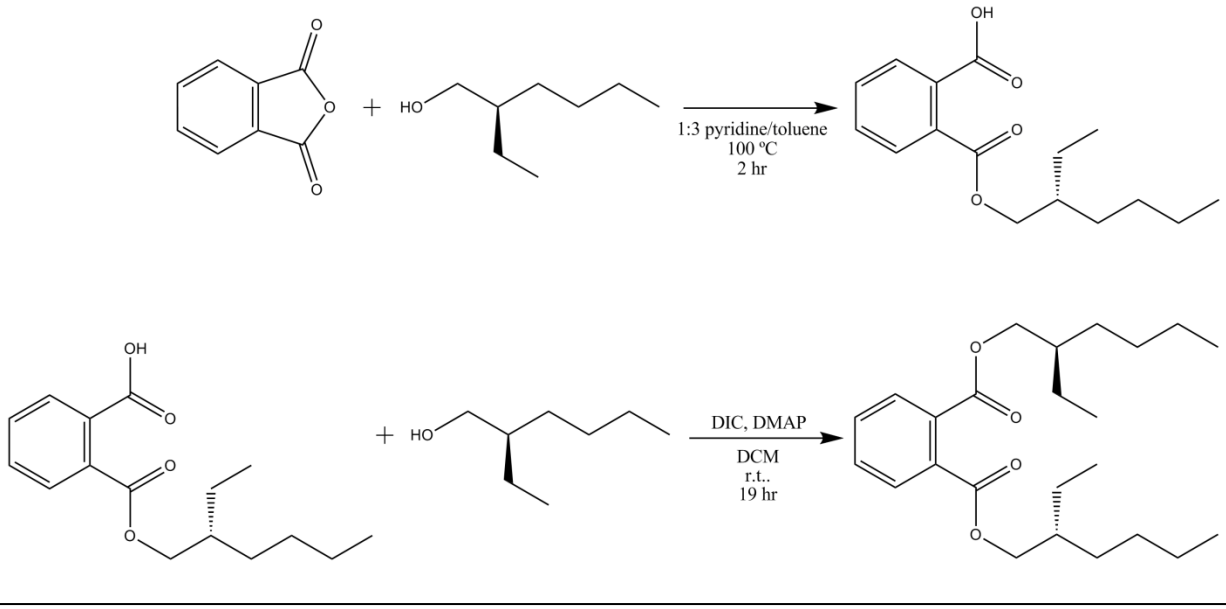
Numerous methods exist for preparing enantiomerically pure samples of primary alcohols. One strategy involves utilizing stereoselective reactions such as the Evans[21,22] and Meyers[23] alkylations. However, these methods have several drawbacks. Syntheses tend to be multistep, more challenging, and require each enantiomer to be synthesized independently. A second method utilizes biologically derived enzymes to kinetically resolve a racemic mixture of 2-ethyl-1-hexanol. This method takes advantage of the stereoselectivity inherent to most biological systems. By lowering the activation energy required to react one of the enantiomers, one enantiomer is chemically modified, leaving the other enantiomer unreacted. This method is particularly attractive because it allows both enantiomers of 2-ethyl-1-hexanol to be isolated simultaneously.

Of particular importance here is the work of Chantal and Krystyna,[24] who used a lipase isolated from the bacterial species *Burkholderia cepacia* (PSBC) to resolve 2-ethyl-1-hexanol in high enantiomeric purity. The enzyme was used to transfer an acyl group from vinyl acetate to (S)-2-ethyl-1-hexanol, leaving behind (R)-2-ethyl-1-hexanol. Purification by column chromatography allows the (R) alcohol and (S) acetate to be separated, with recovery of the (S) acetate only requiring a simple hydrolysis reaction under basic conditions. By varying the solvent, temperature, and reaction time, the researchers were able to maximize the enantiomeric purity of the two enantiomers. Two rounds of acylation and hydrolysis were required to recover the (S) alcohol in high enantiomeric purity. The reactions used by the researchers are summarized in Scheme 3.



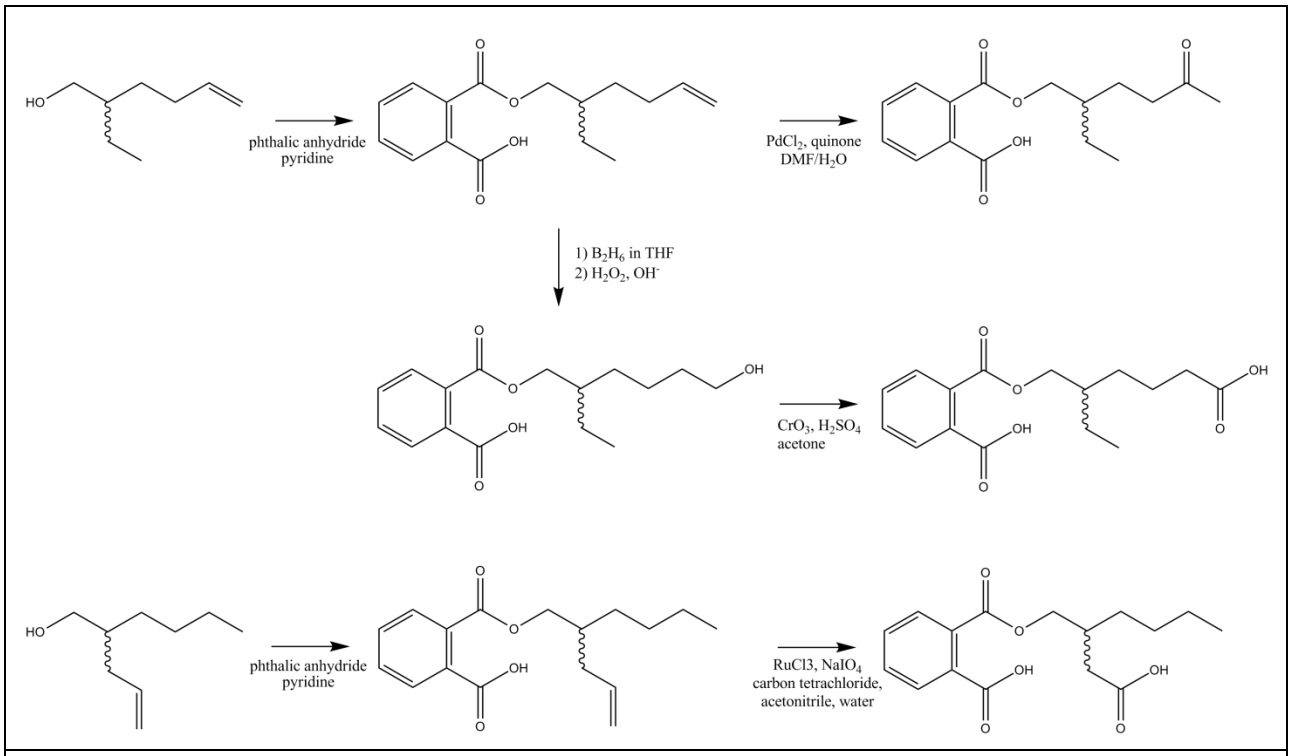
Scheme 3: Enzymatic resolution of 2-ethyl-1-hexanol using PSBC

Once resolved, these samples can be used to synthesize MEHP and DEHP of a known configuration. Reacting one equivalent of phthalic anhydride with 2-ethyl-1-hexanol yields MEHP, which can then be reacted with a second equivalent of 2-ethyl-1-hexanol to produce DEHP. Because the synthesis of DEHP requires the two equivalents of 2-ethyl-1-hexanol to be added stepwise, the configuration of the resulting molecule can be easily controlled. The reactions used to synthesize MEHP and DEHP are shown in Scheme 4, with the synthesis of (R)-MEHP and (R,R)-DEHP shown specifically.



Scheme 4: Proposed synthesis of R-MEHP and R,R-DEHP

The utility of these reactions are not limited to resolving 2-ethyl-1-hexanol and synthesizing MEHP and DEHP. The enzymatic resolution outlined in Scheme 2 and the reactions outlined in Scheme 3 could be used with other alcohols, namely 2-ethyl-5-hexen-1-ol and 2-allyl-1-hexanol. Enantiomerically pure samples of these alcohols could then be used to synthesize chiral versions of several oxidative metabolites of MEHP, such as 6-OH-MEHP, 5-oxo-MEHP, 5cx-MEPP, and 2cx-MHPP. The reactions necessary to synthesize these metabolites from 2-ethyl-5-hexen-1-ol, 2-allyl-1-hexanol and phthalic anhydride are shown in Scheme 5.



Scheme 5: Synthesis of MEHP metabolites

Once synthesized, the chiral samples of DEHP, MEHP, and the oxidative metabolites could be used in a variety of studies. One possibility is to conduct *in vivo* toxicological studies using model organisms, such as mice and zebra fish. In addition, the binding affinity of each compound to PPAR can be analyzed using a thermal shift assay. This assay measures binding affinity by the increased melting temperature of a protein-ligand complex compared to the free protein. During melting, a protein unfolds, exposing its hydrophobic core. When a fluorescent dye is present in the solution, this dye associates with the hydrophobic core and fluoresces at a higher intensity. This allows the melting temperature of the protein to be determined by measuring the fluorescence of the solution. When ligand is bound to the protein, the energy of the complex is lower in energy than the unbound protein. This means that the protein-ligand complex requires more energy to melt than the unbound protein, corresponding to a higher melting temperature. Because binding affinity is a measure of the stability of the protein-ligand

complex, this allows a direct measure of the binding affinity of PPAR for DEHP, MEHP and the oxidative metabolites.[25,26]

In addition, the oxidation of the enantiomers of MEHP by CYP enzymes can also be investigated. Like the lipase used to resolve 2-ethyl-1-hexanol, enzymes in general tend to be stereoselective. This means that one enantiomer of MEHP could take longer to be oxidized. Because excretion of MEHP depends on its oxidation, one enantiomer could have a longer lifetime in the body and exacerbate any toxic effects. In addition, it is possible that CYP enzymes will produce different oxidative metabolites from the two enantiomers. To our knowledge, no research in this area has been conducted. By incubating CYP enzymes with enantiomerically pure MEHP and the required cofactors, the extent of reaction and products formed can be measured and quantitated using GC-MS, HPLC or LC-MS.

Experimental

General: All solvents and solutions were used without further purification unless otherwise indicated. The manufacturers and grades of all solvents and reagents can be found in the appendix. Solutions for NMR spectroscopy were made in deuterated chloroform with 0.1% trimethylsilane as a reference and run on a Varian EM360A NMR (60 MHz). Infrared spectra were obtained on a Perkin-Elmer RX1 FTIR using neat samples pressed between KBr salt plates. All products were purified via flash chromatography on a Biotage Isolera One equipped with SNAP Ultra columns.

Acylation Optimization: Lipases from *Pseudomonas flourescens,* porcine pancreas, and *Burkholderia cepacia* were screened using 2-ethyl-1-hexanol (0.65 g, 5 mmol) and vinyl acetate (1.72 g, 20 mmol, 4 eq) and varying temperatures, solvents and reaction times. To take kinetic data, 3-4 drops of the reaction mixture were filtered over Celite packed in a Pasteur pipette and washed with approximately 2 mL of dichloromethane. These solutions were then analyzed using chiral Gas Chromatography with a flame ionized detector (GC-FID)

Selective Acylation: 2-ethyl-1-hexanol (2.60 g, 20 mmol), vinyl acetate (6.89 g, 80 mmol, 4 eq), and lipase (amano lipase from *Burkholderia cepacia*, 0.300 g, 15 mg/mmol alcohol) were combined in 20 mL dichloromethane and stirred at 0^{o}C for 48 hours. The reaction mixture was then filtered over Celite to remove the lipase, and the solvent evaporated under reduced pressure. The crude product was then purified via automated flash chromatography on silica in an ethyl acetate/hexanes gradient (2-20% ethyl acetate). The fractions were tested by thin layer chromatography (1:9 ethyl acetate hexanes, stained with CAM). The fractions were then combined and evaporated under reduced pressure to yield (R)-2-ethyl-1-hexanol (29% yield, 96:4 e:r) and (S)-2-ethyl-hexyl acetate (55%, 75:25 e:r). 2-ethyl-1-hexanol: FTIR: 3337, 2958, 2928, 2869, 1462, and 1041 cm^{-1}. ^{1}H NMR (60 MHz): δ=0.73-1.42 ppm, m, 16H, Me, Et and OH

protons; 3.54 ppm, d, 2H, HO-Et protons. m/z: 29, 41, 57, 70, 83, 98, 112. 2-ethylhexyl acetate: FTIR: 2959, 1743, 1644, 1037 cm^{-1}. ^{1}H NMR (60 MHz): δ=0.77-1.59 ppm, m, 15 H, Me and Et protons; 2.01 ppm, s, 3 H, Ac-Me H; 3.91-3.99 ppm, d, 2H, HO-Et protons. m/z: 29, 43, 57, 70, 83, 112.

Hydrolysis of 2-ethylhexyl acetate: 2-ethylhexyl acetate was then combined with aqueous potassium hydroxide (5.93 M, 2 eq) in absolute ethanol (to 0.33 M acetate) and stirred at room temperature for 30 minutes. The reaction mixture was tested by thin layer chromatography (9:1 hexanes/ethyl acetate and visualized with CAM). Once deemed complete by TLC, the reaction was then acidified with hydrochloric acid (50 mL, 1 M) and diluted with deionized water (100 mL) to form a cloudy solution. The mixture was then extracted with four 20 mL portions of ether. The ether layers were then combined, washed with brine, and dried with magnesium sulfate. The solution was then evaporated under reduced pressure to yield (S) enriched 2-ethyl-1-hexanol (82% yield, 75:25 e.r.)

Further purification of (S)-2-ethyl-1-hexanol: The acylation and hydrolysis procedures detailed above were run a second time, scaling down for the small amount of (S)-2-ethyl-1-hexanol. (39% yield, 91:9 e.r.)

Synthesis of MEHP: 2-ethyl-1-hexanol (0.26 g, 2 mmol) and phthalic anhydride (0.30 g, 2mmol, 1 eq) were combined in 1:3 pyridine/benzene (0.8 mL, to 2.5 M) The mixture was stirred at 100^{o}C for 2 hours, then quenched with hydrochloric acid (1 M, 10 mL). The heterogeneous mixture was then extracted with three 10 mL portions of dichloromethane, washed with 10 mL brine, and dried with magnesium sulfate. The solution was then evaporated under reduced pressure to yield the crude product. Various purification methods were attempted and detailed in the Results and Discussion section. FTIR: 3075, 2960, 2931, 2873, 1702, 1600, 1581, 1290,

1075 cm^{-1}. ^{1}H NMR: δ=0.82-1.47 ppm, m, 17 H, Me and Et H; 4.17-4.25 ppm, d, 2H, COOR-Et H; 7.60-7.94 ppm, 4H, Ar-H; 10.36 ppm, s, COO-H. m/z: 29, 41, 43, 57, 70, 83, 149, 167.

Synthesis of DEHP: 2-ethyl-1-hexanol (0.12 g, 0.91 mmol) was combined with MEHP (0.28 g, 1 mmol, 1.1 eq), diisopropylcarbodiimide (0.14 g, 1.1 mmol, 1.2 eq), and 4-dimethylaminopyridine (0.0022 g, 0.02 mmol, 0.002 eq) in dichloromethane (5.2 mL, 0.2 M alcohol). The mixture was stirred at room temperature for 19 hours. The cloudy mixture was diluted with 25 mL dichloromethane, filtered over Celite, and then evaporated under reduced pressure. The crude product was then purified via flash chromatography in a hexanes/ethyl acetate gradient (2-20% ethyl acetate). The fractions were tested via thin layer chromatography (1:1 ethyl acetate/hexanes, stained with CAM). The fractions were then combined and evaporated under reduced pressure to yield DEHP (39% yield) FTIR: 2959, 2931, 2867, 1730, 1462, 1279, and 1126 cm^{-1}. NMR (60 MHz): δ=0.80-1.59 ppm, m, 32 H, Me, Et and OH protons; 4.26-4.17 ppm, d, 4H, Et H; 7,42-7.80 ppm, 4 H, Ar-H. m/z: 29, 43, 57, 71, 83, 149, 167, 279, 391.

Gas Chromatography Mass Spectroscopy: MEHP and DEHP were analyzed on a Shimadzu GC-2010plus equipped with a QP2010SE MS with a Shimadzu SH-Rxi-5Sil column and the following method: ramp from 40^{o}C to 300^{o}C at 20.00^{o}C/min with a 5 minute hold at 300^{o}C. The injector and interface temperatures were 300^{o}C, and the ion source temperature was 200^{o}C. The 1 µL injection was split 20.00:1, with a pressure of 91.5 kPa , linear velocity of 46.1 cm/s, and helium as the carrier gas. The MS scanned from m/z 25-400 with a scan speed of 1428 three minutes after injection.

Chiral Gas Chromatography Flame Ionization Detection: 2-ethyl-1-hexanol and 2-ethylhexyl acetate were analyzed on an Agilent 7820A GC-FID with a Restek βdex SA or SE column according to the following method: ramp from 50^{o}C to 180^{o}C at 1^{o}C/min, no hold, with an

injector temperature of 230^{o}C. The 1 µL injection was split 50:1 with a pressure of 10.621 psi, linear velocity of 37 cm/s, and helium as the carrier gas.

Results and Discussion

Optimization of Acylation

In order to determine the optimum conditions and catalyst best suited to resolve the enantiomers of 2-ethyl-1-hexanol**,** the lipase, temperature, and solvent were varied. Vinyl acetate was used as the acyl donor in all reactions based upon previous research by Chantal *et al*.,[24] as well as similar enzymes to those used by the researchers. Lipase from *Pseudomonas flourescens* (PLF) was first tested with 3.5 mg lipase per mmol 2-ethyl-1-hexanol at room temperature for 72 hours in THF. These conditions proved too reactive, with only 2-ethylhexyl acetate isolated after purification by column chromatography. To try and improve the stereoselectivity of PLF, the temperature was lowered to 0^{o}C. Though some alcohol was isolated, PLF was still deemed too reactive for effective separation, as the majority was reacted to form acetate.

Lipase from porcine pancreas (PPL) was then tested at 15 mg per mmol alcohol and 0^{0}C for 24 hours in dichloromethane. The enzyme loading was increased relative to PLF based on previous research,[24] which had found PPL to be less reactive compared to PLF. Unlike PLF, there was a fairly even split of 2-ethyl-1-hexanol and 2-ethylhexyl acetate, with 2 mmol of both the acetate and alcohol isolated after workup. To test the selectivity of PPL, the reaction was monitored by GC-FID equipped with a chiral column able to separate the enantiomers of 2-ethyl-hexanol. The enantiomeric ratio and acetate yield over time of PPL is shown below in Figures 1 and 2.

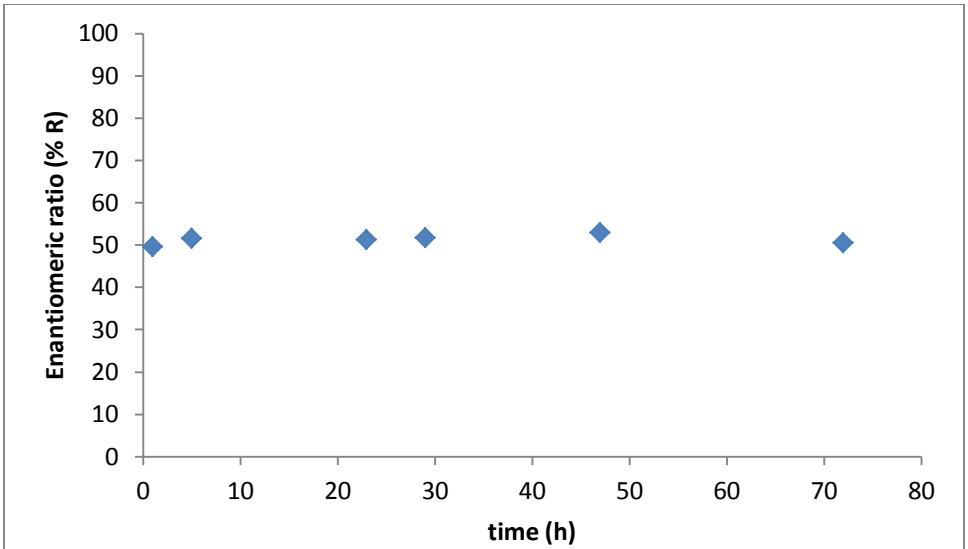


Figure 1: Plot of percent (R)-2-ethyl-1-hexanol versus time for PPL. The enantiomeric ratio stays around 50% for the duration of the reaction, meaning that PPL shows no preference between the enantiomers of 2-ethyl-1-hexanol.

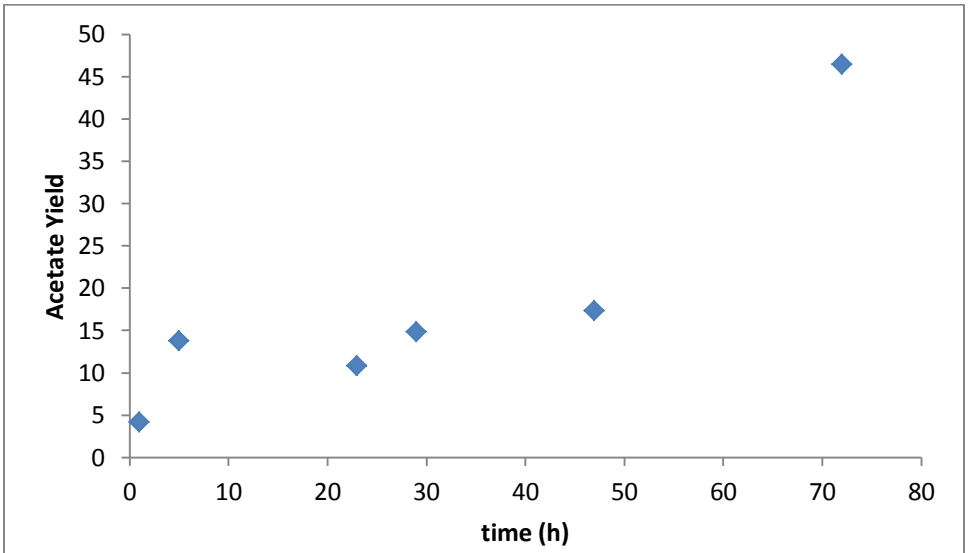


Figure 2: Plot of acetate yield ($100 \cdot A_{Ac}/(A_{(R)}+A_{(S)}+A_{Ac})$) versus time for PPL. Yields for this reaction remain low, with under 20% for most of the reaction, and only 48% after 72 hours.

It should be noted that the acetate yield was calculated using uncorrected peak areas. This causes no error for the two enantiomers of 2-ethyl-1-hexanol, as they have the same response factor towards the flame detector. However, it is very likely that 2-ethylhexyl acetate has a different response factor than 2-ethyl-1-hexanol. Some experiments were attempted with an inert internal standard added to the reaction mixture which would allow for quantitation and response factor determination. However, evaporation of both solvent and the internal standard over the 48 hour reaction time prevented quantitative analysis of the reactants. Though there is almost certainly a response factor difference between the two compounds, the difference is likely small due to their structural similarity.

This being said, the kinetic study still indicates that PPL was unsuitable for resolving 2-ethyl-1-hexanol. The enantiomeric ratio of the unreacted alcohol did not change significantly from 50%, meaning that the lipase was reacting (R) and (S)-2-ethyl-1-hexanol in equal proportions. This renders PPL useless for kinetic resolution due to a lack of stereoselectivity toward 2-ethyl-1-hexanol.

Finally, lipase from the bacterium *Burkholderia cepacia* (PSBC) was tested. As for PPL, a kinetic study was performed to determine the stereoselectivity of PSBC toward 2-ethyl-1-hexanol. PSBC was loaded with 15 mg per mmol of 2-ethyl-1-hexanol and reacted in dichloromethane at 0^{o}C. Like PPL, the enzyme loading of PSBC was increased compared to PLF due to lower reactivity of PSBC, requiring higher catalyst concentrations to achieve conversion in roughly the same reaction times. From this study, the enantiomeric ratio and yield versus time, as well as the enantiomeric ratio of versus yield were determined. These are plotted below in Figures 3, 4 and 5.

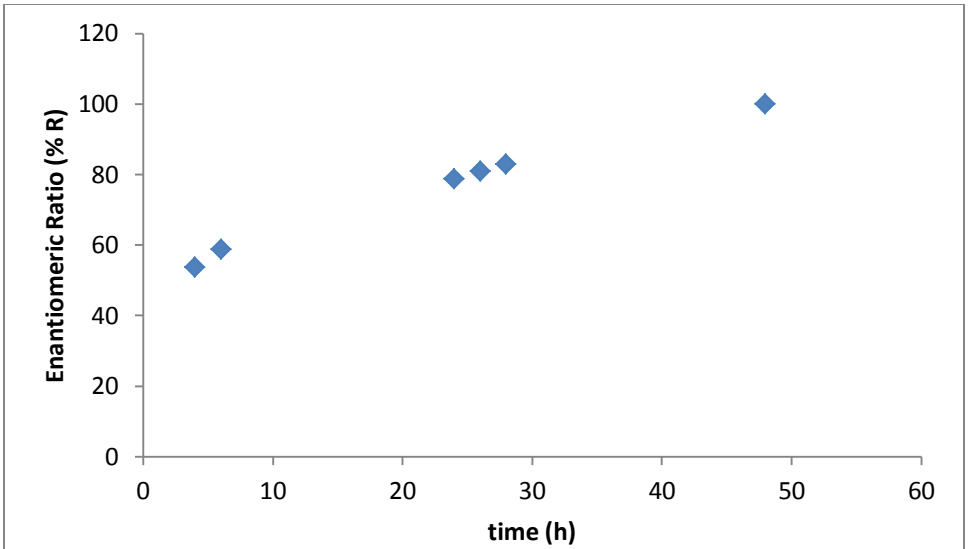


Figure 3: Plot of percent (R)-2-ethyl-1-hexanol versus time for PSBC. Unlike PPL, this enzyme showed an increase in enantiomeric ratio versus time, with the enzyme approaching nearly pure (R) alcohol after 48 hours.

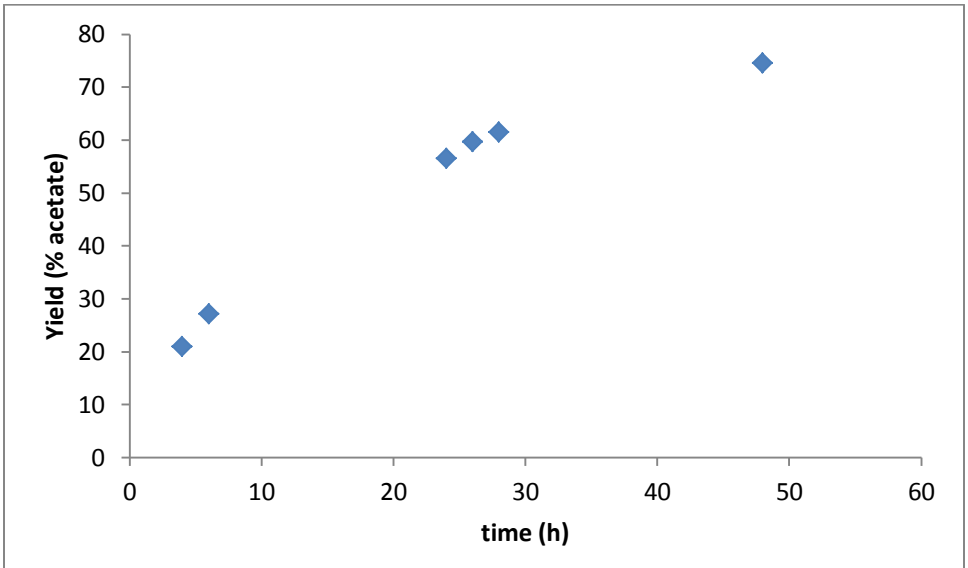


Figure 4: Plot of percent acetate yield ($100 \cdot A_{Ac}/(A_{(R)}+A_{(S)}+A_{Ac})$) versus time for PSBC. At 48 hours, the yield of acetate is over 70%, meaning that some of the (R) alcohol is reacting in addition to the (S).

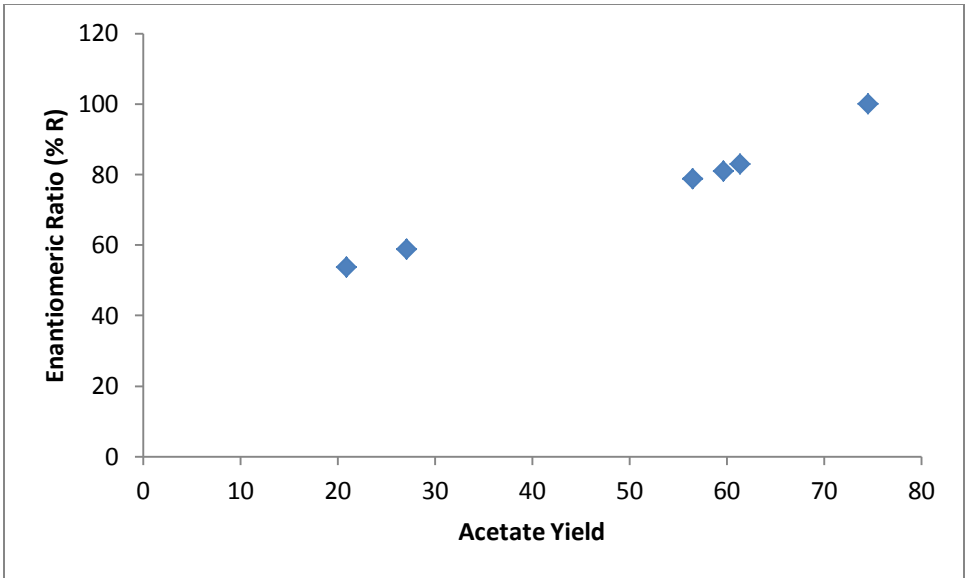


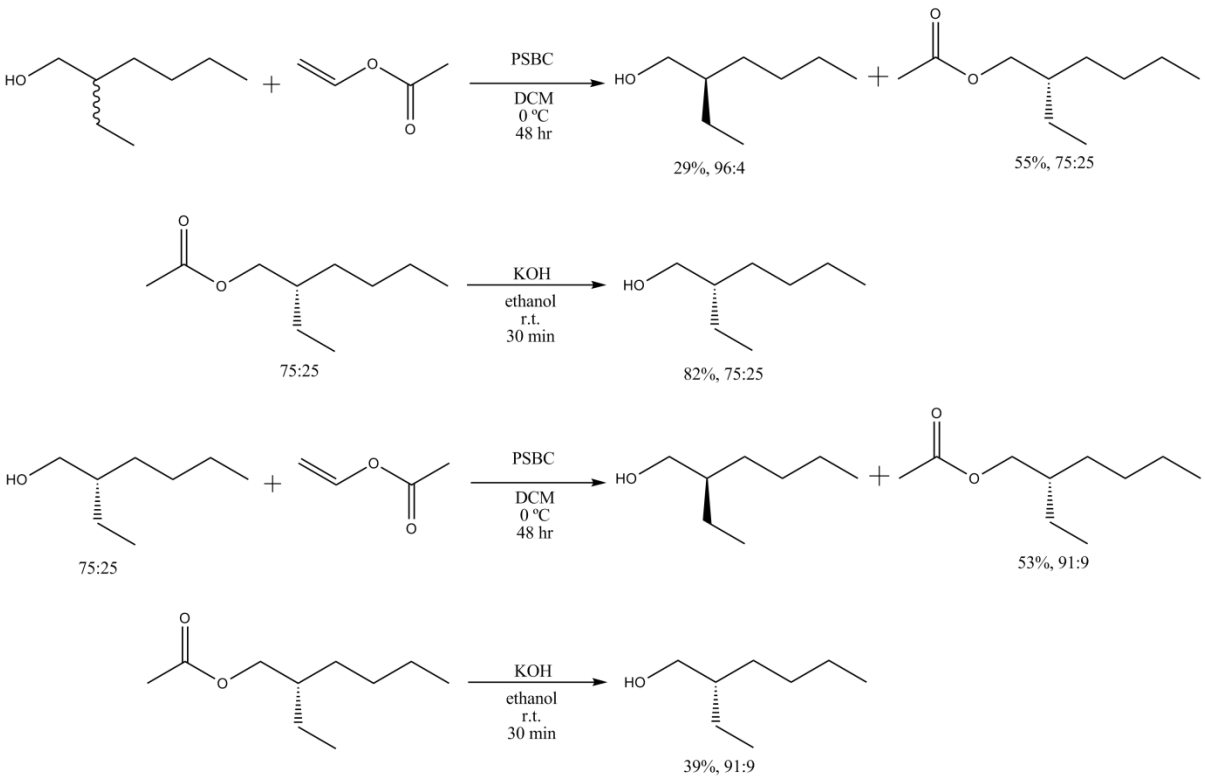
Figure 5: Plot of enantiomeric percent versus percent acetate yield ($100 \cdot A_{Ac}/(A_{(R)}+A_{(S)}+A_{Ac})$) for PSBC. A fairly linear trend of ER versus acetate yield is observed, meaning that 48 hours is near or at the optimum reaction time for this enzyme.

PSBC showed significant selectivity for (S)-2-ethyl-1-hexanol. At 48 hours, the unreacted (R) alcohol was nearly completely pure, with enantiomeric purity approaching 100%. Unfortunately, the acetate yield after about 12 hours was greater than 50%, with a yield of 75% at 48 hours. If PSBC were perfectly stereoselective, an acetate yield of 50% would be the highest observed. Above 50%, the enzyme is also reacting some proportion of both enantiomers. Indeed, it was found that (S)-2-ethyl-1-hexanol sample was only 68% enantiomerically pure, indicating that a significant portion of (R)-2-ethyl-1-hexanol was also acylated as well. However, the ability to isolate nearly pure (R)-2-ethyl-1-hexanol in a single reaction is valuable. Moreover, 2-ethyl-1-hexanol and 2-ethyl-1-hexyl acetate were easily separated by column chromatography, with R_f's of 0.11 and 0.41, respectively in 9:1 hexanes/ethyl acetate. Subsequent acylations typically resolved (R)-2-ethyl-1-hexanol in 94-96% enantiomeric purity, while (S)-2-ethylhexyl acetate had an enantiomeric purity of 70-75%.

The absolute configuration of the resolved enantiomers was determined by comparison with Chantal *et al.*[27]

To recover the (S) enriched samples of 2-ethyl-1-hexanol, the acetate group was hydrolyzed using potassium hydroxide in both absolute ethanol and methanol. The reaction proved easy and recovered the (S)-alcohol in relatively high yields. Only two equivalents of base and 30 minutes of reaction time at room temperature were required, and the (S)-alcohol was typically isolated in 80-90% yield. Moreover, the choice of solvent did not have an appreciable effect on (S)-2-ethyl-1-hexanol recovery. The (S) enriched alcohol was not enantiomerically pure enough to use in synthesizing the stereoisomers of MEHP and DEHP. However, a second round of acylation followed by hydrolysis could be easily used to further purify the (S)-alcohol. After acylating and hydrolyzing a second time, (S)-2-ethyl-1-hexanol was isolated in 91% yield. Though not as enantiomerically pure as the (R) alcohol or as pure as desired, these samples could still be used to synthesize MEHP and DEHP.

From these experiments, a scheme to resolve the two enantiomers of 2-ethyl-1-hexanol was developed and is outlined in Scheme 6.



Scheme 6: Resolution scheme for 2-ethyl-1-hexanol, with the (R) enriched alcohol recovered as the unreacted starting material during the first round of acylation, and two rounds of acylation and hydrolysis to recover the (S)-enriched alcohol.

It should be noted that during acylation, an unexpected product was observed via TLC with an R_f higher than 2-ethylhexyl acetate and considerable streaking. At first, this product was assumed to be unreacted vinyl acetate and was mostly ignored. Moreover, the product was not present after hydrolysis and was deemed unimportant in the resolution scheme. However, GC-MS analysis of 2-ethylhexyl acetate revealed a second peak with a significantly longer retention time and similar area to 2-ethylhexyl acetate. This peak had an M^+ peak of 271 and was identified by a similarity search as 1,1'-[ethylidenebis(oxy)]bisoctane. Because n-octyl and 2-ethyl-1-hexyl substituents would likely have similar mass fragmentation patterns, this product is likely 1,1'-[ethylidenebis(oxy)]bis(2-ethyl)hexane, shown below in Figure 6.

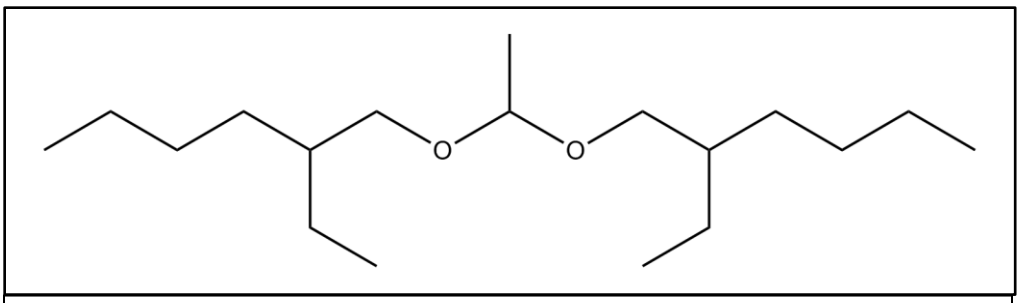
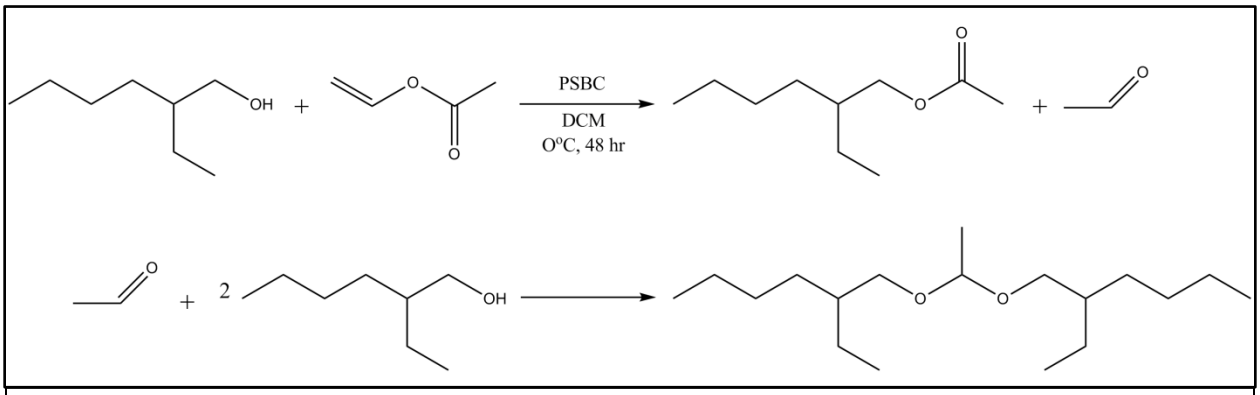


Figure 6: Structure of 1,1'-[ethylidenebis(oxy)]bis(2-ethyl)hexane, the acetal of acetaldehyde and 2-ethyl-1-hexanol.

How this product formed is not entirely clear. During the enzymatic reaction, the acyl group of vinyl acetate is transferred to 2-ethyl-1-hexanol, forming 2-ethylhexyl acetate and vinyl alcohol. This vinyl alcohol quickly tautomerizes to acetaldehyde. The most likely explanation is that two equivalents of 2-ethyl-1-hexanol reacts with acetaldehyde to form the acetal 1,1'-[ethylidenebis(oxy)]bis(2-ethyl)hexane (Scheme 7). More importantly, how this side product affects the enantiomeric purity of the products is also not known. Further work is needed to purify and characterize this side product as well as the enatiomeric purity of any 2-ethyl-1-hexanol recovered from it.



Scheme 7: Formation of acetaldehyde during acylation of 2-ethyl-1-hexanol, and the reaction of acetaldehyde with 2-ethyl-1-hexahol to form 1,1'-[ethylidenebis(oxy)]bis(2-ethyl)hexane.

Despite this unexpected result, the scheme outlined in Scheme 6 was used to resolve larger amounts of 2-ethyl-1-hexanol. Scaling up the reaction was fairly straightforward, with all reagents scaled in the same proportions. Some difficulty was had in producing sufficient

amounts of each enantiomer due to losses at each step but could be overcome by increasing the initial amount of 2-ethyl-1-hexanol used in the first acylation.

Synthesis of MEHP and DEHP

Using the enantiomerically pure samples of 2-ethyl-1-hexanol, the two enantiomers of MEHP were synthesized by combining one equivalent of 2-ethyl-1-hexanol with one equivalent of phthalic anhydride. Though this reaction was fairly facile, an unexpected challenge arose in purifying MEHP from the two starting materials. GC-MS analysis of the reaction mixture showed that the two starting materials were still present after purification by flash chromatography. Separation of the unreacted 2-ethyl-1-hexanol was important in the subsequent reaction to form DEHP, as another equivalent of 2-ethyl-1-hexanol was added to MEHP in an esterification reaction. Any unreacted alcohol from the previous step would have decreased the enantiomeric purity of the resulting DEHP. In addition, this impurity would hamper effective biological testing, as there would be uncertainty as to whether any biological effects are due to MEHP or the unreacted 2-ethyl-1-hexanol. Testing by thin layer chromatography revealed that 2-ethyl-1-hexanol and MEHP had very similar R_f's, precluding purification by column chromatography. Numerous solvent systems were tested in an attempt to improve separation, among them ethyl acetate/hexanes, dichloromethane/acetone, dichloromethane/methanol, and chloroform/ethyl acetate. Streaking caused by the carboxylic acid group in MEHP exacerbated the R_f similarity, making separation impossible. Though addition of 0.5% acetic acid to all of the solvent systems mentioned above did reduce streaking significantly and allow separation of phthalic anhydride in certain cases, it still did not allow for effective separation of 2-ethyl-1-hexanol and MEHP.

In addition to chromatography, a chemically active extraction was attempted to exploit the carboxylic acid present in MEHP. The reaction mixture was first dissolved in 20 mL ether, then extracted with 20 mL water treated with 10% (w/v) potassium carbonate until strongly

alkaline (pH 8-9). The aqueous layer was then separated, acidified, and extracted with a fresh portion of ether (20 mL). Ideally, the first extraction should have deprotonated MEHP and caused the negatively charged species to transfer to the aqueous layer. Alcohols have pK_a's several orders of magnitude larger than carboxylic acids, meaning that 2-ethyl-1-hexanol should not have been deprotonated and remained in the first ether layer. In the second extraction, MEHP should have been protonated again to form a neutral species and transfer to the new portion of ether. Unfortunately, analysis by GC-MS and TLC revealed that MEHP and 2-ethyl-1-hexanol were present in both ether layers, meaning that even when deprotonated, MEHP did not have a very large preference for the aqueous layer. The presence of 2-ethyl-1-hexanol in the second ether portion was likely due to basic hydrolysis of the ester bond between the phthalate and 2-ethyl-1-hexanol moieties.

Vacuum distillation was also attempted to try and separate MEHP and the unreacted alcohol. 2-ethyl-1-hexanol has a boiling point of 186.2^{o}C, while MEHP boils at a temperature greater than 300^{o}C.[27] This difference in boiling point should have been high enough to allow easy separation by vacuum distillation using a Kugelrohr apparatus. Unfortunately, 2-ethyl-1-hexanol was also present in the sample after the attempted purification. It is unclear whether 2-ethyl-1-hexanol is actually present in the sample, or if it an artifact of GC-MS analysis. Because MEHP has a high boiling point, an inlet temperature of 300^{o}C was used to move the sample onto the column. One possibility is that MEHP was degrading in the inlet into 2-ethyl-1-hexanol and phthalic anhydride. To test for this, the inlet temperature was lowered to 200^{o}C. It was found that a significantly lower amount of both 2-ethyl-1-hexanol and phthalic anhydride were detected by the GC-MS, indicating that the high temperature of the inlet was responsible for some degradation. Moreover, comparing retention times with pure samples of the starting material rules out degradation on the column itself, as the retention times are exactly the same. If degradation were occurring on the column, one would observe broad, poorly resolved peaks

with retention times different than the pure materials. However, the extent of degradation is unclear, and it is still possible that some of the starting material is present in the MEHP sample. Compounding this problem is that the instrument response factors for MEHP, 2-ethyl-1-hexanol, and phthalic anhydride are also unknown. Without a way to purify MEHP and unambiguously determine its purity, the stereoisomers of DEHP cannot be synthesized with reliability.

Before the issues in purity had been fully realized, impure samples of (R)-MEHP were used to synthesize (R,R)-DEHP and (R,S)-DEHP from (R)-MEHP and an equivalent of either (S) or (R)-2-ethyl-1-hexanol. This was accomplished through the reaction of MEHP with stoichiometric quantities of diisopropylcarbodiimide (DIC) and catalytic amounts of 4-dimethylaminopyridine (4-DMAP). This reaction proved straightforward, with DEHP formed in decent yields and isolated easily from the starting materials. Though the MEHP reaction has proved problematic, the subsequent reaction to form DEHP will be no barrier to synthesizing the three different stereoisomers. One issue of note is that the enantiomeric purity of MEHP and DEHP could not be determined using the chiral GC column used to analyze 2-ethyl-1-hexanol and 2-ethylhexyl acetate. The chiral columns used had maximum set temperature of 180^{o}C. Unfortunately, this temperature was too low for MEHP and DEHP to move on the column.

Conclusion

An effective resolution scheme for the enantiomers of 2-ethyl-1-hexanol was developed using lipase from *Burkholderia cepacia*, with the solvent and reaction time optimized. (R)-2-ethyl-1-hexanol was isolated in 95% or greater enantiomeric purity as the unreacted alcohol in the first acylation, and (S)-2-ethyl-1-hexanol was isolated in 91% enantiomeric purity after two acylation/hydrolysis reactions. However, further optimization could be still pursued. One of the main drawbacks to this method is that a total of four reactions are required to isolate pure (S)-2-ethyl-1-hexanol, resulting in considerable loss during resolution. The ability to resolve 2-ethyl-2-hexanol in one step would be ideal, as well as higher enantiomeric purity of the resolved enantiomers. In addition, the formation of the acetal 1,1'-[ethylidenebis(oxy)]bis(2-ethyl)hexane is a major issue, as its co-elution with 2-ethylhexyl acetate almost certainly impacts the enantiomeric purity of the resulting (S)-2-ethyl-1-hexanol upon hydrolysis. Several different avenues could be used to address these issues. One possibility is to use isopropenyl acetate as the acyl donor rather than vinyl acetate. Acyl transfer and tautomerization of isopropenyl acetate will produce acetone rather than acetaldehyde. Because acetal formation is slower for ketones than aldehydes, it would likely decrease the amount of acetal formed during the reaction. In addition, the change in acyl donor may result in an increase in stereoselectivity towards 2-ethyl-1-hexanol. A second way to improve the stereoselectivity of the acylation reaction would be to test different reaction conditions. The temperatures and solvents used were based mostly on the work of Chantal *et al*. without much modification.[27] A more thorough survey of different solvents could be done, as well as decreasing the temperature of the reaction. Kinetic resolution relies on a difference in activation energy between the two enantiomers of a compound in a reaction. By lowering the temperature, fewer molecules have sufficient energy to overcome the higher activation barrier, leading to a better resolution of the enantiomers. Though the reaction

would likely take longer due to the lower temperature, the gain in enantiomeric purity would be valuable.

The reaction of 2-ethyl-1-hexanol with phthalic anhydride to form MEHP was successful, though the need to ensure purity of the product is still an outstanding problem. More specifically, determining to what extent MEHP is degrading during GC-MS analysis is paramount. Derivatization reactions could be used to remove the carboxylic group from MEHP, which would allow for lower inlet and oven temperatures to analyze. This in turn would hopefully eliminate any degradation during analysis. Secondly, an internal standard could be used to determine the response of 2-ethyl-1-hexanol, phthalic anhydride, and the MEHP derivative. From this, the actual concentrations of each compound in the reaction mixture could be determined. Alternatively, LC-MS could be used to bypass degradation entirely. LC-MS is better suited for higher molecular weight compounds which are not volatile enough for easy GC-MS analysis, as the ambient temperatures used during LC-MS analysis would eliminate any degradation occurring. Once operational, the LC-MS may be the best option for analyzing MEHP. Moreover, if equipped with a chiral column, it would allow for direct measurement of enantiomeric purity of MEHP and DEHP.

If analysis reveals that MEHP is still impure, the problem of purification once more will come to the forefront. Numerous solvent systems were used in an attempt to purify MEHP. Though other solvent systems could be tested, nearly all common TLC solvent systems have been exhausted. A chemically active extraction proved ineffective as well, with basic conditions causing hydrolysis of the ester bond between the phthalate and 2-ethyl-1-hexyl moieties. Vacuum distillation holds the most promise, despite it initially not succeeding. A relatively low temperature of 60^{o}C was used to try and distill 2-ethyl-1-hexanol. Though this proved sufficient to distill pure 2-ethyl-1-hexanol, it proved insufficient in the reaction mixture. Higher temperatures were not attempted for fear of degrading MEHP. If MEHP proves stable at higher

temperatures, the reaction mixture could be purified by vacuum distillation alone; separating phthalic anhydride (a solid), 2-ethyl-1-hexanol (b.p.186.2^{o}C) and MEHP (b.p. above 300^{o}C).[27]

Luckily, the reaction of MEHP with 2-ethyl-1-hexanol to form DEHP is fairly straightforward, with easy chromatographic separation of the starting materials from the product. Once purification of MEHP has been accomplished, the synthesis of the different stereoisomers of DEHP should be fairly simple. In addition, the resolution scheme, MEHP and DEHP reactions should be applicable to other alcohols, such as 2-allyl-1-hexanol, to form the chiral samples of the different metabolites of DEHP.

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Appendix

Materials

Chemical	Purity/Grade	Distributor
Lipase from *Burkholderia cepacia*	≥30,000 U/g	Sigma-Aldrich
Lipase from Porcine Pancreas	100-400 U/mg	Sigma-Aldrich
Lipase from *pseudomonas fluorescens*	≥20,000 U/g	Sigma-Aldrich
Vinyl acetate	99+%, stabilized	Acros
2-ethyl-1-hexanol	-	J.T. Baker Chemical Co.
Potassium hydroxide	ACS ceritifed	Fisher
Phthalic anhydride	99%	Acros Organics
Diisopropylcarbodiimide	99%	Acros Organics
4-dimethylaminopyridine	99%	Acros Organics
Dichloromethane	HPLC grade	Fisher
Ethyl acetate	HPLC grade	Fisher
Hexanes	HPLC grade	Fisher
Methanol	HPLC grade	Fisher
Ethanol	Absolute, ACS grade	Pharmco-AAPER

List of Abbreviations

DEHP: di(2-ethylhexyl) phthalate

MEHP: mono(2-ethylhexyl) phthalate

PLF: Lipase from the species *Pseudomonas fluorescens*

PPL: Lipase from porcine pancreas

PSBC: Lipase from *Burkholderia cepacia*

NMR: Nuclear magnetic resonance spectroscopy

IR: infrared spectroscopy

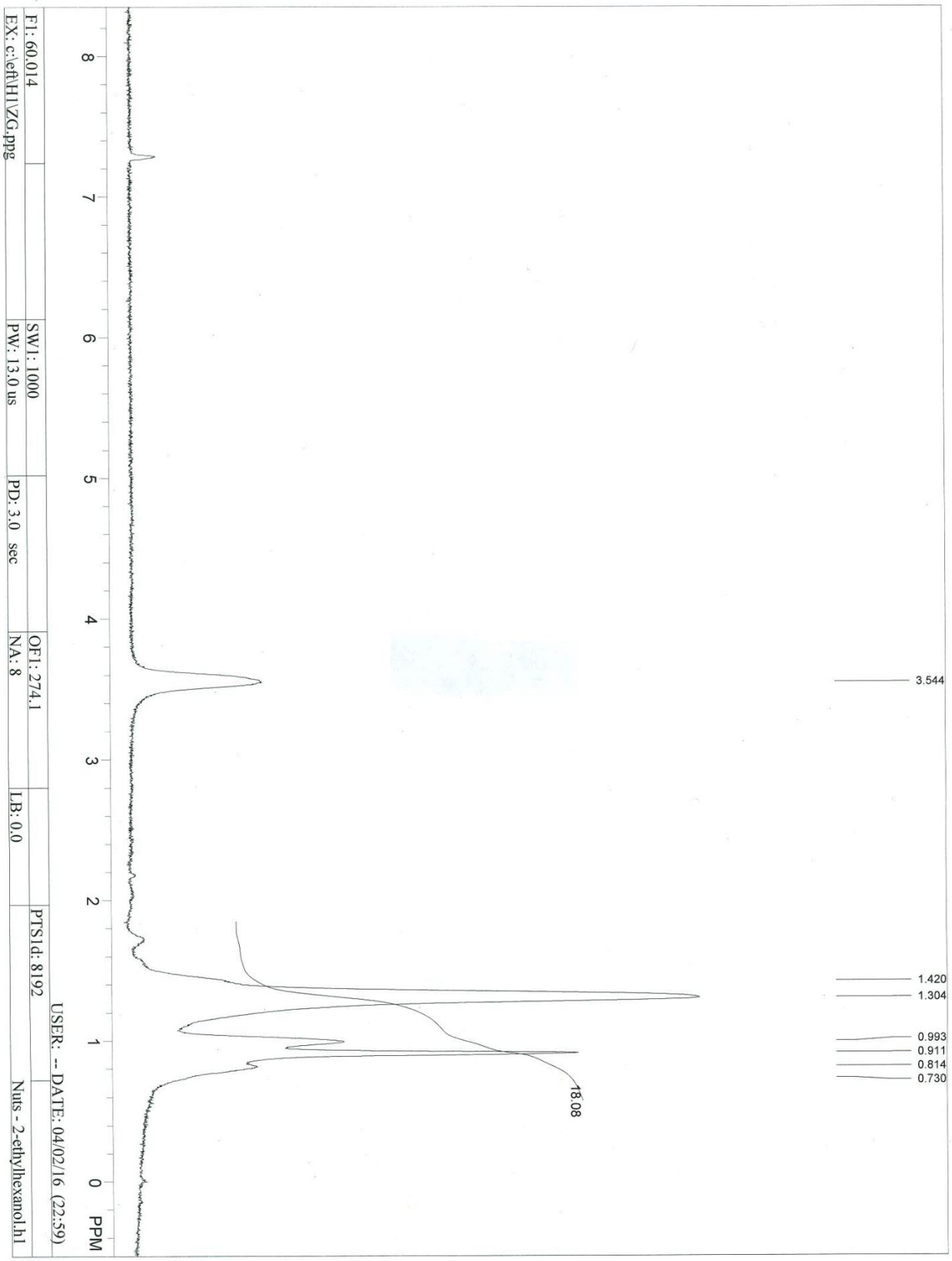
GC-FID: Gas chromatography flame ionization detection

GC-MS: Gas chromatography mass spectrometry

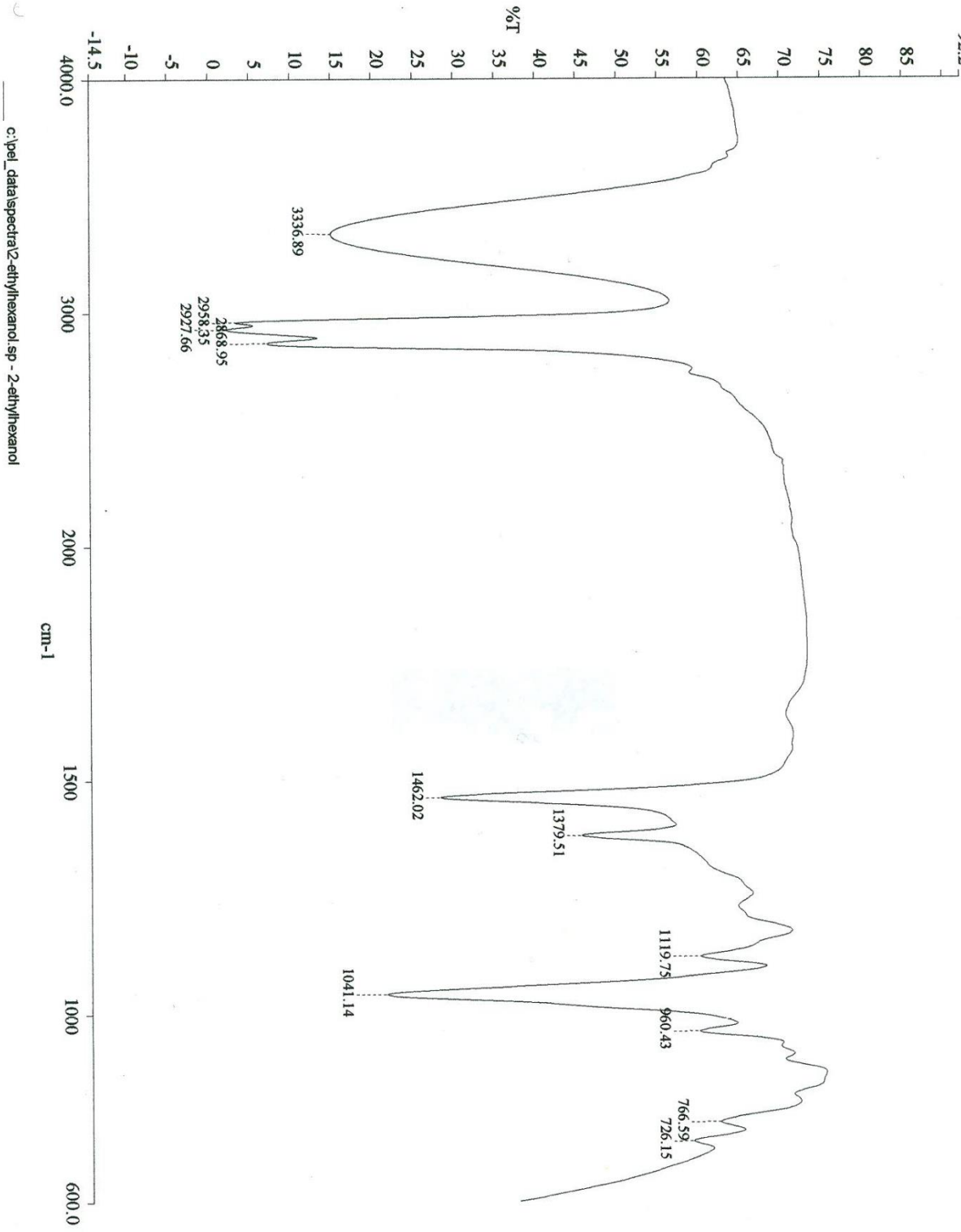
LC-MS: Liquid chromatography mass spectrometry

TLC: thin layer chromatography

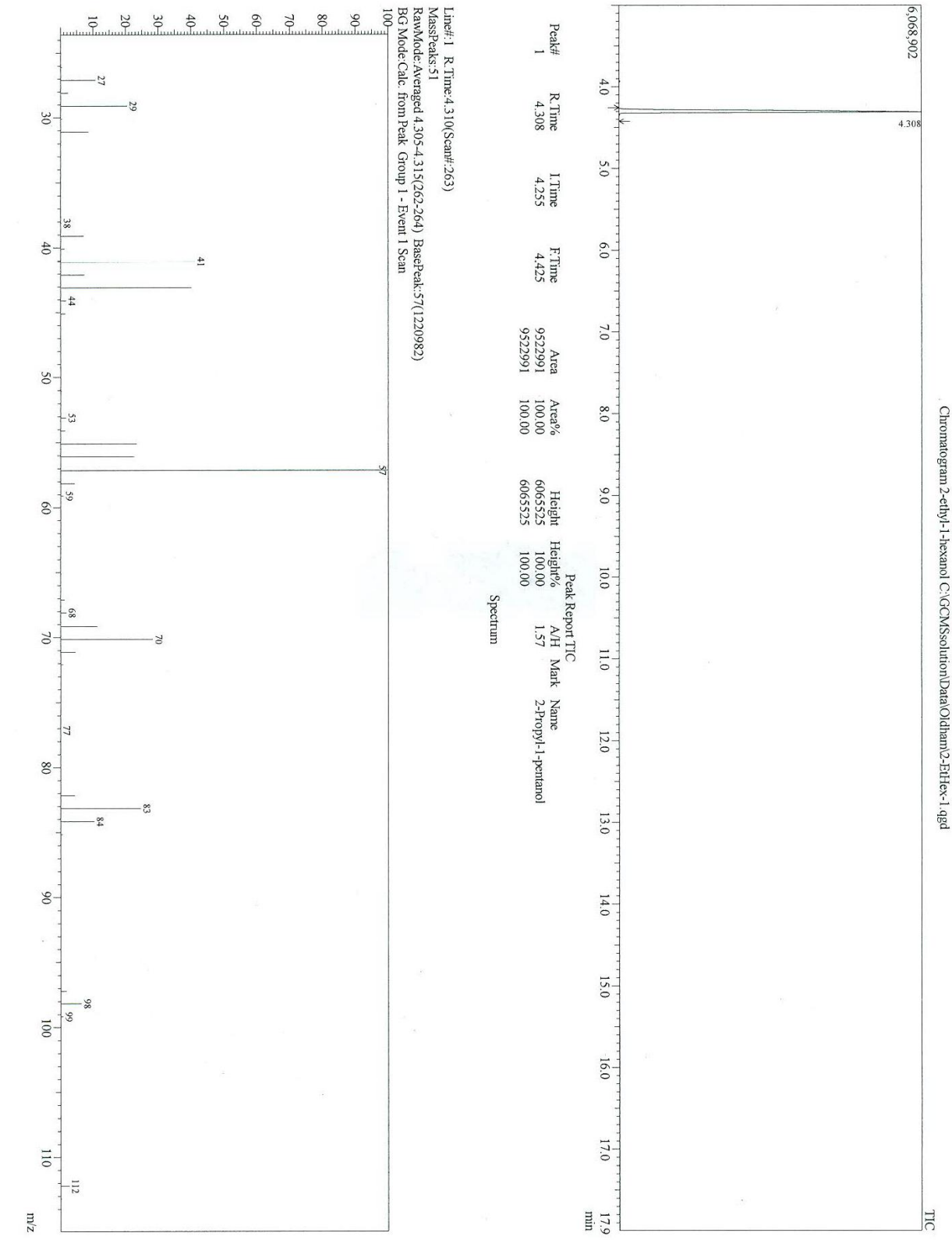
NMR spectrum of 2-ethyl-1-hexanol



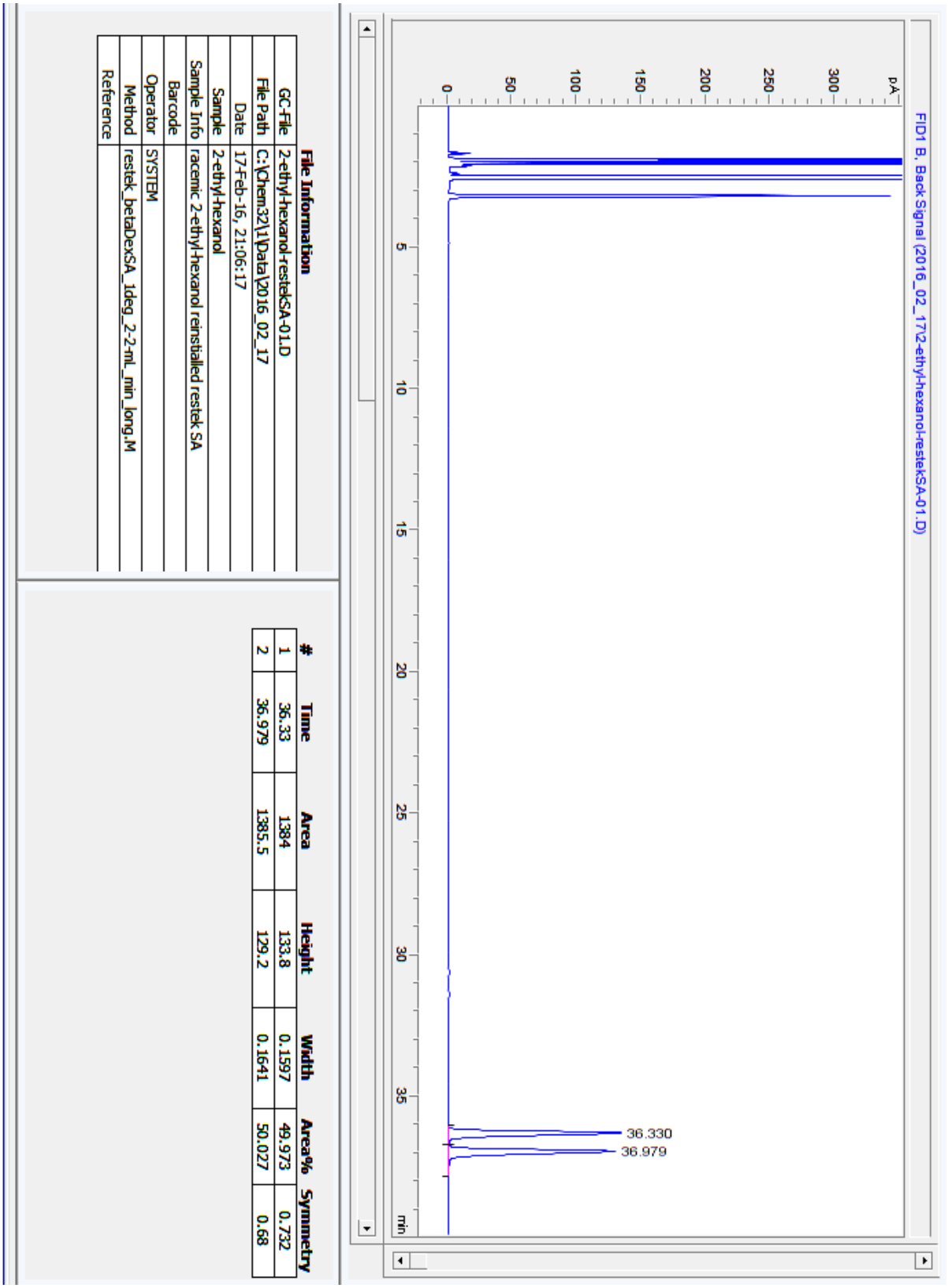
IR spectrum of 2-ethyl-1-hexanol



GC-MS chromatogram and mass spectrum of 2-ethyl-1-hexanol



Chiral GC-FID chromatogram of racemic 2-ethyl-1-hexanol



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Barcode	
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Method	restek_betaDexSA_1deg_2-2-mL_min_long.M
Reference	

#	Time	Area	Height	Width	Area%	Symmetry
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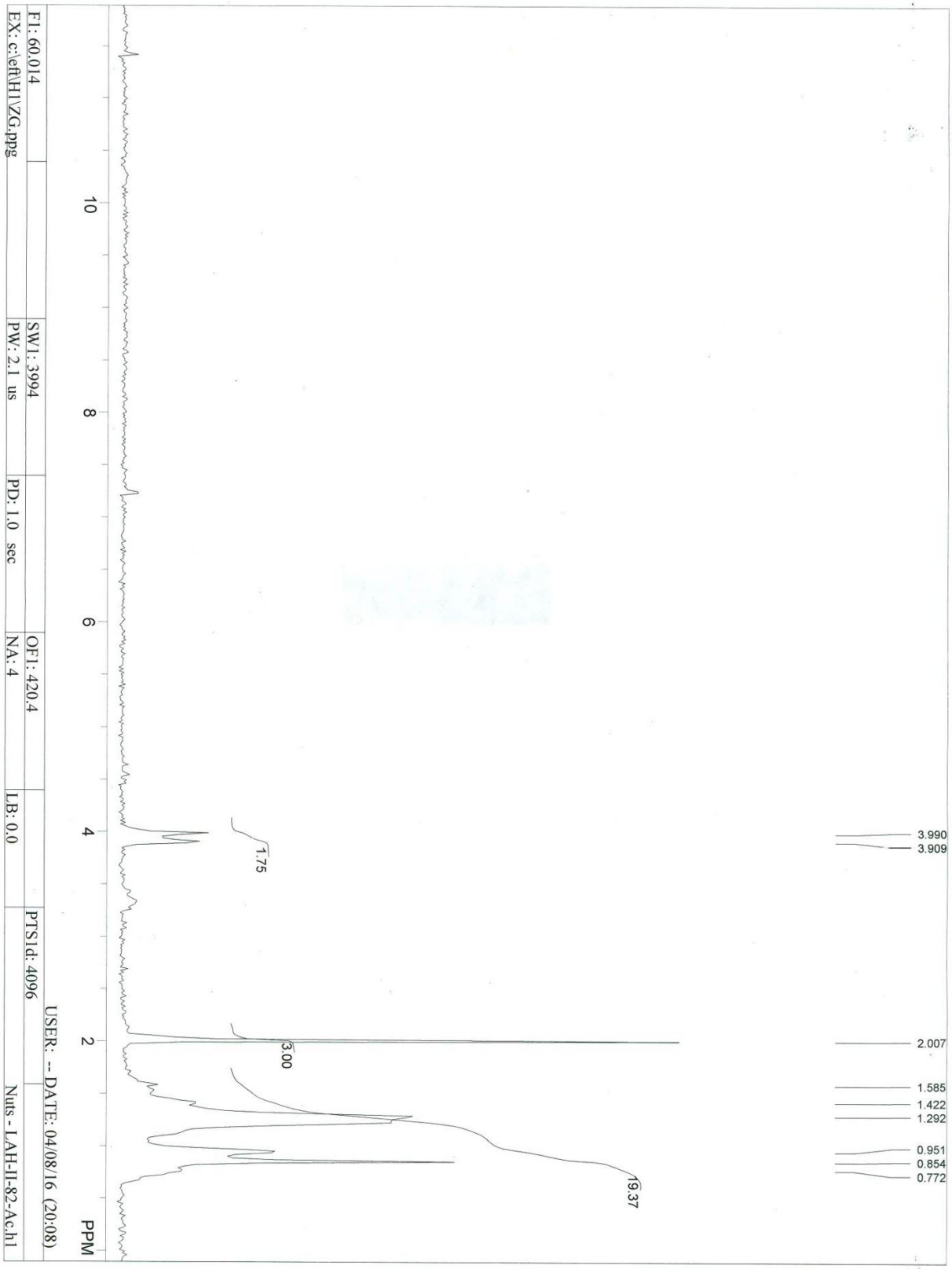
NMR spectrum of 2-ethylhexyl acetate



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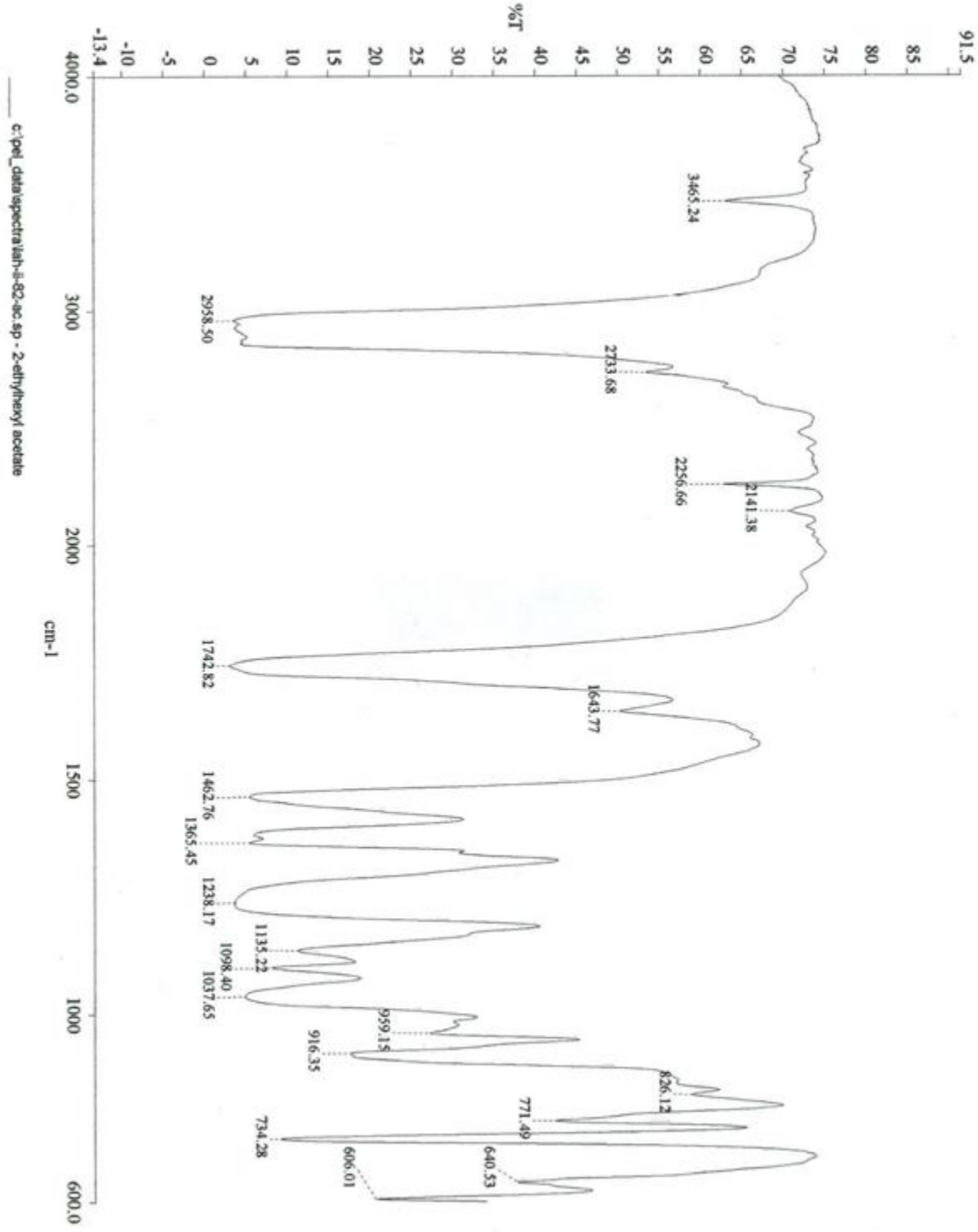
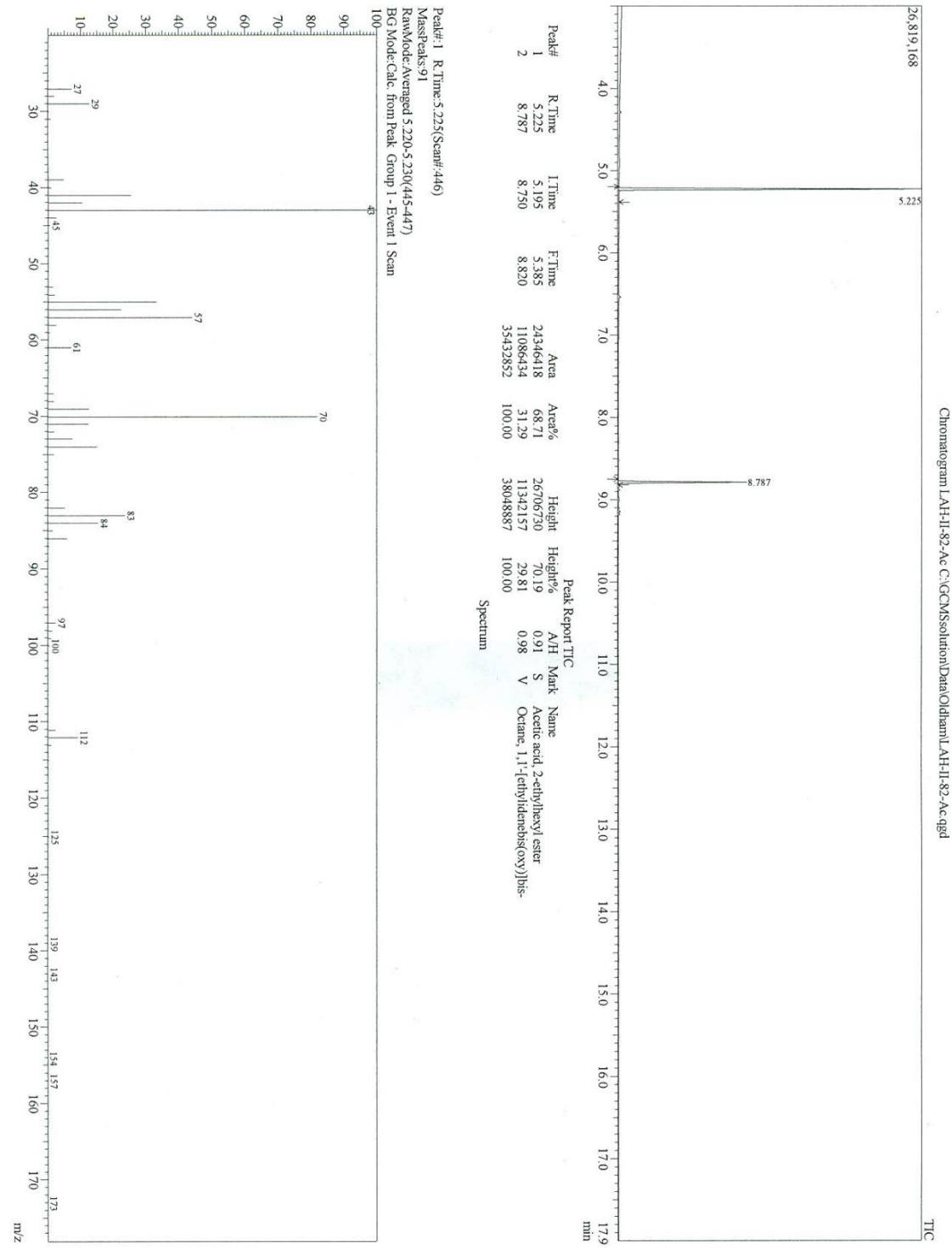


GC-MS chromatogram and mass spectrum of 2-ethylhexyl acetate



Chiral GC-FID Chromatogram of 2-ethylhexyl acetate after first the first acylation with PSBC

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Sample Info	
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Operator	SYSTEM
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Reference	

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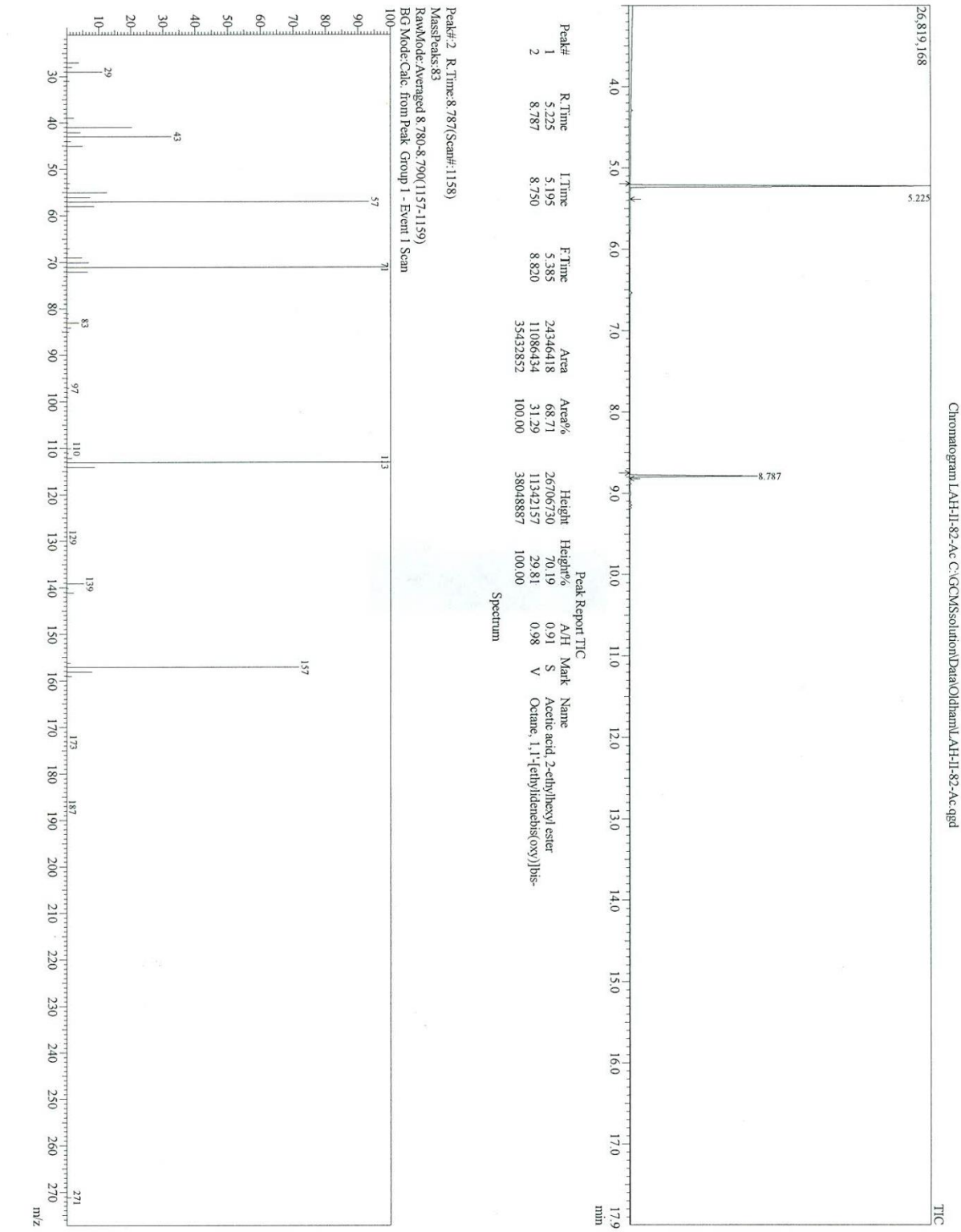
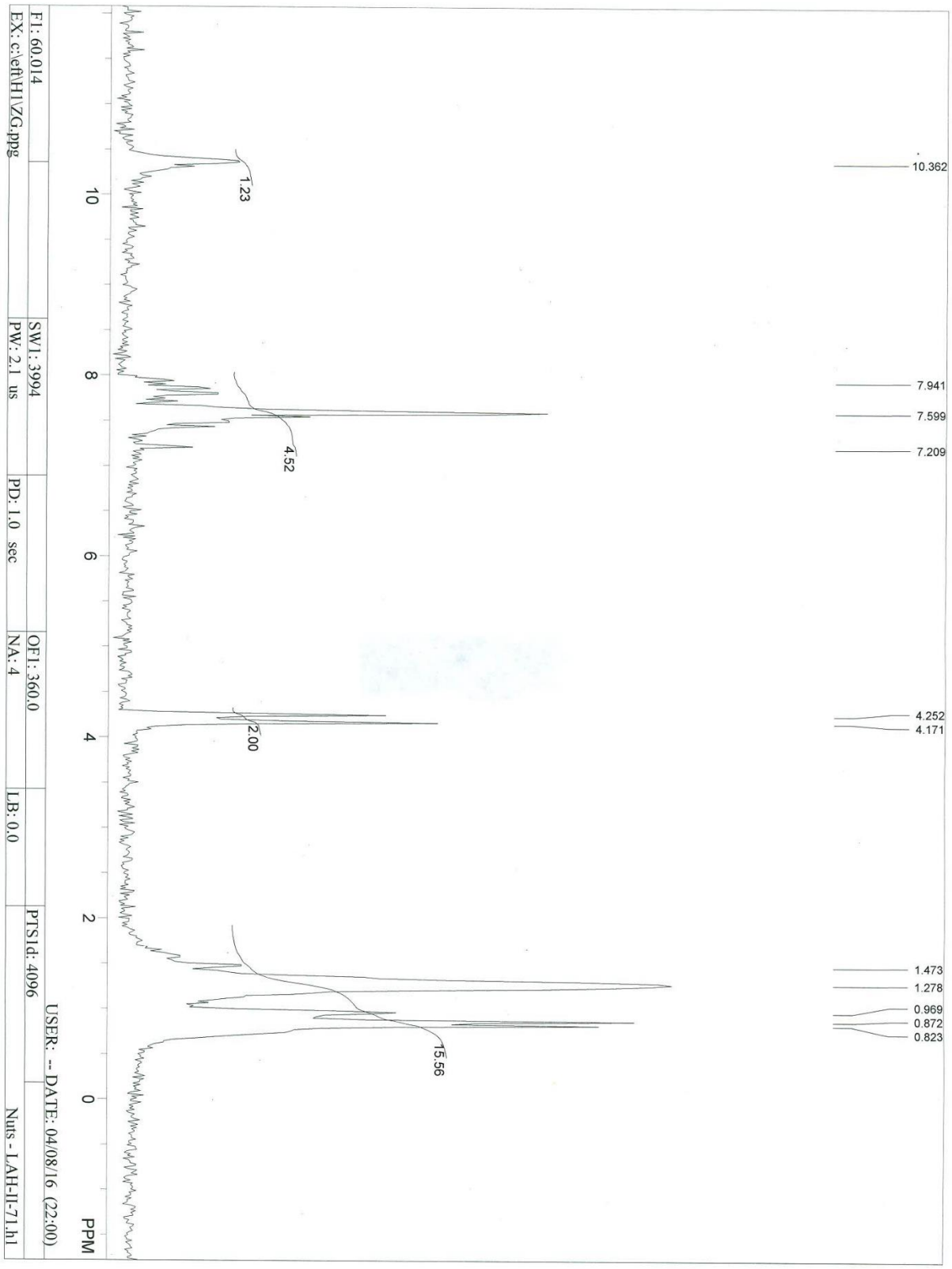
1	33.105	871.6	50.7	0.2117	73.992	0.656
2	33.881	306.4	18.8	0.2214	26.008	0.508

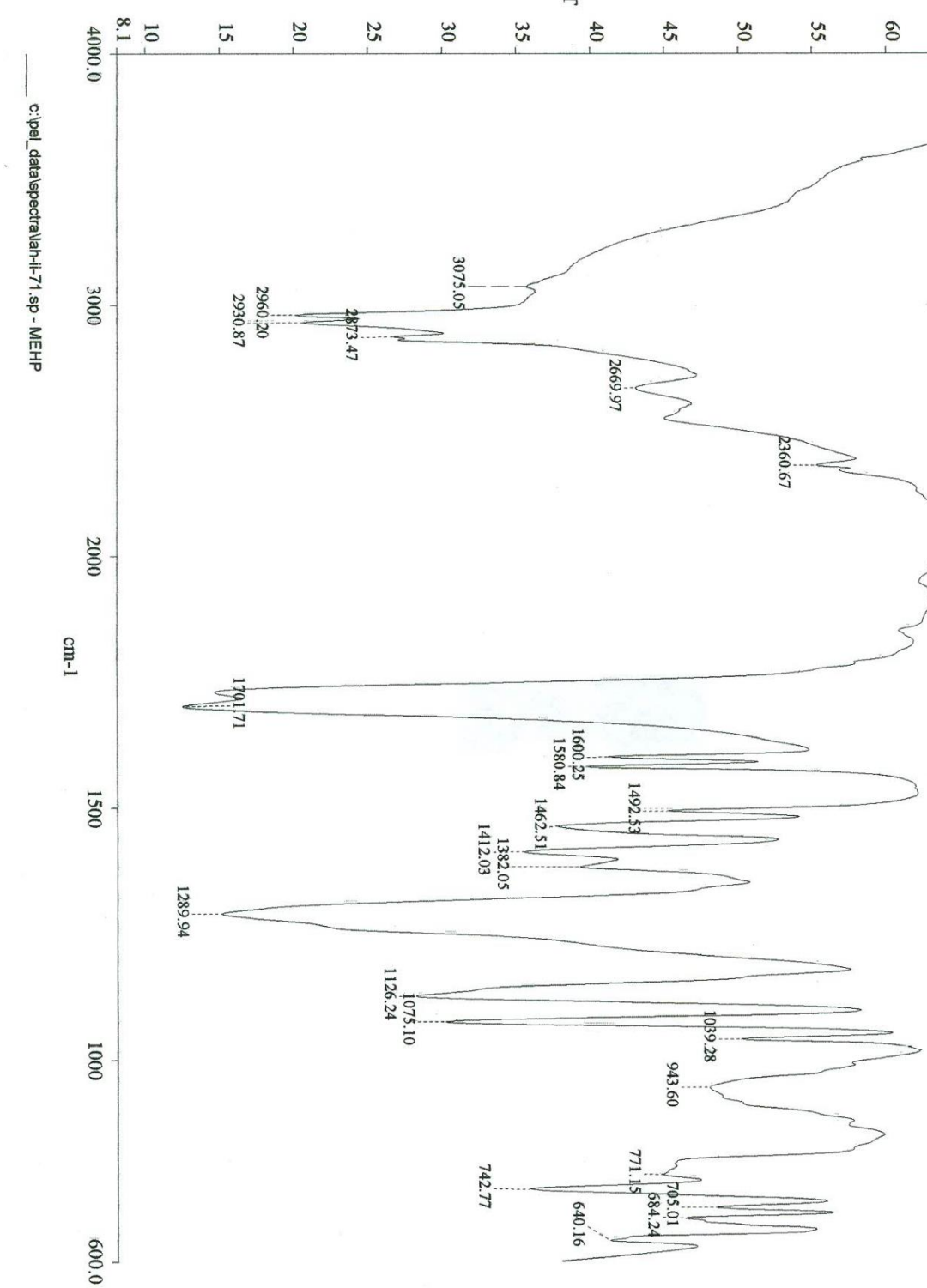
GC-MS Chromatogram of 1,1'-[ethylidenebis(oxy)bis(2-ethyl)hexane



NMR spectrum of mono(2-ethylhexyl)phthalate



IR spectrum of mono(2-ethylhexyl)phthalate

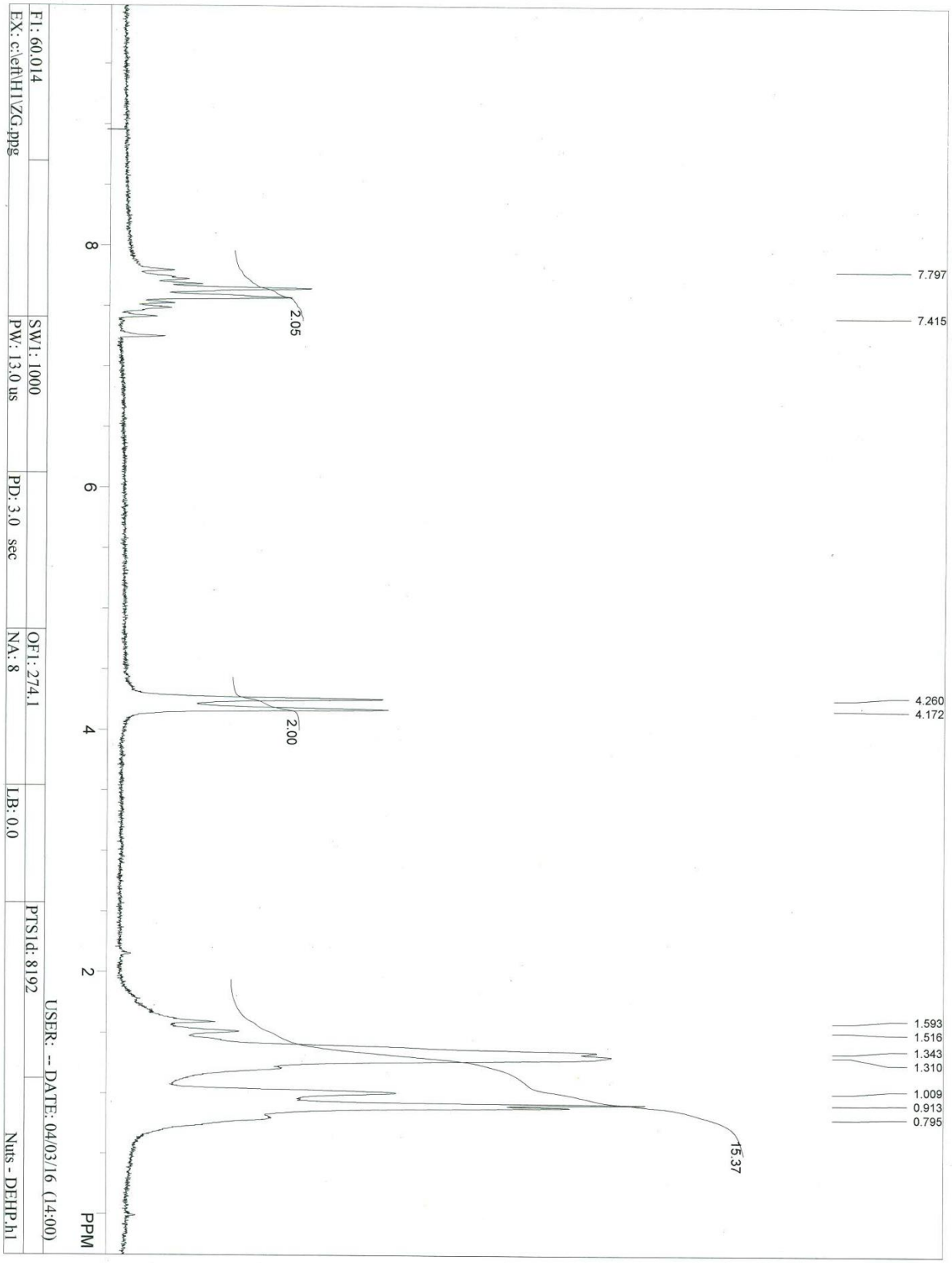


GC-MS chromatogram and mass spectrum of mono(2-ethylhexyl)phthalate

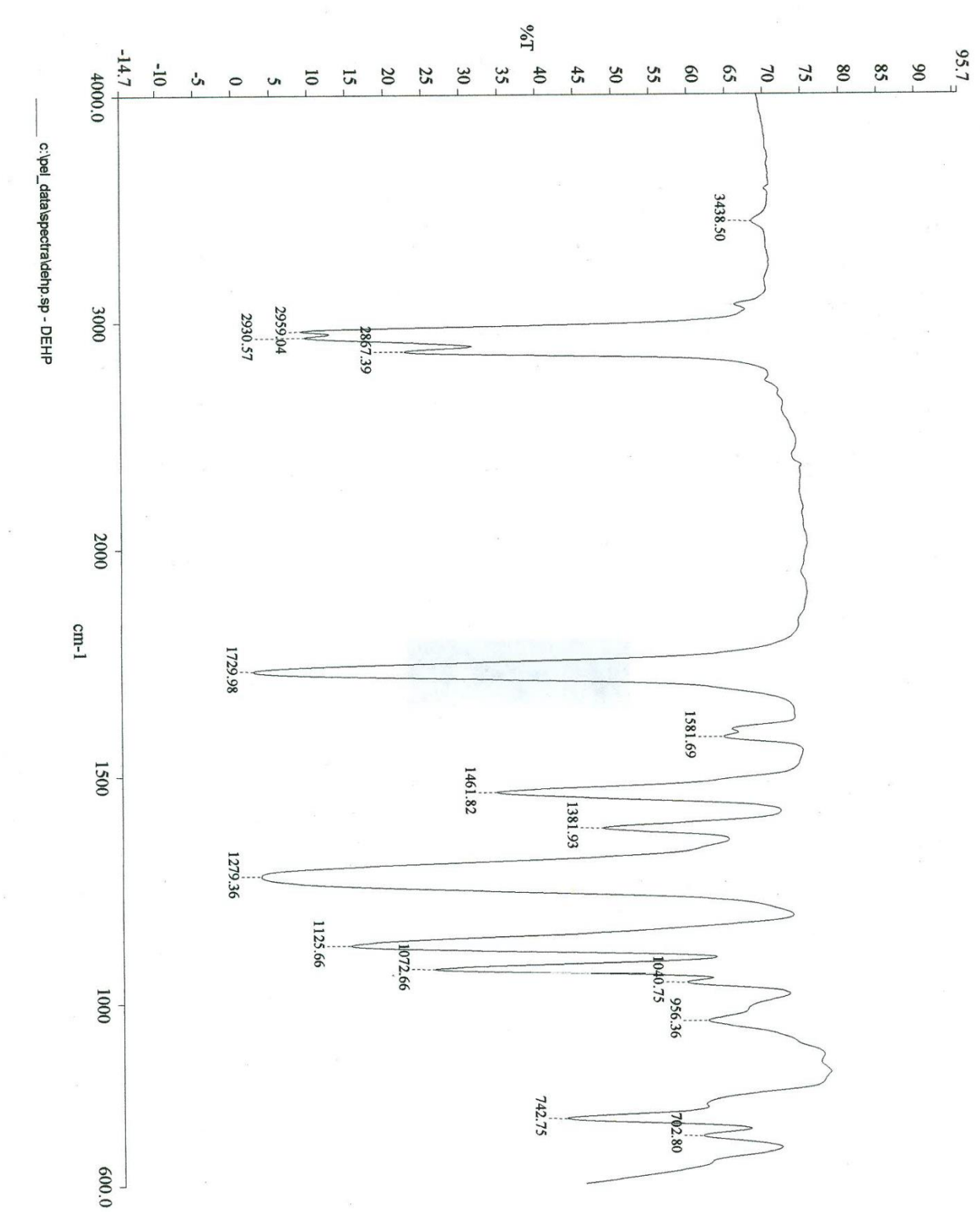
Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	4.302	4.280	4.445	1516166	14.04	1477358	31.68	1.03		1-Hexanol, 2-ethyl-
2	6.549	6.520	6.670	667945	6.19	581293	12.46	1.15		Phthalic anhydride
3	11.173	11.030	11.670	8614980	79.78	2604816	55.86	3.31	SV	1,2-Benzenedicarboxylic acid, mono(2-ethylhe
				10799091	100.00	4663467	100.00			

Peak Report TIC

NMR spectrum of di(2-ethylhexyl)phthalate



IR spectrum of di(2-ethylhexyl)phthalate



GC-MS chromatogram and mass spectrum of di(2-ethylhexyl)phthalate