TECHNIUM

Technium Vol. 27, pp.48-62 (2025)
ISSN: 2668-778X
www.techniumscience.com

DETECTION AND SEPARATION METHODS FOR RESOLUTION OF PRODUCTS OF ENZYMATIC REACTIONS

Meyer Elazar, Ph.D
214 Haalon St., Matta, Israel
Correspondence e-mail: elime11g@gmail.com

Abstract. Special analytical methods have been developed for the detection and separation of the resolved products of several kinetic enzymatic resolutions employing enzymes such as lipases and transglutaminase, which are useful as catalysts in nearly anhydrous organic solvents to carry out stereospecific and selective enzymatic resolutions of compounds such as (+/-)-2-bromohexadecanoic acid (2-Br-HDA), (R,S)-mandelic acid, DL-β-phenyllactic acid and (R,S)-2-phenylpropionic acid.

Keywords: Analytical methods, detection and separation, lipases and transglutaminase, catalysts, organic solvents, stereospecific and stereoselective enzymatic resolutions, bio-transformations, conversion, enantiomeric excess (ee).

Introduction

Since the early experiments reported by Klibanov[1], the use of enzymes in organic solvents rather than in their natural aqueous reaction media was widely recognized revealing that change in solvent from water to an organic solvent is feasible and that in such allegedly hostile environment, enzymes can catalyze reactions that are impossible in water. A broad spectrum of experiments showed that enzymes exhibit higher stability and unexpected behaviors such as molecular memory[2,3,4,5]. In addition, it has been discovered that enzymatic selectivity, including substrate, stereo-, regio- and chemo-selectivity, can be affected and inverted by the solvent. Enzyme-catalyzed reactions in organic solvents, which can be named bio-transformations, have found some commercial applications. In some cases, bio-transformations can be good alternative to chemical synthesis for the production of optically active compounds by using non-aqueous enzymology, representing an important area of research and biotechnological development. This methodology is especially suitable for the modification of precursors of pharmaceutical compounds and fine chemicals, which, in most cases, are insoluble or poorly soluble in water. In a previous article published in Technium Science[6], the stereo-selective and/or stereospecific enzymatic esterification of several carboxylic acid derivatives and hydrolysis of esters have been described. The present article includes data relating to methods of detection and separation of the products of enzymatic reactions carried out in organic solvents as well as detailed synthetic protocols of obtaining reference standards for said enzymatic reactions.

Materials and methods

Several enzymes have been implemented as catalysts, such as, lipases selected from Aspergillus niger lipase, Candida antartica lipase, Candida cylindracea lipase, Candida rugosa lipase, Chromobacterium lipase, Mucor miehei lipase, Porcine pancreatic lipase, Pseudomonas fluorescence lipase, Pseudomonas cepacia and Wheat germ lipase and transglutaminase in a number of nearly anhydrous organic solvents such as diisopropyl ether (DIPE) and hexane, subjecting several compounds to stereospecific or selective esterification. Some of these reactions proceed to an appreciable extent only in non-aqueous solvents. Several enzymes were used as is and part were immobilized on inorganic insoluble matrices selected from alumina, calcium carbonate, diatomaceous earth and celite, silica, ion exchange resins and the like. The analytical methods that have been developed for the detection and separation of these compounds include, inter alia, using Gas Chromatography (GC) or High-Pressure Liquid Chromatography (HPLC) for detection and separation of said reaction products in addition to using liquid extraction, preparative HPLC or applying liquid chromatography using a column packed with silica gel in a mixture of organic solvents. The enantiomeric excess (ee) of the resolved products was checked by using an HPLC apparatus equipped with a chiral column or by using a polarimeter in cases where the value of the specific optical rotation of the enantiomer is mentioned in the literature.

Results and Discussion

Detecting and separating the products of enzymatic resolution of (+/-)-2-bromohexadecanoic acid

The enantiomers of the compound (+/-)-2-bromohexadecanoic acid [(+/-)-2-Br-HDA)], which is also named (R,S)-2-bromohexadecanoic acid can be used as Active Pharmaceutical Ingredients (APIs). It has been discovered that while reacting (+/-)-2-Br-HDA with n-butanol in hexane in the presence of the enzyme Pseudomonas cepacia, immobilized on a solid support, (R)-(+)-2-Br-HDA was isolated in 50% yield and ee of about 80%.

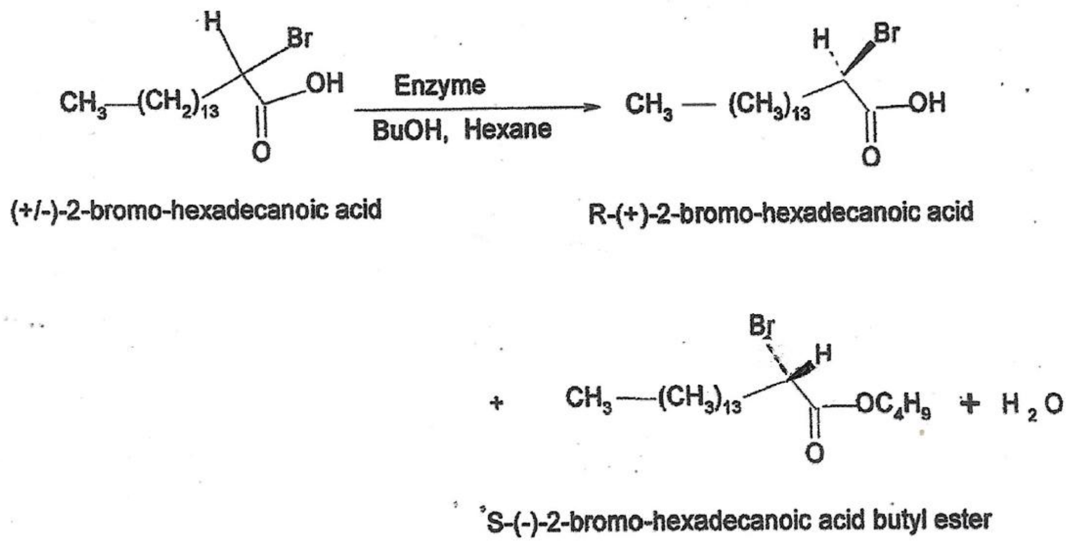
In one experiment, resolution of (+/-)-2-Br-HDA by enzymatic esterification using the substrate n-butanol in hexane was carried out by dissolving 1.025 grams of (+/-)-2-Br-HDA (3 millimoles) in 10 ml of hexane followed by addition of 2 ml of n-butanol (0.022 moles) and 1 gram of immobilized Pseudomonas cepacia shaken in a benchtop shaker at 40°C for 9 hours. The reaction proceeded slowly for 4 hours, after which time 100 μl of water (about

0.1%) was added to activate the enzyme, so the reaction could be concluded within further 5 hours.

The immobilized enzyme was prepared as follows. First, 0.1 M phosphate buffer (pH 5.8) was prepared by weighing 1.2 grams of KH_2PO_4 and 0.15 grams of K_2HPO_4 into a glass vessel under stirring to complete dissolution and the solution was transferred into a volumetric flask and the volume was adjusted to 100 ml with water. The pH was adjusted to 5.8 by adding NaOH solution and tested by using a pH meter. Then, 1 gram of the ion exchange resin Duolite was mixed with 10 ml of said buffer and the slurry was stirred for about 3 hours until the pH reached the value of 5.8. Then, 1 gram of enzyme was added and the slurry was shaken in a benchtop shaker at room temperature overnight after which time it was lyophilized to dryness.

Figure 1 below depicts the one-step reaction scheme for the enzymatic resolution of (+/-)-2-Br-HDA by esterification using the substrate n-butanol in hexane.

Figure 1



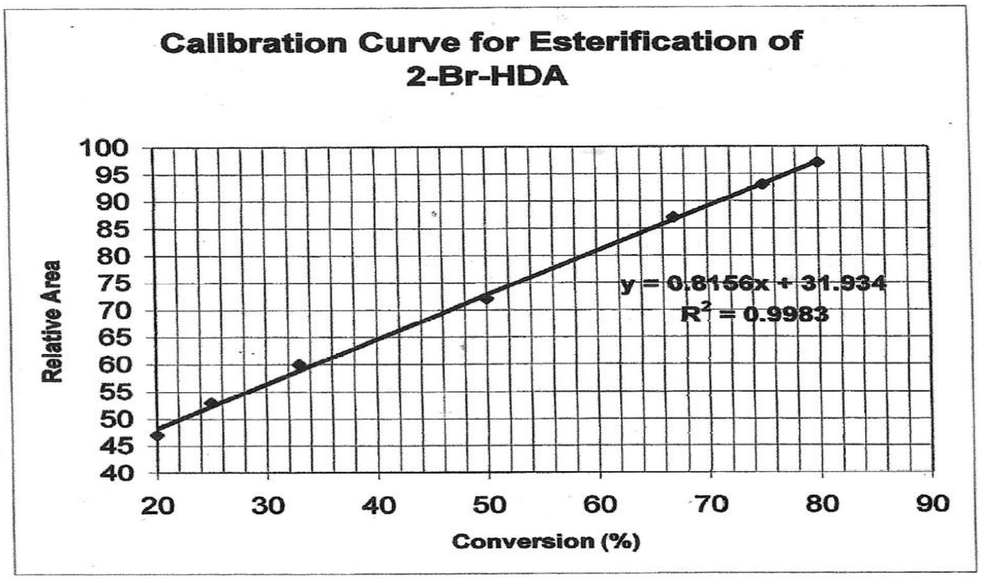
The reaction conversion rate was determined by using a calibration curve in order to terminate the reaction at about 60% conversion. Samples of mixtures of different molar combinations of racemic 2-Br-HDA and 2-Br-HDA butyl ester, which relate to different conversion rates, were injected to a gas chromatograph (GC) analyzer and the percent of the GC peak relative areas were correlated to the known molar concentrations of the two components to obtain the calibration curve. The relative area was calculated by dividing the GC measured peak area of the ester to the sum of the peak areas of the ester and the acid, e.g., for a molar ratio of acid: ester of 1:1, the relative GC area was 126,500/(48,330 + 126,500) X 100 = 72.4%, as detailed in Table 1 below.

Table 1

Molar ratio of acid: ester of samples that were prepared for injection to the GC analyzer	Calculated conversion rate relating to the given molar ratio	Relative area, % according to GC
1:4	20%	47.2
1:3	25%	52.7
1:2	33%	60.0
1:1	50%	72.4
2:1	67%	87.5
3:1	75%	93.5
4:1	80%	97.2

Figure 2 demonstrates the calibration curve.

Figure 2



The GC method used for detection and separation of the products of the kinetic enzymatic resolution of (+/-)-2-Br-HDA is as follows:

Column: Restek MTX-1

Initial temperature: 80°C

Final temperature: 360°C

ΔT= 20°C/minute

Retention Time (RT) of 2-Br-HDA: 4.05 minutes

RT of 2-Br-HDA butyl ester: 4.50 minute.

The reaction products, which are (R)-(+)-2-Br-HDA and (S-(-)-2-Br-HDA butyl ester, were separated by extraction. After termination of the enzymatic reaction, the immobilized enzyme was filtered off and the volume of the resulting solution was diminished to a minimum by using a rotary evaporator. Fatty acids tend in some cases to form emulsions and in order to prevent that, 20 ml of saturated NaCl solution was added in addition to adding 20 ml of saturated sodium bicarbonate solution and 10 ml of diethyl ether and extraction was carried out. The organic phase was separated, dried over sodium sulfate and evaporated to afford 0.38 grams (32.3%) of (S-(-)-2-Br-HDA butyl ester. (R)-(+)-2-Br-HDA free acid was obtained by acidifying the aqueous phase with 1M HCl solution followed by addition of 20 ml of diethyl ether and carrying out extraction. The layers were separated and the organic layer was dried over anhydrous sodium sulfate. The solvent was evaporated using a rotary evaporator to afford 0.57 grams (56.7%) of (R)-(+)-2-Br-HDA, thus the total yield was 89%.

The racemic compound 2-Br-HDA butyl ester was synthesized for usage as a standard for the calibration curve. This was carried out by adding 3.5 grams of 2-Br-HDA (10.4 millimoles) into a 100 ml flask and 14 ml of n-butanol followed by adding 1.75 ml of concentrated sulfuric acid drop-wise under stirring and the mixture was refluxed for two hours. Then, the mixture was transferred into a one-liter Erlenmeyer and 80 ml of saturated sodium bicarbonate solution was added and the mixture was stirred for 15 minutes. Then, the mixture was transferred to an extraction flask and the aqueous phase was extracted with 100 ml of diethyl ether. The organic phase was separated and dried over anhydrous sodium sulfate and the solvent was evaporated. 4.02 grams of residue was obtained and dissolved in ethyl acetate and loaded into a column packed with 70 grams of silica gel Merck Kieselgel 60 (0.063-0.200 mm) in a solvent mixture of 5% ethyl acetate and 95% hexane. Eight volumes were collected comprising 2.25 liters of solvent mixture containing the requested product, as determined by Thin Later Chromatography (TLC) analysis using 0.2 mm silica gel 60 plate in a solvent mixture of 5% ethyl acetate and 95% hexane. The Retention factor (R_f) of 2-Br-HDA: 0.36, R_f of 2-Br-HDA butyl ester: 0.83.

The solvent mixture was evaporated to afford 3.3 grams of chemically pure (+/-)-2-Br-HDA butyl ester (81.4%), according to GC analysis.

The ee of the resolved chiral 2-Br-HDA was checked by HPLC system according to the following method:

Chiral column: Astek Chirobiotic R

Detector: UV/VIS, $\lambda = 220$ nm

Flow = 0.6 ml/min

Mobile phase: Methanol: acetic acid: triethyl amine 100: 0.1: 0.1.

RT of (S)-(-)-2-Br-HDA: 4.4 minutes, RT of (R)-(+)-2-Br-HDA: 5.0 minutes.

The optical rotation of (R)-(+)-2-Br-HDA was checked by using a polarimeter. A solution having known concentration of (R)-(+)-2-Br-HDA in chloroform was prepared and filtered to obtain a clear liquid. Chloroform was inserted into a 1/2 dm measurement cell and the apparatus was calibrated. The solvent was discarded from the measurement cell and the solution of (R)-(+)-2-HDA in chloroform was inserted and the optical rotation measured was $[\alpha]_{D,25}$ (5.0, $CHCl_3$) = + 10.7.

The opposite enantiomer of (R)-(+)-2-Br-HDA can be obtained by chemical hydrolysis of (S)-(-)-Br-2-HDA butyl ester. Thus, 0.7 grams of said ester (1.8 millimoles) were dissolved in 15 ml of methanol and 4 ml of 1 M KOH solution were added drop-wise and mixing was maintained over a period of 1 hour followed by addition of additional 1 ml of 1 M KOH solution and mixing was maintained for further 1 hour.

In order to check the reaction completion, a 50 µl sample of the reaction mixture was withdrawn and 50 µl of 1 M HCl solution was added followed by addition of 0.5 ml of water and 0.5 ml of hexane in a small vial, which was shaken well to afford extraction. The upper organic layer was separated, dried over anhydrous sodium sulfate, filtered and injected to the GC analyzer.

After reaction completion, the solvent was evaporated using a rotary evaporator to afford a white solid which was (S)-(-)-2-Br-HDA sodium salt. 10 ml of water was added under mixing and titration was carried out with 7 ml of 1 M HCl solution to pH 2. 10 ml of saturated NaCl solution was added (to avoid formation of emulsions) followed by addition of 20 ml of diethyl ether and extraction was carried out. The organic layer was separated and dried over anhydrous sodium sulfate to afford (S)-(-)-2-Br-HDA in 75% yield having ee of 80%.

It may be noted that the opposite enantiomer (S)-(-)-2-Br-HDA can be also obtained in ee of about 50% while applying the enzyme Candida antartica lipase in the kinetic resolution of (+/-)-2-Br-HDA.

Detecting and separating the products of kinetic enzymatic resolution of (R,S)-mandelic acid

The kinetic enzymatic resolution of (R,S)-mandelic acid using the substrates methanol, ethanol or n-butanol in an organic solvent was carried in a similar way. In one experiment, 152 mg of (R,S)-mandelic acid (10 millimoles) were dissolved in 10 ml of DIPE followed by addition of 1.5 ml of n-butanol (16 millimoles) employing the enzyme Candida cylindracea lipase shaken in a benchtop shaker at 50°C for 12 hours.

The rates of the conversions were checked using a calibration curve, as described for the resolution of (+/-)-2-Br-HDA, employing an HPLC method having the following parameters:

Column: Phenomenex Luna RP-18

Detector UV/VIS , $\lambda = 254$ nm

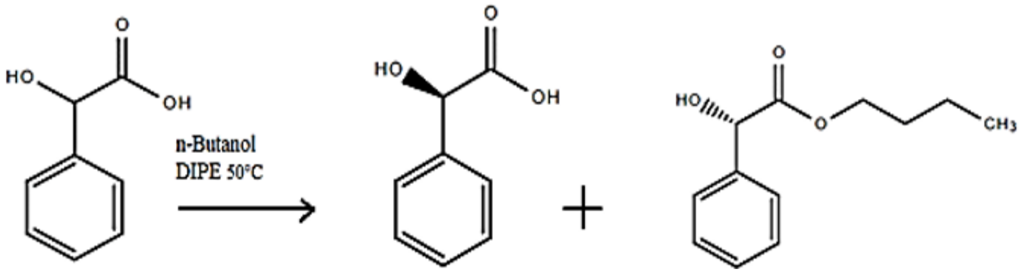
Flow= 0.6 ml/min

Mobile phase: 70% acetonitrile, 30% H_2O

Run time: 12 minutes.

The measured RTs of the analyzed substrates using said HPLC method were the following: mandelic acid: 1.87 minutes, methyl mandelate: 3.11 minutes, ethyl mandelate: 3.53 minutes, O-acetyl mandelate: 3.60 minutes, butyl mandelate: 5.0 minutes. Figure 3 below depicts the one-step kinetic enzymatic resolution of (R,S)-mandelic acid using an alcohol as substrate, e.g., n-butanol in an organic solvent. The enzymatic resolution of (R,S)-mandelic acid with n-butanol forms (S)-butyl mandelate, which can be named also (S)-mandelic acid butyl ester and (R)-mandelic acid, which is left intact. The (S)-mandelic acid enantiomer can also be left intact in this reaction by changing the enzyme and/or the solvent. Methanol and ethanol were also successfully used in this reaction as substrates in addition to n-butanol.

Figure 3



(R,S)-mandelic acid **(R)-mandelic acid** **(S)-mandelic acid butyl ester**

Separating the products of the kinetic enzymatic resolution of (R,S)-mandelic acid, which are mainly (R)-mandelic acid and (S)-mandelic acid butyl ester was carried out by filtering off the enzyme and adding to the resulting solution 25 ml of saturated sodium bicarbonate solution to pH 7.8 and extracting the aqueous phase with 30 ml of DIPE. The organic phase was washed with 25 ml of saturated sodium bicarbonate solution and with 25 ml of water and dried over anhydrous sodium sulfate. The solvent was evaporated in vacuum to obtain the chemically pure (S)-mandelic acid butyl ester. 30 ml of HCl 0.5M solution were added to the aqueous phase and mixing was maintained for 15 minutes followed by extraction with 50 ml of DIPE. The organic phase was dried over anhydrous sodium sulfate and solvent evaporated to afford (R)-mandelic acid.

While using the enzyme Candida antartica immobilized on Duolite ion exchange resin and ethanol instead of n-butanol the conversion after 3 hours of enzymatic reaction was about 46% and the ee of the isolated (R)-ethyl mandelate was 85%. While using the same enzyme immobilized on Lewatit ion exchange resin and methanol instead of n-butanol the conversion after 18 hours of enzymatic reaction was about 34% and (R)-methyl mandelate was isolated having ee of 60%.

The ee of the chiral mandelic acid and its esters was checked using the following HPLC method:

HPLC column: Merck's ChiraDex

Mobile Phase: 90% methanol, 10% H_2O

Flow rate: 0.8 ml/minute

Detector UV/VIS, λ= 220 nm

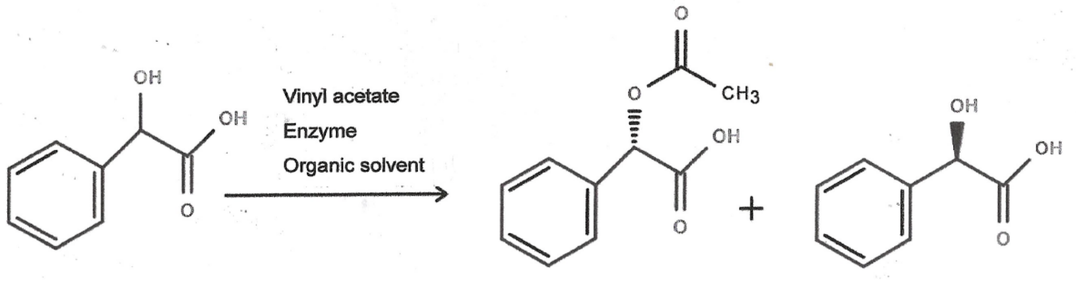
Run Time: 25 minutes

RT of, e.g., (R)-(-)-ethyl mandelate: 16 minutes, RT of (S)-(+)-ethyl mandelate: 18 minutes.

The mandelic acid esters were synthesized for usage as standards for the calibration curves, which were needed in order to determine the rates of the reaction conversions. The synthesis of, e.g., butyl mandelate was carried out by adding 0.685 grams of mandelic acid (4.5 millimoles) into a 100 ml flask followed by addition of 17 ml of n-butanol and 250 µl of concentrated sulfuric acid and the mixture was refluxed for two hours. Then, 25 µl of the reaction mixture were withdrawn, diluted to a volume of 1 ml in acetonitrile, filtered and injected to the HPLC apparatus to check the conversion which was about 85%. The mixture was transferred to a 250 ml flask and 20 ml of saturated sodium bicarbonate solution was added and the mixture was stirred for 15 minutes. Then, the mixture was transferred to an extraction flask and the aqueous phase was extracted with 20 ml of diethyl ether; the organic phase was separated and dried over anhydrous sodium sulfate and the solvent was evaporated to afford 0.58 grams of mandelic acid butyl ester in 63% yield. The synthesis of methyl or ethyl mandelate was carried out in a similar manner.

Figure 4 below depicts the one-step enzymatic O-acylation of (R,S)-mandelic acid using vinyl acetate in an organic solvent.

Figure 4

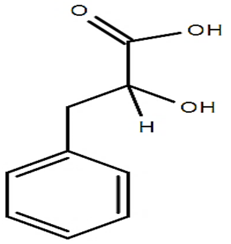


(R,S)-mandelic acid **O-acetyl-(S)-mandelic acid** **(R)-mandelic acid**

In one experiment, the enzymatic O-acylation of (R,S)-mandelic acid with vinyl acetate in an organic solvent was carried out by dissolving 15 mg of the acid (0.1 mmol) in 1 ml of DIPE in a small vial followed by addition of 43 mg of vinyl acetate (0.5 millimoles) and 2 mg of the enzyme Candida antarctica (enzyme substrate ratio of 1: 7.5) and the mixture was shaken in a benchtop shaker at 50°C for 12 hours. The reaction conversion was checked by using an HPLC method in order to terminate it at about 60% conversion. Thus, by choosing the enzyme and the acyl donor (n-butanol or vinyl acetate), it is possible to direct the enzymatic reaction to the main product either butyl mandelate or O-acetyl mandelic acid although the O-acylation reaction is a bit slower (12 hours in comparison to 3 hours respectively).

<u>Detecting and separating the products of kinetic enzymatic resolution of β-phenyl-lactic acid and β-phenyllactic acid ethyl ester</u>

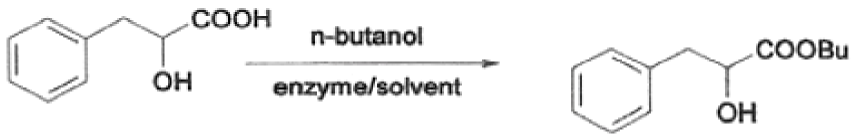
The compound β-phenyllactic is a substituted α-hydroxy carboxylic acid having the following chemical formula:



β-phenyllactic

Figure 5 below depicts the enzymatic esterification of DL-β-phenyllactic acid with the alcohol substrate n-butanol in an organic solvent.

Figure 5

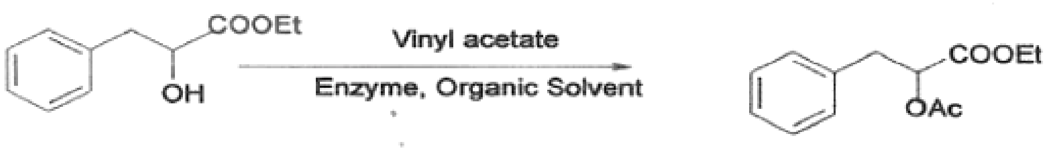


DL-β-phenyllactic acid **D or L-β-phenyllactic acid butyl ester**

Esterification of DL-β-phenyllactic acid with n-butanol was carried out by dissolving 17 mg of the acid (0.1 millimoles) in 1 ml of DIPE followed by addition of 45µl of n-butanol (0.5 millimoles) employing 35 milligrams of the enzyme Candida antartica lipase immobilized on Duolite ion exchange resin shaken in a benchtop shaker at 50°C for 20 hours. After separation and work-up each of β-phenyllactic acid and its ester were obtained in 30% yield.

Enzymatic O-acylation of DL-β-phenyllactic acid methyl or ethyl esters was carried out by reaction with vinyl acetate in an organic solvent. O-acylation of DL-β-phenyllactic acid ethyl ester with vinyl acetate is depicted in Figure 6 below.

Figure 6



DL-β-phenyllactic acid ethyl ester **O-acetyl-D or L-β-phenyllactic acid butyl ester**

The enzymatic reaction was carried out by dissolving 20 mg of DL-β-phenyllactic acid ethyl ester (0.1 millimoles) in 1 ml of DIPE followed by addition of 45µl of vinyl acetate (0.49 millimoles) employing 35 milligrams of the un-immobilized enzyme Candida antartica lipase shaken in a benchtop shaker at 50°C for 20 hours to afford O-acetyl-β-phenyllactic acid ethyl ester in 47% yield.

The synthesis of the starting material β-phenyllactic acid ethyl ester was carried out by adding 1.2 grams of β-phenyllactic acid (7.2 millimoles) into a 100 ml flask followed by addition of 10 ml of ethanol and 1 ml of concentrated sulfuric acid drop-wise and the mixture was refluxed for two hours. Then, 100 µl sample of the reaction mixture was withdrawn and 0.5 ml of saturated sodium bicarbonate solution and 0.5 ml of methanol were added and the mixture was stirred and filtered through a 0.2 µm filter. 0.5 ml of methanol was added and a sample was injected to the HPLC apparatus to check the conversion which was about 80%. The mixture was transferred into a 250 ml flask and 50 ml of saturated sodium bicarbonate solution was added and the mixture was stirred for 15 minutes. Then, the mixture was transferred to an extraction flask and the aqueous phase was extracted with two portions each of 50 ml of diethyl ether. The organic phases were collected and dried over anhydrous sodium sulfate and the solvent was evaporated in vacuum to afford 1.1 grams of β-phenyllactic acid ethyl ester in about 75% yield.

The same HPLC method used for detection and separation of mandelic acid and its esters was applied for detection and separation of the products of the enzymatic esterification of β-phenyllactic acid.

RT of β-phenyllactic acid: 3.15 minutes

RT of O-acetyl-β-phenyllactic acid methyl ester: 4.30 minutes

RT of O-acetyl-β-phenyllactic acid ethyl ester: 4.80 minutes

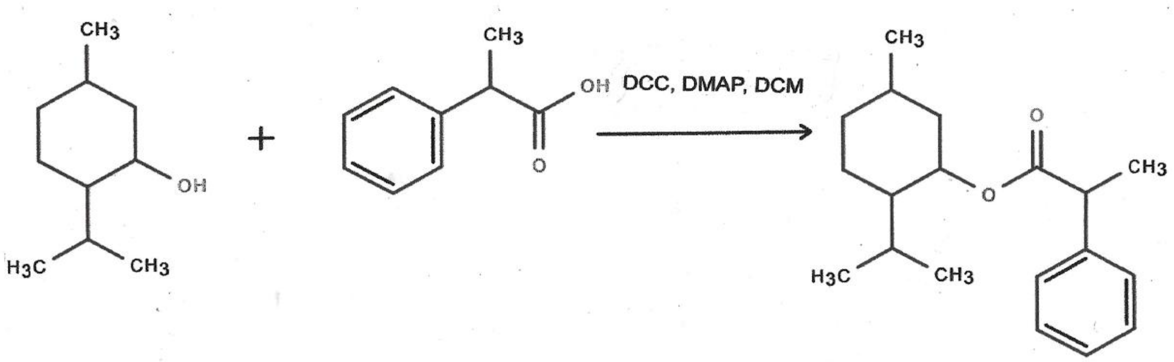
RT of β-phenyllactic acid butyl ester: 6.50 minutes.

Detecting and separating the products of kinetic enzymatic resolution of (R,S)-2-phenylpropionic acid (2-PPA) by esterification with menthol

The enzymatic resolution of (R,S)-2-phenylpropionic acid (2-PPA) by esterification with menthol was carried out by dissolving 23 mg of 2-PPA (0.15 millimoles) in 1 ml of hexane in a small vial followed by addition of 47 mg of menthol (0.30 millimoles) and 20 mg of the enzyme Candida antarctica lipase, immobilized on a solid support, and the mixture was shaken in a benchtop shaker at 45°C for 72 hours. A 50 µl sample was withdrawn and diluted to a volume of 5 ml, filtered and injected to the HPLC apparatus and the concentration of 2-PPA in the solution was calculated using a calibration curve correlating the concentration of 2-PPA in the solution to the HPLC chromatogram area. The calibration curve was determined by preparing several solutions of 2-PPA with known concentrations, e.g., 0.05, 0.1, 0.15, 0.2 and 0.25 molar, diluting the samples and injecting them to the HPLC apparatus. This was carried out in order to correlate each of the specific concentration and dilution factor to the chromatogram area. The initial concentration of 2-PPA was 0.15 molar while the final concentration was about 0.07 molar. Several other enzymes are active carrying out the reaction such as Pseudomonas cepacia.

Figure 7 below depicts the chemical synthesis of the reference compound 2-PPA menthyl ester.

Figure 7



menthol **2-phenylpropionic acid** **2-phenylpropionic acid menthyl ester**

The determination of the reaction conversion was carried out by using an HPLC method as described for the resolution of (+/-)2-Br-HDA. The synthesis of the compound 2-PPA menthyl ester, used as reference standard for calculation of the reaction conversion, was carried out by adding 0.3 grams of 2-PPA (2 millimoles) into a 250 ml flask equipped with a calcium chloride drying tube followed by addition of 25 ml of dichloromethane (DCM).

Then, 0.375 grams of menthol (2.4 millimoles) were added followed by addition of 40 mg of 4-dimethylaminopyridine (DMAP) under stirring. The solution was cooled in an ice bath to 0°C while 0.536 grams (2.6 millimoles) of dicyclohexylcarbodiimide (DCC) dissolved in 25 ml of DCM were added over a period of 5 minutes. The molar ratio between the acid (2-PPA), the alcohol (menthol) and DCC was 1: 1.2: 1.3 respectively. After a further 15 minutes at 0°C, the ice bath was removed and the reaction mixture was stirred overnight at room temperature. The precipitated dicyclohexylurea was removed by filtration and the filtrate was washed twice with 20 ml of HCl 0.5M solution followed by washing with saturated sodium bicarbonate solution to afford additional precipitation of dicyclohexylurea, which was removed by filtration. The layers were separated and the organic layer was dried over anhydrous sodium sulfate and concentrated using a rotary evaporator to afford the crude product, which was purified by preparative HPLC.

Menthol consists of 8 diastereomeric isomers. One of these diastereomers is l-menthol, which is also named (1R, 2S, 5R)-5-methyl-2-(1-methylethyl)cyclohexanol. l-menthol occurs naturally as the main stereoisomer in natural oils such as mint and peppermint oils. Menthyl esters of, e.g., valine and isoleucine exhibit anti-inflammatory properties.

The following are the operating HPLC method parameters used for separation and detection of 2-PPA:

Column: Phenomenex Luna RP-18

Detector: UV/VIS, $\lambda = 202$ nm

Flow = 0.75 ml/min

Mobile phase: 30% acetonitrile, 70% of 20 millimolar KH_2PO_4 solution

RT of 2-PPA: 2.7 minutes, RT of 2-PPA menthyl ester: 5.6 minutes.

The ee measurement of the resolved 2-PPA was carried out as described in the literature[7].

Analysis of the esterification products of citric acid

Citric acid is not a chiral compound but it's esterification can be carried out enzymatically avoiding the use of acids such as sulfuric acid or other hazardous chemicals, so the preparation of, e.g., tributyl citrate in this way can be regarded as natural or "green". Thus, the synthesis of a reference material such as tributyl citrate was carried out in the traditional method as follows: 1 gram (5.2 millimoles) of citric acid was administered into a 100 ml round flask and 17 ml of n-butanol were added followed by addition of 2 ml of concentrated sulfuric acid drop-wise under mixing and the mixture was refluxed for 2 hours. Then, a 50 µl of sample was withdrawn and diluted to the desired volume and injected to the HPLC apparatus to check the degree of conversion. Tributyl citrate was obtained by transferring the reaction mixture into a flask followed by addition of 30 ml of saturated sodium bicarbonate solution and the mixture was stirred for 15 minutes. The reaction mixture was transferred to an extraction flask and 30 ml of diethyl ether were added. The layers were separated and the organic layer was dried over anhydrous sodium sulfate and the solvent was evaporated to afford 1.5 grams of tributyl citrate in 83% yield.

The following HPLC method was used for detection and separation of citric acid and its esters:

HPLC column: Phenomenex Luna RP-18

Mobile Phase: 50% acetonitrile, 50% potassium hydrogen phosphate solution 0.05M, pH 2.25

Flow rate: 1.2 ml/minute

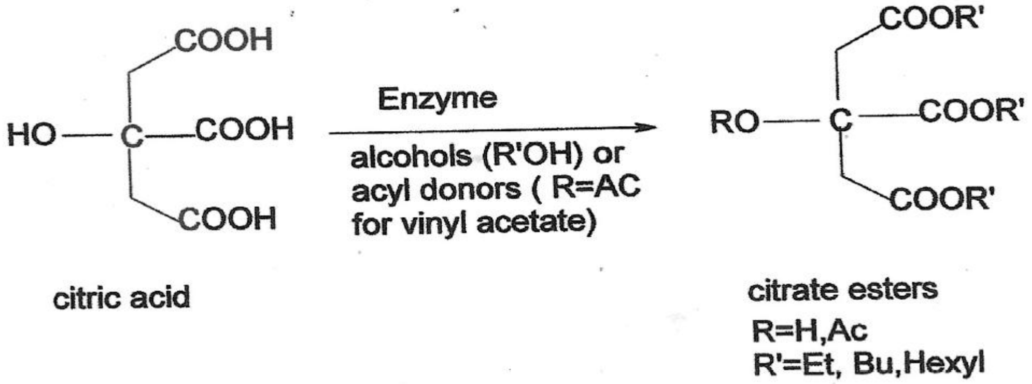
Detector UV/VIS, λ= 206 nm

Run Time: 12 minutes

RTs, citric acid: 1.05 minutes, monobutyl citrate: 1.5 minutes, dibutyl citrate: 3.4 minutes, tributyl citrate: 4.8 minutes.

The enzymatic esterification of citric acid can be carried out as described for mandelic acid, as demonstrated in Figure 8 below.

Figure 8



<u>List of analytical instruments that have been used for the detection and separation of said compounds:</u>

Freeze Dryer: Christ Alpha 1-2

GC: Shimadzu GC Model 2030

HPLC Apparatus: HP Model 1090

Karl Fischer Titrator: Metrohm's KF 701 Titrino

Polarimeter: Jasko Model P-1020

pH meter: Mettler Toledo Model 1120.

Conclusion

Analytical methods have been developed that are useful for the detection and separation of resolved products of several kinetic enzymatic resolutions that have been carried out in nearly anhydrous organic solvents. Enzymes such as transglutaminase and lipases were used as catalysts in carrying out stereospecific and selective enzymatic resolutions of compounds such as (+/-)-2-bromohexadecanoic acid, (R,S)-mandelic acid, DL-β-phenyllactic acid and DL-β-phenyllactic acid ethyl ester and (R,S)-2-phenylpropionic acid. The corresponding esters of said comounds have been synthesized to be used as reference standards.

References

[1] Zaks A., Klibanov A.M., "Enzyme-catalyzed processes in organic solvents", Proc.Natl.Acad.Sci., USA, 82, 3192, 1985.

[2] Kirchner G., Scholar M.P., Klibanov A.M., "Resolution of racemic mixtures via lipase catalysis in organic solvents", J. Amer. Chem. Soc., 107, issue 24, 7072, 1985.

[3] Klibanov A.M., "Enzymatic catalysis in anhydrous organic solvents", Trends in Biochem. Sci., Vol. 14, issue 4, 1411-1414, 1989.

[4] Klibanov A.M., "Asymmetric transformations catalyzed by enzymes in organic solvents", Acc. Chem. Res., 23, 114-120, 1990.

[5] Gutman A.L., Shkolnik E., Meyer E., Polak F., Brenner D., Boltansky A., "Practical enzymatic resolution of racemic alcohols and amines in organic solvents", Annals of the N.Y Academy of Science, 799 (Enzyme Engineering XIII), 620-632, 1996.

[6] Meyer E., Technium BioChemMed, Vol. 5, 2023.

[7] Shengqiang T., Zheng Y., Yan J. , "Application and comparison of high-performance liquid chromatography and high-speed counter-current chromatography in enantio-separation of (±)-2-phenylpropionic acid", Journal of chromatography, Vol. 1281, 70-86, 2013.