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유기용매내에서 리파제의 입체특이성 반응기질로서 이용하기 위한 Trifluoroethylmandelate의 이성질체 합성 및 입체특이성

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Synthesis of Stereoisomeric Trifluoroethylmandelates and Their Stereospecificity for the Uses as the Substrate of Lipases in Organic Solvent

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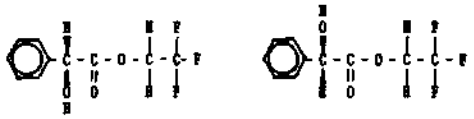
요 약. 유기용매에서 리파제의 입체특이성 반응연구를 위하여 리파제의 기질로서 trifluoroethylmandelate를 도안하고 이를 mandelic acid와 trifluoroethanol를 사용하여 알코올과 산에서 에스텔을 합성하는 방법을 도입하여 합성하였다. 합성된 물질이 trifluoroethylmandelate임을 ^{1}H NMR과 원소 분석을 통하여 확인하였다. (+)-와 (−)-trifluoroethylmandelate의 specific optical rotation($[\alpha]_{25}^D$)은 각각 74.0°과 75.4°이었다. 이 합성된 기질을 이용하여 유기용매내에서의 리파제의 입체이성질체에 대한 transesterification 속도는 서로간에 상당한 차이가 나타났다. 반면에 $[\alpha]_{25}^D$가 낮은 입체 이성질체인 (+)-와 (−)-methylchloropropionate에서는 리파제의 활성은 있었으나 차이는 없었으며, 높은 $[\alpha]_{25}^D$를 갖는 methylmadelate는 리파제의 활성도 없었다.

ABSTRACT. Stereoisomers of trifluoroethylmandelate(mandelic acid trifluoroethylester) were synthesized from each isomer of mandelic acid and trifluoroethanol with *p*-toluene sulfonic acid in order to study the enantioselectivity of lipase in organic solvent. The products were identified by ^{1}H NMR and elemental analysis and their physical properties such as melting point, densities and specific optical rotations($[\alpha]_{25}^D$) were also characterized. $[\alpha]_{25}^D$ of (+)- and (−)-trifluroethylmandelate were +74° and −75.4°, respectively. The trifluoroethylmandelate was found out to be as a good substrate for the transesterfication stereoselectivity of lipases in organic solvent. Any significant difference of the lipase catalyzed transesterification activity between (+)- and (−)-methylchloropropionate was not found, and even lipase activity of transesterfication was not found with high optical polar (+)- and (−)-methylmandelate.

INTRODUCTION

Enzyme can function as a catalyst in anhydrous organic solvents instead of their conventional aqueous reaction medium[1,2], and when placed in this unnatural medium, they exhibit novel catalytic properties such as greatly enhanced thermostability[3], radically altered specificity[4], and stereoselectivity[5~8]. It has been found out especially that subtilisin (from *Bacillus subtilis*) had a remarkable enantioselectivity of oxidation of steroids in organic solvent[9]. The enantioselectivity of enzyme in organic solvent, which did not take place in natural aqueous media, is much advantageous for the enzymatic transformation of lipase reaction[5,6]. In order to investigate the enantioselectivity of lipase in organic solvent, a commercially available ste-

reoisomeric substrates such as methyl-2-chloropropionate and methylmandelate were used as a preliminary step. Unfortunately, however, any of these substrates did not show any enantioselectivity of lipase in organic solvent (see the results). Therefore, an activated enantiomeric substrate, trifluoroethylmandelate (TFEM; mandelic acid trifluoroethylester, $C_6H_5CHOHCO_2CH_2CF_3$), was designed by substituting three hydrogens (H) of methyl group ($-CH_3$) with three fluorides ($-CF_3$) in ethylmandelate. The structural formula of each TFEM is shown as the follows:



R(−)-trifluoroethylmandelate S(+)-trifluoroethylmandelate

Because stereoisomer of each TFEM can not be obtained commercially and because its synthetic methods and the properties were never reported anywhere else, we inevitably each (+)- and (−)-TFEM with each isomers of methylmandelate and trifluoroethanol for the study of enantioselectivity of lipase in organic solvent.

In this paper, the synthetic method of each enatiomer S(+)- and R(−)-TFEM and their identification and characterization were reported. Simultaneously, lipase catalyzed enantioselective transesterification using these stereospecific substrates in organic solvent was also shown for the five lipases.

MATERIALS AND METHODS

Materials. R(−)- and S(+)-mandelic acid(α-hydroxybenzeneacetic acid; $C_6H_5CHOHCO_2H$), 2,2,2-trifluoroethanol(CF_3CH_2OH). *p*-toluenesulfonic acid anhydrous(PTSA), R(−)- and S(+)-ethylmandelate, and racemic methylmandelate were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). Methyl-2-chloropropionate(2-chloropropionic acid methylester) was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of HPLC pure grade purchased from commercial suppliers, Aldrich and Sigma. Lipases from *Humicola lanuginosa* and *Pseudomonas sp.* (LPL) were purchased from Sigma, those from *Aspergillus sp.* (K30) and *Mucor javanicus* were obtained from Amano International Enzyme Co. (Troy, VA), and lipase from *Chromobacterium viscosum*(CV) was supplied from Finn Sugar Biochemicals (EIK Grove Village, IL).

Methods. Since the synthetic method of trifluoroethylmandelate (TFEM, $C_6H_5CHOHCO_2CH_2CF_3$) was not described in any paper and book before, general methods[10,11] of ester synthesis from alcohol and acid was modified to synthesize the TFEM, considering the rare possibility of racemization of products during the synthesis[12]. The modified method for synthesizing the TFEM was as follows; 0.1 mole of each mandelic acid was sampled into the round bottomed flask and added 2.0 mole of trifluoroethanol and small amount of PTSA (0.5%) as a catalyst[13]. Mixture was refluxed at 70℃ for about 4 hrs. On confirming the total conversion, stopped the reaction, and evaporated the solvent using rotary evaporator. After adding 0.1 N sodium bicarbonate solution, TFEM was extracted using diethylether from the mixture by 3 or 4 times. And magnesium sulfate anhydrous was added to remove the water from solvent and diethylether was evaporated. To get higher purity the products was recrystallized using hexane.

The purity and property of product was tested by GC. The properties and purity of product was tested by TLC with Silica Gel IB-F(Baker) and 10% methanol in dichloromethane with 1 or 2 drops of acetic acid. The spots were developed using concentrated H_2SO_4 followed by heating. The structure of TFEM was identified by ^1H NMR ($CDCl_3$, 0.1 TMS as the internal reference) of Department of Chemistry, MIT (250 MHz), by elemental analysis (performed by Robertson Laboratory, Inc., Madison, NJ), and by fast atomic bombardment mass spectrometry analysis at MIT mass spectrometry NIH Facility. Specific optical rotation, $[\alpha]_{25}^{D}$, was measured at 589 nm (sodium line) at 25℃ in Perkin-Elmer (Model 253B, West Germany) polarimeter using a standard cell with 10 cm light path in 1.6 c (1.6 mg/100 ml) acetone. Melting temperature and density of product were

also determined by conventional methods.

Enzymatic transesterification activity of lipases to the substrates in organic solvent using octane[14,15] for five lipases from *H. lanuginosa, M. javanicus, C. viscosum, Pseudomonas sp*(LPL), and *Aspergillus sp*. (K30) dissolved in 0.01 *M* phosphate buffer solution and lyophillized enzyme after adjusted pH to 7.0 (pH memory theory)[16]. Typically lyophillized enzyme (1 mg/ml) was added to ester substrate solution (TFEM) in the presence of methanol nucleophile (40 mM) in octane (1 ml). After 10 sec sonication, the vial was placed on an orbital shaker at 7℃ and 20 rpm. Periodically, aliquots were withdrawn and assayed for the accumulation of the methylmandelate using GC. To compare the enantioselectivity of TFEM, $V_{max}K_m$(min^{-1}) for transesterification in organic solvent system was determined from the Lineweaver-Burk plot of lipase activity[14,17]. Enzyme activity of lipase in organic solvent was defined as one micromole of methylmandelate produced per 1 min. The stereoselectivity of lipase for each isomeric substrate was defined by enantioselective factor, $(kcat/K_m)^+/(kcat/K_m)^-$, which was derived directly from $(V_{max}/K_m)^+/(V_{max}/K_m)^-$ assuming the concentrations of each active enzyme, $[E_o]$, between the two isomeric substrate reaction are the same[14].

RESULTS AND DISCUSSION

The product, trifluoroethylmandelate($C_6H_5CHOHCO_2CF_3$) was white crystalline solid, the purity of compound after two recrystallization was over 99.9% by gas chromatography. The molecular mass measured by mass spectrometry was 234.1 which was consistent with the expected molecular mass of TFEM (the calculated mass is 234.2). Elemental analysis data for the product was C, 51.48 %; H, 3.84%; F, 24.58% (weight ratio), corresponding to the calculated data (C, 51.2%; H, 0.4%;

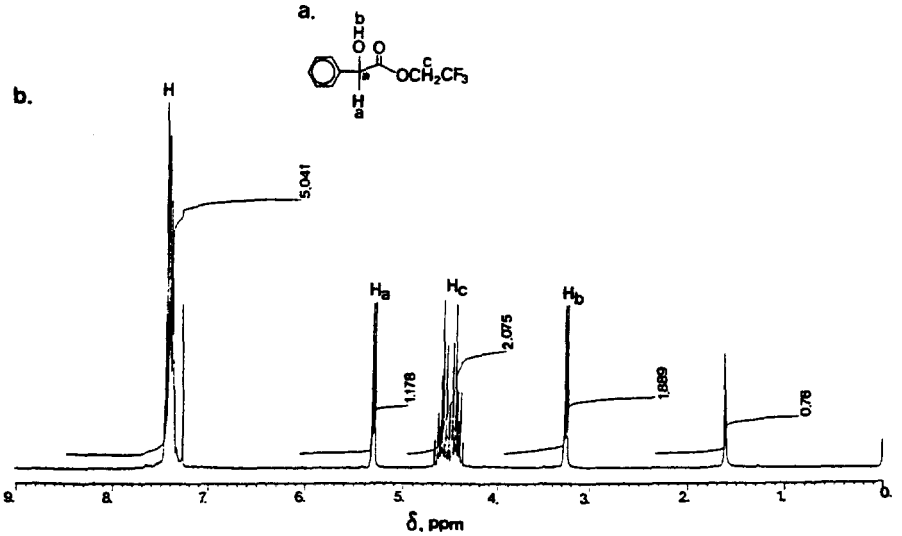


Fig.. 1. Structure formula of trifluoroethylmandelate (a) and its one dimensional proton NMR spectrum (b). Trifluoroethylmandelate was dissolved in $CDCl_3$ (deuterium trichloride). TMS (tetramethylsilane; $(CH_3)_4Si$) was used as an internal reference. ^{1}H NMR(250 Hz) at Department of Chemistry, MIT, was used. H_a, H_b, H_c and H are corresponding to the protons hydrogen (1H) and hydroxide (1OH) at asymmetric (optical) carbon (C* : Cγ), two hydrogens of Cβ ($-CH_2$) and five hydrogenes at benzene ring, respectively. The peak at 1.60 ppm was from the portion of TMS. The data show δ 3.24(1H, d($-$OH), J=6.0 Hz), 4.37~4.67(2H, m($-CH_2$)), 5.31 (1H, d($-$H), J=5.8 Hz), and 7.32~7.46(5H, m). δ, Chemical shift parameter(ppm); s, single peak; brs, broaden single peak; d, dual splitting pleak; m, multiple splitting peak; J, spin spin splitting constant. The numbers on the right side of peak are integrals of proton intensity at starting point of integration. For H_c is the suplitting number of peak is 15(m=15) and H (last peak for five phenol proton) has 18 peaks.

Table 1. Characteristics of synthessized (+)- and (−)-trifluoroethylmandelate

Characters	(+)-TFEM[a]	(−)-TFEM
Purity(%)[b]	over 99.9	over 99.9
Melting point(℃)	52~53	52~54
Density (g/ml)	1.184	1.207
R_f value of TLC[c]	0.90	0.90
Specific optical rotation(°)[d]	+74.0	−75.4

[a]TFEM is an abbreviation of trifluoroethylmandelate, [b]Purity determined by GC : Hewlett-Packard, Model HP5890 with capillary column, [c]TLC condition : developing solvent, 10% methanol in dichloromethane with 1 or 2 drops of acetic acid; TLC plate, Silica Gel IB-F(Baker), [d]$[\alpha]_{25}^{D}$ for 1.6 c in acetone at 589 nm (sodium line).

F, 24.3%) of TFEM (molecular formula of trifluoroethylmendelate is $C_{10}H_9F_3O_3$. NMR data of trifluoroethylmandelate was performed for confirming the structure as shownin *Fig.* 1, ^{1}H NMR ($CDCl_3$, 0.1% TMS) showed δ(chemical shift), 3.24(1H, *d*, (−OH), J=6.0 Hz)), 4.37~4.67(2H, m), 5.31(1H, *d*, J=5.8 Hz), 7.32~7.46(5H, m), confirming the product had a structural formula as $C_6H_5CHOHCO_2CH_2CF_3$. As a result, the product synthesized must be a TFEM. This synthetic method has an advantage of no racemization of stereoisomeric products during the synthesis of TFEM[12].

The physical properties of TFEM is shown in *Table* 1. The melting point of each isomeric product was 52~54℃. The densities (d) of (+)- and (−)-TFEM were 0.184 and 1.207 g/ml, respectively. R_f value of TLC for TFEM, mandelic acid and methylmandelate was 0.90, 0.28 and 0.85, respectively. R_f values indicating that TFEM was much more volatile than mandelic acid as expected, on the other hand, which was slightly more volatile than methylmandelate. Synthesized TFEM on TLC also showed clear one spot showing high purity, and HPLC peaks of products showed high pure single peaks. Specific optical rotations ($[\alpha]_{25}^{D}$) are very important factors for characterizing whether the products are optical or not. *Table* 2 showed $[\alpha]_{25}^{D}$ of each isomer for TFEM, methylmandelate, and mandelamide. The $[\alpha]_{25}^{D}$ for (+)- and (−)-TFEM was +74.0° and −75.4°, respectively. Mandelamide ($C_6H_5CHOHCONH_2$) is an inhibitor of transesterification of lipase which was synthesized from melthylmandelate by conventional method[18] and identified by ^{1}H NMR: δ; 5.02(1H, s), 5.61(1H, br s), 6.39(1H, br s), 7.26~7.42(3H, m), 7.44~7.55(2H, m) (see the Legend of *Fig.* 1). To confirm indirectly whether the observed optical rotation data were reliable or not, the specific optical rotation of each methylmandelate and mandelamide were also determined for comparison with the reported data. For the case of mandelamide observed data were almost the same as those of reported data[19], whereas $[\alpha]_{25}^{D}$ of methylmandelate observed were slightly greater than those of reported data[19] (see *Table* 2). Although specific rotation data of methylmandelate were slightly deviated from reported data, the data of TFEM, mandelamide and methylmandelate were highly reliable. This indicated the synthetic each isomeric TFEM can be high optical stereoisomeric products.

Table 2. Specific optical rotation; $[\alpha]_{25}^{D}$ (1.6 c in acetone) at 589 nm (sodium line), of each isonomer trifluoroethylmandelate, mandelic acid methylester (methylmandelate) and mandelamide

Compounds	$[\alpha]_{25}^{D}$(°)
(+)-trifluoroethylmandelate	+74.0
(−)-trifluoroethylmandelate	−75.4
(+)-methylmandelate	+119.8 (103.6)[a]
(−)-methylmandelate	−117.6 (103.6)
(+)-mandelamide	+79.7 (+73.0)
(−)-mandelamide	−76.7 (−73.0)
(+)-methylchloropropionate	+25.1 (+2.15)
(−)-methylchloropropionate	−21.3 (−26.83)

[a]The data closed by paranthesis are reported data in Dictionary of Organinc Compounds[19].

Using these high stereoisomeric substrates and methanol (as nucleophile), the enantioselectivities of lipase in octane for the transesterification of the five lipases were investigated expressed in terms of enantioselective factor[14]; $(kcat/K_m)^+/(kcat/K_m)^-$, as shown in *Table* 3. Enzyme specificity factor $kcat/K_m(M^{-1}min^{-1})$ can be calculated from V_{max}/K_m with the known refers to the free

Table 3. V_{max}/K_m (min^{-1})[a] in transesterification and enantioselective ratio[b]; $(kcat/K_m)^+/(kcat/K_m)^-$, of five lipase[c] from *H. langinosa, Aspergilus sp.* (K30), *M. javanicus, C. viscosum*, and *Pseudomonas sp.* (LPL) to trifluoroethylmandelate and 40 mM methanol in 1 ml of octane[d] at 7℃[e] and 250 rpm shaking

Lipase origin	V_{max}/K_m (min^{-1}) on the		$(kcat/K_m)^+$
	(+)-TFEM	(−)-TFEM	$(kcat/K_m)^-$
H. lanuginosa	0.045	1.08	0.41
Aspergilus sp. (K30)	0.148	0.044	3.36
M. javanicus	0.353	0.853	0.41
C. viscosum	1.60	0.529	3.02
Psedomonas sp.	0.188	0.077	2.44

[a]V_{max}/K_m of each lipase activity was obtained from Linweaver-Burk plot(1/[S] *vs.* 1/[v]). Enzyme activity of lipase in organic solvent was determined by assaying the methylmandelate using gas chromatography with 5-HP1 capillary column coated methylsilicone gum (Hewlett-packard, Model HP5890, N_2 as carrier gas, 30 ml/min, detector and port temperatures 300℃ and 250℃, respectively. [b]Enantioselective factor; $(kcat/K_m)^+/(kcat/k_m)^-$, is referred to the lipase relative transesterification activity of $(kcat/K_m)$ for (+)-isomer of $TFEM^+$ and $(kcat/K_m)$ for (−)-isomer of TFEM. [c]These five lipases were selected by determining the transesterification activity among 19 lipases using methylchloropropionate as substrate. [d]Lipase was dried by lyophillization at pH7.0 and octane were dried with 3 Å molecular sieves (Linde) before reaction[16]. [e]To eliminate the possible nonenzymatic transesterification the reaction was performed at 7℃ instead of 30℃.

enzyme with the free substrate[20], and hence it is indenpendent of the nature of the nucleophile (alcohol and water). Therefore, this factor could be applied to determine the enantioselectivity of lipase catalyzed reaction in organic solvent. To compare the enantioselective transesterification activity of the lipase between low and high optical polar substrates in the organic solvents, (+)- and (−)-methylchloropropionate and propanol were used as low optical substrates (see *Table* 2)[18]. An significant difference of the lipase catalyzed transesterification activity between (+)- and (−)-methylchloropropionate was not found (data not shown) probably due to the low optical polarity of methylchloropropionate. To use the higher optical polar substrates (see *Table* 2) for this study, high optical polar substrate such as (+)- and (−)-methylmandelate was tried to use[13] (synthesized from each (+)- and (−)-mandelic acid by conventional method[17]) with propanol. However, lipase did not show any activity of transesterification to this substrate at 30℃ (data not shown) because methylmandelate has a very inactive structure. Therefore such an activated high polar substrate, i.e., TFEM, is necessary for this study. Enantioselective activity of lipase on TFEM (*Table* 3) showed that not only increased the transesterification activity of lipases but also enantioselectivity of transesterification were increased dramatically with the TFEM in organic solvent. In fact, fluoride were reported to activate the substrate molecule in other enzyme catalyzed reaction in organic solvent[21].

In conclusion, (+)- and (−)-TFEM were synthesized firstly and characterized as high pure optical polar compounds firstly, which were good substrates for investigating the enantioselective lipase catalyzed transesterification in the octane organic solvent.

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