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Gelozymes in organic synthesis. Part IV: Resolution of glycidate esters with crude Mung bean (*Phaseolus radiatus*) epoxide hydrolase immobilized in gelatin matrix☆

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Abstract—A crude extract of Mung bean meal (*Phaseolus radiatus*) possessing epoxide hydrolase activity immobilized in gelatin gel (gelozyme) is employed in the stereoselective epoxide ring opening of glycidate esters. Thus, ethyl *trans*-(±)-3-phenyl glycidate **1a** and methyl *trans*-(±)-3-(4-methoxyphenyl) glycidate **1b** gave (2*S*,3*R*)-glycidate esters (ee >99% and 45% yield) with gelatin immobilized enzyme in diisopropyl ether. The corresponding (2*R*,3*S*)-enantiomer of **1a** was hydrolyzed by an epoxide hydrolase to predominantly give the *anti*-product, ethyl (2*R*,3*R*)-2,3-dihydroxy-3-phenylpropanoate, with a diastereomeric excess of 78% and ee 94% (40%). A small amount (5%) of racemic *syn*-product was also obtained as a result of the spontaneous hydrolysis. In the case of **1b**, the hydrolysis product was racemic due to high reactivity of the glycidate toward water.
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1. Introduction

Over the last decade, a number of epoxide hydrolases (EC 3.3.2.3) performing hydrolytic kinetic resolution of epoxides have been discovered.[1] Examples of bacterial epoxide hydrolases include those isolated from *Agrobacterium radiobacter*, *Rhodococcus* sp., *Corynebacteriuim* sp., *Mycobacterium paranicasm*, *Nocardia* sp., *Pseudomonas* NRRL B-2994, and some *Streptomyces* strains. Fungal epoxide hydrolases have also been found in *Aspergillus niger*, *Helminthosporum sativum*, *Diploida gossypina*, *Beauveria sulfurescens*, and some *Fursarium* strains. The best-known yeast epoxide hydrolase is *Rhodotorulla glutinis* enzyme.[2] Although the applications of epoxide hydrolases in the preparation of chiral epoxides and diols have been well demonstrated,[1,2] these enzymes are essentially obtained through fermentation and hence are not easily accessible to organic chemists. Recently, epoxide hydrolases have been discovered in Mung beans (*Phaseolus radiatus* L.), a commonly available commodity from supermarkets. The enzyme from these beans has been found to provide chiral (*R*)-diols from racemic styrene oxide and related compounds in excellent yields and enantiomeric purities.[3] Herein, we report the application of the crude enzyme preparation from Mung beans for the resolution of racemic *trans*-ethyl phenyl glycidate **1a** and *trans*-methyl (4-methoxyphenyl)glycidate **1b** to obtain enantiomerically pure (2*S*,3*R*)-glycidate esters in high yields (90% of theoretical) and high enantiomeric purity (ee >99%). (Scheme 1). Although enantiomerically pure glycidate esters are of great interest to organic chemists,[4] we have surprisingly not come across any reference work where epoxide hydrolase has been used to resolve a glycidate ester. Furthermore, several procedures are available to prepare the (2*R*,3*S*)-enantiomers of **1** but very few efficient enzymatic procedures[4e] are available for the (2*S*,3*R*)-enantiomer.

2. Results and discussion

2.1. Epoxide hydrolase in Mung beans

Enantiomerically pure *trans*-ethyl phenyl glycidate **1a** and *trans*-methyl (4-methoxyphenyl)glycidate **1b** are important drug intermediates. Both the (2*S*,3*R*)- and (2*R*,3*S*)-enantiomers of ethyl phenyl glycidate **1a** are useful in the synthesis of the Taxol side chain,[5] while the (2*R*,3*S*)-enantiomer

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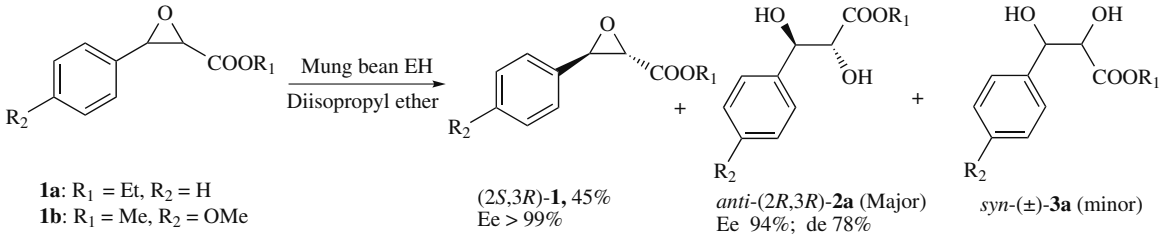


Scheme 1.

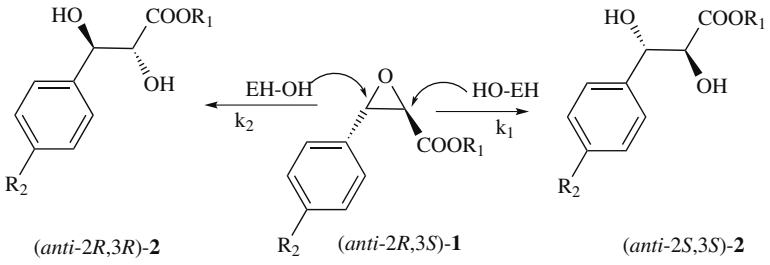
of **1b** is used in the synthesis of Diltiazem, a drug used to treat hypertension.[6] The resolution of racemic glycidate esters by an esterase/lipase catalyzed enantioselective hydrolysis is a well-known process.[6] However, the glycidic acid, which forms as the hydrolysis product in this process is unstable and quickly decomposes to 4-(methoxy) phenyl acetaldehyde, which acts as an inhibitor of the enzyme. It is necessary to design a special bioreactor that allows the continuous removal of the aldehyde as a bisulfite adduct.[6e] Furthermore, this aldehyde cannot be used as raw material for the same process. On the other hand, the product of enantioselective opening of the epoxide ring would produce the corresponding diol which can be converted to the starting aldehyde via a periodate reaction and reused. Thus a resolution process based on epoxide hydrolase would be economically and environmentally more favorable than a lipase based process. The report of the presence of epoxide hydrolase activity in Mung beans which are readily available in supermarkets led us to investigate the application of the enzyme in the resolution of glycidate esters.

It has been reported[3] that Mung beans contain at least 2 epoxide hydrolases, which prefer to attack different carbon atoms and with opposing enantioselectivities. For example, in the case of styrene oxide, one epoxide hydrolse (EH_A) attacks the primary carbon of the (*R*)-epoxide to produce the (*R*)-diol. The other epoxide hydrolase (EH_B) attacks the secondary carbon at the benzylic position of the (*S*)-epoxide, which again results in the formation of (*R*)-diol with the inversion of configuration at the benzylic carbon. Thus, for a compound such as styrene oxide with a single stereogenic center, this situation actually leads to high product yield (>85%) since both reactions are complimentary and produce the same enantiomer.[3] The reaction of two different epoxide hydrolases with opposing regio- and enantioselectivities on a glycidate ester possessing two stereogenic centers can theoretically give a mixture of products. For example, if the enzyme is completely stereoselective toward only the (2*R*,3*S*)-isomer of **1**, and the attack of nucleophilic water molecule results in the inversion of configuration, the epoxide hydrolase EH_A opening of the epoxide ring at C_2 (k_1) would produce *anti*-(2*S*,3*S*)-diol **2**, while attack at the benzylic position (k_2) would produce the *anti*-(2*R*,3*R*) diol **2**. Similarly, EH_B selective toward (2*S*,3*R*)-isomer would produce either the (2*R*,3*R*) or (2*S*,3*S*)-enantiomer of **2** depending upon the site of attack (Scheme 2).

Thus, the products of the enzymatic ring opening were expected to be *anti*-diols, either (2*R*,3*R*) or (2*S*,3*S*) or a mixture of theirs depending upon the relative activities and selectivities of the two enzymes. Experimentally, the reaction of **1** with crude Mung bean paste following the procedure described by Xu et al.[3] indicated a selectivity toward the (2*R*,3*S*)-enantiomer (ee 40% at 80% conversion). The Mung bean paste was hence stirred with an aqueous buffer and fractionated using ultrafiltration equipment to obtain a concentrated enzyme solution.

2.2. Enzyme preparation

The Mung beans were soaked overnight in water, dehusked, grounds, and stirred with a phosphate buffer (0.05 M, pH 7.5) containing EDTA (1 mM). The crude extract was found to be active toward the (2*R*,3*S*)-isomers of both **1a** and **1b**, although most of the ester (>85%) was converted to a mixture of diols, due to the spontaneous ring opening of the epoxide in water. The crude extract was then subjected to fractionation with membranes of different molecular weight cutoffs (0.2 μm, 100–10 kD). After incubation with racemic ester **1b**, we observed that the epoxide hydrolase activity was present in the retentates of 0.2 μm and 100 kD filters. The combined retentate was freeze-dried to obtain a crude enzyme powder (145 mg protein/g).



Scheme 2.

2.3. Enzyme immobilization

The epoxide ring of the glycidate ester **1b** was found to hydrolyze readily in aqueous buffers (pH 6–7.5). This necessitated the use of a water-immiscible solvent such as diisopropyl ether or toluene for the reaction. However, being a hydrolase, the enzyme does require a small amount of water for enzyme activity. The addition of an aqueous buffer to the freeze-dried powder suspended in diisopropyl ether or using water saturated solvent caused the formation of lumps of the biocatalyst that reacted very sluggishly. Attempts were then made to disperse the enzyme by a simple adsorption on supports such as Celite, activated carbon, starch, and carboxymethyl cellulose (CMC). However, the enzyme activity was rapidly lost on these supports when they came into contact with organic solvents. Over the past few years, we have successfully demonstrated the use of lipases immobilized in gelatin organogels (gelozymes) for use in organic solvents.[7] The procedure provides the technique of enzyme immobilization at low temperature and the gelatin matrix provides a porous support. The water content of the matrix can be controlled to a level just sufficient enough for an enzyme to be active. We have thus carried out the immobilization of the crude enzyme preparation in gelatin organogel. The crude enzyme powder is then mixed with a solution of gelatin in a microemulsion of sodium bis(2-ethylhexyl dioctyl sulfosuccinate, AOT)–isooctane–water, and crosslinked with glutaraldehyde. The gelozyme thus obtained was allowed to set overnight, crushed into small pieces, washed with diisopropyl ether until the organic layer was free of impurities as evidenced from HPLC analysis, and dried under vacuum at room temperature.

2.4. Effect of gelatin concentration on enzyme stability

Protection offered to the enzyme by gelatin matrix strongly depends upon the concentration of gelatin. Excess gelatin however increases the bulk without any advantage. We have thus studied the effect of gelatin concentration on the stability and reusability of the enzyme. A fixed amount (100 mg crude powder) was immobilized with varying gelatin concentration (varying volumes of gelatin solution in AOT microemulsion with a fixed amount of enzyme powder), and the reactivity of the enzyme preparation was studied using **1a** as a standard substrate. Optimum enzyme activity was observed when 2 g of gelatin was used for the immobilization of 1 g of crude powder containing 145 mg of protein. The enzyme preparation could be recycled three times without the loss of activity or change in selectivity.

2.5. Effect of concentration of water on enzyme activity

Glycidate esters are susceptible to spontaneous epoxide ring opening in the presence of excess water causing a decrease in the product yield and enantiomeric purity. It is thus necessary to control the amount of water present in the gel matrix. The vacuum-dried gelozyme did not show any hydrolase activity although the determination of water content by Karl-Fisher titration showed the presence of 7% water in the gel. After studying the enzyme activity at increasing water content, we concluded that the addition of an extra 1 mL of phosphate buffer (0.05 M, pH 7.2) to a suspension of 10 g of gelozyme in 100 mL of diisopropyl ether was adequate enough for enzyme activity. The gel strength of the matrix was not significantly reduced and very little spontaneous hydrolysis took place.

2.6. Enzyme reaction

In a typical experiment, the glycidate ester **1a** (960 mg, 5 mmol) dissolved in diisopropyl ether (20 mL) was stirred with gelozyme powder (2.5 g) at room temperature on a magnetic stirrer. The reaction was followed by an analysis on a chiral stationary phase. The (2*R*,3*S*)-ester slowly disappeared. The ee of the unreacted ester reached >99% in 72 h. Interestingly, the glycidate ester **1b** was far more reactive than **1a** and the reaction was complete in 12 h. The organic layer was then separated, the enzyme powder was extracted with diisopropyl ether, and the combined organic layer was evaporated on a rotavapor. In the case of **1a**, the oily residue was purified by column chromatography to separate the unreacted glycidate ester **1a** and the diastereomeric mixture of diol products **2a** and **3a**. In the case of **1b**, the oily residue was extracted with hot hexane to obtain unreacted (2*S*,3*R*)-**1b** (45%), in the hexane fraction. The diol formed during the reaction was quite insoluble in hexane and hence easily separated.

2.7. Determination of the configuration of the products

The configuration of the unreacted glycidate esters was established as (2*S*,3*R*) for both substrates **1a** and **1b** on the basis of their specific rotations, retention times on a chiral HPLC column, and comparison with literature values (ee >99%). In the case of unsubstituted ethyl phenyl glycidate, **1a**, the diastereomeric mixture of diols obtained after chromatography was converted to the corresponding acetonides to determine diastereomeric excesses. In the ^{1}H NMR spectra of acetonide of *syn*-ethyl-2,3-dihydroxy-3-phenylpropanoate **3**, H-2 resonated as a doublet at δ 5.15 ($J = 16.7$ Hz) and for the *anti*-ethyl-2,3-dihydroxy-3-phenylpropanoate **2** acetonide, it resonated at δ 4.75 ($J = 15$ Hz) and 5.4 ($J = 15$ Hz). The ratio of *anti* to *syn* acetonide was found to be 8:1 (de 77.8%). From the negative specific rotation of the acetonide,[8] the configuration of the *anti*-diol was assigned as (2*R*,3*R*). The ee of *anti*-diol was found to be 94% based on chiral HPLC analysis. The *syn*-diol was obtained as a racemic mixture. In the case of **1b**, the diol obtained after the reaction was found to be racemic.

2.8. Product distribution

The unreacted glycidate esters recovered in near quantitative yields (45%, 90% of theoretical) have (2*S*,3*R*)-configuration in both cases. This indicates that the epoxide hydrolase present in our preparation is selective toward only the (2*R*,3*S*)-enantiomer. In case of slow reacting **1a**, the major product is *anti*-(2*R*,3*R*)-diol. This suggests that only one epoxide hydrolase is active in the enzyme preparation and that it opens the epoxide ring of the (2*R*,3*S*)-glycidate ester by attacking at the benzylic carbon. The

racemic *syn*-diastereomer is most probably the result of non-specific ring opening of epoxide ring by water molecules present in the enzyme preparation. This is further supported by the observation that the diol obtained after the reaction of **1b**, which is highly susceptible to spontaneous hydrolysis, is racemic.

3. Conclusion

Herein, we have reported the use of easily extractable epoxide hydrolase from Mung beans for the preparation of (2*S*,3*R*)-enantiomers of glycidate esters such as **1**. Enantiomerically pure glycidate esters (ee >99%) are obtained in high yield (90% of theoretical). The enzyme can be immobilized in a gelatin matrix using water-in-oil microemulsion system. The technique provides an enzyme preparation, in which the amount of water present in the reaction medium can be easily controlled and the enzyme can be recycled for repeated use.

4. Experimental

Glycidate ester **1a** was purchased from Aldrich. Glycidate **1b** was a gift from M/s Godavari Drugs Ltd, Hyderabad. Mung beans (*P. radiatus*) were purchased from supermarkets. HPLC analyses were carried out on a Hewlett Packard HP1090 unit with diode array detector and HP Chem Station software. Ultrafiltration was carried out on Sartorius Minisart.

4.1. Preparation of the crude epoxide hydrolase from Mung beans

The beans (500 g) were soaked in distilled water (5 L) in a refrigerator overnight, dehusked, and ground to a fine paste. The paste was suspended in a Tris–HCl buffer (5 L, 0.05 M containing 1 mM EDTA, pH 7.5) and stirred in the cold for 6 h with a mechanical stirrer. The mixture was then filtered through fine muslin cloth and the sediment was discarded. The supernatant containing a fine suspension of the meal was filtered first through a 0.45 μm filter on a Sartorius ultrafiltration unit (Minisart). The residue was discarded. The filtrate was then filtered through a 0.22 μm filter. The concentrate (0.5 L) was collected and stored in a refrigerator. The supernatent (4.5 L) was then subjected to ultrafiltration with membranes of different molecular weight cutoff (100 kD, 50 kD, and 30 kD). After each filtration, the retentate (500 mL) was diluted with phosphate buffer (5 L, 0.01 M, pH 6.5) and again concentrated to 500 mL. The retentates were collected after each filtration step and stored in a refrigerator for the assay of enzyme activity.

The combined retentate after filtration with 0.22 μm and 100 kD (1 L) where the maximum enzyme activity was observed, was concentrated in a stirred cell with a 10 kD membrane filter. The concentrated slurry was freeze-dried to obtain a pale yellow powder of crude epoxide hydrolase (70 g, protein content 145 mg/g).

4.2. Determination of the enzyme activity in aqueous fractions

The activity of the enzyme in different fractions collected after membrane filtration was determined using methyl 4-methoxyphenyl glycidate **1b** as the substrate. In a typical experiment, 100 μL of a stock solution (0.1 M in ethanol) was added to an enzyme solution (1 mL, pH 6.5) and the mixture was stirred at room temperature for 1 h. A sample of the reaction mixture (100 μL) was diluted with an equal volume of acetonitrile, centrifuged for 5 min at 5000 rpm, filtered through a 0.2 μm syringe filter, and analyzed by reverse phase HPLC to determine the conversion. Another sample of the reaction mixture (100 μL) was mixed with ethyl acetate (100 μL) and centrifuged. The ethyl acetate layer was evaporated in a vacuum concentrator, and the volume was made up with a mobile phase and analyzed by chiral HPLC to determine the enantioselectivity of the reaction.

4.3. Immobilization of crude Mung bean epoxide hydrolase

Gelatin (5 g) was heated with distilled water (8.5 mL) at 60 °C for 15 min to complete gelation. AOT solution (35 mL, 0.3 M in isooctane) was added with vigorous stirring. The viscous and turbid gel thus obtained was cooled in ice with shaking for 10 min to give a transparent free flowing liquid. The crude enzyme powder (2.5 g) was slowly added to the cold solution with vigorous stirring to achieve a uniform distribution of the enzyme powder. The cooling bath was removed and glutaraldehyde (1 mL, 25% solution) was added. The contents were stirred at room temperature with a glass rod until the contents started to become viscous, poured into petri dish, and left at room temperature overnight. The dry gel was cut into small pieces and washed with isooctane and diisopropyl ether until the supernatant was free of impurities (HPLC analysis). The gel was then vacuum-dried at room temperature for 12 h to obtain immobilized enzyme (9 g). Determination of water content by Karl-Fisher titration showed that the water content of the gel was 7.7% (w/w).

4.4. Ethyl (2*S*,3*R*)-phenyl glycidate 1a

The racemic glycidate ester **1a** (960 mg, 5 mmol) dissolved in diisopropyl ether (20 mL) was stirred with crosslinked enzyme powder (0.5 g) at room temperature. The reaction was followed by an analysis on a chiral stationary phase. The ee of the unreacted ester reached >99% in 72 h. The organic layer was then separated, the enzyme powder was washed with diisopropyl ether (10 mL), and the combined organic layer was evaporated. The oily residue was chromatographed over silica gel (ethyl acetate/hexane, 5:95) to recover the unreacted (2*S*,3*R*)-**1a** (432 mg, 45%) and a diastereomeric mixture of ethyl 2,3-dihydroxy-3-phenylpropanoate (435 mg, 45%).

1a: ^{1}H NMR ($CDCl_3$): δ 1.2 (t, 3H, –OCH_2CH_3), 2.1 (d, 1H, $J = 2.5$ Hz, C(2)H); 2.6 (d, 1H, $J = 2.5$ Hz, C(3)H); 4.15 (q, 2H, OCH_2CH_3), 7.2–7.4 (br s, 5H, aromatic). IR (neat): 3560–3360, 2960, 2840, 1723, 1690. MS (EI, M^+)

$m/z = 178, 121, 91$ (base peak), 77, 51, 39. $[\alpha]_D^{25} = 156$, (c 1, $CHCl_3$); (lit.[4c] $= +155$ (c 1, $CHCl_3$)).

4.5. Ethyl 2,3-dihydroxy-3-phenylpropanoate

^{1}H NMR ($CDCl_3$): δ 1.2 (t, 3H, –OCH_2CH_3), 2.9–3.2 (br, OH), 4.15 (q, 2H, OCH_2CH_3), 4.4 (d, $J = 4$ Hz, 1H, CH(OH)COOEt), 5.0 (d, $J = 4$ Hz, PhCH(OH)), 7.3 (m, 5H, aromatic). IR (neat): ν_{max} 3450–3350, 2957, 1736, 1452, 1198, 1092, 703 cm^{-1}.

4.6. Ethyl *anti*-(2*R*,3*R*)-2,3-isopropylinyloxy-3-phenyl proanoate, 2a, and ethyl *syn*-(±) isopropylidinyloxy-3-phenyl propanoate 3a

To a stirred solution of **2a** and **3a** (420 mg, 2 mmol) and camphor sulfonic acid (1 mg) in dichloromethane (10 mL) was added 2,2-dimethoxypropane (1 mL, 6 mmol). After stirring for 12 h at room temperature, aqueous sodium bicarbonate solution (5 mL) was added. The organic layer was separated, and the aqueous layer was extracted with dichloromethane (2 × 20 mL). The combined organic layers were washed with brine (10 mL), dried over anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was purified over column chromatography [ethyl acetate/hexane, 1:100] to afford *syn* (24 mg, 5%) and *anti* (390 mg, 78%). *syn* **3a**. ^{1}H NMR ($CDCl_3$) δ 1.2 (t, 3H, –OCH_2CH_3), 1.6 (2s, 6H, –$C(CH_3)_2$), 4.3 (m, 3H, –OCH_2CH_3 + CH(O)COOEt), 5.15 (d, $J = 16.7$ Hz, 1H, PhCH(O)), 7.3 (m, 5H, aromatic). *anti*-(2*R*,3*R*)-**2a**: ^{1}H NMR ($CDCl_3$) δ 0.75 (t, 3H, –OCH_2CH_3), 1.5 (s, 3H, –$C(CH_3)_2$), 1.8 (s, 3H, –$C(CH_3)_2$); 3.45–3.8 (m, 2H, –OCH_2CH_3), 4.75 (d, $J = 15.0$ Hz), 5.4 (d, $J = 15$ Hz, 1H, PhCH(O)), 7.3 (m, 5H, aromatic). IR (neat) ν_{max} 2990, 1760, 1200 cm^{-1}. $[\alpha]_D = -52$ (c 1, CH_2Cl_2) [lit. = −67.3 (c 1.0, CH_2Cl_2)]. MS (EI, M^+) $m/z = 210$, 141, 135, 122, 105, 91, 71, 57, 43, 41.

4.7. (2*S*,3*R*)-Methyl (4-methoxyphenyl) glycidate

Reaction was performed (with 1.11 g, 5 mmol) as described in Section 4.6. The reaction was complete within 12 h and enantiomerically pure glycidate ester (466 mg, 42%) was obtained. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 3.5 (d, 1H, $J = 2.5$ Hz, C(2)H); 3.9 (s, 3H, $COOCH_3$), 4.1 (d, 1H, $J = 2.5$ Hz, C(3)H); 6.8–7.2 (dd, 4H, aromatic). IR (KBr): 3560–3360, 2960, 2840, 1720, 1600, 1520, 1440, 1280, 1240, 1100, 1000, 840. MS (EI, M^+) $m/z = 208$, 151, 148, 121 (base peak), 120, 105, 91, 77, 51, 39. $[\alpha]_D^{25} = +196$ (c 1, ethanol); {lit.[4b] $= -196.2$ (c 1, ethanol) for the (2*R*,3*S*)-enantiomer}.

4.8. Methyl 2,3-dihydroxy-3-(4-methoxyphenyl) propanoate

^{1}H NMR ($CDCl_3$, 200 MHz): 2.57 (d, $J = 6.70$ Hz, 1H, CH(OH)COOMe), 3.04 (d, $J = 6.04$ Hz, 1H, PhCH(OH)), 3.79 (s, 6H, COOMe + PhOMe), 4.24–4.29 (dd, $J_1 = 3.02$ Hz, $J_2 = 6.04$ Hz, 1H, CH(OH)COOMe), 4.84–4.91 (dd, $J_1 = 3.02$ Hz, $J_2 = 6.04$ Hz, 1H, PhCH(OH)), 6.84 (d, $J = 8.30$ Hz, 2H, aromatic), δ 7.25 (d, $J = 8.30$ Hz, 2H, aromatic). IR (neat) ν_{max} 3497, 3383, 3012, 2958, 2842, 1713, 1612, 1516, 1447, 1105 cm^{-1}. MS (EI, M^+) $m/z = 249$, 149, 137, 121, 109, 94, 77, 66, 41, 51, 41.

4.9. Reverse phase HPLC analysis

The disappearance of glycidate ester **1b** was followed by reverse phase HPLC. Column C-18 (250 × 5 mm), Chrompack, The Netherlands. Mobile phase 70% acetonitrile–water. Flow rate 0.7 mL/min. Detection wavelength 230 nm. Retention times: **1b** 6.24 min, diol (**2** + **3**) 4.39 min.

4.10. HPLC with chiral stationary phase

Enantiomeric purity was determined by HPLC analysis on Chiralcel AD-H column (250 × 5 mm), Daicel Chemical Industries, Japan. Mobile phase 15% isopropyl alcohol in hexane. Flow rate 0.7 mL/min; detection wavelength 230 nm. Retention times **1a**: (2*S*,3*R*) 8.1 min, (2*R*,3*S*) 8.8 min; **1b**: (2*R*,3*S*) 9.52, (2*S*,3*R*) 10.74 min. **2a**: (2*S*,3*S*) 15.4, (2*R*,3*R*) 16.21, (2*S*,3*R*) 18.0, (2*R*,3*S*) 19.5 min. **2b**: Peaks at 20.2, 20.9, 22.5 and 24.6 min (configurations not assigned since the product was racemic).

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