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Enzyme-catalyzed resolution of 3,8-dioxatricyclo[3.2.1.0^{2,4}]octane-6-carboxylic esters and the application to the synthesis of 3-epishikimic acid

Manabu Hamada [a], Yoshikazu Inami [b], Yasuhito Nagai [c], Toshinori Higashi [b], Mitsuru Shoji [b], Seiichiro Ogawa [d], Kazuo Umezawa [a], Takeshi Sugai [b,*]

[a] *Department of Applied Chemistry, Keio University, 3-14-1 Hiyoshi, Yokohama 223-8522, Japan*
[b] *Faculty of Pharmacy, Keio University, 1-5-30, Shibakoen, Minato-ku, Tokyo 105-8512, Japan*
[c] *Department of Chemistry, Keio University, 3-14-1 Hiyoshi, Yokohama 223-8522, Japan*
[d] *Department of Bioscience and Bioinformatics, Keio University, 3-14-1 Hiyoshi, Yokohama 223-8522, Japan*

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ABSTRACT

3,8-Dioxatricyclo[3.2.1.0^{2,4}]octane-6-carboxylic acid, whose racemic form is readily available on a large scale, is a versatile starting material for the synthesis of carbasugars and carbocyclic biologically active natural products. In this study, the enzyme-catalyzed kinetic resolution was attempted on a variety of corresponding carboxylic esters. The hydrophobic and hydrophilic properties of ester substituents greatly affected the rate of reaction and the enantioselectivity. Hydrolysis of the corresponding 2′-chloroethyl ester with pig liver esterase worked well in a highly enantioselective manner (E = 116) to give the hydrolyzate (90.6% ee) and unreacted ester recovery (99.4% ee). The hydrolyzate is a precursor for (−)-oseltamivir phosphate, and a route to (3*S*,4*S*,5*R*)-(−)-3-epishikimic acid was developed from the recovered ester.

1. Introduction

3,8-*endo*-Dioxatricyclo[3.2.1.0^{2,4}]octane-6-carboxylate (acid or ester) **1** is a polyoxygenated cyclohexenecarboxylate with many controlled stereocenters which has been developed as a starting material for carbasugars, including carba-Neu5Ac and carba-KDO as shown in Scheme 1.[1] In the synthesis of epoxyquinols and related compounds, Hayashi et al. have explored new entries. An efficient selective ring-opening reaction was a key step to provide cyclohexenecarboxylate.[2,3] The racemic form of **1** is available in large quantity.[1,6] So far, however, the supply of the enantiomerically pure form has been rather limited, except for asymmetric Diels–Alder reactions controlled by chiral auxiliary[4,5] or tedious preferential crystallization of the diastereomeric salts of a certain precursor.[6]

Herein, we report the kinetic resolution by the action of hydrolytic enzymes on esters **1b**–**g** (Scheme 1). The hydrolysis of carboxylic esters **1** has a clear and significant advantage, for the large-scale enantiomeric resolution. In this approach, the hydrolyzates, carboxylic acids, and the unreacted recoveries, the esters, are separable by only extractive workup under properly adjusted pH conditions. If these attempts are successful, the unreacted recoveries, the esters would be converted to (3*S*,4*S*,5*R*)-(−)-3-epishikimic acid **2**.

2. Results and discussion

The preparation of racemic esters **1b**–**g** is shown in Scheme 2. The Diels–Alder reaction between furan and acrylic acid, and the subsequent iodolactonization of the crude *endo*–*exo* mixture (8:2) yielded racemic iodolactone **5**.[1] The hydrolysis of the lactone followed by the direct alkylation of the resulting carboxylate provided esters **1b**–**g**.

Our first candidate was the ethyl ester **1b**, as it showed a lower melting point (mp 52–53 °C) than the corresponding methyl ester **1c** (mp 75 °C). The lower melting point of the crystalline substrate has been observed to be advantageous for enzyme-catalyzed hydrolysis under aqueous conditions.[7] So far, attempts at the kinetic resolution of the similar *endo*-carboxylic acid **3**, by the action of hydrolytic enzymes on the corresponding ester, have only resulted in moderate enantioselectivity.[8]

Our substrate **1b** reacted poorly with many kinds of hydrolytic enzymes, probably due to high steric hindrance around the *endo*-oriented ester moiety. Among the proteases (*Rhizopus* sp., XP-415; *A. melleus*, XP-488, Nagase ChemteX Co.), lipases (*Candida rugosa*, Meito OF; *Candida antarctica*, Roche diagnostics, Chirazyme L-2), and esterases (*Klebsiella oxytoca*, SNSM-87, Nagase ChemteX Co.; pig liver esterase, Sigma), only pig liver esterase (PLE) showed substantial hydrolysis (Scheme 3). However, the conversion and enantioselectivity (E-value) were as low as 33% and 11%, respectively, as shown in entry 1, Table 1.

The decrease in bulkiness from ethyl to methyl **1c** (entry 2) only resulted in lower selectivity (E = 7). We then introduced an

* Corresponding author. Tel./fax: +81 3 5400 2665.
E-mail address: sugai-tk@pha.keio.ac.jp (T. Sugai).

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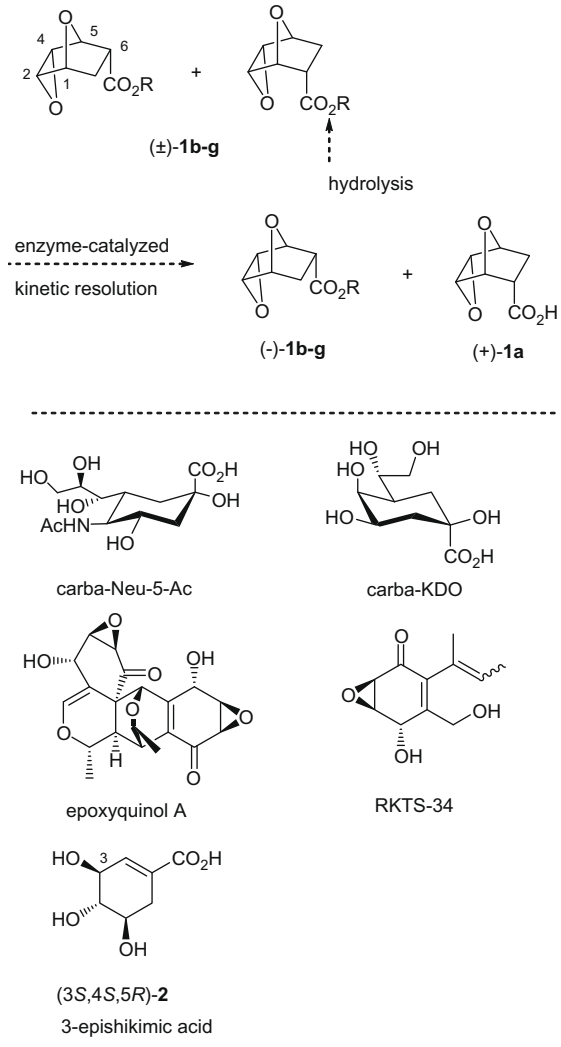
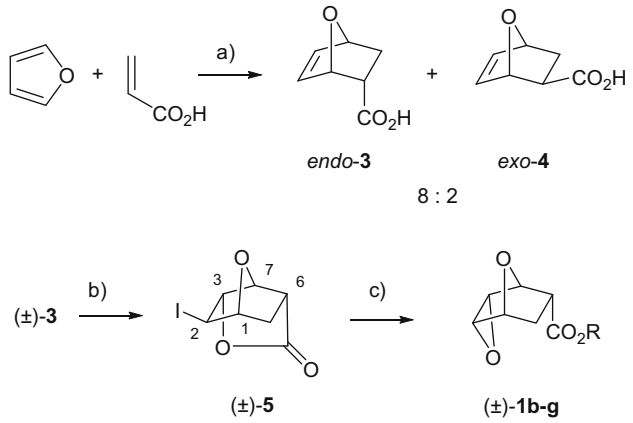
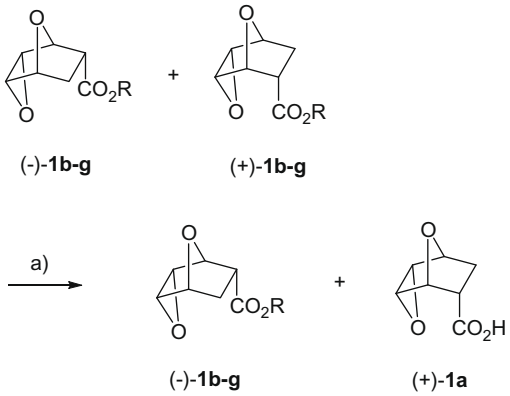


Scheme 1. Enzyme-catalyzed kinetic resolution of 3,8-dioxatricyclo[3.2.1.$0^{2,4}$]-octane-6-carboxylic ester **1** and utilization thereof.



Scheme 2. Preparation of *endo*-epoxy esters. Reagents and conditions: (a) see Ref. 6. The ratio between *endo*- and *exo*-adducts (8:2) was determined by ^{1}H NMR; (b) $NaHCO_3$, H_2O/I_2, THF, rt, 24 h (quant.); (c) KOH, DMF, 40 °C, 24 h/RI, 50 °C, 6 h (yields: see Section 4).

electron-withdrawing group on the ester moiety, expecting that the rate of the 'fast' (1*S*,2*R*,4*S*,5*R*,6*S*)-isomer would be enhanced, so that the enantioselectivity becomes higher. Our first attempt



Scheme 3. Enzyme-catalyzed hydrolysis of *endo*-epoxyesters. Reagents and conditions: (a) pig liver esterase (Sigma E2884), rt, 24 h.

Table 1
PLE-catalyzed hydrolysis of (±)-**1b**–**g**[a]

Entry	Substrate	R	Conv. (%)	*E*-value
1	**1b**	CH_2CH_3	33	11
2	**1c**	CH_3	32	7
3	**1d**	CH_2Cl	NA	NA
4	**1e**	CH_2CH_2Cl	51	78
5	**1f**	CH_2CONH_2	26	5
6	**1g**	CH_2CF_3	50	9

[a] For reaction conditions and evaluation of enantioselectivity, see Section 4.

to directly replace one hydrogen atom with chlorine **1d** (entry 3) turned out to be unsuccessful, because **1d** was too unstable. The introduction of 2-chloroethyl[9] (**1e**, entry 4) and carbamylmethyl[10] **1f** (entry 5) groups brought about contrasting effects. The former showed a great increase in selectivity ($E = 78$) and the conversion reached nearly 50%.

This result suggested that the reactivity of the 'fast' isomer was enhanced, this is well supported by applying an empirical model of the catalytic site of PLE as proposed by Jones (Fig. 1, top).[11] The tricyclic skeleton of the substrate occupies a large hydrophobic pocket (L). In turn, the 2-chloroethyl group fits a small hydrophobic site (S) of the model so that the attack by the hydroxyl group of a serine residue easily takes place. The ^{1}H NMR spectra of **1e** prompted us to hypothesize that an extended conformation is advantageous to fit in the hydrophobic site of PLE. The two geminal protons of Ha and Hb (Fig. 1, top) were not equivalent (Ha: 4.44 ppm, Hb: 4.32 ppm, $\Delta\delta$ 0.12 ppm), and one of them would be located in the eclipsed position of the ester carbonyl group make the chloroethyl group fit into the hydrophobic site (S) (Fig. 1, top).

On the other hand, the lower selectivity in the carbamylmethyl ester **1f** (entry 5, $E = 5$), as well as the lower conversion, means that the 'fast' isomer is not preferable in an enzyme-catalyzed reaction. The ^{1}H NMR measurement suggested that **1f** has a different conformation from **1e**, as judged by a smaller $\Delta\delta$ value between Hc and Hd (Fig. 1, bottom) (Hc: 4.69 ppm, Hd: 4.62 ppm, $\Delta\delta$ 0.07 ppm). When **1f** in this conformation occupies the catalytic site, a carbamylmethyl group fits the hydrophilic site, while the ester carbonyl group is far away from the nucleophilic serine hydroxyl group (Fig. 1, bottom). The crooked conformation of **1f** is supported by the fact that **1f** is prone to epimerization to an *exo*-ester, when under treatment with base followed by an intramolecular delivery of protons from $CONH_2$ group. In contrast, β-elimination exclusively occurs very quickly in **1e** even under low temperatures, as described later.

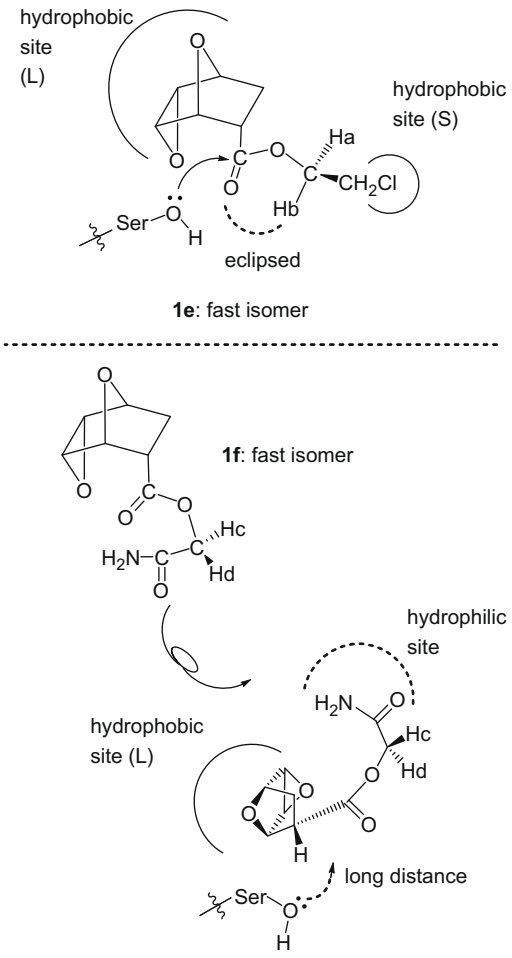
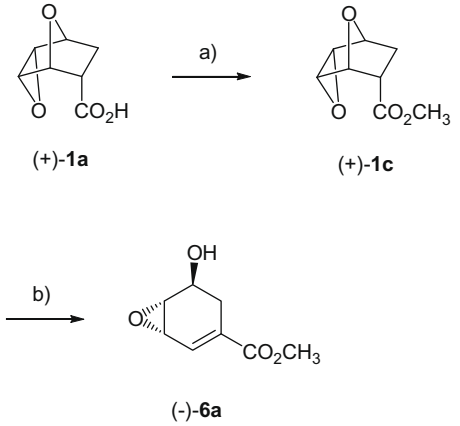


Figure 1. Behavior of 2-chloroethyl **1e** and carbamylmethyl **1f** esters in Jones' empirical catalytic site model of PLE.

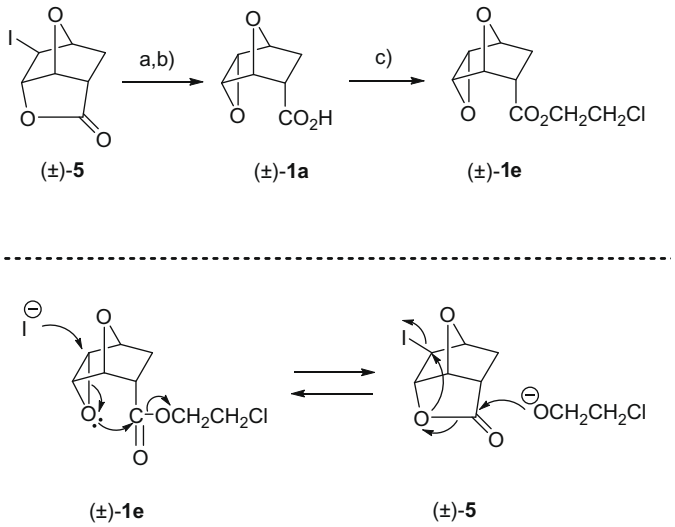
Based on these observations, the more electron-withdrawing and hydrophobic trifluoromethylester **1g** was prepared with the aim of obtaining improved results. However, **1g** was again substantially unstable even in neutral buffer solution. The lower enantioselectivity ($E = 9$) was probably due to enzyme-uncatalyzed spontaneous hydrolysis.

The absolute configuration of the 'fast' isomer in **1e** was determined as follows in Scheme 4. Esterification of (+)-acid **1a**, the hydrolyzate, followed by β-elimination[2] of the resulting ester **1c** using LHMDS gave (−)-**6a**. The absolute configuration of this sample was unambiguously determined to be (1*S*,5*S*,6*R*) by the minus sign of the rotation value compared with literature data.[2] This result is consistent with the stereochemical preference of the 'fast' isomer being similar to that in the case of the ester of *endo*-**3** (Scheme 2).[8]



Scheme 4. Absolute configuration of (+)-**1a**. Reagents and conditions: (a) Cs_2CO_3, MeI, DMF, 50 °C, 24 h (59%); (b) LHMDS, THF, −78 °C, 1 h (70%).

Since the combination of highly enantioselective catalyst and substrate was optimized, the next task was the establishment of suitable reaction conditions for the large-scale preparation of (±)-**1e** from iodolactone (±)-**5**. The conventional method for ester synthesis is the hydrolysis of iodolactone to give the salt of acid **1a** with concomitant epoxide ring formation, and subsequent treatment with an alkylating agent in one pot (Scheme 5).



Scheme 5. Preparation of 2-chloroethyl ester **1e**. Reagents and conditions: (a) KOH, EtOH, 70 °C, 5 h; (b) 1 M HCl (quant.); (c) $ClCH_2CH_2X$, base, additive. For the conditions, see Table 2.

Esterification with 1-chloro-2-iodoethane smoothly proceeded to give **1e** in 80% yield (Table 2, entry 1). However, this mixed dihalide is quite expensive and not suitable for large-scale preparation. The replacement of this iodide with inexpensive 1,2-dichloroethane resulted in only a 33% yield (Table 2, entry 2). Next, KI (4 equiv) was added with the expectation of the in situ formation of iodide, which caused the yield to drop to 3% (Table 2, entry 3). By analysis of the by-products, iodolactone **5** appeared again during the alkylation, even after confirmation of completion of the hydrolysis in the first step (Scheme 5 and **5**→**1a**). At this stage we became aware that the presence of iodide ions in the reaction mixture has a deleterious effect, as the direct attack of an iodide ion opens the epoxide ring of 2-chloroethyl ester itself and the reaction reaches an equilibrium between iodolatone **5** and 2-chloroethyl ester **1e** (Scheme 5, bottom). Thus we switched the procedure into the following conditions. Acid **1a** was extracted after acidic workup, and then it was separately incubated with 1,2-

Table 2
Alkylation of free carboxylic acid (±)-**1a**

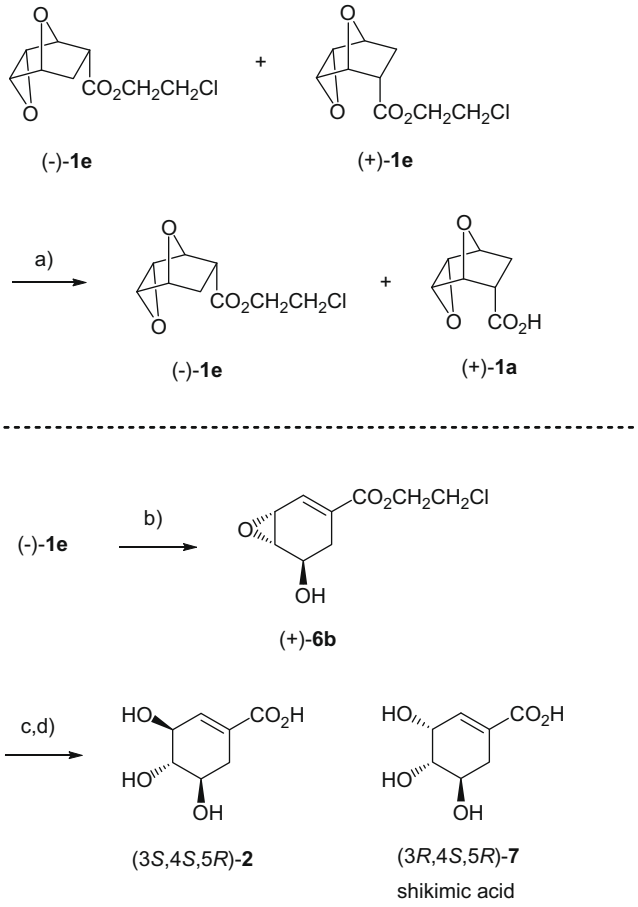
Entry	Base	Alkylating agent X (equiv)	Additive	Yield (%)
1	KOH	I (1.5)	None	80
2	KOH	Cl (3.0)	None	33
3	KOH	Cl (3.0)	KI	3
4	K_2CO_3	Cl (4.0)	None	71

dichloroethane and K_2CO_3. Under these iodide-free conditions, the reaction was successful and a 71% yield was recorded (Table 2, entry 4). In the case of scaled-up conditions, the iodide-free potassium salt of **1a** became accessible via an alternative method by careful observation during hydrolysis. When iodolactone **5** was hydrolyzed in ethanol, first, a precipitate appeared. This was proven by NMR analysis to be a potassium salt of iodohydrin carboxylic, an intermediate acid. If the hydrolysis was continued under prolonged heating at 70 °C, the precipitate disappeared. The resulting potassium salt and all of the by-products were soluble in ethanol. Then the reaction mixture was dried onto silica gel, and elution with methanol provided the desired carboxylate salt. Most of the contaminant, especially inorganic salts such as KI, was removed by adsorption on silica gel.

The PLE catalyzed hydrolysis in the scaled-up experiment proceeded in a reproducible manner, and (+)-**1a** (54.7% yield) and (−)-**1e** (42.7% yield) were obtained with only an extractive work-up separation. In this case, both ees of (+)-**1a** (90.6% ee) and (−)-**1e** (99.4% ee) were unambiguously determined (see Section 4) ($E = 116$). The simple recrystallization of acid (+)-**1a** from EtOAc enhanced the enantiomeric excess to 96.4%. It is noteworthy that acid (+)-**1a** has been reported as the starting material for oseltamivir phosphate by Terashima and Ujita.[12]

Our product, epoxyester **1e**, has the same level of oxygen functionality in suitable positions as shikimic acid and related compounds. Naturally occurring shikimic acid (3*R*,4*S*,5*R*)-**7** is essential for the industrial synthesis of (−)-oseltamivir phosphate.[13,14] The process for fermentative production has already been established,[15,16] as (3*R*,4*S*,5*R*)-**7** is a biosynthetic key intermediate for the well-known shikimate pathway. An epimeric form, (3*S*,4*S*,5*R*)-**2** (3-epishikimic acid), has recently gained attention as the starting material for vitamin D_3 precursors,[17] the synthon of natural products,[18,19] and as a template for combinatorial synthesis.[20] The availability of this epi-form, however, is very low due to no direct biosynthetic pathway. An attempted acid-catalyzed epimerization under harsh conditions only results in a stereoisomeric mixture with parent (3*R*,4*S*,5*R*)-shikimic acid **7**, accompanied with the dehydrated 4-hydroxybenzoic acid.[18] Otherwise, a tedious multi-step conversion was required, involving selective protection of 4,5-*trans* diol and inversion at C-3.[19]

The above-mentioned situation prompted us to establish a route from (−)-**1e** to epishikimate (3*S*,4*S*,5*R*)-**2**. Epoxy ester (−)-**1e** was submitted to LHMDS-mediated β-elimination[2] to give (+)-**6b** (Scheme 6). It is noteworthy that the desired reaction occurred in as high as 94% yield without any damage on the 2-chloroethyl ester, which would also suffer from β-elimination. For the transformation of **6b** to (3*S*,4*S*,5*R*)-**2**, the electron-withdrawing property of the chloroethyl group was quite advantageous. The alkaline hydrolysis of **6b** proceeded very smoothly, and the following stereoselective epoxide ring opening[21] gave (3*S*,4*S*,5*R*)-**2a** (79.7%); $[\alpha]_D^{25} = -33.1$ (*c* 0.34, H_2O) {lit.[21] $[\alpha]_D = -31.0$ (*c* 0.1, H_2O)}; whose spectroscopic data coincided with those reported previously.[21]



Scheme 6. Derivation of hydrolyzate to (3*S*,4*S*,5*R*)-3-epishikimic acid **2a**. Reagents and conditions: (a) pig liver esterase, 0.2 M phosphate buffer (pH 7.0), [42.7% for (1*R*,2*S*,4*R*,5*S*,6*R*)-(−)-**1e** (99.4% ee)], [54.7% for (1*S*,2*R*,4*S*,5*R*,6*S*)-(+)-**1a** (90.6% ee)]; (b) LHMDS, THF, −78 °C, 1 h, (96%); (c) KOH, THF, H_2O, 50 °C, 1 h; (d) TFA, H_2O, 50 °C, 3 h (79.7%).

3. Conclusion

Based on the pig liver esterase-catalyzed kinetic resolution of 2-chloroethyl 3,8-dioxatricyclo[3.2.1.$0^{2,4}$]octane-6-carboxylate **1e**, an expeditious route for polyhydroxylated cyclohexenoids has been established. The design of the substrate structure was supported by conformational analysis and fitness in an enzyme catalytic site model. The reaction conditions for the synthesis of optimized substrate, excluding the formation of by-products necessary to simplify the workup procedure, which is indispensable for preparative-scale synthesis have been elucidated. 3-Epishikimic acid should be a more promising starting material in organic synthesis following our establishment of a scalable supply.

4. Experimental

4.1. Materials and methods

Merck Silica Gel 60 F_{254} thin-layer plates (1.05744, 0.5 mm thickness) and Silica Gel 60 (spherical and neutral; 100–210 μm, 37560-79) from Kanto Chemical Co. were used for preparative thin-layer chromatography and column chromatography, respectively. The commercial PLE preparation was purchased from Sigma.

4.2. Analytical methods

All melting points are uncorrected. IR spectra were measured as thin films for oils or ATR for solid on a Jeol FT-IR SPX60 spectrometer. ^{1}H NMR spectra were measured in $CDCl_3$ or D_2O at 270 MHz on a Jeol JNM EX-270 or at 400 MHz on a Jeol JNM GX-400 spectrometer or at 400 MHz on a VARIAN 400-MR spectrometer, and ^{13}C NMR spectra were measured in $CDCl_3$ or D_2O at 100 MHz on a Jeol GX-400 spectrometer or at 100 MHz on a VARIAN 400-MR spectrometer. High resolution mass spectra were recorded on a Jeol JMS-700 MStation spectrometer. HPLC data were recorded on Jasco MD-2010 multi-channel detectors and SHIMADZU SPD-M20A diode array detector. Optical rotation values were recorded on a Jasco P-1010 polarimeter. Silica Gel 60 (spherical, 100–210 μm, 37558-79) of Kanto Chemical Co. was used for column chromatography. Preparative TLC was performed with E. Merck Silica Gel 60 F_{256} plates (0.5 mm thickness, No. 5744).

4.3. Screening of hydrolytic enzymes

The screening of hydrolytic enzymes were performed as follows. A 2 mL sample tube was charged with an appropriate amount of racemic ethyl ester **1b** (10.0 mg) and potassium phosphate buffer (0.2 M, 0.25 mL, pH 7.0) at room temperature for 24 h in the presence of several lipases and protease at an amount of 80–100 mg/mL of phosphate buffer, in the case of pig liver esterase 0.2 mg/mL of phosphate buffer. The progress of the reaction was monitored by TLC analysis [silica gel, developed with hexane–EtOAc (1:1)]. The reaction mixture was quenched with citric acid to pH 2, and extracted with EtOAc. The combined organic phases were dried over Na_2SO_4 and concentrated in vacuo. Among the seven enzymes tested, only pig liver esterase showed the progress of hydrolysis.

4.3.1. (±)-7-*endo*-Oxabicyclo[2.2.1]hept-5-carboxylic acid 3

The known procedure[6] was slightly modified in regard to the reaction temperature. Furan (250 mL) and acrylic acid (250 mL) were mixed and kept for 6 days at room temperature, for 17 days at 4 °C, and then for 28 days at 7 °C. The precipitated solids were recovered by filtration to give carboxylic acid (±)-**3** (41.7 g, *endo*:*exo* = 8:2) as a colorless solid, mp 95–96 °C, lit.[6] mp 97–100 °C. To the above mentioned filtrate was added furan and acrylic acid and kept for one month at 7 °C to give another crop of crystal. The NMR spectrum was identical with that reported previously.[1,6]

4.3.2. (±)-(1*R**,2*R**,3*R**,6*R**,7*S**)-2-Iodo-4,8-dioxatricyclo[4.2.1.0^{3,7}]-nonan-5-one 5

To a solution of the acid (±)-**3** (20.0 g, 142 mmol) in $NaHCO_3$ aq solution (300 mL) was added dropwise a solution of I_2 (40.0 g, 157 mmol) in THF (80 mL) under ice-cooling, and the mixture was stirred for 68 h at room temperature. To the mixture was added saturated $Na_2S_2O_3$ aq solution, and the precipitates were collected in filtration to give crude iodolactone (±)-**5** (25.1 g). The filtration was extracted with EtOAc (three times). The organic layer was washed with brine, dried over Na_2SO_4 and concentrated in vacuo, to give another crop of iodolactone (±)-**5** (2.00 g) as the sample for NMR measurement. Its NMR spectrum was identical with that reported previously.[1] The combined yield of above-mentioned (±)-**5** (27.0 g) was 71%, and this was employed for the next step without further purification.

4.3.3. (±)-3,8-Dioxatricyclo[3.2.1.0^{2,4}]octane-6-carboxylic acid 1a

To a solution of iodolactone (±)-**5** (1.01 g, 3.80 mmol) in DMF (15 mL) was added KOH (0.54 g, 9.98 mmol) and stirred for 24 h at room temperature. After removal of water in vacuo, the residue was added 1 M HCl to pH 2. The mixture was extracted with EtOAc (10 times), and the combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography with $CHCl_3$–MeOH (6:1) to afford carboxylic acid (±)-**1a** (193 mg, 33.5%) as a colorless solid, mp 153–154 °C. Its NMR spectrum was identical with that reported previously.[12]

4.3.4. Ethyl (±)-3,8-dioxatricyclo[3.2.1.0^{2,4}]octane-6-carboxylate 1b

To a solution of iodolactone (±)-**5** (0.51 g, 1.87 mmol) in DMF (10 mL) was added with KOH (0.37 g, 6.60 mmol) and stirred for 24 h at room temperature. After removal of water in vacuo, the residue was dissolved anhydrous DMF. The mixture was added EtI (0.96 g, 6.56 mmol) at 40 °C, and stirred for 6 h. After removal of volatile materials in vacuo, the reaction was quenched with NH_4Cl aq solution, and extracted with EtOAc (three times). The combined organic phases were washed with brine and dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography with hexane–EtOAc (2:1) to afford ethyl ester (±)-**1b** (316 mg, 91.8%) as a colorless solid, mp 52–53 °C. Its NMR spectrum was identical with that reported previously.[12]

4.3.5. Methyl (±)-3,8-dioxatricyclo[3.2.1.0^{2,4}]octane-6-carboxylate 1c

In a similar manner as described for **1b**, a solution of iodolactone (±)-**5** (0.80 g, 3.01 mmol) in DMF (10 mL) was treated with KOH (0.40 g, 7.13 mmol) and MeI (1.28 g, 6.56 mmol) to give methyl ester (±)-**1c** (390 mg, 78.3%) as a colorless solid; mp 75 °C. Its NMR spectrum was identical with that reported previously.[2]

4.3.6. 2-Chloroethyl (±)-3,8-dioxatricyclo[3.2.1.0^{2,4}]octane-6-carboxylate 1e

In a similar manner as described for **1b**, a solution of iodolactone (±)-**5** (266 mg, 1.00 mmol) in DMF (3 mL) was treated with KOH (132 mg, 2.35 mmol) and $ClCH_2CH_2I$ (300 mg, 1.58 mmol), to give 2-chloroethyl ester (±)-**1e** (171 mg, 78.5%) as a colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.97 (1H, ddd, J = 4.9, 11.3, 11.3 Hz, H-7$_{exo}$), 2.07 (1H, dd, J = 4.9, 11.3 Hz, H-7$_{endo}$), 2.93 (1H, dt, J = 4.9, 11.3 Hz, H-6), 3.69 (2H, t, J = 5.7 Hz, CH_2Cl), 4.01 (1H, dd, J = 2.4, 4.3 Hz, H-4), 4.11 (1H, dd, J = 2.2, 4.3 Hz, H-2), 4.32 (1H, dt, J = 5.7, 11.3 Hz, C*H*HCH$_2$Cl, Ha in Fig. 1), 4.44 (1H, dt, J = 5.7, 11.3 Hz, CH*H*CH$_2$Cl, Hb in Fig. 1), 4.50 (1H, dt, J = 2.2, 4.9 Hz, H-5), 4.70 (1H, dt, J = 2.2, 4.9 Hz, H-1); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 29.5, 41.8, 44.7, 64.1, 66.6, 66.7, 77.5, 78.2, 171.0; IR ν_{max} 3006, 2962, 2358, 1732, 1449, 1334, 1207, 1176, 883 cm^{-1}. Anal. Calcd for $C_9H_{11}ClO_4$: C, 49.44; H, 5.07. Found: C, 49.19; H, 5.04.

This ester was also able to be prepared by the action of $ClCH_2CH_2Cl$. To a solution of the acid (±)-**1a** (15.6 mg, 0.10 mmol) in anhydrous DMF was added K_2CO_3 (55.2 mg, 0.40 mmol) and $ClCH_2CH_2Cl$ (59.4 mg, 0.60 mmol), and the mixture was stirred at 40 °C for 24 h. The same workup as above provided (±)-**1e** (16.5 mg, 75.7%).

4.3.7. Scaled-up and preparative synthesis of 1e

A solution of iodolactone (±)-**5** (3.20 g, 12.0 mmol) in EtOH (20 mL) was added KOH (2.00 g, 35.6 mmol) and the mixture was stirred for 5 h at 70 °C. After removal of volatile materials in vacuo, the residue was re-dissolved in MeOH. To the mixture was added silica gel (50 g), and stirred for 30 min. After concentration in vacuo, the residual solid was charged on a glass column, and that was eluted with ethanol to give carboxylic acid (±)-**1a** (86.4 mg) as a colorless solid. Further elution with MeOH afforded potassium salt (±)-**1a** (2.40 g).

To a solution of the above potassium salt (±)-**1a** (2.07 g) in DMF (10 mL) was added $ClCH_2CH_2Cl$ (5.28 g, 53.4 mmol), and the mixture was stirred at 60 °C for 24 h. The same workup provided (±)-**1e** (1.55 g, 71.1%).

4.3.8. Carbamylmethyl (±)-3,8-dioxatricyclo[3.2.1.0^{2,4}]octane-6-carboxylate 1f

In a similar manner as described for **1b**, a solution of iodolactone (±)-**5** (0.80 g, 3.01 mmol) in DMF (5 mL) was treated with KOH (0.40 g, 7.13 mmol) and $ClCH_2CONH_2$ (0.84 g, 8.98 mmol), gave carbamylmethyl ester (±)-**1f** (423 mg, 65.9%) as a colorless solid. Further purification by recrystallization from EtOAc afforded (±)-**1f**: mp 131.0–133.0 °C: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.03 (1H, ddd, J = 4.6, 11.2, 11.6 Hz, H-7$_{exo}$), 2.09 (1H, dd, J = 4.3, 11.6 Hz, H-7$_{endo}$), 3.02 (1H, dt, J = 4.6, 11.2 Hz, H-6), 4.11 (1H, dd, J = 1.9, 4.4 Hz, H-4), 4.18 (1H, dd, J = 2.2, 4.4 Hz, H-2), 4.55 (1H, dt, J = 2.2, 4.6 Hz, H-5), 4.62 (1H, d, J = 15.6 Hz, C*H*HCONH$_2$, Hc in Fig. 1), 4.69 (1H, d, J = 15.6 Hz, CH*H*CONH$_2$, Hd in Fig. 1), 4.77 (1H, dt, J = 1.9, 4.4 Hz, H-1), 5.81 (1H, br s, NH$_2$), 6.69 (1H, br s,

NH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 29.6, 44.7, 62.8, 67.0, 67.7, 77.4, 78.1, 169.9, 170.4; IR ν_{max} 3400, 3190, 2958, 1753, 1680, 1417, 1306, 1197 cm^{-1}. Anal. Calcd for $C_9H_{11}NO_5$: C, 50.70; H, 5.20; N, 6.57. Found: C, 50.53; H, 5.15; N, 6.47.

4.3.9. 2,2,2-Trifluoroethyl (±)-3,8-dioxatricyclo[3.2.1.0^{2,4}]-octane-6-carboxylate 1g

A mixture of carboxylic acid (±)-**1a** (156 mg, 1.00 mmol), DMAP (245 mg, 2.00 mmol), EDC-Cl (384 mg, 2.00 mmol), CF_3CH_2OH (150 mg, 1.50 mmol), and triethylamine (202 mg, 2.00 mmol) in DMF (1 mL) was stirred at room temperature under argon. The reaction was monitored by silica gel TLC, developed with hexane–EtOAc (1:4). After stirring for 10 h at room temperature, the mixture was quenched by the addition of EtOAc–water. The organic materials were extracted with EtOAc, and the combined organic phases were washed with brine and dried over Na_2SO_4. The organic phase was concentrated in vacuo. The residue was purified by silca gel column chromatography with hexane–EtOAc (1:1) to afford trifluoroethyl ester (±)-**1g** (192 mg, 80.6%) as a colorless oil; ^1H NMR (270 MHz, $CDCl_3$): δ = 1.93 (1H, ddd, J = 4.6, 11.3, 11.6 Hz, H-7$_{exo}$), 2.04 (1H, dd, J = 4.3, 11.6 Hz, H-7$_{endo}$), 2.94 (1H, dt, J = 4.6, 11.3 Hz, H-6), 3.98 (1H, dd, J = 2.4, 4.6 Hz, H-4), 4.05 (1H, dd, J = 2.2, 4.6 Hz, H-2), 4.44 (1H, dddd, J = 8.4, 12.7 Hz, CH_2CF_3), 4.53 (1H, dt, J = 1.9, 4.9 Hz, H-5), 4.58 (1H, dddd, J = 8.4, 12.7 Hz, CH_2CF_3), 4.69 (1H, dt, J = 1.9, 4.9 Hz, H-1); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 29.5, 44.4, 60.2, 60.6, 66.5, 66.6, 77.4, 77.5, 121.5, 124.3, 169.7; IR ν_{max} 3010, 2969, 2368, 2337, 1747, 1411,1276, 1155, 879 cm^{-1}. HRMS (EI): calcd for $C_9H_9F_3O_4$: [M^+]: 238.0453; found: m/z = 238.0453.

4.3.10. PLE-catalyzed hydrolysis of esters 1b–1g

The hydrolysis of each substrate was carried out under the same conditions as described for the screening of enzymes with ethyl ester **1b**. The *E*-value of the each substrate was uniformly calculated from the conversion and ee(P) as follows. The conversion was determined by ^1H NMR analysis of crude reaction mixture. Ee(P) was determined by the HPLC analysis at the stage of **6a**, after methylation of hydrolyzate and following β-elimination as described later.

4.3.11. PLE-catalyzed hydrolysis of 2-chloroethyl ester 1e

To a stirred solution of 2-chloroethyl ester (±)-**1e** (373.3 mg, 1.71 mmol) in a phosphate buffer (0.2 M, pH 7.0; 8.5 mL), PLE (Sigma, E2884, 850 μL) was added and the mixture was stirred for 24 h at room temperature. The reaction was quenched with 1 M HCl to pH 2, and extracted with EtOAc (10 times). The combined organic layer was dried over Na_2SO_4 and concentrated in vacuo, and the ratio between unreacted recovery **1e** and hydrolyzate **1a** was determined by ^1H NMR measurement. The above mentioned crude mixture was washed with saturated $NaHCO_3$ aq solution. The organic layer was washed with brine and dried over Na_2SO_4, concentrated in vacuo to give (−)-**1e** (159.3 mg, 0.73 mmol) as the unreacted recovery. The aqueous layer was acidified to pH 3 and extracted with EtOAc (10 times). The extract was dried over Na_2SO_4 and concentrated in vacuo to give (+)-**1a** (145.4 mg, 0.93 mmol, mp 107–108 °C). These samples were employed for the next step without further purification.

Ester (−)-**1e**: $[\alpha]_D^{23} = -5.3$ (c 1.02, $CHCl_3$), 99.4% ee as shown below. Its IR and NMR spectra were in good accordance with those of racemic sample. Acid (+)-**1a**: $[\alpha]_D^{23} = +11.7$ (c 1.00, MeOH), 90.6% ee as shown below. This was further purified by recrystallization from EtOAc to give (+)-**1a** (93.6 mg, 71%, mp 114–115 °C) $[\alpha]_D^{23} = +14.4$ (c 0.75, MeOH). The sample obtained by recrystallization as above (15.0 mg) was treated with CH_2N_2 to give (+)-**1c** (15.6 mg, 96%); mp 63–64 °C, $[\alpha]_D^{23} = +11.7$ (c 0.75, MeOH). This was further converted to (−)-**6a** (11.1 mg, 74%, 95.6% ee); $[\alpha]_D^{23} = -207$ (c 0.55, MeOH). HPLC analysis was performed in the same manner: t_R (min) = 15.1 (97.8%), 33.1 (2.2%).

Further recrystallization provided a sample of (+)-**1a** (49% recovery, mp 112–113 °C), $[\alpha]_D^{23} = +15.8$ (c 0.76, MeOH). This sample was revealed to be 96.4% ee by the HPLC analysis at the subsequent stage of **6a** as below, and we concluded that the enantiomeric excess of the acid **1a** reaches constant value by repetition of the recrystallization from EtOAc.

The scaled-up experiment by applying (±)-**1e** (1.00 g, 4.59 mmol) worked well in a reproducible manner to give (−)-**1e**: (230 mg, 22.9%) $[\alpha]_D^{24} = -5.3$ (c 1.00, $CHCl_3$); 99.7% ee after derivatization to **6b** and its HPLC anlalysis. Acid (+)-**1a**: (428 mg, 59.8%) $[\alpha]_D^{24} = +11.0$ (c 1.00, MeOH); 77.3% ee by HPLC analysis of corresponding **6a**.

4.3.12. Methyl (1*S*,2*R*,4*S*,5*R*,6*S*)-(+)-3,8-dioxatricyclo[3.2.1.0^{2,4}]-octane-6-carboxylate 1c

To a solution of the acid (+)-**1a** (30.6 mg, 0.20 mmol) as above in anhydrous DMF was added Cs_2CO_3 (163 mg, 0.50 mmol) and CH_3I (85.1 mg, 0.60 mmol). The mixture was stirred at 50 °C for 24 h. After concentration to dryness in vacuo, the residue was extracted with EtOAc (three times), and the combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by preparative TLC with hexane–EtOAc (1:1) to afford methyl ester (+)-**1c** (20.1 mg, 59%) as a colorless solid. Mp 67–68 °C, $[\alpha]_D^{23} = +11.7$ (c 0.57, MeOH). Its IR and NMR spectra were identical with that of the authentic specimen.[2]

4.3.13. Methyl (1*S*,5*S*,6*R*)-(−)-5-hydroxy-7-oxabicyclo[4.1.0]-hept-2-en-3-carboxylate 6a

To a solution of lithium hexamethyldisilazide [$(TMS)_2NLi$, 0.20 mL, 0.20 mmol] was added in THF (0.20 mL) at −78 °C. To a solution of methyl ester (−)-**1c** (20.1 mg, 0.13 mmol) in THF (0.20 mL) was added the LHMDS solution above dropwise at −78 °C, and the mixture was stirred for 1 h at that temperature. The reaction was quenched with saturated NH_4Cl aq solution, and extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by preparative TLC with hexane–EtOAc (1:1) to afford methyl ester (−)-**6a** (14.0 mg, 70%, 90.6% ee) as a colorless solid. $[\alpha]_D^{23} = -200$ (c 0.70, MeOH) [lit.:[2] $[\alpha]_D$ = +213 (c 0.56, MeOH), for (1*R*,5*R*,6*S*)-**6a**]. The product (−)-**6a** was analyzed by HPLC [column, Daicel Chiralcel OD-H, 0.46 cm × 25 cm; hexane–2-propanol (5:1); flow rate 0.5 mL/min]: t_R (min) = 15.1 (95.3%), 33.1 (4.7%).

Enantiomerically enriched acid (+)-**1a** (15.6 mg, 0.10 mmol) by recrystallization in twice was treated with CH_2N_2 to give (+)-**1c** (15.7 mg, 89%); mp 63–64 °C, $[\alpha]_D^{23} = +11.7$ (c 0.75, MeOH). This was converted to (−)-**6a** (11.1 mg, 74%, 96.4% ee); $[\alpha]_D^{23} = -207$ (c 0.55, MeOH). HPLC analysis was performed in the same manner: t_R (min) = 15.1 (98.2%), 33.1 (1.8%).

4.3.14. 2-Chloroethyl (1*R*,5*R*,6*S*)-(+)-5-hydroxy-7-oxabicyclo-[4.1.0]hept-2-en-3-carboxylate 6b

In a similar manner as described for (±)-**6b**, 2-chloroethyl ester (−)-**1e** (47.2 mg, 0.21 mmol) in THF (0.30 mL) was added with a solution of lithium hexamethyldisilazide [$(TMS)_2NLi$, 0.31 mL, 0.31 mmol] in THF (0.30 mL), gave (+)-**6b** (36.4 mg, 77%, 99.4% ee: $[\alpha]_D^{23} = +233$ (c 1.08, MeOH); The product (+)-**6b** was analyzed by HPLC analysis [column, Daicel Chiralcel OD-H, 0.46 cm × 25 cm; hexane–2-propanol (5:1); flow rate 0.5 mL/min]: t_R (min) = 18.0 (0.3%), 38.0 (99.7%); ^1H NMR (400 MHz, $CDCl_3$): δ = 2.32 (1H, ddd, J = 3.3, 5.2, 17.6 Hz, H-6β), 2.80 (1H, dt, J = 2.1, 17.6 Hz, H-6α), 3.48 (1H, t, J = 3.9 Hz, H-3), 3.57 (1H, ddd, J = 2.1, 2.8, 3.9 Hz, H-4), 3.69 (2H, t, J = 5.7 Hz, CH_2Cl), 4.38 (2H, t, J = 5.7 Hz, CO_2CH_2), 4.57 (1H, br m, H-5), 7.19 (1H, dd, J = 3.3, 3.9 Hz, H-2); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 29.3, 41.5, 46.2, 56.1, 63.5, 64.5, 130.3, 134.4, 165.5; IR ν_{max} 3425, 2964, 1709, 1641, 1417, 1392, 1250,

1099, 918 cm^{-1}. Anal. Calcd for $C_9H_{11}ClO_4$: C, 49.44; H, 5.07. Found: C, 49.43; H, 5.36.

4.3.15. (3*S*,4*S*,5*R*)-(−)-3,4,5-Trihydroxy-1-cyclohexene-1-carboxylic acid 2

To a solution of (+)-**6b** (55.0 mg, 0.25 mmol) in THF and water (1:1, 4 mL) was added KOH (21.2 mg, 0.38 mmol). After stirring for 1 h at 50 °C, the mixture was neutralized with 1 M HCl to pH 3, and concentrated in vacuo. The solid was dissolved in water (1 mL) and trifluoroacetic acid (400 μL, 5.39 mmol) was added to the solution with stirring. The mixture was stirred for 3 h at 50 °C. The reaction mixture was concentrated in vacuo to remove volatile materials. The residue was purified by silica gel column chromatography with $CHCl_3$–MeOH (10:1) to afford carboxylic acid (−)-**2**: $[\alpha]_D^{25} = -33.1$ (*c* 0.34, H_2O) [lit.:[20] $[\alpha]_D = -31.0$ (*c* 0.1, H_2O)]; ^1H NMR (400 MHz, D_2O): $\delta = 2.06$ (1H, dddd, $J = 2.8$, 4.0, 10.0, 16.8 Hz, H-6 β), 2.61 (1H, ddd, $J = 1.6$, 6.0, 16.8 Hz, H-6α), 3.33 (1H, dd, $J = 8.4$, 10.0 Hz, H-4), 3.62 (1H, dt, $J = 6.0$, 10.0, 10.0 Hz, H-5), 4.11 (1H, dddd, $J = 1.6$, 2.4, 4.0, 8.4 Hz, H-3), 6.51 (1H, dd, $J = 2.4$, 2.8 Hz, H-2); ^{13}C NMR (100 MHz, D_2O): $\delta = 31.7$, 68.6, 71.5, 76.2, 128.2, 139.2, 169.7; IR ν_{max} 3261, 1556, 1409, 1072 cm^{-1}. Its ^1H NMR spectrum was identical with that reported previously.[21] As this product **2** is a trihydroxy acid and shows highly hydrophilic property and is susceptible to an irreversible adsorption on silica gel, the yield was estimated to be 79.7%, at the stage just before the final purification, based on ^1H NMR with an internal standard [methyl β-D-glucoside, Tokyo Kasei Co., M709, analytically pure grade, standard signal at $\delta = 4.23$ (1H, d, $J = 7.6$ Hz)].

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