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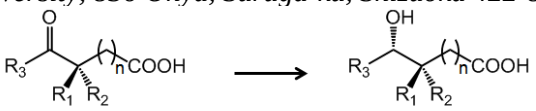

Except (*R*)-**5** Major

R_1 ,R_2 : (*R*)-**1-6** = OH,H; (*S*)-**1-6** = H,OH; **7-10** = H,H

R_3 : **1-8** = ; **9-10** =

n=3: **5**,**6**; n=5: **3**,**4**; n=7: **1**,**2**,**7**,**8**,**9**,**10**

Enantio-selective reduction of the flowering related compound KODA and its analogues in *Pharbitis nil* cv. Violet

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ABSTRACT

Plant oxylipins are an important class of signaling molecules in plants. The cyclic adducts of epinephrine or norepinephrine with the naturally occurring oxylipin (12*Z*,15*Z*)-9-hydroxy-10-oxo-octadeca-12,15-dienoic acid (KODA, **1**) or its synthetic analogues (**2**-**6**) have been reported to possess flower-inducing activity towards *Lemna paucicostata*. By *in vivo* and *in vitro* experiments with seedlings of *Pharbitis nil* cv. Violet carbonyl groups of the α-ketols (**1** and **3)** and the ketones (**7** and **9)** were enantio-selectively reduced to give their corresponding *vicinal* diols (**2** and **4**) and alcohols (**8** and **10**). The stereo-chemistry at the oxymethine carbon was determined based on the long-range C-H coupling constants and the modified Mosher's method. Orientation of the adjacent hydroxyl group in (**1** and **3)** did not affect the enantio-selectivity, whereas the conversion was slightly affected and higher yields were obtained with the *R* enantiomers of the α-ketols.

1. Introduction

KODA (**1**) was isolated as stress-mediated compound from *Lemna paucicostata.* The reaction products of **1** with norepinephrine and epinephrine showed strong flower-inducing activities towards *Lemna*.[1,2] **1** has been also identified in the leaves of *Pharbitis nil* cv. Violet[3] and in immature flower buds of *Dianthus caryophyllus* L., *Dendranthema grandiflorum Kitam*, and *Eustoma russellianum Griseb*.[4] *P. nil,* an well-known model plant for flowering-studies, is classified as a short-day plant which develops flower buds when cotyledons of 4- to 5-days old seedlings are exposed to a single 16 h dark period.[4] We have earlier reported that the endogenous level of **1** in cotyledons of *P. nil* seedlings sharply increased when the seedlings were grown under the continuous dark conditions for 16 h, and dropped rapidly to the basal level by an exposure of light at the end of the flower-inducing dark period. Flower bud formation was also correlated with elevated levels of **1** within 11 h to 16 h following the start of inductive dark period.[4] This transient accumulation of **1** was not observed when seedlings were grown under continuous light.[4] Little is known about chemical changes in plants during the exposure to flower-inductive photoperiod and points to a hypothesis of possible role of **1** in the flowering of *P. nil* as well as *Lemna*. Exogenously applied **1** did not induce the flowering in *P. nil*, however, it promoted the formation of flower buds even though the plants were exposed to shorter inductive dark periods. **1** is metabolized *via* two main reactions, namely α-ketol reduction and β-oxidation of straight-chain acids in *Lemna*,[5] *P. nil*,[6] and *Citrus unshiu* (unpublished) (Fig. 1). These results were revealed by feeding experiments of [U-^{13}C]-**1** to these plants.

We identified **2**-**6** as endogenous metabolites of **1** in these plants by LC-MS and NMR.[6] These pathways seemed to be important to regulate flowering in *P. nil*, because the metabolites **2**-**6** with norepinephrine exhibited lower flower inducing activities. However the enantio-selectivity of the keto-reduction of **1**, **3**, and **5** was not clear. In this study, we have determined the absolute configuration of these metabolites, and demonstrated the enantio-selectivity of the reduction. We also analyzed substrate selectivity of the *P. nil* reductases towards **1** and its synthetic analogues by *in vivo* and *in vitro* experiments.

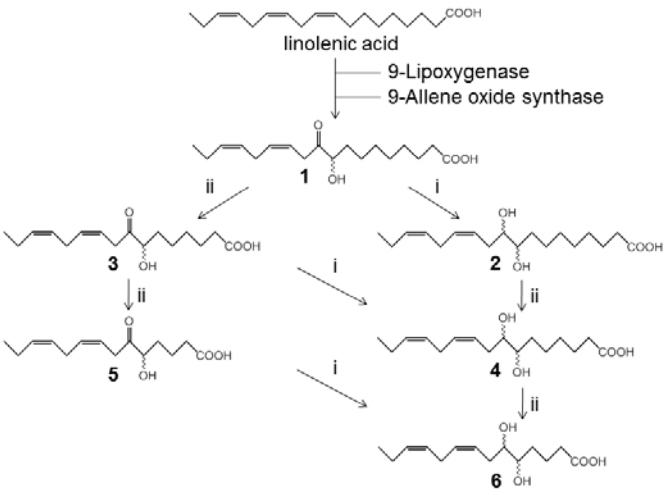
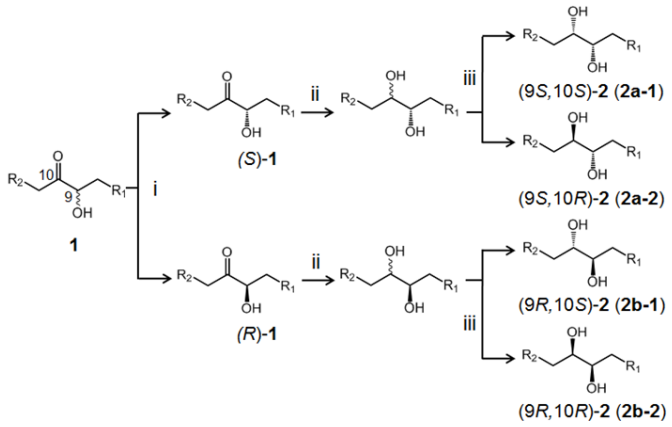


Fig. 1. Proposed metabolic transformations of **1** *via* α-ketol reduction (i) and β-oxidation (ii).[5]

2. Results and discussions

2.1. Stereo-selective synthesis and purification of enantiomeric diols derived from 1

In order to elucidate the stereochemistry of the reduction at C-10 *in vivo* and *in vitro* feeding experiments with **1**, diastereomers of **2** were prepared from **1** by reduction with $NaBH_4$ and their absolute configurations were determined by the NMR analyses. Initially, (*R*)-**1** and (*S*)-**1** were separated by chiral HPLC. The (*R*)-**1** and (*S*)-**1** were separately converted into the diols **2a**-**1**, **2a**-**2** and **2b**-**1**, **2b**-**2** respectively (Scheme 1). The C-10 enantiomers were purified by HPLC on ODS and chiral columns (Fig. S1).



Scheme 1: Synthesis and purification of the four stereoisomers of **2**.
i) chiral phase HPLC separation
ii) $NaBH_4$/ethanol
iii) chiral HPLC separation.

The enantiomers could only be separated and assigned based on their retention times on the chiral column (Fig. 2). We determined configuration of **2b-2** by long range coupling constants and modified Mosher's method.

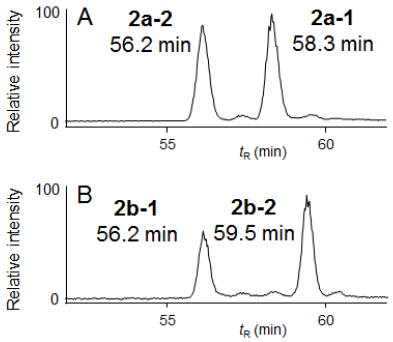


Fig. 2. LC-MS chromatograms (*m/z* 311 $[M-H]^-$) of **2** diastereomers synthesized from (*S*)-**1** (A) and (*R*)-**1** (B) separeted on the chiral HPLC column.

2.2. Determination of the absolute configurations by the measurement of long range coupling constants

Recently, heteronuclear long range coupling constants have been utilized in stereo-chemical investigations of acyclic systems.[7,8] This method has a number of advantages in elucidating the absolute configurations of acyclic asymmetric carbons and is applicable for determining configurations of consecutive asymmetric carbons without chemical derivatizations. These approaches rely upon the determination of coupling constants between protons and carbons separated by two or three chemical bonds. It is well established that $^3J_{H\text{-}H}$ and $^3J_{C\text{-}H}$ values depend on the dihedral angle between proton and carbon at the *vicinal* position and $^2J_{C\text{-}H}$ depends on the dihedral angle between proton and oxygen atom at the *vicinal* position. As a result, **2b-1** and **2b-2** form six possible rotamers around C-9 and C-10 showing different coupling constant values (Fig. S2).

The analyses of the width of spectral line of C-11 at the slice A and the coupling constant between H-9 and C-11 $^3J_{H9\text{-}C11}$ which was determined to be less than 3 Hz suggested that the relationship between H-9 and C-11 is *gauche*. In the same manner, $^3J_{H10\text{-}C8}$, $^2J_{H10\text{-}C9}$, and $^2J_{H9\text{-}C10}$ were determined to be less than 3 Hz were designated as *gauche* conformation. Although we measured $^3J_{H9\text{-}H10}$ by ^{1}H-NMR, it was not appropriate to elucidate the actual *vicinal* coupling constant because the chemical shifts of protons were not resolved. As shown in Fig. 3-A the signals of H-9 and H-10 were partially overlapping.

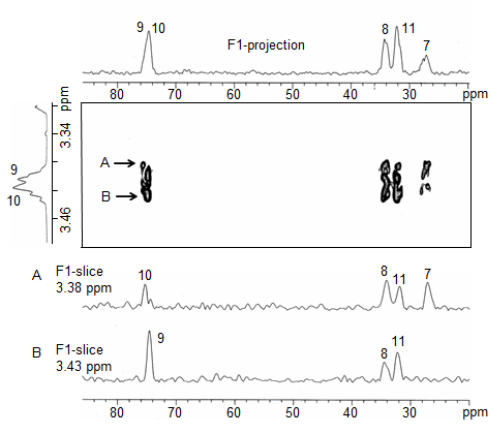


Fig. 3-A. *J*-resolved HSQC spectrum of C-9 and C-10 of **2b-2**. A slice at δ 3.38 (H-9); B slice at δ 3.43 (H-10).

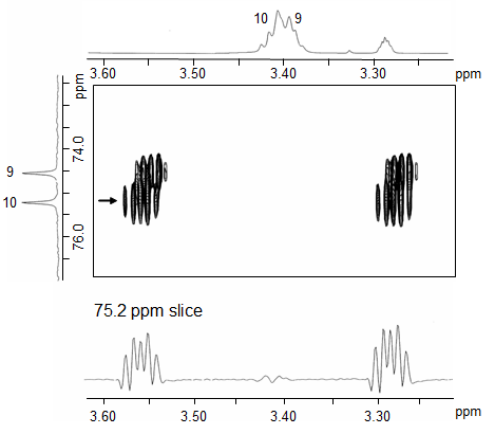


Fig. 3-B. *J*-resolved HSQC spectrum of C-9 and C-10 between satellite signals of H-9 and H-10 of **2b-2**.

Therefore we measured the *J*-resolved HSQC spectrum at C-10 position (δ 75.2) showing cross peaks based on the satellite signals separated by $^1J_{C10\text{-}H10}$ (Fig. 3-B). Based on these results, spectral data of H-10 could be satisfactorily analyzed. The spectral pattern showed that $^3J_{H9\text{-}H10}$ is 4 Hz. In addition, the $^3J_{H10\text{-}H11a}$ and $^3J_{H10\text{-}H\text{-}11b}$ were measured by the decoupling experiments at H-11 signals and were 5 and 7 Hz, respectively. Although $^3J_{H9\text{-}C10}$ with a value of 4 Hz was slightly higher than that for the *gauche* form, it indicated *gauche* conformation. Moreover, there were no divergent results for $^{2,3}J_{C\text{-}H}$ coupling, and therefore H-9 and H-10 was designated to be *gauche*. Based on the $^{2,3}J_{C\text{-}H}$, $^3J_{H\text{-}H}$ data, we successfully determined the stereochemistry at C-10 position of **2b-2** to be *R* configuration by measurement of long range coupling constants.

2.3. Determination of the absolute configurations by modified Mosher's method

Modified Mosher's method is effective in determination of the absolute configurations of secondary alcohols.[9] This method relies on the fact that the protons in diastereomeric mono-α-methoxy-α-(trifluoromethyl) phenylacetyl (MTPA) esters display different chemical shifts in their ^{1}H-NMR spectra. Firstly, **2b-2** was converted to its methyl ester which was subsequently derivatized to its MTPA ester and purified by HPLC prior NMR analysis. The (*S*)-MTPA ester of **2b-2** was obtained similarly. The proton and carbon signals of **2b-2** were assigned on the bases of ^{1}H-^{1}H COSY and HMBC spectra. Alterations of chemical shifts were calculated based on the modified Mosher's method and higher frequency (+Δδ) shifted for C-11 to C-18 and lower frequency (-Δδ) shifted for C-7, C-8, and C-3 (Fig. 4).

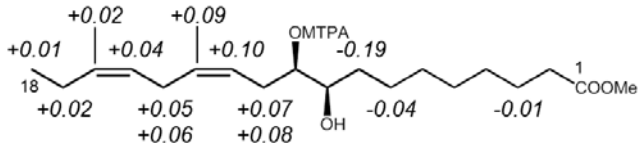


Fig. 4. Chemical shift differences Δδ (*S*–*R*) of MTPA esters derived from **2b-2**.

Based on these results, the absolute configuration at C-10 of **2b-2** was determined to be *R*. Thus, we could unambiguously determine the absolute configuration at C-9 and C-10 of one of the diastereomers based on the long range coupling constants and the modified Mosher's method. Therefore, the absolute configuration at C-10 of the other diol isomer **2b-1** from (*R*)-**1** was assigned to be *S*. The results of the absolute configurations of **2b-2** by Mosher's method were the same as the results obtained by the measurement of long range coupling constants and enabled us to study the stereochemistry of the reduction of **1** and its analogues *in vivo* and *in vitro*.

2.4. Stereo-selective conversion of 1 into 2 *in vivo*

In the *in vivo* system, exogenously-applied (*R*)-**1** and (*S*)-**1** were metabolized to their respective diols (Fig. 5). As a result, seedlings gave **2a-1** (9*S*,10*S*) mainly upon feeding with (*S*)-**1**, likewise, (*R*)-**1** was converted mainly into **2b-1** (9*R*,10*S*) (Fig. 6). These results indicate that α-ketol reduction at C-10 of **1** predominantly yielded 10*S* regardless of the C-9 configuration. In fact, the proportion of the conversion into *S* configuration at C-10 was *ca.* 90% for both.

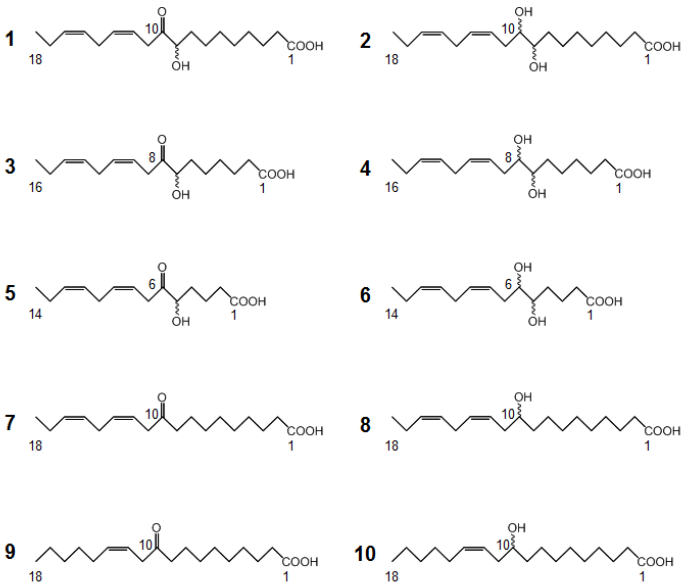


Fig. 5. Structures of keto-oxylipins (odd numbers) and related alcohols (even numbers) investigated in this study.

2.5. Enantio-selective reduction of carbonyl groups of 1 and its analogues *in vitro*

We extracted crude enzymes containing keto-reductases from seedlings of *P.nil* to confirm the enantio-selective conversion of **1** and its analogues. Various oxylipins, including **1** and other β-oxidative metabolites of *P. nil* have carbonyl moieties (unpublished). We investigated the *in vitro* enantio-selective reduction with keto oxylipins, **1**, **3**, **5**, **7**, and **9**. The *R*- and *S*-enantiomers of **1**, **3**, and **5** and ketones **7** and **9** were separately incubated with crude enzymes of the *P. nil*. The enantio-selectivity of the keto-reduction was determined by comparison of the retention times of tolal ion traces of their corresponding alcohols based on the LC-MS analysis (Table S1). *S* enantio-selective reduction occurred with **1**, **3**, **7**, and **9** (Table 1). The orientation of alcohol moiety did not affect the enantio-selectivity.

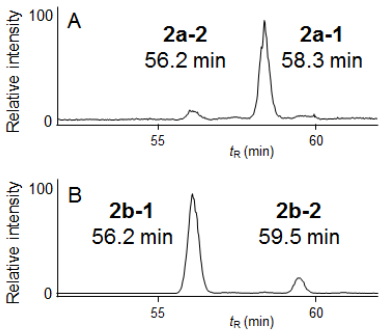


Fig. 6. LC-MS chromatograms (*m/z* 311 [M-H]$^-$) of **2** diastereomers extracted from *P.nil* administered with (*S*)-**1** (A) and (*R*)-**1** (B) separated on the chiral HPLC column.

Table 1. Stereo-selectivity of the keto-reduction of **1** and its analogues

Substrate	Selectivity (%)[a,b]	
	S	*R*
(*R*)-**1**	89	11
(*S*)-**1**	89	11
(*R*)-**3**	76	24
(*S*)-**3**	86	14
(*R*)-**5**	25	75
(*S*)-**5**	72	28
7	19	81
9	17	83

[a] All the products were characterized by ^{1}H-NMR spectroscopy and mass spectroscopy and were in accordance to previously published data.[10]
[b] Selectivity was determined by co-chromatography with authentic reference substances (Table S1).

Although the opposite enantio-selectivity was observed for (*R*)-**5**, (*S*)-**5** was reduced with *S* enantio-selectivity. In *planta* huge numbers of reductases have been reported,[11,12] these enzymes are classified into sub families which catalyze reactions enantio-selectively. The crude enzymes prepared for use in these experiments may contain several types of enzymes involved in the keto-reduction, despite that in the present study *S* enantio-selectivity was observed *in vivo* and *in vitro* experiments. Although the enantio-selectivity showed *R* selectivity by feeding **7** and **9**, but the face-selectivity was the same as for **1** and **3**. In addition, neither orientation of α-keto-hydroxyl groups nor presence of alcohol groups or double bonds affected the enantio-selectivity of the *in vitro* reaction. We also investigated the turnover rates *in vitro*. (*R*)-**1** was approximately two fold easier converted into **2** than (*S*)-**1**. Similarly *R* analogues were more easily reduced than their *S* analogues (Fig. 7). Faster turnover rates were observed with longer aliphatic chains of the substrates in the keto-reduction. As there were no differences in the conformational energy (data not shown) between C-9 and C-10 of **2b-1** and **2b-2**, orientation of 9-OH substantially influenced the enantio–selectivity of the keto-reduction.

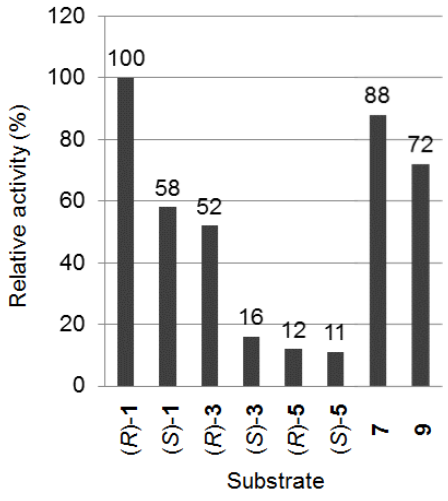


Fig. 7. Keto-reducase activities toward **1** and its analogues. The activities were expressed as reactivity (%) relative to (*R*)-**1**.

3. Conclusion

The previous study demonstrated that exogenously-applied **1** is mainly metabolized *via* reduction and β-oxidation.[6] This study shows that **1** and all analogues except (*R*)-**5** are converted to their corresponding alcohols in same face-selective keto-reduction *in vivo* and *in vitro*. All the *R*-enantiomer of α-ketols (**1** and **3**) were easily metabolized to their *S* alcohols. Compounds lack in the 9-hydroxyl group (**7** and **9**) were metabolized with lower turnover rates. Compounds **3** and **5**, in which the fatty acid chain length is shortened, were reduced with lower rates than **1**. These results show the *R*-enantiomers of C18 α-ketol oxylipin (**1**) are the most favorable substrates for the enantio-selective keto-reduction. In the plants, compounds that have physiological significance activities are controlled by biosynthetic enzymes and metabolic enzymes. Therefore **1** needs to be metabolized immediately in plants after flower-inducing activity. Our clarified results indicated presence of some important metabolic enzymes in *P. nil*, and these enzymes are suit for metabolizing **1**.

4. Experimental

4.1. General procedures

^{1}H, ^{13}C NMR, and 2D NMR spectra were recorded on a JNM-λ500α spectrometer and λ270 α spectrometer (JEOL, Tokyo, Japan). Long range couplings for *J*-resolved HMBC-2 and *J*-resolved HSQC spectra were recorded with a resolution of $J_{C\text{-}H}$=2.4 Hz with JEOL JNM-λ500 NMR spectrometer. High-resolution mass spectra were obtained with a JMS-T100LC AccuTOF mass spectrometer (JEOL). Recycle HPLC was performed with a JASCO PU-9986 LC pump equipped with JASCO 875-UV detector (Tokyo, Japan). LC-MS analyses were carried out with a LC-10VP system equipped with LC-MS 2010A mass spectrometer (Shimadzu, Kyoto, Japan). HPLC separation was performed with a JASCO (Tokyo, Japan) LC system. Solvents for HPLC were purchased from Kanto Chemical (Tokyo, Japan). A two-solvent system was used to generate the mobile phase for HPLC: solvent A, aqueous 0.05% trifluoroacetic acid; solvent B, acetonitrile.

4.2. Plant materials

P. nil cv. Violet (Marudane, Kyoto, Japan) was germinated as described previously.[6] Seeds of *P. nil* were immersed in concentrated H_2SO_4 at 35 °C for 25 min and were subsequently washed in running water overnight. The seeds were placed on soil and incubated at 25 °C under continuous light (84 μmol/m^{2}s^1). Three day-old plants were transferred to Nakayama's liquid culture and incubated at 25 °C under light (84 μmol/m^{2}s^1) for 5–6 h to synchronize the growth of the seedlings. Seeds were exposed to dark-period for 14 h to elevate enzymatic activity after the light period, a mandatory step as shown in the previous study.[4]

4.3. Purification of *R* and *S* enantiomers of keto-alcohols 1, 3, and 5 and alcohols 8 and 10

Compound **1** (*R*/*S*=3/1) was obtained from Shiseido as enantiomeric mixture. Further purification was carried out to isolate (*R*)-**1** and (*S*)-**1** by using a HPLC equipped with chiral column (CHIRALCELL OD-RH 4.6×150 mm, DAICEL, Japan).[10] The recycle HPLC conditions were as follows: flow rate, 5.0 mL/min; mobile phase, A: H_2O (0.05% TFA), B: MeCN; solvent, isocratically B/(A+B) 1:1 (v/v); temperature, 4 °C. Each enantiomer (*R*)-**1** and (*S*)-**1** fraction was evaporated, and the

remaining aqueous layer was extracted with trichloromethane thrice. Thereafter, the organic layer was washed with anhydrous NaCl and dehydrated with anhydrous sodium sulfate prior to evaporation of the solvent to yield (*R*)-**1**[10] (160 mg, *ee* 98%; $[\alpha]^{23}_{D}$ -13.8°, *c* 0.63, CH_3OH) and (*S*)-**1**[10] (50 mg, *ee* 97%; $[\alpha]^{23}_{D}$ +12.5°, *c* 0.63, CH_3OH) as colorless amorphous solids from **1 (**400 mg). In the same manner enantiomers of **3** and **5** were isolated to give (*R*)-**3** (0.9 mg), (*S*)-**3** (1.5 mg) from **3** (*R*/*S*=1/1, 10.3 mg), and (*R*)-**5** (1.9 mg) and (*S*)-**5** (2.4 mg) from **5** (*R*/*S*=1/1, 11.9 mg), respectively. The absolute configuration of the enantiomers were determined based on the optical rotation (described in *4.9.2*).

In the same manner (*R*)-**8** (21.2 mg), (*S*)-**8** (19.4 mg) from **8** (*R/S*=1/1, 60.1 mg), and (*R*)-**10** (20.0 mg) and (*S*)-**10** (19.4 mg) from **10** (*R/S*=1/1, 61.3 mg) were obtained. The absolute configuration of the enantiomers were determined based on modified Mosher's method (described in Fig. S3) after methylation of each compound as described in 4.5.

4.4. Conditions of the measurement of long range coupling constants

$^{2,3}J_{C\text{-}H}$ of **2a-2** were detected by *J*-resolved HMBC-2 spectra (Fig. 3-B) with a scaling factor (n) of 25 and the digital resolution $J_{C\text{-}H}$=2.4 Hz; the slice of position A (at δ3.38) showing coupling constants between H-9 and each carbon; the slice of B (at δ3.43) showing coupling constants between H-10 and each carbons. All conditions were identically to the established method by Furihata and Seto.[8]

4.5. Derivatization by modified Mosher's method

2a-2 ((9*R*,10*R*)-**2,** 13.6 mg, 43.6 μmol) in methanol was treated with trimethylsilyl diazomethane (200 μmol) to give **2a-2** (9*R*,10*R*)-**2** methyl ester (14.0 mg, 36.8 μmol). The methyl ester (7.0 mg, 21.5 μmol) in dichloromethane was treated with 1.5 equivalents of (*R*)-MTPA chloride in the presence of 4-dimethylaminopyridine, and the mixture was stirred at room temperature for 12 h. The mixture was purified by column chromatography on silica gel (hexane:ethyl acetate/4:1 (v/v)) and HPLC on ODS column (B:A/4:1 (v/v)). The target compound 10-mono-MTPA ester (1.4 mg, yield 17.0%) was obtained together with 9,10-di- (2.0 mg, yield 17.0%) and 9-mono-MTPA ester (2.9 mg, yield 35.4%). The 9-mono-(*S*)-MTPA (1.2 mg, yield 14.6%) was obtained together with 9,10-di- (1.5 mg, yield 13.0%) and 10-mono-MTPA (2.1 mg, yield 25,0%) from *S*-MTPA under the same methods. The chirality at C-10 position was determined based on the ^{1}H-NMR spectra.

The MTPA esters of (*R*)-8 and (*S*)-**8** methyl esters and those of (*R*)- and (*S*)-**10** methyl esters were prepared in an usual manner and purified by successive column chromatography and HPLC on ODS column as mentioned above. (*R*)-**8**-(*R*)-MTPA ester (2.3 mg; yield 32%), (*R*)-**8**-(*S*)-MTPA ester (6.0 mg; yield 84%), (*R*)-**10**-(*R*)-MTPA ester (36.1 mg; yield 87%), (*R*)-**10**-(*S*)-MTPA ester (6.2 mg; yield 85%) were obtained. The configuration was determined as described in *4.9.4* and *4.9.5*

4.6. *In vivo* experiments and LC-MS analyses of the metabolites of (*R*)-1 and (*S*)-1

Three cotyledons (0.3 g fresh weight each) of *P. nil* were immersed in a solution (5 mL) of (*R*)-**1** (320 μmol/mL) or (*S*)-**1** (320 μmol/mL) under reduced pressure (30 mmHg) for 1 min. The seedlings were incubated at 25°C under continuous light (84 $\mu mol/m^2s^1$) for 10 min. After incubation the seedlings were homogenized in liquid N_2 and extracted with ethyl acetate five times by sonication for 1 min. The extracts were combined and evaporated to dryness. The residue was dissolved in 20% (v/v) acetonitrile. **1** and its metabolites **2a-1**, **2a-2**, **2b-1**, and **2b-2** were eluted with 80% (v/v) acetonitrile. The *in vivo* conversion of (*R*)-**1** and (*S*)-**1** were examined by the LC-MS analyses. The conversion rates of (*R*)-**1** and (*S*)-**1** to the respective diols **2** were 6% and 3.2% of the amounts (*ca* 0.8%) of the administered in the seedlings. The LC-MS analysis was carried out in selected ion monitoring mode, the conditions were as follows: CAPCELLPAK AS-RH 4.6×150 mm (Shiseido, Japan); flow rate, 0.2 mL/min; gradient, keep 30% (v/v) B/(A+B) 30 min and 30-100% (v/v) B/(A+B) within 90 min; column temperature, 30°C. The MS conditions were set as follows: probe voltage -3.5 kV and gas flow rate 1.5 mL/min. Enantio excess (*ee*) and diasteromeric excess (*de*) were determined based on the peak area of each enantiomer or diastereomer. Enantiomeric compounds **2a-1** and **2b-2** gave different retention times of 58.3 min and 59.5 min, respectively. **2a-2** and **2b-1** co-eluted at 56.2 min from the chiral column.

4.7. Preparation of crude enzyme extract from *P.nil*

Cotyledons of *P. nil* were kept in darkness for 16 h and incubated at 25°C under continuous light (84 $\mu mol/m^2s^1$) for two weeks. The whole plant material 40 g was crushed with liquid nitrogen and homogenized in 400 mL 0.01 M phosphate buffer (pH 8.0) containing 5 mM dithiothreitol (DTT), 0.05% 3-((3-Cholamidopropyl)dimethylammonium)-1-propanesulfonate (CHAPS), 5% glycerine), 30 g polyvinylpyrrolidone (PVPP) and 3 mL of 0.2 M phenylmethylsulfonyl fluoride (PMSF) at 4 °C. After extraction, the crude enzyme extract was centrifuged at 12,000 *g* for 20 min at 5 °C and 380 mL supernatant was obtained. The supernatant was filtered and 30% $(NH_4)_2SO_4$ was deed up to concentration of 50%. After incubation for 30 min on ice, the crude enzyme was centrifuged 12,000 *g* for 20 min at 5 °C. The supernatant was filtered and $(NH_4)_2SO_4$ up to a concentration of 50% was added. Finally, another precipitation step to the final $(NH_4)_2SO_4$ concentration of 60% was carried out. The obtained protein pellet from the precipitation step from 50% to 60% $(NH_4)_2SO_4$ was dissolved in 5 mL 0.1 M phosphate buffer (pH 7.5, 5 mM DTT) and was used for the cell free assays.

4.8. Stereo-selective conversion of 1 into 2 *in vitro* and its analogues LC-MS analysis

Ten microlitter of the substrate (10 mM) dissolved in DMSO, 100 μL crude enzyme solution and 25 μL NADPH (Oriental yeast) dissolved in 0.1 M phosphate buffer (pH 7.5, 5 mM DTT) were reacted for 2 h at 30°C. After addition of 100 μL 2 M HCl to stop the enzyme reaction, reactants were extracted with ethyl acetate (1.5 mL × 4). The extracted reaction products were analyzed by LC-MS. The LC-MS analysis was operated in selected ion monitoring mode. The chromatographic conditions were as follows: CAPCELLPAK AS-RH 4.6×150 mm (Shiseido, Japan); flow rate, 0.2 mL/min; gradient, keep 30% (v/v) B/(A+B) 30 min and 30-100% (v/v) B/(A+B) within 90 min; column temperature, 30°C. The MS was operated under the conditions described under 4.6. Enantio-selectivity of keto-reduction of **1**, **3**, **5**, **7**, and **9** were elucidated based on the LC-MS profiles by direct comparison with those of respective keto-ruduced compounds **2**, **4**, **6**, **8**, and **10**. (Tables S1, S2).

4.9. Synthesis of Compounds 2-10

4.9.1. Compound 2

(*R*)-**1** and (*S*)-**1**[10] (*R*)-**1** (20.0 mg, 64.5 μmole) was dissolved in 1.0 mL ethanol and $NaBH_4$ (7.3 mg, 194 μmol)) were stirred for 30 min at room temperature. After addition of 500 μL 1 M HCl and 30 mL H_2O, the reactants were washed with ethyl acetate 15 mL (×3). The combined ethyl acetate layers were washed with saturated NaCl solution and dehydrated by anhydrous Na_2SO_4 and concentrated under vacuum to give the diastereomers of **2** (20.0 mg, 46.0 μmol). The diasteromers were purified by preparative HPLC under the conditions as follows: CHIRALCELL OD-RH 20×150 mm (DAICEL, Japan); flow rate, 5.0 mL/min; mobile phase, A: H_2O (0.05% TFA), B: MeCN; solvent, isocratically B/(A+B) 1:1 (v/v); column temperature, 15°C. (9*R*,10*R*)-**2** (14.6 mg, yield 42%) and (9*R*, 10*S*)-**2** (14.6 mg, yield 42%) were obtained as colourless amorphous solid. In the similar manner, (9*S*,10*R*)-**2** (5.9 mg, yield 29%) and (9*S*, 10*S*)-**2** (8.2 mg, yield 42%) were obtained from (*S*)-**1**.

(9*S*,10*S*)-**2** $[\alpha]^{26}_D$ -17.9° (*c* 0.56 CH_3OH); HR-MS (ESI^+) *m/z* 335.2198 $[M+Na]^+$ (calcd for $C_{18}H_{32}NaO_4$, 335.2199); ^{13}C NMR (125.0 MHz, CD_3OD) δ 14.6, 21.5, 26.1, 26.6, 27.0, 30.2, 30.4, 30.7, 32.1, 34.1, 35.1, 74.6, 75.2, 127.3, 128.3, 130.9, 132.7, 177.8.

(9*R*,10*R*)-**2** $[\alpha]^{26}_D$ +14.6°(*c* 0.63 CH_3OH); HR-MS (ESI^+) *m/z* 335.2200 $[M+Na]^+$ (calcd for $C_{18}H_{32}NaO_4$, 335.2199); ^{13}C NMR (125.0 MHz, CD_3OD) δ 14.6, 21.5, 26.6, 27.0, 27.8, 30.5, 30.6, 30.7, 32.1, 34.2, 39.3, 74.6, 75.2, 127.3, 128.3, 130.9, 132.7, 177.8.

(9*R*,10*S*)-**2** $[\alpha]^{26}_D$ -0.7°(*c* 0.37 CH_3OH); HR-MS (ESI^+) *m/z* 335.2200 $[M+Na]^+$ (calcd for $C_{18}H_{32}NaO_4$, 335.2199); ^{13}C NMR (125.0 MHz, CD_3OD) identical with (9*S*,10*R*)-**2**.

(9*S*,10*R*)-**2** $[\alpha]^{26}_D$ +1.2°(*c* 0.39 CH_3OH); HR-MS (ESI^+) *m/z* 335.2201 $[M+Na]^+$ (calcd for $C_{18}H_{32}NaO_4$, 335.2199); ^{13}C NMR (125.0 MHz, CD_3OD) identical with (9*R*,10*S*)-**2**.

10-mono-(*R*)-MTPA ester of (9*R*,10*R*)-**2** methyl ester
HR-MS (ESI^+) *m/z* 565.2375 $[M+Na]^+$ (calcd for $C_{29}H_{41}F_3NaO_6$, 565.2753); ^1H-NMR (500 MHz,$CDCl_3$): δ 0.96 (3H, t, *J*=6.5 Hz, 18-H), 1.25~1.35 (6H, m, 4-H, 5-H, 6-H), 1.29 (2H, m, 7-H), 1.44 (2H, m, H-8), 1.61 (2H, m, 3-H), 2.04 (2H, m, 17-H), 2.30 (2H, t, *J*=7.6 Hz, 2-H), 2.46 (1H, m, 11a-H), 2.49 (1H, m, 11b-H), 2.72 (1H, m, 14a-H), 2.76 (1H, m, 14b-H), 3.53 (3H, s, MTPA-OMe), 3.65 (1H, m, 9-H), 3.67 (3H, s, OMe), 5.01 (1H, m, 10-H) , 5.23 (1H, m, 15-H), 5.29 (1H, m, 12-H), 5.38 (1H, m, 16-H), 5.44 (1H, m, 13-H), 7.40 (3H, m, bz), 7.54 (2H, m, bz).

10-mono-(*S*)-MTPA ester of (9*R*,10*R*)-**2** methyl ester
HR-MS (ESI^+) *m/z* 565.2390 $[M+Na]^+$ (calcd for $C_{29}H_{41}F_3NaO_6$, 565.2753); ^1H-NMR (500 MHz, $CDCl_3$) : δ 0.97 (3H, dd, *J*=7.6 Hz, 18-H), 1.25 (2H, m, 8-H), 1.20~1.35 (6-H, m, 4-H , 5-H , 6-H), 1.62 (2H, m, 3-H), 2.06 (2H, m, 17-H), 2.30 (2-H, t, *J*=6.3 Hz, 2-H), 2.54(1H, m,11a-H), 2.56 (1H, m, 11b-H), 2.78 (1H, m,14a-H), 2.81 (1H, m,14b-H),3.53 (3H, s, MTPA-OMe), 3.62 (1H, m, 9-H), 3.67 (3H, s, OMe), 5.03 (1H, dt, *J*=6.3 , 4.0Hz, 10-H), 5.27 (1H, m, 15-H), 5.39 (1H, m, 12-H), 5.40 (1H, m, 16-H), 5.53 (1H, m, 13-H), 7.40 (3H, m, bz), 7.54 (2H, m, bz).

*4.9.2. Compounds **3** and **5***

Compounds **3** and **5** were obtained as described in our previous study and comfirmed the structures based on the HR-MS, ^1H- and ^{13}C-NMR.[10] Enantiomers of **3** and **5** were isolated by chiral HPLC as described in *4.3* and the configurations were determined based on the optical rotation measurement. $[\alpha]^{26}_D$ +8.4° (*ee* 96%; *c* 0.80, CH_3OH) for (*S*)-**3**; $[\alpha]^{26}_D$ -6.7° (*ee* 94%; *c* 0.60, CH_3OH) for (*R*)-**3**; $[\alpha]^{26}_D$ +1.5° (*ee* 96%; *c* 0.79, CH_3OH) for (*S*)-**5**; $[\alpha]^{26}_D$ -1.5° (*ee* 92%; *c* 0.62, CH_3OH) for (*R*)-**5**.

*4.9.3. Compounds **4** and **6***

(9*R*,10*R*)-, (9*R*, 10*S*)-, (9*S*,10*R*)-, (9*S*, 10*S*)-**2** (100 μg each) was treated with β-oxidation enzyme (Asai Kasei, Shizuoka, Japan)[13], comprising with acyl-CoA synthetase (ACS) ,acyl-CoA oxidase (ACOD), 2-enoylacyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase multienzyme (HDT). After the incubation for 12 hr at 30 °C, the reaction mixture was extracted with ethyl acetate and the organic layer was subjected to HPLC analysis on an analytical chiral column under the conditions described in Fig. S1. As the conversion rate of each diastereomer **2** was *ca*. 10%, only trace amount of the corresponding diastereomers (7*R*,8*R*)-, 7*R*,8*S*)-, (7*S*,8*R*)-, (7*S*,8*S*)-**4** and (5*R*,6*R*)-, 5*R*,6*S*)-, (5*S*,6*R*)-, (5*S*,6*S*)-**6** were produced from (9*R*,10*R*)-, (9*R*, 10*S*)-, (9*S*,10*R*)-, (9*S*, 10*S*)-**2**, respectively without any epimerization. (Table S1) Enantio-selectivity of *in vitro* conversion was determined by directly comparing with these compounds.

*4.9.4. Compound **8***

Compound **7** (22.6 mg, 76.8 μmol) dissolved in 3.0 mL ethanol and $NaBH_4$ (10.0 mg, 265 μmol) were stirred for 30 min at room temperature. After addition of 500 μL 1 M HCl and 30 mL H_2O, the reactants were washed with ethyl acetate 15 mL (×3). The combined ethyl acetate layers were washed with saturated NaCl solution and dehydrated by anhydrous Na_2SO_4 and concentrated under vacuum to give the respective enantiomer of **8** (20.0 mg, 68.0 μmol). ^1H-NMR (270 MHz, $CDCl_3$) δ0.97 (3H, t, 7.4 Hz, 18-H), 1.26-1.40 (10H, m, 4~8-H), 1.43-1.50 (2H, m, 9-H), 1.63 (2H, m, 3-H), 2.07 (2H, m, 17-H), 2.27 (2H, t-like, *J*=6.6 Hz, 11-H), 2.35 (2H, t, *J*=7.6 Hz, 2-H), 2.81 (2H, m, 14-H), 3.69 (1H, m, 10-H), 5.25-5.67 (4H, m, 12, 13, 15 and 16-H). ^{13}C-NMR (67.5 MHz, CD_3OD) δ14.2, 20.6, 24.6, 25.5, 29.0, 29.1, 29.3, 29.4, 34.0, 35.0, 36.5, 72.1, 125.0, 126.7, 131.8, 132.3, 180.1. HRMS (ESI^+) *m/z* 319.2243 $[M+Na]^+$ (calcd for $C_{18}H_{32}NaO_3$, 319.2249). Absolute configurations were elucidated based on the modified Mosher's method. The LC-MS analysis on a chiral HPLC was described in **4.8**.
(*S*)-**8** *ee* 96%; $[\alpha]^{26}_D$ -5.2° (*c* 1.29, CH_3OH); (*R*)-**8** *ee* 94%; $[\alpha]^{26}_D$ +6.2° (*c* 1.41, CH_3OH)

Methyl esters of (*R*)-**8** and (*S*)-**8** were prepared in the same manner as describe in **4.5.**

(*S*)-**8**-(*R*)-MTPA ester
^1H-NMR (500 MHz, $CDCl_3$): δ 0.96 (3H, t, *J*=7.6Hz, 18-H), 1.15-1.32 (8H, m, 4-7H), 1.18 (2H, m, H-8), 1.56 (2H, m, 9-H), 1.59 (2H, m, 3-H), 2.05 (2H, m, 17-H), 2.30 (2H, t, *J*=7.6Hz, 2-H), 2.38 (1H, m, 11a-H), 2.48 (1H, m, 11b-H), 2.76 (2H, m, 14-H), 3.55 (3H, s, MTPA-OMe), 3.67 (3H, s, -OMe), 5.12 (1H, m, 10-H), 5.26 (1H, m, H-15), 5.38 (2H, m, 12 and 16-H), 5.50 (1H, m, H-13), 7.39 (3H, m, bz-H), 7.54 (2H, m, bz-H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ14.2, 20.6, 24.9×2, 25.7, 29.1×2, 29.2×2, 29.7, 30.3, 31.8, 33.3, 34.1, 51.4, 55.5, 77.0, 124.0, 126.5, 127.4, 128.3, 129.5, 131.5, 132.3, 132.5, 166.3, 174.3; HRMS (ESI^+) *m/z* 549.2798 $[M+Na]^+$ (calcd for $C_{29}H_{41}F_3NaO_5$, 549.2804).

(*R*)-**8** (*S*)-MTPA ester
^1H-NMR (500 MHz, $CDCl_3$) δ 0.96 (3H, t, *J*=7.6Hz, 18-H), 1.23-1.33 (8H, m, 4-7H), 1.30 (2H, m, H-8), 1.61 (2H, m, 3-H), 1.63 (2H, m, 9-H), 2.04 (2H, m, 17-H), 2.30 (2H, t, *J*=7.6Hz, 2-H),

2.33 (1H, m, 11a-H), 2.39 (1H, m, 11b-H), 2.72 (2H, m, 14-H), 3.54 (3H, s, MTPA-OMe), 3.69 (3H, s,-OMe), 5.10 (1H, m, 10-H), 5.23 (1H, m, H-15), 5.25 (1H, m, 12-H), 5.38 (2H, m, 16-H), 5.42 (1H, m, H-13), 7.39 (3H, m, bz-H),7.54 (2H, m, bz-H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ14.2, 20.6, 25.0×2, 25.6, 29.1×2, 29.3×2, 29.7, 30.3, 31.5, 33.4, 34.1, 51.4, 55.5, 77.0, 123.7, 126.6, 127.4, 128.3, 129.5, 131.4, 132.2, 132.5, 166.3, 174.3; HRMS (ESI^+) *m/z* 549.2799 $[M+Na]^+$ (calcd for $C_{29}H_{41}F_3NaO_5$, 549.2804).

*4.9.5. Compound **10***

An enantiomeric mixture of **10** was obtained from **9** (19.9 mg, 67.1 μmol) under the conditions given in 4.9.3.

Compound **10** (21.0 mg, 70.5 μmol): ^{1}H-NMR (270 MHz, $CDCl_3$) δ0.86 (3H, t, *J*=7.0 Hz, 18-H), 1.31-1.50 (18H, m, 4-9-H and 15-17-H), 1.63 (2H, m, 3-H), 2.04 (2H, m, 17-H), 2.21 (2H, t-like, *J*=6.5 Hz, 11-H), 2.34 (2H, t, *J*=7.6 Hz, 2-H), 3.64 (1H, m, 10-H), 5.35-5.42 (1H, m, 12-H), 5.52-5.62 (1H, m, 13-H); HRMS (ESI^+) *m/z* 321.2404 $[M+Na]^+$ (calcd for $C_{18}H_{34}NaO_3$, 321.2406).

(*R*)-**10** *ee* 98%; $[\alpha]^{26}_D$ +12.3° (*c* 1.33, CH_3OH); (*S*)-**10** *ee* 98%; $[\alpha]^{26}_D$ -11.5° (*c* 1.29, CH_3OH).

Methyl ester of **10**

^{1}H NMR (270 MHz, $CDCl_3$) δ 0.88 (3H, t, *J*=6.6 Hz), 1.31-1.52,(18H, m), 1.63 (2H, m), 2.06 (2H, m), 2.21 (2H, t, *J*=6.6 Hz), 2.30 (2H, t, *J*=7.6 Hz), 3.61 (1H, m), 3.67 (3H, s), 5.40 (1H, m), 5.57 (1H, m); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 14.0, 22.5, 24.9, 25.7, 27.4, 29.1, 29.2, 29.3, 29.4, 29.6, 31.5, 34.1, 35.4, 36.8, 51.4, 71.5, 125.1, 133.6, 174.3; HRMS (ESI^+) *m/z* 335.2545 $[M+Na]^+$ (calcd for $C_{19}H_{36}NaO_3$, 335.2562).

(*R*)-**10**-(*R*)-MTPA ester

^{1}H-NMR (500 MHz, $CDCl_3$) δ0.88 (3H, t, *J*=7.0 Hz, 18-H), 1.13-1.37 (10H, m, 4-7, 15-H), 1.17 (2H, m, 8-H), 1.25 (2H, m, 16-H), 1.28 (2H, m, 17-H), 1.57 (2H, m, 9-H), 1.59 (2H, m, 3-H), 2.01 (2H, m,14-H), 2.29 (2H, t, *J*=7.3 Hz, 2-H), 2.36 (1H, m, 11a-H), 2.44 (1H, m, 11b-H), 3.54 (3H, s, MTPA-OMe), 3.67 (3H, s, -OMe), 5.11 (1H, m, 10-H), 5.33 (1H, m, 12-H), 5.52 (1H, m, 13-H), 7.40 (3H, m, bz), 7.54 (2H, m, bz); ^{13}C-NMR (125 MHz, $CDCl_3$) δ14.0, 22.5, 24.9, 24.9, 29.1-29.2, 31.5, 31.7, 33.3, 34.1, 51.5, 55.7, 77.2, 123.3, 133.3, 174.3; HRMS (ESI^+) *m/z* 551.2964 $[M+Na]^+$ (calcd for $C_{29}H_{43}F_3NaO_5$, 551.2960).

(*R*)-**10**-(*S*)-MTPA ester

^{1}H-NMR (500 MHz, $CDCl_3$) δ0.88 (3H, t, *J*=7.0 Hz, 18-H), 1.13-1.32 (10H, m, 4-7, 15-H), 1.24 (2H, m, 16-H), 1.28 (2H, m, 17-H), 1.30 (2H, m, 8-H), 1.61 (2H, m, 3-H), 1.63 (2H, m, 9-H), 1.95 (2H, m,14-H), 2.30 (2H, t, *J*=7.3 Hz, 2-H), 2.31 (1H, m, 11a-H), 2.35 (1H, m, 11b-H), 3.54 (3H, s, MTPA-OMe), 3.67 (3H, s, -OMe), 5.09 (1H, m, 10-H), 5.22 (1H, m, 12-H), 5.43 (1H, m, 13-H), 7.40 (3H, m, bz), 7.54 (2H, m, bz); ^{13}C-NMR (125 MHz, $CDCl_3$) δ14.0, 22.5, 24.9, 25.3, 29.1-29.3×4, 31.5×2, 33.3, 34.1, 51.4, 55.7, 77.2, 123.3, 133.3, 174.3; HRMS (ESI^+) *m/z* 551.2963 $[M+Na]^+$ (calcd for $C_{29}H_{43}F_3NaO_5$, 551.2960).

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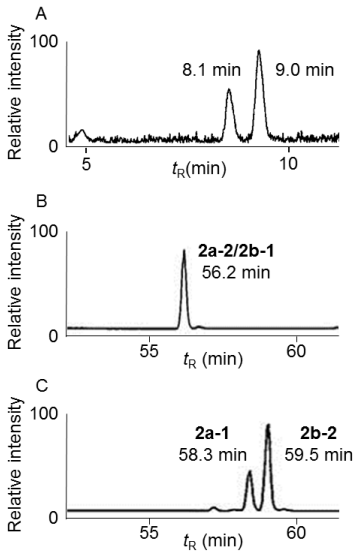
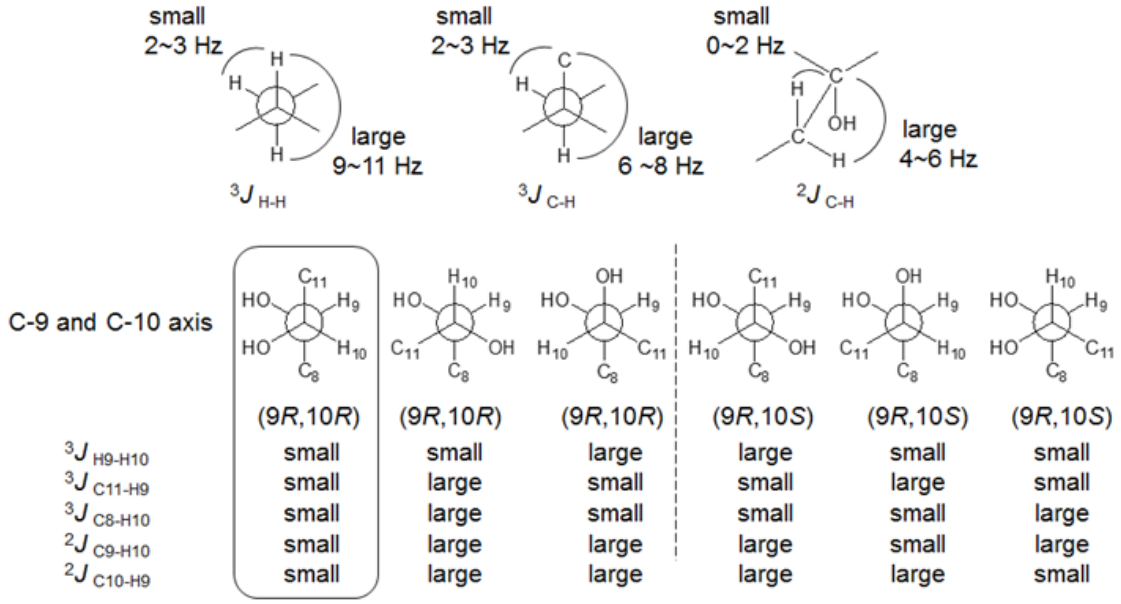


Supplementary Fig. 1. LC-MS analysis of the four diastereomeric isomers of **2** after $NaBH_4$-reduction of **1** (*R*/*S*=3/1). The products were traced at *m/z* 311 $[M-H]^-$. The mixtures of **2a-1**, **2a-2** and **2b-1**, **2b-2** were separated into their respective enantiomers on the ODS column. **2a-2** and **2b-1** showed identical retention times on the ODS column and the chiral column, indicating that these isomers are enantiomeric. **2a-1** and **2b-2** could not be distinguished by comparing chromatographic characteristic on ODS columns, on the other hand, separated on the chiral column.

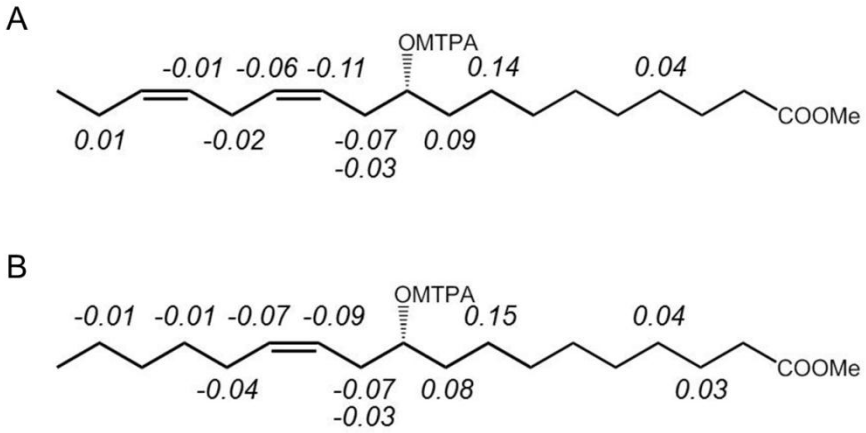
(**A**) Separation of the four diastereomers of **2** on the ODS column. Each of enantiomeric mixture was eluted at t_R=8.1 min and t_R=9.0 min.

(**B**) Analysis of peak with t_R=8.1 min on the chiral column. The two enantiomers 2a-2 and 2b-1 were co-eluted at t_R=56.2 min.

(**C**) Analysis of compounnds detected at t_R 9.0 on the chiral column traced at *m/z* 311 $[M-H]^-$.

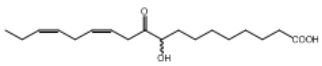
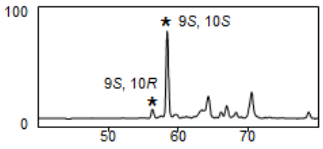
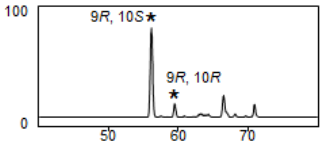
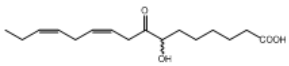
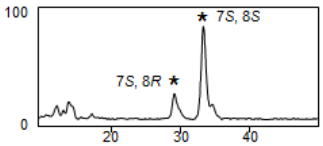
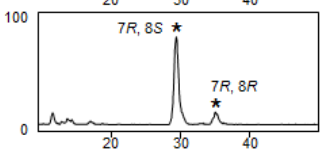
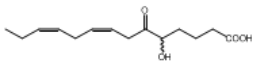
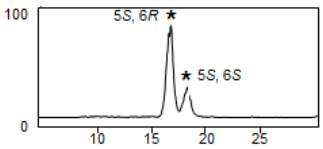
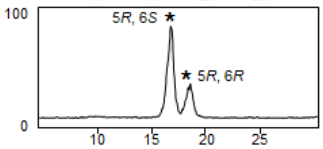
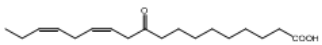
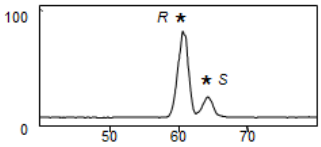
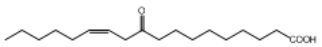
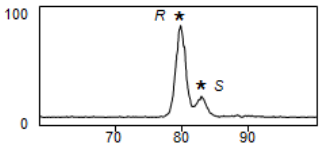


Supplementary Fig. 2. Coupling constants in the staggered system and six possible rotamers around C-9 and C-10 of **2b-2**.



Supplementary Fig. 3. Chemical shift differences Δδ (*S*−*R*) of MTPA esters derived from (*R*)-**8** (A) and (*R*)-**10** (B)

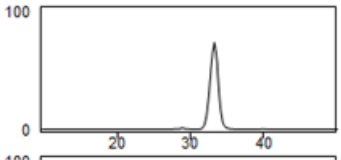
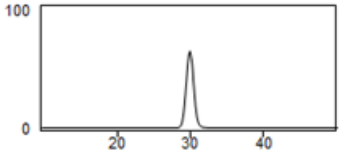
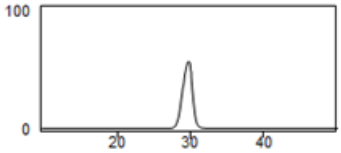
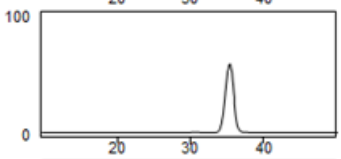
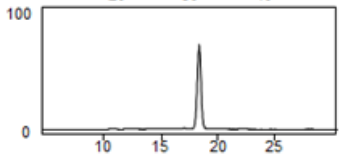
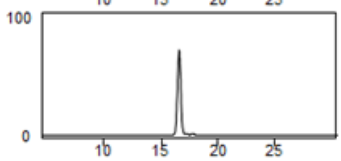
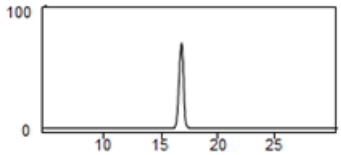
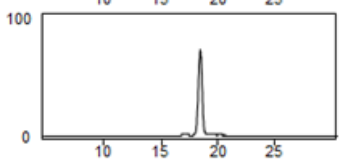
Supplementary Table 1. The stereoselectivity ratio of **1**, **3**, **5**, **7**, and **9**.

Fed compound		Reduced product		Selectivity (%)	
Substrate	Name	Name	MS chromatgram	*S*	*R*
	(*S*)-**1**	9*S*-**2**		89	11
	(*R*)-**1**	9*R*-**2**		89	11
	(*S*)-**3**	7*S*-**4**		76	24
	(*R*)-**3**	7*R*-**4**		86	14
	(*S*)-**5**	5*S*-**6**		25	75
	(*R*)-**5**	5*R*-**6**		72	28
	**7**	**8**		19	81
	**9**	**10**		17	83

We prepared *R* and *S* enantiomers of **1**, **3**, and **5** and obtained corresponding diastereomeric alcohols as the authentic compouns to identify the chirality of each diol and alcohol produced by *vitro* experiments. The reaction mixtures after the enzymatic treatment were separated on the chiral HPLC column and detected by LC-MS.

The stereoselectivity of each respective keto-reduction was calculated based on the peak areas traced at *m/z* 311 $[M-H]^-$ for **2**, *m/z* 283 $[M-H]^-$ for **4**, and *m/z* 255 $[M-H]^-$ for **6**. We prepared **8** and **10** from corresponding ketones **7** and **9** and determined their absolute configurations by modified Mosher's method (Fig. S3). The stereoselectivity of each respective keto-reduction was calculated based on the peak areas traced at *m/z* 295 $[M-H]^-$ for **8** and *m/z* 297 $[M-H]^-$ for **10**.

Supplementary Table 2. The LC-MS/MS analysis of each diastereomer of **4** and **6**.

Substrate	β-oxidation products	
	Name	MS chromatgram
9*S*,10*S*-**2**	7*S*,8*S*-**4**	
9*S*,10*R*-**2**	7*S*,8*R*-**4**	
9*R*,10*S*-**2**	7*R*,8*S*-**4**	
9*R*,10*R*-**2**	7*R*,8*R*-**4**	
9*S*,10*S*-**2**	5*S*,6*S*-**6**	
9*S*,10*R*-**2**	5*S*,6*R*-**6**	
9*R*,10*S*-**2**	5*R*,6*S*-**6**	
9*R*,10*R*-**2**	5*R*,6*R*-**6**	

(7*R*,8*R*)-, (7*R*,8*S*)-, (7*S*,8*R*)-, (7*S*,8*S*)-**4** and (5*R*,6*R*)-, (5*R*,6*S*)-, (5*S*,6*R*)-, (5*S*,6*S*)-**6** were produced from the corresponding (9*R*,10*R*)-, (9*R*,10*S*)-, (9*S*,10*R*)-, (9*S*,10*S*)-**2** by the action of the *β*-oxidation enzyme. Each diaseteomer was detected by LC-MS analyses. The chromatograms are ion traces at *m/z* 283 $[M-H]^-$ for **4**, and *m/z* 255 $[M-H]^-$ for **6,** respectively. Retention times were 35.6 min, 29.8 min, 29.8 min, 33.6 min for (7*R*,8*R*)-, (7*R*,8*S*)-, (7*S*,8*R*)-, (7*S*,8*S*)-**4**, and 18.6 min, 16.9 min, 16.9 min, 18.2 min for (5*R*,6*R*)-, (5*R*,6*S*)-, (5*S*,6*R*)-, (5*S*,6*S*)-**6**.

Enantiomers (7*R*,8*R*)- ,(7*S*,8*S*)-**4** and (5*R*,6*R*)-, (5*S*,6*S*)-**6** were well separated each other, whereas the retention times of enantiomers (7*R*,8*S*)- ,(7*S*,8*R*)-**4** and (5*R*,6*S*)-, (5*S*,6*R*)-**6** were almost identical as those observed in the analyses of each diastereomer, (9*R*,10*R*)-, 9*R*,10*S*)-, (9*S*,10*R*)-, (9*S*,10*S*)-**2**. Based on these analytical data, the enantio-selective reduction of (*R*)-**3**, (*S*)-**3**, (*R*)-**5**, (*S*)-**5** were confirmed as shown in Supplementary Table 1.