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Stereoselective chemoenzymatic synthesis of sitophilate: a natural pheromone

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Abstract—The aggregation pheromone of the granary weevil *Sitophilus granarius*, (2*S*,3*R*)-1-ethylpropyl 3-hydroxy-2-methylpentanoate, has been synthesized in 63% total isolated yield and high chemical and enantiomeric purity (98% de, >99% ee) from readily available methyl 3-oxopentanoate. A stereoselective ketone reduction followed by an ester hydrolysis, were the two key steps of the synthesis and were both performed by commercially available enzymes.

1. Introduction

Sitophilus granarius (L.), the granary weevil,[1] is a major cosmopolitan pest of stored cereal grains responsible for serious economic losses annually. It is, therefore, critical to monitor and control the pest's presence in stored grain to avoid further damages. Due to the dry conditions and cool temperatures of stored grain, these insects have evolved to alter these conditions by forming aggregation spots and by producing their own suitable moisture for warmth, through their metabolic activities.[2] The aggregation spots of male and female adults are partly due to the pheromone of *S. granarius*, which is male-produced and attracts both the sexes.[1] This pheromone was isolated by Burkholder et al.[3] and was identified as 1-ethylpropyl 3-hydroxy-2-methylpentanoate, with *syn* (*erythro*) relative configuration between the hydroxy and the methyl group (Fig. 1).

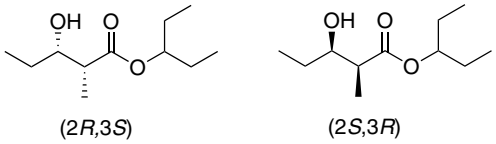

Figure 1. *syn*-1-Ethylpropyl 3-hydroxy-2-methylpentanoate.

In an effort to identify the absolute configuration of the active form of the pheromone, both *syn*-enantiomers (Fig. 1) were synthesized.[4] A comparison of spectral, chromatographic, and biological properties between the natural product and both synthesized enantiomers, showed that the active form of this compound is the (2*S*,3*R*)-configuration (Fig. 1), which was named sitophilate. Numerous approaches have been published for the synthesis of racemic or enantiomerically pure sitophilate, however, either low enantiomeric purities were reported or multi step reaction schemes were required to obtain enantiomerically pure sitophilate.[3,5–8] Clearly, a simple, scalable, and economic method for the synthesis of this weevil attractant pheromone is still lacking.

Since sitophilate is an optically active α-methyl-β-hydroxy ester, it could be easily produced by the stereoselective reduction of an α-methyl-β-keto ester by asymmetric reduction via dynamic kinetic resolution (DKR)[9] (Fig. 2). Ketoreductases are very powerful tools for stereoselective reduction via DKR. We have recently shown that a number of α-alkyl-1,3-diketones[10] and α-alkyl-β-keto esters[11] can be reduced to enantiomerically pure keto alcohols

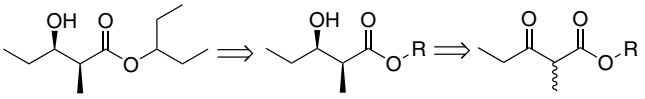

Figure 2. Retrosynthesis of sitophilate.

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and keto esters, respectively, using commercially available ketoreductases. Enzymatic reductions provide a simple, highly stereoselective, and quantitative method for the synthesis of different diastereomers of these valuable chiral synthons.

Using a similar enzymatic reduction, we recently reported the synthesis of another pheromone, (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone (sitophilure), which is produced from the rice weevil (*Sitophilus oryzae* L.) and maize weevil (*Sitophilus zeamais* M.).[12]

Herein, we report a facile chemoenzymatic synthesis of sitophilate using two sequential enzymatic steps, a reduction followed by hydrolysis, as the key reactions.

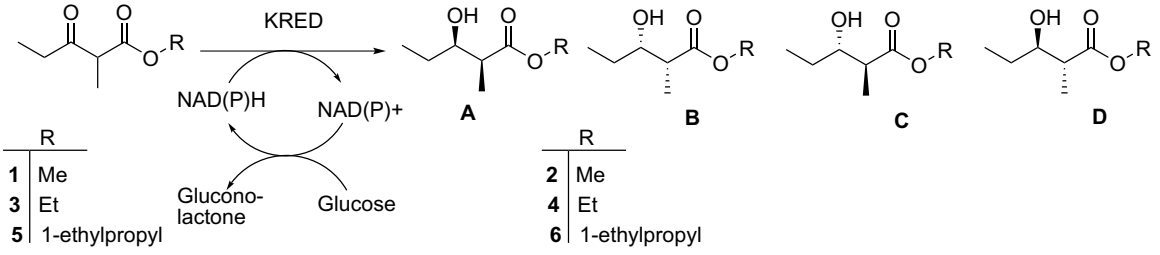
2. Results and discussion

Three different α-methyl-β-keto esters, **1**, **3**, and **5**, were screened for reduction using over 100 NADPH and NADH-dependent commercially available ketoreductases (KREDs). All reactions were performed in the presence of a glucose/glucose dehydrogenase system[13] for in situ recycling of the nicotinamide cofactors (Table 1).

Since the reduction of 1-ethylpropyl 2-methyl-3-oxopentanoate **5** will give sitophilate in a single step, this substrate was first screened for reduction using commercially available ketoreductases. Methyl 2-methyl-3-oxopentanoate **1** and ethyl 2-methyl-3-oxopentanoate **3** were also screened because of their structural similarity to 4-methyl-3,5-heptanedione, the substrate that was successfully reduced to (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone by KRED-A1B, in our previous work.[12] Both keto esters **3** and **5** have been previously used in the synthesis of sitophilate by the microbial reduction with whole cells of *Geotrichum candidum*[6] and *Pichia farinosa* IAM 4682,[7] respectively; however, the wrong (2*S*,3*S*)-stereoisomer was the major product in both cases.

Many enzymes showed activity toward the reduction of keto esters **1**, **3**, and **5**, and the results in Table 1 only show the best and most representative of these reactions. Irrespective of the ester group, two out of the four stereoisomers of each hydroxy ester were produced in high yield by a number of enzymes, with one of them having the same

Table 1. Enzymatic reduction of α-methyl-β-keto esters **1**, **3**, and **5** by isolated ketoreductases



KRED	Substrate	Diastereomeric ratio[a] (%)				Conversion (%)	Time (h)
		A (2*S*,3*R*)	B (2*R*,3*S*)	C (2*S*,3*S*)	D (2*R*,3*R*)		
107	**1**	4	—	93	3	>99	2
	3	—	—	93	7	>99	12
	5	—	—	60	40	32	24
118	**1**	3	3	94	—	>99	24
	3	8	—	92	—	>99	12
	5	—	29	71	—	>99	24
A1B[b]	**1**	91	—	—	9	>99	2
	3	78	—	9	13	>99	2
	5	16	1	22	61	>99	24
A1F	**1**	—	—	98	2	>99	24
	3	—	—	98	2	>99	24
	5	—	1	65	34	82	24
B1B	**1**	—	—	>98	—	>99	24
	3	—	—	>99	—	>99	24
	5	—	—	>99	—	>99	24
B1C	**1**	—	—	>98	—	90	24
	3	—	1	99	—	90	24
	5	—	1	99	—	90	24
B1E	**1**	—	—	98	2	>99	24
	3	—	—	>99	—	>99	24
	5	—	—	>99	—	22	2

[a] Diastereomeric ratio A/B/C/D is categorized in every reaction according to their increasing retention time on chiral GC, with isomer A eluting first. All the enzymatic reactions were performed at pH 6.9, 37 °C.

[b] KRED-A1C and KRED-A1D gave very similar yields and product ratios as KRED-A1B.

absolute configuration as sitophilate [2*S*,3*R*, **A**, Table 1]. Interestingly, this stereoisomer is the result of an enzymatic reduction with *anti*-Prelog selectivity. These results suggest that an enzymatic reduction might be a viable method for producing sitophilate, however, medium to low enantiomeric excesses of the desired (2*S*,3*R*)-hydroxy ester were obtained in all enzymatic reductions that gave this isomer as the major product (KRED-A1B, A1C, and A1D, Table 1). An inverse correlation of the stereoselectivity of the enzymatic reduction with KRED-A1B (as well as KRED-A1C and A1D) and the size of the ester group was observed. Decreasing the size of the ester group from 1-ethylpropyl to ethyl to methyl gave yields of 16%, 78%, and 91%, respectively, for the desired (2*S*,3*R*)-stereoisomer (KRED-A1B, Table 1). In contrast, the yield and enantiomeric purity of stereoisomer C (2*S*,3*S*) was not affected by the size of the ester group in all enzymes that were selective in its synthesis. It should be noted that the absolute configuration of every diastereomer was assigned by comparing their ^{1}H NMR spectrum with the literature data[4,6,8,14] and by transforming it into its corresponding MPA [(*R*)- or (*S*)-methoxy-phenylacetic acid] esters.[15]

The results so far indicate that the synthesis of sitophilate using an enzymatic reduction is possible. Enzymes that give the desired (2*S*,3*R*)-stereoisomer were identified, and the methyl ester derivative gave high, though not excellent, diastereomeric purity of the product. To complete the synthesis of sitophilate using methyl 2-methyl-3-oxopentanoate **1** (Table 1), a transesterification step will be required, as well as an increase in the diastereomeric purity from the 82% de that is currently obtained (Table 1, KRED-A1B, substrate **1**). For this reason, two different approaches were investigated to complete the synthesis. In the first approach, we examined the effect of temperature and pH in the reduction of **1** with KRED-A1B, A1C, and A1D, hoping that a change in the conditions will increase the diastereoselectivity of these transformations (Table 2, Fig. 3, pathway 1). In the second approach, an enzymatic hydrolysis of the hydroxy ester **2** was investigated (Fig. 3, pathway 2).

Reaction temperature and pH changes affected both the selectivity and the activity of KRED-A1B, A1C, and A1D for the reduction of methyl 2-methyl-3-oxopentanoate **1**. Representative results for the reductions with KRED-A1B are shown in Table 2. The most dramatic effect was observed when the pH was increased from 6.0 to 6.9 resulting in an increase of the diastereoselectivity from 48% de to 82% de, respectively. A further increase of the pH to 8.0 considerably reduced the activity of the enzyme without a significant improvement in its stereoselectivity (Table 2). The enantiomeric purity was increased even more to 92% de when the reaction temperature dropped to 0 °C (Table 2). At this temperature and pH, however, the reaction was not completed even after 24 h of incubation.

To this end, we decided that 92% de was the best enantiomeric purity we can achieve with the existing enzymes, so

Table 2. Reaction optimization of KRED-A1B, A1C, and A1D with methyl 2-methyl-3-oxopentanoate **1**

KRED	Reaction conditions	Diastereomeric ratio (%)				Yield (%)	Time (h)
		A (2*S*,3*R*)	B (2*R*,3*S*)	C (2*S*,3*S*)	D (2*R*,3*R*)		
A1B[a]	pH 6.0, 37 °C	74	—	1	25	>99	2
	pH 6.9, 25 or 37 °C	91	—	—	9	>99	2
	pH 8.0, 37 °C	92	—	1	7	>99	20
	pH 8.0, 25 °C	93	—	1	6	>99	24
	pH 8.0, 0 °C	96	—	—	4	95	24

[a] Enzymes KRED-A1C and A1D showed very similar behavior.

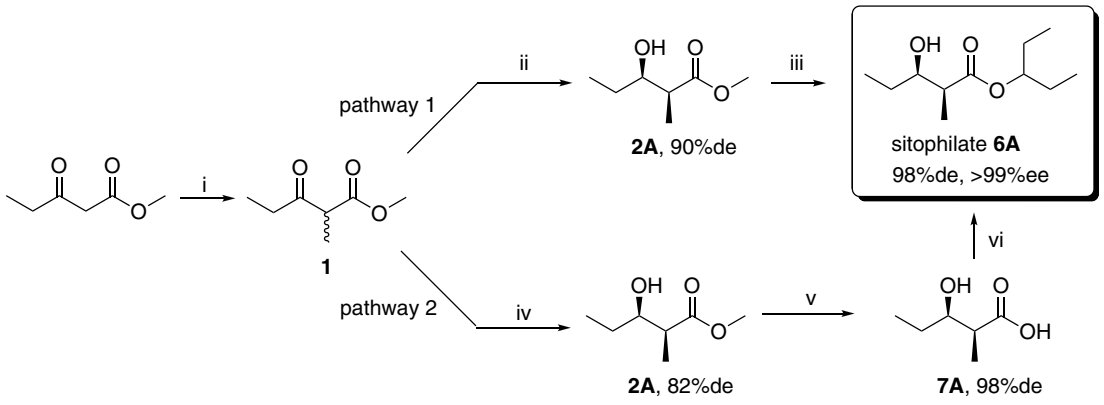


Figure 3. Chemoenzymatic total synthesis of sitophilate. Reagents and conditions: (i) K_2CO_3, MeI, dry acetone, reflux, >99% yield; (ii) KRED-EXP-A1B, NADPH, pH 8.0, 0 °C (12 h), 25 °C (12 h), 90% yield, 90% de, >99% ee; (iii) (a) NaOH, MeOH/H_2O, 1:1 v/v; (b) 3-bromopentane, dry DMF, 50 °C, 65% yield, 98% de, >99% ee; (iv) KRED-EXP-A1B, NADPH, pH 6.9, 25 °C, 95% yield, 82% de, >99% ee; (v) ICR-112, 25 °C, 83% yield, 98% de, >99% ee; (vi) KOH, 3-bromopentane, dry DMF, 50 °C, 80% yield, 99% de, >99% ee.

an overall synthesis based on this enzymatic step was worked out (Fig. 3, pathway 1). Commercially available methyl 3-oxopentanoate was transformed quantitatively to methyl 2-methyl-3-oxopentanoate, **1**, by treatment with methyl iodide in the presence of potassium carbonate (Fig. 3, step i). An enzymatic reduction using the conditions that give the highest enantiomeric excess of the product was then performed in larger scale to produce hydroxy ester **2A**. Under these conditions, the enzymatic reaction was kept at pH 8.0, and 0 °C for the first 12 h, before additional quantities of enzyme and coenzyme were added and the temperature was raised to 25 °C. After another 12 h of incubation, the product was isolated in 90% yield, 90% de, and >99% ee (Fig. 3, step ii). Transesterification of hydroxy ester **2A** by nucleophilic substitution of its sodium salt with 3-bromopentane followed by silica gel chromatographic separation of the diastereomeric mixture gave sitophilate **6A** in 65% isolated yield, 98% de, >99% ee (Fig. 3, step iii). The overall chemical yield calculated from methyl 3-oxopentanoate was 58.0% (Fig. 3, pathway 1).

Even though the previous synthesis (Fig. 3, pathway 1) gave the target molecule in high purity, a silica gel chromatographic separation was required in addition to performing the enzymatic reduction in sub-optimal (in terms of enzyme activity) conditions. Such a process requires longer reduction times and higher enzyme loadings to maximize the diastereomeric purity and make the final chromatographic separation possible. In a second approach, we investigated the possibility of increasing the enantiomeric purity by hydrolyzing hydroxy ester **2**, with an enzyme. For this purpose, 15 commercially available hydrolases (ICR)[13] were screened. Hydroxy ester **2** at 82% de was synthesized on a larger scale by KRED-EXP-A1B under its optimal conditions for activity (pH 6.9, 25 °C, Table 2).

Three enzymes (ICR-110: Lipase B from *Candida antarctica*: CALB, ICR-112: Lipase A from *C. antarctica*: CALA and ICR-123: pig liver esterase PLE) that were able to selectively hydrolyze (2*S*,3*R*)-methyl 3-hydroxy-2-methyl pentanoate **2** were identified (Table 3). In particular, ICR-112 gave a high yield (83% of the maximum 91%) and enantiomeric purity for the hydrolysis of the sitophilate precursor **7A** (Table 3). With this enzyme in hand, an improved synthesis of the natural product was worked out (Fig. 3, pathway 2). The overall yield calculated from methyl 3-oxopentanoate was 63%. Introducing two consecutive enzymatic steps proved to be a very successful strategy in improving the enantiomeric and diastereomeric purity of the product and eliminating the chromatographic separations of diastereomers.

3. Conclusion

An isolated NADPH-dependent ketoreductase (KRED-EXP-A1B) and an isolated hydrolase (ICR-112) were used for the synthesis of sitophilate, the aggregation pheromone of the granary weevil *S. granarius*, in high enantiomeric (98% de, >99% ee), and chemical purity (>99%), with an overall yield of 63%. The starting materials for this synthesis involved achiral and readily commercially available chemicals, such as methyl 3-oxopentanoate. Every reaction in this synthesis can be easily scaled to larger amounts, thus an efficient method for the synthesis of this natural pheromone is provided.

4. Experimental

4.1. Materials and general methods

Methyl 2-methyl-3-oxopentanoate, **1**, and ethyl 2-methyl-3-oxopentanoate, **3**, were prepared from commercially available methyl 3-oxopentanoate and ethyl 3-oxopentanoate, respectively, by alkylation with methyl iodide. 1-Ethylpropyl 2-methyl-3-oxopentanoate, **5**, was prepared from methyl 2-methyl-3-oxopentanoate with transesterification reaction with 3-pentanol.

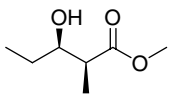
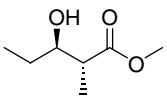
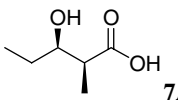
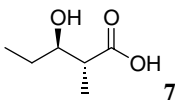
All enzymes utilized in this report are commercially available from Codexis Inc.

Racemic α-methyl-β-hydroxy esters were prepared as standards by the reduction of their corresponding α-methyl-β-keto esters with sodium borohydride.

The absolute configuration of the hydroxy esters was assigned by comparing the ^{1}H NMR spectra with the literature data and by transforming them into their corresponding MPA [(*R*) or (*S*)-methoxy-phenylacetic acid] esters.[15]

The progress of the enzymatic reactions and the selectivities were determined by gas chromatography (HP5890II gas chromatograph equipped with an FID detector; column: 30 m × 0.25 mm × 0.25μm chiral capillary column,

Table 3. Enzyme-catalyzed stereoselective hydrolysis of methyl 3-hydroxy-2-methyl entanoate

Diastereomeric ratio % of hydroxy ester **2**		ICR	Reaction time (yield)	Product **7**	
 **2A**	 **2C**			 **7A**	 **7B**
91	9	110	24 h (nd)	92	8
		112	24 h (83%)	99	1
		123	1 h (nd)	95	5

20% permethylated β-cyclodextrin, HP Part No 19091G-8233). ^{1}H NMR spectra were recorded on 500 MHz Bruker spectrometer in $CDCl_3$ solutions, by using Me_4Si as the internal standard. Chemical shifts are reported in ppm downfield from Me_4Si.

Optical rotation values were measured on a DIP 360 Jasco polarimeter at 360 mmHg.

MS were taken on a GC–MS (Shimadzu GCMS-QP5050 equipped with a SPB-5 column and CI mass detector).

4.2. Methyl 2-methyl-3-oxopentanoate 1

Keto ester **1** was prepared from commercially available methyl 3-oxopentanoate according to the following procedure: under a nitrogen atmosphere, methyl 3-oxopentanoate (1.88 cm^3, 15 mmol) was dissolved in anhydrous acetone (20 cm^3) and pre-dried potassium carbonate (1.932 g, 14 mmol) was added. After stirring the solution at room temperature for 5 min, methyl iodide (1.146 cm^3, 18.4 mmol) was added with a syringe and the reaction mixture was refluxed for 6 h. After completion of the reaction, diethyl ether was added (30 cm^3), the mixture was filtered and the solvent was evaporated under a reduced pressure to afford **1** (2.16 g, >99%). Without any further purification, keto ester **1** was subjected to enzymatic reduction. δ_H (500 MHz; $CDCl_3$; Me_4Si) 3.72 (3H, s, CO_2CH_3), 3.53 (1H, q, $J = 7.5$, $CHCH_3$), 2.46–2.64 (2H, m, $COCH_2CH_3$), 1.34 (3H, d, $J = 7.0$, $CHCH_3$), 1.07 (3H, t, $J = 7.5$, CH_2CH_3).

4.3. Ethyl 2-methyl-3-oxopentanoate 3

Keto ester **3** was prepared by following the same procedure as in the preparation of keto ester **1**, by alkylation of ethyl 3-oxopentanoate (2.16 g, 15 mmol) with methyl iodide. Isolated yield 2.37 g, >99%. δ_H (500 MHz; $CDCl_3$; Me_4Si) 4.18 (2H, q, $J = 7.0$, CO_2CH_2), 3.51 (1H, q, $J = 7.0$, $CHCH_3$), 2.46–2.65 (2H, m, $COCH_2CH_3$), 1.34 (3H, d, $J = 7.0$, $CHCH_3$), 1.27 (3H, t, $J = 7.0$, $CO_2CH_2CH_3$), 1.08 (3H, t, $J = 7.5$, CH_2CH_3).

4.4. 1-Ethylpropyl 2-methyl-3-oxopentanoate 5

In a solution of 3-pentanol (184 mg, 225 μL, 2.084 mmol) and DMAP (13 mg, 0.1 mmol) in dry toluene (8 cm^3), methyl 2-methyl-3-oxopentanoate, **1**, was added (150 mg, 1.042 mmol) and the mixture was refluxed for four days. Saturated NH_4Cl (15 cm^3) was then added and the solution was extracted with EtOAc. The organic layer was washed with 1 M HCl, dried over $MgSO_4$, and concentrated in vacuo to give substrate **5** (176 mg, 85%). Without any further purification the product was subjected to enzymatic reduction. δ_H (500 MHz; $CDCl_3$; Me_4Si) 4.76–4.82 (1H, m, CO_2CH), 3.53 (1H, q, $J = 7.5$, $CHCH_3$), 2.47–2.67 (2H, m, $COCH_2CH_3$), 1.53–1.62 (2H, m, $CHCH_2CH_3$), 1.34 (3H, d, $J = 7.0$, $CHCH_3$), 1.08 (3H, t, $J = 7.5$, CH_2CH_3), 0.88 (3H, t, $J = 7.5$, $CO_2CHCH_2CH_3$), 0.87 (3H, t, $J = 7.5$, $CO_2CHCH_2CH_3$).

4.5. Racemic methyl 3-hydroxy-2-methylpentanoate 2

Under a nitrogen atmosphere, sodium borohydride (24.4 mg, 0.646 mmol) was added in dry ethanol (10 cm^3) and the mixture was cooled to 0 °C. After stirring for 5 min, a solution of dry ethanol (5 cm^3) containing methyl 2-methyl-3-oxopentanoate **1** (303 mg, 2.1 mmol) was added dropwise. After stirring for 3 h at 0 °C, the reaction was quenched with saturated ammonium chloride and the ethanol was evaporated under reduced pressure. Distilled water (15 cm^3) was then added and extracted twice with ethyl acetate (2×15 cm^3). The organic layer was dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by flash column chromatography (silica gel, hexane/EtOAc, v/v, 6:1), to afford **2** (246 mg, 80%). δ_H (500 MHz; $CDCl_3$; Me_4Si) 3.79–3.83 (1H, m, $CHOH$), 3.71 (6H, s, $2 \times CO_2CH_3$), 3.56–3.61 (1H, m, $CHOH$), 2.51–2.58 (2H, m, $2 \times CHCO_2$), 1.39–1.61 (4H, m, $2 \times CH_2CH_3$), 1.20 (3H, d, $J = 7.5$, $CHCH_3$), 1.17 (3H, d, $J = 7.5$, $CHCH_3$), 0.98 (3H, t, J 7.0, CH_2CH_3), 0.96 (3H, t, $J = 7.5$, CH_2CH_3).

4.6. Racemic ethyl 3-hydroxy-2-methylpentanoate 4

The preparation of racemic ethyl 3-hydroxy-2-methylpentanoate **4** was accomplished by following the same procedure as in the preparation of hydroxy ester **2**, using ethyl 2-methyl-3-oxopentanoate **3** (316 mg, 2 mmol). Pure product **4** was obtained after flash column chromatography (silica gel, hexane/EtOAc, v/v, 6:1) (262 mg, 82%). δ_H (500 MHz; $CDCl_3$; Me_4Si) 4.16 (4H, q, $J = 7.0$, $2 \times CO_2CH_2CH_3$), 3.78–3.83 (1H, m, $CHOH$), 3.56–3.60 (1H, m, $CHOH$), 2.49–2.56 (2H, m, $2 \times CHCO_2$), 1.40–1.61 (4H, m, $2 \times CH_2CH_3$), 1.27 (6H, t, $J = 7.0$, $2 \times CO_2CH_2CH_3$), 1.20 (3H, d, $J = 7.5$, $CHCH_3$), 1.17 (3H, d, $J = 7.0$, $CHCH_3$), 0.98 (3H, t, $J = 7.5$, CH_2CH_3), 0.96 (3H, t, $J = 7.0$, CH_2CH_3).

4.7. Racemic 1-ethylpropyl 3-hydroxy-2-methylpentanoate 6

The preparation of racemic 1-ethylpropyl 3-hydroxy-2-methylpentanoate **6** was accomplished by following the same procedure as in the preparation of hydroxy ester **2**, using 1-ethylpropyl 2-methyl-3-oxopentanoate **5** (400 mg, 2 mmol). Pure product was obtained after flash column chromatography (silica gel, hexane/EtOAc, v/v, 6:1) (343 mg, 85%). δ_H (500 MHz; $CDCl_3$; Me_4Si) 4.76–4.83 (2H, m, $2 \times CO_2CH$), 3.78–3.83 (1H, m, $CHOH$), 3.54–3.60 (1H, m, $CHOH$), 2.71 (1H, d, $J = 7.0$, OH), 2.61 (1H, d, $J = 4.0$, OH), 2.49–2.56 (2H, m, $2 \times CHCO_2$), 1.41–1.63 (12H, m, $6 \times CH_2CH_3$), 1.22 (3H, d, $J = 7.5$, $CHCH_3$), 1.18 (3H, d, $J = 7.0$, $CHCH_3$), 0.98 (3H, t, $J = 7.5$, CH_2CH_3), 0.97 (3H, t, $J = 7.5$, CH_2CH_3), 0.89 (12H, t, $J = 7.5$, $4 \times CO_2CHCH_2CH_3$).

4.8. Enzymatic reductions

76 NADPH-dependent ketoreductases from KRED-7600 kit and 29 NADH-dependent ketoreductases from KRED-NADH-2900 kit were screened for the selective reduction of keto esters **1**, **3**, and **5**. In addition to the ketoreductases, both NAD(P)H and glucose dehydrogenase

(GDH) that were used for cofactor recycling are products available from Codexis.

4.9. Small-scale screening of enzymatic reductions

The screening was performed on small-scale reactions where each substrate (keto esters **1**, **3**, **5**) (25 mM) was mixed with NAD(P)H (2 mg, 2.5 mM), each ketoreductase (2–3 mg/cm^3), glucose (18 mg, 100 mM), glucose dehydrogenase (GDH, 2 mg/cm^3) for cofactor recycling and sodium phosphate buffer (1 cm^3, 200 mM, pH 6.9). The reactions were incubated at 37 °C and reaction aliquots taken every hour. After extraction with ethyl acetate the crude extracts were analyzed by chiral GC chromatography. In the case of anti-Prelog enzymes (KRED-EXP-A1B, A1C, A1D) the small-scale enzymatic reductions of keto ester **1** were repeated in two different pH values (pH 6.0 or pH 8.0) and at two different temperatures (0 °C and 25 °C) with the same quantities.

4.10. Hydroxy ester 2C

δ_H (500 MHz; $CDCl_3$; Me_4Si) 3.71 (3H, s, CO_2CH_3), 3.57–3.61 (1H, m, C*H*OH), 2.51–2.58 (1H, m, C*H*CO_2), 1.53–1.62 (1H, m, CH_2CH_3), 1.39–1.48 (1H, m, CH_2CH_3), 1.20 (3H, d, $J = 7.0$, $CHCH_3$), 0.98 (3H, t, $J = 7.5$, CH_2CH_3) [lit. data in Ref. 5g].

4.11. Hydroxy ester 4A

δ_H (500 MHz; $CDCl_3$; Me_4Si) 4.16 (2H, q, $J = 7.0$, $CO_2CH_2CH_3$), 3.78–3.83 (1H, m, C*H*OH), 2.50–2.56 (1H, m, C*H*CO_2), 1.40–1.53 (2H, m, CH_2CH_3), 1.27 (3H, t, $J = 7.0$, $CO_2CH_2CH_3$), 1.17 (3H, d, $J = 7.0$, $CHCH_3$), 0.96 (3H, t, $J = 7.5$, CH_2CH_3) [lit. data in Ref. 6].

4.12. Hydroxy ester 4C

δ_H (500 MHz; $CDCl_3$; Me_4Si) 4.17 (2H, q, $J = 7.5$, $CO_2CH_2CH_3$), 3.55–3.61 (1H, m, C*H*OH), 2.59 (1H, d, $J = 7.0$, O*H*), 2.49–2.55 (1H, m, C*H*CO_2), 1.53–1.61 (1H, m, CH_2CH_3), 1.40–1.49 (1H, m, CH_2CH_3), 1.27 (3H, t, $J = 7.0$, $CO_2CH_2CH_3$), 1.20 (3H, d, $J = 7.0$, $CHCH_3$), 0.98 (3H, t, $J = 7.5$, CH_2CH_3) [lit. data in Ref. 6].

4.13. Hydroxy ester 6C

δ_H (500 MHz; $CDCl_3$; Me_4Si) 4.78–4.83 (1H, m, CO_2CH), 3.54–3.60 (1H, m, C*H*OH), 2.69 (1H, d, $J = 7.0$, O*H*), 2.50–2.56 (1H, m, C*H*CO_2), 1.42–1.64 (6H, m, $3 \times CH_2CH_3$), 1.23 (3H, d, $J = 7.0$, $CHCH_3$), 0.99 (3H, t, $J = 7.5$, CH_2CH_3), 0.89 (6H, t, $J = 7.5$, $2 \times CO_2CHCH_2CH_3$) [lit. data in Ref. 5g].

4.14. Larger-scale enzymatic reductions

4.14.1. (2*S*,3*R*)-Methyl 3-hydroxy-2-methylpentanoate 2A (pH 8.0, 0–25 °C). A phosphate-buffered solution (20 cm^3, pH 8.0, 200 mM) containing 69.5 mM (200 mg, 1.39 mmol) of methyl 2-methyl-3-oxopentanoate, **1**, glucose (432 mg, 120 mM), NADPH (12.3 mg, 0.7 mM, 0.014 mmol,), glucose dehydrogenase (10 mg) and KRED-EXP-A1B (50 mg) was stirred at 0 °C. After 12 h were added KRED-EXP-A1B (20 mg), glucose dehydrogenase (5 mg), and NADPH (8 mg) and the mixture was stirred at 25 °C for 12 h until GC analysis of the extracts showed complete reaction. Periodically the pH was readjusted to 7.7–8.0 with NaOH (2 M). The product was isolated by extracting the crude reaction mixture with EtOAc (2×20 cm^3). The combined organic layers were washed with saturated NaCl solution (15 cm^3), dried over $MgSO_4$, and evaporated to dryness to provide pure (2*S*,3*R*) methyl 3-hydroxy-2-methylpentanoate **2A** (183 mg, 90% yield, 90% de, >99% ee). δ_H (500 MHz; $CDCl_3$; Me_4Si) 3.79–3.84 (1H, m, C*H*OH), 3.71 (3H, s, CO_2CH_3), 2.54–2.59 (1H, m, C*H*CO_2), 1.40–1.53 (2H, m, CH_2CH_3), 1.18 (3H, d, $J = 7.0$, $CHCH_3$), 0.97 (3H, t, $J = 7.5$, CH_2CH_3) [lit. data in Ref. 5g].

4.14.2. (2*S*,3*R*)-Methyl 3-hydroxy-2-methylpentanoate 2A (pH 6.9, 25 °C). A phosphate-buffered solution (100 cm^3, pH 6.9, 200 mM) containing methyl 2-methyl-3-oxopentanoate **1** (62.5 mM, 900 mg, 6.25 mmol), glucose (2.16 g, 120 mM), NADPH (0.5 mM, 44 mg, 0.05 mmol), glucose dehydrogenase (15 mg), and KRED-EXP-A1B (50 mg) was stirred at 25 °C until GC analysis of the extracts showed complete reaction. Periodically the pH was readjusted to 6.9 with NaOH (2 M). The product was isolated by extracting the crude reaction mixture with EtOAc (2×100 cm^3). The combined organic layers were washed with saturated NaCl solution (50 cm^3), dried over $MgSO_4$, and evaporated to dryness to provide pure (2*S*,3*R*)-methyl 3-hydroxy-2-methylpentanoate (866 mg, 95% yield, 82% de, >99% ee). δ_H (500 MHz; $CDCl_3$; Me_4Si) 3.79–3.84 (1H, m, C*H*OH), 3.71 (3H, s, CO_2CH_3), 2.54–2.59 (1H, m, C*H*CO_2), 1.40–1.53 (2H, m, CH_2CH_3), 1.18 (3H, d, $J = 7.0$, $CHCH_3$), 0.97 (3H, t, $J = 7.5$, CH_2CH_3) [lit. data in Ref. 5g].

4.14.3. (2*S*,3*R*)-1-Ethylpropyl 3-hydroxy-2-methylpentanoate 6A, sitophilate. To a solution of (2*S*,3*R*)-methyl 3-hydroxy-2-methylpentanoate **2A** (90% de, >99% ee), derived from enzymatic reduction with KRED-EXP-A1B at pH 8.0 and 0 °C–rt, (183 mg, 1.26 mmol,) in MeOH–H_2O (1:1 v/v, 15 cm^3) was added sodium hydroxide (50 mg, 1.25 mmol) and the mixture was stirred overnight at room temperature. After completion of the reaction, the solution was concentrated under vacuum to give a white solid. After desiccation to dryness, in the same flask were added 10 cm^3 of dry DMF. At room temperature, the addition of 3-bromopentane (172 μL, 1.39 mmol, in 5 cm^3 dry DMF) was followed and the solution was stirred at 50 °C for 24 h. Next 20 cm^3 of water were added to the reaction mixture and the mixture was extracted three times with $CHCl_3$ (3×20 cm^3). The combined extracts were washed with water twice (2×20 cm^3) and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography (silica gel, hexane/EtOAc, v/v, 10:1) to give pure sitophilate (156 mg, 65%, 98% de, >99% ee). $[\alpha]_D^{25} = -3.1$ (*c* 1.7 $CHCl_3$) (it compares to the literature data).[4] δ_H (500 MHz; $CDCl_3$; Me_4Si) 4.76–4.82 (1H, m, CO_2CH), 3.78–3.84 (1H, m, C*H*OH), 2.60 (1H, d, $J = 4.0$, O*H*), 2.51–2.57 (1H, m, C*H*CO_2), 1.41–1.64 (6H, m,

$3 \times CH_2CH_3$), 1.19 (3H, d, $J = 7.0$, $CHCH_3$), 0.98 (3H, t, $J = 7.5$, CH_2CH_3), 0.89 (3H, t, $J = 7.5$, $CHCH_2CH_3$), 0.88 (3H, t, $J = 7.5$, $CHCH_2CH_3$). ^{13}C NMR ($CDCl_3$ 75 MHz, δ ppm): 176.20 (CO_2CH), 76.94 (CO_2CH), 73.20 ($CHOH$), 44.08 ($CHCO_2$), 26.69 (CH_2CH_3), 26.42 (CH_2CH_3), 26.37 (CH_2CH_3), 10.75 (CH_3), 10.36 (CH_3), 9.58 (CH_3), 9.52 (CH_3). MS (m/z): 173 (2), 144 (6), 115 (40), 103 (20), 97 (10), 85 (12), 74 (100).

4.15. Enzymatic hydrolysis

Fifteen lipases and esterases from NZL and NZP kits were screened for the selective hydrolysis of hydroxy ester **2**.

4.16. Small-scale enzymatic hydrolysis

In a phosphate-buffered solution (4,5 cm^3, pH 7.0, 200 mM) were dissolved enzyme (ICR) (5 mg) and (2*S*,3*R*)-methyl 3-hydroxy-2-methylpentanoate (25 mg, 82% de, >99% ee), derived from enzymatic reduction with KRED-EXP-A1B at pH 6.9 and 25 °C. The mixture was stirred at 37 °C. At certain time points (1 h, 2 h, 4 h, and 1 day), 1 cm^3 of the reaction aliquots were treated with NaOH 2 M and after extraction with EtOAc were analyzed by GC, to determine the enantiomeric purity of the remaining ester. If the enantiomeric purity of the ester was changed, the mixture was treated with HCl 1 M, and the produced acid was isolated by the extraction of the aliquots with EtOAc (2×5 cm^3). The pure acid was esterified with diazomethane to the corresponding methyl ester, which was analyzed by GC and the diastereomeric excess was determined.

4.17. Larger-scale hydrolysis of (2*S*,3*R*)-methyl 3-hydroxy-2-methylpentanoate with ICR-112

In a phosphate-buffered solution (15 cm^3, pH 7.0, 200 mM) were dissolved ICR-112 (23 mg) and (2*S*,3*R*)-methyl 3-hydroxy-2-methylpentanoate (102 mg, 0,699 mmol, 82% de, >99% ee), derived from enzymatic reduction with KRED-EXP-A1B at pH 6.9 and 25 °C. The mixture was stirred at 25 °C for 24 h. The product was isolated by extracting the crude reaction mixture with Et_2O (15 cm^3), after treatment with NaOH 2 M until basic pH. Then at acidic pH (treatment with HCl 1 M), the aqueous phase was extracted with EtOAc (3×15 cm^3), the organic phase was dried over $MgSO_4$ and evaporated to dryness to provide pure (2*S*,3*R*) 3-hydroxy-2-methylpentanoic acid, **7A**, which is a known compound[5h] (77 mg, 83% yield, 98% de, >99% ee). The diastereomeric excess was determined by transforming a small quantity of the acid into its methyl ester using diazomethane. δ_H (500 MHz; $CDCl_3$; Me_4Si) 3.84–3.91 (1H, m, $CHOH$), 2.59–2.65 (1H, m, $CHCO_2$), 1.45–1.57 (2H, m, CH_2CH_3), 1.21 (3H, d, $J = 7.2$, $CHCH_3$), 0.99 (3H, t, $J = 7.5$, CH_2CH_3).

4.18. Esterification of (2*S*,3*R*)-3-hydroxy-2-methylpentanoic acid with 3-bromo pentane. Synthesis of sitophilate 6A

To a solution of (2*S*,3*R*)-3-hydroxy-2-methylpentanoic acid (75 mg, 0.568 mmol) in anhydrous DMF (10 cm^3) was added KOH (32 mg, 0.568 mmol) and the mixture was stirred at 80 °C for 3 h. Then 3-bromo pentane (78 mm^3, 0.625 mmol) was added and the mixture stirred overnight at 50 °C. After the completion of the reaction, water (20 cm^3) was added to the reaction mixture and the mixture was extracted three times with $CHCl_3$ (3×20 cm^3). The combined organic extracts were washed with distilled water twice (2×20 cm^3) and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography (silica gel, hexane/EtOAc, v/v, 9:1) to afford pure sitophilate **6A** (92 mg, 80%, 98% de, >99% ee).

4.19. Preparation of MPA esters

4.19.1. General method for the synthesis of MPA esters of 3-hydroxy-2-methyl pentanoate esters. To a solution of the corresponding hydroxy ester (0.1 mmol) in dry CH_2Cl_2 were added 1.1 equiv of DCC (0.11 mmol) and 1.1 equiv of the corresponding (*R*)- or (*S*)-MPA (0.11 mmol) and the reaction mixture was stirred at 0 °C for 4–6 h. After completion of the reaction, the urea produced was filtered, and the filtrate was evaporated and then chromatographed with 5:1 Hex/EtOAc. The produced corresponding MPA ester was isolated in 90% isolated yield.

4.19.2. (*R*)-MPA ester of hydroxy ester 2A. δ_H (500 MHz; $CDCl_3$; Me_4Si) 7.30–7.45 (5H, m, Ar*H*), 5.11–5.16 (1H, m, C*H*OCO), 4.74 (1H, s, Ph*H*), 3.43 (3H, s, CO_2CH_3), 3.41 (3H, s, OCH_3), 2.53–2.59 (1H, m, $CHCO_2$), 1.55–1.66 (2H, m, CH_2CH_3), 0.92 (3H, d, $J = 7.0$, $CHCH_3$), 0.86 (3H, t, $J = 7.5$, CH_2CH_3).

4.19.3. (*S*)-MPA ester of hydroxy ester 2A. δ_H (500 MHz; $CDCl_3$; Me_4Si) 7.30–7.45 (5H, m, Ar*H*), 5.11–5.16 (1H, m, C*H*OCO), 4.74 (1H, s, Ph*H*), 3.63 (3H, s, CO_2CH_3), 3.42 (3H, s, OCH_3), 2.65–2.70 (1H, m, $CHCO_2$), 1.43–1.51 (2H, m, CH_2CH_3), 1.12 (3H, d, $J = 7.0$, $CHCH_3$), 0.59 (3H, t, $J = 7.5$, CH_2CH_3).

4.19.4. (*R*)-MPA ester of hydroxy ester 2C. δ_H (500 MHz; $CDCl_3$; Me_4Si) 7.30–7.45 (5H, m, Ar*H*), 5.07–5.12 (1H, m, C*H*OCO), 4.73 (1H, s, Ph*H*), 3.61 (3H, s, CO_2CH_3), 3.42 (3H, s, OCH_3), 2.72–2.79 (1H, m, $CHCO_2$), 1.50–1.58 (1H, m, CH_2CH_3), 1.40–1.49 (1H, m, CH_2CH_3), 1.12 (3H, d, $J = 7.0$, $CHCH_3$), 0.57 (3H, t, $J = 7.5$, CH_2CH_3).

4.19.5. (*S*)-MPA ester of hydroxy ester 2C. δ_H (500 MHz; $CDCl_3$; Me_4Si) 7.31–7.45 (5H, m, Ar*H*), 5.08–5.12 (1H, m, C*H*OCO), 4.73 (1H, s, Ph*H*), 3.41 (3H, s, OCH_3), 3.36 (3H, s, CO_2CH_3), 2.62–2.68 (1H, m, $CHCO_2$), 1.64–1.71 (1H, m, CH_2CH_3), 1.54–1.61 (1H, m, CH_2CH_3), 0.95 (3H, d, $J = 7.0$, $CHCH_3$), 0.87 (3H, t, $J = 7.5$, CH_2CH_3).

4.19.6. (*R*)-MPA ester of hydroxy ester 4A. δ_H (500 MHz; $CDCl_3$; Me_4Si) 7.30–7.45 (5H, m, Ar*H*), 5.13–5.17 (1H, m, C*H*OCO), 4.74 (1H, s, Ph*H*), 3.91–3.98 (1H, m, CO_2CH_2), 3.83–3.89 (1H, m, CO_2CH_2), 3.42 (3H, s, OCH_3), 2.50–2.57 (1H, m, $CHCO_2$), 1.67–1.75 (1H, m, CH_2CH_3), 1.55–1.62 (1H, m, CH_2CH_3), 1.12 (3H, t, $J = 7.0$, $CO_2CH_2CH_3$), 0.91 (3H, d, $J = 7.0$, $CHCH_3$), 0.86 (3H, t, $J = 7.5$, CH_2CH_3).

4.19.7. (*S*)-MPA ester of hydroxy ester 4A. δ_H (500 MHz; $CDCl_3$; Me_4Si) 7.31–7.45 (5H, m, Ar*H*), 5.12–5.16 (1H, m, C*H*OCO), 4.74 (1H, s, Ph*H*), 4.09 (2H, q, $J = 7.0$, CO_2CH_2), 3.42 (3H, s, OCH_3), 2.62–2.68 (1H, m, $CHCO_2$), 1.52–1.60 (1H, m, CH_2CH_3), 1.43–1.49 (1H, m, CH_2CH_3), 1.23 (3H, t, $J = 7.0$, $CO_2CH_2CH_3$), 1.12 (3H, d, $J = 7.0$, $CHCH_3$), 0.58 (3H, t, $J = 7.5$, CH_2CH_3).

4.19.8. (*R*)-MPA ester of hydroxy ester 4C. δ_H (500 MHz; $CDCl_3$; Me_4Si) 7.31–7.45 (5H, m, Ar*H*), 5.08–5.13 (1H, m, C*H*OCO), 4.72 (1H, s, Ph*H*), 4.03–4.11 (2H, m, CO_2CH_2), 3.41 (3H, s, OCH_3), 2.70–2.76 (1H, m, $CHCO_2$), 1.52–1.59 (1H, m, CH_2CH_3), 1.40–1.48 (1H, m, CH_2CH_3), 1.22 (3H, t, $J = 7.0$ Hz, $CO_2CH_2CH_3$), 1.11 (3H, d, $J = 7.5$, $CHCH_3$), 0.56 (3H, t, $J = 7.5$, CH_2CH_3).

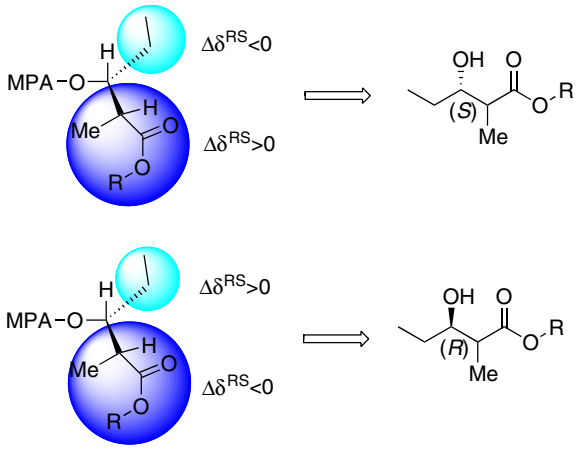
4.19.9. (*S*)-MPA ester of hydroxy ester 4C. δ_H (500 MHz; $CDCl_3$; Me_4Si) 7.31–7.45 (5H, m, Ar*H*), 5.10–5.14 (1H, m, C*H*OCO), 4.73 (1H, s, Ph*H*), 3.82–3.88 (1H, m, CO_2CH_2), 3.74–3.80 (1H, m, CO_2CH_2), 3.41 (3H, s, OCH_3), 2.61–2.67 (1H, m, $CHCO_2$), 1.64–1.72 (1H, m, CH_2CH_3), 1.52–1.60 (1H, m, CH_2CH_3), 1.10 (3H, t, $J = 7.0$, $CO_2CH_2CH_3$), 0.94 (3H, d, $J = 7.0$, $CHCH_3$), 0.86 (3H, t, $J = 7.5$, CH_2CH_3).

4.19.10. (*R*)-MPA ester of hydroxy ester 6A. δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.29–7.48 (5H, m, Ar*H*), 5.09–5.16 (1H, m, C*H*OCO), 4.75 (1H, s, Ph*H*), 4.58–4.67 (1H, m, CO_2CH), 3.42 (3H, s, OCH_3), 2.48–2.58 (1H, m, $CHCO_2$), 1.55–1.67 (2H, m, CH_2CH_3), 1.36–1.55 (4H, m, $2 \times CO_2CHCH_2CH_3$), 0.86 (3H, d, $J = 7.2$, $CHCH_3$), 0.81 (3H, t, $J = 7.5$, CH_2CH_3), 0.79 (6H, t, $J = 7.5$, $2 \times CO_2CHCH_2CH_3$).

4.19.11. (*S*)-MPA ester of hydroxy ester 6A. δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.30–7.48 (5H, m, Ar*H*), 5.07–5.14 (1H, m, C*H*OCO), 4.74 (1H, s, Ph*H*), 4.69–4.78 (1H, m,CO_2CH), 3.42 (3H, s, OCH_3), 2.62–2.72 (1H, m, $CHCO_2$), 1.42–1.59 (6H, m, $3 \times CH_2CH_3$), 1.12 (3H, d, $J = 6.9$, $CHCH_3$), 0.87 (6H, t, $J = 7.5$, $2 \times CO_2CHCH_2CH_3$), 0.56 (3H, t, $J = 7.5$, CH_2CH_3).

4.19.12. (*R*)-MPA ester of hydroxy ester 6C. δ_H (500 MHz; $CDCl_3$; Me_4Si) 7.31–7.45 (5H, m, Ar*H*), 5.11–5.15 (1H, m, C*H*OCO), 4.72–4.77 (1H, m, CO_2CH), 4.70 (1H, s, Ph*H*), 3.40 (3H, s, OCH_3), 2.72–2.79 (1H, m, $CHCO_2$), 1.40–1.60 (6H, m, $3 \times CH_2CH_3$), 1.12 (3H, d, $J = 7.5$, $CHCH_3$), 0.89 (3H, t, $J = 7.5$, $CO_2CHCH_2CH_3$), 0.87 (3H, t, $J = 7.5$, $CO_2CHCH_2CH_3$), 0.52 (3H, t, $J = 7.0$, CH_2CH_3).

4.19.13. (*S*)-MPA ester of hydroxy ester 6C. δ_H (500 MHz; $CDCl_3$; Me_4Si) 7.31–7.46 (5H, m, Ar*H*), 5.11–5.15 (1H, m, C*H*OCO), 4.75 (1H, s, Ph*H*), 4.58–4.63 (1H, m, CO_2CH), 3.41 (3H, s, OCH_3), 2.65–2.70 (1H, m, $CHCO_2$), 1.39–1.65 (6H, m, $3 \times CH_2CH_3$), 0.91 (3H, d, $J = 7.5$, $CHCH_3$), 0.85 (3H, t, $J = 7.5$, CH_2CH_3), 0.83 (3H, t, $J = 7.5$, $CO_2CHCH_2CH_3$), 0.80 (3H, t, $J = 7.5$, $CO_2CHCH_2CH_3$).



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