

A Two-Step Chemoenzymatic Synthesis of the Natural Pheromone (+)-Sitophilure Utilizing Isolated, NADPH-Dependent Ketoreductases

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Dedicated to the memory of Professor Christopher S. Foote

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Isolated, NADPH-dependent ketoreductases were used for the synthesis of the aggregation pheromone of the pests rice weevil (*Sitophilus oryzae* L.) and maize weevil (*Sitophilus zeamais* M.). This is the easiest and most straight forward synthesis of pheromone (+)-Sitophilure in two steps and an

overall yield of 81 %, starting from commercially available 3,5-heptanedione.

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Introduction

Optically active α -alkyl- β -hydroxy ketones are very important compounds in asymmetric organic synthesis because of their presence, as building blocks, in many natural products and pharmaceuticals.^[1] Several methods have been developed for their synthesis^[2] including the stereoselective reduction of the corresponding α -alkyl 1,3-diketone using chiral chemical catalysts^[3] or whole-cell microbial catalysts.^[4] Recently, we published the stereoselective reduction of α -alkyl 1,3-diketones^[5] utilizing twenty different isolated, NADPH-dependent ketoreductases.^[6] This method was proven to be very efficient for the synthesis of various optically and chemically pure α -alkyl- β -hydroxy ketones. Isolated ketoreductases offer many advantages compared to chemical or whole-cell biocatalytic reductions and have been utilized for the stereoselective reduction of a variety of ketones.^[7] Scaling of successful enzymatic reductions to preparative scale (5–50 g) and high titers of ketone (0.7 M to 1.4 M) is usually straightforward and requires catalytic amounts of ketoreductase and cofactor.^[7a]

In this paper we present a simple, scalable, two-step chemo-enzymatic synthesis of the natural pheromone

(4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone, commonly known as (+)-Sitophilure. The key step of this synthesis relies on the stereoselective reduction of the chemically synthesized precursor of (+)-Sitophilure, 4-methyl-3,5-heptanedione, by an isolated NADPH-dependent ketoreductase, (KRED-EXP-A1C, Table 1) in the presence of a glucose/glucose dehydrogenase system for cofactor recycling (Figure 1).

In 1984, Burkholder and co-workers isolated the male-produced aggregation pheromone of the pests rice weevil (*Sitophilus oryzae* L.) and maize weevil (*Sitophilus zeamais* M.), which is named Sitophilure.^[8] This biologically-active compound was first identified as (4*R*,5*S*)-5-hydroxy-4-methyl-3-heptanone, from the extracts of thousands of insects. All four stereoisomers of this pheromone were synthesized and it was proved that the active form of this compound is the (4*S*,5*R*) enantiomer.^[9] Since then, several total syntheses of racemic,^[10] or other stereoisomers^[11] and the natural form^[12] of this pheromone have been published.

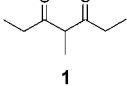
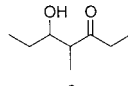
Serious economic losses of stored cereal grains (maize, rice and grain) are mainly caused by three weevils of the genus *Sitophilus* (*Sitophilus zeamais*, *Sitophilus oryzae*, *Sitophilus granarius*, respectively) throughout the world. Early detection of infestations is critical in order to avoid further damage to the grains and the subsequent economic losses. Traps that contain very small amounts of synthetic (+)-Sitophilure have been shown to be very effective in the early detection of all three species of weevils;^[13] however, a simple scalable and economic method for the synthesis of this weevil attractant pheromone is still lacking.^[9–12] As a result all the commercially available traps for the early detection of these weevils are food-based.

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Table 1. Enzyme-catalyzed stereoselective reduction of 4-methyl-3,5-heptanedione.

Substrate	KRED	Diastereomeric ratio % ^[a]				Conversion (time)	U/mg ^[b]	Product
		A	B	C	D			
	101	3	-	6	91	100% (6h)	0.042	
	114	8	4	-	88	90% (24h)	0.042	
	115	4	-	4	92	100% (6h)	0.016	
	118	4	-	-	96	93% (24h)	0.069	
	119	<1	-	-	>99	100% (12h)	0.204	
	123	20	-	-	80	100% (6h)	0.032	
	128	3	-	1	96	100% (3h)	0.180	
	130	6	-	-	94	100% (16h)	0.046	
	A1A	<2	-	-	>98	20% (24h)	0.058	
	A1B	-	97	3	-	100% (40min)	0.184	
	A1C	-	98	2	-	100% (1h)	0.168	
	A1D	-	97	3	-	100% (1h)	0.131	

[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. [b] Unit definition: 1 U/mg = formation of 1 μ mol alcohol per mg enzyme per minute. Activity was calculated on continuous assays by measuring the rate of NADPH absorption loss at 340 nm after adding 20 μ L of each enzyme lysate into 980 μ L of reaction buffer (NADPH, diketone 10 mM, and potassium phosphate 100 mM, pH 6.9).

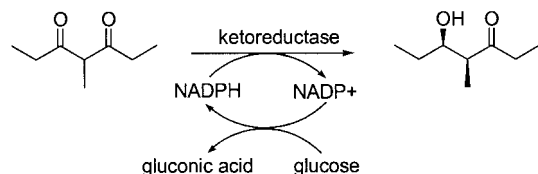


Figure 1. Enzymatic reduction of 4-methyl-3,5-heptanedione with NADPH-dependent ketoreductases.

Results and Discussion

Because Sitophilure is an optically active keto alcohol, it can be easily produced by the stereoselective reduction of the corresponding diketone 4-methyl-3,5-heptanedione (**1**, Figure 1). The diketone **1** is also a naturally occurring aggregation pheromone of *Sitona lineatus*.^[14]

Positive enzymes for the reduction of 4-methyl-3,5-heptanedione were identified after the screening of 64 isolated commercially available ketoreductases.^[6] Among them, three ketoreductases successfully produced (+)-Sitophilure with high diastereo- and enantioselectivity. Many enzymes showed activity towards the reduction of 4-methyl-3,5-heptanedione, and the best results of these enzymatic reductions are shown in Table 1. Note that all enzymes selectively produced the keto alcohol and not the diol even after longer incubation times.

It is clearly demonstrated that two out of the four stereoisomers, B and D, of the 5-hydroxy-4-methyl-3-heptanone are formed in optically pure form using five different enzymes, in very short reaction time, without the formation of the corresponding diol. In particular, KRED-119 completed the reaction in 12 hours forming diastereomer D, whereas KRED-A1B, KRED-A1C and KRED-A1D completed the reaction in only 40 and 60 minutes, respectively,

forming the diastereomer B. To the best of our knowledge there is no other biocatalyst or chemical catalyst that can produce this keto alcohol in optically pure form, from the corresponding diketone, in such a short reaction time. The diastereomeric ratio and reaction time, presented in Table 1, were derived from chiral GC analysis.

In order to determine the absolute configuration of the two stereoisomers B and D, we accomplished larger-scale reductions and isolated the keto alcohol **2**, in high yield and optical purity (87% yield, 99% *de*, >99% *ee* with KRED-119 and 85% yield, 96% *de*, >99% *ee* with KRED-A1C). The ¹H NMR^[15] and ¹³C NMR^[16] of the isolated products indicate that the relative stereochemistry of the product D (Table 1) is *anti* and that of the product B is *syn*. The absolute stereochemistry of these hydroxy ketones was determined by assigning first the stereochemistry of the hydroxy group by the use of chiral derivatizing agents.^[17]

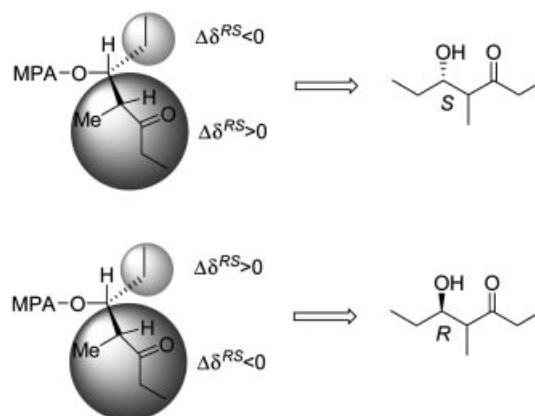


Figure 2. Determination of the stereochemistry of the hydroxy group of 5-hydroxy-4-methyl-3-heptanone.

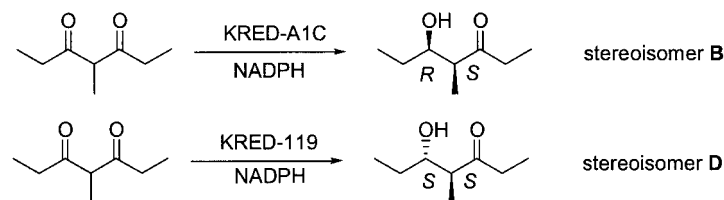


Figure 3. The absolute stereochemistry of the B, D stereoisomers of 5-hydroxy-4-methyl-3-heptanone.

Therefore, by the use of α -methoxyphenylacetic acid (MPA) the stereoisomers B and D were transformed into the corresponding MPA esters (Figure 2). The absolute stereochemistry of the enantiomers B and D (Figure 3) was found to be (4S,5R) and (4S,5S), respectively, taking into account that the relative stereochemistry of the product D is *anti* and of the product B *syn*.

As we can see in Figure 3, the product from the reduction of 4-methyl-3,5-heptanedione with KRED-A1C has the same stereochemistry with that of the natural pheromone (+)-Sitophilure. These results clearly indicate that ketoreductases KRED-A1B, KRED-A1C and KRED-A1D showed unusual *anti*Prelog selectivity, concerning reduction of the 5-keto group and successfully produced the keto alcohol with the desired stereochemistry (4S,5R). So the natural product can be produced easily from the corresponding diketone.

In large scale, the reaction completed in 24 hours producing the pheromone with chemical yield 85%, *de* 96%, *ee* >99% and chemical purity >99%, utilizing catalytic amounts of the NADPH cofactor (0.81% relative to the substrate), which was recycled in situ using Glucose Dehydrogenase (GDH). The corresponding 4-methyl-3,5-heptanedione can be readily produced from the commercially available 3,5-heptanedione (Figure 4).

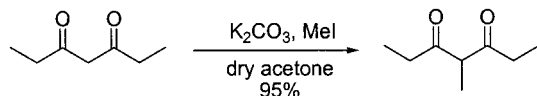


Figure 4. Preparation of Sitophilure's precursor.

Conclusion

In conclusion, an isolated, NADPH-dependent ketoreductase was used for the synthesis of the aggregation pheromone of the pests rice weevil (*Sitophilus oryzae* L.) and maize weevil (*Sitophilus zeamais* M.). To the best of our knowledge this is the easiest and most straight forward synthesis of pheromone (+)-Sitophilure in two steps and overall yield 81%, starting from commercially available 3,5-heptanedione.

Experimental Section

General Methods: 4-Methyl-3,5-heptanedione was prepared from commercially available 3,5-heptanedione by alkylation with methyl iodide. Racemic 5-hydroxy-4-methyl-3-heptanone was prepared

from 4-methyl-3,5-heptanedione by reduction with sodium borohydride. 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 $\times$ 0.25 mm $\times$ 0.25 μ m chiral capillary column, 20% permethylated cyclodextrin). ¹H NMR and ¹³C NMR spectra were recorded with Bruker spectrometers in CDCl₃ solutions (300, 500 MHz), with Me₄Si as internal standard. Chemical shifts are reported in ppm downfield from Me₄Si. Yields refer to isolated and spectroscopically pure materials.

Synthesis of 4-Methyl-3,5-heptanedione: The substrate was prepared from commercially available 3,5-heptanedione according to the following procedure: 3,5-Heptanedione (5 mmol, 640 mg, 676 μ L) was dissolved in anhydrous acetone (20 mL) under nitrogen and pre-dried potassium carbonate (4.7 mmol, 642 mg) was added. After stirring the solution at room temperature for 5 min, methyl iodide (6.15 mmol, 873 mg, 383 μ L) was added with syringe, and the reaction mixture was refluxed for 20 hours. After completion of the reaction, diethyl ether (30 mL) was added, the mixture was filtered and the solvent was evaporated to dryness. Without any further purification, 4-methyl-3,5-heptanedione was subjected to enzymatic reduction. Isolated yield 95% (674 mg) in equilibrium with enolic form. ¹H NMR (CDCl₃ 300 MHz, ppm): δ = 6.66 (q, *J* = 6.9 Hz, 1 H), 2.33–2.55 (m, 8 H), 1.80 (s, 3 H), 1.28 (d, *J* = 7.2 Hz, 3 H), 1.11 (t, *J* = 7.5 Hz, 3 H), 1.02 (t, *J* = 7.2 Hz, 3 H).

Synthesis of Racemic 5-Hydroxy-4-methyl-3-heptanone: Sodium borohydride (0.098 mmol, 4 mg) was added in dry ethanol (10 mL) under nitrogen, and the mixture was cooled to 0 °C. After stirring for 5 min a solution of dry ethanol (5 mL) containing 4-methyl-3,5-heptanedione (0.3 mmol, 43 mg) was added dropwise. After stirring for 2 hours at 0 °C, the reaction was quenched with saturated ammonium chloride, and the ethanol was evaporated in the rotor evaporator. Then water (15 mL) was added and extracted twice with ethyl acetate (2 $\times$ 10 mL). The organic layer was dried with MgSO₄, and the solvents evaporated to dryness. Pure products obtained using silica gel chromatography (hexane/EtOAc, v/v, 6:1), 80% isolated yield. ¹H NMR (CDCl₃ 300 MHz, ppm): δ = 3.73–3.84 (m, 1 H), 3.54–3.64 (m, 1 H), 2.71 (s, OH), 2.36–2.68 (m, 6 H), 1.28–1.59 (m, 4 H), 1.10 (d, *J* = 7.2 Hz, 3 H), 1.09 (d, *J* = 7.2 Hz, 3 H), 1.03 (t, *J* = 7.2 Hz, 3 H), 1.02 (t, *J* = 7.2 Hz, 3 H), 0.95 (t, *J* = 7.2 Hz, 3 H), 0.92 (t, *J* = 7.2 Hz, 3 H). ¹³C NMR (CDCl₃ 300 MHz, ppm): δ = 216.8, 216.7, 75.0, 72.6, 50.5, 49.3, 36.0, 35.1, 27.5, 26.9, 14.2, 10.4, 9.9, 9.8, 7.6, 7.5. GC data: (column: 30 m $\times$ 0.25 mm $\times$ 0.25 μ m chiral capillary column, 20% permethylated cyclodextrin 65 °C for 100 min, rate: 1 °C/min, final temp.: 100 °C; carrier gas: N₂, press 70 kPa). *t*_R = 93.3 min and 98.5 min (*syn*-5-hydroxy-4-methyl-3-heptanone), *t*_R = 102.5 min and 115.1 min (*anti*-5-hydroxy-4-methyl-3-heptanone).

Enzymatic Reductions: Sixty-four different ketoreductases (KRED-101–131 and KRED-EXP-A1C; BioCatalytics, Inc. Pasadena, CA USA) were screened to determine the best enzymes for the selective reduction of substrate 4-methyl-3,5-heptanedione. In

addition to the ketoreductases, both NADPH and glucose dehydrogenase (GLDH) are products available from BioCatalytics.

Small-Scale Enzymatic Reductions: 4-Methyl-3,5-heptanedione (25 mM) was mixed with NADPH (2.5 mM, 2 mg), each ketoreductase (2 mg/mL), glucose (100 mM, 18 mg), glucose dehydrogenase (GDH = 2 mg/mL) for cofactor recycling, NaCl (100 mM, 6 mg) and sodium phosphate buffer (1 mL, 200 mM, pH 6.5–6.9). The reactions were incubated at 25 °C–37 °C and reaction aliquots were taken every one hour and after extraction with ethyl acetate they were analyzed by GC chromatography.

Larger-Scale Enzymatic Reductions

Synthesis of (4*S*,5*S*)-5-Hydroxy-4-methyl-3-heptanone: A phosphate-buffered solution (20 mL, pH 6.9, 200 mM) containing 4-methyl-3,5-heptanedione (50 mM, 1 mmol, 142 mg), NaCl (200 mM, 234 mg), glucose (120 mM, 432 mg), NADPH (0.5 mM, 0.01 mmol, 9 mg), glucose dehydrogenase (10 mg) and KRED-119 (10 mg) was stirred at 37 °C for 24 hours, until GC analysis of the crude 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 (15 mL $\times$ 2). The combined organic layers were then extracted with saturated NaCl solution, dried with MgSO₄ and the solvents evaporated to dryness. Pure (4*S*,5*S*)-5-hydroxy-4-methyl-3-heptanone (125 mg) was obtained in 87% yield. ¹H NMR (CDCl₃ 300 MHz, ppm): δ = 3.54–3.65 (m, 1 H), 2.36–2.70 (m, 3 H), 1.29–1.61 (m, 2 H), 1.10 (d, J = 7.2 Hz, 3 H), 1.03 (t, J = 7.2 Hz, 3 H), 0.95 (t, J = 7.2 Hz, 3 H). ¹³C NMR (CDCl₃ 300 MHz, ppm): δ = 216.8, 75.0, 50.5, 36.0, 27.6, 14.2, 10.0, 7.5.

Determination of the Enantiomeric Purity of (4*S*,5*S*)-5-Hydroxy-4-methyl-3-heptanone: GC (column: 30 m $\times$ 0.25 mm $\times$ 0.25 μ m chiral capillary column, 20% permethylated cyclodextrin 65 °C for 100 min, rate: 1 °C/min, final temp.: 100 °C; carrier gas: N₂, press 70 kPa). t_R = 93.3 min [$<1\%$, (4*R*,5*S*)-5-hydroxy-4-methyl-3-heptanone], t_R = 115.4 min [$>99\%$, (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone]. The enantiomeric purity was estimated to be $>99\%$ and the diastereomeric purity 99%.

Synthesis of (4*S*,5*R*)-5-Hydroxy-4-methyl-3-heptanone: A phosphate-buffered solution (16 mL, pH 6.5, 200 mM) containing 4-methyl-3,5-heptanedione (84 mM, 1.35 mmol, 192 mg), NaCl (200 mM, 200 mg), glucose (130 mM, 375 mg), NADPH (0.69 mM, 0.011 mmol, 10 mg), glucose dehydrogenase (10 mg) and KRED-A1C (10 mg) was stirred at 25 °C for 24 hours, until GC analysis of the crude extracts showed complete reaction. Periodically the pH was readjusted to 6.5 with NaOH (2 M). The product was isolated by extracting the crude reaction mixture with EtOAc (15 mL $\times$ 2). The combined organic layers were then extracted with saturated NaCl solution, dried with MgSO₄ and the solvents evaporated to dryness. Pure (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone (165 mg) was obtained in 85% yield. ¹H NMR (CDCl₃ 500 MHz, ppm): δ = 3.77–3.85 (m, 1 H), 2.72 (s, OH), 2.41–2.64 (m, 3 H), 1.32–1.58 (m, 2 H), 1.12 (d, J = 7.1 Hz, 3 H), 1.05 (t, J = 7.3 Hz, 3 H), 0.95 (t, J = 7.4 Hz, 3 H). ¹³C NMR (CDCl₃ 300 MHz, ppm): δ = 216.7, 72.6, 49.3, 35.1, 26.9, 10.4, 9.9, 7.6.

Determination of the Enantiomeric Purity of (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone: GC (column: 30 m $\times$ 0.25 mm $\times$ 0.25 μ m chiral capillary column, 20% permethylated cyclodextrin 65 °C for 100 min, rate: 1 °C/min, final temp.: 100 °C; carrier gas: N₂, press 70 kPa). t_R = 100.0 min [98%, (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone], t_R = 105.1 min [2%, (4*R*,5*R*)-5-hydroxy-4-methyl-3-heptanone]. The enantiomeric purity was estimated to be $>99\%$ and the diastereomeric purity 96%.

Preparation of MPA Esters

Synthesis of (R)-MPA Ester of (4*S*,5*S*)-5-Hydroxy-4-methyl-3-heptanone: To a solution of (4*S*,5*S*)-5-hydroxy-4-methyl-3-heptanone (0.11 mmol, 16 mg) in dry CH₂Cl₂ was added 1.1 equiv. of DCC (0.121 mmol, 25 mg) and 1.1 equiv. of the (R)-MPA ester (0.11 mmol, 20 mg) and the reaction mixture was stirred at 0 °C for 3 h. After completion of the reaction the produced urea was filtered, the filtrate was evaporated and then chromatographed with Hex/EtOAc (5:1), and the produced corresponding MPA ester was isolated (27 mg). Yield 89%. ¹H NMR (CDCl₃ 500 MHz, ppm): δ = 7.33–7.47 (m, 5 H), 5.11 (m, 1 H), 4.72 (s, 1 H), 3.44 (s, 3 H), 2.87 (m, 1 H), 2.37–2.52 (m, 2 H), 1.41–1.59 (m, 2 H), 1.05 (d, J = 7 Hz, 3 H), 1.02 (t, J = 7 Hz, 3 H), 0.59 (t, J = 7.5 Hz, 3 H).

Synthesis of (S)-MPA Ester of (4*S*,5*S*)-5-Hydroxy-4-methyl-3-heptanone: To a solution of (4*S*,5*S*)-5-hydroxy-4-methyl-3-heptanone (0.056 mmol, 8 mg) in dry CH₂Cl₂ was added 1.1 equiv. of DCC (0.0616 mmol, 13 mg) and 1.1 equiv. of the (S)-MPA ester (0.0616 mmol, 10 mg) and the reaction mixture was stirred at 0 °C for 3 h. After completion of the reaction the produced urea was filtered, the filtrate was evaporated and then chromatographed with Hex/EtOAc (5:1), and the produced corresponding MPA ester was isolated (13 mg). Yield 87%. ¹H NMR (CDCl₃ 500 MHz, ppm): δ = 7.32–7.46 (m, 5 H), 5.14 (m, 1 H), 4.73 (s, 1 H), 3.41 (s, 3 H), 2.73 (m, 1 H), 2.07–2.29 (m, 2 H), 1.49–1.73 (m, 2 H), 0.89 (d, J = 7 Hz, 3 H), 0.86 (t, J = 7.5 Hz, 3 H), 0.83 (t, J = 7.5 Hz, 3 H).

Synthesis of (R)-MPA Ester of (4*S*,5*R*)-5-Hydroxy-4-methyl-3-heptanone: To a solution of (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone (0.076 mmol, 11 mg) in dry CH₂Cl₂ was added 1.1 equiv. of DCC (0.0836 mmol, 17 mg) and 1.1 equiv. of the (R)-MPA ester (0.0836 mmol, 14 mg), and the reaction mixture was stirred at 0 °C for 3 h. After completion of the reaction the produced urea was filtered, the filtrate was evaporated and then chromatographed with Hex/EtOAc (5:1) and the produced corresponding MPA ester was isolated (18 mg). Yield 85%. ¹H NMR (CDCl₃ 500 MHz, ppm): δ = 7.33–7.48 (m, 5 H), 5.12 (m, 1 H), 4.76 (s, 1 H), 3.44 (s, 3 H), 2.65 (m, 1 H), 2.17 (q, J = 7 Hz, 2 H), 1.52–1.62 (m, 2 H), 0.88 (t, J = 7.5 Hz, 3 H), 0.87 (d, J = 7 Hz, 3 H), 0.86 (t, J = 7.5 Hz, 3 H).

Synthesis of (S)-MPA Ester of (4*S*,5*R*)-5-Hydroxy-4-methyl-3-heptanone: To a solution of (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone (0.125 mmol, 18 mg) in dry CH₂Cl₂ was added 1.1 equiv. of DCC (0.138 mmol, 28 mg) and 1.1 equiv. of the (S)-MPA ester (0.138 mmol, 23 mg), and the reaction mixture was stirred at 0 °C for 3 h. After completion of the reaction the produced urea was filtered, the filtrate was evaporated and then chromatographed with Hex/EtOAc (5:1), and the produced corresponding MPA ester was isolated (30 mg). Yield 88%. ¹H NMR (CDCl₃ 500 MHz, ppm): δ = 7.31–7.48 (m, 5 H), 5.15 (m, 1 H), 4.76 (s, 1 H), 3.44 (s, 3 H), 2.78 (m, 1 H), 2.36–2.57 (m, 2 H), 1.45 (m, 2 H), 1.05 (d, J = 7 Hz, 3 H), 1.03 (t, J = 7.5 Hz, 3 H), 0.58 (t, J = 7.5 Hz, 3 H).

Supporting Information (see footnote on the first page of this article): ¹H and ¹³C NMR spectra as well as GC analytical data.

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