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# Solvent-free Lipase-Catalyzed Preparation of Long-Chain Alkyl Phenylpropanoates and Phenylpropyl Alkanoates

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An enzymatic method was developed for the preparation of medium- or long-chain alkyl 3-phenylpropenoates (alkyl cinnamates), particularly alkyl hydroxy- and methoxy-substituted cinnamates such as oleyl *p*-coumarate and oleyl ferulate. The various alkyl cinnamates were formed in high to moderate yield by lipase-catalyzed esterification of cinnamic acid and its analogues with fatty alcohols in vacuo at moderate temperatures in the absence of drying agents and solvents. Immobilized *Candida antarctica* lipase B was the most effective biocatalyst for the various esterification reactions. The relative esterification activities were of the following order: dihydrocinnamic > cinnamic > 3-methoxycinnamic > dihydrocaffeic ≈ 3-hydroxycinnamic > 4-methoxycinnamic > 2-methoxycinnamic > 4-hydroxycinnamic > ferulic ≈ 3,4-dimethoxycinnamic > 2-hydroxycinnamic acid. With respect to the position of the substituents at the phenyl moiety, the esterification activity increased in the order meta > para > ortho. *Rhizomucor miehei* lipase demonstrated moderate esterification activity. Compounds with inverse chemical structure, that is, 3-phenylpropyl alkanoates such as 3-(4-hydroxyphenyl)propyl oleate, were also obtained in high yield by esterification of fatty acids with the corresponding 3-phenylpropan-1-ols.

**KEYWORDS: Alkyl cinnamates; alkyl 3-(hydroxyphenyl)propenoates; oleyl coumarates; alkyl 3-(methoxyphenyl)propenoates; oleyl ferulate; palmityl ferulate; 3-phenylpropyl alkanoates; 3-(4-hydroxyphenyl) oleate; immobilized *Candida antarctica* lipase B; lipase-catalyzed esterification**

## INTRODUCTION

Minor constituents of plants are gaining importance due to their proposed health benefits (*1*, *2*). For example, epidemiological and nutritional studies suggest that the consumption of foods containing high proportions of phenolic antioxidants may prevent chronic diseases such as coronary heart disease and colorectal cancer (*3*−*5*). The beneficial effects of phenolic antioxidants, such as 3-(hydroxyphenyl) propenoates (hydroxy cinnamic acids), on health have been attributed to their antioxidant capacity, particularly their ability to protect low-density lipoproteins from oxidative attack (*6*). The antioxidant capacity and biological availability of several of these compounds may be further improved by increasing their lipophilicity (*7*−*12*), and the range of applications of such lipophilic antioxidants may be extended by their possible use as additives for food and nutraceutical (*12*, *13*) as well as cosmetic and technical applications (*14*−*18*).

Hydroxy cinnamic acids and their methoxy derivatives such as 4-hydroxycinnamic (*p*-coumaric), ferulic, caffeic and sinapic acids are common constituents of plants, particularly of cereals such as rice, rye, and wheat, in which they are predominantly esterified to cell wall polysaccharides (*19*−*24*). Recently, Mendez (*25*) demonstrated the occurrence of dihydrocinnamic acid derivatives, for example, dihydro-*p*-coumaric and dihydroferulic acids, in bracken fern (*Pteridium aquilinum*). Although hydroxy cinnamic acids esters of polysaccharides occur most commonly in plants, various other esters of cinnamic acid and its analogues including medium- or long-chain alkyl esters have also been detected, for example, alkyl ferulates (**Figure 1**), which are predominantly found in the cork layers of several plant species (*26*−*32*). In addition, similar compounds with inverse chemical structure, that is, 3-(4-hydroxyphenyl)propenyl (*p*-coumaryl) alkanoates, were isolated from apple (*Malus domestica*) fruits (*33*, *34*).

Various methods are known for the chemical esterification of cinnamic acid and its analogues, for example, esterification of hydroxy cinnamic acids or their tetrahydropyranyl derivatives with various alcohols in the presence of *N*,*N*-dicyclohexylcarbodiimide in tetrahydrofuran or pyridine (*9*, *35*). Moreover, esterification of hydroxy cinnamic acids including ferulic acid with alkanols in the presence of acid catalysts such as sulfuric acid or *p*-toluenesulfonic acid has been described using benzene, toluene, or chlorobenzene as the solvents (*18*). Sitostanyl ferulate was prepared by esterification of acetylated ferulic acid with sitostanol in the presence of *N*,*N*-dicyclohexylcarbodiimide and 4-(dimethylamino)pyridine (*36*). Enzymatic esterification is preferred, however, over chemical esterification for the prepara-

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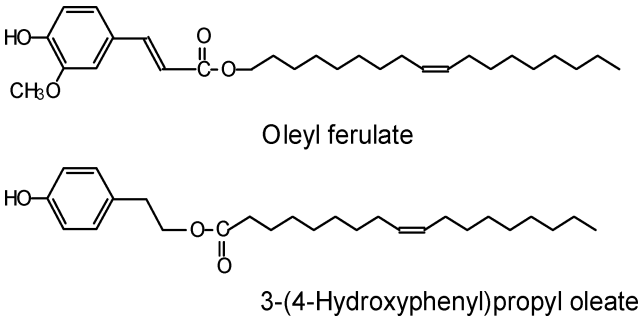


**Figure 1.** Chemical structures of the alkyl cinnamate (alkyl 3-phenylpropenoate) derivative oleyl ferulate and the structurally similar inverse dihydrocinnamyl (3-phenylpropyl) alkanoate derivative 3-(4-hydroxyphenyl)-propyl oleate.

tion of hydroxy cinnamic acids esters that may be used in foods. Little is known about lipase-catalyzed esterification of cinnamic acid derivatives, particularly hydroxy cinnamic acids (*12*). Enzymatic esterification procedures requiring organic solvents or using the alcohol component as the solvent have been reported for the preparation of short- and medium-chain alkyl esters of hydroxy cinnamic acids including alkyl ferulates (*37*−*43*). Similarly, the corresponding compounds with inverse chemical structure, that is, 3-phenylpropyl and 3-phenylpropenyl alkanoates, may be formed by esterification of fatty acids with various dihydrocinnamyl and cinnamyl alcohol derivatives (3-phenylpropan-1-ols and 3-phenylpropen-1-ols). Such "retro" compounds may also be of interest because they are expected to have antioxidant properties equivalent to those shown for alkyl hydroxycinnamates (*33*, *34*).

The aim of the present work was to develop a simple and environmentally friendly method for the preparation of various lipophilic medium- and long-chain alkyl 3-phenylpropanoates, particularly hydroxy- and methoxycinnamates, by esterification of 3-phenylpropanoic and 3-phenylpropenoic acids and their analogues with medium- or long-chain fatty alcohols as well as of the structurally inverse 3-phenylpropyl alkanoates by esterification of medium- or long-chain fatty acids with 3-phenylpropan-1-ols using lipases as biocatalysts. The esterification reactions were performed at moderate temperatures without solvents or drying agents using reduced pressure to remove reaction water.

## MATERIALS AND METHODS

**Materials.** 1-Dodecanol, 1-hexadecanol, and *cis*-9-octadecen-1-ol (oleyl alcohol) as well as *trans*-cinnamic acid [(*E*)-3-phenylpropenoic acid], dihydrocinnamic acid (3-phenylpropanoic acid), 2- and 3-hydroxy- as well as 2-, 3-, and 4-methoxycinnamic acids, 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 3,4-dihydroxycinnamic acid (caffeic acid), 3,4-dihydroxydihydrocinnamic acid (dihydrocaffeic acid), 4-hydroxy-3,5-dimethoxycinnamic acid (sinapic acid), methyl *trans*-cinnamate, cinnamyl alcohol (3-phenylpropen-1-ol), dihydrocinnamyl alcohol (3-phenylpropan-1-ol), 3-(4-hydroxyphenyl)propan-1-ol, and 3-(3,4-dimethoxy)propan-1-ol were obtained from Sigma-Aldrich-Fluka (Deisenhofen, Germany). 4-Hydroxycinnamic acid (*p*-coumaric acid) was a product of Alfa Aesar (Karlsruhe, Germany). For comparison, *trans*-ferulic acid was partially isomerized to *cis*-ferulic acid by UV irradiation in methanolic solution (*44*).

Immobilized lipase preparations from *Candida antarctica* (lipase B, Novozym 435), *Rhizomucor miehei* (Lipozyme RM IM), and *Thermomyces lanuginosus* (Lipozyme TL IM) were kindly provided by Novozymes, Bagsvaerd, Denmark. Various other enzymes were also checked for esterification activity using cinnamic acid derivatives and *cis*-9-octadecen-1-ol as the substrates, including lipases from *Candida rugosa* (Sigma), *Candida lipolytica* (Fluka), *Chromobacterium viscosum* (Biocatalysts, Pontypridd, Mid Glamorgan, U.K.), *Rhizopus arrhizus*, *Rhizopus niveus*, *Rhizopus javanicus* (all from Fluka), and *Fusarium oxysporum* (Lipopan F BG, Novozymes), and pectinase from *Aspergillus niger* (Serva, Heidelberg, Germany) as well as plant proteases/esterases including bromelain from *Ananassa comosus*, papain from *Carica papaya*, and ficin from *Ficus carica* (all from Sigma).

**Lipase-Catalyzed Reactions.** As a typical example, 4-methoxycinnamic acid (53.4 mg, 0.3 mmol) was esterified with *cis*-9-octadecen-1-ol (80.4 mg, 0.3 mmol) in the presence of 50 mg of the various lipase preparations by magnetic stirring in a screw-capped tube in vacuo at 80 °C for periods of up to 144 h with water-trapping in the gas phase using potassium hydroxide pellets. A moderate vacuum (80 kPa) was used to prevent substantial loss of substrates. Samples of the reaction products were withdrawn at various intervals, extracted with diethyl ether, and filtered through a 0.45 $\mu$m syringe filter to remove the biocatalyst. An aliquot of the filtrate was analyzed as given below.

Similar reaction conditions were used for the preparation of phenylpropyl alkanoates with inverse chemical structure. As a typical example, oleic acid (84.8 mg, 0.3 mmol) was esterified with cinnamyl alcohol (40.2 mg, 0.3 mmol) under identical conditions as described above for the esterification of cinnamic acid derivatives using Novozym 435 lipase preparation as biocatalyst.

Enzyme units were calculated from the initial rates (4 or 24 h) of esterification of the various 3-phenylpropanoic and 3-phenylpropenoic acids with alkanols as well as the corresponding esterification of fatty acids with various 3-phenylpropanol and 3-phenylpropenol derivatives. One unit of enzyme activity was defined as the amount of enzyme (grams) that produced 1 $\mu$mol of the respective alkyl or phenylpropyl ester per minute.

**Thin-Layer Chromatography (TLC).** Aliquots were withdrawn from the reaction mixtures, and free carboxy groups of the various compounds of the reaction mixture were methylated using a solution of diazomethane in diethyl ether. The conversion was checked by TLC on 0.3 mm layers of silica gel H (E. Merck, Darmstadt, Germany), and spots were located by iodine staining and charring by spraying with 30% sulfuric acid followed by heating (200 °C).

The different methods of development (A and B) used led to the following $R_f$ values of the various compounds: (A) isohexane−diethyl ether (1:1), one run to the top, long-chain alkyl cinnamates and dihydrocinnamates, 0.55−0.6; long-chain methoxy-substituted 3-phenylpropyl alkanoates, 0.5−0.55; long-chain 3-(3,4-dimethoxy-phenyl)-propyl alkanoates, 0.4; fatty acid methyl esters, 0.75; saturated and unsaturated long-chain fatty alcohols, 0.3; unesterified fatty acids and 3-phenylpropanoic acids, 0.4−0.45; (B) isohexane−diethyl ether (1:1), developed up to half of the plate, followed by the same solvent system to the top, long-chain alkyl 3-(hydroxyphenyl)propenoates, 0.6−0.65; medium- and long-chain alkyl ferulates, 0.5; long-chain 3-(4-hydroxyphenyl)propyl oleate, 0.6; 3-phenylpropan-1-ol (dihydrocinnamyl alcohol) and 3-phenylpropen-1-ol (cinnamyl alcohol) 0.3; 3-(4-hydroxyphenyl)propan-1-ol and 3-(3,4-dimethoxyphenyl)propan-1-ol, 0.1−0.15; unesterified fatty acids and 3-phenylpropanoic acids, 0.45−0.55.

Similarly, 0.5 mm layers of silica gel H were used for the separation of reaction products by preparative TLC. The various fractions were scraped off the plates and extracted from silica gel using water-saturated diethyl ether. Reaction mixtures containing phenolic hydroxy compounds were separated under similar conditions as described above using method B.

**Purification by Crystallization.** Alkyl 3-phenylpropanoates and 3-phenylpropyl alkanoates were extracted from the immobilized biocatalysts with diethyl ether and purified by column chromatography and/or crystallization from isohexane or mixtures of isohexane−diethyl ether (1:1). The following melting points were determined using a Kofler heating block: oleyl 2-hydroxycinnamate, 37−39 °C; palmityl 2-hydroxycinnamate, 84−85 °C; palmityl 3-hydroxycinnamate, 78−79 °C; oleyl 4-hydroxycinnamate, 38−39 °C; palmityl 4-hydroxycinnamate, 89−90 °C; palmityl 2-methoxycinnamate, 46−47 °C; palmityl 3-methoxycinnamate, 47−48 °C; palmityl 4-methoxycinnamate, 59−60 °C; lauryl ferulate, 45−48 °C; palmityl ferulate, 61−63 °C; oleyl ferulate, 30−33 °C; oleyl dihydrocaffeate, 21−23 °C; oleyl caffeate, 78−79 °C; cinnamyl palmitate (3-phenylprop-2-en-1-yl palmitate), 45−

46 °C; 3-(4-hydroxyphenyl)propyl palmitate, 60−61 °C; 3-(3,4-dimethoxyphenyl)propyl palmitate, 35−36 °C. Other long-chain alkyl cinnamates or cinnamyl alkanoates such as oleyl cinnamate, oleyl dihydrocinnamate, oleyl 3-hydroxycinnamate, oleyl 2-methoxycinnamate, oleyl 3-methoxycinnamate, oleyl 4-methoxycinnamate, oleyl 3,4-dimethoxycinnamate, 2-ethylhexyl ferulate, oleyl sinapate, cinnamyl oleate, 3-phenylpropyl palmitate, 3-(4-hydroxyphenyl)propyl oleate are liquids at room temperature.

**Column Chromatography.** As a typical example, the reaction mixture resulting from the esterification of ferulic acid with oleyl alcohol (around 400 mg) was dissolved in 2 mL of diethyl ether and applied to a 25 × 2 cm i.d. silica gel 60 column (Merck). The column was eluted first with 30 mL of isohexane and then with 30 mL portions of various isohexane−diethyl ether mixtures (95:5, 9:1, 8:2, 7:3). Elution with isohexane−diethyl ether (1:1) yielded around 300 mg of oleyl ferulate, which was crystallized from isohexane−diethyl ether (1:1).

**Gas Chromatography (GC).** Aliquots of esterification products were removed from the reaction mixture, dissolved in dichloromethane, and filtered through a 0.45 μm PTFE syringe filter to remove the lipase catalysts. The filtrate was concentrated, dissolved in diethyl ether, and treated with an ethereal solution of diazomethane to convert the unreacted carboxy groups to the corresponding methyl esters. The resulting mixture of methyl esters, unreacted alkanols or 3-phenylpropanols, and alkyl cinnamates or 3-phenylpropyl alkanoates was analyzed by GC. The phenolic hydroxy group of 2-hydroxycinnamic acid derivatives was methylated to the 2-methoxy compounds by treatment with an ethereal solution of diazomethane in the presence of catalytic amounts of silica gel (*45*) to avoid coumarin formation during high-temperature GC. A Hewlett-Packard (Böblingen, Germany) HP-5890 series II gas chromatograph equipped with a flame ionization detector was used. Separations were carried out on a 15 m × 0.25 mm i.d., 0.1 μm 400-1HT fused silica capillary column (Quadrex Corp., New Haven, CT) using hydrogen as the carrier gas (column pressure of 50 kPa).

Three different temperature programs were used to separate the various compounds of reaction mixtures: (method A) initially at 150 °C for 2 min, followed by linear programming from 150 to 180 °C at 2 °C/min, then from 180 to 360 °C at 20 °C/min, and finally kept at 360 °C for 2 min; (method B) initially at 140 °C for 2 min, followed by linear programming from 140 to 180 °C at 5 °C/min, then from 180 to 250 °C at 10 °C/min (5 min isothermally), then from 250 to 410 °C at 20 °C/min, and finally kept at 410 °C for 2 min; and (method C) initially at 80 °C for 2 min, followed by linear programming from 80 to 280 °C at 10 °C/min, and finally kept at 280 °C for 5 min. Injector and detector temperatures were maintained at 380 °C. Retention times of the various reaction products and starting materials are given in **Table 1**.

Peaks in gas chromatograms were assigned by comparison of their retention times with those of peaks from TLC fractions that had been identified by GC-MS. Response factors of FID were determined using purified compounds, and percentages of peak areas were calculated using a Hewlett-Packard GC ChemStation software. For the determination of enzyme activities, small proportions of methoxy compounds that had been formed during carboxymethylation of hydroxycinnamic acids with diazomethane were calculated as the original hydroxy compounds.

**Reversed Phase High-Performance Liquid Chromatography (RP-HPLC).** Aliquots of purified reaction products were dissolved in diethyl ether, filtered as described for GC, concentrated, and dissolved in acetonitrile. Samples were analyzed for their composition by RP-HPLC as follows: the HPLC system consisted of a Merck-Hitachi pump L-6200 (E. Merck) equipped with a column oven (VDS Optilab, Berlin, Germany) set at 30 °C, a Knauer K 2501 UV−vis detector (Knauer, Berlin, Germany) set to 220 nm, and a PL-ELS 2100 (Polymer Laboratories, Darmstadt, Germany) ELSD detector (thermostated to 30 °C for acetonitrile), which were used in series. UV and mass traces were monitored and evaluated in a Knauer Eurochrom for Windows, Preparative Version, data acquisition unit (Knauer).

Long-chain alkyl 3-phenylpropanoates, alkyl 3-phenylpropenoates, and 3-phenylpropyl alkanoates were separated on a 250 mm × 4 mm i.d., 5 μm LiChrospher RP-18e column (Phenomenex, Aschaffenburg, Germany) and precolumn using (method A) acetonitrile isocratically at a flow rate of 1.0 mL/min, (method B) acetonitrile isocratically at a flow rate of 0.6 mL/min, and (method C) methanol−water−acetic acid (50:50:0.5) isocratically at a flow rate of 1 mL/min and a UV detector set at 320 nm. Injections (around 20−60 μg) of the reaction products were carried out with a Rheodyne 7161 sample injector (Cotati, CA) equipped with a 20-μL sample loop. Peak areas and percentages were calculated using the Eurochrom software. Retention times of the various reaction products and starting materials are given in **Table 2**.

**Table 1.** GC Retention Times of Various Medium- and Long-Chain Alkyl 3-Phenylpropanoates, Alkyl 3-Phenylpropenoates, and 3-Phenylpropyl Alkanoates and Starting Materials

| alkyl 3-phenylpropanoates, alkyl 3-phenylpropenoates, and 3-phenylpropyl alkanoates as well as starting materials[a] | retention time (min) |
|---|---|
| Method A[b] | |
| methyl dihydrocinnamate | 1.0 |
| methyl cinnamate | 1.2 |
| methyl 2-methoxycinnamate | 1.6 |
| methyl 3-methoxycinnamate | 1.6 |
| methyl 4-methoxycinnamate | 1.8 |
| methyl 3-hydroxycinnamate | 1.8 |
| methyl 4-hydroxycinnamate | 1.9 |
| oleyl alcohol | 6.6 |
| oleyl dihydrocinnamate | 12.5 |
| oleyl cinnamate | 13.8 |
| oleyl 2-methoxycinnamate | 13.6 |
| oleyl 3-methoxycinnamate | 13.7 |
| oleyl 4-methoxycinnamate | 13.8 |
| oleyl 3-hydroxycinnamate | 13.3 |
| oleyl 4-hydroxycinnamate | 13.4 |
| Method B[b] | |
| methyl ferulate | 0.9 |
| lauryl alcohol | 1.0 |
| palmityl alcohol | 3.6 |
| oleyl alcohol | 5.5 |
| lauryl ferulate | 15.3 |
| palmityl ferulate | 18.6 |
| oleyl *cis*-ferulate | 19.7 |
| oleyl *trans*-ferulate | 20.3 |
| oleyl sinapate | 19.0 |
| Method C[b] | |
| 3-phenylpropan-1-ol | 1.6 |
| cinnamyl alcohol | 2.1 |
| 3-(4-hydroxyphenyl)propan-1-ol | 6.2 |
| 3-(3,4-dimethoxyphenyl)propan-1-ol | 6.8 |
| methyl oleate | 10.7 |
| 3-phenylpropyl oleate | 17.4 |
| 3-phenylpropenyl oleate (cinnamyl oleate) | 17.7 |
| 3-(4-hydroxyphenyl)propyl oleate | 19.2 |
| 3-(3,4-dimethoxyphenyl)propyl oleate | 19.9 |

[a] Carboxylic acids were converted to the methyl esters by reaction with diazomethane. [b] For details see Materials and Methods.

**GC-MS Analyses.** The fragmentation of the various alkyl 3-phenylpropanoates or 3-phenylpropyl alkanoates formed by lipase-catalyzed esterification reactions was studied by GC-MS (EI mode). Compounds containing carboxy groups were analyzed after derivatization to the corresponding methyl esters using an ethereal solution of diazomethane and purification by preparative TLC or column chromatography. GC-MS analyses were carried out on a Hewlett-Packard model 5890 series II/5989A apparatus equipped with a 15 m × 0.25 mm i.d., 0.25 μm Rtx-5MS fused silica capillary column (Restek Germany, Bad Homburg, Germany), using the electron impact ionization (EI, 70 eV) mode. Alternatively, separations were carried out on a 15 m × 0.25 mm i.d., 0.1 μm 400−1HT (Quadrex Corp.) fused silica capillary column. The separation conditions were identical for both columns. The carrier gas was He at a flow rate of 1.0 mL/min. The column temperature was initially kept at 140 °C for 2 min and then programmed from 140 to 180 °C at 5 °C/min, held there for 5 min, and then programmed from 180 to 360 °C at 20 °C/min; the final temperature was held for 9 min. Other operating conditions were split/splitless injector in split mode

**Table 2.** RP-HPLC Retention Times of Various Medium- and Long-Chain Alkyl 3-Phenylpropanoates, Alkyl 3-Phenylpropenoates, and 3-Phenylpropyl Alkanoates and Starting Materials

| alkyl 3-phenylpropanoates, alkyl 3-phenylpropenoates, 3-phenylpropyl alkanoates, and starting materials[a] | retention time (min) |
|---|---|
| Method A[b] | |
| oleyl dihydrocinnamate | 14.9 |
| oleyl cinnamate | 16.6 |
| oleyl 2-methoxycinnamate | 15.7 |
| oleyl 3-methoxycinnamate | 15.0 |
| oleyl 4-methoxycinnamate | 14.2 |
| oleyl 3,4-dimethoxycinnamate | 11.7 |
| oleyl ferulate | 4.7 |
| methyl ferulate | 2.7 |
| oleyl alcohol | 5.9 |
| 3-(4-hydroxyphenyl)propyl oleate | 8.7 |
| 3-(4-hydroxyphenyl)propyl palmitate | 9.0 |
| 3-phenylpropyl (dihydrocinnamyl) palmitate | 15.0 |
| 3-phenylpropenyl (cinnamyl) palmitate | 13.2 |
| 3-(3,4-dimethoxyphenyl)propyl palmitate | 10.7 |
| Method B[b] | |
| oleyl 2-hydroxycinnamate | 18.6 |
| oleyl 3-hydroxycinnamate | 17.5 |
| oleyl 4-hydroxycinnamate | 17.4 |
| 2-ethylhexyl ferulate | 5.4 |
| dodecyl ferulate | 9.3 |
| palmityl ferulate | 18.3 |
| oleyl ferulate | 17.3 |
| oleyl dihydrocaffeate | 12.5 |
| oleyl caffeate | 14.2 |
| oleyl sinapate | 15.4 |
| Method C[b] | |
| *cis*-ferulic acid[c] | 6.0 |
| *trans*-ferulic acid[c] | 5.6 |

[a] Carboxylic acids were converted to the methyl esters by reaction with diazomethane. [b] For details see Materials and Methods. [c] Separated as free carboxylic acids.

(split 1:10, temperature 360 °C), interface temperature of 360 °C, and ion source temperature of 200 °C.

For example, the following molecular ions and important mass fragments were observed: *m*/*z* oleyl cinnamate 398 $[M]^+$, 149 $[M - C_{18}H_{33}O]^+$, 131 $[M - C_{18}H_{35}O_2]^+$; oleyl dihydrocinnamate 400 $[M]^+$, 149 $[M - C_{18}H_{35}]^+$, 133 $[M - C_{18}H_{35}O]^+$; oleyl 2-hydroxycinnamate, 414 $[M]^+$, 165 $[M - C_{18}H_{33}]^+$, 146 $[M - C_{18}H_{36}O]^+$; 3-hydroxy- and 4-hydroxycinnamates 414 $[M]^+$, 164 $[M - C_{18}H_{34}]^+$, 147 $[M - C_{18}H_{35}O]^+$; palmityl 2-hydroxy-, 3-hydroxy-, and 4-hydroxycinnamates 388 $[M]^+$, 164 $[M - C_{16}H_{32}]^+$, 147 $[M - C_{16}H_{33}O]^+$; palmityl 2-methoxy-, 3-methoxy-, and 4-methoxycinnamates 402 $[M]^+$, 178 $[M - C_{16}H_{32}]^+$, 161 $[M - C_{16}H_{33}O]^+$; oleyl 2-methoxy-, 3-methoxy-, and 4-methoxycinnamates 428 $[M]^+$, 178 $[M - C_{18}H_{34}]^+$, 161 $[M - C_{18}H_{35}O]^+$; oleyl 3,4-dimethoxycinnamate 458 $[M]^+$, 208 $[M - C_{18}H_{34}]^+$, 191 $[M - C_{18}H_{35}O]^+$; oleyl sinapate 474 $[M]^+$, 224 $[M - C_{18}H_{34}]^+$, 207 $[M - C_{18}H_{35}O]^+$, 167 $[M - C_{20}H_{35}O_2]^+$; 2-ethylhexyl ferulate 306 $[M]^+$, 194 $[M - C_8H_{16}]^+$, 177 $[M - C_8H_{16}O]^+$; dodecyl ferulate 362 $[M]^+$, 194 $[M - C_{12}H_{24}]^+$, 177 $[M - C_{12}H_{25}O]^+$; palmityl ferulate 418 $[M]^+$, 194 $[M - C_{16}H_{32}]^+$, 177 $[M - C_{16}H_{33}O]^+$; oleyl ferulate 444 $[M]^+$, 194 $[M - C_{18}H_{34}]^+$, 177 $[M - C_{18}H_{35}O]^+$; oleyl dihydrocaffeate 432 $[M]^+$, 182 $[M - C_{18}H_{34}]^+$, 165 $[M - C_{18}H_{35}O]^+$; oleyl caffeate 430 $[M]^+$, 180 $[M - C_{18}H_{34}]^+$, 163 $[M - C_{18}H_{35}O]^+$; 3-phenylpropyl oleate 400 $[M]^+$, 118 $[M - C_{18}H_{34}O_2]^+$; 3-phenylpropyl palmitate 374 $[M]^+$, 118 $[M - C_{16}H_{32}O_2]^+$; 3-phenylprop-2-enyl palmitate (cinnamyl palmitate) 372 $[M]^+$, 239 $[M - C_9H_9O]^+$, 133 $[M - C_{16}H_{31}O]^+$, 117 $[M - C_{16}H_{31}O_2]^+$; 3-(3,4-dimethoxyphenyl)propyl palmitate 434 $[M]^+$, 196 $[M - C_{16}H_{30}O]^+$, 178 $[M - C_{16}H_{31}O_2]^+$; 3-(4-hydroxyphenyl)propyl oleate 416 $[M]^+$, 134 $[M - C_{18}H_{34}O_2]^+$; 3-(4-hydroxyphenyl)propyl palmitate 390 $[M]^+$, 134 $[M - C_{16}H_{32}O_2]^+$.

## RESULTS AND DISCUSSION

3-Phenylpropenoic (cinnamic) acid analogues such as 4-hydroxycinnamic (*p*-coumaric), ferulic, and sinapic acids are natural phenolic acids occurring ubiquitously in all parts of plants. These hydroxylated cinnamic acids gain importance because of their antioxidant capacity and their beneficial effects on human health. Phenolics including hydroxylated cinnamic acids predominantly occur as hydrophilic compounds. Their use as food additives is limited, therefore, to hydrophilic phases in foods. Lipophilization of hydroxy cinnamic acids derivatives is of great importance in extending their field of applications to fatty food phases (*12*).

Lipase-catalyzed lipophilization of phenolic antioxidants, particularly hydroxycinnamic acids and similar compounds with analogous or inverse chemical structure (**Figure 1**), by esterification of various 3-phenylpropanoic acids with long-chain fatty alcohols or of fatty acids with various 3-phenylpropanols is of special interest for their application as lipophilic antioxidants in oil-based food and feed as well as in cosmetic and technical products. Recently, we have shown that plant steryl and stanyl esters can be efficiently prepared from sterols and stanols via lipase-catalyzed esterification with fatty acids under environmentally friendly conditions, particularly in the absence of organic solvents and drying reagents such as molecular sieves or sodium sulfate (*46*, *47*). In continuation of the above work, we have developed an enzymatic method for the preparation of lipophilic alkyl esters of 3-phenylpropanoic (dihydrocinnamic) and 3-phenylpropenoic (cinnamic) acid derivatives by esterification with medium- or long-chain fatty alcohols. Similarly, compounds with inverse chemical structure, that is, 3-phenylpropyl alkanoates, are prepared by lipase-catalyzed esterification of long-chain fatty acids with various 3-phenylpropan-1-ols. The reactions proceed in vacuo at moderate temperature in the absence of organic solvents and drying reagents using immobilized lipases as biocatalysts.

**Enzyme Screening.** Various esterases, particularly lipases, were checked for the esterification reaction of 4-methoxycinnamic acid with oleyl alcohol (*cis*-9-octadecen-1-ol) as the screening system. The conversions were performed for 24 h at 50 and/or 80 °C in vacuo (80 kPa) without solvent using 50 mg of the respective enzyme preparation. The results of these experiments demonstrated that immobilized lipase B from *Candida antarctica* (Novozym 435) was the biocatalyst with highest enzyme activity for the above esterification reaction (data not shown). A similar esterification activity was determined for immobilized lipase from *Rhizomucor miehei* (Lipozyme RM IM), whereas all other enzymes of the screening, including immobilized lipase from *Thermomyces lanuginosus* (Lipozyme TL IM), lipases from *Candida rugosa*, *Candida lipolytica*, *Chromobacterium viscosum*, *Rh. arrhizus*, *Rh. niveus*, *Rh. javanicus*, *F. oxysporum*, and pectinase from *Aspergillus niger* as well as plant esterases/proteases including papaya (*Carica papaya*) latex, bromelain from *Ananassa comosus*, and ficin from *F. carica*, demonstrated very low esterification activity, if any, under the screening conditions described above (data not shown).

**Lipase Specificities.** The time course of the esterification of cinnamic acid with oleyl alcohol at 80 °C using three commercial immobilized lipases, Novozym 435, Lipozyme RM IM, and Lipozyme TL IM, is shown in **Figure 2**. It is obvious from these results that Novozym 435 shows the highest conversion rates, whereas moderate and very low esterification rates were obtained for Lipozyme RM IM and Lipozyme TL IM, respectively, as biocatalysts.

**Figure 3** shows the time course of the esterification of various dihydrocinnamic (3-phenylpropanoic) and cinnamic (3-phenylpropenoic) acids with oleyl alcohol at 80 °C in vacuo using

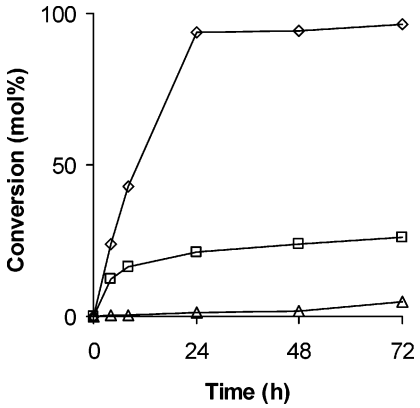


**Figure 2.** Time course of the formation of oleyl cinnamate by esterification of cinnamic acid with oleyl alcohol catalyzed by various immobilized microbial lipases (◇, Novozym 435; □, Lipozyme RM IM; △, Lipozyme TL IM). Reaction conditions: molar ratio of cinnamic acid to oleyl alcohol, 1:1; 50 mg of immobilized lipase; 80 °C. Values are the mean of two determinations. For details see Materials and Methods.

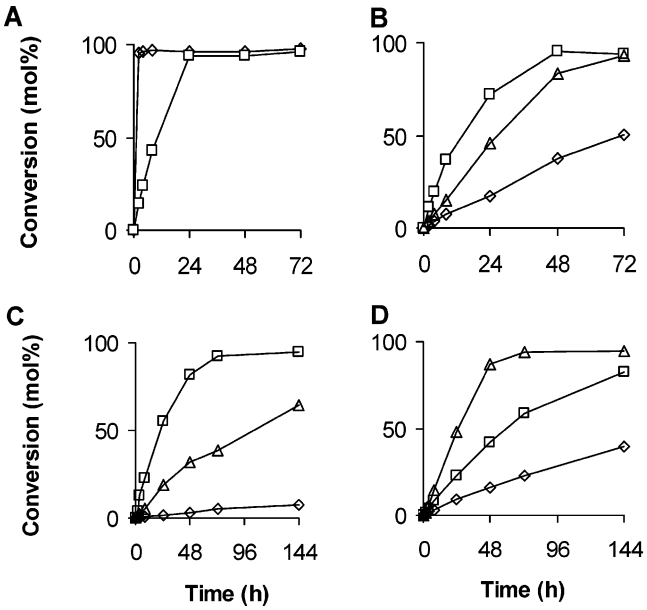


**Figure 3.** Time course of the formation of oleyl esters of cinnamic acid and its analogues including ◇, oleyl dihydrocinnamate, and □, oleyl cinnamate (**A**); ◇, 2-methoxy-, □, 3-methoxy-, and △, 4-methoxycinnamate (**B**); and ◇, 2-hydroxy-, □, 3-hydroxy-, and, △, 4-hydroxycinnamate (**C**), by Novozym 435-catalyzed esterification of dihydrocinnamic, cinnamic, methoxycinnamic, and hydroxycinnamic acids with oleyl alcohol. Time course of the formation of oleyl ferulate (**D**) by esterification of ferulic acid with oleyl alcohol using different amounts of Novozym 435 lipase (◇, 50 mg; □, 100 mg; △, 250 mg). Values are the mean of two determinations. For details see Materials and Methods.

Novozym 435 as biocatalyst. In particular, the formation of oleyl dihydrocinnamate and oleyl cinnamate (**Figure 3A**), various oleyl methoxy- and hydroxycinnamates, for example, 2-methoxy-, 3-methoxy-, and 4-methoxycinnamates (**Figure 3B**), 2-hydroxy-, 3-hydroxy-, and 4-hydroxycinnamates (**Figure 3C**), and oleyl ferulate (4-hydroxy-3-methoxycinnamate) (**Figure 3D**), by Novozym 435-catalyzed esterification of the corresponding cinnamic acids with oleyl alcohol is demonstrated over a period of 72 or 144 h. It is obvious from these results that (a) highest esterification activity is observed with dihydrocinnamic acid (3-phenylpropanoic acid) as the substrate (**Figure 3A**); (b) introduction of a >C═C< double bond to form cinnamic acid (3-phenylprop-2-enoic acid) leads to a large decrease of lipase activity (**Figure 3A**); (c) introduction of a methoxy group (**Figure 3B**) or a hydroxy group (**Figure 3C**) on the phenyl ring leads to further loss of lipase activity; and (d) the presence of more than one methoxy and/or hydroxy group in the substrate molecule (e.g., ferulic, caffeic, and sinapic acids) results in an additional reduction of enzyme activity as demonstrated in **Figure 3D** and **Table 3**.

**Table 3** shows the enzyme activities of three commercial immobilized lipases and maximum conversions for the esterification of various dihydrocinnamic and cinnamic acids with medium- or long-chain fatty alcohols and of fatty acids with various 3-phenylpropanols and 3-phenylpropenols under different reaction conditions. These data demonstrate that immobilized lipase B from *C. antarctica* (Novozym 435) is generally superior to immobilized lipase preparations from *Rh. miehei* (Lipozym RM IM) and, particularly, *Th. lanuginosus* (Lipozyme TL IM) in the production of alkyl dihydrocinnamates and cinnamates. Together with the results shown in **Figure 3** the following relative esterification activities are found for Novozym 435: dihydrocinnamic > cinnamic > 3-methoxycinnamic > dihydrocaffeic ≈ 3-hydroxycinnamic > 4-methoxycinnamic > 2-methoxycinnamic > 4-hydroxycinnamic > ferulic ≈ 3,4-dimethoxycinnamic > 2-hydroxycinnamic acid. With respect to the position of substituents on the phenyl moiety, the esterification activity of Novozym 435 increases in the order 3- > 4- > 2-position (meta > para > ortho) (**Figure 3B**,**C**). It is worth noting that the enzyme activity of Lipozyme RM IM is similar to or even higher than that of Novozym 435 for the esterification of meta-substituted cinnamic acids including 3-hydroxy-, 3-methoxy-, and 3,4-dimethoxycinnamic acids with oleyl alcohol. However, conversions found for Novozym 435-catalyzed esterifications of cinnamic acid analogues are clearly higher than those observed for Lipozyme RM IM-catalyzed ones (**Table 3**). The Novozym 435-catalyzed esterification of long-chain fatty acids with various 3-phenylpropanols generally produces the corresponding 3-phenylpropyl alkanoates in high yield (**Table 3**).

**Reaction Conditions.** Generally, enzyme activity for the esterification of cinnamic acid and its analogues with oleyl alcohol was highest using immobilized lipase B from *C. antarctica* (Novozym 435) at 80 °C in vacuo. Moderate esterification activity is observed for immobilized lipase from *Rh. miehei* (Lipozyme RM IM) and rather low activity for immobilized lipase from *Th. lanuginosus* (Lipozyme TL IM) (**Table 3** and **Figure 2**). All other lipases and esterases screened as described above show far lower esterification activities, if any (data not shown).

Depending on the reaction conditions, conversions to alkyl dihydrocinnamates and cinnamates as well as 3-phenylpropyl alkanoates of up to 98 mol % were obtained by esterification of various dihydrocinnamic and cinnamic acids with medium- and long-chain fatty alcohols such as lauryl, palmityl, and oleyl alcohol or of 3-phenylpropanols with long-chain fatty acids such as palmitic and oleic acids catalyzed by Novozym 435 lipase in vacuo. Increasing the reaction temperature from 60 to 80 °C increased the enzyme activity for the formation of various oleyl 3-phenylpropanoates by 2−6-fold as shown for the esterification of cinnamic and dihydro-, methoxy-, and hydroxycinnamic acids with oleyl alcohol (**Table 3**).

Under the reaction conditions described, equimolar mixtures of substrates or relatively low excess of one of the substrates is necessary to obtain acceptable yields of methoxy- and/or hydroxy-substituted alkyl cinnamates, for example, oleyl 4-hy-

**Table 3.** Enzyme Activities of Various Lipases for the Esterification of 3-Phenylpropenoic (Cinnamic) Acid and Its Analogues with Medium- or Long-Chain Saturated and Unsaturated Fatty Alcohols and for the Esterification of Alkanoic Acids with 3-Phenylpropanol Analogues

| 3-phenylpropenoic (cinnamic) acid and its analogues and fatty acids | medium- and long-chain fatty alcohols and 3-phenylpropanols | lipase | max conversion[a] (mol %) after [h] | enzyme activity[a] (units/g) | ±SEM ($n = x$) |
|---|---|---|---|---|---|
| dihydrocinnamic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 98 [4] | 98[b] | ±0.28 ($n = 2$) |
| dihydrocinnamic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 96 [72][c] | 22.9[c] | ±1.6 ($n = 2$) |
| dihydrocinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme RM IM | 91 [4] | 22.8[d] | ±0.03 ($n = 2$) |
| dihydrocinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme TL IM | 57 [72] | 2.8 | ±0.05 ($n = 2$) |
| cinnamic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 94 [24] | 6.0 | ±0.48 ($n = 3$) |
| cinnamic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 96 [72][c] | 2.7[c] | ±0.79 ($n = 2$) |
| cinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme RM IM | 26 [72] | 3.1 | ±0.64 ($n = 2$) |
| cinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme TL IM | 5 [72] | nd[i] | – ($n = 2$) |
| 2-methoxycinnamic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 51 [72] | 0.9 | ±0.15 ($n = 3$) |
| 2-methoxycinnamic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 12 [72][c] | nd[c] | – ($n = 2$) |
| 2-methoxycinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme RM IM | 7 [72] | nd | – ($n = 2$) |
| 2-methoxycinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme TL IM | <1 [72] | nd | – ($n = 2$) |
| 3-methoxycinnamic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 96 [48] | 4.9 | ±0.56 ($n = 4$) |
| 3-methoxycinnamic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 95 [72][c] | 1.8[c] | ±0.15 ($n = 2$) |
| 3-methoxycinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme RM IM | 58 [72] | 5.6 | ±0.39 ($n = 3$) |
| 3-methoxycinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme TL IM | 4 [72] | nd | – ($n = 2$) |
| 4-methoxycinnamic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 94 [72] | 1.8 | ±0.25 ($n = 3$) |
| 4-methoxycinnamic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 22 [72][c] | 0.3[c] | ±0.01 ($n = 2$) |
| 4-methoxycinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme RM IM | 35 [72] | 0.9 | ±0.14 ($n = 2$) |
| 4-methoxycinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme TL IM | 2 [72] | nd | – ($n = 2$) |
| 2-hydroxycinnamic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 8 [144] | nd | – ($n = 2$) |
| 2-hydroxycinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme RM IM | <1 [144] | nd | – ($n = 2$) |
| 2-hydroxycinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme TL IM | <1 [144] | nd | – ($n = 2$) |
| 3-hydroxycinnamic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 94 [144] | 3.3 | ±0.40 ($n = 2$) |
| 3-hydroxycinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme RM IM | 28 [144] | 1.9 | ±0.26 ($n = 4$) |
| 3-hydroxycinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme TL IM | <1 [144] | nd | – ($n = 2$) |
| 4-hydroxycinnamic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 65 [144] | 0.6 | ±0.01 ($n = 2$) |
| 4-hydroxycinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme RM IM | 10 [144] | 0.6 | ±0.03 ($n = 2$) |
| 4-hydroxycinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme TL IM | <1 [144] | nd | – ($n = 2$) |
| 3,4-dimethoxycinnamic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 25 [72] | 0.3 | ±0.06 ($n = 4$) |
| 3,4-dimethoxycinnamic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 4 [72][c] | nd[c] | – ($n = 2$) |
| 3,4-dimethoxycinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme RM IM | 27 [72] | 1.3 | ±0.26 ($n = 3$) |
| 3,4-dimethoxycinnamic acid | *cis*-9-octadecen-1-ol | Lipozyme TL IM | 2 [72] | nd | – ($n = 2$) |
| ferulic acid | 2-ethylhexan-1-ol | Novozym 435 | 73 [144] | 2.0 | ±0.17 ($n = 3$) |
| ferulic acid | dodecan-1-ol | Novozym 435 | 27 [72] | 0.3[e] | ±0.03 ($n = 5$) |
| ferulic acid | hexadecan-1-ol | Novozym 435 | 15 [72] | 0.3[e] | ±0.04 ($n = 3$) |
| ferulic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 25 [72] | 0.4[e] | ±0.03 ($n = 2$) |
| ferulic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 82 [144][f] | 0.5[e,f] | ±0.03 ($n = 4$) |
| ferulic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 94 [144];[g] 98 [48][h] | 0.4[e,g] | ±0.02 ($n = 2$) |
| ferulic acid | *cis*-9-octadecen-1-ol | Lipozyme RM IM | 27 [72][f] | 0.3[e,f] | ±0.01 ($n = 2$) |
| dihydrocaffeic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 30 [72] | 3.4 | ±0.15 ($n = 2$) |
| caffeic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 33 [144] | 0.1[g] | 0.01 ($n = 2$) |
| sinapic acid | *cis*-9-octadecen-1-ol | Novozym 435 | 23 [144][g] | nd | – ($n = 2$) |
| oleic acid | cinnamic alcohol | Novozym 435 | 79 [4] | 19.7 | ±0.21 ($n = 2$) |
| palmitic acid | cinnamic alcohol | Novozym 435 | 85 [24] | 11.5 | ±2.3 ($n = 2$) |
| oleic acid | 3-phenylpropan-1-ol | Novozym 435 | 93 [4] | 23.3 | ±0.03 ($n = 2$) |
| palmitic acid | 3-phenylpropan-1-ol | Novozym 435 | 93 [4] | 23.3 | ±0.19 ($n = 2$) |
| oleic acid | 3-(4-hydroxyphenyl)propan-1-ol | Novozym 435 | 90 [4] | 22.4 | ±0.61 ($n = 2$) |
| palmitic acid | 3-(4-hydroxyphenyl)propan-1-ol | Novozym 435 | 96 [8] | 22.0 | ±0.25 ($n = 2$) |
| oleic acid | 3-(3,4-dimethoxyphenyl)propan-1-ol | Novozym 435 | 91 [8] | 21.8 | ±2.6 ($n = 2$) |
| palmitic acid | 3-(3,4-dimethoxyphenyl)propan-1-ol | Novozym 435 | 85 [8] | 19.4 | ±3.2 ($n = 2$) |

[a] Standard assay conditions, if not otherwise indicated: 0.3 mmol of carboxylic acid + 0.3 mmol of alcohol; immobilized lipase/assay, 50 mg; 80 °C; 80 kPa; 4 h. [b] Twelve and a half milligrams of Novozym 435. [c] Maximum conversion and enzyme activity at 60 °C. [d] Enzyme activity after 2 h: 45 units/g. [e] Enzyme activity after 24 h. [f] One hundred milligrams of immobilized lipase preparation. [g] Two hundred and fifty milligrams of Novozym 435. [h] Molar ratio ferulic acid to oleyl alcohol, 1:2 or 2:1; 250 mg of Novozym 435. [i] nd, not determined.

droxycinnamate and oleyl ferulate (**Table 3**), which differs from the enzyme-catalyzed esterification methods developed by others (*12*, *37*−*43*). Moreover, the procedure described above for the Novozym 435-catalyzed esterification of various substituted cinnamates including hydroxycinnamates requires much less time as compared to those reported by others (*37*, *39*−*42*). Large amounts of immobilized lipase B from *C. antarctica* (Novozym 435) are necessary, however, to obtain satisfactory yields, particularly of alkyl cinnamates bearing more than one methoxy or hydroxy group (**Table 3** and **Figure 3**). This was found in previous studies as well (*12*, *37*, *39*, *40*). Further research will deal with experiments reducing the enzyme concentration for the preparation of methoxy- and hydroxy-substituted alkyl dihydrocinnamates and cinnamates as well as 3-phenylalkyl alkanoates.

The molecular ions and typical mass fragments of the esterification products, that is, alkyl cinnamates and cinnamyl alkanoates, obtained by GC-MS confirmed the chemical structures of these compounds as given above. These data agree well with those given in the literature (*32*).

In conclusion, we have developed a lipase-catalyzed esterification procedure for the preparation of lipophilic alkyl cinnamates and similar compounds with inverse chemical structure, particularly hydroxy- and/or methoxy-substituted alkyl

3-phenylpropanoates and alkyl 3-phenylpropenoates as well as 3-phenylpropyl alkanoates, in high to moderate yield. Immobilized lipase B from *C. antarctica* (Novozym 435) and, to a lesser degree, immobilized lipase from *Rh. miehei* (Lipozyme RM IM) were found to be the most effective biocatalysts for these reactions, which were performed at moderate temperatures in vacuo in the absence of solvents and drying agents. The reaction products, particularly hydroxy- and/or methoxy-substituted medium- and long-chain alkyl 3-phenylpropenoates and 3-phenylpropyl alkanoates such as oleyl ferulate and 3-(4-hydroxyphenyl) palmitate, may be useful lipophilic antioxidants for food and feed as well as cosmetic and technical applications.

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