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Effect of Molecular Sieves on Lipase-Catalyzed Esterification of Rutin with Stearic Acid

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Rutin was acylated with stearic acid in the esterification reaction catalyzed by immobilized *Candida antarctica* lipase B (Novozym 435) in *tert*-amyl alcohol with and without molecular sieves. The lipophilic rutin stearate was synthesized by this method, which had a potential use in food, cosmetics, and pharmacy. The structure of rutin stearate was characterized by spectral methods of ^{1}H NMR and ^{13}C NMR, Fourier transform infrared, and UV−vis. The results suggested that the regioselectivity of the lipase-catalyzed esterification of rutin was specific at the $C_{4''}$-position of the rhamnose moiety. It was found that the addition of molecular sieves increased both the reaction rate and the yield. The time effect of adding molecular sieves in the reaction system on the conversion of rutin stearate was further examined. Instead of adding molecular sieves at the beginning of the reaction, the addition of molecular sieves at 5, 18, 24, 31, and 44 h after the beginning of the reaction was also applied. The final conversion for the case to add molecular sieves at 24 h after the beginning of reaction was the highest, with the conversion yield about 46%.

KEYWORDS: Lipase-catalysis; esterification; rutin; rutin stearate; Novozym435; molecular sieves; spectrometry; regioselectivity

INTRODUCTION

Flavonoids are widely distributed in different parts of miscellaneous plants. It was well-known that there are at least 6000 name-given flavonoids in nature (*1*). Animals and human beings cannot synthesize flavonoids by themselves but take these compounds by eating plants. In fact, flavonoids are also significantly present in many common foods and drinks, such as vegetable, fruit, tea, beer, and red wine (*2*, *3*). Rutin is a famous and typical one in the family of flavonoids. These flavonoids contained in foods and drinks are an integral part of the human diet. They exhibit a wide range of biological activities, such as anticancer (*4*), anti-inflammatory (*5*, *6*), antivirus (*7*), anticoagulant (*8*, *9*), antiatherosclerosis (*10*), and inhibitory effects on low-density lipoprotein (LDL) oxidation (*3*, *10*−*12*). The flavonoids are also known as potential cell growth inhibitors (*13*) and multidrug resistance modulators (*14*). Because of their biological properties, the use of flavonoids in food, cosmetic, and pharmaceutical preparations is currently a subject of great interest (*15*, *16*). Unfortunately, most of the naturally occurring flavonoids (mainly flavonoid glycosides) show low solubility and stability in lipophilic media (*17*) and ineffectiveness in stabilizing fats and oils (*18*). These properties limit the development of nutritional food and commodity-containing flavonoids. Most of biological activities of flavonoids may arise from their resistance to oxidation including scavenging free radicals, chelating transition metal ions, and protecting the lipid from peroxidization (*19*). Generally, flavonoid aglucones show more excellent antioxidation as compared with their glucosides because the former exhibit strong lipotropy and partition preferentially into the hydrophobic core of the biomembrane (*20*). These results are confirmed by protecting LDL (*21*) and the complex of carotenoids and unsaturated fatty acids from oxidation (*22*, *23*) by flavonoids. Generally, the polyhydroxyl and saccharide groups of flavonoid glucosides lead to some hydrophilic property of glucosides, which will compress their antioxidative activity.

Esterification of the hydroxyl groups by fatty acids is a possible way to improve the hydrophilic property of flavonoid glucosides. In the classical esterification catalyzed by chemical catalyst, most of hydroxyl groups may be acylated, which leads to a mixture of products with various degrees of esterification. Once the active hydroxyls are acylated, the biological activity of flavonoids will be reduced or even lost (*24*, *25*). However, the enzymatic catalysis is an alternative because enzymes are very regioselective. More attention has recently been paid to enzymatic esterification of flavonoid glucosides. Daniele and Riva (*26*) have reported that in subtilisin-catalyzed esterification, the acylation of isoquercitrin and luteolin-7-glucoside takes place at both the sugar primary and a secondary OH, affording mainly the corresponding 6″-*O*- esters accompanied by the 3″-*O*-monoesters and 3″,6″-*O*-diester; however, the rhamnoside of quercitrin is not acylated. Rutin and hesperidin give single monoesters at 3″ of their glucose moieties. *Candida antarctica* lipase exhibit regioselectivity at $C_{3''}$, $C_{6''}$-OH of glucose moiety and $C_{4''}$-OH of rhamnose moiety, giving corresponding 6″-*O*-

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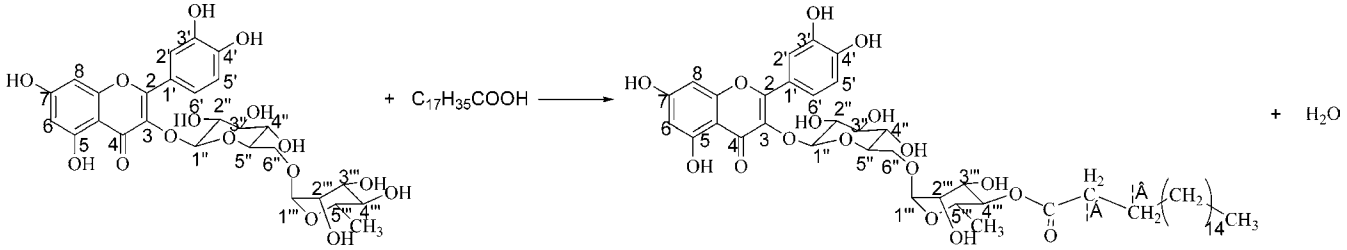
Scheme 1



mono-, 3″-*O*-mono-, and 3″, 6″-*O*-diesters of isoquercitrin, a 4″-*O*-ester of quercitrin, and a 3″,4‴-*O*-diester of rutin (*27*). Nakajima et al. (*28*) have obtained 6″-*O*-caffeate, 6″-*O*-*p*-coumarate, 6″-*O*-ferulate, and 6″-*O*-cinnamate, using the cultured cells as biocatalysts. These results indicate that the sugar moiety nature and enzyme origin affect the acylation position of flavonoid glucosides. They also suggested that acetone and acetonitrile are the more appropriate solvents for *C. antarctica* lipase-catalyzed esterification (*29*). Kontogianni et al. (*18*) have reported that higher conversions were achieved when using acetone or *tert*-butanol as a solvent system but tertrahydrofuran is incompatible with the lipase used. Gayot et al. (*30*) have reported that the hydration of the reaction medium leads to low conversion yield and specific activity of lipase during the synthesis of plamitate naringin by *C. antarctica* lipase. This result has also been confirmed by Ardhaoui et al. (*31*), who have suggested that a small variation of the water content of the medium leads to a drastic change of the water activity and the equilibrium shifts toward the hydrolysis even if the water content is low. The kinetic study of naringin esterification with decanoic acid has been reported by Kontogianni et al. (*18*), who have indicated that the acylation reaction follows Michaelis−Menten kinetics. The results of acyl donor consumption kinetics in rutin and naringin esterification suggest that the concentration decreases quickly in the beginning and then gradually slows down until a plateau is reached, which corresponds to the thermodynamic equilibrium (*31*).

It was suggested that the lipophilic rutin stearate was synthesized by this method, which had a potential to be used in food, cosmetics, and pharmacy. In this work, the structure of rutin stearate was characterized by spectral methods of ^{1}H NMR and ^{13}C NMR, Fourier transform infrared (FT-IR), and UV−vis. These results suggested that the regioselectivity of the lipase-catalyzed esterification of rutin was specific at the $C_{4'''}$-position of the rhamnose moiety.

Water is very important in esterification, the nature of which is to reverse reaction. Generally, the removal of water from the reaction system is necessary for the formation of ester by a feasible way. An application of molecular sieves to the esterification is an efficient way to remove water formed in the reaction system. By now, a lot of literature have reported the effect of water content, acyl donor structure, flavonoid backbone, enzyme origin, and solvent nature on the results of the acylation (*18*, *26*−*31*). However, the effect of the addition of molecular sieves at different times on the conversion in the esterification has not yet been reported in detail. In the present paper, the lipophilic rutin stearate was obtained by acylation of rutin with stearic acid using the immobilized *C. antarctica* lipase B (Novozym 435) as a catalyst. We are particularly interested in determining experimentally the effect of the addition of molecular sieves on the conversion of the esterification. The experimental design included the lipases-catalyzed synthesis with and without molecular sieves, as well as with the addition of molecular sieves at different times after the beginning of the reaction.

MATERIALS AND METHODS

Enzymes and Chemicals. The immobilized lipase B was from *C. antarctica* (Novozym 435) (enzyme activity: 10470 PLU/g), and it was purchased directly from Novo-Nordisk Co. (China). Rutin (≥95%) was from Beijing Biochemical Reagent Co. (China). Both of them were used as received. The acyl donor, stearic acid, was analytical grade and provided by Chengdu Kelong Chemical Plant. The *tert*-amyl alcohol was from Beijing Chemical Plant (China). Molecular sieves (4 Å) were purchased from Sino-American Global Molecular Sieves Co. (Shanghai, China). Silica gel (44−74 μm) was purchased from Qingdao Haiyang Chemical Plant (China).

Syntheses of Rutin Stearate. A 762 mg amount of rutin was dissolved in 85 mL of *tert*-amyl alcohol in a 100 mL three-necked flask. The stearic acid in a 1:5 (rutin:stearic acid) molar ratio was added, and the solution was heated to 60 °C with constant stirring at 300 rpm with a mechanical stirrer. Direct esterification reactions were started by the addition of 340 mg of Novozym 435. Activited molecular sieves (4 Å) were added to the reactor with 100 g/L after some time of reaction to control the water content in the reaction medium. Finally, the reaction was stopped and the enzyme was filtered off. The solvent was removed by the vacuum rotary evaporator.

Drying Conditions of Chemicals. Rutin and stearic acid were dried in a desiccator under vacuum for 1 week. The *tert*-amyl alcohol was dried for 5 days with 100 g/L of molecular sieves (4 Å). Molecular sieves were activated by heating at 150 °C for 24 h before use.

Purification of Rutin Stearate. The products of esterification reactions were loaded onto a silica gel column (22 cm × 2.5 cm, i.d.) and eluted with ethyl acetate/ethanol/water (15:1:1) (v/v/v). The elutes were subjected to thin-layer chromatography (TLC) (silica gel 60, purchased from Merck, Germany) eluted ethyl acetate/ethanol/water/formic acid (15:1:2:1) (v/v/v/v), and then analyzed at a wavelength of 254 nm in the ultraviolet region. The combination of fractions having the same value of retardation factor R_f (0.67) was loaded on Sephadex LH-20 (Merck) and eluted with pure methanol to give yellow powders. The powders were recrystallized from methanol, and the pure rutin esters were obtained.

FT-IR and NMR of Rutin Stearate. The FT-IR spectrum was performed on a Nicolet 17OSX spectrometer with a scanning range from 4000 to 400 cm^{-1}. The ^{1}H NMR spectrum was measured on a Varian INOVA400 spectrometer operating at 400 MHz, and the ^{13}C NMR spectrum was measured on a Bruker AVANCE300 spectrometer operating at 300 MHz, using Me_4Si as an internal standard.

High-Performance Liquid Chromatography (HPLC). The reactions were monitored quantitatively by HPLC. Analyses were conducted with a Shimadzu System (Shimadzu, Japan) equipped with a column (Shimadzu RP-18, 150 mm × 4.6 mm, 5 μm, Japan). The separation of the different components of the reaction products was performed using the following gradient: from 10 to 60% methanol in H_2O in 15 min, then from 60 to 100% methanol in H_2O in 10 min, and 100% methanol for 20 min. The flow rate was 1 mL/min. The oven temperature was 30 °C. The different compounds were quantified by a UV−vis detector at 280 nm.

RESULTS AND DISCUSSION

The chemical reaction of the enzyme synthesis of rutin stearate was expressed in **Scheme 1**.

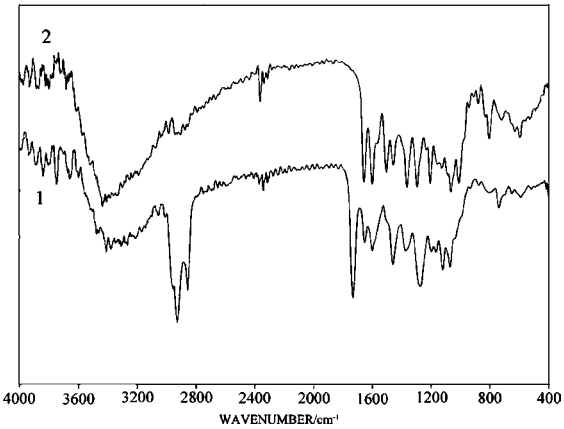


Figure 1. FT-IR spectra of rutin stearate (**1**) and rutin (**2**).

Table 1. ^{1}H NMR Chemical Shifts of the Rutinose Moiety of Rutin Stearate, the Rutinose Moiety of Rutin, and Stearic Acid

atom	rutinose moiety of rutin stearate	rutinose moiety of rutin	stearic acid
H-1″	5.32	5.32	
H-2″	3.73–2.65	3.74–3.04	
H-3″	3.73–2.65	3.74–3.04	
H-4″	3.73–2.65	3.74–3.04	
H-5″	3.73–2.65	3.74–3.04	
H-6″	3.73–2.65	3.74–3.04	
H-1‴	4.48	4.42	
H-2‴	3.73–2.65	3.74–3.04	
H-3‴	3.73–2.65	3.74–3.04	
H-4‴	4.66	3.74–3.04	
H-5‴	3.73–2.65	3.74–3.04	
fatty chain $CH_2\alpha$	2.21–2.13		2.18–2.13
fatty chain $CH_2\beta$	1.5		1.47–1.43
fatty chain $-CH_2-$	1.25–1.13		1.21
fatty chain CH_3	0.95		0.83
CH_3 rhamnosyl	0.85	1.01	

Structural Characterization of Rutin Stearate Synthesized by Enzyme. The formation of rutin stearate was confirmed by comparison of FT-IR spectra of rutin and its ester. **Figure 1** shows FT-IR spectra of rutin stearate (curve 1) and rutin (curve 2). A peak at 1732 cm^{-1} was attributed to vibration of carbonyl group and the peaks around 2950−2850 cm^{-1} were attributed to vibration of C−H of methyl and methylene groups. The chemical change in functional groups of the samples indicated the formation of rutin stearate.

Figure 2 and **Figure 3** show ^{1}H NMR spectrum of rutin stearate and rutin, respectively. The number of residuals of stearic acid linked on rutin and their position on the glycoside moiety or the aglucone moiety could be determined by the comparison of ^{1}H NMR spectra between the rutin and the rutin stearate. **Table 1** shows the selected ^{1}H NMR chemical shifts of the rutinose moiety of rutin stearate, the rutinose moiety of rutin, and stearic acid. For the flavone protons, no significant change in chemical shift was observed. Only a downfield at 4.66 ppm for rutin stearate was observed in the spectra of the glycoside moiety. The glycoside moiety of rutin was monoacylated.

Danieli et al. (*27*) have reported that isoquercitrin acylation takes place at both the sugar primary and a secondary OH, and the acylation of quercitrin occurs on the secondary 4‴-OH of the rhamnose. These results suggested that the selectivity of *C. antarctica* lipase is precisely on the primary and a secondary OH of the glucose moiety and the secondary 4‴-OH of the rhamnose moiety. The precise position, on which the acylation took place, was obtained from the results of ^{13}C NMR spectra.

Table 2. ^{13}C NMR Chemical Shifts of the Rutinose Moiety of Rutin Stearate, the Rutinose Moiety of Rutin, and Stearic Acid

atom	rutinose moiety of rutin stearate	rutinose moiety of rutin	stearic aicd
$C_{2''}$	71.6	72.2	
$C_{3''}$	76.9	76.8	
$C_{4''}$	76.3	76.2	
$C_{2'''}$	70.9	70.9	
$C_{3'''}$	69.3	70.3	
$C_{4'''}$	74.7	70.4	
$C_{5'''}$	67	67.3	
CH_3 rhamnosyl	18.1	18.1	
C═O ester	174.0		174.5
fatty chain $CH_2\alpha$	34.7		34.0
fatty chain $CH_2\beta$	31.8		31.8
fatty chain $-CH_2-$	29.7–15.2		29.7–22.5
fatty chain CH_3	12.1		14.0

Figure 4 shows ^{13}C NMR spectrum of rutin stearate. **Table 2** shows the selected ^{13}C NMR chemical shifts of the rutinose moiety of rutin stearate, the rutinose moiety of rutin, and stearic acid. As shown in the table, the chemical shifts of C4″ and C3″ of glucose moiety of rutin stearate were similar to those of the rutin. Apparently, the stearic acid was not acylated to either of the positions. In the rhamnose moiety, there is a significant downfield shift at the $C_{4'''}$-position (δ = 74.7 ppm) of the rutin stearate as compared to the chemical shift of the $C_{4'''}$-position of the rutin (δ = 70.4 ppm). However, there is no significant change in chemical shifts at $C_{3'''}$ between rutin stearate and rutin. Therefore, it is obvious that acylation takes place on the secondary 4‴-OH of the rhamnose moiety (**Scheme 1**).

Selected ^{1}H NMR data of rutin stearate (DMSO-d_6): δ 7.53 (2H, m, $H_{2'}$ and $H_{6'}$), 6.78 (1H, d, J = 9 Hz, $H_{5'}$), 6.28 (1H, s, H_8), 6.11 (1H, s, H_6), 5.32 (1H, d, J = 7.4 Hz, $H_{1''}$), 4.66 (1H, t, J = 9 Hz, $H_{cacylated}$), 4.48 (1H, s, $H_{1'''}$), 3.73−2.65 (9H, m, $H_{rhamnoglucosyl}$), 2.21−2.13 (2H, m, $CH_2\alpha$ fatty chain), 1.5 (2H, m, $CH_2\beta$ fatty chain), 1.25−1.13 (28H, m, $-CH_2$ fatty chain), 0.95 (3H, t, J = 7.0 Hz, CH_3 fatty chain), 0.85 (3H, d, J = 6.7 Hz, CH_3 rhamnosyl).

Selected ^{1}H NMR data of rutin (DMSO-d_6): δ 12.48 (1H, s, OH_5), 7.52 (2H, m, $H_{2'}$ and $H_{6'}$), 6.80 (1H, d, J = 9 Hz, $H_{5'}$), 6.36 (1H, s, H_8), 6.18 (1H, s, H_6), 5.32 (1H, d, J = 7 Hz, $H_{1''}$), 4.42 (1H, s, $H_{1'''}$), 3.74−3.04 (10H, m, $H_{rhamnoglucosyl}$), 1.01 (3H, d, J = 7.2 Hz, CH_3, rhamnosyl).

Selected ^{13}C NMR data of rutin stearate (DMSO-d_6): δ 177.5 (C_4), 174.0 (C═O, ester), 168.3 (C_7), 157.7 (C_9 or C_2), 150.2 ($C_{4'}$), 133 (C_3), 122.5 ($C_{6'}$), 116.7 ($C_{5'}$), 77.9 ($C_{3''}$), 75.3 ($C_{4''}$), 74.7 ($C_{4'''}$), 71.6 ($C_{2''}$), 70.9 ($C_{2'''}$), 69.3 ($C_{3'''}$), 67 ($C_{5'''}$), 34.7 ($CH_2\alpha$ fatty chain), 31.8 ($CH_2\beta$ fatty chain), 29.7−15.2 ($-CH_2-$ fatty chain), 12.1 (CH_3 fatty chain).

HPLC of Reaction Mixtures. In order to follow the enzyme synthesis of rutin stearate, we analyzed the reaction mixture by HPLC during the reaction. The typical HPLC chromatograms of the reaction mixture are shown in **Figure 5**. In the figure, there are two main peaks, which represent the reaction starting material and product, respectively. The former was rutin, and the latter was rutin stearate on the basis of the difference in retention time. The retention times of HPLC for rutin and rutin stearate were 17.5 and 29.4 min, respectively. This was an expected result because the hydrophobicity of rutin stearate is much larger than that of the rutin and its retention time should be longer than that of rutin in the inversed phase chromatography. UV−vis spectra of both compounds were obtained by utilizing the HPLC equipped with diode array detection, as

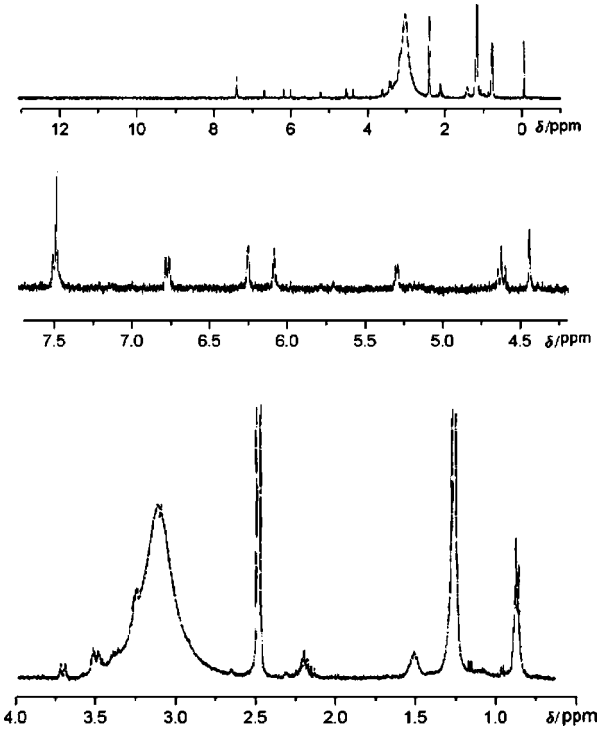


Figure 2. ^{1}H NMR spectrum of rutin stearate.

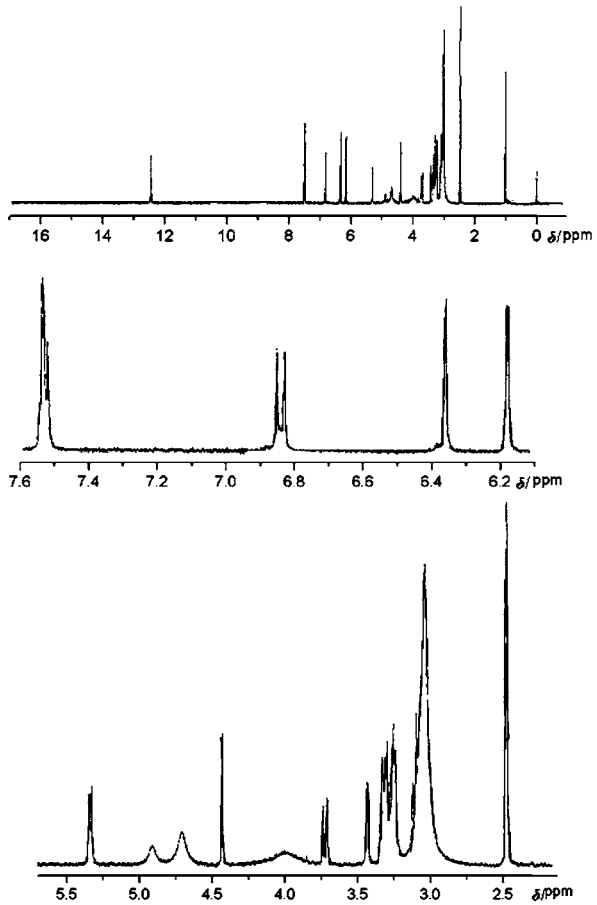


Figure 3. ^{1}H NMR spectrum of rutin.

shown in **Figures 6** and **7**, respectively. The UV−vis spectrum of rutin in **Figure 6** is the same as any standard reference. As shown in the figures, the band maxima (256 and 357 nm) of the rutin stearate are almost the same as those of the rutin (256 and 356 nm). This was quite plausible because the residual of

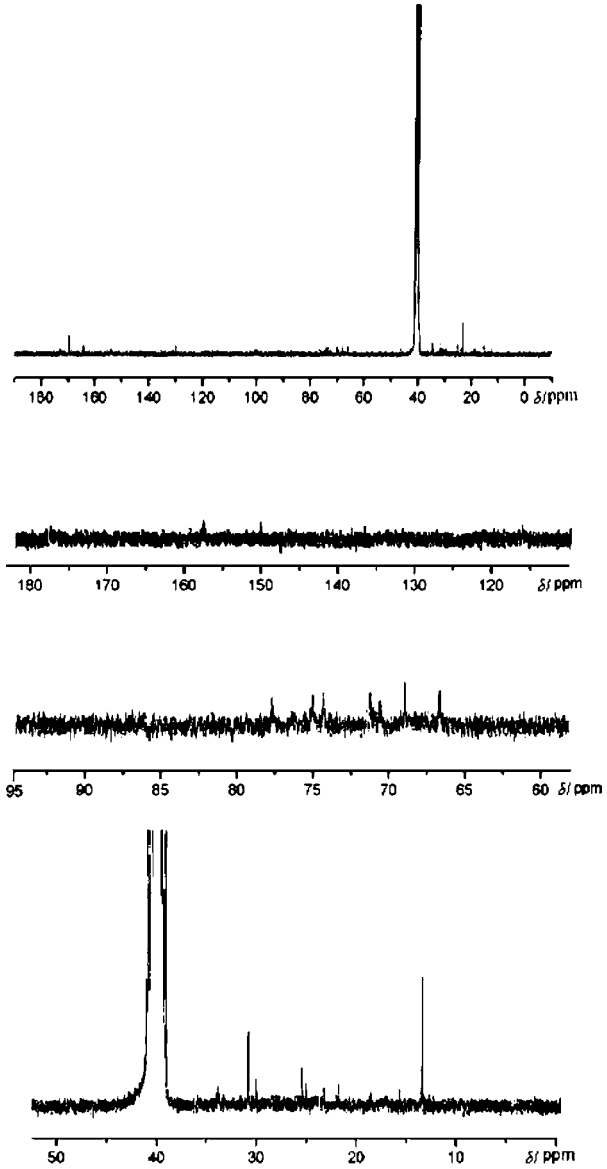


Figure 4. ^{13}C NMR spectrum of rutin stearate.

the stearic acid in rutin stearate only gave a little disturbance to the main rings of flavonoids. The chemical structure of stearate rutin has been confirmed previously by the studies of IR and NMR spectra for the pure sample, which was obtained from the purification by the silica gel column.

It was noteworthy that in the HPLC chromatograms two other peaks following peaks of rutin and stearate rutin were observed, and their retention times were about 18.9 and 30.2 min, respectively. The corresponding UV−vis spectra are listed in **Figure 8**. The peak at about 18.9 min had two absoptions at 255 and 356 nm (**Figure 8a**), and the absorptions of the peak at about 30.2 min were at 256 and 357 nm (**Figure 8b**). These absorptions were usually special for flavonoids. It was suggested that the first peak represented an impurity of flavonoids in the raw material of rutin and the second its lipase-catalyzed product, which will be a subject of our future study.

Role of Molecular Sieves 4 Å in Enzymatic Synthesis of Rutin Stearate. Humeau et al. (*32*) and Patti et al. (*17*) have reported that the enzymatic esterification in the organic media is favored by an excess of the acyl donor. Moreover, Novozym 435 is known to be highly active at high temperatures (*33*, *34*). Therefore, in the present study, the molar ratio of 1:5 (rutin:

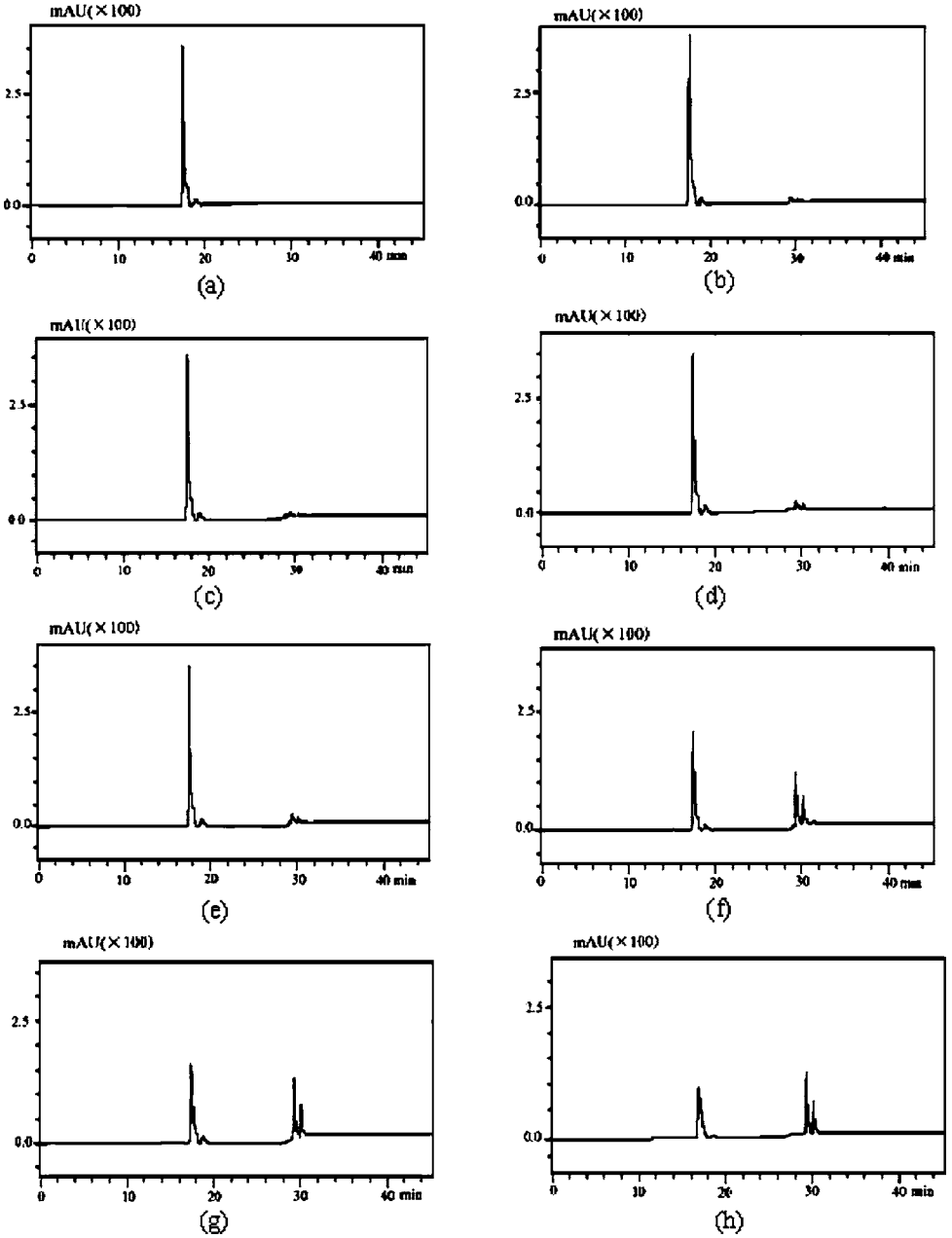


Figure 5. HPLC chromatograms of the lipase-catalyzed reaction mixture of rutin and stearic acid at different reaction times: (**a**) 0, (**b**) 3, (**c**) 7, (**d**) 19, (**e**) 31, (**f**) 55, (**g**) 79, and (**h**) 96 h. The retention time of the first peak is 17.5 min (for rutin), the second is 18.9 min (for impurity of rutin), the third is 29.4 min (for impurity of rutin stearate), and the last is 30.2 min (for possible product of impurity of rutin).

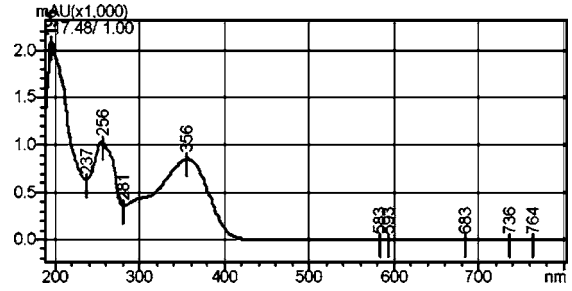


Figure 6. UV–vis spectrum of rutin.

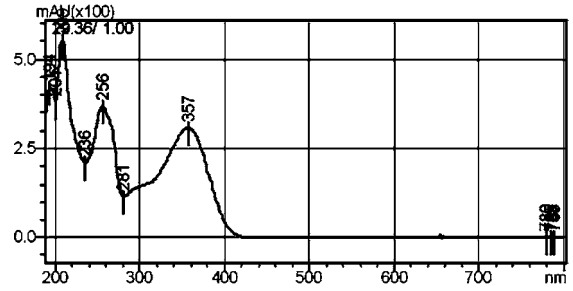


Figure 7. UV–vis spectrum of stearate rutin.

stearic acid) and the temperature of 60 °C were chosen as the basic parameters in the synthesis.

The water content is a very important parameter in the enzymatic esterification in organic medium. On the other hand, water surrounding enzyme further reduces the rigidity of protein in the enzyme by forming hydrogen bonds, which allow extension of the protein to expose the active section (*35*). The loss of this hydration layer leads to low enzymatic activity. Gayot et al. (*30*) have reported that drying of the enzyme leads to a sharp decline in the conversion by 60% and the specific activity by 40% as compared to the nondried Novozym 435. On the other hand, excess water leads to lower esterification

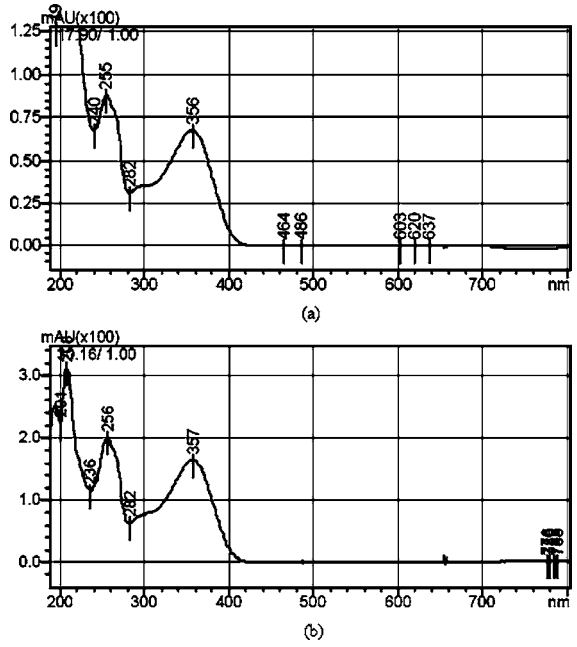


Figure 8. UV–vis spectra of unknown impurity (**a**) and its possible product (**b**).

conversion yield because water can compete with the hydroxyl groups of rutin and hydrolyzes the acyl-enzyme intermediates, which are critical in enzymatic synthesis. Ardhaoui et al. (*31*) have indicated that as the water content increases from 200 to higher than 400 ppm, the acid conversion yield decreases from 76 to 55%. The effect of the water content has also been reported by Gayot et al. (*30*) in the case of the naringin acylation with palmitic acid catalyzed by *C. antarctica* lipase. They indicated that the conversion yield of naringin increases by 63% and the specific activity by 60% as compared to the nondried medium.

Taking into account the role played by the water content in esterification reactions, we dried the reaction medium components except Novozym 435 before rutin stearate synthesis. An amount of molecular sieves (4 Å) was added to remove the water produced during the reaction.

Molecular sieves are framework compounds and are usually made of synthetic zeolites. They are used to adsorb or separate molecules. The adsorbed molecules are trapped in "cages", the size of which can be selected to suit solvent. The cage size determines the performance of these adsorbents. For example, when the 3 Å molecular sieves are added as a desiccant, they will adsorb molecules having a critical diameter less than 3 Å, and the enzyme is inactivated presumably due to stripping of essential water (*36*). In the work of Giacometti, 5 Å molecular sieves were used, and they claimed that these molecular sieves will not decrease the enzyme activity (*36*). In the present work, 4 Å molecular sieves were used and they adsorbed molecules having a critical diameter less than 4 Å, such as water and various gases. The water adsorption capacity of 4 Å molecular sieves was about 22% (by weight).

The curves in **Figure 9** show the conversion in the lipase-catalyzed esterification with and without molecular sieves. The upper curve represents the esterification with the addition of molecular sieves at the beginning of the reaction and the lower curve in the absence of molecular sieves. The conversion was increased quickly first after adding molecular sieves and then gradually approached the equilibrium. However, for the reaction without molecular sieves, after the rapid increase of conversion in the first 20 h of the reaction, a very slow increase of conversion could be observed between about 21 and 55 h of

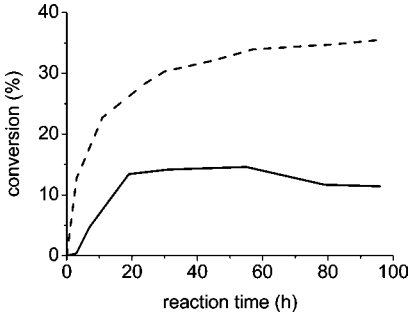


Figure 9. Time dependence of conversion of rutin in lipase-catalyzed esterification with or without molecular sieves. To add molecular sieves to the reaction mixture at the beginning of the reaction. Key: —, without molecular sieves; - - -, with molecular sieves.

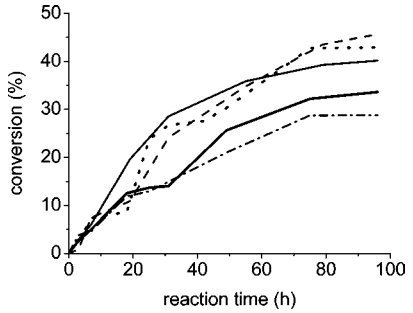


Figure 10. Time dependence of conversion of rutin in lipase-catalyzed esterification with adding molecular sieves at different times after the beginning of the reaction. Key: —, 5 h; • • •, 18 h; – – –, 24 h; - • -, 31 h; and –, 44 h.

reaction. A decrease of the conversion was observed after a reaction time longer than 55 h, as a result of the hydrolysis of esters. Because both reactions were carried out under the same conditions, it was obvious that the slopes of the conversion curves represented the reaction rate. As shown in **Figure 9**, at the very beginning of the reaction, the slope of the upper curve was much greater than that of the lower one, which indicated that the addition of molecular sieves could increase the rate of lipase-catalyzed esterification significantly. A similar effect of adding molecular sieves on the reaction rate also was observed for the enzymatic esterification of glycerol with oleic acid by Giacometti et al. (*36*).

In addition to the addition of molecular sieves at the beginning of the reaction, we also carried out a series of experiments by adding the molecular sieves at 5, 18, 24, 31, and 44 h after the beginning of the reactions. The results are shown in **Figure 10**. In all cases, we found that the addition of molecular sieves led to the abrupt increase in the reaction rate. Moreover, the increase in rate upon the addition of the molecular sieves also resulted in the increase in the yield of conversion. As a consequence, the final yield of conversion was greater than that of the addition of molecular sieves at the beginning of the reaction. As shown in **Figure 10**, the highest yield of conversion was obtained when the addition of the molecular sieves was at 24 h after the reaction, which amounted to a total yield of 46%.

When the molecular sieves were added at 31 and 44 h after the beginning of the reaction, the rate of conversions began to decrease after 20 h upon the addition of molecular sieves. Gayot et al. have a similar observation in their study of the acylation of naringin by palmitic acid (*30*). They found that the acylation rate decreases significantly 24 h after the reaction with no addition of molecular sieves. This decrease in rate may arise from the hydrolysis of the ester by the water formed in the solution as a result of esterification. When the content of water

was high in the solution, the enzyme catalyzed more easily the hydrolysis of the ester linkage as compared with their synthesis.

It is concluded that the optimization time of addition of molecular sieves is about 24 h after the beginning of reaction, and in this optimization condition, the conversion of rutin stearate by lipase-catalyzed synthesis arrives at the maximum.

LITERATURE CITED

(1) Harborne, J. B.; Baxter, H. *The Handbook of Natural Flavonoids*; Wiley: New York, 1999; Vols. 1 and 2.

(2) Hollman, P. C. H.; Katan, M. B. Absorption, metabolism and health effects of dietary flavonoids in man. *Biomed. Pharmacother.* **1997**, *51*, 305−310.

(3) Hollman, P. C. H.; Katan, M. B. Dietary flavonoids: Intake, health effects and bioavailability. *Food Chem. Toxicol.* **1999**, *37*, 937−942.

(4) Birt, D. F.; Hendrich, S.; Wang, W. Dietary agents in cancer prevention: Flavonoids and isoflavonoids. *Pharmacol. Ther.* **2001**, *90*, 157−177.

(5) Na, L.; Takashi, S.; Yuji, T.; Yoshihiro, M.; Yutaka, S.; Masamichi, Y. Novel anti-inflammatory actions of nobiletin, a citrus polymethoxy flavonoid, on human synovial fibroblasts and mouse macrophages. *Biochem. Pharmacol.* **2003**, *65*, 2065−2071.

(6) Guardia, T.; Rotelli, A. E.; Juarez, A. O.; Pelzer, L. E. Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. *Farmaco* **2001**, *56*, 683−687.

(7) Li, B. Q.; Fu, T.; Dongyan, Y.; Mikovits, J. A.; Ruscetti, F. W.; Wang, J. M. Flavonoid baicalin inhibits HIV-1 infection at the level of viral entry. *Biochem. Biophys. Res. Commun.* **2000**, *276*, 534−538.

(8) Gäälnur, T.; Esra, K.; Merve, M.; Erdem, Y. C. Flavonoids with antinociceptive and anti-inflammatory activities from the leaves of Tilia argentea (silver linden). *J. Ethnopharmacol.* **2004**, *95*, 393−397.

(9) Ramesh, M.; Nageshwar Rao, Y.; Appa Rao, A. V. N.; Prabhakar, M. C.; Seshagiri Rao, C. Antinociceptive and anti-inflammatory activity of a flavonoid isolated from Caralluma attenuate. *J. Ethnopharmacol.* **1998**, *62*, 63−66.

(10) Aviram, M.; Fuhrman, B. Polyphenolic flavonoids inhibit macrophage-mediated oxidation of LDL and attenuate atherogenesis. *Atherosclerosis Suppl.* **1998**, *137*, S45−S48.

(11) Belinky, P. A.; Aviram, M.; Fuhrman, B.; Rosenblat, M.; Vaya, J. The antioxidative effects of the isoflavan glabridin on endogenous constituents of LDL during its oxidation. *Atherosclerosis* **1998**, *137*, 49−61.

(12) Zhu, Q. Y.; Huang, Y.; Chen, Zh. Y. Interaction between flavonoids and alpha-tocopherol in human low density lipoprotein. *J. Nutr. Biochem.* **2000**, *11*, 14−21.

(13) Casagrande, F.; Darbon, J. M. Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: Regulation of cyclin-dependent kinases CDK2 and CDK1. *Biochem. Pharmacol.* **2001**, *61*, 1205−1215.

(14) Leslie, E. M.; Mao, Q.; Oleschuk, C. J.; Deeley, R. G.; Cole, S. P. C. Modulation of multidrug resistance protein 1 (MRP1/ABCC1) transport and ATPase activities by interaction with dietary flavonoids. *Mol. Pharmacol.* **2001**, *59*, 1171−1180.

(15) Foti, M.; Piattelli, M.; Tiziana Baratta, M.; Ruberto, G. Flavonoids, coumarins, and cinnamic acids as antioxidants in a micellar system. Structure-activity relationship. *J. Agric. Food Chem.* **1996**, *44*, 497−501.

(16) Harboren, J. B.; Williams, C. A. Advances in flavonoid research since 1992. *Phytochemistry* **2000**, *55*, 481−504.

(17) Patti, A.; Piattelli, M.; Nicolosi, G. Use of Mucor miehei lipase in the preparation of long chain 3-O-acylcatechins. *J. Mol. Catal. B: Enzym.* **2000**, *10*, 577−590.

(18) Kongogianni, A.; Skouridou, V.; Sereti, V.; Stamatis, H.; Kolisis, F. N. Lipase-catalyzed esterification of rutin and naringin with fatty acids of medium carbon chain. *J. Mol. Catal. B: Enzym.* **2003**, *21*, 59−62.

(19) Rice Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−951.

(20) Arora, A.; Byrem, T. M.; Nair, M. G.; Strasburg, G. M. Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids. *Arch. Biochem. Biophys.* **2000**, *373*, 102−109.

(21) Hou, L.; Zhou, B.; Yang, L.; Liu, Zh. L. Inhibition of human low density lipoprotein oxidation by flavonols and their glycosides. *Chem. Phys. Lipids* **2004**, *129*, 209−219.

(22) Schubert, S. Y.; Lansky, E. P.; Neeman, I. Antioxidant and eicosanoid enzyme inhibition properties of pomegranate seed oil and fermented juice flavonoids. *J. Ethnopharmacol.* **1999**, *66*, 11−17.

(23) Vaya, J.; Belinky, P. A.; Aviram, M. Antioxidant constituents from licorice roots: isolation, structure elucidation and antioxidative capacity toward LDL oxidation. *Free Radical Biol. Med.* **1997**, *23*, 302−313.

(24) Chen, Sh. R.; Jin, Ch. F.; Yang, X. Q. Molecule modification of catechins. *J. Tea* **1999**, *25*, 76−79.

(25) Wang, Q. E.; Tang, An. B.; Shi, B. The synthesis of liposoluble tea polyphenols(LTP) and its resistance to autoxidation of oil. *Nat. Prod. Res. Deve.* **2001**, *13*, 12−15.

(26) Danieli, B.; Riva, S. Enzyme-mediated regioselective acylation of polyhydroxylated natural products. *Pure Appl. Chem.* **1994**, *66*, 2215−2218.

(27) Danieli, B.; Luisetti, M.; Sampognaro, G.; Carrea, G.; Riva, S. Regioselective acylation of polyhydroxylated natural compounds catalyzed by *Candida antarctica* lipase B (Novozym 435) in organic solvents. *J. Mol. Catal. B*: *Enzym.* **1997**, *3*, 193.

(28) Hakajima, N.; Ishihara, K.; Hamada, H.; Kawabe, S. I.; Furuya, T. Regioselective acylation of flavonoid glucoside with aromatic acid by an enzymatic reaction system from cultured cells of *Ipomoea batatas. J. Biosci. Bioeng.* **2000**, *90*, 347−349.

(29) Nakajima, N.; Ishihara, K.; Itoh, T.; Furuya, T.; Hamada, H. Lipase-catalyzed direct and regioselective acylation of flavonoid glucoside for mechanistic investigation of stable plant pigments. *J. Biosci. Bioeng.* **1999**, *87*, 105−107.

(30) Gayot, S.; Santarelli, X.; Coulon, D. Modification of flavonoid using lipase in non-conventional media: Effect of the water content. *J. Biotechnol.* **2003**, *101*, 29−36.

(31) Ardhaoui, M.; Falcimaigne, A.; Ognier, S.; Engasser, J. M.; Moussou, P.; Pauly, G.; Ghoul, M. Effect of acyl donor chain length and substitutions pattern on the enzymatic acylation of flavonoids. *J. Biotechnol.* **2004**, *110*, 265−272.

(32) Humeau, C.; Girardin, M.; Rovel, B.; Miclo, A. Enzymatic synthesis of fatty acid ascorbyl esters. *J. Mol. Catal. B: Enzym.* **1998**, *5*, 19−23.

(33) Guyot, B.; Bosquette, B.; Pina, M.; Graille, B. Esterification of phenolic acids from green coffee with an immobilized lipase from *Candida antarctica* in solvent-free medium. *Biotechnol. Lett.* **1997**, *19*, 529−532.

(34) Coulon, D.; Girardin, M.; Engasser, J. M.; Ghoul, M. Investigation of keys parameters of fructose oleate enzymatic synthesis catalyzed by an immobilized lipase. *Ind. Crop Prod.* **1997**, *6*, 375−381.

(35) Laane, C.; Boeren, S.; Vos, K. Rules for optimization of biocatalysis in organic solvents. *Biotechnol. Bioeng.* **1986**, *30*, 81−87.

(36) Giacometti, J.; Giacometti, F.; Milin, Č.; Vasić;-Rački, D. Kinetic characterisation of enzymatic esterification in a solvent system: Adsorptive control of water with molecular sieves. *J. Mol. Catal. B: Enzym.* **2001**, *11*, 921−928.

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