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Enzymatic Synthesis of a Capsinoid by the Acylation of Vanillyl Alcohol with Fatty Acid Derivatives Catalyzed by Lipases

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Capsinoids are a novel group of compounds produced by the *Capsicum* plant. We synthesized a capsinoid by the lipase-catalyzed esterification of vanillyl alcohol with fatty acid derivatives in an organic solvent. The use of seven out of 17 commercially available lipases, especially Novozym 435, was applicable to the synthesis of vanillyl nonanoate, a model compound of capsinoids. The yield of vanillyl nonanoate under the optimum conditions of 50 mM vanillyl alcohol and 50 mM methyl nonanoate in 500 μl of dioxane, using 20 mg of Novozym 435 and 50 mg of 4 Å molecular sieves at 25°C, was 86% in 20 h. Several capsinoid homologues having various acyl chain lengths (C6–C18) were synthesized at 64–86% yields from the corresponding fatty acid methyl ester. The natural capsinoids, capsiate and dihydrocapsiate, were obtained by a 400-fold-scale reaction at these optimum conditions in 60% and 59% isolated yields, respectively.

Key words: capsinoid; enzymatic synthesis; lipase; vanillyl alcohol; capsaicin

Capsinoids are a novel group of compounds of the *Capsicum* plant.[1–3] The fundamental chemical structure of capsinoids is an ester of an aliphatic hydroxyl group in vanillyl alcohol with a fatty acid. Capsinoids have a structural resemblance to capsaicinoids, the well-known pungent principal in the *Capsicum* plant, which is a fatty acid amide of vanillylamine. Capsiate and dihydrocapsiate, natural major capsinoids, have the same acyl residue as that in the corresponding natural major capsaicinoids, capsaicin and dihydrocapsaicin, respectively. Despite the structural similarity between capsinoids and capsaicinoids, there is no or only slight pungency in the natural capsinoids.[2] Yazawa *et al*. have reported that the intake of a non-pungent pepper containing natural capsinoids raises the human skin temperature without marked perspiration or languidness.[4] Great interest in non-pungent analogues of capsaicin and its related substances has arisen in respect of their various biological and physiological activities without noxious stimuli.[5–11]

Capsinoids can be chemically synthesized by the condensation of vanillyl alcohol and a fatty acid chloride.[11] However, there are some drawbacks to this method: for example, the toxicity of the condensing and catalytic agents, the formation of by-products, and such complicated processes as the protection and release of the aromatic hydroxyl group. On the other hand, numerous reports concerning lipase-catalyzed *O*-acylation in an organic solvent have appeared. We have previously reported the enzymatic synthesis of capsaicinoids by the condensation of vanillylamine with fatty acid derivatives catalyzed by lipases.[12,13] In this reaction, several lipases catalyzed *N*-acylation of a primary amino group in vanillylamime, rather than *O*-acylation of an aromatic hydroxyl group in it. Furthermore, several lipases are known to selectively acylate a primary alcohol rather than phenol.[14,15] It was therefore expected that selective *O*-acylation of the aliphatic, but not aromatic, hydroxyl group in vanillyl alcohol would give a capsinoid.

In the present study, we found that a capsinoid could be synthesized by using lipases. We first attempted to optimize several factors for the lipase-catalyzed capsinoid synthesis by selective *O*-acylation of vanillyl alcohol with fatty acid derivatives in an organic solvent. Several homologues of the capsinoid having different acyl chain lengths were then synthesized from the corresponding fatty acid derivatives under optimum conditions. Finally, a scaled-up synthesis of the natural capsinoid was carried out. Vanillyl nonanoate was chosen as a model product for this optimization (Scheme 1) because of its structural resemblance to the natural capsinoid in terms of the carbon chain length in the acyl residue.

Materials and Methods

Enzymes. The lipases used in this study are summarized in Table 1. Lipase from *Candida cylindracea* (900 units/mg of solid) was purchased from Sigma-Aldrich Japan (Tokyo, Japan). The other lipases were kindly presented by Novo Nordisk Bioindustry

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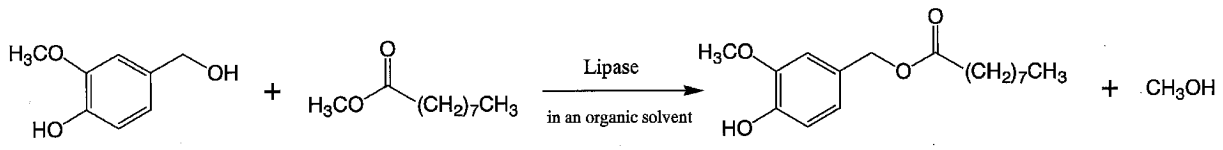


Vanillyl alcohol (VA) Methyl nonanoate (MN) Vanillyl nonanoate

Scheme 1

(Chiba, Japan), Meito Sangyo Co. (Tokyo, Japan) and Amano Pharmaceutical Co. (Nagoya, Japan).

Chemicals and preparation. Vanillyl alcohol of 98% purity and trinonanoin of 99% purity were purchased from Sigma-Aldrich Japan. Capsaicin of first-class reagent grade, containing approx. 60% capsaicin and 40% dihydrocapsaicin, and acyl donors of first class reagent grade were obtained from Wako Pure Chem. Ind. (Osaka, Japan). The other compound of special reagent grade were purchased from Wako. Vanillyl nonanoate, an authentic sample, was prepared chemically by the condensation of vanillyl alcohol (10 mmol) and nonanoyl chloride (5 mmol) in dry pyridine (10 ml) at 0°C for 2 h. The reaction mixture was partitioned with ethyl acetate to remove pyridine, and repeated chromatography of the ethyl acetate-soluble portion afforded a pure compound in a 32.7% yield. The structure of the compound was confirmed by comparing its ^{1}H-NMR data with those in the literature.[11]

Enzymatic reaction. Vanillyl alcohol and an acyl donor were dissolved in an organic solvent (500 μl), before adding a lipase and molecular sieves under dry conditions. The mixture was shaken at 200 rpm by a vortex shaker (Bio-Shaker BR-15, Taitec Co., Saitama, Japan) under a regulated temperature. The yield of the product was determined by an HPLC analysis. At the end of the reaction, the structure of the product was confirmed by an NMR analysis after preparative HPLC purification.

HPLC analysis. The HPLC sample was prepared in two ways: 1) A portion (5 μl) of the reaction mixture was added to a stock solution (45 μl) of ethyl acetate containing 1% trifluoroacetic acid to terminate the reaction. An aliquot of this solution was then subjected to an HPLC analysis. 2) After the whole reaction mixture had been centrifuged at 1500 × *g* for 30 sec to precipitate the enzyme, 1 μl of the supernatant was injected into the HPLC system under the following conditions: reversed-phase silica gel column, J'sphere ODS-H80, 150 mm × 4.6 mm i.d. (YMC, Kyoto, Japan); eluent, methanol for the analysis of vanillyl octadecanoate, 90% methanol for vanillyl nonanoate and tetradecanoate, and 80% methanol for vanillyl hexanoate; flow rate, 1 ml/min; detection, fluorescence with excitation at 280 nm and emission at 320 nm.

HPLC purification and NMR analysis of the products. Each capsinoid homologue was synthesized from 50 mM vanillyl alcohol and 50 mM methyl ester of a fatty acid (hexanoic (C6), nonanoic (C9), tetradecanoic (C14) or octadecanoic (C18) acid) in 500 μl of dioxane with 20 mg of Novozym 435 and 50 mg of 4 Å molecular sieves at 25°C for 20 h. At the end of the reaction, the reaction mixture was condensed by a nitrogen stream to about 50 μl and subjected to preparative HPLC: column, J'sphere ODS-H80, 150 mm × 20 mm i.d.; flow rate, 5–10 ml/min; the other conditions were the same as those already mentioned. The HRMS spectrum was measured by a JMS-AX500 instrument (Jeol, Japan), and the NMR spectra were measured by a Jeol α-400 instrument (^{1}H-NMR at 399.65 MHz, ^{13}C-NMR at 100.40 MHz, $CDCl_3$ as the solvent, TMS as the internal standard).
Vanillyl hexanoate was obtained in a 3.6 mg (59% isolated) yield as a colorless oil. HRMS *m/z* (M^+): Calcd. for $C_{14}H_{20}O_4$: 252.1361, Found: 252.1408. NMR δ_H ($CDCl_3$): 6.91–6.86 (3H, m, phenyl), 5.03 (2H, s, -COOCH_2Ph), 3.90 (3H, s, OCH_3), 2.33 (2H, t, J=7.2 Hz, COCH_2), 1.64 (2H, quint, J=7.2 Hz, COCH$_2$CH_2), 1.3–1.2 (4H, m, CH_2 × 2 of acyl chain), 0.88 (3H, t, J=6.8 Hz, CH_3 of acyl terminal). NMR δ_C ($CDCl_3$): 173.8 (*C*=O), 146.5, 145.8, 128.1, 122.0, 114.3 and 111.2 (phenyl), 66.3 (ph-*C*H$_2$-OCO), 55.9 (O*C*H$_3$), 34.4, 31.3, 24.7 and 22.3 (acyl chain), 13.9 (acyl terminal).
Vanillyl nonanoate was obtained in a 4.8 mg (71%) yield as a colorless oil. The spectral data for this compound agreed with those of an authentic chemically synthesized sample.
Vanillyl tetradecanoate was obtained in a 6.8 mg (77%) yield as colorless needles, mp 45–46°C. HRMS *m/z* (M^+): Calcd. for $C_{22}H_{36}O_4$: 364.2614, Found: 364.2627. NMR δ_H ($CDCl_3$): 6.91–6.86 (3H, m, phenyl), 5.03 (2H, s, -COOCH_2Ph), 3.90 (3H, s, OCH_3), 2.33 (2H, t, J=7.2 Hz, COCH_2), 1.63 (2H, quint, J=7.2 Hz, COCH$_2$CH_2), 1.3–1.2 (20H, CH_2 × 10 of acyl chain), 0.88 (3H, t, J=6.8 Hz, CH_3 of acyl terminal). NMR δ_C ($CDCl_3$): 173.8 (*C*=O), 146.5, 145.8, 128.1, 122.0, 114.3 and 111.2 (phenyl), 66.3 (ph-*C*H$_2$-OCO), 55.9 (O*C*H$_3$), 34.4, 31.9, 29.7 × 3, 29.6, 29.5, 29.4, 29.3, 29.2, 25.0 and 22.7 (acyl

Table 1. Effect of Enzymes (20 mg) on the Yield of Vanillyl Nonanoate Synthesized from Vanillyl Alcohol (VA) and Methyl Nonanoate (MN) in an Organic Solvent (500 μl) at 25°C

Lipase	Origin	Supplier	VA:MN = 50 mM:1000 mM in dioxane Reaction time and yield (%)		VA:MN = 200 mM:10 mM in acetone Reaction time and yield (%)	
			0.5 h	20.0 h	0.5 h	20.0 h
Novozym 435	*Candida antarctica*	Novo	71.3	70.7	56.8	70.9
Lipase	*C. cylindracea*	Sigma	0.0	2.9	0.0	0.0
Lipase OF	*C. cylindracea*	Meito	0.9	11.9	0.0	6.2
Lipase AY	*C. rugosa*	Amano	0.0	1.4	0.0	0.0
Lipase L	*C. lipolytica*	Amano	0.0	0.0	0.0	0.0
Lipase A	*Aspergillus niger*	Amano	0.0	0.0	0.0	0.0
Lipase M	*Mucor javanicus*	Amano	0.0	8.4	0.0	0.5
Lipozyme IM20	*M. miehei*	Novo	14.3	66.9	0.0	25.4
Lipase F	*Rhizopus oryzae*	Amano	0.0	0.0	0.0	0.0
Lipase D	*R. delemar*	Amano	0.0	0.9	0.0	0.0
Lipase R	*Penicillum roqueforti*	Amano	0.0	3.2	0.0	0.0
Lipase G	*Pe. camembertii*	Amano	0.6	0.6	0.0	1.3
Lipase PS	*Pseudomonas cepacia*	Amano	11.2	75.4	0.5	21.7
Lipase AH	*Ps. cepacia*	Amano	10.0	73.9	1.3	32.5
Lipase AK	*Ps. fluorescens*	Amano	38.0	67.7	4.3	62.1
Lipase PL	*Alcaligenes* sp.	Meito	27.9	66.8	8.2	58.6
Lipase QL	*Al.* sp.	Meito	64.5	62.7	18.6	63.8

chain), 14.1 (acyl terminal).

Vanillyl octadecanoate was obtained in a 6.6 mg (66%) yield as a colorless amorphous solid, mp 58–59°C. HRMS m/z (M^+): Calcd. for $C_{26}H_{44}O_4$: 420.3240, Found: 420.3216. NMR δ_H ($CDCl_3$): 6.91–6.86 (3H, m, phenyl), 5.03 (2H, s, –COOCH_2Ph), 3.90 (3H, s, OCH_3), 2.33 (2H, t, J=7.2 Hz, COCH_2), 1.63 (2H, quint, J=7.2 Hz, COCH$_2$CH_2), 1.3–1.2 (28H, CH_2×14 of acyl chain), 0.88 (3H, t, J=6.8 Hz, CH_3 of acyl terminal). NMR δ_C ($CDCl_3$): 173.8 (C=O), 146.5, 145.8, 128.1, 122.0, 114.3 and 111.2 (phenyl), 66.3 (ph–CH$_2$–OCO), 55.9 (OCH$_3$), 34.4, 31.9, 29.7×7, 29.6, 29.5, 29.4, 29.3, 29.2, 25.0 and 22.7 (acyl chain), 14.1 (acyl terminal).

Scaled-up synthesis of natural capsinoids. A mixture of acyl donors for the natural capsinoid synthesis, methyl 8-methylnon-6-enoate and methyl 8-methylnonanoate, was prepared by methanolysis of first-class reagent grade capsaicin. In brief, three grams of the capsaicin in 1 liter of 4 N–HCl aq. containing 67% methanol were refluxed for 48 hours. Petroleum ether was added to the solution to extract the acyl donors. The mixture of acyl donors was obtained in an almost quantitative yield of 1.9 g. Eight grams of Novozym 435 and 20 g of 4 Å molecular sieves were added to a solution of 1,4-dioxane (200 ml) containing vanillyl alcohol (1.54 g, 50 mM) and the mixture of acyl donors (1.9 g, 50 mM). After being incubated while shaking at 25°C for 40 hours, the reaction mixture was filtered to remove the enzyme and molecular sieves and then condensed by a rotary evaporator *in vacuo* to give a residue. This residue was purified by MPLC (YFLC 540-GR II, Yamazen Co., Osaka, Japan) under the following conditions: silica gel column, Ultrapack SI-40C (300 mm × 37 mm i.d.); eluent, hexane-ethyl acetate stepwise system; flow rate, 20 ml/min; detection, UV at 280 nm. The fraction from the hexane-ethyl acetate (75:25) eluent was a mixture of capsiate and dihydrocapsiate (2.4 g). Part (198.7 mg) of this mixture was rechromatographed by MPLC in an ODS-silica gel column (Ultrapack ODS-S-50B, 300 mm × 26 mm i.d.) with 80% methanol to afford capsiate (98.4 mg, 60% isolated yield) and dihydrocapsiate (48.6 mg, 59%). The ^{1}H- and ^{13}C-NMR spectra of these compounds completely matched those of naturally occurring capsiate and dihydrocapsiate, respectively.[2)]

Results and Discussion

Screening of the enzymes

Table 1 shows the conversion yields for the vanillyl nonanoate synthesis by 17 commercially available lipases conducted under two different reaction conditions. The lipases used in this work were native enzymes, except for Novozym 435 and Lipozyme IM20 that were lipases immobilized on a macroporous acrylic resin and an ion-exchange resin, respectively. Among them, Novozym 435, Lipozyme IM20, and Lipase PS, AH, AK, PL and QL gave relatively high yields of the product of 66.8–75.4% after 20 h in the excess acyl donor condition with dioxane as a solvent. Novozym 435 and Lipase QL gave particularly high yields in a short reaction time, their initial conversion rates being 148 and 102 μmol/min/g, respectively. Lipase AK and PL showed moderate rates of 28 and 18 μmol/min/g, respectively. The activities of

the lipases for capsinoid synthesis were correlated with those for the acylation of *p*-nitrophenol and the hydrolysis of its acyl ester.[16] In brief, the potency of the enzymes for capsinoid synthesis was closely related with the general activities of the lipases. The activities of the lipases markedly decreased under the excess aromatic donor condition and by using acetone as a solvent (Table 1). The catalytic efficiency of an enzyme in an organic solvent generally decreases as the hydrophilicity of the solvent increases.[17] However, only Novozym 435 retained high potency in the capsinoid synthesis with acetone. We selected Novozym 435 as the most suitable catalyst for the reaction because of its high activity and also its advantages as an immobilized enzyme such as convenient handling, reusability and high stability.

Effect of the amount of the enzyme

The effect of the amount of the enzyme was investigated when using Novozym 435 under the excess acyl donor condition just described. As shown in Table 1 and Fig. 1, when 20 mg of the enzyme was used, the equilibrium yield of the product was about 70% and was attained after 30 min. The equilibrium yields when using 5 mg and 50 mg of the enzyme were the same as that when using 20 mg of the enzyme, these yields being attained after 120 and 15 min, respectively. In brief, an increase in the amount of the enzyme did not alter the equilibrium yield, but accelerated the reaction rate. However, more than 50 mg of the enzyme would probably be unsuitable for this batch system because too much swelling of the enzyme would take up too much space in the reaction solution.

Choice of organic solvent

There have been many studies on the effect of an organic solvent on enzyme activities.[17–20] We tested nine organic solvents for capsinoid synthesis when catalyzed by Novozym 435 (Table 2). The conversion yields reached equilibrium at 70.9–78.0% within 0.5 h with most of the solvents used except for hexane and chloroform. The low activity with hexane was probably due to the low solubility of vanillyl alcohol in it, although the reason for the low activity with chloroform is not clear. The catalytic potency of an enzyme is generally influenced by the polarity of the solvent.[17] However, Novozym 435 accepted a wide range of polarity for the solvent in the capsinoid synthesis. It should be noted that capsinoids are unstable to high-polar solvents such as water, alcohol and dimethyl sulfoxide.[21] Furthermore, esters and primary or secondary alcohols are unsuitable as solvents because they would be likely to be used as substrates.

Effect of reaction temperature

Novozym 435 is a heat-tolerant preparation with maximum activity for esterification in the range of 70–90°C.[22,23] Additionally, dioxane allows a wide range of reaction temperature because of its relatively high boiling point of 101.5°C. Therefore, we investigated the effect of the reaction temperature on capsinoid synthesis by using Novozym 435 in dioxane. Figure 1 shows the time-course plots of the yield of vanillyl nonanoate at various reaction temperatures. As expected, the conversion rate increased in proportion to increase in reaction temperature, whereas the equilibrium yield was not influenced by a change in the reaction temperature. From the viewpoint of the practical use of the synthesis, the most appropriate temperature for the reaction was at around ambient.

Table 2. Effect of Organic Solvents (500 μl) on the Yield of Vanillyl Nonanoate Synthesized from 50 mM Vanillyl Alcohol and 1000 mM Methyl Nonanoate Using 20 mg of Novozym 435 at 25°C

Organic solvent	Reaction time and yield (%)	
	0.5 h	20.0 h
Hexane	19.1	28.7
Diethyl ether	74.1	74.4
Diisopropyl ether	70.9	75.4
Dioxane	71.3	70.7
Tetrahydrofuran	71.6	71.8
Chloroform	14.9	62.1
Acetone	72.2	72.1
Acetonitrile	78.0	78.0
t-Amyl alcohol	73.5	73.4

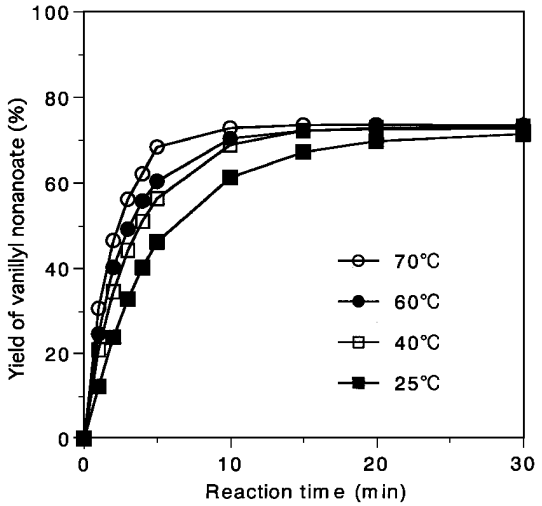


Fig. 1. Time-course Profiles of the Yield of Vanillyl Nonanoate at Various Temperatures.

Reaction condition: 50 mM vanillyl alcohol and 1000 mM methyl nonanoate in dioxane (500 μl) with 20 mg of Novozym 435.

Effect of molar ratio of the substrates

The effect of molar ratio of the substrates for capsinoid synthesis was investigated in dioxane because both substrates possessed high solubility in the sol-

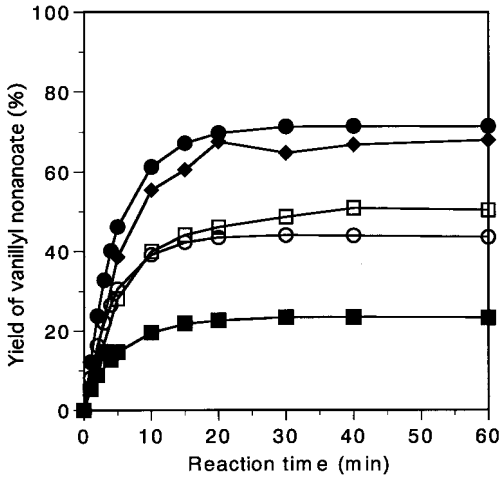


Fig. 2. Effect of Substrate Ratio on the Yields of Vanillyl Nonanoate Synthesized from Vanillyl Alcohol (VA) and Methyl Nonanoate (MN) in Dioxane (500 μl) Using 20 mg of Novozym 435 at 25°C.

Symbols: VA:MN (mM) = ●, 50:1000; ○, 50:200; ■, 50:50; □, 200:50; ◆, 1000:50.

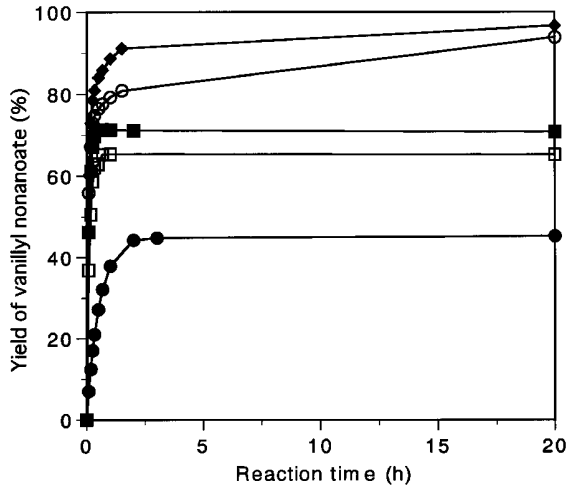


Fig. 3. Effects of Water Content and Additives on the Yield of Vanillyl Nonanoate.

Symbols: ■, no additive; □, 0.1% H_2O; ●, 1% H_2O; ○, 3 Å molecular sieves, 50 mg; ◆, 4 Å molecular sieves, 50 mg. Reaction conditions: 50 mM vanillyl alcohol and 1000 mM methyl nonanoate in dioxane (500 μl) with 20 mg of Novozym 435 at 25°C.

vent. As shown in Fig. 2, as the molar ratio of vanillyl alcohol to methyl nonanoate approached 1 by reducing the concentration of one of the substrates, the equilibrium yield of vanillyl nonanoate decreased. Vanillyl alcohol had lower solubility in a hydrophobic solvent and was easier to crystallize (having a higher melting point of 113–115°C) than the acyl donors. These characteristics allowed easy separation of surplus vanillyl alcohol from the reaction mixture as precipitation could be induced by pouring in a large quantity of hexane and/or by evaporating the solvent at the end of the reaction. Moreover, the separated vanillyl alcohol was reusable. The excess vanillyl alcohol condition was therefore better than the excess acyl donor condition if an excess of the substrate were used. However, it is desirable that the starting materials should be in equivalent amounts to apply the reaction to practical manufacture, and we solved this problem by adjusting the water content in the reaction mixture as mentioned next.

Effects of water content and additives

It is generally known that a small amount of water plays a crucial role in lipase-catalyzed esterification in an organic solvent.[24–28] The effect of the water content in the reaction mixture on the yield from capsinoid synthesis was investigated by using Novozym 435 (Fig. 3). The addition of 0.1% water decreased the equilibrium yield from 71% (under the non-additive condition) to 65%, while the addition of 1% water drastically suppressed not only the equilibrium yield to 43% but also the conversion rate. In contrast, an enhanced conversion yield was observed with the addition of molecular sieves. 4 Å molecular sieves gave particularly good results; the conversion yield reached 84% in 0.5 h and 97% in 20 h. The addition of molecular sieves to an organic solvent generally suppresses the catalytic activity where the water content is almost zero. However, Novozym 435 had high catalytic activity in the capsinoid synthesis even in the presence of molecular sieves. Furthermore, the non-immobilized enzyme for Novozym 435, *Candida antarctica* lipase B, retained high activity in the acylation of cyclohexanol, even after drying with molecular sieves.[20] Therefore, it is likely that Novozym 435 prefers the dry or nearly dry condition for acylation in an organic solvent. The water content of the intact reaction mixture (in the absence of molecular sieves) was less than 0.005% as measured by the Karl Fisher method. In spite of such a nearly dry condition, the addition of molecular sieves further increased the conversion yield. These results indicate that the molecular sieves acted as an eliminator of methanol, a concomitant product of the reaction, rather than as a desiccant of the possible trace amount of water. Consequently, the elimination of methanol probably induced a shift in the equilibrium of the reaction, and this shift raised the conversion yield for capsinoid synthesis.

Effect of structure of the acyl donor

Figure 4 shows the effect of various forms of acyl donor under the condition of an equivalent amount of substrates. Under the non-additive condition, the most suitable donor was trinonanoin because of its high equilibrium yield of the product at 74% (Fig. 4D). A free form of the acyl donor (nonanoic acid) gave a higher equilibrium yield at 39% (Fig. 4A) than those of the simple ester forms (Figs. 4B and C). High temperature did not affect the

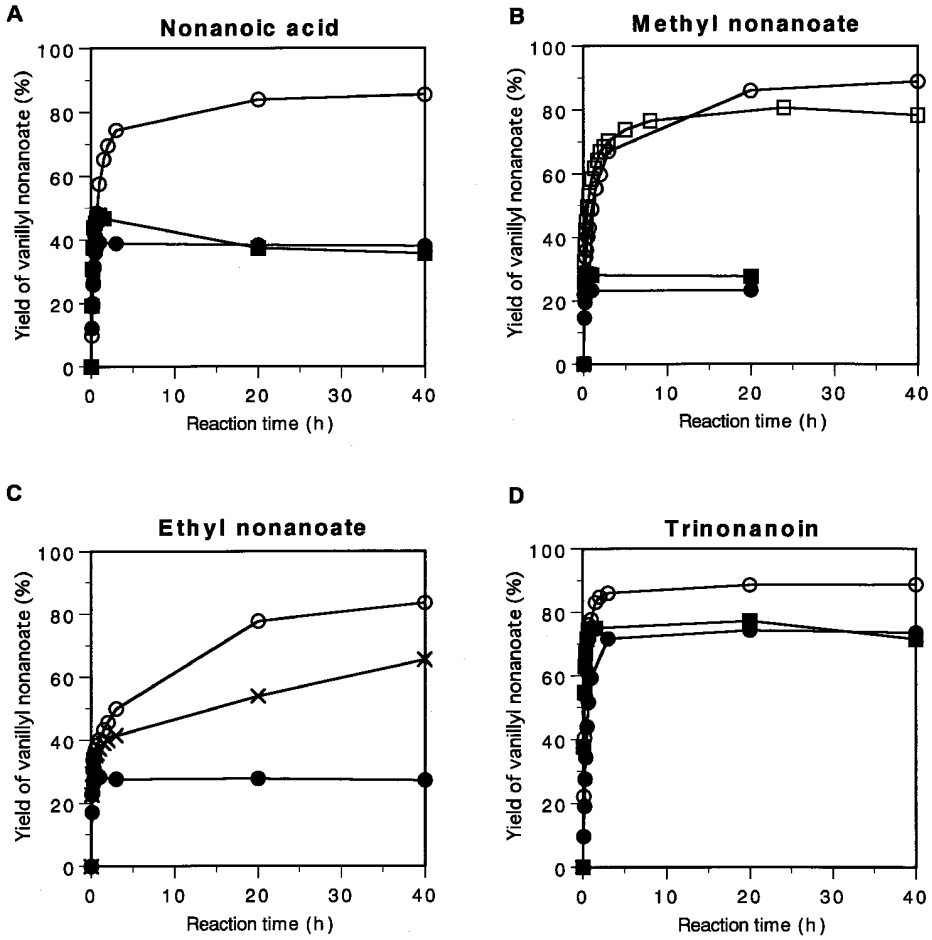


Fig. 4. Effect of the Form of Acyl Donor on the Yield of Vanillyl Nonanoate.
Symbols: ●, 25°C; ○, 25°C + 4 Å molecular sieves; ■, 70°C; □, 70°C + 4 Å molecular sieves; ×, 25°C + 3 Å molecular sieves. Reaction conditions: 50 mM vanillyl alcohol and 50 mM acyl donor in dioxane (500 μl) with 20 mg of Novozym 435.

equilibrium yield when the ester forms were used (Figs. 4B and D), while the conversion yield from the free form increased rapidly and then declined gradually (Fig. 4A). This phenomenon was probably due to the lability of capsinoid against water which was a concomitant product when the free form was used. On the other hand, the addition of molecular sieves to the reaction mixture dramatically increased the conversion yield for all the donors. The conversion yield after 20–40 h increased two-fold for the free form (Fig. 4A) and three times for the simple ester form (Figs. 4B and C). The maximum conversion yield was recorded as 90% after 40 h when methyl nonanoate was used with 4 Å molecular sieves (Fig. 4B). When ethyl nonanoate was used, enhancement of yield by 3 Å molecular sieves was weaker than that by 4 Å. This was probably due to the lower adsorption ability of ethanol to 3 Å molecular sieves than 4 Å. This enhancement of the conversion yield by the addition of molecular sieves can be explained by the elimination of concomitant products such as water, methanol and ethanol from the reaction. When trinonanoin was used, the additive probably acted only as a desiccant of a possible trace amount of water. In summary, the addition of 4 Å molecular sieves to the reaction mixture gave a sufficiently high yield from any form of acyl donor of 78–89% in 20 h, even in the condition when an equivalent amount of the substrate was used.

Considering all the foregoing results, the following optimum conditions for capsinoid synthesis could be inferred: substrate concentration, 50 mM vanillyl alcohol and 50 mM acyl donor; reaction medium, dioxane; enzyme, Novozym 435, 20 mg/500 μl of solvent; additive, 4 Å molecular sieves, 50 mg/500 μl of solvent; temperature, 25°C; reaction time, 20–40 h.

Effect of chain length of the acyl donor

Capsaicin analogues having a longer acyl chain length than that of capsaicin, for example vanillyl tetradecanamide (C14) and vanillyl octadecanamide (C18), have no pungency, but some of them enhance epinephrine secretion from adrenal medulla in rodents as well as capsaicin dose.[6,7] Vanillyl (*Z*)-9-octadecenamide (C18:1), named olvanil, possesses capsaicin-like biological and physiological properties without any noxious stimuli.[8,9] We were therefore interested in capsiate analogues with various lengths of

the acyl moiety, and attempted their lipase-catalyzed synthesis under the optimum conditions just described. Vanillyl hexanoate (C6), nonanoate (C9), tetradecanoate (C14) and octadecanoate (C18) were obtained from the methyl ester of the corresponding acyl donors, and their conversion yields in 20 h as estimated by an HPLC analysis were 78%, 86%, 83% and 64%, respectively (Fig. 5). At the end of the reaction, the reaction mixtures were subjected to preparative HPLC to purify the homologues, and the structures were confirmed by NMR measurements (see the Materials and Methods section). Consistent with a small loss of each product during purification, the isolated yields (see the Materials and Methods section) were consistent with the HPLC analyzed yields. The relatively low yield of the C18 product was probably due to impurities, mainly methyl hexadecanoate (C16), in the acyl donor. Indeed, a C16 homologue was detected by the HPLC analysis at *ca.* 10% of the total products. Thus, there was substantially no specificity for acyl chain length within C6–C18 in the reaction. Since Novozym 435 can accept a wide range of acyl chain length for the esterification of a fatty acid with an alcohol,[23] various homologues for the acyl moiety of capsinoid would be easily given in high yields by this synthetic method.

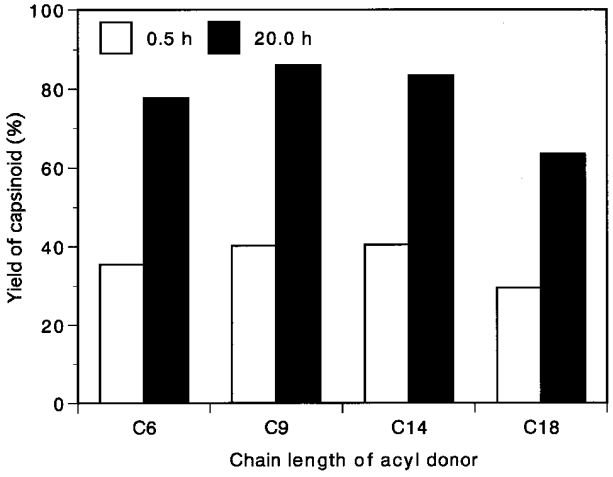
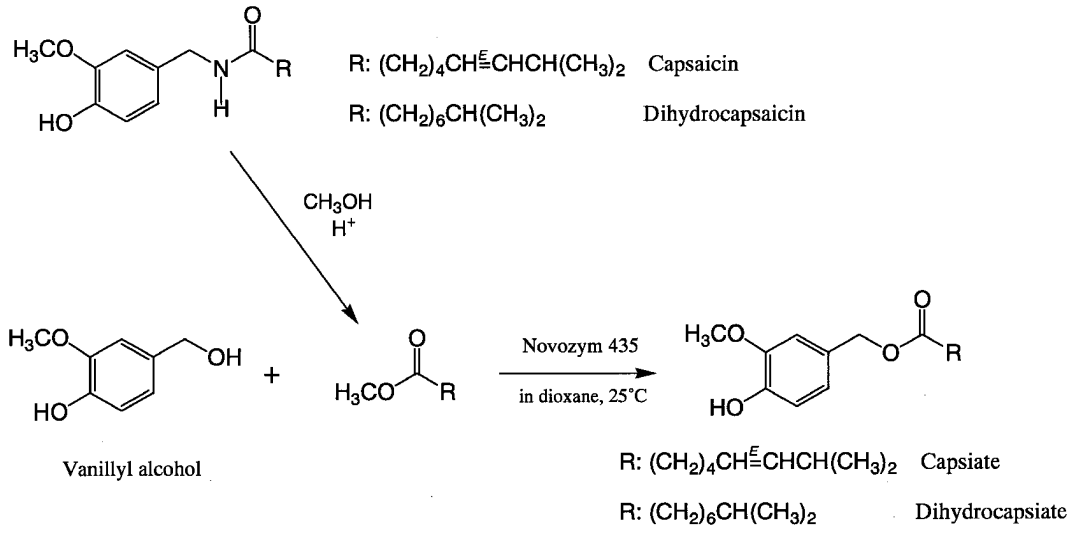


Fig. 5. Effect of Chain Length of the Acyl donor on the Yield of the Corresponding Capsinoid.

Reaction conditions: 50 mM vanillyl alcohol and 50 mM fatty acid methyl ester in dioxane (500 μl) with 20 mg of Novozym 435 and 50 mg of 4 Å molecular sieves.

Scaled-up synthesis of natural capsinoids

Finally, we attempted a scaled-up synthesis of the natural capsinoids, capsiate and dihydrocapsiate (Scheme 2). The acyl moiety of capsiate is an 8-methylnon-6-enoic acid, and that of dihydrocapsiate is an 8-methylnonanoic acid. The acyl donors can be produced by chemical synthesis,[29,30] although the process is complicated and the yield is low. Furthermore, commercial products are too expensive for application to practical use. We therefore prepared the acyl donors by methanolysis of the first-class reagent grade of capsaicin that is commercially available and contains both capsaicin and dihydrocapsaicin. The molar ratio of capsaicin and dihydrocapsaicin in this reagent was determined to be *ca.* 2:1 by our HPLC analysis. The methanolysis of capsaicin proceeded quantitatively, and the molar ratio of the methyl esters of 8-methylnon-6-enoic and 8-methylnonanoic acids was estimated to be *ca.* 2:1 by an NMR analysis. Lipase-catalyzed synthesis of natural capsinoids was then carried out from the mixture of the acyl donors on a 400-fold scale under the optimum conditions already mentioned. The conversion yield of the natural capsinoids was calculated as *ca.* 80% in 40 h by an HPLC analysis. After purifying the products by repeated MPLC separation, capsiate and di-



Scheme 2

hydrocapsiate were given in 60% and 59% isolated yields, respectively. Although negligible by-products were present, there was no phenyl ester of vanillyl alcohol with the acyl donor.

Conclusions

To achieve a sufficient supply of capsinoids, we carried out the lipase-catalyzed esterification of vanillyl alcohol with fatty acid derivatives in an organic solvent. Several factors in the synthesis of vanillyl nonanoate, a model compound for capsinoids, were investigated to elucidate the optimum conditions. Among 17 commercially available lipases, seven lipases possessed relatively high activity for the reaction. A good catalyst for general esterification and/or transesterification rather than for hydrolysis was suitable for the capsinoid synthesis. Novozym 435 was selected as the most suitable catalyst for the reaction because of both its high activity and its advantages as an immobilized enzyme. Moreover, Novozym 435 allowed a wide range of reaction temperature and several common organic solvents to be used as a reaction medium. Increasing the amount of enzyme did not alter the equilibrium yield, but accelerated the reaction rate. As the amount of one of the substrates exceeded that of the other, the equilibrium yield was markedly increased. Increasing the water content of the reaction mixture negatively affected the equilibrium yield, while the addition of molecular sieves dramatically raised the equilibrium yield, even when the initial amounts of the substrates were equivalent. Although the equilibrium yield was influenced by different forms of the acyl donor, the addition of molecular sieves raised the yield with any form to a sufficient level. The effect of this additive was probably due to a shift in the equilibrium toward esterification by elimination of concomitant products such as water and alcohol. The optimum conditions for capsinoid synthesis were thus elucidated as follows: 50 mM vanillyl alcohol and 50 mM acyl donor in 500 μl of dioxane, using 20 mg of Novozym 435 and containing 50 mg of 4 Å molecular sieves as an additive at 25°C for 20–40 h. Homologues having various acyl chain lengths (C6–C18) were synthesized in high yields from the corresponding fatty acid methyl ester under the optimum conditions. Furthermore, syntheses of the natural capsinoids, capsiate and dihydrocapsiate, were achieved in a scaled-up reaction. We plan in the near future to study the biological and physiological properties of the various capsinoids prepared by this enzymatic method.

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