(2*S*,3*S*)- and (2*R*,3*S*)-3-[(+)-α-Methoxy-α-((trifluoromethyl)phenyl)acetoxy]-2-methyl-2-(2-propynyl)cycloheptanone (21a, 23a). Purified by fc (30% ether in hexane). **21a**: ^{1}H NMR ($CDCl_3$, 470 MHz) 1.25 (3 H, s, CH_3), 1.5–2.0 (6 H, m), 2.02 (1 H, t, J = 2.5 Hz), 2.3 (1 H, dd, J = 15, 2.5 Hz), 2.39 (1 H, dd, J = 15, 2.5 Hz), 2.4–2.7 (2 H, m), 3.53 (3 H, s, OCH_3), 5.43 (1 H, d, J = 8 Hz), 7.4 (3 H, m), 7.55 (2 H, m). **23a**: ^{1}H NMR ($CDCl_3$, 470 MHz) 1.11 (3 H, s, CH_3), 1.5–2.0 (6 H, m), 2.10 (1 H, t, J = 2.5 Hz) 2.21 (2 H, d, J = 2.5 Hz), 2.4–2.7 (2 H, m), 3.58 (3 H, s, OCH_3), 5.70 (1 H, t, J = 6.5 Hz), 7.4 (3 H, m), 7.55 (2 H, m).

(2*R*,3*S*)- and (2*S*,3*R*)-3-[(+)-α-Methoxy-α-((trifluoromethyl)phenyl)acetoxy]-2-methyl-2-(2-propynyl)cycloheptanone (23a, 24a; 1:1). Purified by fc (30% ethyl acetate in hexane): ^{1}H NMR ($CDCl_3$, 470 MHz) 1.11 (3 H, s, CH_3), 1.12 (3 H, s, CH_3), 1.5–2.0 (12 H, m), 2.15 (1 H, t, J = 2.5 Hz), 2.21 (2 H, d, J = 2.5 Hz), 2.35 (1 H, dd, J = 14, 2.5 Hz), 2.37 (1 H, dd, J = 14, 2.5 Hz), 2.5–2.7 (4 H, m), 3.51 (3 H, s, OCH_3), 3.58 (3 H, s, OCH_3), 5.64 (1 H, dd, J = 8, 2 Hz), 5.71 (1 H, t, J = 6 Hz), 7.4 (6 H, m), 7.55 (4 H, m).

(2*S*,3*S*)-3-[(+)-α-Methoxy-α-((trifluoromethyl)phenyl)acetoxy]-2-isobutenyl-2-methylcycloheptanone (26a) and (2*R*,3*S*)-3-[(+)-α-Methoxy-α-((trifluoromethyl)phenyl)acetoxy]-2-isobutenyl-2-methylcycloheptanone (28a). Purified by fc (20–30% ether in hexane). **26a**: ^{1}H NMR ($CDCl_3$, 470 MHz) 1.06 (3 H, s, CH_3), 1.56 (3 H, s, CH_3), 1.5–1.8 (5 H, m), 2.11 (1 H, d, J = 14 Hz), 2.15 (1 H, m), 2.34 (1 H, d, J = 14 Hz), 2.37 (1 H, m), 2.64 (1 H, m), 3.54 (3 H, s, OCH_3), 4.57 (1 H, s), 4.79 (1 H, s), 5.13 (1 H, d, J = 8 Hz), 7.4 (3 H, m), 7.55 (2 H, m). **28a**: ^{1}H NMR ($CDCl_3$, 470 MHz) 1.08 (3 H, s, CH_3), 1.56 (3 H, s, CH_3), 1.5–1.9 (6 H, m), 2.02 (1 H, d, J = 14 Hz), 2.28 (1 H, d, J = 14 Hz), 2.40 (1 H, m), 2.65 (1 H, dt, J = 11, 2.5 Hz), 3.57 (3 H, s, OCH_3), 4.48 (1 H, s), 4.82 (1 H, s), 5.37 (1 H, d, J = 10 Hz), 7.4 (3 H, m), 7.55 (2 H, m).

(2*S*,3*S*)- and (2*R*,3*R*)-3-[(+)-α-Methoxy-α-((trifluoromethyl)phenyl)acetoxy]-2-isobutenyl-2-methylcycloheptanone (26a, 27a; 1:1) and (2*R*,3*S*)- and (2*S*,3*R*)-3-[(+)-α-Methoxy-α-((trifluoromethyl)phenyl)acetoxy]-2-isobutenyl-2-methylcycloheptanone (28a, 29a; 1:1). Purified by fc (20-30% ether in hexane). **26a, 27a**: ^{1}H NMR ($CDCl_3$, 470 MHz) 1.06 (3 H, s, CH_3), 1.11 (3 H, s, CH_3), 1.56 (6 H, br s, 2 CH_3), 1.5–1.8 (10 H, m), 2.07 (1 H, d, J = 13 Hz), 2.11 (1 H, d, J = 14 Hz), 2.15 (2 H, m), 2.34 (1 H, d), J = 14 Hz), 2.37 (2 H, m), 2.43 (1 H, d, J = 13 Hz), 2.64 (2 H, m), 3.54 (3 H, s, OCH_3), 3.56 (3 H, s, OCH_3), 4.55 (1 H, s), 4.57 (1 H, s), 4.79 (2 H, s), 5.07 (1 H, d, J = 8 Hz), 5.13 (1 H, d, J = 8 Hz), 7.4 (6 H, m), 7.55 (4 H, m). **28a, 29a**: ^{1}H NMR ($CDCl_3$, 470 MHz) 1.07 (3 H, s, CH_3), 1.08 (3 H, s, CH_3), 1.56 (3 H, s, CH_3), 1.59 (3 H, s, CH_3), 1.5–1.9 (12 H, m), 2.02 (1 H, d, J = 14 Hz), 2.10 (1 H, d, J = 14 Hz), 2.28 (1 H, d, J = 14 Hz), 2.35 (1 H, d, J = 14 Hz), 2.4 (2 H, m), 2.65 (2 H, br t , J = 11 Hz), 3.5 (3 H, s, OCH_3), 3.57 (3 H, s, OCH_3), 4.48 (1 H, s), 4.55 (1 H, s), 4.82 (1 H, s), 4.85 (1 H, s), 5.36 (1 H, d, J = 10 Hz), 5.37 (1 H, d, J = 10 Hz), 7.4 (6 H, m), 7.55 (4 H, m).

(2*S*,3*S*)- and (2*R*,3*S*)-3-[(+)-α-Methoxy-α-((trifluoromethyl)phenyl)acetoxy]-2-allyl-2-methylcyclooctanone (31a, 33a). Purified by fc (25% ethyl acetate in hexane). **31a**: ^{1}H NMR ($CDCl_3$, 470 MHz) 0.89 (3 H, s, CH_3), 1.5–2.0 (8 H, m), 2.15 (1 H, dd, J = 14, 8 Hz), 2.33 (1 H, m), 2.50 (1 H, dd, J = 14, 6 Hz), 2.77 (1 H, m), 3.55 (3 H, s, OCH_3), 4.90 (1 H, s), 4.93 (1 H, d, J = 6.0 Hz), 5.78 (1 H, dd, J = 9, 4 Hz), 5.82 (1 H, m), 7.4 (3 H, m), 7.55 (2 H, m). **33a**: ^{1}H NMR ($CDCl_3$, 470 MHz) 1.02 (3 H, s, CH_3), 1.5–2.0 (8 H, m), 2.1–2.9 (4 H, m), 3.45 (3 H, s, OCH_3), 4.84 (1 H, d, J = 15 Hz), 4.97 (1 H, d, J = 10 Hz), 5.8 (2 H, m), 7.4 (3 H, m), 7.55 (2 H, m).

(2*S*,3*S*)- and (2*R*,3*R*)-3-[(+)-α-Methoxy-α-((trifluoromethyl)phenyl)acetoxy]-2-allyl-2-methylcyclooctanone (31a, 32a; 1:1). Purified by fc (30% ethyl acetate in hexane): ^{1}H NMR ($CDCl_3$, 470 MHz) 0.89 (3 H, s, CH_3), 1.5–2.0 (16 H, m), 2.15 (2 H, m), 2.33 (2 H, m), 2.50 (2 H, m), 2.77 (2 H, m), 3.55 (3 H, s, OCH_3), 3.58 (3 H, s, OCH_3), 4.92 (4 H, m), 5.8 (4 H, m), 7.4 (6 H, m), 7.55 (4 H, m).

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Supplementary Material Available: 470-MHz ^{1}H NMR spectra of the (+)-MTPA esters of the ketols derived from microbial and $NaBH_4$ reduction (12 pages). Ordering information is given on any current masthead page.

Chemical Reactions by Polyethylene Glycol Modified Enzymes in Chlorinated Hydrocarbons

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Generally native enzymes never dissolve in organic solvents. Recently, we have reported that a modified enzyme, whose surface was covalently attached to polyethylene glycol (PEG), dissolved in various organic solvents such as benzene, toluene, acetone, ethanol, and dimethylformamide in a transparent state.[1] This modified enzyme can be used as a catalyst of chemical reactions that take place in organic solvents such as benzene and toluene. The modified peroxidase, lipase, catalase, and chymotrypsin specifically catalyze oxidation reactions,[2] ester syntheses,[3] hydrogen peroxide decomposition,[4] and acid–amide bond formation,[5] respectively, in a transparent benzene solution.

In the present paper, we have studied the ester synthesis (eq 1), acid–amide bond formation (eq 2), and H_2O_2 decomposition (eq 3) reactions by the polyethylene glycol modified enzymes in chlorinated hydrocarbons, aiming the wide application of the modified enzymes in various organic solvents.

lauryl alcohol + lauric acid → lauryl laurate + H_2O (1)

N-benzoyl-L-tyrosine ethyl ester + *n*-butylamine → *N*-benzoyl-L-tyrosine butylamide + ethanol (2)

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (3)$$

Table I shows the activity and solubility for the reactions (eq 1–3) by PEG-modified enzymes [lipase (eq 1), chymotrypsin (eq 2), and catalase (eq 3)] in chlorinated hydrocarbons. The PEG-modified enzymes dissolved in a

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Table I. Enzymic Activity and Solubility of Polyethylene Glycol Modified Enzymes in Chlorinated Hydrocarbons

	modified lipase		modified chymotrypsin		modified catalase		toxicity
solvent	activity,[a] %	solubility,[b] mg/mL	activity,[a] %	solubility,[b] mg/mL	activity,[a] %	solubility,[b] mg/mL	TLV,[c] ppm
benzene	100	1.00	100	0.90	100	0.48	25 C
chloroform	30	1.00	1	0.48	90	0.59	50 C
1,1-dichloroethane	8	0.90	7	1.00	1	0.60	100
1,1,1-trichloroethane	359	0.94	128	0.92	152	0.44	350
trichloroethylene	38	1.00	114	0.79	30	0.59	100
perchloroethylene	33	0.23	33	0.79	30	0.51	100

[a] Activity in benzene was taken as 100%, which corresponds to 7.9 μmol/min/mg of protein for modified lipase, 0.5 μmol/min/mg of protein for modified chymotrypsin, and 26 000 units/mg of protein at 2.4 mM hydrogen peroxide for modified catalase. [b] These data were taken as indicated in Experimental Section. [c] TLV and C are threshold limit value and ceiling limit, respectively, in the occupational environment recommended by American Conference Government Industrial Hygenist.[6]

transparent state in chlorinated hydrocarbons, while the native enzymes never dissolved. Among the chlorinated hydrocarbons, 1,1,1-trichloroethane was the most effective to promote each reaction, and the rates of the reactions (enzymic activities) were determined as 26 μmol/min/mg of protein (25 °C) for modified lipase and 0.64 μmol/min/mg of protein (37 °C) for modified chymotrypsin. The activity of modified catalase in 1,1,1-trichloroethane was 40 000 units/mg of protein (30 °C) at 2.4 mM hydrogen peroxide, which is 2.6 times higher activity than that of the native one in aqueous system. These reactions proceed in completely transparent solutions. The rate of each reaction was linearly enhanced with increasing amount of the modified enzyme. The yields of ester synthesis (eq 1) and acid–amide bond formation (eq 2) were 90% (25 °C, 100 min) and 90% (37 °C, 20 min), respectively. For the decomposition reaction of hydrogen peroxide (2.4 mM) (eq 3), the reaction was completed (100%) within 1 min at 30 °C. It is noteworthy that, as seen in Table I, the rates of the reactions in 1,1,1-trichloroethane are 1.3–3.6 times higher than those in benzene. Furthermore, this solvent has another advantage, that its toxicity is lowest among the solvents studied here (Table I). Thus, 1,1,1-trichloroethane is profitable for practical application of the modified enzymes for chemical reactions in the organic solvent system.

Experimental Section

Lipoprotein lipase crystallized from *Pseudomonas fluorescens* was kindly donated from Amano Pharmaceutical Ltd. (Nagoya, Japan). Chymotrypsinogen from bovine pancreas was prepared by the method of Kunits and Northrop.[7] Bovine liver catalase were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of a Modifier (Activated PEG_2), 2,4-Bis(ω-methoxypoly(ethyleneoxy))-6-chloro-*s*-triazine.[8] To a benzene solution (200 mL) of monomethoxypolyethylene glycol (40 g, 8 mmol; average molecular weight, 5000) was added 13 g of molecular sieves (3A, Nikka Seiko Co., Tokyo) to remove the water. To the anhydrous benzene, thus prpared, were added anhydrous sodium carbonate (13 g, 123 mmol) and cyanuric chloride (756 mg, 4 mmol) (recrystallized two times). The mixture was refluxed at 80 °C for 120 h. The reaction mixture was cooled and filtered, and then petroleum ether was added to the filtrate. The precipitate formed was redissolved in 400 mL of benzene–acetone (1:1 v/v) and reprecipitated with petroleum ether. A pure activated PEG_2 was obtained by repeating the reprecipitation process three times.

Preparation of Polyethylene Glycol Modified Enzymes. After adding activated PEG_2 (0.8, 1.4, and 0.35 g) to buffer solutions of enzymes (3.2 mL of 0.4 M sodium borate, pH 10, 20 mg of lipase; 2 mL of 0.4 M sodium tetraborate, pH 10, 14 mg of chymotrypsinogen; 2 mL of 0.1 M sodium borate, pH 9.5, 6 mg of catalase, respectively), the reaction mixture was incubated at 37 °C for 1 h, and ultrafiltrated with Amicon Diaflo PM-30 membrane to remove unreacted activated PEG_2. The activated PEG_2 is covalently coupled with amino groups of the enzyme molecules. The degrees of modification of the lipase, the chymotrypsinogen, and the catalase were 55%, 83%, and 42%, respectively, which were estimated by measuring free amino groups with trinitrobenzenesulfonate.[9] A modified chymotrypsin was obtained by activation of the modified chymotrypsinogen with trypsin. The modified enzymes were dialyzed against cold water and lyophilized. A given amount of modified enzyme (1 mg of modified lipase, 1 mg of modified chymotrypsin, and 0.6 mg of modified catalase) was mixed with 1 mL of solvents, and the clear supernatant containing modified enzyme was obtained by centrifugation (1700 × *g*, 10 min). Protein concentration in the supernatant was determined by a biuret method after removing solvents by evaporation, and it was defined as the solubility in Table I.

Synthesis of Lauryl Laurate with the Modified Lipase.[2] After adding 0.1 mL of the modified lipase in water-saturated solvent to a solution (0.3 mL) of 0.12 mmol of the lauric acid and 0.18 mmol of lauryl alcohol, the reaction mixture was incubated at 25 °C for 10 min. The reaction was stopped by adding 0.1 mL of a 0.2N sulfuric acid to the transparent reaction mixture. The remaining fatty acid and synthesized ester were separated as follows. To the reaction mixture were added 2 mL of 0.25 N NaOH, 2 mL of petroleum ether, and 0.8 mL of methanol, and they were shaken vigorously and centrifuged. Ester extracted in the supernatant was determined by a colorimetric method.[10]

Synthesis of *N*-Benzoyltyrosine Butylamide with the Modified Chymotrypsin.[5] To 0.2 mL of a solution of 0.15 μmol of benzoyl-L-tyrosine ethyl ester, 3.3 μmol of *n*-butylamine, and an organic solvent was added 0.1 mL of the modified chymotrypsin. The transparent mixture was incubated at 37 °C, and an aliquot (10 μL) was taken out from the mixture at a given time. The sample was subjected to analysis with Shimadzu high-performance liquid chromatograph LC-3A (Shodex Silicapack E-411 column) with 2.5% methanol in chloroform. The elemental analysis of the product was made by a Perkin-Elmer elemental analyzer Model 240.

Hydrogen Peroxide Decomposition with the Modified Catalase. To 2 mL of a solution of hydrogen peroxide and organic solvents was added 0.02 mL of the modified catalase, and the reaction mixture was incubated at 30 °C. The concentration of hydrogen peroxide in the reaction mixture was colorimetrically determined with titanium sulfate by the method of Pobiner.[11] One unit of catalase activity was calculated as the decomposition of 1.0 μmol hydrogen peroxide per min at the substrate concentration of 2.4 mM.

Registry No. L-PhCOTyrOEt, 3483-82-7; NH_2Bu, 109-73-9; L-PhCOTyrNHBu, 95043-84-8; H_2O_2, 7722-84-1; $CHCl_3$, 67-66-3; Cl_2CHCH_3, 75-34-3; Cl_3CCH_3, 71-55-6; $Cl_2C{=}CHCl$, 79-01-6; $Cl_2C{=}CCl_2$, 127-18-4; 2,4-bis(ω-methoxypoly(ethyleneoxy))-6-chloro-*s*-triazine, 72708-10-2; monomethoxypolyethylene glycol, 9004-74-4; cyanuric chloride, 108-77-0; lauric accid, 143-07-7; lauryl alcohol, 112-53-8; benzene, 71-43-2; lauryl laurate, 13945-76-1.

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