Fatty Acid Vinyl Esters as Acylating Agents: A New Method for the Enzymatic Synthesis of Monoacylglycerols

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ABSTRACT: Lipase-catalyzed synthesis of monoacylglycerols (MAG) was performed by transesterification reactions between fatty acid vinyl esters and either glycerol (**1**) or 1,2-O-isopropylidene-*rac*-glycerol (**2**), without solvents or in the presence of *n*-pentane. Vinyl decanoate, vinyl laurate, vinyl stearate and vinyl palmitate have been converted to the corresponding monoacylglycerols. As expected for the reaction with **1**, a mixture of mono-, di- and triacylglycerols was synthesized. The highest concentrations of MAG were achieved with vinyl stearate (30% 2-MAG and 15% 1-MAG). The reactions of fatty acid vinyl esters with the protected glycerol (**2**) led to the corresponding protected 3-monoacylglycerols with 100% conversion after short reaction times. The subsequent cleavage of these acetonides was performed by four different methods. The fastest cleavage was found with trifluoroacetic acid as catalyst, whereas the highest concentration of MAG (100%) was obtained for the boric acid-catalyzed hydrolysis of the acetonides.
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KEY WORDS: Cleavage, diacylglycerol, 2,2-dimethyl-1,3-dioxolane-4-methanol, fatty acid vinyl ester, 1,2-O-isopropylidene-*rac*-glycerol, lipase, monoacylglycerol, transesterification.

In the last decade, the application of enzymes—especially lipases (triacylglycerol hydrolases, E.C. 3.1.1.3)—in organic syntheses has become more and more important. They have been widely used in the enantioselective synthesis of precursors for pharmaceutically active compounds (1) and the conversion of natural fats and oils into high-value added products, e.g., monoacylglycerols (MAG) (2,3).

MAG are the most widely used emulsifiers in food, pharmaceutical and cosmetic industries. At present, they are manufactured by continuous chemical glycerolysis of fats and oils at high temperatures (220–250°C) with inorganic alkaline catalysts under nitrogen gas atmosphere. The product thus produced has several drawbacks, e.g., low yield, dark color, burnt taste, etc. (2).

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For enzymatic production of MAG, several methods are described in the literature. Beside selective hydrolysis with 1,3-regiospecific lipases (4) and esterification of free fatty acid with glycerol (5,6), high yields of MAG were obtained in glycerolysis reactions of triacylglycerols (TAG) (2,3). As reactions systems, reverse micelles, microemulsions, water-organic biphasic systems, organic solvents and the solid state have been investigated (3–8). The products were often mixtures of MAG with different fatty acid residues, and also small amounts of 2-MAG have been formed.

Pure, racemic MAG have been prepared chemically by esterification of 1,2-O-isopropylidene-*rac*-glycerol or 1,2-O-benzylideneglycerol with acid chlorides and the subsequent selective hydrolysis of the ketal (9). For the chemical synthesis of enantiomerically pure MAG, sugars like D- or L-mannitol, D- or L-arabinose or serine derivatives have been used as starting materials (10–12).

The enantioselective, enzymatic synthesis of MAG was reported for short-chain MAG, such as acetates, propionates and valerates (13), but only low enantiomeric excesses were achieved. By using 1,2-O-isopropylidene-*rac*-glycerol as protecting group, high optical purities were obtained for glycerol butanoates (14). High conversions were reported for the esterification of free fatty acids (15,16) or the corresponding methyl esters (16), but acyl migration occurred in the acid-catalyzed removal of the protecting group.

Here, we report the application of different commercially available fatty acid vinyl esters (FAVE) in the formation of MAG with different properties, such as chainlength and degree of saturation. For comparison, the transesterification reactions were performed in different systems with glycerol or 1,2-O-isopropylidene-*rac*-glycerol (IPG) as substrates in a solvent-free reaction system or in the presence of an organic solvent (Scheme 1).

EXPERIMENTAL PROCEDURES

Lipase. Two thousand units (U) of lipase from *Pseudomonas cepacia* [PCL, crude (40 U/mg); Amano Pharmaceutical Co. Ltd., Nagoya, Japan] were used in each experiment.

Reactions with glycerol. Experiments in pure glycerol: 25

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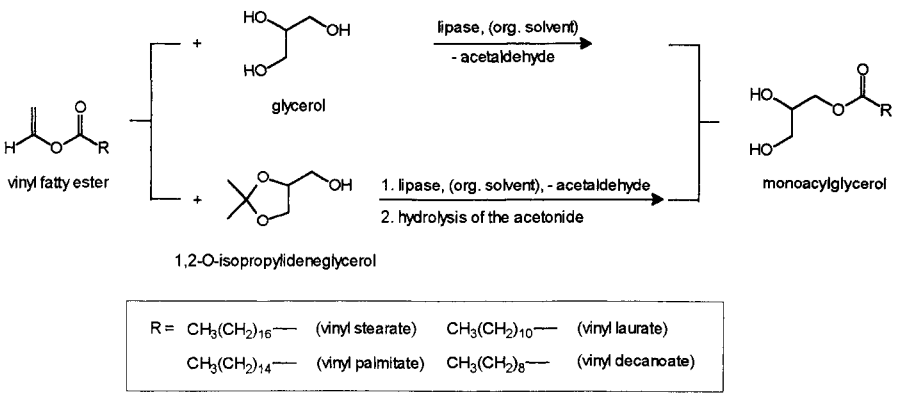

SCHEME 1

mmol glycerol (purity 95%; Wako Pure Chemical Industries Ltd., Osaka, Japan) and FAVE (0.5 mmol) were mixed, and lipase powder was added. In the presence of a solvent: 2.5 mmol glycerol, 0.5 mmol FAVE and 2 mL *n*-pentane were mixed, and lipase powder was added.

Reactions with 1,2-O-isopropylidene-rac-*glycerol.* Without solvent: FAVE (0.5 mmol) and 15 mmol 1,2-O-isopropylidene-*rac*-glycerol (purity 97%; Sigma, St. Louis, MO) were mixed, and lipase powder was added. In the presence of a solvent: FAVE (0.5 mmol) and 1,2-O-isopropylidene-*rac*-glycerol (1.5 mmol) were dissolved in 2 mL *n*-pentane, and lipase powder was added. Reactions were carried out in 10-mL test tubes in a reciprocal shaker at 400 strokes/min. All experiments were performed twice.

The water content of the glycerol was 4% (w/w), which was determined as optimum in previous experiments (3). All solvents were of analytical grade and dried over molecular sieves. Reaction temperature was 25°C. Vinyl palmitate (purity 93%), vinyl stearate (purity 97%), vinyl laurate (purity 98%) and vinyl decanoate (purity 98%) were purchased from Wako.

Analysis of the reaction mixture. When using glycerol, 50-µL samples were withdrawn with a pipette. Inactivation of the enzyme and removal of excess glycerol were achieved by extraction of the reaction mixture into chloroform according to the method of Yamane *et al.* (17). When using 1,2-O-isopropylidene-*rac*-glycerol, 50-µL samples were centrifugated (5 min at 10 000 rpm), and the supernatant was used for analysis.

Analysis was performed by thin-layer chromatography/flame-ionization detection (TLC/FID) (Iatroscan TH-10; Iatron Laboratories Inc., Tokyo, Japan). Chloroform extract (1 µL) was applied to boric acid-treated chromarods and followed by development in chloroform (8 cm) and chloroform/(methanol/ammonia 8:2) (70:0.05) solvent for 10 cm. The rods were dried and scanned under the following conditions: hydrogen, 1.1 kg/cm^2; airflow, 2000 mL/min; and 30 s/scan. TAG, diacylglycerol (DAG), MAG, acetonide, IPG and FAVE were effectively separated. Peak areas were calculated with an SIC Chromatocorder 12 integrator (System Instruments Co., Osaka, Japan). Results are expressed as percentage peak areas, which may vary slightly from the true weight percent. The deviation of all data was *ca.* 3%. Samples were also analyzed by TLC with chloroform or hexane/1,2-dichloroethane/ethanol (40:12:3) as solvents. Dyeing was performed with aqueous $KMnO_4$ solution (1% vol/vol). R_f values are: TAG, 0.72; FAVE, 0.66; acetonides, 0.57; 1,3-DAG, 0.43; 1,2-DAG, 0.32; IPG, 0.20; MAG, 0.04.

Assay of lipolytic activity. Activity of lipase was expressed as its hydrolytic activity measured by the olive oil emulsion method without addition of surfactants (18). Olive oil (Katayama Chemical, Osaka, Japan) (1.5 mL) was incubated with phosphate buffer (pH 7.0) at 37°C for 5 min, and lipase solution in the same buffer was added to a final volume of 10 mL assay solution. After 20 min, the reaction was stopped by the addition of 20 mL acetone/ethanol solution (1:1). Two drops of phenolphthalein solution (1%) were added, and the amount of free fatty acids was titrated in an automatic titrator with 0.05 M NaOH solution. One unit of activity is defined as the amount of enzyme that liberates 1 µmol equivalent of fatty acid from olive oil in 1 min at 37°C. After the reaction, the suspended lipase was isolated from the reaction mixture by filtration through filter paper, and the remaining activity was determined as described above. All samples were measured in triplicate; the deviation of the data was *ca.* 10%.

Water content. Water concentration was determined with a Karl-Fischer moisture meter (MKS-1; Kyoto Electronics, Tokyo, Japan).

Hydrolysis of the acetonide. Four methods were applied: (i) The acetonide (1,2-O-isopropylidene-3-fatty acid glycerol) and finely powdered boric acid (molar ratio 1:10) were heated in the presence of 2-methoxyethanol 70°C until TLC showed the consumption of the acetonide (19). (ii) The acetonide was transferred to a 1.0% solution of iodine in methanol (wt/vol) at room temperature until TLC showed the consumption of the substrate (20). Iodine was reduced with saturated sodium thiosulfate solution. (iii) The acetonide and aqueous HCl [50% (vol/vol)] were incubated at 75°C for 1 h (15). (iv) The acetonide and aqueous trifluoroacetic acid [TFA, 90% (vol/vol)] were incubated for 10 min at room temperature (15). After cleavage, the product was extracted in *t*-butylmethylether and washed with water. The ether solution was

dried over sodium sulfate and filtered. After evaporation of excess solvent, the product was dried first in air and finally in a vacuum desiccator.

RESULTS AND DISCUSSION

As shown in Scheme 1, two different approaches toward the synthesis of MAG have been investigated. In both reactions, FAVE have been used. They offer the advantage of a shift of the reaction equilibrium toward the desired product. This is caused by tautomerization of the vinyl alcohol to the corresponding aldehyde (21). The transesterification reaction of the FAVE with glycerol (Scheme 1a) resulted in a mixture of MAG, DAG and TAG. Although a large excess of glycerol was used in the solvent-free reactions, the concentration of MAG never exceeded more that 45% (Table 1). In glycerolysis reactions of triglycerides, up to 99% of mainly 1-MAG was formed (2,3). There, in the first reaction hours, a maximum of 2% of 2-MAG was synthesized, which was converted into 1-MAG during the progress of the reaction. In contrast, in the transesterification reactions between glycerol and FAVE reported here, considerably higher concentrations of 2-MAG were found for the solvent-free reactions (Table 1). With vinyl stearate 30%, and with vinyl decanoate 19%, 2-MAG was formed.

Several organic solvents were tested with respect to solubility of the substrates and activity of the lipase (data not shown). *n*-Pentane was the most suitable solvent. The concentrations of 1-MAG were considerably lower with all FAVE, compared to the experiments in pure glycerol (Table 1). The synthesis of TAG- and DAG was favored, and no 2-MAG was formed. This was mainly due to the lower concentration of glycerol. The absence of 2-MAG may be caused by a solvent-induced change in the regiospecifity of the lipase.

The remaining activity of the lipase in the reactions with pure glycerol differed significantly from those experiments where *n*-pentane was used as solvent (Table 1). The highest stability was found for the reactions with vinyl laurate, whereas only 7% remaining lipase activity was measured after the reaction of vinyl decanoate with pure glycerol.

TLC and TLC/FID analysis showed that the reactivity of the four different FAVE increased with increasing chainlength from vinyl decanoate to vinyl stearate. The same substrate of lipase from *Pseudomonas cepacia* was reported for the hydrolysis of the corresponding methyl esters of these FAVE (22). It seems that lipase from *P. cepacia* prefers esters of longer-chain fatty acids as substrates.

To overcome the problem of subsequent acylation of the MAG, the reactions were also performed with the protected glycerol, 1,2-O-isopropylidene-*rac*-glycerol (Scheme 1b). Figure 1 shows the time course for the conversion of the FAVE into the corresponding acetonides. In the presence of *n*-pentane, after 1 h reaction time, about 50% conversion was found for all FAVE, and after about 20 h, the FAVE were converted almost quantitatively into the corresponding acetonides. With the exception of vinyl palmitate, the reactions of FAVE with pure IPG gave lower initial reaction rates, and 2 d were necessary for complete conversion. This is in accordance with the remaining lipase activity, which 2.75 to 5 times higher in the presence of *n*-pentane (Table 1, values in parentheses). A comparison of the remaining lipase activity of all experiments shows that the lipase was more stable in *n*-pentane with IPG as substrates than with glycerol as substrate.

Isolation of the MAG was attained by cleavage of the protecting acetone group from the acetonides synthesized in the enzymatic transesterification reaction. Four different methods, from mild hydrolysis with boric acid or iodine in methanol to strongly acidic conditions with half-concentrated HCl or 90% TFA, were investigated. During the cleavage, excess amounts of IPG were hydrolyzed to glycerol, which was easily extracted in the water phase during the downstream process (see Experimental Procedures section), allowing the isolation of pure MAG without further purification steps. The results of the hydrolysis reactions are shown in Table 2 for each acetonide. It is obvious that boric acid was the most effective catalyst with respect to MAG concentration, but long reaction times were necessary. With TFA, complete hydrolysis of the acetonides took place after only 10 min reaction

TABLE 1
Final Concentration of TAG, DAG, MAG and Remaining Lipase Activity for the Reaction of Glycerol with Fatty Acid Vinyl Esters (FAVE) Solvent-Free or in *n*-Pentane

FAVE	TAG conc. (% peak area)	DAG conc. (% peak area)	2-MAG conc. (% peak area)	1-MAG conc. (% peak area)	Remaining lipase activity (%)[a]
Vinyl stearate	25	30	30	15	64 (21)
in *n*-pentane	24	55	0	21	38 (88)
Vinyl palmitate	13	72	7	8	34 (24)
in *n*-pentane	29	62	0	7	37 (66)
Vinyl laurate	5	82	6	7	99 (19)
in *n*-pentane	57	38	0	5	71 (96)
Vinyl decanoate	18	39	19	24	7 (24)
in *n*-pentane	57	37	0	6	42 (88)

[a]Values in brackets represent the remaining lipase activity for the reaction of the FAVE with 1,2-O-isopropylidene-*rac* glycerol. TAG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerols.

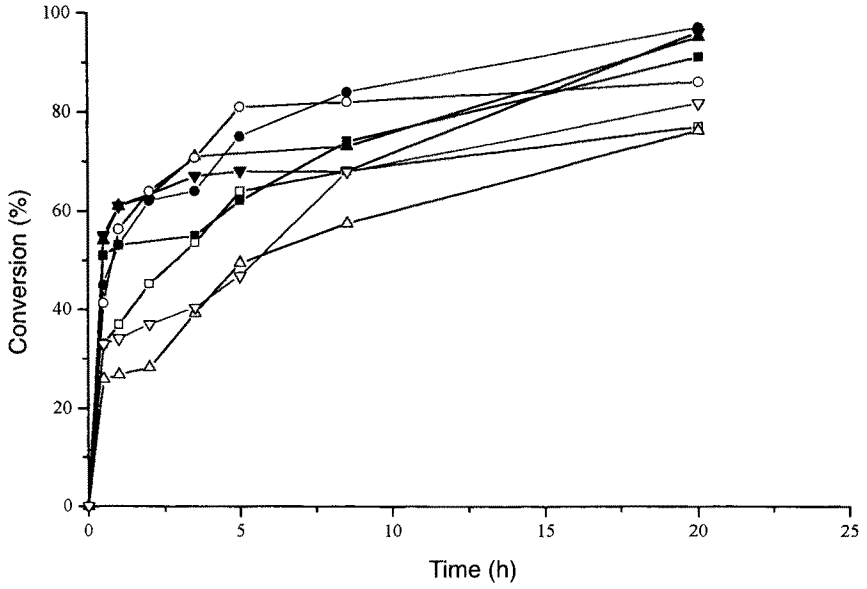

FIG. 1. Time course for the lipase-catalyzed transesterification reactions without solvent (open symbols) or in *n*-pentane (filled symbols) between vinyl palmitate (●), vinyl stearate (■), vinyl laurate (▲) and vinyl decanoate (▼) and 1,2-O-isopropylidene-*rac*-glycerol. The deviation of all data is below 3%.

time, and the concentration of MAG was higher than 90% with the exception of 1,2-O-isopropylidene-3-lauroylglycerol (78%). Iodine in methanol was an acceptable reagent, too, but not for the cleavage of 1,2-O-isopropylidene-3-stearoylglycerol (**3b**, Table 2). Half-concentrated HCl was found unsuitable because it resulted in high concentrations of DAG and TAG in the hydrolysis reaction mixture.

In summary, the transesterification reaction between the FAVE and IPG in *n*-pentane represents an effective method for the synthesis of MAG. Higher yields of MAG and shorter reaction times have been found, compared to the results described in the literature for the direct esterification and transesterification of fatty acid esters (15; Heβ, R., U. Bornscheuer, A. Capewell and T. Scheper, unpublished data). This may be caused by the shift of the reaction equilibrium due to the irreversible formation of acetaldehyde and the choice of the biocatalyst used. Although the reaction with IPG requires subsequent cleavage, this method seems to be superior because MAG are obtained quantitatively. In addition, the high remaining lipase activity and its possible use in a continuous

TABLE 2
Glyceride Composition After the Hydrolysis of the Acetonides by Various Methods

Acetonide[a]	Method	Time (h)	TAG conc. (% peak area)	DAG conc. (% peak area)	MAG conc. (% peak area)
3a	Boric acid	72.0	0	0	100
	I_2/MeOH	48.0	0	19	81
	HCl (50% vol/vol)	1.0	7	72	21
	TFA (90% vol/vol)	0.2	0	10	90
3b	Boric acid	72.0	0	0	100
	I_2/MeOH	48.0	0	67	33
	HCl (50% vol/vol)	1.0	14	65	21
	TFA (90% vol/vol)	0.2	0	6	94
3c	Boric acid	72.0	0	0	100
	I_2/MeOH	48.0	0	17	83
	HCl (50% vol/vol)	1.0	19	67	14
	TFA (90% vol/vol)	0.2	0	22	78
3d	Boric acid	72.0	0	0	100
	I_2/MeOH	48.0	6	24	70
	HCl (50% vol/vol)	1.0	18	62	30
	TFA (90% vol/vol)	0.2	0	8	92

[a]**3a**, 1,2-O-isopropylidene-3-palmitoylglycerol; **3b**, 1,2-O-isopropylidene-3-stearoylglycerol; **3c**, 1,2-O-isopropylidene-3-lauroylglycerol; **3d**, 1,2-O-isopropylidene-3-decanoylglycerol. See Table 1 for abbreviations; TFA, trifluoroacetate acid.

process make this method very attractive. In contrast, the reactions with glycerol and FAVE gave only low to moderate yields of MAG, but the formation of 2-MAG may be of interest. For the high-yield synthesis of 1-MAG—especially from natural fats and oils—other methods, such as glycerolysis reaction of fats and oils in a solid-phase system or esterification in microemulsions, seem to be the better choice.

Enzymic hydrolysis of TAG and subsequent separation of 1-MAG is less effective than glycerolysis and transesterification because 1 mole MAG and 2 moles of free fatty acid per mole of TAG are theoretically formed by hydrolysis, whereas 3 moles MAG per mole of TAG by glycerolysis and one mole MAG per mole 1,2-IPG without free fatty acid can be obtained by our method; the yields of transesterification and glycerolysis are much higher than for hydrolysis.

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