[BIO31] Organic synthesis of 1,2-O-Isopropylidene-3-acyl glycerol by Amberlite XAD-7 adsorbed lipase of *Pseudomonas* sp. AK

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Introduction

Monoacylglycerols (MG) are widely used as emulsifiers mainly in the food, cosmetics and pharmaceutical industries. MG can be chemically synthesized via glycerolysis or hydrolysis of fats and oils at high temperature using inorganic catalysts. Chemical synthesis through esterification of 1,2-0-isopropylidene glycerol with acid chloride, followed by subsequent hydrolysis of the ketal has also been reported (Quinn *et al*., 1967). However, chemical synthesis posed several problems, such as high energy consumption, difficulty in product separation, use of carcinogenic inorganic catalyst and involves non-environmental friendly processes. Thus, enzymatic method has been employed and were found to be more superior than the conventional chemical method (Hess *et al*., 1995).

The enzymatic glycerolysis using triolein or olive oil (McNeill and Yamane, 1991; Chang *et al*., 1991) and selective hydrolysis of triolein or natural fats have been reported for MG production (Holmberg and Osterberg, 1988; Plou *et al*., 1996). Enzymatic esterification between glycerol and fatty acids was also employed (Jansen *et al*., 1993). However, the subsequent formation of mixture of monoglycerides, diglycerides and triglycerides caused low or moderate yield of MG due to the instability of the partial glycerides in aqueous conditions. Therefore, esterification will have to be considered under highly reduced water content in organic phase reaction system. Stability can be achieved through immobilization of enzymes. Various support materials have been developed for many decades using methods based on the principles of adsorption, covalent binding, entrapment and crosslinking. Most of the immobilized enzyme preparations were used in aqueous phase reactions, although a number of these reactions were also used in organic synthesis. Among the methods used, adsorption technique remains the most widely used method for enzyme immobilization mainly for application in organic synthesis not only due to the simplicity of the method, but also the preparation gave high activity and stability. One important characteristic in the adsorption technique is that the enzyme molecules which are adsorbed to the surface of the support materials allow the binding of the monolayer of water molecules which is needed to maintain the enzyme stability and integrity for biocatalysis (Norin *et al*., 1988).

This paper describes the synthesis of 1,2-0-isopropylidene acyl glycerol (Fig. 1) as an intermediate in the formation of MG using the lipase of *Pseudomonas* sp" AK adsorbed onto Amberlite XAD- 7. Some of the governing parameters affecting the esterification reaction were examined including the stability of the immobilized lipase preparation for long term application in the organic phase reaction.

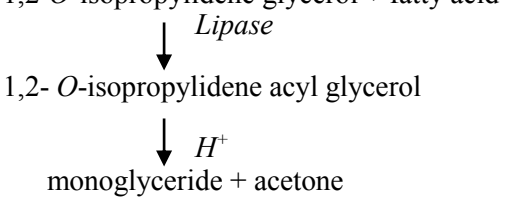

FIGURE 1 Reaction scheme of 1,2-0-isopropylidene acyl glycerol synthesis and MG formation from 1,2-0- isopropylidene acyl glycerol.

Materials and methods

Source of lipase

Crude lipase preparation *of Pseudomonas* sp. AK used in the work was a generous gift *of* Amano Pharmaceutical Co. Ltd (Nagoya Japan). The enzyme preparation was used without prior purification.

Immobilization of lipase

Immobilization of lipase onto Amberlite XAD- 7 (Fluka Chemika) was carried out by the adsorption method as described by Ibrahim *et al.* (1988) (Ibrahim *et al*., 1988) with modifications in the immobilization conditions based on the

characteristics of lipase of *Pseudomonas* sp. Ak as documented by Amano Pharmaceutical Co. Ltd. The *immobilization* of lipase was performed by dissolving 0.5 g of enzyme powder in *50* ml *of* McIlvaine buffer of pH 5.0. The undissolved materials were by filtration using Whatmann No#1 filter paper. The filtrate was was added with 1.0 g of Amberlite XAD-7 and was agitated at 150 rpm, 32°C for120 min. After the agitation, the adsorbed lipase was crosslinked with glutaradehyde at the final concentration of 2.5% (v/v) for 60 min. The adsorbed and cross linked lipase was filtered and washed thrice with distilled water and dried in vacuum overnight at room temperature before the activity was determined. The hydrolytic activity of immobilized lipase was determined by the method *of* polyvinyl alcohol emulsion method as described by Ibrahim *et al.* (1987) (Ibrahim *et al*., 1987). The fatty acid liberated was titrated using the pH stat autotitrator (Titramate *20* Mettler Toledo) at the pH of 9.0. One unit of lipase is defined as the amount of enzyme which liberates 1 µmole of free fatty acids under the assay conditions.

Reaction mature for synthetic reaction

The reaction for the synthesis of 1,2-*O*-isopropylidene acyl glycerol was carried out in a screw-capped conical flasks. The reaction mixture consists of 8 mM of 1,2-*O*-isopropylidene glycerol (Sigma) and 8 mM of fatty acid (Sigma) in 10 ml of n-hexane with 100mg of the immobilized lipase preparation of activity 1300 U/g. The reaction was carried out at 32°C with shaking of 150 rpm for 24 h. Samples of 400 µI were withdrew every 4 h to analyse the residual reactants and product formation using gas chromatography (Shimadzu GC 14A), followed by mass spectrometry.

Determination of conversion degree.

The conversion degree was determined based on the percent composition by weight (%, w/w) of the equilibrium conversion of fatty acids to the corresponding esters. The column used to detect the reactants and product was Permabond SE-54-HKW-l (Macherey and Nagel, Duren, Germany) of size 0.32 mm internal diameter and 25 m in length. The analytical conditions used were carrier gas hydrogen (1 kg/m^2 and nitrogen (3 kg/m^2) of flow rate of 2.0 ml min^{-1}, injection temperature of 280°C, detector temperature 280°C, temperature programme 250°C (2 min) 280°C (15 min) and injection volume of 0.1 µI. The esters produced and the reactants were confirmed with Thermofinnigan Trace gas chromatography mass spectrometry (GCMS) using the conditions of carrier gas helium (50 psi) at the flow rate of 1.0 ml min^{-l} with injection and detector temperature of 280°C.

Effect of physical parameters on the synthetic reaction

The physical parameters which affected the synthetic reaction studied were in the range of 32 -60°C, agitation rate of 50 - 225 rpm, and substrate ratio of 1,2-*O*-isopropylidene glycerol to fatty acid ratio of 3:1, 2:1, 1:1, 1:2 and 1:3 (mol/mol). The organic solvents consisting of isooctane, heptane, hexane, carbon tetrachloride toluene, chloroform, benzene, diisopropyl ether, ethyl acetate, tetrahydrofuran and methanol were used to determine its effect on the synthetic reaction. The substrates and the organic solvents were kept in the presence of molecular sieve pellets (Fluka Chemika) at all times prior to use to keep the water content to the minimum.

The use of anhydrous and hydrated salts and saturated salt solutions for water control in the reaction system

Anhydrous and hydrated salts were used either singly or in pairs as described by Ibrahim and Robb (1995a) (Ibrahim and Robb, 1995a). About 0.4 g of the single salt or 0.2 :0.2 g of the paired salts were added in the reaction mixture. The salt hydrates used were N_a2SO_4 (R & M Chemicals), $Na_2HPO_4.2H_2O$ (a_w 0.177, R & M Chemicals), $Na_2HPO_4.12H_2O$ (a_w 0.85, R & M Chemicals), Na_2HPO_4 (BDH Chemicals), $Na_2HPO_4.0/2H_2O$, $Na_2HPO_4.0/12H_2O$, $Na_2HPO_4.2/12H_2O$, Na_2CO_3, (Riedel de Haen), $Na_2CO_3.10H_2O$ (a_w 0.79, Riedel de Haen), $Na_2CO_3.0/10H_2O$, $Na_2B_40_7$ (Riedel de Haen), $Na_2B_40_7.10H_2O$ (a_w 0.52, Merck)

$Na_2B_4O_7.0/10H_2O$, $CaCl_2$, (Biolab), $CaCl_2.2H_2O$ (a_w 0.043, Fluka Chemika), $CaCl_2.0/2H_2O$ and $CuSO_4.5H_2O$ (a_w 0.1, Cambrian Chemicals). The water activity (a_w) data of the salts at 30°C were obtained from Halling (1992) (Halling, 1992).

The saturated salt solutions were used to pre-equilibrate the immobilized lipase preparation following the procedure described by Ibrahim and temperature Robb (1995b) (Ibrahim and Robb, 1995b). The dried enzyme preparation were pre-equilibrated in various saturated salt solutions for 24 h before the enzyme preparation was added in the reaction mixture to start the reaction. The saturated salt solutions used were $LiCl_2$ (a_w 0.12), CH_3COOK (a_w 0.22), K_2CO_3 (a_w 0.432), NaBr (a_w 0576), KI (a_w 0.689), NaCl (a_w 0.753), $(NH_4)_2SO_4$ (a_w 0.810), KCl (a_w 0.840), KNO_3(a_w 0.963), and K_2SO_4 (a_w 0.973),. The water activity data (a_w) of the saturated salts at 30°C was obtained from Greenspan (1977) (Greenspan, 1977).

Repeated used of Amberlite XAD- 7 adsorbed lipase for synthesis of 1,2-0- isopropylidene lauroyl glycerol

The reaction mixture and conditions used in the repeated reaction consist of the substrates 1,2-*O*-isopropylidene glycerol and lauric acid at the ratio of 2:1 (mol/mol) in 10 ml n-hexane. $CalCl_2.2H_2O$ at 0.4 g was added in the reaction mixture and incubated for 24 h at 30°C with shaking of 150 rpm. After 24 h, the enzyme particles was filtered and the filtrate was analysed using GC. The enzyme particles was washed twice with 20 ml n-hexane and dried under vacuum at room temperature. The dried lipase preparation was reused for the subsequent synthetic reaction.

Results and discussions

Time course profiles of 1,2-O- isopropylidene glycerol synthesis by Amberlite XAD- 7 adsorbed lipase from Pseudomonas sp.

The profiles of 1,2-*O*-isopropylidene lauroyl glycerol conversion from lauric acid and 1,2-*O*-isopropylidene glycerol are shown in Fig. 2. As shown in the figure, the increase in the % composition weight of the ester is correspond with decrease in the total composition of weight of IPG and lauric acid used in the conversion reaction. The product and the reactants were confirmed by GCMS. A maximum esterification of about 75% (w/w) was obtained after 24 hours which remained constant thereafter.

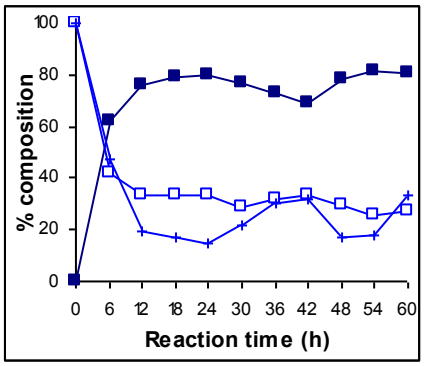

FIGURE 2 Time course profiles of the synthesis of 1,2-O-Isopropylidene-3-lauryl glycerol (■) and the reduction of the reactants, 1,2-*O*-Isopropylidene-*sn*-glycerol (□) and lauric acid (+).

Effect of temperature on synthetic reaction

Fig. 3 shows the effect of temperature on the conversion degree by immobilized lipase preparation. As shown in the figure, the stability of the reaction was observed within a wide temperature range of 30-50°C with the conversion degree in the range of about 75 – 78%. However the conversion degree dropped slightly to about 70% at 50 – 60°C. The result obtained indicated that a significant improved in the temperature stability of the immobilized preparation of 30 – 32°C (data not shown). The immobilized lipase of *Mucor miehei* (Lisozyme TM) also showed a higher optimum temperature of 55°C in organic phase reaction (Pecnik and Knez, 1992).

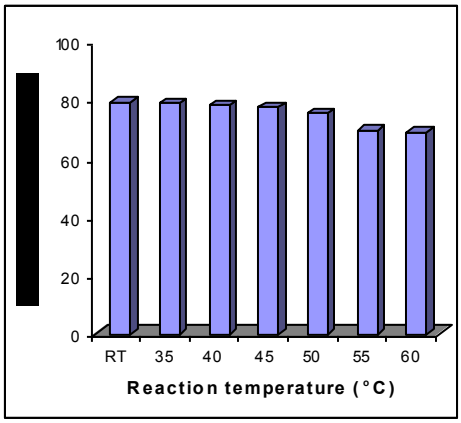

FIGURE 3 Effect of temperature on the synthetic reaction.

Effect of agitation rate on synthetic reaction

The agitation rate was studied using the reaction temperature of room temperature. The aim of agitation was to enhance mass transfer between the reactants and at the same time to disperse the available water content in the reaction mixture. It was observed that the agitation rate of 50 – 125rpm, the conversion degree remains unchanged of about 65% (Fig. 4). Similarly, the conversion degree also remained unaffected at the agitation rate of 175 – 225rpm. However an optimum conversion degree of about 80% was obtained at the agitation rate of 150rpm. Visual observation of the reaction system revealed that at lower agitation rate the biocatalyst was suspended freely in the organic phase and similarly, at higher agitation rate, the biocatalyst particles are highly dispersed or disintegrated. At 150rpm, due to the optimal centrifugal forces, the biocatalyst adhered strongly to the bottom of the flasks giving a good separation between the mixture reaction and the biocatalyst which promote good catalytic activity. On the other hand, high agitation was found to be detrimental to the biocatalyst due to excessive abrasion and disintegration of the support materials resulting in the lost of enzyme activity.

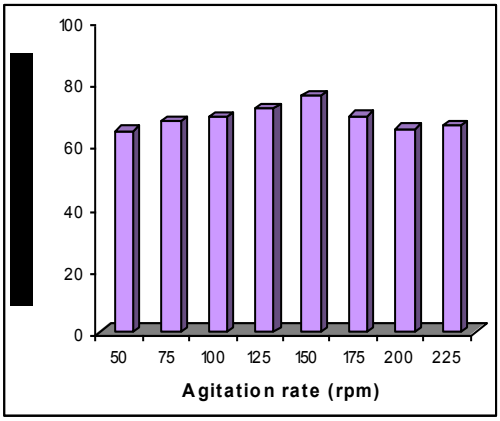

FIGURE 4 Effect of agitation rate on the synthetic reaction.

Effect of the substrate ratio on synthetic reaction

The mol ratio of IPG to lauric acid was examined in the ratio of 3:1, 2:1, 1:1, 1:2 and 1:3. As shown in Fig. 5, higher concentration of IPG (3:1) or lauric acid (1:3) resulted a slight drop in the conversion degree when compared to other ratio of the subsrates. The optimum was 2:1 which is in agreement with the result reported by Ibrahim *et al*. (1989) (Ibrahim *et al*., 1989) who suggested that higher alcohol content reduced the availability of water in the reaction system thus maintaining the equilibrium toward the ester formation.

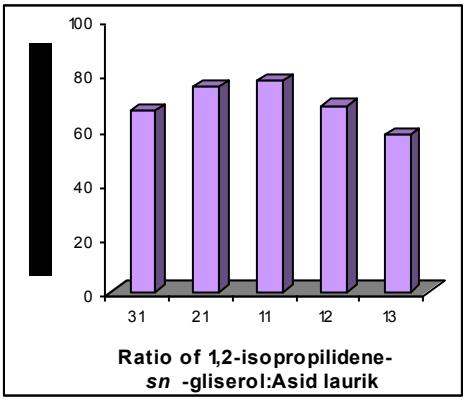

FIGURE 5 Effect of substrate ratio on the synthetic reaction.

Effect of fatty acid chain length on synthetic reaction

The specificity of the immobilized enzyme towards fatty acid chain length was determined using the fatty acids of carbon number ranging from C4 – C18 and C18:1 (Fig. 6). The fatty acid specificity of the enzyme preparation was observed for medium chain length of C8 – C14. Short chain fatty acid such as butyric acid, caproic acid, and caprylic acid showed lower conversion degree of 65 – 75%. Similar result were observed with longer chain length of fatty acid.

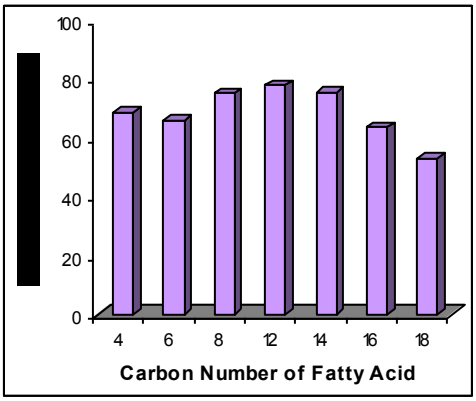

FIGURE 6 Effect of fatty acid chain length on the synthetic reaction.

Effect of various organic solvents on synthetic reaction

The performance of the immobilized lipase preparation in different organic solvents

is shown in Fig. 7. The result indicated that the conversion degree was closely related to the Log P values of the solvents which is an indicator of the solvent's polarity. It was observed that good conversion degree was obtained in organic solvents with the Log P values of 3.0 or more. On the other hand, lower Log P values (2.0 or less), resulted in poorer conversion degree. n-hexane with the Log P values of 3.5 resulted the highest conversion degree of about 85%. Organic solvents with Log P values between 3 -3.5 such as carbon tetrachloride and n-hexane are an extremely low affinity for water. Thus, water molecules will remain bound to the enzymes. On the other hand, organic solvents with lower Log P values are hydrophilic in nature and will strip away the essential water from the enzymes (Gorman and Dordick, 1992). High Log P values of 4.5 as in the case of isooctane, which is extremely immiscible in water (0.00095%, w/v) also showed lower conversion degree due to the low partition coefficient of the products in isooctane (Jansen *et al*., 1992).

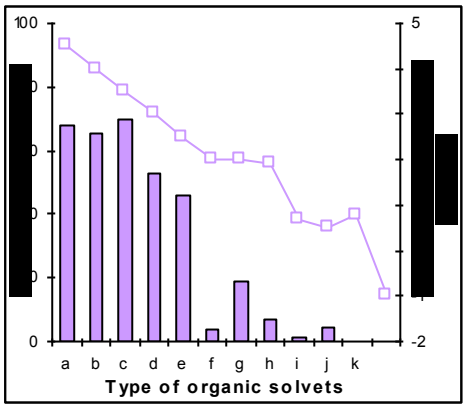

FIGURE 7 Relationship between various organic solvents and the log P values on the synthetic reaction.
a. isooctane, b. heptane, c. hexane, d. carbon tetrachloride, e. toluene, f. chloroform, g. benzene, h. diisopropyl eter, i. ethyl acetate, j. tetrahydrofuran, k. methanol

Effect of salt hydrate addition on synthetic reaction as water donor

Water control is an integral part of a non-conventional reaction under reduced water content. Several methods can be employed as water control systems in organic reactions, such as the use of molecular sieve pellets, dehydrating agents, salt hydrates and saturated salt solutions (Noor Izani and Ibrahim, 1998). The water control system using salt hydrates or also known as *water buffering* was employed in this study to determine the performance of the immobilized lipase preparation. It was observed that the use of salt hydrates either singly or in pairs did not show significant differences in the conversion degree (Fig. 8). However, it was observed that the differences in water activity of the salt hydrates influenced the conversion degree. The salt hydrate $Na_2HPO_4.2H_2O$ (a_w 0.177) showed higher conversion degree than $Na_2HPO_4.12H_2O$ (a_w 0.85). Salt hydrates with high a_w gave lower water buffering effect and must be used with either a lower a_w salt hydrate or an anhydrous salt. Thus, the use of $Na_2HPO_4.0/12H_2O$ resulted a higher conversion degree than the use of only $Na_2HPO_4.12H_2O$. Anhydrous salts such as Na_2SO_4, Na_2HPO_4, $Na_2B_40_7$ and $CaCl_2$ were also found effective in absorbing the water present in the reaction system giving a high conversion degree. A maximum conversion degree of about 90% was obtained using $CaCl_2$ either singly or in pairs ($CaCl_2.2H_2O$ or $CaCl_2.0/2H_2O$, a_w0.043). The results indicated that the synthetic reaction of 1,2-*O*-isopropylidene glycerol and lauric acid occurred under extremely low water content. The use of Na_2CO_3 was found to be detrimental to the immobilized lipase preparation. The salt tends to precipitate and forms aggregates with the biocatalyst particles which prevent the mass transfer between the particles and the reactants. $CuSO_4.5H_2O$ was found to show a relatively high conversion degree comparable to the use of molecular sieve pellets although Cu may cause enzyme protein denaturation. On the contrary, satisfactory stability towards CU^{2+} was observed with the immobilized lipase on Amberlite XAD- 7 in the presence of reaction. Cu^{2+}.

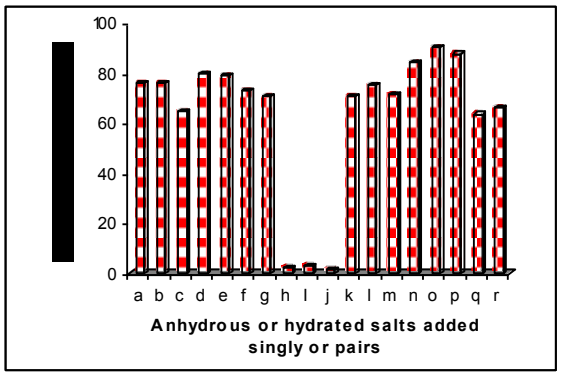

FIGURE 8 Effect of salts hydrated added in the reaction system on the synthetic reaction.
The salts hydrate used were
a. Na_2SO_4, b. $Na_2HPO_4.2H_2O$, c. $Na_2HPO_4.12H_2O$, d. Na_2HPO_4, e. $Na_2HPO_4.0/2H_2O$, f. $Na_2HPO_4.0/12H_2O$, g. $Na_2HPO_4.2/12H_2O$, h.Na_2CO_3, i. $Na_2CO_3.10H_2O$, j. $Na_2CO_3.0/10H_2O$, k. $Na_2B_4O_7$, l. $Na_2B_4O_7$. $10H_2O$, m. $Na_2B_4O_7$. $0/10H_2O$, n. $CaCl_2$, o. $CaCl_2$. $2H_2O$, p. $CaCl_2$. $0/2H_2O$, q. $CuSO_4.5H_2O$ and r. molecular sieve pallets.

Effect of saturated salt solution on synthetic reaction for water control

Saturated salts have been used successfully to pre-equilibrate or to fix the water content in a number of reaction systems (Blanco *et al*., 1992). In this study, only enzyme preparation was pre-equilibrated. As shown in Fig. 9, the change in water activity (a_w) up to 0.84 did not give any significance difference in the conversion degree in the range of 70 – 75%. This conversion degree was similarly observed in the reaction system without pre-equilibration. The result suggested that the water available within the enzyme molecules were inadequate in maintaining the integrity of the enzyme molecules. At the same time, the available water is strongly bound and is not easily transferred to reaction system. However an optimum degree of about 85% was observed when enzyme preparations was pre-equilibrated at the aw of 0.973. A salt of higher water activity (a_w) was needed to ensure adequate water content is made available to enzyme preparation and the reaction system. The water adsorption isotherm of the enzyme may have reached the maximum capacity using the salt solution K_2SO_4 of a_w 0.973. Therefore the access water which was adsorbed and transfer to reaction system may have pushed the equilibrium of the reaction towards the hydrolytic reaction on the formed ester to the corresponding IPG and lauric acid.

Repeated use of Amberlite XAD-7 adsorbed lipase from Pseudomonas sp. for synthetic reaction

Under optimized conditions without the presence of salt hydrate for the water control, the immobilized lipase preparation was then used repeatedly in the shake flasks system for the synthetic reaction. Each reaction was carried out for 24h. As shown in Fig. 10, it was found that the preparation shown excellent stability for more than two month. The maximum conversion degree obtained was in the range of 75 – 85%.

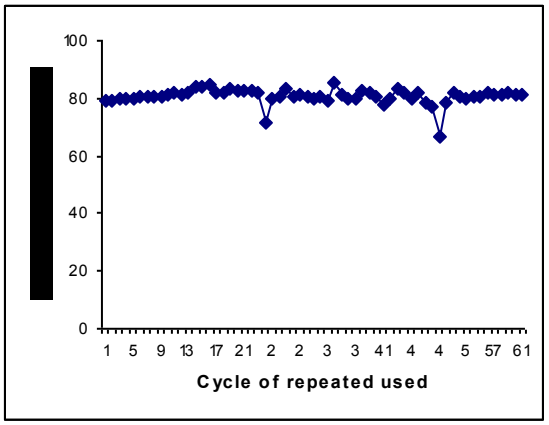

FIGURE 10 Stability of the immobilized lipase preparation for repeated use in the synthesis of 1,2-O-Isopropylidene-3-lauryl glycerol.
Each reaction was performed for 24 h.

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