Enzymatic Preparation of Enantiomerically Pure *sn*-2,3-Diacylglycerols: A Stereoselective Ethanolysis Approach

Weera Piyatheerawong[a], Tsuneo Yamane[b], Hideo Nakano[a], and Yugo Iwasaki[a,*]

[a]Laboratory of Molecular Biotechnology, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan, and [b]Department of Environmental Biology, School of Bioscience and Biotechnology, Chubu University, Aichi 487-8501, Japan

ABSTRACT: Stereoselective ethanolysis of monoacid TAG by immobilized *Rhizomucor miehei* lipase (RML) was studied for preparation of optically pure *sn*-2,3-DAG. Trioctanoylglycerol (TO) was used as a model substrate. The enantiomeric purity of the product, *sn*-2,3-dioctanoylglycerol (*sn*-2,3-DO), was very high (percent enantiomeric excess > 99%) when an excess of ethanol was used. The result indicated that RML was highly stereoselective toward the *sn*-1 position of TO under conditions of excess ethanol. The stereoselectivity of RML depended on the amount of ethanol. The larger the amount of ethanol was, the higher the stereoselectivity became. After optimizing the parameters such as reactant molar ratio, water content, and temperature, (ethanol/TO molar ratio = 31:1 and water content = 7.5 wt% of the reactants at 25°C), optically pure *sn*-2,3-DO was obtained at 61.1 mol% in the glyceride fraction in 20 min. The above conditions were further applied for ethanolysis of monoacid TAG with different acyl groups such as tridecanoylglycerol (C10:0), tridodecanoylglycerol (C12:0), tritetradecanoylglycerol (C14:0) and trioctadecenoylglycerol [triolein, (C18:1)]. The yields and enantiomeric purities of 1,2(2,3)-DAG were dramatically reduced when TAG with FA longer than decanoic acid were used.

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KEY WORDS: *sn*-2,3-Diacylglycerols, ethanolysis, monoacid triacylglycerols, *Rhizomucor miehei* lipase.

DAG are widely used as emulsifiers in the food, cosmetics, and pharmaceutical industries. Frequently, mixtures of MAG and DAG are exploited because they are cheap and give appropriate performance (1). At present, DAG are regarded as healthful oils owing to their activity in preventing obesity (2). In addition, pure isomers of DAG have great potential as intermediates for the organic synthesis of phospholipids, glycolipids, prodrugs, and structured lipids (3–6).

DAG exist in three isomers: *sn*-1,3-, *sn*-1,2-, and *sn*-2,3-DAG. For monoacid DAG, *sn*-1,2- and *sn*-2,3-DAG are enantiomers of each other. Although pure isomers of DAG are difficult to synthesize by traditional chemical synthetic methods, lipase-catalyzed regio- and stereoselective reactions would provide alternative routes. For example, preparation of *sn*-1,3-DAG at high yield and purity was successfully achieved by direct esterification of glycerol and FFA using 1,3-regiospecific lipase (6–8) and by direct transesterification of glycerol and vinyl esters of medium-chain FA using *Candida antarctica* lipase B (9).

In contrast to the preparation *sn*-1,3-DAG, chemoenzymatic methods are used for the preparation of optically pure *sn*-1,2-DAG or *sn*-2,3-DAG. Some recent publications describe syntheses of enantiomerically pure *sn*-1,2-DAG by lipase-mediated sequential transesterification of the racemic *O*-alkyl glycerols (10,11). In another synthetic route, the chiral *sn*-1,2-DAG are chemically synthesized from (*R*)- and (*S*)-enantiomers of *O*-(4-methoxyphenyl)-glycidol, which are obtained from a one-pot reduction of ketone and *in situ* lipase-mediated resolution (12). Although the foregoing synthetic methods are accomplished by using an enzyme-catalyzed reaction as a part of the process, the other parts mainly require chemical reactions, which involve laborious steps and use of toxic reagents. A simple enzymatic process for the preparation of chiral *sn*-1,2- or 2,3-DAG has not been reported. One possible approach for this purpose is lipase-catalyzed asymmetrical deacylation of prochiral monoacid TAG at either the *sn*-1 or *sn*-3 position by alcoholysis, which generates *sn*-2,3- or *sn*-1,2-DAG, respectively. To establish an effective production method for chiral *sn*-1,2- or *sn*-2,3-DAG, lipases with the desired stereoselectivity are required. Indeed, it is reported that some lipases discriminate the *sn*-1 position from the *sn*-3 position of TAG in hydrolysis, interesterification, and ethanolysis (13–15).

In the present study, ethanolysis of trioctanoylglycerol (TO) with immobilized *Rhizomucor miehei* lipase (RML) was examined as a reaction model. Several reaction parameters such as reactant molar ratio, water content, and temperature were investigated. Under the optimized reaction conditions, ethanolysis of monoacid TAG to prepare *sn*-2,3-DAG was demonstrated.

EXPERIMENTAL PROCEDURES

Materials. TO, tridecanoylglycerol, tridodecanoylglycerol, tritetradecanoylglycerol, and trioctadecenoylglycerol (triolein) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Ethanol (99.5%), HPLC-grade *n*-hexane, and HPLC-grade 2-propanol were purchased from Wako Pure Chemicals

*To whom correspondence should be addressed at Nagoya University, Furocho, Chikusa-ku, Nagoya 464-8601, Japan.
E-mail: iwasaki@agr.nagoya-u.ac.jp

(Osaka, Japan). Lipozyme RM IM (immobilized RML) was a gift from Novozymes Japan Ltd. (Chiba, Japan). All other chemicals were of analytical quality or better.

Lipase-catalyzed ethanolysis. In a typical experiment, a reaction mixture consisting of 1.0 g TO, 3.0 g of ethanol, and varying amounts of water (which was necessary to promote the reaction) was incubated at 35°C with stirring (500 rpm) for 15 min to emulsify the mixture. The reaction was started by adding 0.4 g (10% of the reaction mixture) of immobilized RML. Portions (0.1 mL) of the reaction mixture were withdrawn at intervals, mixed with 0.4 mL diethyl ether, and filtered to remove the catalyst.

Analysis of the reaction mixture. The glyceride compositions in the resultant lipid solution were analyzed with a TLC/FID analyzer using Chromarod S III quartz rods (Iatron Laboratories, Tokyo, Japan). The rods loaded with the samples were eluted for 10 cm with *n*-hexane/diethyl ether (9:1), and then the upper 8 cm was scanned for the quantification of FA ethyl ester, TAG, and FFA. 1,3-DAG, 1,2(2,3)-DAG, 1(3)-MAG, and 2-MAG, which remained close to the rod origin after the first development, were separated by a second development with benzene/chloroform/acetic acid (100:5:1) and scanned for quantification.

Estimation of acyl migration. Nonenzymatic acyl migration from the *sn*-2 position to *sn*-1(3) of 1,2(2,3)-DAG or 2-MAG may form 1,3-DAG or 1(3)-MAG, respectively. The formed 1,3-DAG or 1(3)-MAG are expected to be further deacylated by the 1,3-specific lipase, and eventually be converted to glycerol, decreasing the product yield. Since the concentration of 1,3-DAG and 1(3)-MAG were very low throughout the reaction, they would have been rapidly deacylated even if they had been generated. Therefore, the amount of glycerol in the reaction mixture corresponds to the amount of glycerides lost through acyl migration followed by the complete deacylation. Thus, the degree of the acyl migration can be evaluated from the recovery of the glycerides, which can be calculated from the amount of glycerol in the reaction mixture. The amount of glycerol was measured as follows. Sample solution (100 μL) was transferred into a pre-weighed microcentrifuge tube, and the solvent was evaporated completely under a nitrogen stream. After weighing the residual materials, 100 μL of water was added to the tube. The mixture was briefly centrifuged, and the aqueous layer containing glycerol was recovered. The amount of glycerol in the resulting aqueous solution was quantified by an enzymatic assay using Glycerol Assay Kit (R-Biopharm AG, Darmstadt, Germany).

Evaluation of enantiomeric purity. For investigating enantiomeric purity of 1,2(2,3)-DAG, 40 μL of the sample solution was evaporated at low temperature under vacuum and redissolved in 40 μL of *n*-hexane. From the resulting lipid solution, 5 μL was injected onto the tandem column HPLC system (15), in which a silica gel column (Ultrasphere 5 μm column, 4.6 × 250 mm; Beckman Coulter Inc., Fullerton, CA) and a chiral stationary phase column [CHIRALCEL OD™ cellulose-tris-3,5-dimethylphenyl carbamate-impregnated silica, 4.6 × 250 mm; Daicel Chemical, Tokyo, Japan) were connected in series. The mobile phase was *n*-hexane/2-propanol (300:7) at flow rate of 1.0 mL/min using an HPLC pump at 25°C. The peaks were detected using an ELSD. The drift tube temperature was 50°C. Nitrogen was used as evaporation gas at 350 kPa. The weight-based amount (in μg) of each enantiomer was calculated from the corresponding peak area using standard curves drawn with known amounts of authentic 1,2-DAG (15). The enantiomeric purity of 1,2(2,3)-DAG was evaluated from the enantiomeric excess (%ee) value, which was calculated using the weight-based amount of each isomer as follows;

$$\%ee = \frac{[sn\text{-}2,3\text{-}\mathrm{DAG}] - [sn\text{-}1,2\text{-}\mathrm{DAG}]}{[sn\text{-}2,3\text{-}\mathrm{DAG}] + [sn\text{-}1,2\text{-}\mathrm{DAG}]} \times 100 \qquad [1]$$

Analysis of the water content. The water contents in the reaction mixtures were measured by a Karl-Fischer moisture meter and expressed as the weight percentage of the reactants (lipids plus ethanol).

RESULTS AND DISCUSSION

In the presence of an excess amount of ethanol, the lipase-catalyzed deacylation of prochiral monoacid TAG by ethanolysis initially yields chiral *sn*-1,2- and/or *sn*-2,3-DAG as intermediates, which are further converted to 2-MAG as the final product (16–18). Some lipases are known to have stereoselectivity toward the *sn*-1 or *sn*-3 position in hydrolysis, interesterification, and ethanolysis (13–15).

Figure 1A demonstrates the typical time course for the change in glyceride composition during the ethanolysis of TO with immobilized RML at a 3:1 ethanol/TO weight ratio and at 0.8 wt% water content (no exogeneous water added). As the reaction proceeded, TO was consumed, generating first the 1,2(2,3)-dioctanoylglycerol [1,2(2,3)-DO] as the intermediate and then 2-monooctanoylglycerol (2-MO). The content of 1,2(2,3)-DO in the glyceride fraction reached approximately 50% in 6 h. HPLC analysis of the 1,2(2,3)-DO revealed that the enantiomeric purity was absolutely to *sn*-2,3-DO (%ee > 99%). The result was analogous to our previous report that RML greatly prefers the *sn*-1 position over the *sn*-3 position of TO in the presence of an excess amount of ethanol (15).

Since the target product is an intermediate of the reaction, it would be deacylated further to 2-MO, which is a by-product. Thus, we focused on achieving both high yield and high enantiomeric purity of *sn*-2,3-DO. Figure 1B shows the influence of ethanol/TO ratio on the yield and the enantiomeric purity of 1,2(2,3)-DO. The stereoselectivity of RML was found to depend on the amount of ethanol. The larger the amount of ethanol was, the higher the stereoselectivity became. At an ethanol/TO molar ratio of 31:1 (weight ratio = 3:1) or higher, the %ee value was more than 99%. The yield of 1,2(2,3)-DO was not affected very much by the reactant ratio, although the composition of the other glycerides (i.e., the unreacted TO and the "over-deacylated" 2-MO) was different.

A possible explanation for the significant influence of ethanol on the stereoselectivity of lipase could be related to the effect of solvent. In organic solvent, particularly water-removing

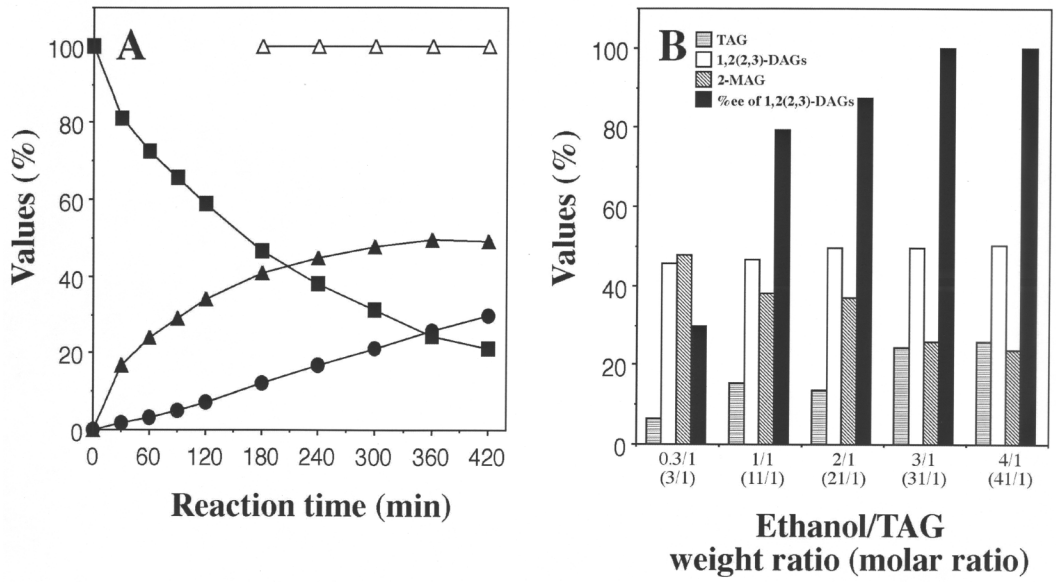

FIG. 1. (A) Time course change of glyceride composition and enantiomeric purity of 1,2(2,3)-DAG during ethanolysis of trioctanoylglycerol (TO). The reaction was performed at an ethanol/TO molar ratio of 31:1 (weight ratio of 3:1) and 35°C with no addition of exogenous water (water content was 0.8 wt% due to the endogeneous water). (■) TAG (mol%), (▲) 1,2(2,3)-DAG (mol%), (●) 2-MAG (mol%), (△) enantiomeric purity (%ee). (B) Influence of reactant ratio on glyceride composition and enantiomeric purity. The values shown are those determined when the contents of the 1,2(2,3)-DAG were maximum.

solvent such as ethanol, the enzymes generally become more rigid (19). In such an environment, the binding pocket of the enzyme is relatively fixed to one geometry, resulting in highly selective catalysis to a specific position of TAG.

Since the reactant molar ratio of 31:1 (weight ratio of 3:1) was somewhat better than the others with respect to the yield, further investigation was made at this reactant ratio. Water content in the reaction is also an important parameter for this reaction system. As shown in Figure 2A, the reaction proceeded slowly in a lower water-content environment, perhaps because ethanol strips off water molecules from the catalyst. Since most of enzymes require some amount of water molecules to maintain their active conformation, addition of exogenous water molecules could improve the reaction rate (20,21). As expected, the reaction proceeded rapidly and selectively to give *sn*-2,3-DO to approximately 60% among the glycerides in 30 min at a water content of 7.5 wt% or above (Fig. 2B). The result was in accordance with our previous report indicating that RML needs a certain amount of water molecules to perform the catalytic reaction (18). When the water content was 4.8 wt% or above, small amounts of FFA were detected because of hydrolysis. The amount of the FFA was less than one-tenth that of the FA ethyl esters throughout the reaction, indicating the dominant reaction that took place was not the hydrolysis but the ethanolysis.

Reaction temperature is another key parameter for the reaction. As illustrated in Figure 3, the reaction progressed rapidly at lower temperatures, and the highest content of *sn*-2,3-DO (61.1%) was achieved in only 20 min at 25°C with high enantiomeric purity. By contrast, the reaction proceeded slowly at high temperature, suggesting that the active conformation of the enzyme was not maintained even though it has water molecules in the system to stabilize its structure.

Table 1 summarizes the ethanolysis of some other monoacid TAG of different chain lengths [tridecanoylglycerol (C10:0), tridodecanoylglycerol (C12:0), tritetradecanoylglycerol (C14:0), and trioctadecenoyl glycerol (C18:1)] as well as TO. The experiments were performed under the optimized conditions, i.e.; ethanol/TAG weight ratio = 3:1, water content = 7.5 wt% at 25°C. In the case of C10:0, the content of *sn*-2,3-DAG reached 58.7% in 50 min with high enantiomeric purity.

In contrast, in the cases of the other TAG having acyl groups of C12 or longer, the accumulation level of 1,2(2,3)-DAG was very low. The reason for this very low accumulation of 1,2(2,3)-DAG is (i) deacylation of TAG was very slow and/or (ii) deacylation of the generated 1,2(2,3)-DAG was too fast. In the cases of TAG with C12 or C18:1, RML deacylated 1,2(2,3)-DAG rapidly to 2-MAG, accumulating smaller amounts of 1,2(2,3)-DAG and larger amounts of 2-MAG. TAG with C14 was not ethanolized very much, and the 1,2(2,3)-DAG generated was quickly deacylated, resulting in little accumulation of 1,2(2,3)-DAG.

The recovery of the glyceride estimated from the amount of glycerol formed was more than 80 mol% for all the different TAG tested. Considering the recovery of the glyceride, the yield of 1,2(2,3)-DAG is 54.0 and 50.5 mol% for the cases of C8:0 and C10:0, respectively, which is satisfactory for practical purposes.

Glyceride recovery of more than 80 mol% also indicates that certain portions (less than 20 mol%) of the glyceride were

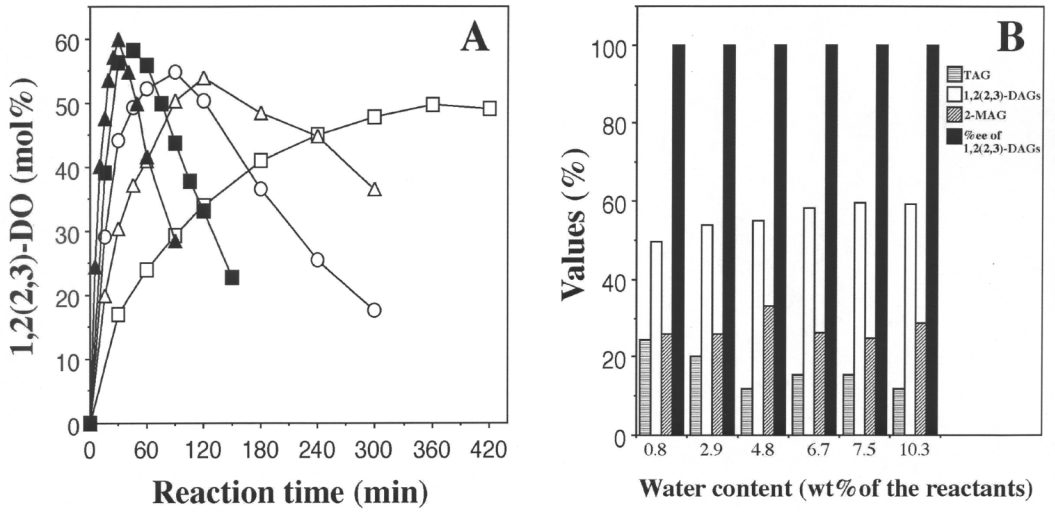

FIG. 2. (A) Comparison of 1,2(2,3)-dioctanoylglycerol [1,2(2,3)-DO] formation during ethanolysis reaction with different water contents. The reaction was performed at an ethanol/TO molar ratio of 31:1 (weight ratio of 3:1) and 35°C. (□) 0.8 wt%, (△) 2.9 wt%, (○) 4.8 wt%, (■) 6.7 wt%, (▲) 7.5 wt%. (B) Influence of water content on glyceride composition and enantiomeric purity. The values shown are those determined when the contents of the 1,2(2,3)-DAG were maximum. For other abbreviation see Figure 1.

lost as glycerol by complete deacylation. Since RML is known to be very strictly 1,3-position specific, it is unlikely that the lipase was able to attack the glycerides randomly at the *sn*-2 position as well as the *sn*-1(3) positions. Therefore, the complete deacylation should involve nonenzymatic acyl migration from the *sn*-2 position to *sn*-1(3) position in 1,2(2,3)-DAG or in 2-MAG to generate 1,3-DAG or 1(3)-MAG, respectively. The generated 1,3-DAG and 1(3)-MAG are the substrates of RML and are thereby further deacylated to glycerol. Since no 1,3-DAG and only a very small amount of 1(3)-MAG (<1 mol%) were detected throughout the reaction, the deacylation of 1,3-DAG and 1(3)-MAG may be much faster than acyl migration itself. From the above consideration, the amount of glycerol (= loss of the glyceride) can be used as a measure to estimate the degree of acyl migration. Judging from the amounts of glycerol (less than 20 mol%), the degree of acyl migration under the reaction conditions is not so high as to reduce the yield of product seriously.

The present study provides a simple enzymatic method for preparation of *sn*-2,3-DAG with an environmentally friendly

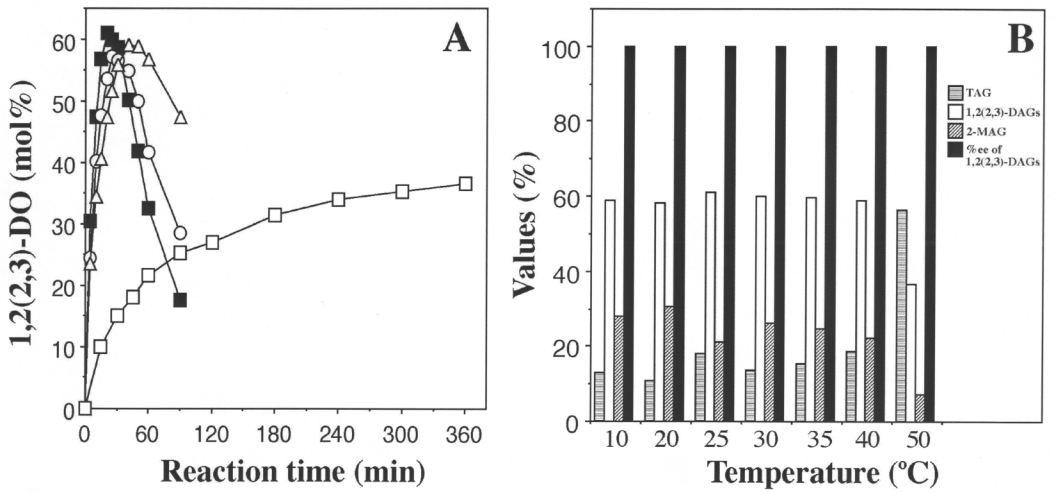

FIG. 3. (A) Comparison of 1,2(2,3)-DO formation during ethanolysis reaction at various temperatures. The reaction was performed at an ethanol/TO molar ratio of 31:1 (weight ratio 3:1) and a water content equal to 7.5 wt%. (■) 25°C, (○) 35°C, (△) 40°C, (□) 50°C. (B) Influence of temperature on glyceride composition and enantiomeric purity. The values shown are those determined when the contents of the 1,2(2,3)-DAG were maximum. For abbreviations see Figures 1 and 2.

TABLE 1
Summary of *Rhizomucor miehei* Lipase-Catalyzed Ethanolysis of TAG with Various Acyl Groups[a]

TAG (chain length)	Time (min)	Glyceride composition (mol%)[b]			Recovery of Glyceride[c] (mol%)	Selectivity and %ee of 1,2(2,3)-DAG
		TAG	1,2(2,3)-DAG	2-MAG		
Trioctanoyl glycerol (C8:0)	20	17.8	61.1	21.1	88.4	*sn*-1, >99%
Tridecanoyl glycerol (C10:0)	50	11.5	58.7	29.8	86.2	*sn*-1, >99%
Tridodecanoyl glycerol (C12:0)	420	1.8	7.2	90.9	81.4	ND[d]
Tritetradecanoyl glycerol (C14:0)	540	68.2	1.3	30.5	87.4	ND[d]
Trioctadecenoyl glycerol (C18:1)	360	17.9	25.0	57.1	86.6	*sn*-1, 67%

[a]The reactions were performed at 25°C, ethanol/TAG weight ratio of 3:1, and water content of 7.5 wt%.
[b]Composition of the reaction mixture when the contents of 1,2(2,3)-DAG were maximum.
[c]Recovery of glycerides [= TAG, 1,2(2,3)-DAG and 2-MAG] was estimated from the amount of the glycerol.
[d]Not determined due to the very low amount of 1,2(2,3)-DAG.

approach. Since *sn*-2,3-DAG is the building block for various organic compounds, it will be useful as a potential intermediate in organic synthesis.

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