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Enantioselective Reduction

Enantioselective Reduction of Ethyl 3-Oxo-5-phenylpentanoate with Whole-Cell Biocatalysts

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Abstract: The biocatalytic stereoselective synthesis of a sterically demanding *sec*-alcohol (ethyl 3-hydroxy-5-phenylpentanoate) was investigated by starting from the corresponding prochiral ketone. Screening of a collection of microorganisms led to the identification of stereocomplementary catalysts suitable for accessing both enantiomers of the target compound. Cofactors, recycling systems and 2-propanol amounts were optimized for selected biocatalysts, leading to excellent enantiomeric excesses for the obtained hydroxy ester with up to 99 % *ee*. The utility of the identified strains was showcased by using preparative-scale reactions.

Introduction

Enantiomerically pure β-hydroxy esters[1] have been used as building blocks for the synthesis of a variety of chemicals, including β-lactams,[2] carotenoids,[3] insect pheromones[4] and 2,3-dihydro-4*H*-pyran-4-ones.[5] To date, only a few reports have described the asymmetric synthesis of 3-hydroxy-5-phenylpentanoic acid derivatives.[6–8] Most of the reported synthetic routes are based on stereoselective reductions by using baker's yeast. Athanasiou and co-workers have reported the use of an organic solvent system for yeast-mediated reductions of a series of β-oxo esters.[9] Some serious drawbacks are associated with this biocatalyst; for instance, low productivity, large amounts of biomass and generation of many side products are often characteristic of yeast-based reductions.[10,11] A few years later, Padhi et al. published the deracemization of aryl-substituted β-hydroxy esters by using immobilized whole cells from *Candida parapsilosis* ATCC 7330; the corresponding (*S*) enantiomers of these compounds were generated with moderate enantiomeric excesses (87 %) and low yields (10 %).[12] Unfortunately, the reported procedures provided only the (*S*) enantiomer. In this work, we focus our attention on new biocatalysts, which may provide access to both enantiomers of ethyl 3-hydroxy-5-phenylpentanoate in optically pure form. To achieve this goal, a series of whole-cell biocatalysts was screened. These efforts are inspired, in large part, by the realization that the application of biocatalysis to create complex molecules offers the opportunity to fulfill the principles of green chemistry.[13] Such alternative methods enable reduced use of organic solvents, diminished production and/or use of toxic metals and diminished environmental footprints.[14]

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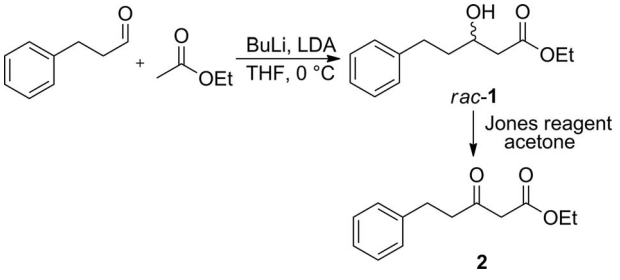
Results and Discussion

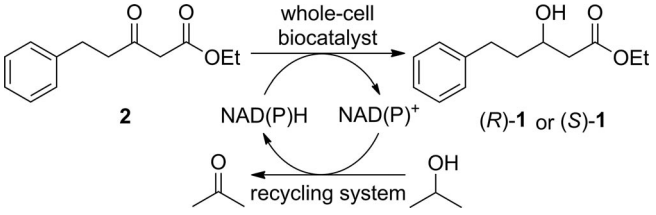
In the present study, we report the biocatalytic synthesis of both enantiomers of ethyl 3-hydroxy-5-phenylpentanoate (**1**). The corresponding ketone substrate **2** was obtained in a two-step synthesis (Scheme 1). C–C bond formation between 3-phenylpropionaldehyde and ethyl acetate afforded racemic alcohol *rac*-**1**, which was oxidized with Jones reagent (CrO_3, H_2SO_4, acetone) to produce β-oxo ester **2**.[15,16]



Scheme 1. Synthesis of ethyl 3-oxo-5-phenylpentanoate (**2**).

In a first approach two recombinant alcohol dehydrogenases (ADHs), heterologously expressed in *E. coli*, were assessed for the ability to catalyze the asymmetric reduction of compound **2** (Scheme 2). Since ADH-catalyzed reactions rely on cofactors, an effective method for cofactor regeneration was required. Many whole-cell biocatalysts have internalized cofactor-regeneration mechanisms that can be enabled upon the addition of co-substrates such as glucose.[17] To have comparable conditions for a broader screening of wild-type microorganisms, glucose/glucose dehydrogenase (GDH) as well as 2-propanol were

used in the screening reactions, whereby the latter may serve not only as the hydride donor but also as co-solvent to improve substrate solubility.[18] To have both nicotinamide cofactors (NADH, NADPH) present in sufficient quantities, a mixture of the two was added. Some ADHs display high cofactor specificity, whereas others are able to accept both cofactors equally well.[19]



Scheme 2. Whole-cell-mediated reduction of ethyl 3-oxo-5-phenylpentanoate.

In comparing the results collected for the two recombinant ADHs it is clear that the ADH from *Sphingobium yanoikuyae*[20,21] did not accept β-oxo ester **2** as a substrate (Table 1, Entry 2). Conversely, the ADH from *Ralstonia sp.*[22,23] (Table 1, Entry 1) afforded the desired (*S*) product with moderate yield (54 % on the basis of HPLC) and in a low enantiomeric excess (49 %). Due to the low enantiomeric excess obtained, further experiments aimed at preparing enantiomerically pure hydroxy ester **1** were performed. Consequently, a collection of wild-type whole-cell biocatalysts was screened for their reactivity with **2**.

Table 1. Reduction of oxo ester **2** with recombinant ADHs.[a]

Entry	ADH	*c* [%]	Yield [%]	*ee* [%]
1	*Ralstonia sp.* DSM 6428	> 99	54	49 (*S*)
2	*Sphingobium yanoikuyae* DSM 6900	n.d.	<1	n.d.[b]

[a] Conditions: 10 mg of freeze-dried *E. coli* cells containing the recombinant ADH, glucose dehydrogenase (1 mg mL^{-1}), 20 mM glucose, 0.5 mM NADH, 0.5 mM NADPH, 10 mM substrate in 500 μL reaction mixture Tris-HCl buffer pH 7.5/2-propanol (90:10, v/v), 30 °C, 120 rpm in a thermoshaker for 20 h. Conversion (i.e., consumption of substrate) and product yield (i.e., formation of **1**) were determined by HPLC analysis on an achiral stationary phase by using calibration curves for **1** and **2**. The *ee* of **1** was determined by HPLC analysis on a chiral stationary phase. [b] n.d. = not determined.

About 250 microorganisms from the in-house culture collection, encompassing mainly commercially available strains,[24,25] have been assessed for their ability to reduce β-oxo ester **2**; only 33 of these were found to be active (see Table 2).

Table 2. Reduction of oxo ester **2** with whole-cell biocatalysts.[a]

Entry	Microorganism	Strain	*c* [%]	Yield [%]	*ee* [%]
1	*Alcaligenes sp.*	DSM 2625	68	36	5 (*S*)
2	*Arthrobacter sp.*	DSM 7325	> 99	82	85 (*R*)
3	isolate *Gordonia sp.* NAM-BN063A	FCC018	> 99	49	77 (*S*)
4	isolate *Gordonia sp.* NAM-BN063B	FCC019	> 99	46	77 (*S*)
5	isolate *Actinomyces sp.* SRB-AN053	FCC027	94	24	96 (*R*)
6	isolate *Actinomyces sp.* SRB-AN019	FCC022	98	11	90 (*R*)
7	isolate *Actinomyces sp.* SRB-AN029	FCC023	95	14	92 (*R*)
8	isolate *Actinomyces sp.* SRB-AN042	FCC026	> 99	12	88 (*R*)
9	isolate *Actinomyces sp.* SRB-AN040	FCC025	90	19	89 (*R*)
10	isolate *Actinomyces sp.* SRB-AN030	FCC024	94	28	70 (*R*)
11	*Coprinus radians*	CSM 888	72	29	45 (*R*)
12	*Comamonas testosteroni*	DSM 1455	85	32	27 (*S*)
13	*Comamonas badia*	DSM 17552	98	65	43 (*R*)
14	*Comamonas denitrificans*	DSM 17887	93	14	23 (*S*)
15	*Kluyveromyces thermotolerans*	DSM 3434	95	34	61 (*S*)
16	*Xanthomonas arboricola pvar. Celebensis*	DSM 50853	> 99	70	48 (*R*)
17	*Xanthomonas campestris*	DSM 1050	> 99	62	49 (*R*)
18	*Xanthomonas sp.*	DSM 1049	> 99	55	47 (*R*)
19	*Gordonia alkanivorans*	DSM 44369	96	51	64 (*S*)
20	*Lactobacillus oris*	DSM 4864	> 99	70	88 (*R*)
21	*Paracoccus pantotrophus*	DSM 11072	60	47	92 (*S*)
22	*Pichia pastoris*	CBS 7435	> 99	79	77 (*R*)
23	*Pseudomonas sp.*	DSM 6978	72	33	49 (*S*)
24	*Pseudomonas thermotolerans*	DSM 14292	98	44	66 (*S*)
25	isolate ARG-AN024	FCC014	94	16	98 (*R*)
26	isolate ARG-AN025	FCC015	94	21	94 (*R*)
27	isolate USA-AN012	FCC021	99	10	96 (*R*)
28	isolate ANTs-AN002	FCC013	91	26	67 (*R*)
29	isolate *Actinomyces sp.* 67-BEN001	FCC028	96	10	90 (*R*)
30	isolate *Actinomyces sp.* ENG-AN033	FCC020	95	23	78 (*R*)
31	*Sphingomonas sp.* HXN200	FCC139	98	7	98 (*R*)
32	*Sphingomonas faenia*	FCC183	76	51	60 (*R*)
33	bacterium (code 0091B)	FCC042	97	8	62 (*R*)

[a] Conditions: 10 mg of lyophilized biocatalyst, glucose dehydrogenase (1 mg mL^{-1}), 20 mM glucose, 0.5 mM NADH, 0.5 mM NADPH, 10 mM substrate in 500 μL reaction mixture Tris-HCl buffer pH 7.5/2-propanol (90:10, v/v), 30 °C, 120 rpm in a thermoshaker for 20 h. Conversion (i.e., consumption of substrate) and product yield (i.e., formation of **1**) were determined by HPLC analysis on an achiral stationary phase by using calibration curves for **1** and **2**. The *ee* of **1** was determined by HPLC analysis on a chiral stationary phase.

Table 3. Optimization of the reaction conditions.[a]

Entry	Microorganism	NADH [mM]	NADPH [mM]	Glucose [mM]	2-PrOH [%]	*c* [%]	Yield [%]	*ee* [%]
1	isolate *Actinomyces sp.* SRB-AN053	0.5	0.5	20	10	94	24	96 (*R*)
2	isolate *Actinomyces sp.* SRB-AN053	0.5	0.5	20	20	92	23	98 (*R*)
3	isolate *Actinomyces sp.* SRB-AN053	0.5	0.5	20	30	24	9	n.d.[b]
4	isolate *Actinomyces sp.* SRB-AN053	–	0.5	20	10	99	16	97 (*R*)
5	isolate *Actinomyces sp.* SRB-AN053	0.5	–	20	10	96	24	95 (*R*)
6	isolate *Actinomyces sp.* SRB-AN053	0.5	0.5	–	10	95	26	> 99 (*R*)
7	*Arthrobacter sp.*	0.5	0.5	20	10	> 99	82	85 (*R*)
8	*Arthrobacter sp.*	–	0.5	20	10	56	18	11 (*R*)
9	*Arthrobacter sp.*	0.5	–	20	10	99	59	96 (*R*)
10	*Arthrobacter sp.*	0.5	0.5	–	10	98	45	96 (*R*)

[a] Conditions: 10 mg of lyophilized biocatalysts, glucose dehydrogenase (1 mg mL^{-1}), 20 mM glucose, 0.5 mM NADH, 0.5 mM NADPH, 10 mM substrate in 500 µL reaction mixture Tris-HCl buffer pH 7.5/2-propanol (90:10; v/v), 30 °C, 120 rpm in a thermoshaker for 20 h. Conversion (i.e., consumption of substrate) and product yield (i.e., formation of **1**) were determined by HPLC analysis on an achiral stationary phase using calibration curves for **1** and **2**. The *ee* of **1** was determined by HPLC analysis on a chiral stationary phase. [b] n.d. = not determined.

Table 4. Reduction of **2** with whole-cell biocatalysts on a 1 mmol scale.

Entry	Microorganism	Substrate recovery [%]	Yield [%][a]	*ee* [%]
1	*Arthrobacter sp.*[b]	6	47	94 (*R*)
2	isolate *Actinomyces sp.* SRB-AN053[c]	5	23	> 99 (*R*)
3	*Paracoccus pantotrophus*[d]	40	41	92 (*S*)

[a] Yield refers to isolated product **1** after column chromatography. [b] Conditions: 30 °C, 120 rpm in a thermoshaker for 48 h, 2 g of whole-cell lyophilized biocatalyst in 100 mL of reaction solution, containing 20 mM glucose, 0.5 mM NADH, 1 mmol substrate in mixture of 100 mM Tris-HCl buffer pH 7.5/2-propanol (90:10, v/v). [c] Glucose dehydrogenase (1 mg mL^{-1}). [d] 0.5 mM NADPH, glucose dehydrogenase (1 mg mL^{-1}).

Out of the tested biocatalysts, isolate *Gordonia sp.* NAMBN063A (Table 2, Entry 3) and *Paracoccus pantotrophus* (Table 2, Entry 21) afforded (*S*)-configured product **1** with enantiomeric excesses of 77 and 92 %, respectively. Moreover, the use of *Arthrobacter* sp. (Table 2, Entry 2), isolate *Actinomyces sp.* SRB-AN053 (Table 2, Entry 5), isolate ARG-AN024 or *Sphingomonas sp.* HXN200 provided the opposite (*R*) enantiomer with excellent enantiomeric excesses of up to 98 %. Due to the presence of various types of enzymes in whole-cell catalysts, various side reactions often occur. Substrate **2**, containing more than one reactive group, was found to be amenable to high conversions (> 60 %), but moderate yields of the desired products (11–82 %). Thus, yields above 70 % of product **1**, were observed only in a few cases (Table 2, Entries 2, 16, 20, 22).

Although the achieved stereoselectivities were often excellent, the chemoselectivities of whole-cell bioreductions with respect to carbonyl bond reduction vs. ester bond hydrolysis were found to be moderate. Driven by this observation, cofactors, recycling systems and the amounts of 2-propanol employed in reactions were consequently optimized for selected biocatalysts.

To slow down the hydrolysis reaction, larger quantities of 2-propanol were investigated. An increase of the 2-propanol concentration from 10 to 20 % did not affect the product yield (Table 3, Entry 2). A larger increase in the 2-proponal concentration diminished the efficiency of both hydrolysis and reduction reactions (Table 3, Entry 3). Whole cells of *Actinomyces sp.* SRB-AN053 were used for the reduction of oxo ester **2** to the corresponding (*R*)-hydroxy ester with excellent stereoselectivity of > 99 % (Table 3, Entry 6). Independent of the cofactor added, high *ee* values were obtained (Table 3, Entries 4 and 5). Notably, the conversions achieved were independent of the presence or absence of glucose and GDH. In contrast, the ability of *Arthrobacter sp.* to catalyze reactions with high *ee* was NADH-dependent (Table 3, Entry 9). We reason that this microorganism may contain a second, less selective dehydrogenase, which is NADPH-dependent (Table 3, Entry 8). Interestingly, combining both NADH and NADPH resulted in the activation of both enzymes leading to higher yields but with lower enantiomeric excesses (Table 3, Entry 7). A conversion of 59 % with an *ee* of 96 % was achieved after 24 h in buffer-containing additional NADH and glucose for internal cofactor regeneration. Reaction supplementation with glucose improved the reaction efficiency but appeared to have no impact upon the *ee* (Table 3, Entry 9 vs. 10).

Finally, to highlight the utility of the developed procedure, experiments were run on a large scale (1 mmol; Table 4). Thus, 220 mg of β-oxo ester **2** was transformed into β-hydroxy ester **1**; the reaction at 30 °C was complete within 24 h. The yields of isolated products on a preparative scale were similar to those determined in analytical-scale experiments. Both enantiomers of compound **1** were obtained with good yields and in excellent enantiomeric excesses of up to 99 %. This finding represents a significant step forward, since suitable enantioselective biocatalysts have not been described for this substrate. The absolute configuration of products was assigned on the basis of optical-rotation measurements.[12,26]

Conclusions

Screening of various enzymes and microorganisms led to the identification of suitable wild-type whole-cell catalysts for the reduction of the sterically demanding ketone **2**. Following reac-

tion optimization, both enantiomers of ethyl 3-hydroxy-5-phenylpentanoate were obtained with excellent optical purities and high yields. The utility of the identified catalysts was showcased by carrying out the reaction on a preparative scale.

Experimental Section

General Remarks: ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solution with a Varian Gemini 400 MHz spectrometer. Chemical shifts are expressed in parts per million (ppm) by using TMS as an internal standard. Recombinant dehydrogenases and whole-cell biocatalysts are from the collection of the Institute of Chemistry – Organic and Bioorganic Chemistry, University of Graz. All chemicals were commercial products of analytical grade. TLC was performed by using Kieselgel 60 F_{254} aluminium sheets (Merck). Optical-rotation measurements were executed with a Jasco P-2000 polarimeter. For the determination of product (**1**) yields and substrate (**2**) conversions, 1.2 mL samples dissolved in methanol were analyzed by HPLC with a Shimadzu Prominence LC-20A series instrument coupled to a UV/Vis detector. A LUNA C-18 column (Phenomenex, 250 mm × 4.6 mm × 5 μm) was used with the following conditions: eluent water/acetonitrile; low-pressure gradient from 100 % of water to 100 % of acetonitrile over 20 min, 100 % of acetonitrile for 5 min, back to 100 % water over 5 min, 100 % water for 2 min; temperature 30 °C; flow 0.5 mL min^{-1}; λ = 210 nm. For the determination of product (**1**) *ee* values, 1.2 mL samples dissolved in methanol were analyzed by HPLC with a Shimadzu Prominence LC-20A series instrument coupled with a UV/Vis detector. A Chiralcel OD-H column (Daicel, 250 mm × 4.6 mm × 5 μm) was used with the following conditions: eluent heptane/2-propanol (90:10, v/v); temperature 30 °C; flow 1.0 mL min^{-1}; λ = 210 nm.

Screening Procedure: Whole-cell biocatalysts (10 mg) were suspended in reaction solution (500 μL), containing glucose dehydrogenase (1 mg mL^{-1}), 20 mM glucose, 0.5 mM NADH, 0.5 mM NADPH, 10 mM substrate in a mixture of 100 mM Tris-HCl buffer pH 7.5/2-propanol (90:10, v/v). Biotransformations were conducted at 30 °C and 120 rpm in a thermoshaker for 20 h. After this time, the reaction mixture was extracted with ethyl acetate, centrifuged, and the supernatant was transferred to an HPLC vial. The solvent was evaporated under an air flow, the residue was re-dissolved in HPLC-grade MeOH, and the samples were analyzed by HPLC.

Preparative-Scale Procedure: Whole-cell biocatalyst (2 g) was suspended in reaction solution (100 mL), containing glucose dehydrogenase (1 mg mL^{-1}), 20 mM glucose, 0.5 mM NADH, 0.5 mM NADPH, 1 mmol of substrate in a mixture of 100 mM Tris-HCl buffer pH 7.5/2-propanol (90:10, v/v). Biotransformations were conducted at 30 °C and 120 rpm in a thermoshaker for 48 h. After this time, the biocatalyst was removed by centrifugation, and the reaction mixture was extracted with ethyl acetate. The organic phase was separated, dried ($MgSO_4$), concentrated under reduced pressure, and the product was purified by column chromatography on silica gel with petroleum ether/ethyl acetate as eluent.

Ethyl 3-Hydroxy-5-phenylpentanoate (1): To a precooled solution (0 °C) of diisopropylamine (9 mL, 63.4 mmol, 1.7 equiv.) in anhydrous THF (280 mL), was added *n*BuLi (2.7 M in heptane, 20.7 mL, 55.8 mmol) under argon. The resulting reaction mixture was stirred at 0 °C for 30 min and then cooled to –78 °C. Ethyl acetate (5.4 mL, 55.8 mmol, 1.5 equiv.) in anhydrous THF was added dropwise, and the mixture was stirred at –78 °C for 1 h. After this time, 3-phenylpropionaldehyde (5 mL, 37.2 mmol, 1 equiv.) in anhydrous THF (10 mL) was added. After stirring at –78 °C for 3 h, the reaction was quenched by the addition of saturated ammonium chloride solution and the mixture left to warm to room temperature. The two layers were separated, and the aqueous layer was extracted with ethyl acetate (3 × 100 mL). The combined organic layers were dried with $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by column chromatography on silica gel by using hexane/ethyl acetate (8:2, v/v). The product was obtained as a colorless oil (16.7 mmol, 3.7 g, 45 %). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.30–7.26 (m, 2 H, Ar), 7.21–7.16 (m, 3 H, Ar), 4.19–4.14 (q, J = 7.1 Hz, 2 H, OCH_2CH$_3$), 4.04–3.99 (td, J_1 = 8.3, J_2 = 4.2 Hz, 1 H, C*H*OH), 3.06 (br. s, 1 H, O*H*), 2.82–2.80 (m, 1 H, CH_2), 2.72–2.66 (m, 1 H, CH_2), 2.52–2.40 (m, 2 H, CH_2), 1.91–1.78 (m, 1 H, CH_2), 1.78–1.64 (m, 1 H, CH_2), 1.28–1.25 (t, J = 7.2 Hz, 3 H, OCH$_2$C*H*$_3$) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 172.9, 141.7, 128.4, 128.4, 125.9, 67.2, 60.7, 41.3, 38.1, 31.7, 14.1 ppm. $C_{13}H_{18}O_3$ (222.3): calcd. C 70.24, H 8.16; found C 70.11, H 8.33. HPLC (C-18 Luna): t_r = 21.5 min. HPLC (OD-H Chiralcel): t_{r1} = 7.1 min, t_{r2} = 8.1 min.

Ethyl 3-Oxo-5-phenylpentanoate (2): Jones reagent was prepared by the addition of concentrated H_2SO_4 (30 mL) to CrO_3 (33.5 g) followed by careful dilution with water to give 250 mL of total solution. Then Jones reagent (17 mL, 17 mmol) was added dropwise to a stirred solution of ethyl 3-hydroxy-5-phenylpentanoate (16 mmol, 3.55 g) in acetone (70 mL) at 0 °C. The solution was left to warm to room temperature and stirred for 3 h. After this time, methanol (7 mL) was added to quench any excess of the Jones reagent. The mixture was extracted with Et_2O (70 mL), the organic layer was washed with water (3 × 50 mL) and brine (50 mL) and dried with $MgSO_4$. The crude product was purified by column chromatography on silica gel by using hexane/ethyl acetate (8:2, v/v). The product was obtained as colorless oil (67 %, 10.7 mmol, 2.36 g). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.30–7.26 (m, 2 H, Ar), 7.21–7.17 (m, 3 H, Ar), 4.20–4.15 (m, 2 H, OCH_2CH$_3$), 3.41 (s, 2 H, COCH_2CO), 2.93–2.86 (m, 4 H, 2 CH_2), 1.28–1.24 (t, J = 7.2 Hz, 3 H, OCH$_2$C*H*$_3$) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 201.8, 167.1, 140.5, 128.5, 128.3, 126.2, 61.4, 49.4, 44.5, 29.4, 14.1 ppm. $C_{13}H_{16}O_3$ (220.3): calcd. C 70.89, H 7.32; found C 70.97, H 7.43. HPLC (C-18 Luna): t_r = 22.8 min.

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Keywords: Biotransformations · Dehydrogenase · Bioreduction · Asymmetric synthesis · Hydroxy esters · Biocatalysis

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