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Enantioselective desymmetrization of prochiral diesters catalyzed by immobilized *Rhizopus oryzae* lipase

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ABSTRACT

The asymmetric hydrolysis of dimethyl 3-phenylglutarate **1** catalyzed by different immobilized preparations of *Rhizopus oryzae* lipase (ROL) has been studied. The Lewatit CNP 105 commercial support was activated to aldehyde groups (Lewatit-aldehyde) and used as a support for the immobilization of ROL using different strategies. Thus, the lipase immobilized in the presence of dithiothreitol at pH 7 (ROL-Lew-pH 7) was the most enantioselective catalyst for the hydrolysis of **1** at pH 7 and 25 °C producing the (*R*)-monoester with an *E* value of 4.2 (ee = 62%) whereas ROL immobilized at pH 10 gave only an *E* value of 1.1 (ee = 4%). The medium engineering was also an interesting tool for improving the lipase selectivity. The addition of a solvent, combined with decreasing temperature improved the *E* value for the reaction from 4.2 to 24 (ee = 62 to ee = 92%). Finally, the application of the ROL-Lew-pH 7 preparation in the presence of 20% dioxane and 5 °C allowed us to obtain the (*R*)-isomer of the monoester with an *E* value of 24 (ee = 92%) in a 97% yield (of monoesters).

1. Introduction

The development of new biocatalysts with high selectivity, activity, and stability for the application of different kinds of chemical reactions is of great interest for the implementation of enzymes in industrial processes.

Currently, lipases are the enzymes mostly used in biocatalysis and organic chemistry.[1,2] In particular, the use of lipases to catalyze asymmetric reactions has attracted great interest[3–5] due to them being able to accept a broad range of substrates with good activity, and in many cases high regio- and enantioselectivity and specificity.[6,7] However, when a lipase is used as a biocatalyst for a given reaction, its application is often hampered by; (a) its difficult recovery and reuse; (b) its low selectivity toward non-natural compounds; and (c) its low stability under processing conditions. Thus, several strategies have been used to improve these drawbacks. In this context, conformational engineering has been described as a very interesting approach toward tuning a lipase's properties by using different immobilization protocols.[8,9] Thus, immobilization of a particular lipase by different orientations, by different rigidity, or in the presence of different micro-environments allows us to generate different biocatalysts from the same lipase with very different catalytic properties.[10–12]

Medium engineering has been used in many cases as a simple and successful strategy to improve the features of these enzymes.[8–11] Using this method, some additives, in particular detergents and solvents have been shown to modulate the properties of lipases. Detergents may shift the equilibrium between closed and open structures of the enzyme by coating the large hydrophobic pocket that surrounds the active center of lipases, thus greatly altering the activity and enantiospecificity of the enzyme.[13–18] Solvents in turn, can promote the opening of the hydrophobic pocket to strengthen the electrostatic interactions, which were often related to the solvent property of log *P*.[13,19,20]

Asymmetric hydrolysis of prochiral compounds, such as the diesters of phenylglutaric acid, important building blocks for the synthesis of several biologically active compounds (Scheme 1), is a simple and noteworthy alternative for the production of chiral compounds.[21–23,4] This strategy has the advantage of permitting 100% conversion to the desired compound, instead of the maximum 50% in the standard resolution of racemic mixtures. In this reaction, neither the substrate (diester) nor the final products (diacid) are chiral compounds. However, the monoester, an intermediate product, is chiral. If the reaction is stopped at the monoester stage and the enantioselectivity of the process is very high, a 100% yield of an enantiomerically pure compound can be produced.[24,25]

Rhizopus oryzae lipase (ROL) has been broadly used in many biotransformations, such as esterifications and *trans*-esterifications.[26–29] However, to date ROL has not been reported as an enantioselective biocatalyst in asymmetric reactions. Therefore

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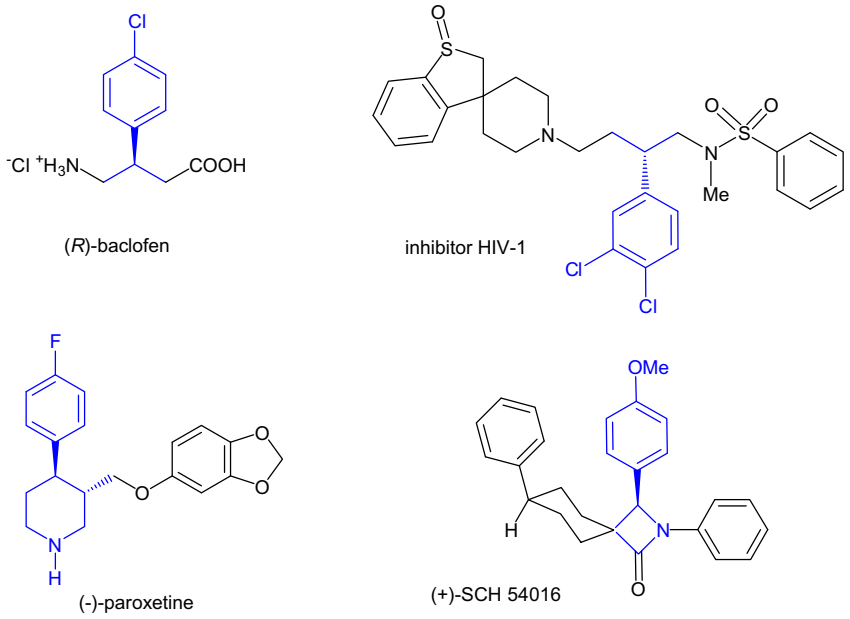
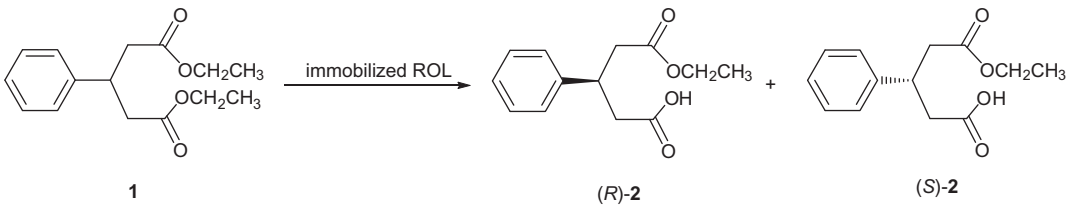


Scheme 1. Biologically active compounds containing a 3-arylglutaric building block.



Scheme 2. Asymmetric hydrolysis of **1**.

its use constitutes an important precedent for its application in such reactions.

Herein, we have studied the potential of ROL to catalyze enantioselective asymmetric reactions, in particular, the hydrolysis of dimethyl 3-phenylglutarate **1** (Scheme 2) catalyzed by three different covalently immobilized preparations on a Lewatit-aldehyde support.

2. Results and discussion

2.1. Immobilization and characterization of the immobilized preparations

The immobilization on a Lewatit-aldehyde support, using different protocols was studied.

The immobilization process was relatively quick, after 4 h the activity of the supernatant was constant. However, the percentages of activity recovered were quite different and dependent upon the immobilization strategy used. For example, ROL-Lew-pH 7, recovered 75% of the initial activity offered, while ROL-Lew-pH 7/pH 10 and ROL-Lew-pH 10 only recovered 50% and 40% of activity, respectively (Table 1).

The stability of the different preparations immobilized of ROL in the presence of 40% (v/v) of dioxane was also studied. Figure 1 shows that the stability was very similar for the three immobilized preparations. However, the enzyme immobilized at pH 10 slightly improved the enzyme stability compared to the enzyme immobilized at pH 7.

Table 1
Specific activity of different immobilized preparations in the hydrolysis of *p*NPB

Biocatalyst	Activity[a]	Recovered activity[b] (%)
ROL-Lew-pH 7	22	75
ROL-Lew-pH 7/pH 10	13	50
ROL-Lew-pH 10	11	40

[a] Activity in μmol mg prot^{-1} min^{-1}.
[b] After the covalent immobilization.

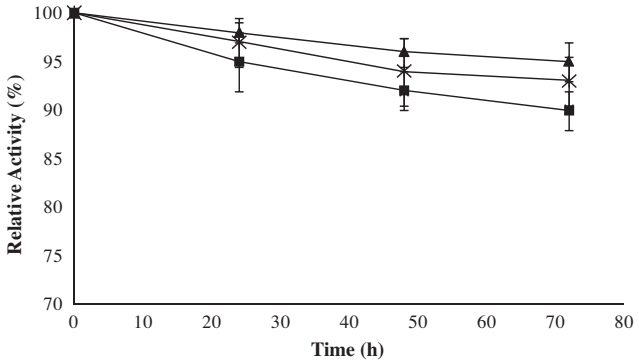


Figure 1. Inactivation courses of ROL immobilized preparations on Lewatit-aldehyde in the presence of 40% (v/v) co-solvent. ROL-Lew-PH 7 (square), ROL-Lew-PH 7/pH 10 (asterisk), ROL-Lew-pH 10 (triangles).

Table 2
Selectivity of different ROL immobilized preparations in the hydrolysis of **1** at pH 7 and 25 °C

Biocatalyst	Activity[a]	Time (h)	Conversion (%)	ee[b] (%)	*E* value (*R*)/(*S*)
ROL-Lew-pH 7	0.036	8	19	62	4.2
ROL-Lew-pH 7/pH 10	0.012	22	17	7	1.2
ROL-Lew-pH 10	0.011	24	18	4	1.1

[a] Activity in μmol mg prot^{-1} h^{-1}.
[b] Enantiomeric excess of monoester (*R*)-**2** calculated at 15–20% conversion.

2.2. Asymmetric hydrolysis of 1 catalyzed by different ROL immobilized preparations

The activities and selectivities of the different ROL immobilized preparations in the hydrolysis of **1** at pH 7 and 25 °C are shown in Table 2. In all cases, the diester was recognized and the (*R*)-monomethyl ester was obtained as the main product.

When using the ROL-Lew-pH 7 immobilized preparation as the catalyst, the reaction proceeded more rapidly (initial activity was threefold higher) than using the ROL-Lew-pH 7/pH 10 or ROL-Lew-pH 10 preparations (Table 2). The selectivity of ROL was strongly affected by the immobilization protocol used. Thus, ROL-Lew-pH 7/pH 10 and ROL-Lew-pH 10 immobilized preparations exhibited very low enantiomeric ratio (*E*) values, *E* = 1.2 (ee = 7%) and *E* = 1.1 (ee = 4%), respectively, although this value could be greatly improved upon by using the ROL-Lew-pH 7 preparation as the biocatalyst, reaching an *E* value of 4.2 (ee = 62%) (Table 2).

Thus, the ROL-Lew-pH 7 appeared to be the optimal immobilized preparation exhibiting the best selectivity and activity during the hydrolysis of **1**. Thus, this immobilized preparation was used in all subsequent experiments.

2.2.1. Influence of the experimental conditions on ROL-Lew-pH 7 in the asymmetric hydrolysis of 1

In order to optimize the selectivity of the reaction, the enzymatic hydrolysis was performed under different conditions. In all cases, the main product was the (*R*)-monoester. The enantioselectivity of the enzyme was strongly altered by the addition of some organic solvents and additives (Table 3). The presence of 20% (v/v) of diglyme, acetone, or dioxane, as co-solvent in the reaction media significantly improved the selectivity of immobilized preparation with an *E* value of 4.2 (ee = 62%) in the absence of solvent, which increased to more than 9 (ee = 80%) when the aforementioned solvent was used. However, in the case of DMSO, enantioselectivity only reached a value of 6.1 (ee = 72%), (Table 3, entries 1, 3, 6, 9 and 12).

The reaction was also carried out in the presence of 40% solvent. Here, the results were not higher than those previously obtained (20% (v/v) solvent), although they were higher than those obtained in the absence of the solvent (*E* = 4.2, ee = 62%), (Table 3, entries 5, 8, 11 and 14).

The enzymatic activity of the ROL immobilized preparation decreased in the presence of co-solvents, although it was more dramatic when 40% (v/v) of the solvent was used. Thus, the ROL activity decreased by 70% with 20% (v/v) acetone while with that obtained with 20% (v/v) of diglyme only 30% activity was lost (Table 3, entries 6 and 9).

Detergents can in some cases alter the enantioselectivity of the lipases. Thus, the effect of SDS, in the presence and absence of co-solvents on the asymmetric reaction was studied (SDS showed very good results with the soluble lipase, results not shown). The enantioselectivity of the ROL immobilized preparation was only slightly influenced by the presence of SDS, with *E* values ranging from 4.2 (ee = 62%) to 5.4 (ee = 69%) without co-solvent (Table 3, entries 1 and 2) or from 6.1 (ee = 72%) to 7.4 (ee = 76%) with 20% (v/v) of DMSO (Table 3, entries 12 and 13). The enzymatic activity in the presence of a detergent was significantly lower (Table 3).

Finally, the influence of temperature on the activity and stereoselectivity of the ROL immobilized preparation was evaluated (Table 4). Thus, when the reaction was conducted at 5 °C, the enantioselectivity of the ROL-Lew-pH 7 immobilized preparation was improved from *E* = 4.2 (ee = 62%) at 25 °C to *E* = 5.4 (ee = 69%) at 5 °C (Table 3, entry 1 and Table 4, entry 1).

When the reaction was catalyzed by ROL preparation in the presence of 20% (v/v) of diglyme, the *E* value also improved from 9 (ee = 80%) to 12.3 (ee = 85%) when the temperature was decreased from 25 to 5 °C (Table 3, entry 6 and Table 4, entry 2). However, the best result was found when the reaction was performed in the presence of 20% dioxane and at 5 °C where *E* = 24 (ee = 92%) was achieved (Table 4, entry 3). The selectivity of the immobilized preparation in the presence of acetone could not be quantified due to the long reaction time required (results not shown).

Under these conditions, the enzymatic activity of the immobilized preparation decreased considerably. However, the ROL immobilized preparation at 5 °C and in the presence of dioxane maintained more than 60% of the activity value of that at 5 °C and without solvent; these were the best conditions to perform the asymmetric hydrolysis of **1** (Table 4).

Table 3
Selectivity of ROL-Lew-pH 7 in the presence of different concentrations of solvent and additives in the hydrolysis of **1** at pH 7 at 25 °C

Entry	Co-solvent	(%)	Additive	Activity[a]	ee[b] (%)	*E* value (*R*)/(*S*)
1	—			0.036	62	4.2
2	—		0.05% SDS	0.008	69	5.4
3	Dioxane	20		0.017	80	9.0
4	Dioxane	20	0.05% SDS	0.009	77	7.7
5	Dioxane	40		0.004	72	6.1
6	Diglyme	20		0.025	80	9.0
7	Diglyme	20	0.05% SDS	0.013	81	9.5
8	Diglyme	40		0.004	83	10.8
9	Acetone	20		0.01	80	9.0
10	Acetone	20	0.05% SDS	nd[c]	nd[c]	nd[c]
11	Acetone	40		0.003	78	8.1
12	DMSO	20		0.026	72	6.1
13	DMSO	20	0.05% SDS	0.022	76	7.4
14	DMSO	40		0.005	74	6.7

[a] Activity in μmol mg prot^{-1} h^{-1}.
[b] Enantiomeric excess of (*R*)-**2** calculated at 15–20% conversion.
[c] nd: not determined.

Table 4
Selectivity of ROL-Lew-pH 7 in the presence of different solvents (20% v/v) in the hydrolysis of **1** at 5 °C and pH 7

Entry	Co-solvent	Activity[a]	ee[b] (%)	*E* value (*R*)/(*S*)
1	—	0.012	69	5.4
2	Diglyme	0.005	85	12.3
3	Dioxane	0.008	92	24.0

[a] Activity in μmol mg prot^{-1} h^{-1}.
[b] Enantiomeric excess of (*R*)-**2** calculated at 15–20% conversion.

2.2.2. Effects of co-solvents on the stability of ROL-Lew-pH 7

The stability of the immobilized preparation ROL-Lew-pH 7 in the presence of 40% of different solvents (acetone, dioxane and diglyme) for long incubation times was studied. In all cases, the immobilized preparation was highly stable, retaining over 80% of its activity after 15 days (Fig. 2).

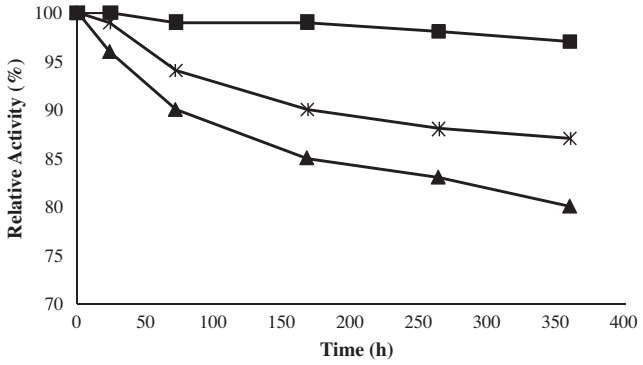


Figure 2. Inactivation courses of ROL immobilized on Lewatit-aldehyde at pH 7 in the presence of 40% solvent. dioxane (triangles), acetone (squares), diglyme (asterisk).

2.2.3. Reuse of ROL-Lew-pH 7 biocatalyst in the asymmetric hydrolysis of 1

Under the optimal conditions, the total conversion of the substrate was performed and the reuse of the biocatalyst was evaluated. The reaction course and evolution of the selectivity of ROL immobilized preparation is shown in Figure 3. The selectivity of ROL-Lew-pH 7 was not altered over the course of the reaction and an E value >20 (ee >90%) was maintained until the end of the process. Another excellent result was the final yield, with 97% of **1** being transformed into **2** with 100% conversion.

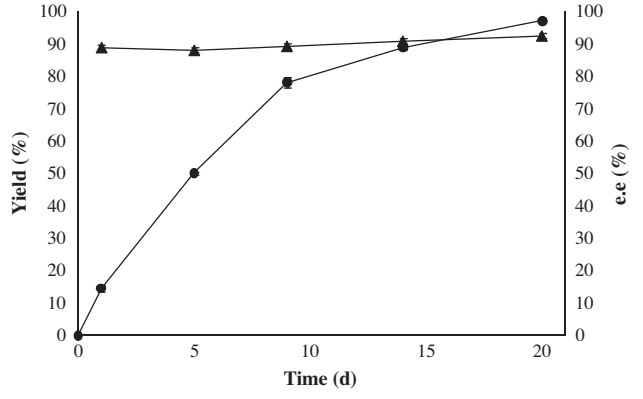


Figure 3. Course of the asymmetric hydrolysis of **1** catalyzed by ROL-Lew-PH 7. Yield (circles), ee (triangles).

At the end of the reaction, the immobilized preparation conserved over 90% of its initial activity, which permits the realization of a second production cycle.

When a second cycle was performed, the results obtained were very similar to those of the first production cycle. Thus, a yield of over 95% was obtained, while the asymmetry slightly decreased, $E = 19$ (ee = 90%). The activity after a second reaction cycle was over 85% of its initial activity. These results suggest that the immobilized enzyme preparation could be reused for three or more production cycles.

3. Conclusion

The results reported herein suggest that *R. oryzae* lipase immobilized on Lewatit-aldehyde has an interesting potential to catalyze enantioselective asymmetric reactions and that the presence of solvents can significantly improve the enantioselectivity of the immobilized preparation. Thus, in the presence of dioxane and at a low temperature, it was possible to obtain the (*R*)-methyl-3-phenylglutarate with an E value of 24 (ee = 92%) and with a yield in monoester of 97%. The high stability of the preparation immobilized in the presence of an organic solvent allows the reuse of the biocatalyst for at least two cycles, while maintaining the selectivity and more than 85% of the activity.

4. Experimental

4.1. General

R. oryzae lipase (ROL), *p*-nitrophenyl butyrate (*p*NPB), DL-dithiothreitol (DTT), dimethyl 3-phenylglutarate **1**, bis(2-methoxyethyl) ether (diglyme), dioxane, acetone and dimethyl sulfoxide (DMSO) were obtained from Sigma. Lewatit CNP 105 was obtained from Lanxess. Lewatit-aldehyde support was prepared as previously described with minor modifications.[30] Other reagents were of analytical or HPLC grade.

4.2. Methods

4.2.1. Lipase activity determination

Activity assay was performed by measuring the increase in absorbance at 348 nm produced by the releasing of *p*-nitrophenol in the hydrolysis of 0.4 mM *p*-nitrophenyl butyrate in 25 mM sodium phosphate at pH 7 and 30 °C, using a thermostatized spectrophotomer with magnetic stirring. To initialize the reaction, 10 mg of immobilized enzyme were added to 10 mL of substrate solution. An international unit of *p*NPB activity is defined as the amount of enzyme necessary to hydrolyze 1 μmol of *p*NPB/min (IU) under the conditions described above.

4.2.2. Immobilization of ROL on Lewatit-aldehyde support

The different ROL immobilized preparations were prepared following the procedures previously described with minor modifications.[31]

(a) Immobilization at pH 7 (ROL-Lew-pH 7): One gram of support was added to 10 mL of 25 mM sodium phosphate pH 7, containing 2 mg of protein/mL, in the presence of 50 mM DTT and 0.05% Triton X-100 and kept under gentle stirring at 25 °C, for 4 h. Immobilization was through the terminal amino group of ROL.

(b) Immobilization at pH 7/pH 10 (ROL-Lew-pH 7/pH 10): After the enzyme immobilization at pH 7 for 4 h, the pH was adjusted to 10.05 using 1 M sodium bicarbonate and kept under gentle stirring at 25 °C, for 2 h. ROL was immobilized by terminal amino group orientation and finally covalent attachment between lysines on the enzyme and aldehydes on the support.

(c) Immobilization at pH 10 (ROL-Lew-pH 10): One gram of support was added to 10 ml of 100 mM sodium bicarbonate pH 10.05, containing 2 mg of protein/mL, in the presence of 0.05% Triton X-100 and kept under gentle stirring at 25 °C, for 4 h. ROL was immobilized directly via covalent attachment by the richest area in lysine and aldehydes on the support.

In all cases, the activities of the supernatants were periodically taken, and the activities were assayed by the method described above. Finally, 10 mg of sodium borohydride were added under gentle stirring at 25 °C to reduce the imino groups.[32] After 30 min, the immobilized enzyme was filtered by vacuum and washed several times with an excess of distilled water.

4.2.3. Inactivation of different ROL preparations

ROL immobilized preparations were incubated in the presence of different solvents. At different times, samples were withdrawn and washed 5 times with water. Finally, the residual activity was measured as described previously.

4.2.4. Asymmetric hydrolysis of 1 catalyzed by different ROL immobilized preparations

The activities of the different ROL preparations in the hydrolysis of **1** were determined by adding 0.1 g of catalyst in 1.5 mL solution of 1.2 mM of substrate in 25 mM sodium phosphate at pH 7 and 25 °C. The degree of hydrolysis was followed by reverse-phase HPLC (Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP 8450) on a Kromasil C18 (15 × 0.4 cm) column supplied by Analysis Vinicos (Spain). In all cases, least triplicates of each assay were made. The elution was performed with a mobile phase of acetonitrile (35% v/v) and 10 mM ammonium phosphate (65% v/v) at pH 3. The flow rate was 1 mL/min. The elution was monitored by recording the absorbance at 225 nm. The enantiomeric excesses were determined at 10–15% conversion.

4.2.5. Reuse of ROL-Lew-pH 7 biocatalyst in the asymmetric hydrolysis of 1

The total conversion of substrate was determined by adding 0.6 g of catalyst in a 3 mL solution of 1.2 mM of substrate in the presence of dioxane (20% v/v) and 25 mM sodium phosphate at pH 7 and 5 °C. The calculation of yield was performed using the following equation:

$$\text{yield} = \frac{n[R-2] + n[S-2]}{n[1]}, \quad n = \text{enantiomer(mol)}.$$

4.2.6. Determination of enantiomeric excess

The enantiomeric excess (ee) of the monoester formed was analyzed by Chiral Reverse Phase HPLC. The column was a Chiracel OD-R. The mobile phase was acetonitrile (25% v/v) and 10 mM ammonium phosphate (75% v/v) at pH 3 and the analyses were performed at a flow of 0.7 mL/min by recording the absorbance at 225 nm. The enantiomeric excesses were determined at 10–15% conversion. The calculation of the ee and enantiomeric ratio (E) was performed using the following equations:

$$\text{ee}(\%) = [\frac{n[R] - n\,[S]}{n[R] + n[S]}] \times 100, \quad E = \frac{n[R]}{n[S]}, \quad n = \text{enantiomer(mol)}.$$

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