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Horseradish peroxidase – a biocatalyst for the one-pot synthesis of enantiomerically pure hydroperoxides and alcohols

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Abstract

The kinetic resolution of racemic hydroperoxides by horseradish peroxidase (HRP)-catalyzed reduction was investigated, with major emphasis on catalytic efficiency and enantioselectivity. The kinetic parameters of the enzymatic reaction were determined, enantiomeric excess (ee) and absolute configurations of hydroperoxides and alcohols were measured, and a broad spectrum of structurally different hydroperoxides were investigated to assess the scope and limitation of this method. Both the catalytic efficiency and the stereoselectivity of HRP highly depend on the structure of the hydroperoxides. The enzyme selectively recognizes sterically unencumbered hydroperoxides, which allows kinetic resolution by means of enantioselective reduction to yield optically active hydroperoxides and alcohols in excellent ee values (up to 99%). Functional groups in the hydroperoxide molecule do not affect the stereoselectivity of the enzyme, which permits a large number of functionalized hydroperoxides to be resolved by HRP.

Keywords: Horseradish peroxidase; Hydroperoxides; Optically active; Kinetic resolution

1. Introduction

Hydroperoxides play an important role as oxidants in organic synthesis [1]. Although several general methods are available for the preparation of racemic hydroperoxides, no convenient method of broad scope is known for the synthesis of optically active hydroperoxides. Such peroxides have potential as oxidants for the catalytic, asymmetric oxidation of organic substrates, currently a subject of intensive investigation in synthetic organic chemistry [2]. Besides chiral metal complexes [3], metalloenzymes play an increasing role as biocatalysts in asymmetric synthesis [4]. They comprise a number of oxidoreductases from microbial sources such as enolate reductase from *Clostridia*, several monooxygenases from *Acinetobacter* and *Pseudomonas* species, as well as lipoxygenases and peroxidases.

The heme-containing peroxidases are a superfamily of enzymes which oxidize a variety of structurally diverse substrates by using hydroperoxides as oxidants. For example, chloroperoxidase (CPO) catalyzes the enantioselective epoxidation of unfunctionalized alkenes [5] and

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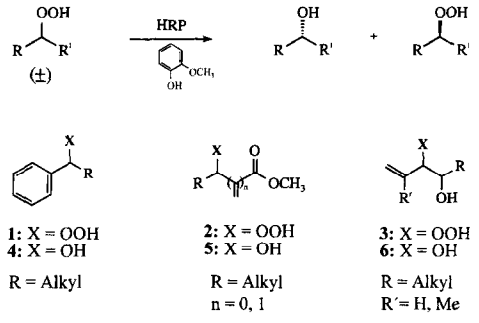
the stereoselective sulfoxidation of prochiral thioether by racemic 1-arylethyl hydroperoxides [6]. The latter reaction results in (*R*) sulfoxides, (*S*) hydroperoxides and the corresponding (*R*) alcohols in good enantiomeric excess. Reports on selective reactions catalyzed by horseradish peroxidase (HRP) are rather scarce; merely the asymmetric sulfoxidation of prochiral sulfides is known [7]. During the last few years we have investigated the kinetic resolution of racemic hydroperoxides by enantioselective reduction of alcohols with HRP. Here we summarize the results of our recent investigations on the synthesis of optically active hydroperoxides by kinetic resolution of racemic derivatives.

2. Results and discussion

Our studies revealed that HRP catalyzes very efficiently the kinetic resolution of secondary, racemic hydroperoxides **1**, **2**, and **3** in the presence of guaiacol (Scheme 1) to afford the hydroperoxides and their alcohols in high optical purities [8]. Three different aspects of the HRP-catalyzed enantioselective reduction of chiral hydroperoxides have been investigated: (i) determination of the kinetic parameters to optimize the reaction conditions, (ii) stereochemical analysis of the substrates and products to determine the enantiomeric excess and the absolute configuration, and (iii) screening of structurally diverse hydroperoxides to assess the scope and limitation of the reaction.



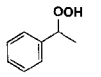
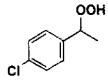
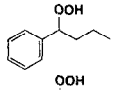
Scheme 1.

2.1. Kinetic parameters of HRP-catalyzed kinetic resolution

In order to assess the catalytic efficiency of the enzymatic transformations, we have determined the kinetic parameters K_m (Michaelis constant) and k_{cat} (turnover number). The K_m value reflects the affinity of the enzyme for the hydroperoxide, whereas k_{cat} measures the number of substrate molecules turned over per enzyme molecule per minute. The ratio k_{cat}/K_m represents the second order rate constant for the enzyme–substrate reaction. The kinetic parameters obtained for alkylaryl hydroperoxides **1** in the presence of guaiacol as electron donor are shown in Table 1. Since similar trends in catalytic efficiency were observed for the functionalized hydroperoxides **2** and **3**, merely the kinetic features of the enzymatic transformation of unfunctionalized hydroperoxides **1** are reported in detail.

Both the K_m and k_{cat} values depend on the R substituent. While H_2O_2 is the most effective oxidant by far, as reflected by the very large k_{cat}/K_m value, of the hydroperoxides **1**, the highest value is obtained for the derivative **1a** (Table 1, entry 2). Introduction of a functional group in the aromatic ring, as in derivative **1b** (entry 3), decreases the rate of the reaction significantly. A stepwise increase of the alkyl chain (entry 4) dramatically decreases the catalytic efficiency, presumably due to steric restrictions at the active site of the enzyme. The k_{cat} value of the bicyclic derivative **1e** is similar to that obtained for 1-phenylethyl hydroperoxide **1a** (entry 2), although the larger affinity constant (K_m) of the former (entry 6) resulted in lower catalytic efficiency in comparison with that of derivative **1a** (811 versus 132 in Table 1, entries 2 and 6). In such cases, the kinetic resolution must be carried out at high hydroperoxide concentrations to ensure optimal reaction rates. In view of the observed trends in the catalytic efficiency (k_{cat}/K_m), the kinetic resolutions were conducted at different substrate concentrations by varying the amounts of HRP

Table 1
Kinetic parameters for horseradish peroxidase-catalyzed kinetic resolution of hydroperoxides **1** in the presence of guaiacol [a]

Entry	Peroxide	K_m[b] (mM)	k_{cat}[c] (min^{-1})	k_{cat}/K_m ($mM^{-1}\,min^{-1}$)
1	H_2O_2	0.05	4600	92000
2	**1a** 	0.7	568	811
3	**1b** 	3.4	376	110
4	**1c** 	26	92	3.5
5	**1d** 	4	2	0.5
6	**1e** 	4.4	584	132
7	**1g** 	18	176	9.8

[a] The kinetic parameters were obtained at a fixed guaiacol concentration of 500 μM; the initial rates were monitored by following the appearance of the guaiacol oxidation product ($\varepsilon = 2.66 \times 10^4$ $M^{-1}\,cm^{-1}$ at 470 nm) in 0.1 M potassium phosphate buffer (pH 6.0); the data were processed with the Duggleby program to obtain K_m and k_{cat} values.
[b] Error limit between 5 and 10% of the stated values.
[c] Error limit between 1 and 5% of the stated values.

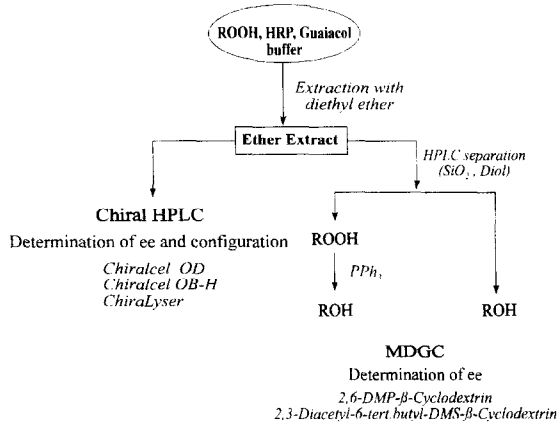
to assess optimal reaction conditions (substrate concentration, reaction time) during the kinetic resolution of the hydroperoxides **1**, **2** and **3**.

2.2. Determination of enantiomeric excess and assignment of absolute configurations

The enantioselectivity of the HRP-catalyzed reactions were determined by employing high performance liquid chromatography (HPLC) and multidimensional gas chromatography (MDGC) on chiral phases. In Scheme 2 the analytical protocol is depicted. For HPLC analysis the diethyl ether extract of the reaction mixture was injected on the chiral column without further purification. The enantiomeric excess was determined from the ratio of areas under the two enantiomer peaks in the HPLC traces. The stereochemistry of the known alcohols and hydroperoxides was assessed by determining the sign of the optical rotation with an online-coupled chiroptical detector. HPLC analysis was performed with hydroperoxides **1**, **2** and the corresponding alcohols **4** and **5**. The ee values of the hydroxy-functionalized hydroperoxides **3** were determined by MDGC analysis. After enzymatic reduction, the temperature labile hydroperoxides **3** were separated from the corresponding alcohols **6** by normal phase HPLC or flash chromatography and reduced by triphenylphosphine prior to MDGC analysis. A representative chromatogram for HPLC and MDGC analysis is given in Fig. 1 for the hydroperoxide **1a** and alcohol **4a** and for hydroxy-functionalized hydroperoxide *threo*-**3c** and corresponding alcohol *threo*-**6c**. As can be seen from these examples, HRP selectively recognized the (+)-(*R*) enantiomer of **1a** and the (*R*,*R*) enantiomer of *threo* diastereomer **3c**. Consequently, in the former case the enantiomerically pure (+)-(*R*)-**4a** alcohol and the (−)-(*S*)-**1a** hydroperoxide and in the latter case the (*R*,*R*)-**3c** alcohol and the (*S*,*S*)-**6c** hydroperoxide were obtained.

2.3. Screening of hydroperoxides

For the screening of hydroperoxides, analytical scale reactions were carried out in aqueous



Scheme 2.

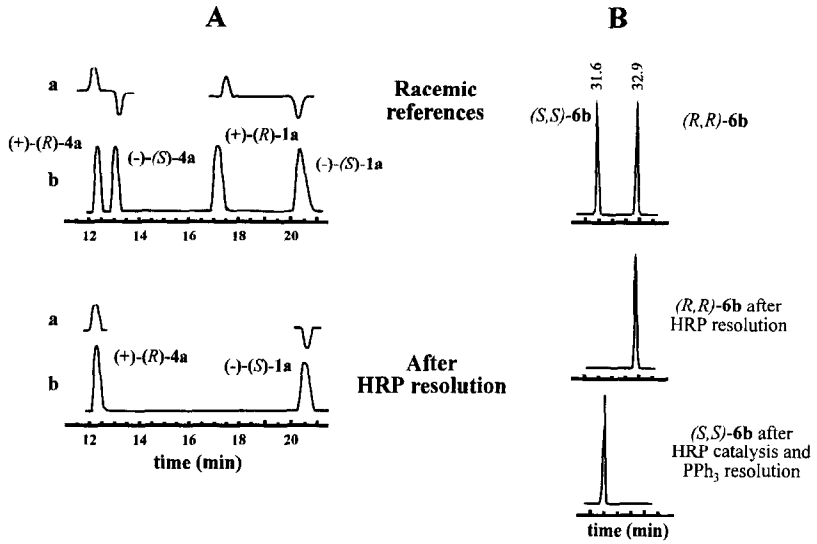


Fig. 1. HPLC and MDGC traces of the HRP-catalyzed kinetic resolution. (A) HPLC traces of the HRP-catalyzed reaction of 1-(1-phenyl)ethyl hydroperoxide (**1a**) with guaiacol. Top: authentic racemic samples of 1-phenylethanol (**4a**) and hydroperoxide **1a**. Bottom: reaction products after enzymatic resolution. The polarimetric detection on the ChiraLyser (a) is placed above the UV signals (b) at 220 nm. Column: Daicel–Chiralcel OD, 250 × 4 cm, eluent: *n*-hexane/isopropyl alcohol (90:10), flow rate: 0.6 mL min^{-1}. (B) MDGC traces of the HRP-catalyzed resolution of *threo*-4-hydroperoxy-5-hexen-3-ol (**3b**). Top: authentic racemic sample of alcohol *threo*-**6b**. Bottom: reaction products after kinetic resolution with HRP and triphenylphosphine reduction. The condition of MDGC analysis are given in Table 4.

buffer solutions at pH 6. Fortunately, all hydroperoxides do not react at room temperature with guaiacol in the absence of HRP, so that the enzymatic reactions could be conducted at about 20°C. To limit the hydroperoxide consumption to 50% and thereby avoid overreduction, equimolar amounts of hydroperoxides and guaiacol were used. Conversion rates, absolute configurations and ee values for hydroperoxides **1** are reported in Table 2. The absolute configurations of the alcohols **4** were assigned by comparison of the HPLC data with those of the authentic substances or according to literature data [9]. The absolute configurations of hydroperoxides **1** were assigned analogously.

The hydroperoxide structure dramatically influenced not only the enantioselectivity but also the enantiomer preference of the enzyme. In the cases of the *n*-alkyl aryl hydroperoxides **1a–d** (Table 2, entries 1–4) and both bicyclic derivatives **1e** and **1f** (entries 5 and 6), HRP preferentially accepts the (*R*) hydroperoxides as substrates with concurrent formation of (*R*) alcohols, while the (*S*) hydroperoxides are left behind. However, the stereochemical course is opposite for the branched hydroperoxides **1g** and **1h** (entries 7 and 8). Consequently, the HRP-catalyzed kinetic resolution affords the (*S*) alcohols **4g,h** and the (*R*) hydroperoxides **1g,h**. Presumably the bulky alkyl chains cause distinct binding of the hydroperoxides in the heme pocket of the enzyme and thereby express opposite enantiomer preference.

High stereoselectivity was observed for the 1-phenylethyl hydroperoxide **1a** and its *para*-chloro derivative **1b** (entries 1 and 2), the 1-phenylpropyl hydroperoxide **1c** (entry 3), and the bicyclic hydroperoxides **1e** and **1f** (entries 5 and 6). Unfortunately, the corresponding alcohol **4c** could not be detected in the HPLC analysis of the crude reaction mixture. Since a control experiment with authentic racemic alcohol **4e** under the enzymatic reaction conditions established that it was completely recovered upon work-up, we suspect that the alcohol **4e** suffers further transformation during enzyme catalysis, but the details of the chemical fate are not known.

Table 2
Enantioselectivities of the HRP-catalyzed kinetic resolution of unfunctionalized hydroperoxides **1** in the presence of guaiacol [a]

Entry	Peroxide	ROOH : HRP (mol)	Time (min)	Enantiomeric excess (%)[b]	
				(-)-(*S*)-ROOH	(+)-(*R*)-ROH
1	**1a** 	12000 : 1	5	>99	>99
2	**1b** 	4720 : 1	90	>95	>95
3	**1c** 	6000 : 1	150	93	95
4	**1d** 	2400 : 1	90	<5	<5
5	**1e** 	10000 : 1	60	>99	c
6	**1f** 	2400 : 1	180	95	97
				(+)-(*R*)-ROOH	(-)-(*S*)-ROH
7	**1g** 	480 : 1	180	15	14
8	**1h** 	1000 : 1	960	36.6	47.7
9	**1i** 			no conversion	

[a] All reactions were conducted on a 0.06 mmol scale; conversion of the peroxides was 50%, determined photometrically; the absolute configurations of the corresponding alcohols were confirmed by comparison with the authentic sample or literature data [8].
[b] The enantiomeric excess was established by HPLC on a Chiralcel OD column by using the area under each enantiomer peak; in all cases a clean separation of the hydroperoxide and alcohol enantiomers was achieved; detection limit $\leq 5\%$.
[c] The alcohol was not detected in the HPLC analysis.

Replacement of the ethyl group by a longer alkyl chain as in the hydroperoxide **1d** (entry 4), dramatically decreased the degree of asymmetric induction; no resolution was observed for **1d**. This result marks one definite limitation in the HRP-catalyzed kinetic resolution of racemic hydroperoxides.

Branching of the alkyl substituent as in the derivatives **1g** and **1h** (entries 7 and 8) results in only moderate enantioselectivities even at 50% conversion. Moreover, the position of the branching point in the alkyl chain exhibits significant differences in the ee values. Thus, α branching, i.e. right next to the stereogenic center as in the hydroperoxide **1g**, reduces the enantioselectivity much more effectively than β branching as in **1h** (Table 2, entries 7 and 8). Furthermore, attempts to resolve tertiary hydroperoxides such as **1i** by HRP failed. As already alluded to above, this speaks for rather sensitive steric effects in the enantiomer recognition by the HRP enzyme, and marks the limitation of this enzymatic method for the preparation of optically active hydroperoxides.

Table 3 lists the results of the HRP-catalyzed kinetic resolution of α- and β-hydroperoxy ester **2**. The enzyme reduces the *n*-alkyl derivatives of both α- and β-hydroperoxy ester (**2a** and **2d**,**c**) with excellent stereoselectivity (Table 3, entries 1, 4, 5). After 50% conversion of racemic hydroperoxides the corresponding alcohol and remaining hydroperoxide enantiomers were obtained in $> 97\%$ optical purity.

Table 3
Enantioselectivities of the HRP-catalyzed kinetic resolution of ester-functionalized hydroperoxides **2** in the presence of guaiacol [a]

Entry	Peroxide	ROOH : HRP (mol)	Time (min)	Enantiomeric excess (%)[b]	
				(*R*)-ROOH	(*S*)-ROH
1	**2a** 	10000 : 1	60	97[c]	97
2	**2b** 	10000 : 1	60	79	64
3	**2c**	10000 : 1	72h	no conversion	
				(*S*)-ROOH[d]	(*R*)-ROH
4	**2d** 	12000 : 1	5	97	>99
5	**2e** 	21000 : 1	10	>99	>99
6	**2f** 	240 : 1	1200	conversion <5%	

[a] All reactions were conducted on a 0.06 mmol scale; conversion of the peroxides was 50%, determined photometrically.
[b] The enantiomeric excess was established by HPLC on a Chiralcel OD column by using the area under each enantiomer peak; in all cases a clean separation of the hydroperoxide and alcohol enantiomers was achieved; detection limit $\leq 5\%$.
[c] The absolute configuration was assigned by the synthesis of an authentic sample of (−)-(*S*)-**2a**.
[d] The absolute configurations were assigned by CD spectroscopy after benzoylation of the hydroxy group.

In the case of alkylaryl hydroperoxides **1** an unfavorable influence of branched alkyl groups in the hydroperoxide molecule on the reactivity and stereoselectivity of the enzymatic reduction was observed. A similar trend was expected for the ester-functionalized hydroperoxides **2**. Indeed, lower ee values were observed for the products derived from kinetic resolution of α-hydroperoxy ester **2b** in comparison to **2a** (entries 1 and 2), while the derivatives **2c** and **2f** did not react at all with HRP/guaiacol (entries 3 and 6). These results clearly demonstrate the steric restrictions in the active site of HRP also in the case of α- and β-hydroperoxy esters. While the isopropyl derivative **2b** is still a substrate for the enzyme, HRP does not accept derivative **2f** (entries 2 and 6). Even after a long reaction time, the conversion rate was below 5%. Apparently, the flanking α-methylene group in hydroperoxide **2f** compared to derivative **2b** impedes its approach to the active site of the enzyme.

The absolute configurations of the α-hydroxy esters **5a,b** were determined by synthesis of an authentic sample of (−)-(*S*)-alcohol **5a** from (+)-(*S*)-α-aminobutyric acid as starting material according to the literature procedure [10]. The absolute configurations of the α-methylene-β-hydroxy esters **5d,e** were established by the CD spectroscopic method worked out by Nakanishi et al. [11]. The ee values and the configurations manifest that the HRP enzyme recognizes preferentially the (+)-(*S*)-α-hydroperoxy esters **2a,b** and the (+)-(*R*)-β-hydroperoxy esters **2d,e**. Consequently, the kinetic resolution of the former substrates affords optically active (−)-(*R*)-α-hydroperoxy esters **2a,b** and (−)-(*S*)-α-hydroxy esters **5a,b**, while the latter ones yield (−)-(*S*)-β-hydroperoxy esters **2d,e** and (+)-(*R*)-β-hydroxy esters **5d,e**. The enantioselectivity of HRP for the chiral oxidant **2a,b** is in contrast to that observed for 1-phenylalkyl hydroperoxides **1**, for which HRP recognizes the (*R*) enantiomer. Presumably such chiral recognition is not only due to the steric demand of the substrate, but also electronic factors through H-bonding between the substrate ester and the amino acid functionalities at the active site of the enzyme appear to play a role.

To assess the influence of hydroxy groups on the HRP-catalyzed reduction of hydroperoxides, we examined furthermore the diastereomeric hydroxy-functionalized allyl hydroperoxides **3**.

Table 4
Enantioselectivities of the HRP-catalyzed kinetic resolution of hydroxy-functionalized hydroperoxides **3** in the presence of guaiacol [a]

Entry	Peroxide	ROOH : HRP (mol)	Time (min)	Enantiomeric excess (%)[b]	
	threo-ROOH			*(S,S)*-ROOH[c]	*(R,R)*-ROH
1	**3a** 	3400 : 1	180	63	67
2	**3b** 	3040 : 1	180	>99	>99
3	**3c** 	1866 : 1	180	>99	>99
4	**3d** 	400 : 1	1440	14	18
	erythro-ROOH			*(S,R)*-ROOH	*(R,S)*-ROH
5	**3a** 	3200 : 1	180	>99[d]	84
6	**3b** 	3040 : 1	180	>99	89
7	**3c** 	1866 : 1	180	>99	92
8	**3d** 	760 : 1	1440	62	73

[a] All reactions were conducted on a 0.06 mmol scale; conversion of the peroxides was 50% as determined photometrically; for the HRP reaction diastereomerically pure *threo*-**3** was used, for *erythro*-**3** a 1:1 threo/erythro mixture was used.

[b] The enantiomeric excess was established by MDGC analysis (area under each enantiomer peak); pre-column: J&W DB-Wax (25 m×0.25 mm), temperature program: started at 60°C and increased by 10°/min to 240°C; main column: heptakis(2,6-dimethyl-3-*O*-pentyl)-β-cyclodextrin in OV 1701 (25 m×0.25 mm), temperature program: started at 60°C held for 15 min and increased by 2°/min to 200°C; the hydroperoxides were reduced to the corresponding alcohols prior to MDGC analysis.

[c] The absolute configurations were assigned by CD spectroscopy after benzoylation of the hydroxyl groups after reduction.

[d] Separation of the enantiomers was achieved on heptakis (30% 2,3-diacetyl-6-*tert*.butyl-dimethylsilyl)-β-cyclodextrin in OV-1701 (0.25 mm×25 m), temperature program: started at 60°C held for 15 min and increased by 3°/min to 200°C.

Conversion rates, absolute configurations and ee values are given in Table 4. The absolute configurations of the hydroperoxides **3a–d** were assigned after triphenylphosphine reduction (retention of configuration [12]) to the corresponding alcohols by employing the CD spectroscopic method developed by Nakanishi et al. [11]. The details of the configurational assignment of hydroxy-functionalized hydroperoxides **3** will be reported elsewhere.

The HRP enzyme exhibits excellent stereoselectivity for reduction of *threo* and *erythro* diastereomers of racemic hydroperoxides **3** with high preference for the (*R*)-configurated hydroperoxy group. Accordingly, the kinetic resolution afforded the (*R,R*) or (*R,S*) allylic diols **6**, whereas the (*S,S*) and (*S,R*) allyl hydroperoxides were left behind enantiomerically pure (ee > 99%) (Table 4, entries 2, 3 and 5–7).

In contrast to *erythro* **3a**, its *threo* diastereomer displayed a significantly lower enantioselectivity (ee > 99 versus 63%) (entries 1 and 5). Presumably, the *erythro* configuration of the functional groups (HOO and HO) facilitates selective binding of the (*R,S*) enantiomer at the active site of the enzyme. Moreover, steric effects on the HRP selectivity are more pronounced in *threo*-configurated substrates than in *erythro* diastereomers, as evidenced by derivative **3d**. Thus, methyl-branching at the α-position of the double-bond results in a significantly lower optical purity in the *threo* (entry 4) compared to the *erythro* diastereomer (entry 8). However, alkyl substituents adjacent to the hydroxy group do not affect the enantioselectivity of HRP (entries 2, 3 and 5–7).

In summary, our study establishes that the reactivity and enantioselectivity of the HRP-catalyzed reduction of chiral hydroperoxides depend mainly on the steric demand of the substrate adjacent to the stereogenic center, but electronic factors also play a significant role. Additional functionalities in the hydroperoxide molecule, e.g. ester and hydroxy groups, do not significantly affect the enantioselectivity of HRP. Thus, HRP is much less substrate specific than any of the previously employed enzymes and accepts a variety of unfunctionalized (**1**) and functionalized hydroperoxides (**2** and **3**), to reduce them enantioselectively to the corresponding alcohols **4**, **5** and **6**. For preparative purposes it is important that the enzyme-catalyzed resolution can be performed on a preparative scale to provide optically active hydroperoxides for asymmetric synthesis.

Acknowledgements

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