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Biocatalytic synthesis of optically active oxyfunctionalized building blocks with enzymes, chemoenzymes and microorganisms

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This work is dedicated to Professor Helmut Werner, on the occasion of his retirement and in appreciation for his continued support of our work in the SFB 347.

Abstract

In this account, we summarize the highlights of our cooperative research work on the biocatalytic preparation of enantiomerically enriched, oxyfunctionalized substances in the 'Sonderforschungsbereich (SFB 347): Selektive Reaktionen Metall-aktivierter Moleküle'. The biocatalytic kinetic resolution of secondary hydroperoxides with the metalloenzymes horseradish (HRP) and *Coprinus* (CiP) peroxidase affords the hydroperoxides and their alcohols in excellent enantiomeric excess. Also the semisynthetic enzyme selenosubtilisin catalyzes effectively the enantioselective reduction of racemic hydroperoxides to produce optically active hydroperoxides and alcohols in high ee values. The asymmetric CH oxidation of long- and medium-chain carboxylic acids by molecular oxygen, catalyzed with the α-oxidase from pea leaves or germinating peas yields enantiomerically pure 2-hydroxy acids. The enzymatic kinetic resolution of racemic 2-hydroxy acids through the enantioselective oxidation by glycolate oxidase (GOX) or the esterification by lipase provides an attractive route to such optically active building blocks. The bacteria *Bacillus subtilis* and *Bacillus megaterium*, isolated from topsoil by selective screening with hydrogen peroxide or allylbenzene, are efficient biocatalysts for the enantioselective reduction of racemic hydroperoxides and the asymmetric CH oxidation of alkanes. The latter enzymatic process is to date still difficult to emulate by chemical catalysis.

Keywords: Biocatalysts; Semisynthetic enzyme; Soil bacteria; Enantioselective transformations; Kinetic resolution; Asymmetric CH oxidation

1. Introduction

The development of environmentally acceptable, efficient chemical processes for the preparation of enantiomerically pure compounds still represents a major challenge in organic synthesis. For this purpose, biocatalytic transformations with enzymes have attracted much attention in the past years. The reasons for the increasing acceptance of enzymes as synthetic reagents rest, on the one hand, on their currently better accessibility and, on the other hand, on the advantages for utilizing them in asymmetric synthesis. Unquestionably, isolated enzymes and whole-cell enzyme systems are efficient catalysts under mild reaction conditions. Moreover, since enzymes are chiral materials and they possess distinct substrate selectivity, optically active compounds may be obtained in high enantiomeric excess from prochiral or racemic substrates by catalytic asymmetric induction or kinetic resolution. Furthermore, biocatalysts are environmentally compatible, and they may perform transformations, which are difficult to realize by chemical catalysts.

In the interdisciplinary structured 'Sonderforschungsbereich (SFB 347): Selektive Reaktionen Metall-aktivierter Moleküle', we have developed in the last years biocatalytic methods for the preparation of optically active, oxyfunctionalized substances such as hydroperoxides, alcohols, α-hydroxy carboxylic acids, β-lactones,

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and β-lactams [1–4]. For this purpose, isolated enzymes as well as cells (microorganisms) were used as biocatalysts. Also, the semisynthetic enzyme selenosubtilisin, which was obtained by chemical modification of the active site of the well-known enzyme subtilisin, was successfully employed as catalyst for the preparation of enantiomerically enriched hydroperoxides. In this account, we present a brief overview of our highlights in the fascinating and timely research area of biocatalytic asymmetric synthesis.

2. Biocatalytic transformations with enzymes

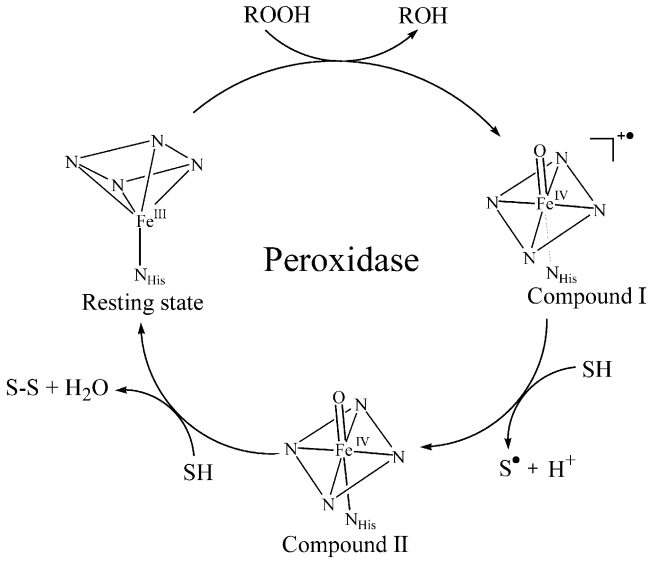
Numerous enzymes are nowadays accessible, which may catalyze a broad spectrum of chemical reactions, e.g. hydrolysis, esterification, addition and elimination, halogenation and dehalogenation, as well as oxidation and reduction. Our research activities in the area of biocatalysis have been mainly focused on redox reactions with oxidoreductases, in particular peroxidases and oxidases. For comparison purposes, readily available lipases were utilized for the preparation of enantiomerically pure oxyfunctionalized substances by kinetic resolution.

2.1. Peroxidase-catalyzed kinetic resolution of hydroperoxides

Peroxidases are a heterogeneous group of redox enzymes, which are ubiquitously found in plants, microorganisms and animals. Although the biological role of peroxidases is quite diverse, these enzymes have in common that they catalyze redox reactions by using hydrogen peroxide or hydroperoxides as oxygen sources [1]. The largest group of peroxidases studied so far are heme enzymes with ferric protoporphyrin as the prosthetic group. The mechanism of heme-dependent peroxidase catalysis has been largely deduced from model studies on the horseradish peroxidase (HRP) [1], which furnished the catalytic cycle in simplified form as shown in Scheme 1. Due to the fact that no cofactor is necessary, peroxidases are highly attractive for biocatalytic enantioselective transformations such as peroxide reduction, olefin epoxidation and sulfide oxidation. Of these, we have extensively studied the enantioselective reduction of racemic hydroperoxides by peroxidases, in particular horseradish and *Coprinus* peroxidase (CiP), to prepare optically active hydroperoxides through kinetic resolution.

2.1.1. Horseradish peroxidase (HRP)

The heme-containing HRP has been extensively used in biocatalytic transformations [1]. This metalloenzyme catalyzes the oxidation of a variety of organic substrates, e.g. phenols, aromatic amines, sulfides, with

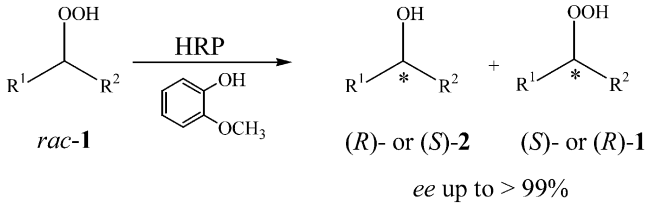


Scheme 1. Catalytic cycle of peroxidases; SH means substrate.

hydrogen peroxide or hydroperoxides as oxygen sources, the latter are reduced to water or alcohols. We have utilized this reactivity of HRP to develop a convenient and versatile biocatalytic method for the preparation of optically active hydroperoxides, which are valuable oxygen donors for asymmetric oxidations [5–7].

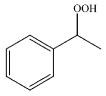
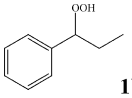
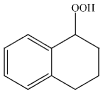
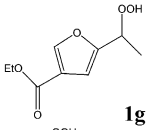
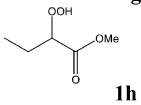
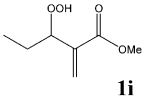
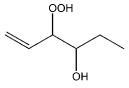
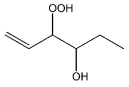
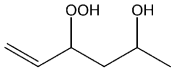
The HRP-catalyzed kinetic resolution of racemic hydroperoxides (**1**) in the presence of guaiacol (2-methoxyphenol) afforded the hydroperoxides and their alcohols (**2**) in high enantiomeric excess (Scheme 2). A representative selection of HRP-catalyzed kinetic resolutions of hydroperoxides is shown in Table 1, in which also the pertinent literature is given [8–15].

Initially, the HRP-catalyzed kinetic resolution of simple alkyl aryl hydroperoxides (substrates **1a**, **b**, **e** in Table 1) was conducted to obtain the corresponding hydroperoxides in up to >99% ee values [8–10]. This efficient biocatalytic method was then successfully extended to various functionalized derivatives such as the furan-derived hydroperoxide **1g** [11], hydroperoxy esters (substrates **1h**, **i**) [12,13,16], α-hydroxy and β-hydroxy allyl hydroperoxides (substrates **1j** and **m**) [14,15,17], to provide the functionalized hydroperoxides and the corresponding alcohols in nearly enantiomerically pure form. Unfortunately, the sterically demanding



Scheme 2. HRP-catalyzed kinetic resolution of racemic hydroperoxides **1**.

Table 1
Enantioselectivities of the HRP-catalyzed kinetic resolution of hydroperoxides **1** in the presence of guaiacol

Hydroperoxide[a]	ROOH : HRP (mol)	Time (min)	Enantiomeric excess (%)[b] Peroxide **1**	Alcohol **2**	Ref
 **1a**	12000 :1	5	> 99 (*S*)	> 99 (*R*)	[8,9]
 **1b**	6000 : 1	150	93 (*S*)	95 (*R*)	[9]
 **1e**	2400 : 1	180	95 (*S*)	97 (*R*)	[10]
 **1g**	2400 : 1	60	> 99 (*S*)	89 (*R*)	[11]
 **1h**	10000 : 1	60	97 (*R*)	97 (*S*)	[12]
 **1i**	21000 : 1	10	> 99 (*S*)	> 99 (*R*)	[13]
 *threo*-**1j**	3040 : 1	180	> 99 (*S,S*)[c]	> 99 (*R,R*)[c]	[14]
 *erythro*-**1j**	3040 : 1	180	> 99 (*S,R*)[c]	89 (*R,S*)[c]	[14]
 *like*-**1m**	5000 : 1	80	98 (*R,R*)	> 98 (*S,S*)	[15]

[a] All reactions were conducted on a 0.06-mmol scale; conversion of the peroxides was ca. 50%.
[b] The enantiomeric excess was determined by HPLC analysis on a Chiralcel OD column.
[c] The ee values were determined by MDGC analysis.

hydroperoxy vinylsilanes (not shown in Table 1) are sluggishly converted by HRP, and only moderate ee values (42–74%) were observed [18]. Also, poor enzymatic recognition was observed for hydroperoxides with branched aliphatic chains, but not at all with chiral tertiary hydroperoxides [1,9].

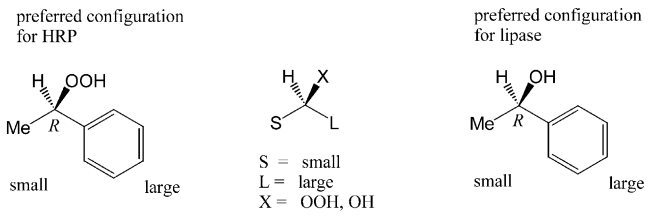
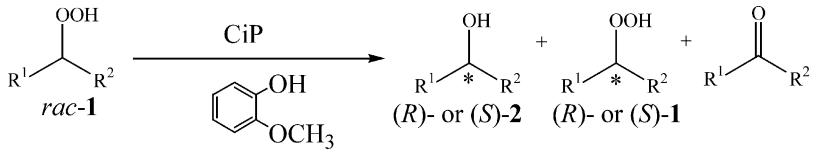


Fig. 1. Preferred enantiomers in the HRP-catalyzed reduction of hydroperoxides and in the lipase-catalyzed esterification.

Our detailed studies on a wide range of secondary racemic hydroperoxides revealed that the empirical model (Fig. 1), established for the enzymatic recognition of the preferred enantiomer by lipases [19], also applies to HRP [15]. Both enzymes convert preferentially the enantiomer, in which the larger substituent at the stereogenic center lies on the right-hand side and the oxyfunctional group points out of the plane of the paper towards the viewer. This remarkable similarity in steric differentiation by these two so distinct enzymes, namely the hydrolases (lipases) and oxidoreductases (peroxidases), has not been recognized prior to our investigations.

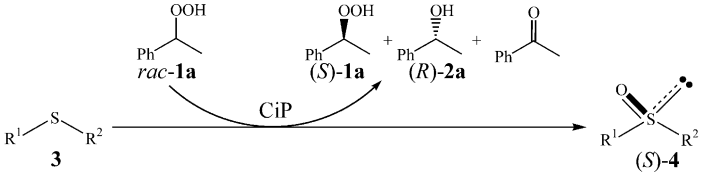
The HRP-catalyzed enzymatic process for the preparation of optically active hydroperoxides and their alcohols has two major advantages: The reaction may be

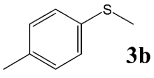
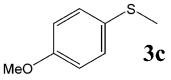
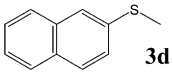


	R^1	R^2	*ee* value (%) 1	2
a	Ph	CH_3	> 98 (*S*)	90 (*R*)
c	Ph	CH_2OH	68 (*R*)	65 (*S*)

Scheme 3. CiP-catalyzed kinetic resolution of hydroperoxides **1a**, **c** in the presence of guaiacol.

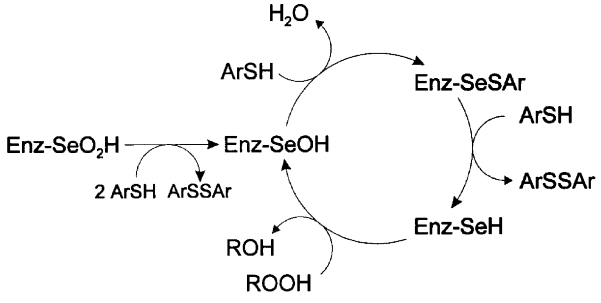
Table 2
CiP-catalyzed sulfoxidation with the raccmic (1-phenyl)ethyl hydroperoxide (**1a**)



Sulfide	Convn (%)[a] sulfide	Product distribution (%)[a] (*S*)-**1a**	(*R*)-**2a**	Ketone	Enantiomeric excess (%)[b] (*S*)-**4**	(*S*)-**1a**	(*R*)-**2a**
 **3b**	68	28	57	15	79	> 98	68
 **3c**	> 95	38	52	10	79	95	65
 **3d**	53	35	46	19	89	81	76

[a] Determined from the ^{1}H-NMR spectrum of the crude product mixture.
[b] Determined by HPLC analysis on a Chiralcel OD-H or OB-H column.

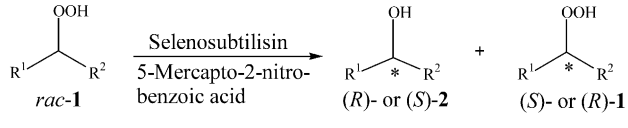
run on the preparative scale (gram quantities), and since additional functional groups in the hydroperoxides do not affect the stereoselectivity of the enzyme, a large number of diverse functionalized hydroperoxides may be resolved by HRP [1].



Scheme 4. Proposed catalytic cycle for selenosubtilisin.

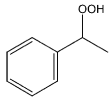
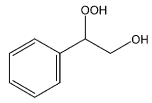
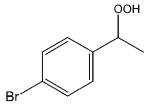
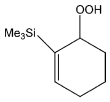
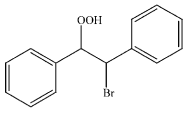
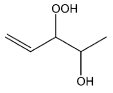
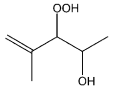
2.1.2. *Coprinus peroxidase (CiP)*

As mentioned in the previous Section 2.1.1, sterically demanding hydroperoxides are not accepted by the enzyme HRP. Therefore, we have searched for other peroxidases, which may enable the enzymatic kinetic resolution of sterically encumbered, racemic hydroperoxides. The CiP, isolated from the basidiomycete *Coprinus cinereus*, is known to have a rather large substrate opening [20] and should, therefore, be a favorable enzyme to accept a broad range of substrates, also sterically encumbered ones.



Scheme 5. Kinetic resolution of racemic hydroperoxides **1** catalyzed by selenosubtilisin.

Table 3
Kinetic parameters and enantioselectivities for selenosubtilisin-catalyzed resolution of racemic hydroperoxides **1**

Hydroperoxide	K_m[a] (mM)	k_{cat}[a] (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	Time (min)	Enantiomeric excess (%)[b] Peroxide **1**	Alcohol **2**
 **1a**	15.7	2125	135	12	52 (*R*)	60 (*S*)
 **1c**	2.1	2443	1150	3	99 (*S*)	99 (*R*)
 **1d**	4.3	592	138	8	34 (*R*)	28 (*S*)
 **1f**	8.5	820	97	13	80[c]	96[c]
 *erythro*-**1n**	0.07	3322	47500	0.4	64[c]	90[c]
 *threo*-**1k**	5.3	449	84	9	14 (*S,S*)	30 (*R,R*)
 *threo*-**1l**	12.2	643	53	16	22 (*R,R*)	38 (*S,S*)

[a] Measured for 0.44 μM selenosubtilisin Carlsberg.
[b] Determined by multidimensional gas chromatography on chiral cyclodextrin columns and corrected for the nonenzymatic background reaction.
[c] Configuration unknown.

In this hope, the enzyme CiP was applied to the kinetic resolution of racemic hydroperoxides in the presence of guaiacol. Unfortunately, sterically encumbered secondary and also tertiary hydroperoxides could not be resolved by this enzyme [21]. Nevertheless, the sterically less demanding alkyl aryl hydroperoxide **1a** and its hydroxy derivative **1c** were enantioselectively reduced by CiP to afford the hydroperoxides and alcohols in up to 98% ee values (Scheme 3).

Peroxidases also possess the ability to catalyze oxygen-transfer reactions; therefore, the oxygen-transfer propensity of CiP was investigated for the enantioselective sulfoxidation of prochiral sulfides as the model reaction [21]. As oxidant, racemic (1-phenyl)ethyl hydroperoxide (**1a**) was used, which was shown to be the best for CiP-catalyzed kinetic resolution. In this enzymatic transformation, a double asymmetric induction occurred and three optically active substrates were prepared simultaneously in very good enantiomeric excess, namely, the (*S*) sulfoxide **4**, the (*R*) alcohol **2a** and the (*S*) hydroperoxide **1a** (Table 2). The best substrate was methyl naphthyl sulfide **3d**, which was oxidized with the highest ee value of 89%; the reason may be the large steric demand of the naphthyl group [21].

2.1.3. Selenosubtilisin

The efficient kinetic resolution of chiral hydroperoxides by peroxidases, in particular HRP, has made available a broad structural variety of optically active hydroperoxides, which are otherwise cumbersome to prepare. Despite this success, the application of the enzymes HRP, CiP and chloroperoxidase (CPO) is limited to sterically not demanding substrates. Moreover, these peroxidases prefer the same absolute configuration of the chiral hydroperoxide; consequently, the

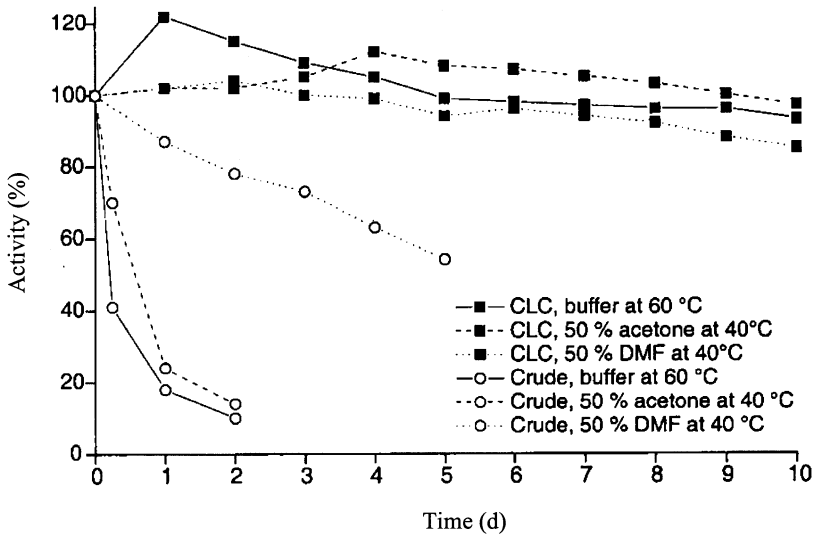


Fig. 2. Stability of amorphous and immobilized (CLC) selenosubtilisin in organic solvents or at high temperature.

opposite enantiomer is not accessible [1]. Therefore, efforts have been expanded to resolve racemic hydroperoxides by semisynthetic enzymes, which may circumvent the short-comings of natural enzymes [3,22,23].

It has been reported that the chemical modification of serine 221 into selenocysteine at the active site of subtilisin imparts this protease with selenoperoxidase activity [24]. The semisynthetic enzyme selenosubtilisin, a mimic for glutathione peroxidase, with the peptide framework of the serine protease subtilisin, catalyzes the reduction of hydrogen peroxide and hydroperoxides in the presence of 5-mercapto-2-nitrobenzoic acid [24]. The catalytic cycle of selenosubtilisin enzyme is shown in Scheme 4 [23].

We have utilized for the first time this semisynthetic peroxidase for the kinetic resolution of racemic hydroperoxides (Scheme 5) [3,22,23]. The catalytic efficiency and enantioselectivity of selenosubtilisin for a set of structurally varied hydroperoxides are shown in Table 3 [23].

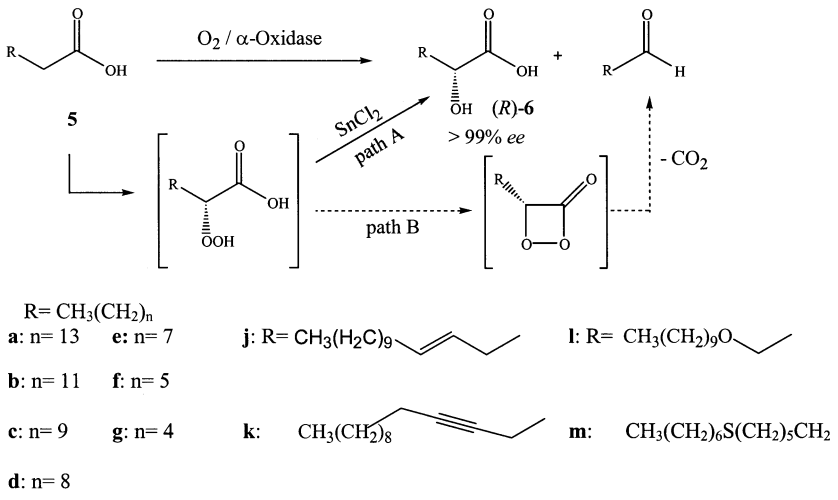
Since the active site of selenosubtilisin peroxidase is located in a groove on the surface of the former *endo*-protease [24], it should be more readily accessible for large substrates. Indeed, the sterically encumbered α-bromo peroxide *erythro*-**1n** displayed an excellent turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m) value. Moreover, the sterically demanding hydroperoxy vinylsilane (**1f**), which is not accepted by HRP [18], exhibited a high k_{cat} value.

The sense in enantioselectivity of the selenosubtilisin-catalyzed reduction of racemic hydroperoxide is opposite to that of the previously used peroxidases HRP and CiP (compare the data in Table 1 and Scheme 3 with Table 3). Clearly, the selectivity of the semisynthetic enzyme is strongly influenced by the structure of the substrate. In the case of alkyl aryl hydroperoxides **1a**, **c**, **d**, the highest selectivity was observed for the α-hydroxy derivative (**1c**) (Table 3, second entry). Its composition of functional groups, a hydrophobic phenyl residue and a polar hydroxy function, provides an optimal fit at the active site and, hence, a high enantioselectivity. The α-bromo hydroperoxide (**1n**) constitutes a similar substrate, and good enantioselectivity was observed despite its sterically encumbered structure. Although the allylic hydroperoxides **1k** and **1l** have a α-hydroxy group as polar functionality, they lack a large hydrophobic phenyl group and, consequently, a lower enantioselectivity ensues. In contrast to the peroxidases CiP [21] and HRP [18], the selenosubtilisin reduces enantioselectively the sterically demanding hydroperoxy vinylsilane (**1f**) (fourth entry), to afford the optically active hydroperoxide and its alcohol with ee values of 80 and 96% [23].

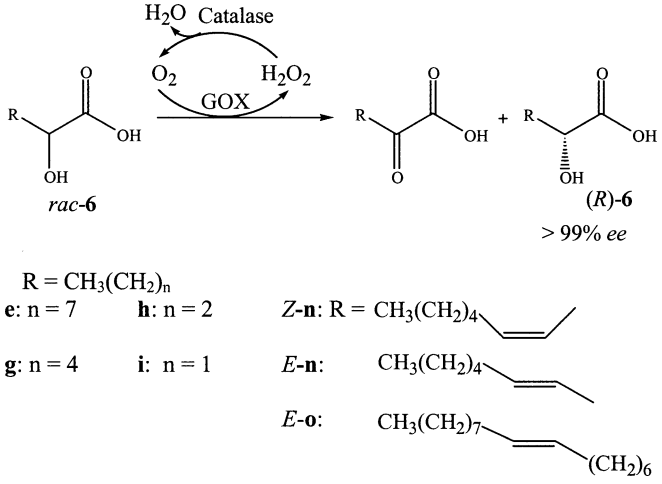
Our results establish the selenosubtilisin as an efficient catalyst for the enantioselective reduction of a variety of functionalized racemic hydroperoxides. Its reactivity towards sterically encumbered substrates is significantly higher than that of the enzymes HRP and CiP. Advantageous for preparative purposes, the selenosubtilisin exhibits the opposite sense in the enantioselectivity compared with the natural peroxidases. The overall synthesis of the immobilized (CLC) semisynthetic peroxidase [25] was achieved in gram scale. Its exceptional stability in organic solvents and at high temperatures is shown in Fig. 2 [26]. Thus, it is demonstrated for the first time that semisynthetic enzymes may complement optimally the set of naturally available biocatalysts for enantioselective synthesis.

2.2. Oxidase-catalyzed CH oxidation

From the synthetic point of view, the selective oxygen transfer to organic substrates is one of the most valuable



Scheme 6. α-Oxidation of carboxylic acids **5** by the crude homogenate of young pea leaves or crude extract of germinating peas with molecular oxygen.



Scheme 7. Oxidation of 2-hydroxy acids (**6**) with molecular oxygen, catalyzed by the GOX from spinach (*S. oleracea*).

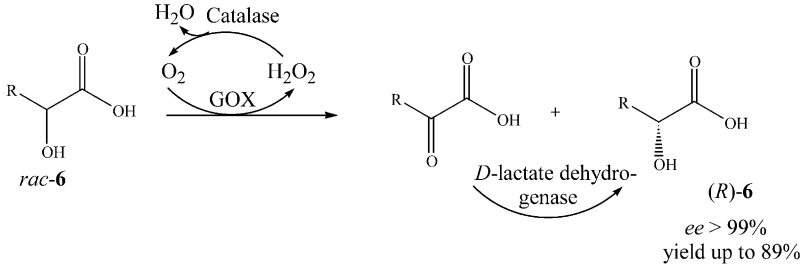
oxidation reactions. Although several efficient metal-catalyzed asymmetric oxygen-transfer processes have been developed in the past decades [27], the selective catalytic CH oxidation still remains a challenge in organic synthesis. Oxidative enzymes [28] offer promising opportunities to meet this challenge. We present in this section the successful application of α-oxidases of higher plants and glycolate oxidase (GOX) for the preparation of enantiomerically pure 2-hydroxy carboxylic acids by asymmetric CH oxidation of carboxylic acids and kinetic resolution of racemic substrates.

2.2.1. α-Oxidase

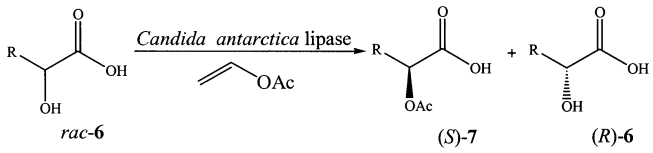
Many chemical and enzymatic methods have been reported on the synthesis of optically active 2-hydroxy acids, but little is known on the direct enantioselective CH oxidation of readily available carboxylic acids [2].

The biochemical formation of 2-hydroxy acids through α-oxidation of fatty acids has been known for higher plants, e.g. pea leaves, since long time. It has been postulated [29,30] that the α-oxidation of fatty acids leads to an intermediary 2-hydroperoxy acid, which preferentially decarboxylates to the corresponding aldehyde (Scheme 6, path B), in competition with reduction to the enantiomerically enriched 2-hydroxy acid (Scheme 6, path A). Recent biochemical and molecular biological studies gave detailed insight into the structure of the α-oxidase [31,32].

Encouraged by the biochemical α-oxidation process, we had examined the possibility to synthesize optically active α-hydroxy carboxylic acids by enzymatic CH oxidation of carboxylic acids with α-oxidases of higher



Scheme 8. Tandem enzymatic transformation of racemic 2-hydroxy acids (**6**) with GOX and D-lactate dehydrogenase.



R = $CH_3(CH_2)_n$

e: n = 7 **l**: R = $CH_3(CH_2)_9O$

g: n = 4 *E*-**n**: $CH_3(CH_2)_4$

h: n = 2 *E*-**o**: $CH_3(CH_2)_7$ $(CH_2)_6$

	Enantiomeric excess (%)					
	e	**g**	**h**	**l**	*E*-**n**	*E*-**o**
(*R*)-**6**	94	> 98	> 98	65	70	80
(*S*)-**7**	77	85	73	43	62	> 98

Scheme 9. Lipase-catalyzed kinetic resolution of racemic 2-hydroxy acids **6**.

plants. Indeed, the α-oxidation of carboxylic acids (**5**) by the crude homogenate of young pea leaves or crude extract of germinating peas with molecular oxygen yielded the (*R*)-2-hydroxy acids (**6**) in enantiomerically pure form and the corresponding next lower homologous aldehyde (Scheme 6) [33,34]. By employing tin(II) chloride as an in-situ-reducing agent, the undesirable competitive decarboxylation of the intermediary 2-hydroperoxy acids to aldehydes (Scheme 6, path B) was circumvented, and the enantiopure 2-hydroxy acids were obtained in up to > 95% yield [35].

Detailed substrate-selectivity studies revealed that the α-oxidase of pea leaves accepts saturated carboxylic acids with 16–7 carbon atoms. In this homologous series **5a**–**g**, the enzyme activity decreases with chain length [36]. Also the unsaturated fatty acids **5j**, **k**, as well as the oxygen (**5l**)- and sulfur (**5m**)-containing saturated acids, are excellent substrates for the enzyme, since they were converted enantioselectively to the corresponding (*R*)-configured (*ee* > 99%) 2-hydroxy acids in high yields [34]. Thus, a biocatalytic α-hydroxylation of carboxylic acids has been made available for the direct synthesis of optically pure (*R*)-2-hydroxy carboxylic acids.

2.2.2. Glycolate oxidase

The GOX is a peroxisomal enzyme, which is found in the leaves of many green plants, e.g. spinach, and catalyzes the oxidation of glycolic acid (Scheme 7, structure **6**, R = H) to glyoxylic acid by molecular oxygen. We have asked the question, whether this readily available enzyme may oxidize enantioselectively racemic 2-hydroxy acids **6** with molecular oxygen to provide optically active oxidation products. Indeed, the enzymatic kinetic resolution of racemic 2-hydroxy acids **6** by enantioselective oxidation with the GOX from spinach afforded the optically active hydroxy acids in up to 99% ee (Scheme 7) [35]. GOX oxidized not only medium-chain (C_{10} and C_7) 2-hydroxy carboxylic acids **6e** and **g**, but also short-chain derivatives **6h** (C_5) and **6i** (C_4). Thus, this novel biotransformation made available enantiopure (*R*)-2-hydroxy acids **6h**, **i**, which are not accessible by CH oxidation of the respective carboxylic acids with α-oxidase and nicely complements the latter process. GOX also accepts *Z*- and *E*-configured unsaturated 2-hydroxy acids *Z*-**6n**, *E*-**6n** and *E*-**6o**, to afford the corresponding enantiomerically pure (*R*)-2-hydroxy acids [35].

An inherent disadvantage of kinetic resolution is that a maximum yield of 50% of one enantiomer may be achieved. Therefore, we have coupled the GOX-catalyzed oxidation of 2-hydroxy acids with the asymmetric reduction of 2-oxo acids by D-lactate dehydrogenase (Scheme 8). Indeed, this tandem enzymatic transformation afforded the optically pure (*R*)-2-hydroxy acids in up to 89% yield, based on the racemate [36].

2.3. Lipase-catalyzed kinetic resolution

Lipases have been extensively used as convenient and efficient biocatalysts for the asymmetric synthesis of a wide range of organic compounds [37]; thus, these biocatalysts are called the 'work horse' in synthetic chemistry. The greatest advantage of these enzymes are that they are readily available, easy to handle, and require no expensive cofactors nor any elaborate experimental setup.

In the past years, we have used lipases for the biocatalytic synthesis of numerous optically active oxyfunctionalized substrates. A few examples are presented below:

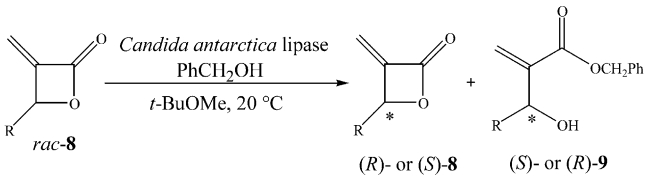
For comparison with other biocatalytic methods employed by us (see Sections 2.2.1 and 2.2.2), we have investigated the lipase-catalyzed kinetic resolution of racemic 2-hydroxy acids **6** to prepare optically active substrates. The enantioselective acetylation of 2-hydroxy acids **6** by lipases from microbes, in particular *Candida antarctica*, afforded the (*R*)-2-hydroxy acids and the corresponding (*S*)-acetoxy acids **7** in up to > 98% ee (Scheme 9) [38]. The medium- and short-chain (C_{10} to C_4) saturated hydroxy acids **6e**, **g**, **h** were resolved by lipase in high ee values. However, the 2-hydroxy acids with an oxygen atom (**6l**) or a double bond (*E*-**6n**) in close proximity to the hydroxy group decreased significantly the enantioselectivity of lipase. In contrast, a double bond remote from the stereogenic center does not diminish the selectivity, as demonstrated for the substrate *trans*-2-hydroxyoctadec-9-enoic acid (*E*-**6o**) [38].

We have also successfully applied the lipase-catalyzed enantioselective acetylation for the kinetic resolution of 2-hydroxy ketones [39], hydroxy vinylsilanes [40], homoallylic alcohols [41], and α,β-unsaturated vicinal diols [42,43]. The structures of these racemic alcohols are

shown in Fig. 3, the details are given in the pertinent references.

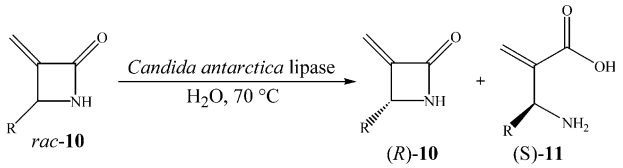
Furthermore, the lipase-catalyzed enantioselective transesterification of α-methylene β-lactones **8** with benzyl alcohol in organic solvent afforded the optically active α-methylene β-lactones and the respective β-hydroxy esters **9** in excellent ee values (Scheme 10) [44].

Also, the nitrogen analogs of the α-methylene β-lactones, namely α-methylene β-lactams **10** and their corresponding β-amino acids **11**, where made available by lipase-catalyzed kinetic resolution in high enantiomeric purity (Scheme 11) [45].



	R	*ee* value (%) 8	*ee* value (%) 9	Enantioselectivity (E)
a	Me	99 (*R*)	95 (*S*)	> 200
b	*i*Pr	99 (*S*)	99 (*R*)	> 200

Scheme 10. Lipase-catalyzed kinetic resolution of α-methylene β-lactones (**8**).



R	*ee* value (%) (*R*)-**10**	*ee* value (%) (*S*)- **11**	Enantioselectivity (E)
Me	96	98	> 200
Et	99	98	> 200

Scheme 11. Lipase-catalyzed kinetic resolution of β-lactams (**10**).

3. Biocatalytic transformations with soil microorganisms

Despite the success with isolated enzymes as catalysts for numerous enantioselective transformations, large quantities of pure enzymes required for preparative applications are not always readily accessible. This disadvantage of pure enzyme may be contrasted with whole-cell systems (bacteria, fungi), which are in principle available in sufficient quantities at preparative scale through self-replication. Indeed, as shall become evident in this section, we have developed biocatalytic methods by using soil bacteria for the preparation of optically active hydroperoxides through kinetic resolution, and for the asymmetric CH oxidation of unfunctionalized alkanes.

3.1. Kinetic resolution of hydroperoxides

As mentioned in Sections 2.1.1 and 2.1.2, optically active hydroperoxides may be prepared in high enantiomeric excess by kinetic resolution of racemic substrates with horseradish and CiP. Nevertheless, the large quantities of pure enzyme necessary for preparative applications, are not readily accessible and certainly expensive. These disadvantages may be circumvented by employing whole-cell systems (bacteria, fungi), which may be made available in the desired quantities through replication.

Since prior to our work, no microbial kinetic resolution of racemic hydroperoxides had been reported, a screening procedure was developed to select suitable soil bacteria for this purpose, with hydrogen peroxide as screening reagent [46,47]. The hydrogen-peroxide-mediated selective screening resulted in three reactive bacterial species, of which the *Bacillus subtilis* strain (Table 4, first entry) gave the best results in regard to the kinetic resolution of the model substrate (1-phenyl)ethyl hydroperoxide (**1a**). For comparison, commercially available fungal species were also tested (last three entries) [46]. Biologically significant, the fungal systems and also HRP (see Section 2.1.1), displayed the opposite enantioselectivity in the kinetic resolution of the hydroperoxide *rac*-**1a** compared with that of the bacterial systems.

By employing the most effective bacterial strain *B. subtilis*, a variety of secondary alkyl aryl hydroperoxides and the tertiary 1-methyl(1-phenyl)propyl hydroperoxide have been resolved in moderate to good enantioselectivities [47]. Furthermore, it was demonstrated that *B. subtilis* cells immobilized in alginate gel may be reused for several cycles without loss of activity, and they displayed even higher peroxidase activity than free cells [47]. These unexpected advantages of the present bacterial biotransformation offer attractive opportunities for further applications.

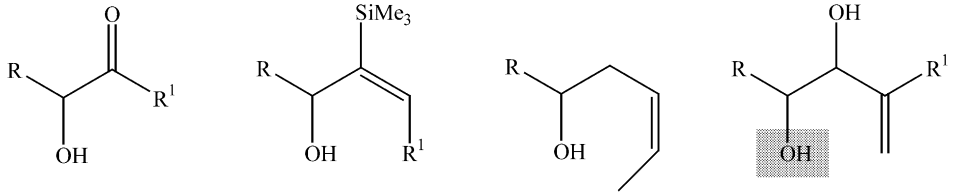
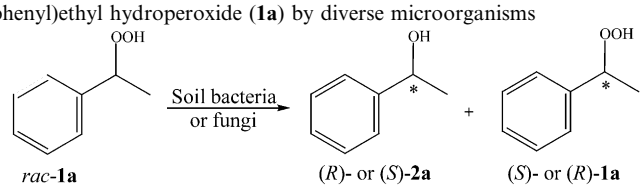


Fig. 3. Structures of racemic alcohols, which were resolved by lipase-catalyzed acetylation.

Table 4
Biotransformation of (1-phenyl)ethyl hydroperoxide (**1a**) by diverse microorganisms



Microorganism	Time Precultivation[a] (d)	Time Incubation[b] (min)	Convn[c] (%)	Enantiomeric excess (%)[d] **2a**	**1a**
B. subtilis[e]	1	30	94	20 (*S*)	> 99 (*R*)
Paecilomyces sp[e].	11	80	92	8 (*S*)	79 (*R*)
Bacillus sp.[e]	3	240	19	-[f]	6 (*R*)
A. niger[g]	8	30	67	25 (*R*)	37 (*S*)
Botrytis cinerea[g]	8	15	57	7 (*R*)	24 (*S*)
Penicillium v.[g]	8	60	23	23 (*R*)	9 (*S*)

[a] Time for which the culture has been pregrown before adding substrate.
[b] Actual time for the incubation of the substrate with the grown culture.
[c] Conversion determined by HPLC analysis.
[d] Determined by multi-dimensional gas chromatography or HPLC analysis.
[e] Bacteria isolated by H_2O_2-mediated selective screening procedure.
[f] Racemic.
[g] Commercially available fungi.

3.2. Asymmetric CH oxidation

Asymmetric CH oxidation provides a powerful method to obtain potentially useful oxyfunctionalized building blocks from readily accessible hydrocarbons. Therefore, much effort has been expended to develop efficient catalytic hydroxylations of hydrocarbons. It should be emphasized that prior to our studies, no biocatalytic process was available for the asymmetric hydroxylation of unfunctionalized hydrocarbons with broad substrate scope. To develop a whole-cell biocatalytic method for the asymmetric CH oxidation, we have selected suitable soil bacteria by employing an adequate screening procedure with allylbenzene (**12e**) as selecting substrate [48]. A *Bacillus megaterium* strain was isolated from topsoil by this screening procedure, which performed chemoselectively (no phenyl hydroxylation nor epoxidation) and enantioselectively the α-hydroxylation of the selecting substrate allylbenzene (Table 5, first entry). As shown in Table 5, the soil bacteria *B. megaterium* hydroxylates a variety of unfunctionalized arylalkanes **12f**–**i** in good to high enantiomeric excess [48].

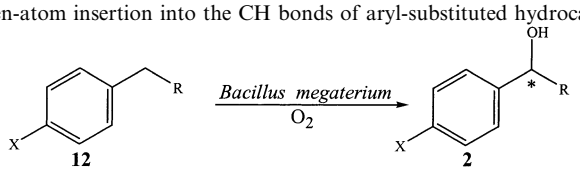
Encouraged by these results, we have explored the biocatalytic CH oxidation of simple hydrocarbons by *B. megaterium*. This soil bacterium hydroxylated the *n*-alkanes **12a**–**d** to afford optically active alcohols in up to > 99% enantiomeric excess (Table 6) [49]. The results in Tables 5 and 6 demonstrate convincingly that the *B. megaterium* strain isolated from topsoil is capable of the chemoselective asymmetric hydroxylation of a broad spectrum of hydrocarbons without further oxidation of the alcoholic products.

Our subsequent work has revealed that the immobilization of *B. megaterium* cells in alginate gel effectively increased the amount of products in such biocatalytic hydroxylations of arylalkanes and prolonged the viability of the cells. Thus, the immobilized cells may be used for up to five catalytic cycles without any significant loss of enzymatic activity [50]. The improved productivity, as well as the easier handling, make immobilized cells the better choice for such biotransformation.

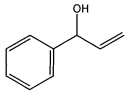
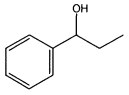
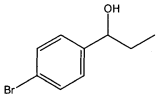
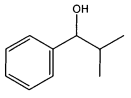
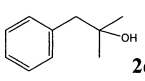
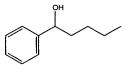
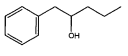
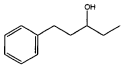
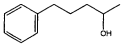
3.3. Enantioselective sulfoxidation

Although several chemical and enzymatic methods have been reported [1,28] for the asymmetric sulfoxidation, little is known on the biocatalytic sulfoxidation by microorganisms [51]. Motivated by our experience in biocatalysis with whole-cell systems, we have recently expended efforts to develop a biocatalytic method for the asymmetric sulfoxidation by microorganisms. For this purpose, two catalytically active strains have been isolated from topsoil by a newly developed screening procedure with phenyl methyl sulfide (**3a**) as selecting substrate. Our preliminary experiments reveal that both strains oxidize the sulfide **3a** to the corresponding sulfoxide **4a** in high ee values, but with opposite enantioselectivity (Scheme 12). Thus, the strain I afforded the (*S*)-configured sulfoxide **4a** in 92% *ee*, while the strain II produced (*R*)-**4a** in 69% ee [52]. Currently, we are investigating in detail the substrate selectivity of these microorganisms and their characterization by DNA sequencing of the specific genes.

Table 5
Microbial asymmetric oxygen-atom insertion into the CH bonds of aryl-substituted hydrocarbons **12e–i** with *Bacillus megaterium*



12	R	X
e	$CH=CH_2$	H
f	C_2H_5	H
g	C_2H_5	Br
h	$CH(CH_3)_2$	H
i	$(CH_2)_3CH_3$	H

Substrate	Convn[a] (%)	Product	Selectivity (%) Regio	Enantio[b]
12e	49	 **2o**	96	70 (*R*)
12f	63	 **2b**	100	74 (*R*)
12g	95	 **2p**	100	68 (*R*)
12h	43	 **2qα**	31	91 (*R*)
		 **2qβ**	69	
12i	89	 **2rα**	29 (4)	42 (*S*)
		 **2rβ**	33 (3)	88 (*R*)
		 **2rγ**	9 (3)	86 (*R*)
		 **2rδ**	14 (5)	> 99 (*S*)

[a] Conversion of hydrocarbons determined by GC analysis.
[b] Enantiomeric excess was determined by multi-dimensional gas chromatography.

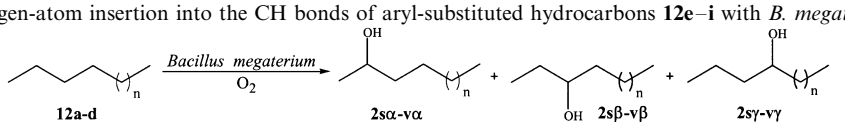
4. Conclusion

The results highlighted here demonstrate that peroxidases, oxidases, and lipases, as well as soil bacteria isolated by adequate selection procedures, are efficient biocatalysts for the preparation of optically active, oxyfunctionalized compounds from racemic or prochiral substrates. A broad spectrum of unfunctionalized and differently functionalized enantiopure hydroperoxides have been made available by kinetic resolution of racemic substrates with isolated peroxidases or microorganisms. These hydroperoxides are to date difficult to acquire enantiomerically pure by nonenzymatic means. Among the biocatalysts employed for the kinetic resolution of hydroperoxides, HRP is superior in terms of enantioselectivity and substrate scope. Nevertheless, the kinetic resolution of sterically encumbered tertiary hydroperoxides remains to be a challenge.

Furthermore, optically active 2-hydroxy acids are accessible by kinetic resolution with GOX and lipases. Although, other enzymatic methods are known for the synthesis of optically active α-oxyfunctionalized carbonyl compounds, the enantioselective, catalytic hydroxylation of prochiral carboxylic acids by plant α-oxidases is a promising method. Since molecular oxygen is the oxygen source, this oxidation constitutes an effective direct oxyfunctionalization without recourse to interconversion of functional groups in the substrate.

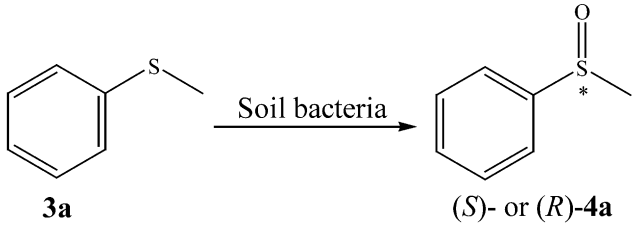
The unprecedented catalytic asymmetric hydroxylation of unactivated hydrocarbons by the soil bacterium *B. megaterium* may encourage further work with this microorganism. Isolation and overexpression of the

Table 6
Microbial asymmetric oxygen-atom insertion into the CH bonds of aryl-substituted hydrocarbons **12e–i** with *B. megaterium*



Alkane	Alcohol	Selectivity (%)	
		Regio	Enantio[a]
12a (n= 1)	**2sα**	19 (11)	93
	2sβ	57 (13)	54
12b (n= 2)	**2tα**	34 (17)	> 99 (*S*)
	2tβ	21 (13)	> 99 (*S*)
	2tγ	15	
12c (n= 3)	**2uα**	53 (19)	> 99 (*S*)
	2uβ	17 (5)	> 99 (*S*)
	2uγ	6	6
12d (n= 4)	**2vα**	34 (27)	> 99
	2vβ	23 (9)	98
	2vγ	7	-[b]

[a] Enantiomeric excess was determined by multi-dimensional gas chromatography on a cyclodextrin column; absolute configurations, when given, were determined by comparison with authentic reference samples or with literature data.
[b] The enantiomeric excess of the minor product was not determined.



Bacterium	*ee* value (%)
Strain I	92 (*S*)
Strain II	69 (*R*)

Scheme 12. Enantioselective sulfoxidation of phenyl methyl sulfide (**3a**) by isolated soil bacteria.

pertinent monooxygenase enzyme in *B. megaterium* strain should significantly improve the biocatalytic efficiency and facilitate preparative applications of this biotransformation for the asymmetric CH oxidation of simple *n*-alkanes.

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