Tetrahedron: Asymmetry Vol. 3, No. 8, pp. 1063-1068, 1992
Printed in Great Britain

0957-4166/92 $5.00+.00
Pergamon Press Ltd

Effects of Substrate Structure on the Enantioselectivity and Stereochemical Course of Sulfoxidation Catalyzed by Cyclohexanone Monooxygenase

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(*Received* 30 *June* 1992)

ABSTRACT. A systematic study of the stereochemistry of oxidation at sulfur catalyzed by cyclohexanone monooxygenase from *Acinetobacter* using as the substrates numerous alkyl aryl sulfides, dialkyl sulfides and dialkyl disulfides has been carried out. It was found that the structure of the sulfide dramatically influenced the enantioselectivity of the enzyme which yielded sulfoxides with optical purities ranging from 99% ee and R-configuration to 93% ee and S-configuration.

INTRODUCTION

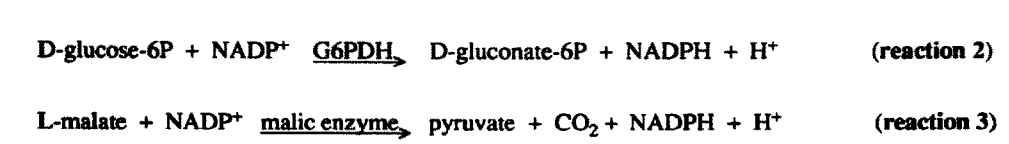
Enantiomerically pure sulfoxides are important chiral synthons for the synthesis of natural products and rank among the most powerful stereodirecting groups.[1] For these reasons both chemical[2] and biological[3] approaches have been tested to stereospecifically oxidize organic sulfides to the correspondent sulfoxides.

Regarding the biological oxidations, *Aspergillus niger*,[3a] *Mortierella isabellina*,[3b,c] *Corynebacterium equi*,[3d] *Helmintosporium sp*,[3e] *Fusarium oxysporum*[3e] and, more recently, chloroperoxidase[3f,g] and horseradish peroxidase[3h] have been employed with numerous alkyl aryl sulfides, vinyl sulfides and allyl aryl sulfides obtaining, in some cases, products of high optical purity. Walsh and coworkers[3i] have described the synthesis of both enantiomers of ethyl *p*-tolyl sulfoxide by the use of purified monooxygenase from hog liver microsomes (R-sulfoxide; 90% ee) or cyclohexanone monooxigenase from *Acinetobacter* (S-sulfoxide; 64% ee). However, the investigation was not extended to other sulfides.

In the present paper, we report on a systematic study of the stereochemistry of oxidation at sulfur by cyclohexanone monooxygenase from *Acinetobacter*, using numerous alkyl aryl sulfides (**A**), dialkyl sulfides (**B**), and dialkyl disulfides (**C**) as the substrates. We found that the structure of the sulfide dramatically influenced not only the enantioselectivity but even the enantiopreference of the enzyme which yielded sulfoxides ranging from 99% ee and R-configuration (methyl phenyl sulfoxide) to 93% ee and S-configuration (ethyl *p*-fluorophenyl sulfoxide).

RESULTS AND DISCUSSION

The oxidation of a large number of sulfides by cyclohexanone monooxygenase (**reaction 1**) was coupled to a second enzymatic reaction in order to regenerate NADPH. As NADPH regenerating system it was used either glucose 6-phosphate and glucose 6-phosphate dehydrogenase (G6PDH) (**reaction 2**), as already reported by Light et al.[3i] or malate and malic enzyme (**reaction 3**).[4]

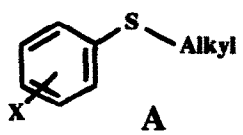


The degrees of conversions of sulfides into sulfoxides, after overnight reaction, are reported in **Table I**.The two NADPH regenerating systems had similar effectiveness; however the malate/malic enzyme system is preferable for preparative-scale synthesis because L-malate is much cheaper than glucose-6-phosphate.

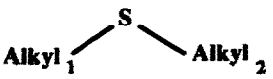
Besides the substrates listed in **Table I**, some sulfides with bulky alkyl chains such as *t*-butyl phenyl sulfide, octyl phenyl sulfide and decyl *p*-tolyl sulfide were tested, but no appreciable oxidation was observed. Also phenyl disulfide, *p*-tolyl disulfide and 2-phenyl-1,3-dithiane were not oxidized,possibly because of the insolubility of these solid compounds in the aqueous reaction medium.

The initial oxidation rates of alkyl aryl sulfides by cyclohexanone monooxygenase were determined spectrophotometrically and the results are shown in **Table II**. The increase in size of the alkyl chain increased enzymatic activity especially when passing from the ethyl to the *iso*-propyl group (entries **1, 8, 11** and **12, 16, 15**). The benzyl was activating compared to the phenyl group (entries **6, 1**) and activation was also induced by the introduction in the aromatic ring of methyl and fluoride and the rates were in the order *para> meta> ortho* (entries **12, 9, 3**).

Regarding the stereoselectivity of enzymatic sulfoxidation, the data in **Table I** indicate that it was highly dependent on substrate structure. Thus, in the case of alkyl aryl sulfides (**A**), the optical purity of the products ranged from 99% ee and R-configuration with methyl phenyl sulfoxide (entry **1**) to 93% ee and S-configuration with ethyl *p*-flurophenyl sulfoxide (entry **17**). The increase in size of the alkyl chain increased the contribution of the S-orienting effect (entries **1, 8, 11**). The same effect was obtained by introducing substituents in the phenyl ring, and this trend was generally more pronounced in the case of bulkier groups (see however the anomalous behaviour of *p*-C_2H_5O-C_6H_4-S-CH_3, entry **5**). The position of substitution in the aromatic ring also played an important role, with an increasing S-orienting influence on passing from *ortho* to *meta* and *para* position (see for instance entries **3, 9, 12**). The above described effects were additive and, therefore, the S-sulfoxides with higher optical purities were those possessing *p*-substituents in the aromatic ring and ethyl or propyl as the alkyl chain (entries **15, 16, 17**). As for ethyl *p*-tolyl sulfide (entry **16**) we found a selectivity remarkably higher than that reported by Light et al.[3i](89% ee instead of 64% ee).



A



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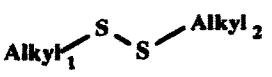


C

Table I. Cyclohexanone monooxygenase catalyzed oxidation of sulfides to sulfoxides

entry	sulfide	yield%	ee%	sulfoxide configuration[a]
1	C_6H_5-S-CH_3	88	99	R
2	*p*-F-C_6H_4-S-CH_3	91	92	R
3	*o*-CH_3-C_6H_4-S-CH_3	90	87	R
4	2-pyridil-S-CH_3	86	87	R
5	*p*-C_2H_5O-C_6H_4-S-CH_3	92	59	R
6	C_6H_5-CH_2-S-CH_3	97	54	R
7	*o*-CH_3O-C_6H_4-S-CH_3	81	51	R
8	C_6H_5-S-C_2H_5	86	47	R
9	*m*-CH_3-C_6H_4-S-CH_3	90	40	R
10	o-Cl-C_6H_4-S-CH_3	35	32	R
11	C_6H_5-S-isopropyl	93	3	S
12	p-CH_3-C_6H_4-S-CH_3	94	37	S
13	p-Cl-C_6H_4-S-CH_3	78	51	S
14	p-CH_3O-C_6H_4-S-CH_3	89	51	S
15	p-CH_3-C_6H_4-S-isopropyl	99	86	S
16	p-CH_3-C_6H_4-S-C_2H_5	89	89	S
17	p-F-C_6H_4-S-C_2H_5	96	93	S
18	t-butyl-S-CH_3	98	99	R
19	butyl-S-S-butyl	85	32	ND[c]
20	methyl-S-S-propyl	92	62;34[b]	ND

[a]The absolute configuration of the sulfoxides was determined by comparison with authentic samples using chiral HPLC or GLC.

[b] For the two regioisomeric thiosulfinates. [c]ND, not determined.

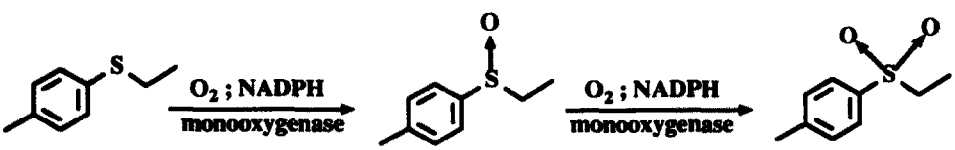
The enzyme showed a very high enantioselectivity (99% ee) in the case of the dialkyl sulfide *t*-butyl methyl sulfide (entry **18**), whereas poor selectivity was found in the case of the two disulfides investigated (entries **19, 20**). It should be noted that with methyl propyl disulfide the two regioisomeric thiosulfinates were formed in similar quantities.

Table II. Initial oxidation rates of alkyl aryl sulfides[a]

entry	sulfides	rel. rate[b]
1	C_6H_5-S-CH_3	14
8	C_6H_5-S-C_2H_5	15
11	C_6H_5-S-*iso*propyl	62
6	C_6H_5-CH_2-S-CH_3	34
12	*p*-CH_3-C_6H_4-S-CH_3	26
16	*p*-CH_3-C_6H_4-S-C_2H_5	34
15	*p*-CH_3-C_6H_4-S-*iso*propyl	100
9	*m*-CH_3-C_6H_4-S-CH_3	22
3	*o*-CH_3-C_6H_4-S-CH_3	12
2	*p*-F-C_6H_4-S-CH_3	44
17	*p*-F-C_6H_4-S-C_2H_5	46
13	*p*-Cl-C_6H_4-S-CH_3	14
10	*o*-Cl-C_6H_4-S-CH_3	5

[a] The initial rates were determined by monitoring NADPH consumption at 340 nm in 0.05 M Tris-HCl buffer, pH 8.6, containing 0.6 mM sulfide and 0.12 mM NADPH. [b] Rate relative to that of entry 15 taken as 100.

The oxidations of methyl phenyl sulfide and ethyl *p*-tolyl sulfide were monitored over a 24 h period. The optical purity of sulfoxide products did not significantly change with the progress of the reaction. In the case of ethyl *p*-tolyl sulfide, as already reported by Light et al.,[3i] the formation of a small amount (about 5% of total products) of the correspondent sulfone was observed (**Scheme**).



When racemic methyl phenyl sulfoxide and ethyl *p*-tolyl sulfoxide were used as the substrates, a very slow oxidation of the S-enantiomer in the first case, and of the R-enantiomer in the second case, was evidenced. The enantioselectivity of cyclohexanone monooxygenase towards racemic sulfoxides, however, cannot be exploited for resolution purposes because of the extremely low activity of the enzyme with these

substrates.

The mechanism proposed by Walsh and Chen[31] for the sulfoxidation catalyzed by cyclohexanone monooxygenase suggests the formation of a substrate-4a-hydroperoxy flavoenzyme complex and an electrophilic oxygen transfer from the activated hydroperoxide equivalent to the nucleophilic substrate.

Regarding the possibility of scaling up the enzymatic synthesis of chiral sulfoxides, there are some encouraging results. For instance, when using malate and malic enzyme as the coenzyme regenerating system, the same conversions were obtained with either native NADP or NADP covalently linked to polyethylen glycol.[4] This should make it possible to carry out the reactions in continuos-flow membrane reactors with retention of both the enzymes and the coenzymes, which would decrease the economical incidence of these factors.[5] One drawback in the enzymatic synthesis is the relative instability of cyclohexanone monooxygenase under our working conditions (half-life 25 h). However, no systematic study aimed at improving the stability of this enzyme has been carried out so far, so it is likely that conditions more suitable for preservation of enzyme activity could be found.

EXPERIMENTAL SECTION

Materials. Sulfides were bought from Aldrich or synthesized as previously reported.[3g] $NADP^+$, NADPH, glucose-6-phosphate, L-malate, glucose-6-phosphate dehydrogenase (type XXIV) and malic enzyme were obtained from Sigma. All other chemicals were reagent grade.

Cyclohexanone monooxygenase. *Acinetobacter* NCIB 9871 (from NCIMB) was grown (20-l colture) as described by Donoghue et al.[6] The cells were disrupted by ultrasonication, and cell debris were removed by centrifugation.[6] The supernatant was subjected to fractionation with $(NH_4)_2SO_4$ and the fraction precipitated between 50 and 80% saturation retained[6]. It was redissolved in 0.02 M potassium phosphate buffer, pH 7, dialyzed against the same buffer and lyophilized. The enzymatic activity was assayed by monitoring NADPH consumption at 340 nm using as the assay buffer 0.05 M Tris-HCl, pH 8.6, containing 0.6 mM methyl phenyl sulfide and 0.12 mM NADPH. The total activity obtained from a 20-l colture was of 260 units.

Enzymatic oxidation: typical procedure. The sulfide (0.8 mmol) was magnetically stirred in 20 ml of 0.05 M Tris-HCl buffer, pH 8.6, containing 3 μmol NADP, 2mmol glucose-6-phosphate, 6 units of cyclohexanone monooxygenase and 50 units of glucose-6-phosphate dehydrogenase. Alternatively, as the NADPH regenerating system it was used 2 mmol L-malate and 12 units of malic enzyme. At a scheduled time, the reaction solution was extracted with 3 portions (20 ml each) of ethyl acetate. The organic extract was dried and evaporated and the sulfoxide purified by flash chromatografy (SiO_2) with mixtures of diethyl ether and methanol as eluents.

Determination of degree of conversion and enantiomeric excess. The degrees of conversion of

sulfides into sulfoxides and sulfones were determined on the ethyl acetate extracts by GLC with a 25 m HP-1 capillary column coated with methylsilicone gum (Hewlett Packard) with H_2 as carrier gas. The enantiomeric excesses of sulfoxides were generally determined by chiral HPLC on a chiralcel OB column (Daicel) using the proper mixture of *n*-hexane-propan-2-ol as the mobile phase. All sulfoxides enantiomers were base-line separated. In the case of *t*-butyl methyl sulfoxide the enantiomeric excesses were determined by chiral GC with a CP-Cyclodextrin-β-2,3,6-M19 column (50 m, 0.25 mm ID, Chrompack) at 75°C and H_2 as carrier gas.

Characterization of the sulfoxides. Sulfoxides were all known in the optically pure form and the physical properties of our specimens were in agreement with those reported.[3c,7]

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