Microalga *Chlorella sorokiniana*: a new sulfoxidation biocatalyst

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Received 28th November 2005, Accepted 10th February 2006
First published as an Advance Article on the web 10th March 2006
DOI: 10.1039/b516813a

Whole cells of the microalga *Chlorella sorokiniana* were evaluated in the oxidation of prochiral thioethers as regards conversion, enantiomeric excess and enantioselectivity.

Biocatalysis is now a well established approach to obtain valuable compounds which are difficult to synthesise by conventional chemistry. Indeed biocatalysts can modify compounds with inherent high regio- and stereoselectivities. Moreover the use of whole cells prevents problems of expensive cofactor recovery in comparison with isolated enzymes.

Bacteria and fungi are commonly used microorganisms because of their rapid growth, ease of handling and well established DNA technology. Many people have also studied biotransformations with plant cultured suspension cells that possess the ability to transform exogeneous substrates.[1,2] However the longer doubling times of undifferentiated plant cells and the lower production of the desired enzymes in comparison with microbial cells are major drawbacks.[2] On the other hand microalgae, phototrophic unicellular microorganisms, are the fastest growing plants on earth and thus constitute an interesting option in comparison with plant cells. Surprisingly little attention has been focused on microalgae until recently. Despite the fact that the estimated 30 000 species possess extraordinary biochemical diversity, they remain largely unexploited. The only activities studied up to date are the reduction of aldehydes[3] and ketones,[4] the reduction and decarboxylation of β-keto esters,[5] N- and O-dealkylation,[6] and the hydroxylation and biotransformation of terpenoids (progesterone).[7] Up to now lack of appropriate technologies for culture[8] and molecular engineering has limited their use in biotransformations. However recent progress in methods for culturing these microorganisms (ultrahigh-density cultures,[9] photobioreactor engineering,[10] use of heterotrophic conditions[11] or even trophic conversion[12] for growth) and in molecular biology[13] (development of new markers, promoters and reporters) should change this fact.

In our efforts to elucidate the mechanism of desaturases[14–16] in the microalga *Chlorella sorokiniana* 211-8k (*Chlorella* species have been used for many years as a higher plant model in the study of lipid metabolism and desaturation), we have demonstrated the ability of whole cells to enantioselectively oxidise thiostearates after intracellular conversion to mono-unsaturated analogues.[14] However we noticed a non-desaturase oxidation of these substrates under particular conditions.[16] In the present paper, we report on a systematic study of the stereochemistry of thioether oxidation by autoheterotrophically grown whole cells (Scheme 1).

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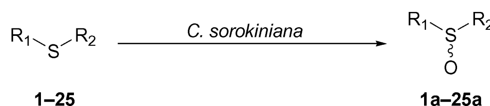

Scheme 1

Sulfoxides were chosen as targets because of their great interest not only as valuable asymmetric starting materials and chiral auxiliaries[17] in synthetic chemistry, but also in biochemistry since sulfoxides occur as natural products (flavour and aroma precursors, antibiotics), as enzyme inhibitors, pharmaceuticals or metabolites.[18,19]

Results and discussion

The thioethers used in this study consisted in alkylarylsulfides (phenyl, benzyl and phenethyl series) and a few dialkylsulfides. *Chlorella sorokiniana* CCAP 211-8k (also known as *Chlorella vulgaris*) is a green unicellular, non motile, photosynthetic microalga (*Chlorophyta*).

The cells were harvested in the middle of the log phase and resuspended in phosphate buffer (pH = 7.4). The auto-oxidation of the substrates was checked with blanks using the same experimental conditions as for the biotransformation assays but without cells; this one was negligible (≤2%). The absence of sulfone formation, which could lead to the kinetic resolution of sulfoxides, *i.e.* to an enantiomeric enrichment, was assessed by TLC referring to sulfone standards. Finally the effect of dead cells on the thioethers was evaluated in the same conditions after heating the cells at 90 °C during 10 min before adding the substrate. Consequently, in this latter case an amount of racemic sulfoxide was formed (from 5% to 78% according to the substrate).

The configuration of the sulfoxides was attributed by HPLC with respect to the order of elution on a Chiralcel OB column (Daicel®). Sulfoxides **1a**, **2a**, **3a**, **4a**, **7a**, **9a**, **10a**, **11a**, **12a**, **14a**, **15a**, **16a**, **17a**, and **20a** have been studied on Chiralcel OB previously.[20] Sulfoxides **6a**, **18a**, **22a**, **23a** and **24a** were synthesised in enantiomerically pure form according to the method developed by Alcudia *et al.* and used as standards for chiral HPLC.[21] Compounds **5a**, **8a**, **19a** and **25a** have not been synthesised in enantiomerically pure form because of the very low enantiomeric excess obtained during this study. Compounds **13a** and **21a** were not synthesised in enantiomerically pure form. Compound **13a** was supposed to elute as all *para*-substituted S-oxide thioanisoles analysed so far on Chiralcel OB. Sulfoxide **21a** could not be obtained in enantiomerically pure form by the method of Alcudia *et al.*; the order of elution was deduced from that of methyl phenethyl sulfide and is subject to discussion.[20]

Table 1 shows the results obtained in the oxidation of sulfides **1–25** in terms of conversion, enantiomeric excess and predominant

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Table 1 Bioconversion of thioethers

	Substrate	Conversion[a] (%)	ee (%)	Configuration
1	Methyl phenyl sulfide	10	58	*R*
2	Ethyl phenyl sulfide	10	44	*S* [d]
3	Propyl phenyl sulfide	15	21	*R*
4	Butyl phenyl sulfide	7	22	*R*
5	Pentyl phenyl sulfide	7	2	—[c]
6	Octyl phenyl sulfide	1	10	*R*
7	Isopropyl phenyl sulfide[b]	5	<1	—[c]
8	Isopentyl phenyl sulfide	5	<1	—[c]
9	Vinyl phenyl sulfide	1	10	*R*
10	*p*-Tolyl methyl sulfide	40	42	*R*
11	*p*-Methoxyphenyl methyl sulfide	49	39	*R*
12	*p*-Bromophenyl methyl sulfide	15	51	*R*
13	*p*-Cyanophenyl methyl sulfide	7	41	*R*[e]
14	*p*-Nitrophenyl methyl sulfide	1	19	*R*
15	Methyl benzyl sulfide	32	57	*S*
16	Butyl benzyl sulfide	17	13	*S*
17	Hexyl benzyl sulfide	26	5	*S*
18	Octyl benzyl sulfide	11	4	*S*
19	Isopropyl benzyl sulfide	10	1	—[c]
20	Isopentyl benzyl sulfide	10	1	—[c]
21	Ethyl phenethyl sulfide	67	22	*S*[e]
22	Heptyl phenethyl sulfide	19	5	*S*
23	Allyl butyl sulfide	3	10	*S*
24	Homoallyl butyl sulfide	17	5	—[f]
25	2-Pentenyl butyl sulfide	—[c]	—[c]	—[c]

[a] 24 hours of incubation. [b] Only one experiment. [c] Not significant. [d] Although sulfoxide **2a** has already been studied on this column,[20] it was synthesised in enantiomerically pure form in order to check the order of elution because of the change in the enzymatic enantioselectivity between compounds **1a** and **2a** on one side, and **2a** and **3a** on the other side. [e] Extrapolated from compounds with very close structures (see text). [f] No separation on Chiralcel OB.

absolute configuration of the products **1a**–**25a**. According to these data, the oxidation of the thioethers proceeded enantioselectively, demonstrating that an enzymatic reaction took place. It must be emphasised that each ee value reported (except for isopropyl phenyl sulfide) is a mean of 3 experiments for which cells were viable during the whole biotransformation, as checked by the green colour (microalgae) of the medium (death of cells leads to a brown medium). In each series the yield was maximal for the smallest alkyl group (R = Me for the phenyl (**1**) and benzyl (**15**) series, R = Et (**21**) for the phenethyl series). We noticed that the longer the alkyl chain was, the lower the conversion was, a phenomenon probably related to aqueous solubility and micelle formation[22] (a non quantified amount of substrate was extracted from the supernatant after 24 hours of incubation, as detected by HPLC). It is noteworthy that the conversion increased with the distance between the aromatic ring and the sulfur atom, *i.e.* from the phenyl to the phenethyl series (substrates **1**, **15** and **21**). The presence of an unsaturation next to the sulfur atom seemed to prevent oxidation since the substrates **9** and **23** showed very low or no conversion, whereas if the unsaturation lay farther away, oxidation took place normally (substrate **24**).

These data also showed the influence of the alkyl group on the enantioselectivity of the sulfoxidation. In the case of unbranched alkyl chains, the enantiomeric purity of the sulfoxides tended to decrease while the chain length increased. For branched alkyl chains (substrates **7**, **8**, **19** and **20**), the sulfoxides obtained were racemic with very low conversion ($\leq$10%). Moreover, we observed a reversed enantioselectivity between the phenyl series on one side ((*R*)-sulfoxides) and the benzyl and phenethyl series on the other side ((*S*)-sulfoxides). The change of stereoselectivity observed for ethylphenylsulfide **2** still remains unexplained at present. Biotransformed dialkylsulfides led to sulfoxides with low (**24a**) or moderate (**23a**) enantiomeric excess.

Different *para*-substituted thioanisoles were also incubated in order to evaluate the influence of electronic effects (substrates **10**–**14**). These effects are usually rationalised by considering the σ_p value of the substituents. This parameter, introduced by Hammett in his equation, represents the electronic effects of substituents, composed of a field/inductive component and a resonance component.[23] In our case compounds with negative values of σ_p (**10**–**11**) were oxidised with fourfold to fivefold higher yields (relative to thioanisole **1** for which $\sigma_p = 0$), whereas compounds with positive values of σ_p showed similar (**12**) or lower yields (**13**–**14**).[24] The very low conversion obtained for *p*-nitrothioanisole **14** is not so surprising. As reported by Noma *et al.*[3c] in the reduction of substituted aromatic aldehydes with *Dunaliella tertiolecta* (a halophilic unicellular green microalga), such compounds with strong electron withdrawing groups in the *para* position seem difficult to transform. Moreover, sulfoxidation studies carried out with different kinds of isolated enzymes (chloroperoxidase from *Caldariomyces fumago* (CPO), lactoperoxidase isolated from mammalian milk (LPO), vanadium peroxidase from *Ascophyllum nodosum* (VBrPO)) usually reveal a lower extent of biotransformation for compounds with *para* nitro substituents in comparison with compounds with other substituents (methoxy, halogens, methyl···).[25,26] This was also true for *p*-cyanothioanisole (CN is also a strong electron withdrawing group) with VBrPO[26] and LiP (lignin peroxidase from *Phanerochaete chrysosporium*).[27] Where the sulfoxidation using whole cells is concerned, a similar phenomenon was observed with the fungus *Mortierella isabellina*[28] (very important for *p*-nitrothioanisole and in a lesser extent for *p*-cyanothioanisole). However the results obtained with another

fungus, *Helminthosporium* sp., did not reveal any significantly different conversion yields between these substrates and the others.[18] All the *para* substituted sulfoxides obtained with whole cells of *C. sorokiniana* (compounds **10a–13a**), with the exception of *p*-nitrophenyl methyl sulfoxide **14a**, showed nearly the same ee (between 39% and 51%).

All these experiments were carried out with intact whole cells of *C. sorokiniana*. Microalgae are supposed to possess several classes of oxidative enzymes (cytochrome P450s or peroxidases for example) which can lead to enantiodivergent sulfoxidations as well as to extensive degradation of the metabolites (unidentified polar compounds from the substrates **6**, **9**, **14**, **20**, **22** and **23** were detected by HPLC, which likely resulted from hydroxylation or epoxidation of the aromatic ring). Moreover moderate yields of sulfoxidation may reflect other problems such as the transport through the membrane or the distribution into the cell. Finally, if uncatalysed oxidation occurred, it could not be responsible for results such as 58% ee since we observed that blanks (medium alone or dead cells) led to racemic sulfoxide.

Sulfoxidation is usually performed by oxygenases (cytochrome P450s, flavin monooxygenases) or peroxidases. This strain of *C. sorokiniana* is assumed to contain cytochrome P450[6] and lipoxygenase activities.[29] Well-known inhibitors of cytochrome P450 such as 1-aminobenzotriazole (ABT) and piperonyl-butoxide (PBO) have been successfully used with whole cells of *C. sorokiniana* for inhibiting O- and N-dealkylations of coumarin, resorufin ethers and Metflurazon.[6] On the basis of this previous work, we realised a few experiments in order to elucidate the enzymatic activities responsible for sulfoxidation. These specific inhibitors added to whole cells of our strain in the same concentrations,[6] did not prevent sulfoxidation. Lipoxygenase inhibitors, *i.e.* nordihydroguaiaretic acid (NDGA) and phenidone, were also ineffective. Nevertheless the implication of lipoxygenase cannot be ruled out since our experiments were done with whole cells whereas Kulkarni and Naidu's experiments concerned the oxidation of thiobenzamide by enzymatic extracts.[30] These results might be explained by the lack of inhibitor availability to the cell. A final duplicate experiment carried out in the darkness induced a decrease of the relative sulfoxidation activity (about 18%) as well as a 20% increase of the sulfoxide enantiomeric excess. This result can be explained by the participation of an NADPH,H^+ dependent enzyme which is partially inhibited in the darkness due to a limited regeneration of the cytosolic NADPH,H^+ (the essential cosubstrate of cytochrome P450 and flavin monooxygenases).

Conclusion

The ability of the microalga *Chlorella sorokiniana* to enantioselectively oxidise prochiral thioethers was demonstrated. The structure of the sulfide deeply influenced the yield, the enantiomeric excess and the enantioselectivity of the oxidation. The enzymatic activity responsible for the sulfoxidation remains to be highlighted since it was not possible to conclude in this regard with inhibition studies. However, the participation of an NADPH,H^+ dependent enzyme different from cytochrome P450 is highly suspected. The inhibition of the lipoxygenase activity has never been studied in *C. sorokiniana* before and more experiments are needed for ruling out its implication in the sulfoxidation. The yields (up to 67%) and the enantioselectivities (up to 58%) remain quite modest in comparison with other microorganisms such as the fungi *Mortierella isabellina* or *Helminthosporium* sp. The low ees can be explained by the lack of enzymatic stereoselectivity, enantiodivergent oxidation by different enzymes, extensive degradation of metabolites or limited uncatalysed racemic oxidation. Nevertheless it is the first example of an enantioselective sulfoxidation of prochiral thioethers carried out by microalgae, microorganisms not often used in biotransformations. It has been demonstrated that the enantiomeric excess and even the stereoselectivity of the reduction of some keto esters by *Chlorella sorokiniana* could be influenced by growth conditions (heterotrophic or autotrophic) and additives in the medium.[31] Such an approach could be used for improving the yield and the enantiomeric excess of sulfoxides, as well as for extending the scope of the application of *Chlorella sorokiniana* as a new oxidative biocatalyst.

Acknowledgements

We gratefully acknowledge the Ministère de l'Education Nationale, de la Recherche et de la Technologie, for a grant to Franck Daligault. We thank Dr José Daniel Carballeira Rodriguez for helpful discussions and advice.

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