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Stereoselective oxidation of sulfides by cloned naphthalene dioxygenase

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Abstract

Washed-cell preparations of recombinant *Escherichia coli* JM109(pDTG141), engineered to express the naphthalene dioxygenase (NDO) gene from *Pseudomonas* sp. NCIB 9816-4, have been used to biooxidise a range of aryl alkyl-, dialkyl- and bicyclic sulfides. A series of 16 phenyl alkyl sulfides was oxidised to equivalent sulfoxides, typically with moderate to high (> 90%) yield and high enantioselectivity (> 85% ee), the (*S*)-enantiomer being the predominant product, with little if any further oxidation. The addition of some electron-donating or electron-withdrawing groups to the phenyl ring decreased yield and/or stereoselectivity of the NDO-catalysed biotransformation, whereas increasing the size of the alkyl chain (nC_3H_7, iC_3H_7 and nC_4H_9) resulted in a notable inversion in selectivity to yield (*R*)-series sulfoxides (> 74% ee) as the predominant products. The addition of one or more methylene groups between the phenyl ring and sulfur atom resulted in notable reductions in both the yield and stereoselectivity of the (*S*)-predominant sulfoxidations. With the exception of cyclohexyl- and *n*-hexyl methyl sulfide which both gave (*S*)-sulfoxides with good stereoselectivity and yield, other dialkyl- and bicyclic sulfides were poor substrates for sulfoxidation by NDO. Both the close agreement with data obtained using purified NDO and the absence of stereoselective sulfoxidation in equivalent controls with the *E. coli* JM109 host support the contribution made by the cloned NDO carried on the pDTG141 plasmid.

Keywords: Naphthalene dioxygenase; Sulfoxidation; Stereoselective oxidation; Aryl alkyl sulfides; *Pseudomonas* sp. NCIB 9816-4

1. Introduction

Bacterial dioxygenases (EC. 1.13.11.x) are multi-component NADPH-dependent enzymes consisting of an iron–sulfur flavoprotein, a ferredoxin and an iron–sulfur oxygenase component, that participate in the aerobic catabolism of various aryl hydrocarbons [1]. Interest in the synthetic utility of these enzymes stems from initial studies on the degradation of benzene and toluene by the toluene dioxygenase (TDO) present in F39/D, a mutant strain of *Pseudomonas putida* deficient in dihydrodiol dehydrogenase, which was shown to oxidise these two hydrocarbons to *cis*-1,2-dihydroxycyclohexa-3,5-diene [2] and *cis*-(1*S*,2*R*)-dihydroxy-3-methylcyclohexa-3,5-diene [3,4] respectively. Studies with equivalent blocked mutants [5,6], including naphthalene dioxygenase (NDO)-containing *Pseudomonas* sp. NCIB 9816/II [7,8], plus recombinant strains of *Escherichia coli* engi-

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neered to express the genes for various bacterial dioxygenases [8–11] have confirmed that a number of these enzymes have relaxed substrate specificities enabling them to yield chiral *cis*-diols with a variety of monocyclic, multicyclic and heterocyclic aromatic compounds. The versatility of bacterial dioxygenases has been further emphasised by the demonstration that in addition to *cis*-dihydroxylation, these enzymes are able to catalyse a wide range of other reaction types including monooxygenation [12–14], *N*- and *O*-dealkylation [15], desaturation [14–16] and sulfoxidation [17,18]. Of particular interest was the observation that with some substrates such as methyl phenyl- and ethyl phenyl sulfide, purified preparations of TDO and NDO yielded homochiral sulfoxides (> 98% ee) in enantiocomplementary series [18]. Because of the increased demand for chiral sulfoxides in synthetic organic chemistry [19], and our prior experience of oxygenase-dependent biotransformations as a means to access this group of chiral auxillaries [20–22], we have studied whether the relaxed substrate specificity of bacterial dioxygenases (vide supra) can be exploited to enhance the available pool of chiral sulfoxide synthons using *E. coli* JM109(pDTG141), a recombinant strain engineered to express the NDO genes from *Pseudomonas* sp. NCIB 9816-4 [9]. This cloned strain, which can be grown conveniently by conventional culture techniques without the use of hydrocarbon-based media, offers an attractive system for yielding NDO-catalysed biotransformation products provided there is no significant conflicting participation of one or more enzymes coded for by the host cell genome.

2. Materials and methods

2.1. Bacterial strains and growth

E. coli JM109(pDTG141) is a recombinant strain containing the structural genes for NDO originating from *P. putida* NCIB 9816-4 on pT7-5, a plasmid possessing an ampicillin resistant marker, the expression of which is under control of the isopropylthiogalactopyranoside (IPTG)-inducible lac promoter.

Cells of JM109(pDTG141) were grown either on nutrient-rich L-broth medium or MSB minimal medium (MSB) supplemented with glucose (20 mM), thiamine (1 mM), and additional ammonium sulfate (0.5 g l^{-1}). In both cases media were supplemented with ampicillin (100 mg l^{-1}) for plasmid selection. Cultures were initially grown at 37°C, pH 7 with continuous agitation (200 rpm). At mid-log phase the growth temperature was reduced to 30°C and cells supplemented with isopropylthiogalactopyranoside (1 mM) and ferrous ammonium sulfate (0.03% w/v). After a further 3–4 h, at a point representing late log/early stationary phase of growth, the resultant biomass was either employed as a growing cell culture to perform biotransformations, or harvested and used to prepare washed-cell suspensions.

2.2. Preparation of washed-cell suspensions

Washed whole-cell preparations were formed by harvesting the growing cells (centrifugation, $10{,}000 \times g$, 10 min), washing the resultant pellet twice with Na_2H/KH_2PO_4 buffer, pH 7.2, and finally resuspending the pellet in the same buffer at a wet weight concentration of 0.2 g ml^{-1}. Preparations were either used directly, or stored at −25°C until required: samples showed no significant lose of activity when used after up to 12 weeks of storage.

2.3. Biotransformation of sulfides

Sulfides (Table 1, Entries 1–20; Table 2, Entries 1–5) were added to growing cells and washed-cell suspensions of *E. coli* JM109-(pDTG141) at a level of 5 and 10 mM respectively unless otherwise stated. The addition of ethanol (1% v/v final concentration) as cosolvent was shown to have negligible effect on the stereochemical outcome and was thus routinely

Table 1
Oxidation of a range of aryl alkyl sulfides by NDO-containing washed-cell preparations of *E. coli* JM109(pDTG141)

Entry	Sulfide	Biotransformation outcome	Sulfoxide product		
			Conversion (%)	ee (%)	Configuration
1	Ph–S–CH_3	sulfoxide	98	98	*S*
2	Ph–S–C_2H_5	sulfoxide	87	86	*S*
3	Ph–S–*n*C_3H_7	sulfoxide	58	76	*R*
4	Ph–S–*i*C_3H_7	sulfoxide	69	74	*R*
5	Ph–S–*n*C_4H_9	sulfoxide	25	97	*R*
6	Ph–S–CH = CH_2	sulfoxide trace sulfone	98	93	unknown (peak 2)
7	*p*(NO_2)–Ph–S–CH_3	sulfoxide major unknown peak	5	> 98	*S*
8	*p*(CN)–Ph–S–CH_3	sulfoxide	13	> 98	*S*
9	*p*(F)–Ph–S–CH_3	sulfoxide	97	98	*S*
10	*p*(Cl)–Ph–S–CH_3	sulfoxide	10	90	*S*
11	*p*(Cl)–Ph–S–C_2H_5	sulfoxide	31	> 98	unknown (peak 1)
12	*p*(Br)–Ph–S–CH_3	sulfoxide	25	90	*S*
13	*p*(CH_3O)–Ph–S–CH_3	sulfoxide	25	92	*S*
14	*p*(CH_3)–Ph–S–CH_3	sulfoxide	96	> 98	*S*
15	*m*(Cl)–Ph–S–CH_3	sulfoxide trace sulfone	21	75	*S*
16	*o*(Cl)–Ph–S–CH_3	sulfoxide	12	31	unknown (peak 1)
17	Ph–CH_2–S–CH_3	sulfoxide	20	< 1	*rac*
18	Ph–CH_2–S–C_2H_5	sulfoxide	11	5	*S*
19	Ph–(CH_2)$_2$–S–CH_3	sulfoxide	10	54	unknown (peak 2)
20	Ph–(CH_2)$_3$–S–CH_3	negligible biotrans	–	–	–

used to increase the solubility of the sulfide in the aqueous reaction mix. Biotransformation reactions were incubated at 25°C with shaking (200 rpm) for 48 h. At designated intervals, aliquots (200 μl) were taken and extracted with an equal volume of ethyl acetate. Extent of reaction was monitored by analysis of the extracted reaction mix (1 μl) using gas liquid chromatography (Shimadzu GC-14A equipped with a BP1 capillary column). Sulfoxide formation was expressed as percent conversion of added sulfide substrate, quantified where possible by the internal standard method.

Two controls were set up for every reaction under standard experimental conditions; a biological control using substrate plus *E. coli* JM109 (devoid of engineered plasmid) as biocatalyst, and a chemical control using substrate plus buffer and no biocatalyst.

2.4. Product isolation, identification and analysis

Biotransformation reactions were directly extracted with ethyl acetate (×3), the organic

Table 2
Oxidation of a range of dialkyl sulfides by NDO-containing washed-cell preparations of *E. coli* JM109(pDTG141)

Entry	Sulfide	Biotransformation outcome	Sulfoxide product		
			Conversion (%)	ee (%)	Configuration
1	CH_3–S–*n*C_6H_{13}	sulfoxide	70	74	*S*
2	CH_3–S–*cyclo*C_6H_{11}	sulfoxide	70	85	*S*
3	CH_3–S–*n*C_7H_{15}	complete sulfide disappearance, unidentified peak	< 5	3	*R*
4	CH_3–S–*n*C_8H_{17}	little sulfide disappearance, 2 unidentified peaks	< 5	4	*R*
5	CH_3–S–*n*C_9H_{19}	small trace of sulfoxide, large unidentified peak	< 5	5	*R*

layers pooled, washed with NaCl, dried with anhydrous magnesium sulfate and concentrated in vacuo. Extracted products were compared to known standards by GLC analysis.

Sulfoxides were purified by silica chromatography using ethyl acetate as eluant. The optical purity of sulfoxide was determined by chiral GC using a Shimadzu GC-14A machine fitted with either a Lipodex D or E column. In the absence of racemic and enantiomerically pure standards, optical purity was determined by polarimetry (AA1000 Optical Activity polarimeter), comparing specific rotation values to standards quoted in the literature.

2.5. Chemicals

All chemicals and solvents were obtained from Aldrich, Lancaster or Sigma. Sulfides, sulfoxides and sulfones were bought where commercially available or synthesised as previously described [23].

3. Results

Initial tests confirmed that NDO was active in both growing cells and washed-cell preparations of *E. coli* JM109(pDTG141) as evidenced by the ability of both cultures to yield indigo, an insoluble blue chromatophore resulting from the oxidation of indole released during tryptophan catabolism. In order to minimise potential problems arising from the toxicity of the range of tested xenobiotic sulfide substrates (Fig. 1) to growing cells, it was decided to concentrate studies on sulfoxidation by washed-cell preparations of the genetically-engineered strain. The NDO in washed-cell preparations of *E. coli* JM109(pDTG141) was shown to catalyse the enantioselective oxidation of a substantial number of aryl alkyl sulfides to the corresponding sulfoxides. (Table 1). NDO exhibited predominantly (*S*)-selectivity with the phenyl alkyl sulfides tested (Entries 1–16), and in the majority of cases the (*S*)-sulfoxide was formed with high optical purity (ee > 85%). For the simple non-substituted phenyl alkyl sulfides (Entries 1–6), an increase in alkyl chain length from methyl to ethyl resulted in some decrease both in yield (98% to 87%) and optical purity (98% ee to 86% ee) of the predominantly (*S*)-sulfoxide product. However, a further increase in chain length (Entry 3) not only resulted in a further decrease in sulfoxide yield (58%), but an inversion in stereochemistry of the predominant sulfoxide product from the (*S*)- to (*R*)-configuration. These trends were characteristic of the biotransformation of the equivalent branched chain sulfide (Entry 4), and also when the alkyl chain of the presented substrate was further extended to the *n*-butyl homologue (Entry 5), both substrates giving moderate yields of predominantly (*R*)-series sulfoxides. Substitution at the *para*-position of the phenyl ring with either electron withdrawing ($R_2 = NO_2$, CN, F, Cl, Br, Entries 7–12) or electron donating (R_2 = MeO, Me, Entries 13–14) groups had little effect on the stereochemical outcome of sulfoxidation In all cases the resultant (*S*)-sulfoxide was formed with high optical purity (> 90% ee) but in variable yield (< 5–98%). Pro-(*S*) NDO-catalysed attack was also observed when the phenyl ring of the sulfide was substituted with a chloro group at either the *meta*- (Entry 15) or *ortho*-positions (Entry 16), although the closer the substituent approached to the sulfur atom of the substrate, the lower the ee value of the resultant sulfoxide (*para* > *meta* > *ortho*).

Benzyl alkyl sulfides (Table 1, Entries 17–18) were also shown to be suitable substrates for the NDO. However, both the yield and selectivity was dramatically reduced compared to the corresponding phenyl alkyl sulfide biotransformations. Extending the distance between the aryl ring and the heteroatom by introducing addi-

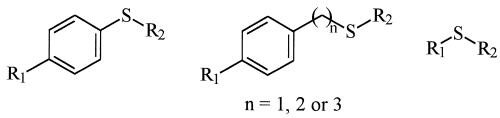

Fig. 1. Sulfide substrates employed for NDO-catalysed sulfoxidations.

tional methylene groups (Entries 19–20) further continued this trend of decreasing the yield of sulfoxide formed.

n-Hexyl methyl- and cyclohexyl methyl sulfide were shown to be the only suitable dialkyl substrates for NDO amongst those tested, both giving moderate yields of the equivalent (*S*)-sulfoxide with moderate to good ee (Table 2, Entries 1–2). Negligible sulfoxidation was detected when the *n*-alkyl chain length was increased further, though significant substrate disappearance linked to the appearance of an uncharacterised product was observed.

In all cases, the observed outcomes were compared to equivalent controls performed to assess the extent, if any, of both non-enzyme catalysed chemical oxidation, and any biological oxidation catalysed by one or more enzymes coded for by the host cell genome, rather than NDO-mediated catalysis. Despite the fact that a wide variety of different microbial monooxygenases, oxidases and peroxidases has been shown to be capable of the biooxidation of various organosulfides [24], no significant evidence was found to suggest that either possibility contributed to the outcomes of the observed biotransformations of any of the tested sulfide substrates with *E. coli* JM109(pDTG141).

4. Conclusions

Recent studies have shown that the NDO enzyme from *P. putida* NCIB 9816-4 is able to catalyse a wide range of mechanistically different reactions including dioxygenation, monooxygenation, desaturation, heteroatom dealkylation and sulfoxidation with a limited range of substrates [25]. By using the NDO-containing recombinant strain *E. coli* JM109-(pDTG141), this study has considerably extended the portfolio of substrates known to undergo stereoselective sulfoxidation by this enzyme purified from the original host, *Pseudomonas* sp. NCIB 9816-4 [18]. It is possible that biotransformations catalysed by whole-cell preparations of recombinant cells could be complicated by reactions catalysed by other enzyme activities coded for by the host cell genome, a possibility apparently not excluded from prior studies performed with this engineered strain [8,9]. However, two principal pieces of evidence support the sole involvement of the cloned NDO in sulfoxidation by this engineered strain. Firstly, control experiments using the non-recombinant parent strain *E. coli* JM109 in most cases failed to yield any detectable oxidation product(s): with the relatively few substrates that did manifest traces of sulfoxide (e.g., Table 1, Entry 1), the yield was always low ($< 5\%$) and sulfoxides were obtained in racemic form. Thus, the contribution made by the host was minimal. Secondly, the data generated in this study compares closely to the results obtained using a partially purified preparation of the NDO from the original host microorganism [18].

NDO-mediated sulfoxidation resulted in predominantly pro-(*S*) attack with the sulfides tested. Notable exceptions to this are the oxidation of aryl alkyl sulfides with either *n*- or *iso*-series alkyl group ≥ 3 carbon atoms, where (*R*)-selectivity is favoured. This, in combination with the ability of NDO to catalyse such a diverse range of oxidative reactions can be used to make some preliminary comments on the nature of the active site of the enzyme. Substituted and non-substituted methyl phenyl sulfides must occupy a similar orientation within the active site, a feature reflected by the consistent formation of (*S*)-sulfoxides of high ee. Electron influences on the phenyl ring present in these substrates do not appear to have a significant effect on the stereochemical outcome of NDO-catalysed sulfoxidation, as evidenced by the fact that substitution of the phenyl ring with a range of either electron-withdrawing and electron-donating groups had negligible effect on the stereoselectivity of the relevant sulfoxidation. However, the significantly higher yield of sulfoxide recorded with *p*(F)–Ph–S–CH_3 (Table 1, Entry 9) compared to equivalent substrates carrying sterically larger substituents (Table 1,

Entries 10 and 12) suggests the presence of some size restrictive pocket within the active site of the enzyme. Despite the predilection of this enzyme to form *cis*-dihydrodiols with various aromatic hydrocarbons and the proposed requirement for at least one aromatic ring for substrate binding by NDO [25], the aromatic ring of the aryl alkyl sulfides tested is not a site for enzymatic oxidative attack, nor can it be a prerequisite for heteroatom biooxidation as evidenced by the outcomes of the biotransformations by the cloned NDO recorded with cyclohexyl methyl sulfide ((*S*)-sulfoxide, ee 85%, yield 70%) and *n*-hexyl methyl sulfide ((*S*)-sulfoxide, ee 74%, yield 70%).

A range of bicyclic sulfides, including thiochroman and thiochroman-4-one, have been tested as substrates for the NDO enzyme (data not shown). These were typically poor substrates giving negligible yields of sulfoxide with low ee values. This is surprising in the light of the known ability of NDO to catalyse *cis*-dihydroxylation of naphthalene [7], which suggests that similar but more conformationally restrained carbocyclic bicyclic compounds must be accommodated within the active site of the enzyme, albeit to undergo a mechanistically different reaction.

This and additional sulfoxidation data not reported in this current study is being used to develop a predictive active site model for the NDO, thus enabling this dioxygenase enzyme to be compared more directly to other types of enzyme able to perform sulfoxidation, which includes Baeyer–Villiger monooxygenases from both *P. putida* NCIMB 10007 [22] and *Acinetobacter calcoaceticus* NCIMB 9871 [26], the cytochrome $P450_{CAM}$ monooxygenase from the same strain of *P. putida* [27], the heme-dependent chloroperoxidase from *Caldariomyces fumago* [28–30], the vanadium-dependent bromoperoxidase from *Corallina officinalis* [31], as well as a number of conventional peroxidases [32–34].

In conclusion, NDO from *Pseudomonas* sp. NCIB 9816-4 cloned into *E. coli* JM109-(pDTG141) has been found to be a useful biocatalyst for the production of a range of (*S*)-sulfoxides of high optical purity, providing an attractive 'green technology' option for the preparation of sulfoxides with the opposite stereochemistry to those obtained from the TDO activity present in *P. putida* UV4 [6] and *P. putida* F1 [18].

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