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**PAPER**

# Dioxygenase-catalysed *cis*-dihydroxylation of *meta*-substituted phenols to yield cyclohexenone *cis*-diol and derived enantiopure *cis*-triol metabolites†

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*cis*-Dihydroxylation of *meta*-substituted phenol (*m*-phenol) substrates, to yield the corresponding cyclohexenone *cis*-diol metabolites, was catalysed by arene dioxygenases present in mutant and recombinant bacterial strains. The presence of cyclohexenone *cis*-diol metabolites and several of their cyclohexene and cyclohexane *cis*-triol derivatives was detected by LC-TOFMS analysis and confirmed by NMR spectroscopy. Structural and stereochemical analyses of chiral ketodiol bioproducts, was carried out using NMR and CD spectroscopy and stereochemical correlation methods. The formation of enantiopure cyclohexenone *cis*-diol metabolites is discussed in the context of postulated binding interactions of the *m*-phenol substrates at the active site of toluene dioxygenase (TDO).

## Introduction

Enzyme-catalysed mono- and poly-hydroxylations of aromatic rings are ubiquitous in nature, occurring in animals, plants, fungi and bacteria. A range of enzymes may be involved including heme and non-heme monooxygenases, dioxygenases, phenol oxidases and peroxidases.[1a,1b] Over the past two decades, many reviews published on this topic indicate that stereoselective bacterial dioxygenase (DO)-catalysed hydroxylations are among the most widely studied types of arene biotransformations.[2a–2n] The role of bacterial dioxygenases in the oxidation of monosubstituted (**A**, R′ = H, R ≠ H) and disubstituted benzenes (**A**, R and R′ ≠ H) and polycyclic arenes, to yield the corresponding *cis*-dihydrodiols (**B**, Scheme 1) is of particular interest because of their numerous applications as chiral precursors in the synthesis of enantiopure natural and unnatural products.[2a–2n] *cis*-Dihydrodiols (**B**) are generally formed as initial intermediates which may

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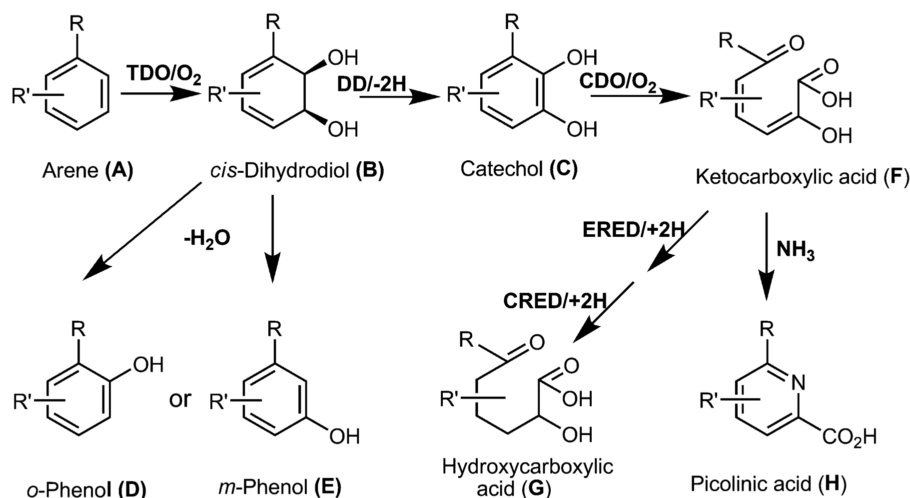



**Scheme 1** Aromatic hydroxylation and mineralization pathways *via* toluene dioxygenase (TDO), *cis*-diol dehydrogenase (DD), catechol dioxygenase (CDO), ene reductase (ERED) and carbonyl reductase (CRED) biocatalysis.

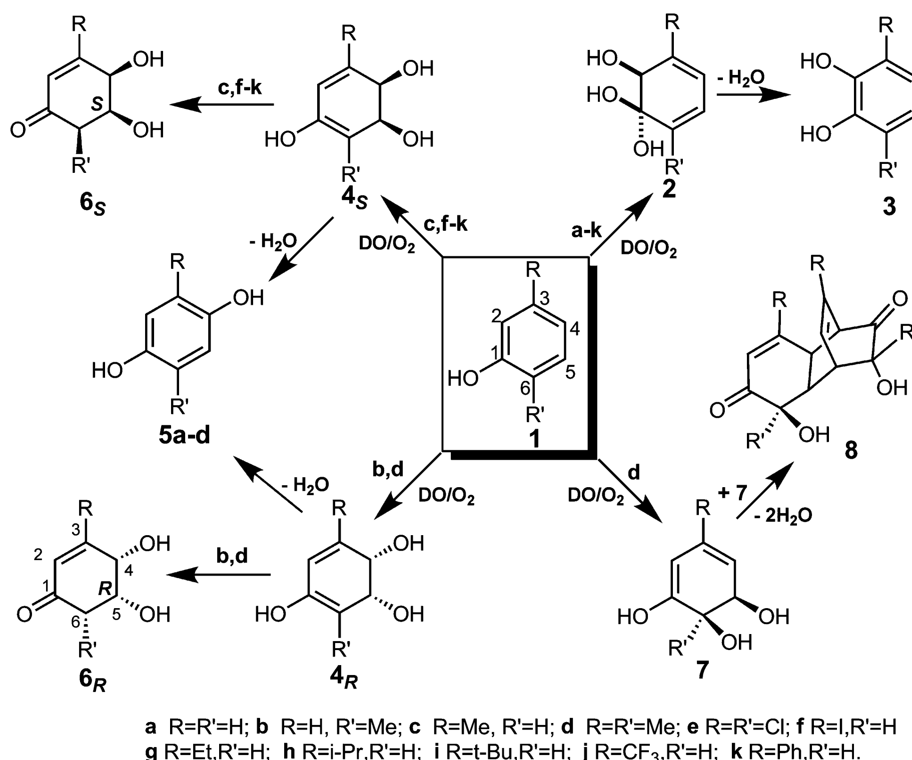
undergo spontaneous dehydration to yield *ortho*- (**D**, R′ = H) or *m*-phenols (**E**, R′ = H) or *cis*-diol dehydrogenase (DD)-catalysed dehydrogenation to yield catechols (**C**, R′ = H). The latter bioproducts can also be produced *via* arene dioxygenase-catalysed oxidation of phenols (**D** or **E**, R′ = H) prior to a ring-opening process catalysed by an extradiol catechol dioxygenase (CDO, Scheme 1) or an intradiol CDO.

As phenols are often found to be the initial metabolites isolated from both arenes **A** and their *cis*-dihydrodiol derivatives **B**, *e.g.* *ortho*-phenols (**A** → **B** → **D**, R′ = H), *m*-phenols (**A** → **B** → **E**, R′ = H), their further metabolism is of considerable importance in relation to the environment. Based on earlier literature reports, on the bacterial metabolism of a range of phenol types, *e.g.* **D** or **E**,[3a–3h] it might be inferred that further aromatic hydroxylation, to yield achiral catechols, *e.g.* **C**, was the major or sole metabolic pathway (Scheme 1). However, studies have indicated that while catechols **C** are the dominant bioproducts, a further range of water soluble metabolites, including ketocarboxylic acids **F**, hydroxycarboxylic acids **G** and picolinic acids **H**, may also be formed during the mineralization process of phenols.

Following the pioneering work of Gibson *et al.*,[4] initially using mutant strains of the soil bacterium *Pseudomonas putida*, and later *Escherichia coli* recombinant strains containing TDO, it became possible to intercept, characterise and utilize the *cis*-dihydrodiol metabolites **B**. Recent biotransformation studies, using whole cells of the *P. putida* UV4 mutant strain, showed that the TDO-catalysed *cis*-dihydroxylation was more efficient for monosubstituted (**A**, R ≠ H, R′ = H) compared with disubstituted benzenes (**A**, R and R′ ≠ H). *meta*-Disubstituted benzene substrates in particular were generally much more resistant to this type of oxidation (the *meta effect*).[5a] Where possible, the latter type of *meta*-substituted benzene substrates were found to undergo alternative types of TDO-catalysed oxidation according to the nature of substituents *e.g.* alkylaryl sulfide sulfoxidation (**A**, R = *S*-Alkyl, *meta*-R′ ≠ H),[5b] alkene dihydroxylation, (**A**, R = vinyl, *meta*-R′ ≠ H)[5c] or benzylic hydroxylation (**A**, R = $CH_2$-Alkyl, *meta*-R′ ≠ H).[5a]

It has been postulated that bacterial dioxygenase-catalysed *cis*-dihydroxylation of phenols (Scheme 2), for example **1e**, at the 1,2-bond to give a transient triol **2e**,[3f] or **1d** at the 4,5-bond to yield the enol *cis*-diol **4d**,[3j] followed in each case by their spontaneous dehydration, could in principle account for the formation of the corresponding catechol **3e** (from **2e**) or hydroquinone **5d** (from **4d**).

To our knowledge, no direct evidence of either of these two types of phenolic *cis*-dihydrodiols, **2** or **4**, has been reported in the literature. However, a recent preliminary communication of this study,[6] showed that a *P. putida* UV4 mediated biotransformation of *ortho*-, *e.g.* **1b**, and *m*-phenols, *e.g.* **1c** or **1f**, and disubstituted phenols, *e.g.* **1d**, could yield, in addition to the expected catechols **3b–3d** and **3f**, enantiopure cyclohexenone *cis*-diols $\mathbf{6b}_R$–$\mathbf{d}_R$ and $\mathbf{6f}_S$, as isolable metabolites, albeit in generally modest yields. Since these compounds are the more stable tautomeric forms of the initial *cis*-dihydrodiols $\mathbf{4b}_R$–$\mathbf{d}_R$, and $\mathbf{4f}_S$, they have provided the first direct evidence that phenols, in common with other mono- and di-substituted benzene substrates, undergo stereoselective TDO-catalysed *cis*-dihydroxylation. Tentative evidence of this type of hydroxylation also occurring at the 5,6-bond of phenols, *e.g.* **1d**, was found when enantiopure dimeric bioproducts, *e.g.* **8d**, were obtained.[6] The pathway presumably involves rapid dehydration of the transient phenolic *cis*-diol metabolite, *e.g.* **7d**, followed by spontaneous cycloaddition between two molecules of the




**Scheme 2** Dioxygenase-catalysed oxidation of phenols **1** to yield catechols **3**, hydroquinones **5**, cyclohexenone *cis*-diols $\mathbf{6}_S$ (or $\mathbf{6}_R$) and cycloadducts **8**.


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**Table 1** Relative proportions of cyclohexenone *cis*-diols **6**, triols **12** and carboxylic acids **15** found by LC-TOFMS analysis[a] and catechols **3** from NMR analysis[b]

| Phenol (**R**) | *cis*-Diol | Relative ratio[a] | Triol | Relative ratio[a] | Acid | Relative ratio[b] | Catechol | Relative ratio[a] |
|---|---|---|---|---|---|---|---|---|
| **1c** (Me) | $\mathbf{6c}_S$ | ++ | **12c** | [c] | **15c** | [c] | **3c** | +++ |
| **1f** (I) | $\mathbf{6f}_S$ | ++++ | **12f** | + | **15f** | — | **3f** | ++ |
| **1g** (Et) | $\mathbf{6g}_S$ | ++ | **12g** | [d] | **15g** | [d] | **3g** | ++ |
| **1h** ($^i$Pr) | $\mathbf{6h}_S$ | +++ | **12h** | + | **15h** | + | **3h** | [d] |
| **1i** ($^t$Bu) | $\mathbf{6i}_S$ | ++++ | **12i** | + | **15i** | ++ | **3i** | [d] |
| **1j** ($CF_3$) | $\mathbf{6j}_S$ | ++ | **12j**[e] | +++ | **15j** | [c] | **3j** | + |
| **1k** (Ph) | $\mathbf{6k}_S$[f] | ++ | **12k** | [d] | **15k** | + | **3k** | [d] |

[a] LC-TOFMS based on peak areas; [b] NMR; [c] Not detected; [d] Trace; [e] Triol **11j** also found (+); [f] *cis*-Dihydrodiol **17** also found (+)

resulting diene to yield a diketone, *e.g.* **8d**. This result, and those obtained from earlier studies,[3f,3j,6] where bacterial metabolism of specific phenols **1** yielded the corresponding achiral catechols **3**, cyclohexenone *cis*-diols $\mathbf{6}_S$ and cycloadducts **8**, could all be accounted for by alternative regioselective *cis*-dihydroxylation pathways (Scheme 2). Thus, the postulated TDO-catalysed oxidation of phenols **1** occurring at the 1,2-bond[3f] (*e.g.* on compound **1d** to give catechol **3d** *via* intermediate **2d**), at the 4,5-bond (to give keto *cis*-diol $\mathbf{6d}_R$ *via* intermediate $\mathbf{4d}_R$)[6] and at the 5,6-bond (to give *bis*-ketol **8d** *via* intermediate **7d**),[6] contrasts with the almost exclusive regioselectivity of TDO-catalysed *cis*-dihydroxylation at the 2,3-bond of monosubstituted benzene substrates (**A** → **B**, R′ = H, Scheme 1).

Following on from our earlier Communication,[6] this more comprehensive study was undertaken in order to establish if:

(i) (a) the new cyclohexenone *cis*-diol family of metabolites $\mathbf{6}_S$ (R′ = H) could be formed using a wider range of *m*-phenol substrates **1** (R′ = H) and (b) any second generation chiral products could be derived from these initial metabolites.

(ii) (a) TDO was the enzyme responsible for catalysing the production of cyclohexenone *cis*-diols $\mathbf{6}_S$ (R′ = H) or other *m*-phenol metabolites by employing a recombinant strain containing TDO and (b) these bioproducts could be formed using alternative dioxygenase enzymes.

(iii) the preferential TDO-catalysed *cis*-dihydroxylation of *m*-phenols **1** (R′ = H), to form enantiopure cyclohexenone *cis*-diols $\mathbf{6}_S$ (R′ = H), which contrasts with the behaviour of most other *ortho*- and *para*-phenols and other non-phenolic *meta*-substituted benzene substrates, could be rationalised in terms of binding interactions of these substrates within the TDO active site.

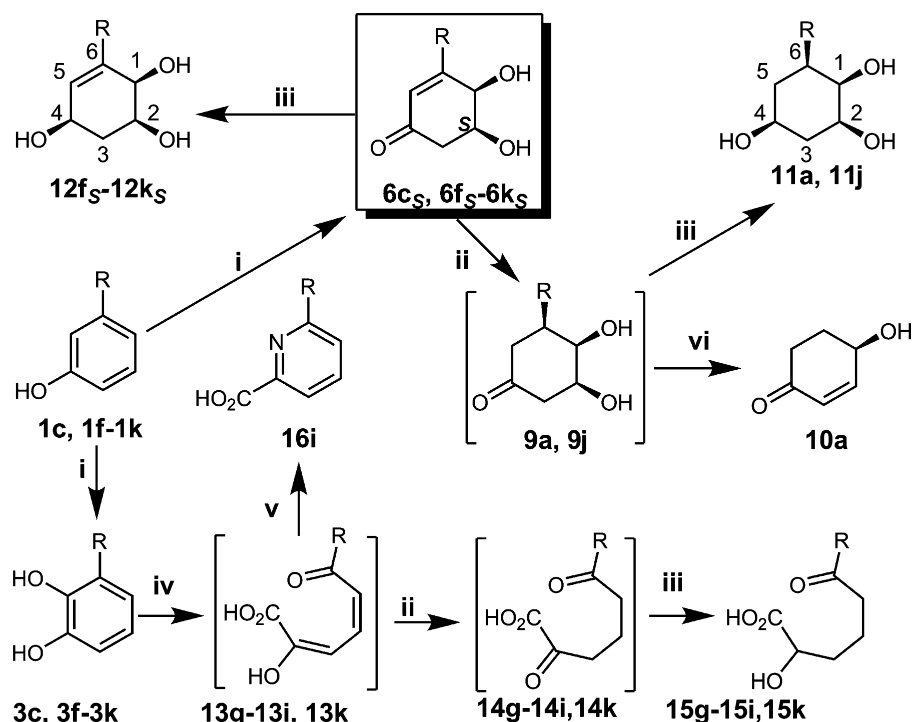
## Results and discussion

### (a) Dioxygenase-catalysed *cis*-dihydroxylation of *m*-phenols 1c, 1f–k to form cyclohexenone *cis*-diols $\mathbf{6c}_S$, $\mathbf{6f}_S$–$\mathbf{k}_S$, their carbonyl reductase-catalysed ketone reductions to yield triols $\mathbf{12f}_S$–$\mathbf{k}_S$ and other metabolites

The first member of the new cyclohexenone *cis*-diol family $\mathbf{6d}_R$ (Scheme 2) was isolated from a serendipitous biotransformation of *p*-xylene (**A**, R = R′ = Me, Scheme 1) using a constitutive mutant strain (UV4) of *P. putida*, a source of TDO.[6] The initial step in the biosynthetic sequence involved the TDO-catalysed formation of the corresponding *p*-xylene *cis*-dihydrodiol (**B**, R = R′ = Me, Scheme 1). Dehydration of a small proportion of this diol during biotransformation yielded phenol **1d** (Scheme 2). Further TDO-catalysed *cis*-dihydroxylation of phenol **1d** then gave enol $\mathbf{4d}_R$ which preferentially exists as the keto tautomer, *i.e.* cyclohexenone *cis*-diol $\mathbf{6d}_R$ (Scheme 2).[6] When phenol **1d** was added as substrate to whole cells of *P. putida* UV4, a higher yield of *cis*-diol $\mathbf{6d}_R$ was obtained, compared with that found using *para*-xylene as substrate.[6] Preliminary studies have shown that alternative *para*-disubstituted phenolic substrates, *e.g.* **1d** (R′ = Cl or Et and R = Me, or R′ = Me and R = F), for *P. putida* UV4, also yield the corresponding keto *cis*-diols $\mathbf{6}_R$. The structures and absolute configurations of keto *cis*-diols $\mathbf{6}_R$ (R = Me, R′ = Cl or Et and R = F, R′ = Me), each containing three chiral centres, derived from the corresponding disubstituted phenols **1d**, will be discussed elsewhere.

When the *m*-phenols **1c** (and **1f**) were previously examined as substrates for *P. putida* UV4,[6] it was found that the corresponding cyclohexenone *cis*-diols, $\mathbf{6c}_S$ (and $\mathbf{6f}_S$) were formed as the major metabolites (15–70% isolated yield) along with the expected catechols **3c** (and **3f**) as minor bioproducts. *ortho*-Cresol **1b** also proved to be a substrate for *P. putida* UV4 producing mainly catechol **3** (R = Me, R′ = H) and a low yield of cyclohexenone *cis*-diol $\mathbf{6b}_R$ (*ca.*: 1%).[6] When *para*-cresol was used as substrate, the corresponding 4-methylcatechol was the only identifiable bioproduct formed. Thus, the biotransformation of these *ortho*- and *para*-substituted phenols strongly favoured the formation of catechols. As *m*-phenols **1c** and **1f** yielded enantiopure cyclohexenone *cis*-diols, preferentially, a wider range of *m*-phenols was examined to extend the study.

Following the earlier report of cyclohexenone *cis*-diol formation from *meta*-cresol **1c** (R = Me, R′ = H),[6] three further alkyl-substituted *m*-phenols **1g** (R = Et, R′ = H), **1h** (R = *iso*-Pr, R′ = H) and **1i** (R = *tert*-Bu, R′ = H) were initially found to yield the corresponding cyclohexenone *cis*-diols ($\mathbf{6g}_S$–$\mathbf{i}_S$) as bioproducts (>5% isolated yield, Scheme 3 and Table 1) accompanied by minor or trace amounts of the corresponding known catechols **3g–i** which were not isolated. NMR spectroscopic analysis was used to identify the structures of the new keto *cis*-diols $\mathbf{6g}_S$–$\mathbf{i}_S$. It is noteworthy that the relative yields of these cyclohexenone *cis*-diols varied between experiments but generally diminished with decreasing substituent size (*tert*-Bu > *iso*-Pr > Et > Me, Table 1). The variable isolated yields of both cyclohexenone *cis*-diols $\mathbf{6g}_S$–$\mathbf{i}_S$, and catechols **3g–i** formed during repeated biotransformations were attributed to further metabolism of these bioproducts.




**Scheme 3** Dioxygenase-catalysed oxidation of phenols **1c**, **1f–k**, to yield cyclohexenone *cis*-diols **6c**$_S$, **6f**$_S$–**k**$_S$, cyclohexene *cis*-triols **12f**$_S$–**k**$_S$, cyclohexane *cis*-triols **11a** and **11j**, catechols **3c**, **3f–k**, hydroxycarboxylic acid **15g–i,15k**, and picolinic acid **16i**.

The enantiopurity of each of the metabolites **6g**$_S$–**i**$_S$ was established as ≥98% *ee* by formation of chiral boronate derivatives, using (*R*)- and (*S*)-2-(1-methoxymethyl)benzene boronic acid (MEBBA) and $^1$H-NMR analysis as previously reported for the cyclohexenone *cis*-diols **6c**$_S$ or **6f**$_S$[6] and for standard arene *cis*-dihydrodiols.[7a–c] The absolute configuration of the iodo-substituted cyclohexenone *cis*-diol **6f**$_S$ had earlier been assigned as (4*S*,5*S*) on the basis of X-ray crystallographic analysis and was stereochemically correlated with the (4*R*,5*S*) configuration of the parent cyclohexenone *cis*-diol **6a**$_S$ following hydrogenolysis.[6] Recent studies, employing both experimental and calculated circular dichroism spectra, have shown that cyclohexenone *cis*-diols **6a**$_S$, **6c**$_S$, **6g**$_S$–**i**$_S$ all have similar bisignate CD spectra, with positive Cotton effects at shorter wavelengths and negative Cotton effects at longer wavelengths, indicative of identical absolute (4*R*,5*S*) configurations.[8]

Initial analysis of the bioproducts in the crude aqueous culture medium obtained from *P. putida* UV4 biotransformations of *m*-phenols **1c**, **1f–k**, based on the HPLC/ESI-TOF/MS (LC-TOF/MS) method, provided further evidence of the alkyl-substituted cyclohexenone *cis*-diols **6c**$_S$, **6f**$_S$–**k**$_S$, being formed as the most significant metabolites prior to extraction (Table 1). Although this method was not found to be suitable for the detection of catechols **3**, which were only identified after extraction and NMR analysis, it resulted in the detection of several other minor metabolites (Scheme 3). This prompted a large-scale biotransformation of the *meta*-substituted phenol **1f**, which was selected as substrate since it possesses a readily replaceable iodine atom. In addition to the major product, cyclohexenone *cis*-diol **6f**$_S$, bioproducts **10a**[6] and **11a**[6] and triol **12f**$_S$ were also isolated as minor metabolites. Compounds **10a** and **11a** were assumed to be formed *via* the cyclohexanone intermediate **9a** which could only be detected by LC-TOF/MS. The metabolite **12f**$_S$ was fully characterized using NMR spectroscopy and other methods. The absolute configuration shown in Scheme 3 was determined by stereochemical correlation *via* hydrogenolysis/hydrogenation to yield triol **11a** of established absolute configuration. LC-TOF/MS analysis of the crude culture medium resulting from biotransformation of phenols **1g–k** with *P. putida* UV4 detected the presence of the corresponding triol metabolites **12g–k**. Only the triol diastereoisomers **12f**$_S$, **12i**$_S$ and **12j**$_S$, were obtained in sufficient quantity and purity for complete characterization. The relative *cis* stereochemistry of the three chiral centres in each of the triols **12f**$_S$, **12i**$_S$ and **12j**$_S$, was determined by NMR spectroscopy. The pseudo axial proton on C3 exhibited a strong geminal coupling and two strong diaxial couplings, thereby establishing that the hydroxyl groups on C2 and C4 were diequatorial and, hence, confirmed the relative stereochemistry. It is thus presumed that all the triol metabolites, **12g–i**, are likely to possess similar relative and absolute configurations. Chemoenzymatic synthesis studies, using the corresponding, more readily available, *cis*-dihydrodiols (**B**, R′ = H, Scheme 1) are in progress and should provide adequate samples of all the triols for unequivocal confirmation of their structures and stereochemistry. It was assumed that each of the minor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triol metabolites, $\mathbf{12f}_S$–$\mathbf{k}_S$, was a second generation metabolite derived from the corresponding enantiopure cyclohexenone *cis*-diols $\mathbf{6f}_S$–$\mathbf{i}_S$. This premise was confirmed when compounds $\mathbf{6f}_S$ and $\mathbf{6i}_S$, were used as substrates for *P. putida* UV4 and yielded the corresponding triols $\mathbf{12f}_S$ and $\mathbf{12i}_S$ as the major metabolites; it also confirmed that a carbonyl reductase (CRED) was present in this organism.

In addition to the cyclohexenone *cis*-diols, $\mathbf{6g}_S$–$\mathbf{k}_S$, and triols, $\mathbf{12g}_S$–$\mathbf{k}_S$, obtained from biotransformation of the phenols **1g–k** and **1k** in *P. putida* UV4 cultures, LC-TOF/MS analysis revealed a third family of bioproducts. These were tentatively identified as carboxylic acids, **15g–i** and **15k**, based on the parent ion data ($[M + H]^+$, $[M + Na]^+$,$[M + NH_4]^+$, $[M + K]^+$ or $[MH - H_2O]^+$) and their relative retention times (Table 1). Although compounds **15g–i** and **15k**, (Scheme 3), appeared to be new metabolites, bioproduct **15h** and several other carboxylic acids (**15**, R = *n*-Pr, *n*-Bu and *iso*-Bu) had been observed as metabolites of the parent alkylbenzenes using wild-type *Pseudomonas* strains.[9a–9d] Carboxylic acid **15k** was not isolated as a bacterial metabolite from biphenyl, but its immediate metabolic precursor **14k** was identified.[9d] In our studies only carboxylic acid **15i** was obtained in sufficient quantity for full structure determination by spectroscopic methods.

It was assumed that each of the carboxylic acids **15g–i** and **15k** resulted from ring-opening of the corresponding catechol metabolites **3g–i** and **3k**. An extradiol catechol dioxygenase, CDO, appeared to be responsible for this oxidative ring-opening process leading to the undetected conjugated ketocarboxylic acids **13g–i** and **13k**, *via* the corresponding lactone intermediates.[10a,b] Earlier work[9a–d] had shown that the next step involved an ene reductase (ERED)-catalysed hydrogenation of an alkene group to give the corresponding non-conjugated diketocarboxylic acids; a similar metabolic sequence is proposed for the formation of diketocarboxylic acids **14g–i** and **14k**. This ERED-catalysed hydrogenation was similar to the conversion of *cis*-diol $\mathbf{6a}_S$ into cyclohexane *cis*-triol **11a**. CRED-catalysed ketone reduction, similar to the conversion of keto *cis*-diols **6** into triols **12**, occurred in the final biosynthetic step to give the carboxylic acids **15g–i** and **15k**. Further metabolism of cyclohexenone *cis*-diols, $\mathbf{6g}_S$–$\mathbf{i}_S$ to give triols, **12g–i**, catechols **3g–i**, **3k** and then carboxylic acids, **15g–i** and **15k**, may explain the relatively low but variable yields obtained. The presence of these carboxylic acids also provides indirect evidence of catechols **3g**, **3h**, **3i** as precursors of value, since the LC-TOF/MS analysis protocol used in his study was found to be unsuitable for the detection of catechols in general. This study, has extended the known range of carboxylic acid metabolites (**15**, R = Et, *n*-Pr, *iso*-Pr, *n*-Bu, *iso*-Bu, *tert*-Bu, Ph)[9a–d] derived from the corresponding catechols **3**.

An authentic sample of picolinic acid derivative **16i**, available from independent studies allied to LC-TOF/MS analysis, was used to confirm the presence of trace amounts of this acid, as a further metabolite of catechol **3i** (Scheme 3). Earlier work on the biotransformation of a series of monosubstituted benzenes, *e.g.* diphenylacetylene,[11a] and biphenyl (**A**, R = Ph, R′ = H),[11b] involving the sequence: (i) TDO-catalysed *cis*-dihydroxylation (to give *cis*-dihydrodiols **B**, R = Ph, R′ = H), (ii) DD-catalysed dehydrogenation (to form catechols, **C**, R = Ph, R′ = H), (iii) CDO-catalysed synthesis (to yield ketocarboxylic acids, **F**, R = Ph, R′ = H) and (iv) non-enzymatic cyclization as the final step to yield picolinic acids (**H**, R = Ph, R′ = H, Scheme 1).

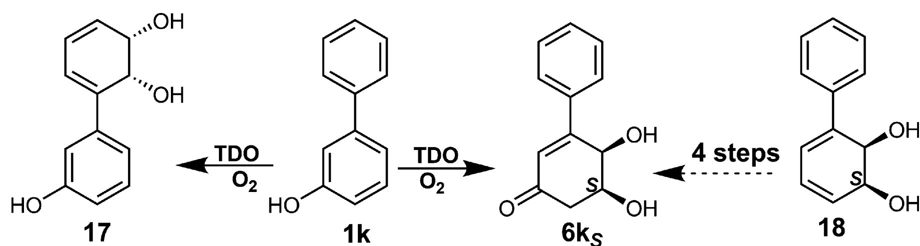
The presence of extradiol catechol dioxygenase (CDO), ene reductase (ERED), and carbonyl reductase (CRED) and other enzymes in whole cells of *P. putida* UV4, would be expected to significantly reduce the isolated yields of both catechols **C** and derived metabolites, *e.g.* **G** and **H** (Scheme 1). Thus, the relative proportions of these metabolites, as determined by LC-TOFMS or NMR analysis, were often present only in trace amounts (Table 1).

Earlier experiments had shown that *m*-phenols bearing bulky substituents, *e.g. tert*-Bu or I, gave better yields of cyclohexenone *cis*-diols. Phenols **1j** and **1k**, having large $CF_3$ and Ph groups, were therefore selected for further study as substrates for *P. putida* UV4. 3-Trifluoromethylphenol **1j**, on biotransformation, gave the expected enantiopure cyclohexenone *cis*-diol $\mathbf{6j}_S$ but only as a minor metabolite after column chromatography (4% yield). A second more polar fraction proved to be a mixture of two triol metabolites (16% yield) that was then separated by multi-elution preparative layer chromatography (PLC) with MeOH–$CHCl_3$ (1 : 5) into triols $\mathbf{12j}_S$ and triol **11j**; the structure, relative and absolute stereochemistry of each triol was assigned by NMR spectroscopy. The proportion of the major triol $\mathbf{12j}_S$, relative to keto *cis*-diol $\mathbf{6j}_S$, was much higher than that observed for other phenol substrates (Table 1). This observation suggests that a ketone bearing a vinylogous $CF_3$ group [-CH=C($CF_3$)-] in metabolite $\mathbf{6j}_S$ is more readily reduced by a CRED from *P. putida* UV4 compared with the other substituted vinyl ketones ($\mathbf{6c}_S$, $\mathbf{6f}_S$–$\mathbf{i}_S$).

The metabolic pathway (**1** → **6** → **12**) shown in Scheme 3 is an example of a tandem redox biotransformation *i.e.* a TDO/CRED-catalysed *cis*-dihydroxylation/ketone reduction of phenols **1**. An earlier biotransformation of $\alpha,\alpha,\alpha$-trifluoromethyl acetophenone ($CF_3COPh$) using *P. putida* UV4 resulted in the reverse redox tandem sequence, *i.e.* a CRED/TDO-catalysed ketone reduction/*cis*-dihydroxylation, indicative of the facile reduction of the ketone bearing a $CF_3$ group.[12] Only trace amounts of catechol **3j**, but no evidence of the carboxylic acid **15j**, were detected by LC-TOF/MS (Table 1).

3-Phenylphenol **1k**, proved to be among the most interesting of the *m*-phenol substrates; it provided the TDO enzyme present in *P. putida* UV4 with the option of catalysing *cis*-dihydroxylation of either the monosubstituted phenyl ring or the disubstituted *m*-phenol ring. *m*-Disubstituted benzenes are normally much poorer substrates for TDO.[5a] Thus, we anticipated preferential *cis*-dihydroxylation of the monosubstituted ring in compound **1k** to give *cis*-dihydrodiol **17**. Surprisingly, more cyclohexenone *cis*-diol $\mathbf{6k}_S$ was formed relative to metabolite **17** in a readily separable mixture (Scheme 4, Table 1). Triol **12k** was only detected by LC-TOFMS as a very minor metabolite (<1%). This observation suggests that a substrate with a *m*-phenol ring presents a more attractive *cis*-dihydroxylation site for TDO than its monosubstituted phenyl group. $^1$H-NMR analysis of MEBBA derivatives was used to determine the *ee* values of *cis*-diols $\mathbf{6k}_S$ and **17** and, as anticipated, they were both enantiopure (≥98% *ee*).

The absolute configuration of *cis*-dihydrodiol **17** was determined by comparison of its CD spectrum with that of the *cis*-dihydrodiol of biphenyl **18**. Due to the presence of the additional phenyl chromophore in compound $\mathbf{6k}_S$, determination of the absolute configuration by comparison of its CD spectrum with those of cyclohexenone *cis*-diols $\mathbf{6a}_S$, $\mathbf{6c}_S$, $\mathbf{6f}$–$\mathbf{6i}_S$, was considered to be less reliable. However, as part of a separate study designed




**Scheme 4** Dioxygenase-catalysed oxidation of phenol **1k** to yield *cis*-dihydrodiol **17** and cyclohexenone *cis*-diol **6k**$_S$ with absolute configuration assignment by stereochemical correlation with *cis*-dihydrodiol **18**.

to provide synthetic routes to keto *cis*-diols, *e.g.* **6k**$_S$, and triols, *e.g.* **12k**, from the corresponding arene *cis*-dihydrodiols (**B**, R′ = H, Scheme 1), it was possible to provide unequivocal confirmation of its absolute configuration *via* stereochemical correlation with the biphenyl *cis*-dihydrodiol metabolite **18** (unpublished data). As shown in Table 1, other very minor metabolites from 3-hydroxybiphenyl **1k**, tentatively identified, included catechol **3k** (detected by NMR spectroscopy), and the derived carboxylic acid metabolite **15k** (detected by LC-TOF/MS).

It is noteworthy that biotransformation of the six *m*-phenols **1f–k** in *P. putida* UV4 generally yielded the corresponding cyclohexenone *cis*-diols **6f**$_S$**–k**$_S$, as major metabolites and triols, **12f–k**, as minor bioproducts based on LC-TOF/MS analyses (Table 1). No evidence of the corresponding triol **12c**$_S$ or carboxylic acid metabolites **15c** or **15j** was observed.

The relatively low but variable yields of the initial cyclohexenone *cis*-diol bioproducts, **6f**$_S$**–k**$_S$, obtained could be accounted for by several factors. These include:

(i) the toxicity of phenolic substrates which are often used as antimicrobial agents

(ii) the competition from alternative metabolism routes of phenols, particularly formation of catechols **3**

(iii) the inhibition effect of catechols **3** on the toluene dioxygenase enzyme[13]

(iv) the CDO-catalysed ring opening of catechols **3** to yield ketocarboxylic **15** and picolinic acids **16** prior to mineralization

(v) the ERED-catalysed hydrogenations of cyclohexenone *cis*-diols **6** to yield cyclohexanone *cis*-diols **9** and CRED-catalysed ketone reductions of both cyclohexenone *cis*-diols **6** and cyclohexanone *cis*-diols **9** to yield triols **12** and **11**.

The CRED-catalysed reduction of the keto group to yield cyclohexene triols **12** and ERED/CRED-catalysed hydrogenation/reduction to yield cyclohexane *cis*-triols **11** have been identified as further steps in the biodegradation sequence of the new family of cyclohexenone *cis*-diol metabolites **6**. The possibility that *cis*-diol dehydrogenases, already known to be present in the environment, can also biodegrade this new family of phenol metabolites remains to be investigated.

### (b) Enzymes responsible for the production of cyclohexenone *cis*-diols 6 from the corresponding *m*-phenols 1

Preliminary results obtained during the biotransformation of *m*-phenols **1** needed further work to establish if:

(i) TDO is the enzyme responsible for the formation of the new family of cyclohexenone *cis*-diols **6** in *P. putida* UV4

(ii) other types of arene dioxygenases can also catalyse this *cis*-dihydroxylation process

(iii) the strong preference shown by TDO for *m*-phenols could be rationalized in terms of favourable substrate binding interactions at the active site

As part of the study, a new *E. coli* recombinant strain bearing the TDO genes was constructed. The X-ray crystal structure of TDO derived from *P. putida* F1 has recently been reported[14] and a cloned TDO obtained from *P. putida* UV4 has now been shown to be identical to the *P. putida* F1 enzyme. A plasmid, pCL-4t, containing all four *tod*-genes from *P. putida* UV4, was constructed using a pBAD-ET vector. *E. coli* TOP10 cells were transformed with the pCL-4t plasmid to produce an *E. coli* TOP10 (pCL-4t) clone. The complete sequence of the cloned *P. putida* UV4 TDO $ISP_{TOL}$ large and small subunits, is exactly equivalent to the *tod* C1 and C2 components respectively from *P. putida* F1.[15]

The *E. coli* TOP10 (pCL-4t) recombinant strain, hereafter described as *E. coli* CL-4t, was tested with the *m*-phenols **1c**, **1f–k** as substrates. In each case the corresponding cyclohexenone *cis*-diol, **6c**$_S$**–k**$_S$, was found to be present using LC-TOF/MS analysis. The isolated yields of cyclohexenone *cis*-diols obtained using the clone, were however, consistently lower than those found using *P. putida* UV4 under the reaction conditions used. We speculate that this may reflect the increased toxicity of the phenolic substrates **1c**, **1f–k** against the *E. coli* host cell. In addition, the known inhibition effect of some catechols [13] on TDO, possibly including catechols **3c**, **3f–k**, would be much stronger in *E. coli* CL-4t compared with *P. putida* UV4, since they would not be readily removed in the absence of the extradiol catechol dioxygenase enzyme. Significantly, this result confirms unequivocally that the same TDO enzyme, known to be present in *P. putida* F1 and now *P. putida* UV4, was responsible for the formation of cyclohexenone *cis*-diols **6**. In contrast to the earlier biotransformations of the *m*-phenols, **1f–k**, using *P. putida* UV4, no evidence of the formation of triol **12** or carboxylic acid **15** was found using *E. coli* CL-4t. This was consistent with the CRED, responsible for ketone reductions, and the ring opening CDO enzyme in *P. putida* UV4 both being absent in the recombinant strain.

The question of what other types of dioxygenase enzyme could catalyse the *cis*-dihydroxylation of phenol **1**, to yield the *cis*-diols **6** or **17**, was addressed using four dioxygenase-containing bacterial strains (Table 2). These were *P. putida* UV4 (a constitutive mutant source of TDO), *E. coli* CL-4t (a recombinant source of TDO), *P. putida* 9816/11 (an inducible mutant source of naphthalene dioxygenase, NDO) and *Sphingomonas yanoikuyae* B8/36 (an inducible mutant source of biphenyl dioxygenase, BPDO). 3-Hydroxybiphenyl **1k** was used as substrate since earlier studies


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**Table 2** Relative ratios[a] of metabolites $6k_S$ and **17** obtained *via* biotransformation using different dioxygenase enzymes

| Dioxygenase | Cyclohexenone-*cis* diol $6k_S$ | *cis*-Dihydrodiol **17** |
|---|---|---|
| TDO (UV4) | 61 | 39 |
| TDO (CL-4t) | 63 | 37 |
| NDO | 48 | 52 |
| BPDO | 29[b] | 71[b] |

[a] Based on peak areas using LC-TOF/MS analysis. [b] The corresponding triol **12k** was also observed as a minor metabolite.

had shown that biaryls, *e.g.* biphenyl[16] or phenyl pyridines,[17] were the only types of monocyclic arenes acceptable to each of these dioxygenases (Table 2).

LC-TOF/MS analysis showed that in each case a mixture of the *cis*-diols **17** and $6k_S$ was formed (Table 2). Whilst the protonated molecular ion, $(M + H)^+$ at $m/z$ 205 was readily detected for the cyclohexenone *cis*-diol $6k_S$, the corresponding ion for the dihydrodiol **17** was not. The sodiated molecular ion, $(M + Na)^+$, at $m/z$ 227 was strong for both metabolites $6k_S$ and **17**, and in all cases this allowed the relative ratios to be estimated from their peak areas (calibrated using pure samples of diols $6k_S$ and **17** of known concentration) in the crude aqueous culture medium (Table 2).

It should be emphasised that, while *cis*-diols $6k_S$ and **17** were detected as metabolites using all three enzymes (TDO, NDO and BPDO), the strongest preference (61–63%) for *cis*-dihydroxylation of the phenolic ring was found with TDO. Thus, the relative yields of cyclohexenone *cis*-diol $6k_S$, produced by individual strains under different culture conditions, decreased in the sequence TDO > NDO > BPDO.

In the preliminary communication[6] we reported that the TDO enzyme present in *P. putida* UV4 was responsible for the formation of these previously undetected but stable intermediate cyclohexenone *cis*-diol metabolites **6**. This study has now demonstrated that other bacterial strains and dioxygenases, can yield more members of this new family of metabolites. When the large number of anthropogenic or natural product phenols, and phenolic metabolites resulting from direct aromatic hydroxylation,[1] dehydration of *cis*-dihydrodiols,[18a] or isomerisation of arene oxides,[18b] are combined with the diverse range of bacterial strains containing arene dihydroxylating dioxygenases, present in the environment, it is anticipated that a much larger family of cyclohexenone *cis*-diols will be identified in the future.

In light of the recently available X-ray crystal structure of TDO,[14] it was timely to re-examine the earlier observation that *meta*-disubstituted benzene substrates were generally found to be poorer substrates for TDO compared with mono-substituted, *ortho*-disubstituted and *para*-disubstituted benzenes (*meta-effect*). A representation of the natural substrate, toluene, within the active site is shown in Fig. 1. This image has been generated from the published X-ray crystal structure of the enzyme[14] associated with the toluene substrate, first described in the same reference, using the PDB database Ligand Explorer (see www.pdb.org; database file code is 3EN1). Coordination of the substrate in the active site is a result of many hydrophobic interactions between the arene and active site amino acid residues. In Fig. 1 only interactions with calculated bond lengths below 1.7 Å are shown.

The binding site consists of an ellipse-shaped substrate pocket lined by 17 mainly hydrophobic residues. The *ortho* and *meta* carbon atoms on one side of the bound toluene substrate are proximate to the mononuclear non-heme iron atom. The toluene structure is held within the active site by a series of Van der Waals and hydrophobic interactions, including possibly edge-to-face bonding, to the surrounding amino acids bearing non-polar phenyl or alkyl groups, *e.g.* Phe 366, Phe 216, Ile 32. This spatial arrangement is fully consistent with the regio- and stereo-selective delivery of two Fe-bound oxygen atoms at the 2,3-bond and to only one face of the benzene ring. Of particular note, in the context of this study, is the proximity of the positively charged imidazole ring of His 311 to the *meta* position of the toluene

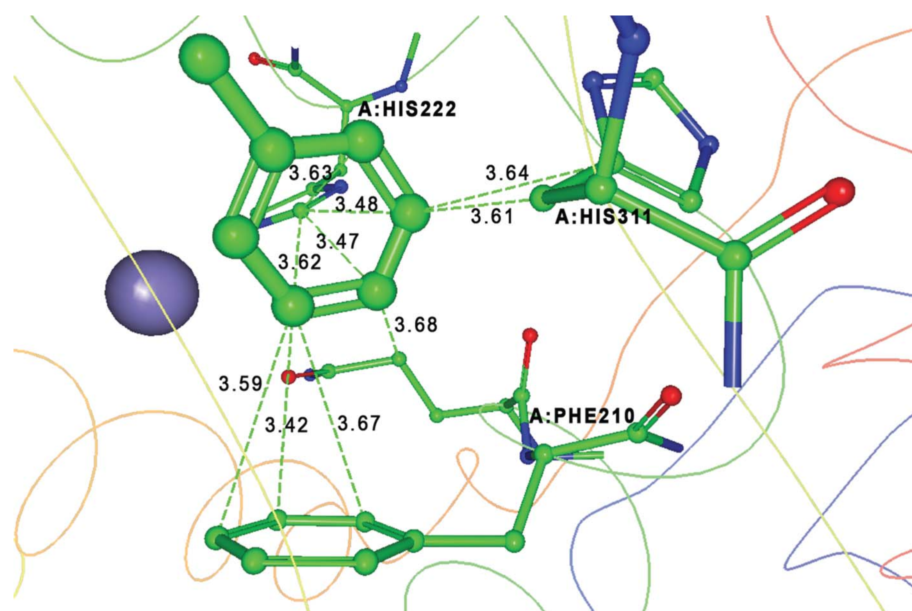


**Fig. 1** Toluene bound at the active site of TDO showing all its hydrophobic interactions with amino acid residues with bond distances calculated below 3.7 Å.

substrate furthest from the dihydroxylation site. The basicity of the imidazole ring would then result in an attractive interaction with an acidic phenolic OH group at a *meta* position and similarly a repulsive interaction when non-hydroxylic *meta*-alkyl groups are present.

Precedents for histidine-ligand hydrogen bonding/electrostatic interactions have also been revealed through analysis of X-ray crystal structures of the active sites for other enzyme-phenolic ligand complexes. These include (i) a catechol dioxygenase with pyrogallol,[19a] (ii) a toluene monooxygenase with 4-bromophenol,[19b] (iii) a *para*-hydroxyphenyl acetate hydroxylase with the natural substrate,[19c] and (iv) 1,2-dihydroxynaphthalene dioxygenase with 4-methylcatechol.[19d] A role for histidine residues in coordinating phenolic substrates in TDO, may provide an explanation for this preference for *meta*-disubstituted, and to a lesser extent *ortho*-disubstituted[6] benzene substrates bearing a phenolic hydroxyl group, and for the production of cyclohexenone *cis*-diols **6**.

While X-ray crystallography shows that there are many similarities between the active sites of TDO, BPDO and NDO there are also significant differences. Thus the histidine (His 311) in TDO is replaced by an aspartic acid (Asp 204) in BPDO and by an asparagine (Asn 297) in NDO. The stronger attractive H-bonding interaction between the *m*-phenolic OH group and the proximate imidazole ring of His 311 in TDO could explain the higher proportion (62%) of cyclohexenone *cis*-diol $\mathbf{6k}_S$ found. These attractive interactions would be weaker with proximate Asn 297 and Asp 204 amino acids and are consistent with the lower proportions (48% and 29% respectively, Table 1) of keto *cis*-diol $\mathbf{6k}_S$ observed.

## Conclusion

TDO-catalysed biotransformation of the *m*-phenols **1c**, **1f–k**, using *P. putida* UV4, yielded the enantiopure cyclohexenone *cis*-diols $\mathbf{6c}_S$, $\mathbf{6f}_S$–$\mathbf{k}_S$, which were structurally and stereochemically characterized. CRED-catalysed reductions of the cyclohexenone *cis*-diols, $\mathbf{6f}_S$–$\mathbf{j}_S$, to yield the corresponding cyclohexene *cis*-triols, **12f–j**, and ERED/CRED-catalysed reductions to give cyclohexane *cis*-triols, **11a** and **11j**, appeared to be further steps in the biodegradation of phenols by *P. putida* UV4. Other phenol metabolites identified by LC-TOF/MS analysis include the corresponding catechols **3**, $\alpha$-hydroxyacids **15** and picolinic acids **16**.

The ability of three different dioxygenases, TDO, NDO and BPDO, to catalyse the *cis*-dihydroxylation of both the phenyl and phenol rings in 3-hydroxybiphenyl **1k** has been established. The role of the TDO enzyme in the production of cyclohexenone *cis*-diols **6** has been confirmed by the use of a new *E. coli* recombinant strain over-expressing this enzyme. The ability of TDO to accept *meta*-substituted phenols as substrates, in preference to *ortho*- and *para*-phenols, may be rationalized in terms of their optimal binding and catalysis within the active site, facilitated by binding between the phenolic OH group and a proximate histidine amino acid.

## Experimental

$^1$H and $^{13}$C NMR spectra were recorded on Bruker Avance 400, DPX-300 and DRX-500 instruments. Chemical shifts ($\delta$) are reported in ppm relative to $SiMe_4$ and coupling constants ($J$) are given in Hz. Mass spectra were run at 70 eV, on a VG Autospec Mass Spectrometer, using a heated inlet system. Accurate molecular weights were determined by the peak matching method, with perfluorokerosene as the standard. CD spectra were recorded in spectroscopic grade acetonitrile using a JASCO J-720 instrument. A PerkinElmer 341 polarimeter was used for optical rotation ($[\alpha]_D$) measurements (*ca.* 20 °C, $10^{-1}$ deg cm$^2$ g$^{-1}$). Flash column chromatography and preparative layer chromatography (PLC) were performed on Merck Kieselgel type 60 (250–400 mesh) and $PF_{254/366}$ plates respectively. Merck Kieselgel type $60F_{254}$ analytical plates were employed for TLC. *m*-Phenol substrates **1c**, **1g–k**, were purchased from Aldrich and used as received. Authentic samples of catechol bioproducts derived from the corresponding *cis*-dihydrodiols and two cyclohexenone *cis*-diols $\mathbf{6c}_S$ and $\mathbf{6f}_S$ were available from earlier studies.[6,20]

Liquid chromatography-time of flight mass spectrometry (LC-TOF/MS) analyses were conducted using an Agilent 1100 series HPLC coupled to an Agilent 6510 Q-TOF (Agilent Technologies, USA). Separation was performed using a reverse phase column (Agilent Eclipse Plus C18, 5 μm, 150 × 2.1 mm) together with the corresponding guard column (5 μm, 12.5 × 2.1 mm). The mobile phase consisted of 95% methanol containing 0.1% formic acid in channel A, and 5% methanol containing 0.1% formic acid in channel B. The system was programmed to perform an analysis cycle consisting of 100% B for 1 min, followed by gradient elution from 100% to 5% B over a 14 min period, hold at 5% B for 10 min, return to initial conditions over 2 min and then hold these conditions for a further 8 min. The flow rate was 0.20 ml min$^{-1}$ and the injection volume was 5 μl. MS experiments were carried out using ESI in positive ion mode with the capillary voltage set at 4.0 kV. The desolvation gas was nitrogen set at a flow rate of 8 L min$^{-1}$ and maintained at a temperature of 350 °C.

### Construction of recombinant biocatalyst strain

A derivative of the arabinose $P_{BAD}$ promoter vector pBAD24 was used.[21] This vector, pBADET (obtained from Dr V Ksenzenko), has a translation start codon within a unique NdeI restriction site. Primers were designed for cloning of TDO genes from *Pseudomonas putida* F1 (Accession number for the DNA sequence used is J04996). Forward primer (611–636 nt): 5′-GAG AAG <u>CAT</u> <u>ATG</u> AAT CAG ACC GAC ACA TC-3′ and Reverse primer (4199–4229 nt): 5′-GC<u>G</u> <u>AAT</u> <u>TC</u>G CCT TCA AGT CTC AGC TTA GGT C-3′ *NdeI* restriction site was introduced into the 5′- part of the forward primer and *Eco*RI site into the reverse primer (sites are underlined). A DNA fragment amplified with these primers included all four TDO genes: *todC1*, *todC2*, *todA* and *todB*. PCR amplification was performed with PfuTurbo polymerase obtained from Stratagene. Reaction conditions were as follows: 95 °C for 3 min and then 32 cycles of 95 °C (30 s), 55 °C (30 s) and 72 °C (4.5 min). Fragments were purified using PCR DNA and a Gel Band Purification kit (Amersham), and digested with the corresponding enzymes. The PBAD-ET DNA fragment was digested with both NdeI and *Eco*RI restriction enzymes prior to cloning. Ligated DNA was transformed into *E. coli* TOP10 cells (Invitrogen). Transformation mixtures were plated onto 2YT media containing ampicillin (100 μg ml$^{-1}$) and arabinose (0.02%). Colonies were analysed after incubation at 30 °C for 24–30 h.


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The *E. coli* TOP10 clones containing TDO genes were initially selected by their ability to convert indole to indigo and then the activity of TDO was confirmed in biotransformation experiments. The pCL-4t plasmid, was constructed with the complete TDO sequence from *P. putida* UV4. The cloned genes were under a tight regulation by the $P_{BAD}$ promoter (Fig. 2) that allowed efficient modulation of its expression by addition of an inducer (arabinose) to cultivation media. *E. coli* TOP10 cells containing this plasmid were used in all subsequent biotransformation experiments.

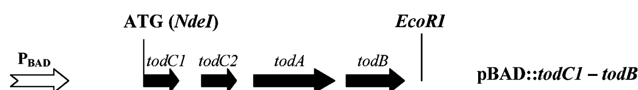


**Fig. 2** Construction of the TDO biocatalyst. Genes encoding subunits of the F1/UV4 TDO are shown as black arrows. The transcription of the system is determined by a $P_{BAD}$ promoter and indicated by the white arrow.

## General procedure

Small scale (0.2–4.00 g) shake flask biotransformations, with the whole cells of *P. putida* UV4 (TDO), *P. putida* 9816/11 (NDO), *S. yanoikuyae* B8/36 (BPDO) and the *E. coli* CL-4t recombinant strains were performed using methods described earlier[22–24] for non-phenolic aromatic substrates. The biotransformation conditions were not optimised for the phenol substrates used in this study and isolated yields (5–70%) of cyclohexenone *cis*-diols, **6f**$_S$–**j**$_S$, varied between different strains and repeat biotransformations. The aqueous culture medium, obtained after the biotransformation, was concentrated under reduced pressure at *ca.* 40 °C, the concentrate repeatedly extracted (EtOAc), and the extract concentrated under reduced pressure to give the crude mixture of bioproducts. $^1$H-NMR spectra of the bioproduct mixtures were routinely recorded, before further purification. The bioproducts were separated either by flash column chromatography and/or PLC. The enantiomeric excess (*ee*) values of metabolites **6c**$_S$, **6f**$_S$–**k**$_S$, were indirectly estimated from NMR spectroscopic analysis of their boronate derivatives. The (–)-(*S*)- and (+)-(*R*)-2-(1-methoxyethyl)benzene boronic acids were synthesised and used according to the literature method.[7a–7c] Phenol metabolism also yielded the corresponding known 2,3-catechols, ($^1$H-NMR analysis) but these were not investigated further.

## Biotransformations of phenols and enone *cis*-diols using *P. putida* UV4

**(i) Biotransformation of *m*-cresol 1c.** Chromatographic separation and purification from the crude mixture of bioproducts from substrate **1c**, yielded enone *cis*-diol **6c**$_S$ (*ca.* 13% yield) as reported earlier.[6]

**(4*R*,5*S*)-4,5-Dihydroxy-3-methyl-cyclohex-2-enone 6c$_S$.** $[\alpha]_D$ –115 (*c* 1.0, MeOH); LC-TOF/MS: Retention time 4.89 min; *m/z* obs. 143.06977 calcd. 143.07027 for $[M + H]^+$; *m/z* obs. 165.05221 calcd. 165.05222 for [M + Na].$^+$ Spectroscopic data was identical to that reported.[6]

**(ii) Biotransformation of 3-iodophenol 1f.** The crude mixture of bioproducts yielded four compounds. Three of these, *viz*, (4*R*,5*S*)-4,5-dihydroxy-3-iodocyclohex-2-enone **6f**$_S$, (4*R*)-hydroxycyclohexenone **10a** and (1*R*,2*S*,4*S*)-cyclohexane-1,2,4-triol **11a** have already been reported[6] and the fourth, iodo-1,2,3-triol **12f**$_S$, isolated after PLC, was a new bioproduct.

**(1*S*,2*S*,4*R*)-6-Iodo-cyclohex-5-ene-1,2,3-triol 12f$_S$.** White crystals (*ca.* 2% yield), m.p. 102 °C (EtOAc); $R_f$ (0.48, EtOAc); $[\alpha]_D$ –60 (*c* 0.85, MeOH); LC-TOF/MS: Retention time 7.08 min; *m/z* obs. 256.9681 calcd. 256.9669 for $[M + H]^+$; *m/z* obs. 278.9480 calcd. 278.9489 for [M + Na]; HRMS (EI): Found: $[M - H_2O]^+$ 237.9505 requires $C_6H_7O_2I$ 237.9491; $^1$H-NMR (500 MHz, $CD_3OD$) $\delta$ 6.49 (1H, td, $J$ = 2.2, 1.1 Hz, 5-H), 4.13 (2H, m, 1-H, 4-H), 3.69 (1H, ddt, $J$ = 12.4, 5.4, 3.8 Hz, 2-H), 2.04 (1H, m, $J$ = 12.3 Hz, 3-H), 1.75 (1H, dddd, $J$ = 12.1, 12.1, 9.9, 2.2 Hz, 3-$H_a$); $^{13}$C-NMR (125 MHz, $CD_3OD$) $\delta$ 146.2, 101.7, 77.1, 69.8, 68.6, 35.1; *ee* ≥ 98%.

**(iii) Biotransformation of 3-ethylphenol 1g.** After separation and purification by PLC, enonediol **6g**$_S$ was isolated as the major product with two other metabolites, **12g** and **15g**, in trace amounts.

**(4*R*,5*S*)-4,5-Dihydroxy-3-ethyl-cyclohex-2-enone 6g$_S$.** White silky needles (*ca.* 60% yield), m.p. 77–78 °C ($Et_2O$–hexane); $R_f$ (0.24, EtOAc–hexane, 3 : 1); $[\alpha]_D$ –103 (*c* 0.94, MeOH); LC-TOF/MS: Retention time 11.4 min; *m/z* obs. 157.0856 calcd. 157.0859 for $[M + H]^+$; *m/z* obs. 179.0678 calcd. 179.0679 for [M + Na].$^+$; HRMS (EI): Found 156.0797 requires $C_8H_{12}O_3$ 156.0786; $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$ 5.91 (1H, s, 2-H), 4.35 (1H, m, 4-H), 4.24 (1H, m, 5-H), 3.671 (2H, m, 2 x OH), 2.74 (1H, dd, $J$ = 7.3, 16.4 Hz, 6-H), 2.57 (1H, dd, $J$ = 3.3, 16.4 Hz, 6″-H), 2.43 (2H, m, $J$ = 7.0 Hz, C$H_2$Me), 1.14 (3H, t, $J$ = 7.0 Hz, $CH_2$*Me*); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$ 198.3, 165.1, 125.5, 69.6, 69.1, 42.4, 27.4, 11.5; IR (KBr) $\nu_{max}$/cm$^{-1}$ 1664 ($\alpha,\beta$ unsaturated ketone); *ee* ≥ 98%.

**6-Ethylcyclohex-5-ene-1,2,3-triol 12g.** LC-TOF/MS: Retention time 11.98 min; *m/z* obs. 181.08373 calcd. 181.08352 for [M + Na].$^+$

**2- Hydroxyl-6-oxooctanoic acid 15g.** LC-TOF/MS: Retention time 14.42 min; *m/z* obs. 197.0782 calcd. 197.0783 for $[M + Na]^+$, *m/z* obs. 213.0519 calcd. 213.0524 for $[M + K]^+$, 157.0859 calcd. 157.0859 for $[MH - H_2O]$.$^+$

**(iv) Biotransformation of 3-*iso*-propylphenol 1h.** The crude mixture of bioproducts yielded enonediol **6h**$_S$ as the major product (after PLC) and the following two metabolites, **12h** and **15h**, in trace amounts.

**(4*R*,5*S*)-4,5-Dihydroxy-3-*iso*-propylcyclohex-2-enone 6h$_S$.** White crystals (*ca.* 65% yield), m.p. 39–40 °C; $R_f$ (0.24, EtOAc–hexane, 3 : 1); $[\alpha]_D$ –79 (*c* 1.00, MeOH); LC-TOF/MS: Retention time 13.62 min; *m/z* obs. 171.1022 calcd. 171.1016 for $[M + H]^+$; *m/z* obs. 193.0841 calcd. 193.0835 for [M + Na].$^+$; HRMS (EI): Found 170.0943 requires $C_9H_{14}O_3$ 170.0943; $^1$H-NMR (400 MHz, $CDCl_3$) $\delta$ 5.92 (1H, s, 2-H), 4.42 (1H, dd, $J$ = 3.4, 3.4 Hz, 4-H), 4.28 (1H, dq, $J$ = 7.9, 4.0 Hz, 5-H), 3.50 (1H, d, $J$ = 3.5 Hz, OH), 3.43 (1H, d, $J$ = 3.4 Hz, OH), 2.78 (1H, m, -C*H*$Me_2$), 2.74 (1H, m, $J$ = 17.0 Hz, 6-H), 2.57 (1H, dd, $J$ = 3.8, 17.0 Hz, 6″-H), 1.16 (3H, d, $J$ = 6.7 Hz, Me), 1.10 (3H, d, $J$ = 7.0 Hz, Me); $^{13}$C-NMR (100 MHz, $CDCl_3$) $\delta$ 198.5, 168.8 124.5, 69.6, 68.3, 42.1, 32.4, 21.7, 20.2; IR (KBr) $\nu_{max}$/cm$^{-1}$ 1666 ($\alpha,\beta$ unsaturated ketone); *ee* ≥ 98%.

**6-*iso*-Propylcyclohex-5-ene-1,2,3-triol 12h.** LC-TOF/MS: retention time 14.25 min; *m/z* obs. 195.0982 calcd. 195.0992 for $[M + Na]^+$.

**2- Hydroxyl-7-methyl-6-oxooctanoic acid 15h.** LC-TOF/MS: Retention time 15.37 min; *m/z* obs. 189.1125 calcd. 189.1121 for $[M + H]^+$, *m/z* obs. 211.0933 calcd. 211.0941 for $[M + Na]^+$, *m/z* obs. 206.1390 calcd. 206.1387 for $[M + NH_4]^+$, *m/z* obs. 227.0680 calcd. 227.0676 for $[M + K]^+$, 171.1018 calcd. 171.1016 for $[MH - H_2O]^+$.

**(v) Biotransformation of 3-*tert*-butylphenol 1i.** The crude mixture of bioproducts yielded enonediol $\mathbf{6i}_S$ as the major product and its corresponding triol $\mathbf{12i}_S$ and $\alpha$-hydroxyacid **15i** as the minor bioproducts. All proved to be separable using PLC.

**(4*R*,5*S*)-4,5-Dihydroxy-3-*tert*-butylcyclohex-2-enone $\mathbf{6i}_S$.** Colourless crystals (*ca.* 68% yield), m.p. 57–58 °C (acetone–hexane); $R_f$ (0.36, EtOAc–hexane, 3 : 1); *ee* ≥ 98%; $[\alpha]_D$ −9 (*c* 0.8, MeOH); LC-TOF/MS: Retention time 15.41 min; *m/z* obs. 185.1164 calcd. 185.1172 for [M + H];$^+$ *m/z* obs. 207.0992 calcd. 207.0992 for [M + Na].$^+$; HRMS (EI): Found 184.1096 requires $C_{10}H_{16}O_3$ 184.1099; $^1$H-NMR (400 MHz, $CDCl_3$) $\delta$ 6.02 (1H, s, 2-H), 4.49 (1H, m, 4-H), 4.00 (1H, m, 5-H), 3.11 (1H, m, OH), 2.96 (1H, m, OH), 2.77 (1H, dd, *J* = 11.4, 16.3 Hz, 6-H), 2.60 (1H, dd, *J* = 5.3, 16.3 Hz, 6′-H), 1.21 (9H, s, C*Me*$_3$); $^{13}$C-NMR (100 MHz, $CDCl_3$) $\delta$ 199.0, 168.6, 126.0, 69.1, 66.3, 40.4, 36.4, 28.9; IR (KBr) $\nu_{max}$/cm$^{-1}$ 1651 ($\alpha$,$\beta$ unsaturated ketone); *ee* ≥ 98%.

**(1*R*,2*S*,4*R*)-6-*tert*-Butylcyclohex-5-ene-1,2,4-triol $\mathbf{12i}_S$.** White crystals (*ca.* 5% yield), m.p. 107 °C ($CH_2Cl_2$); $R_f$ (0.57, MeOH–$CHCl_3$, 1 : 4); $[\alpha]_D$ −21 (*c* 1.25, MeOH); LC-TOF/MS: Retention time 15.78 min; *m/z* obs. 209.1148 calcd. 209.1148 for $[M + Na]^+$; *m/z* obs. 204.1590 calcd. 204.1594 For $[M + NH_4]^+$; HRMS (EI): Found: $[M - H_2O]^+$ 168.1161 requires $C_{10}H_{16}O_2$ 168.1150; $^1$H-NMR (400 MHz, $CD_3OD$) $\delta$ 5.57 (1H, dd, *J* = 2.9, 2.9 Hz, 5-H), 4.08 (1H, m, *J* = 10.2, 2.9, 2.9 Hz, 4-H), 4.05 (1H, dd, *J* = 6.7, 2.9 Hz, 1-H), 3.11 (1H, ddd, *J* = 12.2, 2.9, 2.9 Hz, 2-H), 1.90 (1H, m, *J* = 10.2, 2.9 Hz, 3-$H_e$), 1.68 (1H, ddd, *J* = 12.2, 12.2, 10.2 Hz, 3′-$H_a$); $^{13}$C-NMR (100 MHz, $CD_3OD$) $\delta$ 148.3, 127.8, 70.0, 67.9, 67.3, 35.8, 34.6, 30.3; *ee* ≥ 98%.

**2-Hydroxy-7,7-dimethyl-6-oxooctanoic acid 15i.** Light yellow viscous oil (< 1% yield); $R_f$ (0.26, MeOH–$CHCl_3$, 1 : 3); $[\alpha]_D$ +8.5 (*c* 0.41, MeOH); MS (ES$^-$) Found: 202.1128 requires $C_{10}H_{17}O_4$ 201.1127; LC-TOF/MS: Retention time 16.98 min; *m/z* obs. 203.12808 calcd. 203.12779 for $[M + H]^+$, *m/z* obs. 220.1543 calcd. 220.1543 for $[M + NH_4]^+$, *m/z* obs. 225.1091 calcd. 225.1097 for $[M + Na]^+$, *m/z* obs. 241.0840 calcd. 241.0837 for $[M + K]^+$, *m/z* obs. 185.1168 calcd. 185.1172 for $[MH - H_2O]$; $^1$H-NMR (400 MHz, $CD_3OD$) $\delta$ 3.94 (1H, dd, *J* = 3.8, 7.1 Hz, 2-H), 2.62 (2H, t, *J* = 7.3 Hz, 5-H), 1.68 (4H, m, 2 × 3-H, 2 × 4-H), 1.17 (9H, s, -C*Me*$_3$); $^{13}$C-NMR (100 MHz, $CD_3OD$) $\delta$ 218.7, 181.6, 73.4, 45.2, 37.6, 35.8, 26.7, 21.0.

**Biotransformation of 3-trifluoromethylphenol 1j.** The mixture of bioproducts was separated by PLC and was found to contain enonediol $\mathbf{6j}_S$ as the minor product and the corresponding triols $\mathbf{12j}_S$ and **11j** as the major metabolites.

**(4*R*,5*S*)-4,5-Dihydroxy-3-trifluromethylcyclohex-2-enone $\mathbf{6j}_S$.** Light yellow viscous oil (*ca.* 4% yield); $R_f$ (0.31, MeOH–$CHCl_3$, 7 : 93); $[\alpha]_D$ −65 (*c* 1.00, MeOH); LC-TOF/MS: Retention time 10.58 min. *m/z* obs. 197.0425 calcd. 197.0420 for $[M + H]^+$; *m/z* obs. 219.0246 calcd. 219.0240 for [M + Na].$^+$; HRMS (EI): Found: 196.0344 requires $C_7H_7O_3F_3$ 196.0347; $^1$H-NMR (400 MHz, $CD_3OD$) $\delta$ 6.41 (1H, s, 2-H), 4.58 (1H, d, *J* = 3.3 Hz, 4-H), 4.28 (1H, ddd, *J* = 9.6, 4.1, 3.3 Hz, 5-H), 2.83 (1H, dd, *J* = 9.6, 16.8 Hz, 6-H), 2.66 (1H, dd, *J* = 4.1, 16.8 Hz, 6″-H); $^{13}$C-NMR (100 MHz, $CD_3OD$) $\delta$ 198.7, 146.9 (q, *J* = 30.8 Hz), 130.9 (q, *J* = 5.2 Hz), 124.4 (q, *J* = 272.4 Hz, $CF_3$), 69.7, 65.9, 42.7; IR(liquid film) $\nu_{max}$/cm$^{-1}$ 1694 (α,β unsaturated ketone); *ee* ≥ 98%.

**(1*R*,2*S*,4*R*)-6-Trifluoromethylcyclohex-5-ene-1,2,4-triol $\mathbf{12j}_S$.** White crystals (*ca.* 6% yield), m.p. 144–45 °C (EtOAc); $R_f$ (0.46, MeOH–$CHCl_3$, 1 : 4); $[\alpha]_D$ −29 (*c* 0.68, MeOH); LC-TOF/MS: Retention time 9.21 min; *m/z* obs. 221.0383 calcd. 221.0396 for $[M + Na]^+$; *m/z* obs. 216.0831 calcd. 216.0842 for $[M + NH_4]$.$^+$; HRMS (EI): Found: $[M - H_2O]^+$ 180.0405 requires $C_7H_7O_2F_3$ 180.0398; $^1$H-NMR (500 MHz, $CD_3OD$) $\delta$ 6.47 (1H, s, 5-H), 4.32 (1H, m, *J* = 12.3, 2.6 Hz, 4-H), 4.05 (1H, d, *J* = 3.6 Hz, 1-H), 3.61 (1H, ddd, *J* = 3.6, 12.3, 3.6 Hz, 2-H), 2.09 (1H, m, *J* = 3.6, 10.3, 2.6 Hz, 3-H), 1.68 (1H, ddd, *J* = 10.3, 12.3, 12.3 Hz, 3′-H); $^{13}$C-NMR (100 MHz, $CD_3OD$) $\delta$ 139.4 (q, *J* = 5.3 Hz), 130.6 (q, *J* = 29.2 Hz), 125.4 (q, *J* = 270.1 Hz, $CF_3$), 68.4, 66.8, 64.7, 34.5; *ee* ≥ 98%.

**(1*R*,2*S*,4*S*,6*R*)-6-Trifluoromethylcyclohexane-1,2,4-triol 11j.** Colourless viscous liquid (*ca.* 10% yield); $R_f$ (0.35, MeOH–$CHCl_3$, 1 : 4); $[\alpha]_D$ −3.0 (*c* 1.0, MeOH); LC-TOF/MS: Retention time 5.73 min. *m/z* obs. 201.0743 calcd. 201.0733 for $[M + H]^+$; *m/z* obs. 223.0565 calcd. 223.0553 for $[M + Na]^+$; *m/z* obs. 218.1006 calcd. 218.0999 for $[M + NH_4]^+$; $^1$H-NMR (400 MHz, $CD_3OD$) $\delta$ 3.93 (1H, m, 1-H), 3.54 (1H, tt, *J* = 11.7, 3.8 Hz, 4-H), 3.41 (1H, ddd, *J* = 12.0, 4.4, 2.8, 2-H), 2.18 (1, m, 6-H), 1.82 (1H, m, 3-$H_e$), 1.72 (1H, m, 5-$H_e$), 1.59 (2H, q, 12.0, 3-$H_a$ and 5-$H_a$); $^{13}$C-NMR (100 MHz, $CD_3OD$) $\delta$ 128.6 (q, *J* = 278.1 Hz, $CF_3$), 70.7, 67.8, 67.3 (q, *J* = 2.7 Hz), 43.4 (q, 26.4), 37.9, 28.5 (q, *J* = 2.1 Hz).

**(vii) Biotransformation of 3-phenylphenol 1k.** PLC separation of the mixture of bioproducts yielded enonediol $\mathbf{6k}_S$ as the major compound, dihydrodiol **17** as the minor metabolite and two metabolites, **12k** and **15k**, in trace amounts.

**(4*R*,5*S*)-4,5-Dihydroxy-3-phenylcyclohex-2-enone $\mathbf{6k}_S$.** White crystals (*ca.* 28% yield), m.p. 83–84 °C ($CHCl_3$); $R_f$ (0.40, EtOAc–hexane, 3 : 1); $[\alpha]_D$ −20 (*c* 0.74, MeOH); LC-TOF/MS: Retention time 15.52 min; *m/z* obs. 205.0853 calcd. 205.08592 for $[M + H]^+$; *m/z* obs. 227.0672 calcd. 227.0679 for [M + Na].$^+$; HRMS (EI): Found 204.0788 requires $C_{12}H_{12}O_3$ 204.0786; $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$ 7.65 (2H, m, Ar-H), 7.46 (3H, m, Ar-H), 6.42 (1H, s, 2-H), 4.92 (1H, m, 4-H), 4.33 (1H, m, 5-H), 2.88 (1H, dd, *J* = 9.8, 16.3 Hz, 6-H), 2.72 (2H, m, *J* = 4.4, 16.3 Hz, 6′-H and OH), 2.55 (1H, d, *J* = 5.8, OH); $^{13}$C-NMR (100 MHz, $CDCl_3$) $\delta$ 197.4, 156.1, 136.5, 130.5, 129.1, 127.0, 126.8, 68.7, 68.0, 41.1; IR (KBr) $\nu_{max}$/cm$^{-1}$ 1648 (α,β unsaturated ketone); *ee* ≥ 98%.

**(1*S*,2*R*)-1,2-Dihydroxy-3-(3′-hydroxyphenyl)cyclohexa-3,5-diene 17.** White crystals (*ca.* 10% yield); m.p. 137–38 °C


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(EtOAc–hexane); $R_f$ (0.38, EtOAc–hexane, 3 : 1); $[\alpha]_D$ +222 ($c$ 1.09, MeOH); LC-TOF/MS: Retention time 15.90 min; $m/z$ obs. 227.0672 calcd. 227.0679 for [M + Na].$^+$; MS (ES$^-$) Found: 203.0697 requires $C_{12}H_{11}O_3$ 203.0708; $^1$H-NMR (400 MHz, $CD_3OD$) $\delta$ 7.05 (1H, dd, $J$ = 7.8, 7.8 Hz, 5′-H), 6.95 (1H, m,, $J$ = 7.8, 2.2, 1.0 Hz, 4′-H), 6.91 (1H, dd, $J$ = 2.2, 1.0 Hz, 2′-H), 6.59 (1H, ddd, $J$ = 7.8, 2.2, 1.0 Hz, 6′-H), 6.27 (1H, d, $J$ = 6.0 Hz, 4-H), 5.96 (1H, ddd, $J$ = 9.5, 6.0, 2.9 Hz, 5-H), 5.73 (1H, m, $J$ = 9.5, 6.0 Hz, 6-H), 4.41 (1H, m, $J$ = 6.0 Hz, 1-H), 4.26 (1H, d, $J$ = 6.0 Hz, 2-H); $^{13}$C-NMR (100 MHz, $CD_3OD$) $\delta$ 158.7, 142.2, 139.5, 132.3, 130.5, 125.0, 122.8, 118.0, 115.6, 113.3, 72.7, 69.9; CD (MeCN) $\lambda$ 208 nm ($\Delta\varepsilon$ −11.37), $\lambda$ 212.6 nm ($\Delta\varepsilon$ −9.14), $\lambda$ 229.8 nm ($\Delta\varepsilon$ −13.57), $\lambda$ 320 nm ($\Delta\varepsilon$ −6.34); $ee \geq 98\%$.

**6-Phenylcyclohex-5-ene-1,2,3-triol 12k.** LC-TOF/MS: Retention time 15.16 min; $m/z$ obs. 229.0838 calcd. 229.0835 for [M + Na].$^+$

**2-Hydroxyl-6-phenyl-6-oxohexanoic acid 15k.** LC-TOF/MS: Retention time 19.70 min; $m/z$ obs. 223.0972 calcd. 223.0965 for [M + H]$^+$, $m/z$ obs. 245.0793 calcd. 245.0784 for [M + Na].$^+$

**(viii) Biotransformation of (4*R*,5*S*)-4,5-dihydroxy-3-iodocyclohex-2-enone 6f$_S$.** The mixture of bioproducts obtained from substrate **6f**$_S$ (0.100 g), was separated by a combination of column chromatography (hexane → EtOAc → 10% MeOH in EtOAc) and PLC (EtOAc–hexane, 1 : 1) to give the reported metabolites (+)-(*R*)-4-hydroxycyclohex-2-enone **10a** (0.011 g)[6], (−)-(1*R*,2*S*,4*S*)-cyclohexane-1,2,4-triol **11a** (0.015 g)[6] and (−)-(1*S*,2*S*,4*R*)-6-iodo-cyclohex-5-ene-1,2,3-triol **12f**$_S$ (0.018 g) having identical properties to those obtained using phenol **1f** as substrate.

**(ix) Biotransformation of (4*R*,5*S*)-4,5-dihydroxy-3-*tert*-butylcyclohex-2-enone 6i$_S$.** The only compound obtained after PLC of the crude mixture from substrate **6i**$_S$ (0.030 g) was identified as (−)-(1*R*,2*S*,4*R*)-6-*tert*-butylcyclohex-5-ene-1,2,3-triol **12i**$_S$ (0.02 g). This product was indistinguishable from the sample produced earlier using the phenol **1i**.

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