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Enzyme-Catalysed Synthesis of Cyclohex-2-en-1-one cis-Diols from Substituted Phenols, Anilines and Derived 4-Hydroxycyclohex-2-en-1-ones


Abstract: Toluene dioxygenase-catalysed cis-dihydroxylation of substituted aniline and phenol substrates, with a Pseudomonas putida UV4 mutant strain and an Escherichia coli pCL4-1t recombinant strain, yielded identical arene cis-dihydrodiols, which were isolated as the preferred cyclohex-2-en-1-one cis-diol tautomers. These cis-diol metabolites were predicted by preliminary molecular docking studies, of anilines and phenols, at the active site of toluene dioxygenase. Further biotransformations of cyclohex-2-en-1-one cis-diol and hydroquinone metabolites, using Pseudomonas putida UV4 whole cells, were found to yield 4-hydroxycyclohex-2-en-1-ones as a new type of phenol bioproduct. Multistep pathways, involving ene reductase and carbonyl reductase-catalysed reactions, were proposed to account for the production of 4-hydroxycyclohex-2-en-1-one metabolites. Evidence for the phenol hydrate tautomers of 4-hydroxycyclohex-2-en-1-one metabolites was shown by formation of the corresponding trimethylsilyl ether derivatives.

Keywords: aniline biotransformations; cyclohex-2-en-1-one cis-diols; 4-hydroxycyclohex-2-en-1-ones; phenol hydrates.

Introduction

Enzyme-catalysed formation of phenol metabolites, from aromatic substrates, can proceed directly [1a] or indirectly, via transient intermediates. Phenols have been obtained indirectly by: (a) monoxygenase- or peroxoxygenase-catalysed arene epoxidation and spontaneous isomerization of arene oxide-oxepine intermediates [1b–c] or (b) dioxygenase-catalysed cis-dihydroxylation of arenes [2a–m] and dehydration of cis-dihydrodiol intermediates [3a,b]. Since their discovery by Gibson et al. in 1968 [2a] more than four hundred cis-dihydrodiol metabolites of substituted monocyclic and polycyclic arenes have been isolated and identified, mainly, Pseudomonas putida mutant and Escherichia coli recombinant strains, expressing ring-hydroxylating dioxygenases [2b–m]. Synthetic applications of enantiopure substituted benzene cis-dihydrodiols 2, obtained as toluene dioxygenase (TDO)-catalysed cis-dihydroxylation products, from monosubstituted benzene substrates 1, continue to be widely reported, despite their limited stability [2b–m]. Dehydration of most cis-dihydrodiols 2, to yield a mixture of meta- and ortho-phenols 3 and 4, can occur at ambient temperature. The rate of dehydration and ratio of phenol isomers depend on the type of
Since phenols are widely distributed in the environment, as natural products, arene metabolites and environmental pollutants, their biodegradation pathways have been extensively studied. Bacterial metabolism of phenols often results in a wider range of metabolites, compared with most non-phenolic aromatic substrates. Recent biotransformation results of TDO-catalysed oxidations of phenols, e.g. 3 and 4, using P. putida UV4, showed that, in addition to the expected, catechol and hydroquinone metabolites, the corresponding cyclohex-2-en-1-one cis-diols 6a-d, 8a and 8b were also isolated, as the preferred keto tautomers of the initially formed enolic cis-dihydrodiols 5a-d, 7a and 7b. The dihydroxylation was regio- and stereo-selective and cyclohex-2-en-1-one cis-diols 6a-d, 8a and 8b were often found to be the major isolated metabolites. More than twenty members of this new cis-diol family have now been isolated.

The biodegradation pathways, for anilines, have also been studied, due to their presence in the environment, as a result of the partial combustion of tobacco and automotive fuels, the application of pesticides / herbicides and the production of pharmaceuticals, dyestuffs and textiles. Many aniline derivatives are known to be genotoxic and cytotoxic, severely inhibiting cell growth in soil bacteria and slowing their mineralization.

Similar to the metabolism of electron-rich phenols, ring hydroxylating dioxygenase-catalysed biotransformations of electron-rich anilines (using Pseudomonads and other bacterial species) have also been reported, to yield catechol and hydroquinone metabolites. Aniline-, biphenyl-, diphenylamine- and toluene-dioxygenases, have been reported to catalyse the oxidation of aniline substrates. Although cis-diol metabolites were often postulated as intermediates leading to the formation of catechol, hydroquinone and phenol metabolites of anilines, to date none have been detected or isolated. Thus, TDO-catalysed cis-dihydroxylation, at the 1,2- and 2,3-bonds of 4-chloroaniline, was proposed (using P. putida T57) as a possible initial step in the formation of both catechol and phenol metabolites. Biotransformations using other substituted anilines and bacterial strains, were also found to yield catechol and hydroquinone metabolites. In silico molecular docking studies, on aniline substrates, were thus conducted to: (a) predict the most favourable structures of expected metabolites and (b) compare the substrate docking results with the experimental results of TDO-catalysed cis-dihydroxylation of aniline substrates.

**Results and Discussion**

**P. putida UV4 biotransformations of aniline and phenol substrates, to yield cyclohex-2-en-1-one cis-diols and 4-hydroxycyclohex-2-en-1-ones**

(i) Molecular docking of meta-substituted aniline substrates 9a-d with TDO

Recent molecular docking studies, of the meta substituted phenols 3a and 3b, at the active site of TDO, were based on a comparison with an X-ray crystal structure of TDO and docked toluene substrate. These studies of TDO, without dioxygen incorporation (3EN1M model), provided preferred orientations of phenol substrates 3a and 3b (Scheme 1)
Although BPDO aniline the prediction that the active site of biphenyl dioxygenase (BPDO) model for diphenylamine interactions. The site, might also apply to aniline phenols with His 321, Ile 366) groups. It was speculated that similar binding of phenols with His-311 and Gln-215, at the TDO active site, might also apply to aniline substrate interactions.\(^{(56)}\) An earlier \textit{in silico} molecular binding model for diphenylamine 1 (R=NHPh, Scheme 1), at the active site of biphenyl dioxygenase (BPDO) led to the prediction that cis-dihydroxylation would yield aniline cis-dihydrodiol intermediate 2 (R=NHPh).\(^{(6c,6d)}\) Although BPDO-catalysed cis-dihydroxylation of substituted aniline 1 (R=NHPh)\(^{(6d)}\) did not result in the detection of cis-diol 2 (R=NHPh), it was postulated that formation of the major metabolite, phenol 4 (R=NHPh), had resulted from dehydration of this transient intermediate.\(^{(6d)}\)

The qualitative nature of docking results recorded may not quantitatively reflect the experimental results (using \textit{P. putida} UV4 whole cells), due to further metabolism by the co-induced enzymes. Apart from this caveat, the 3EN1M and 3EN1M-O\(_2\) models, employed for TDO docking studies\(^{(6)}\) of phenol substrates 3, were found to be useful predictors of the preferred regiochemistry and stereocchemistry of cis-diol metabolites. These models have now been applied to cis-dihydroxylation of aniline substrates 9a-d (Scheme 2). From analysis of the data collected, it was predicted that the NH-imines 11a-d would be the preferred tautomers of the initially formed enamine cis-diols 10a-d. It was also assumed that: (i) this type of imine would readily hydrolyse, during the biotransformation, to yield the corresponding cyclohex-2-en-1-one tautomers, derived from phenols, e.g. 6a and 6b, were single enantiomers, having an (S) absolute configuration at C-5. The main attractive interactions, at the TDO active site, involved: (i) hydrogen bonding of the phenol OH group with the C=O group of Gln-215 and the imidazole ring of His-311, (ii) van der Waals interactions of the hydrophobic Me group of the phenol with the proximate alkyl (Ala-223, Val-309, Leu-321, Ile-324) and aryl (Phe-366) groups.

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\textit{cis-Dihydroxylation}, at the 4,5-bond and % formation of enamine \textit{cis}-diols 10a-d, was predicted (Table 1), from TDO docking orientations (using 3EN1M model), for \textit{meta} substituted anilines 9a (Fig. 1A, 47%), 9b (Fig. 1B, 90%), 9c (Fig. 1C, 80%) and 9d (Fig. 1D, 100%). The substrate binding, according...
Table 1. Predicted, using the 3EN1M model of TDO, initial metabolites 11a-d, catechols 13a-c and isolated cis-diol 6a-d products of 3-substituted aniline substrates 9a-d.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Figure</th>
<th>Predicted</th>
<th>Product</th>
<th>%φ</th>
<th>ΔGd</th>
<th>K_Dφ</th>
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<tr>
<td>9a 3-Methoxyaniline</td>
<td>1A</td>
<td>11a</td>
<td>6a</td>
<td>47</td>
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<tr>
<td></td>
<td></td>
<td>13a</td>
<td></td>
<td>26</td>
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<td>216.49</td>
</tr>
<tr>
<td>9b 3-Methylaniline</td>
<td>1B</td>
<td>11b</td>
<td>6b</td>
<td>90</td>
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<td></td>
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<td>13b</td>
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<td>4</td>
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<td>11d</td>
<td>6d</td>
<td>100</td>
<td>-6.13</td>
<td>31.88</td>
</tr>
</tbody>
</table>

* Predicted metabolite; b Detected product; c Orientation occurrence; d Binding energy (kJ/mol); e Dissociation constant (µM)

Figure 1. Molecular docking of meta substituted anilines 9a-d (Figs 1A-1D) at the active site of TDO

to the in silico docking, was facilitated by two major interactions: (i) van-der- Waals interaction of Ala-223, Val-309, Leu-321, Ile-324, Phe-366 and Phe-372 amino acid residues with the substituents (Me, OMe, CF₃, I) and (ii) H-bonding of the NH₂ group to Gln 215, and sometimes to His-311, in a similar manner to the docking of phenols.⁵⁰
The docking experiments (3EN1M model) of anilines 9a-d also led to the prediction that catechols 13a-c might also be formed, via a minor pathway (4-26% docking orientations), by dihydroxylation at the 1,2-position, to yield intermediate cis-diols 14a-c. Employing the 3EN1M-O2 model of TDO, catechols 13a-d were predicted to be the major metabolites (91-100% docking orientations), without evidence of orientations of anilines 9a-d, leading to the corresponding cis-diols 10a-d (Supporting Information Figs. S1-S4). These results (3EN1M-O2 model) were similar to those found earlier for phenol substrates 3a and 3b, where catechols 13a and 13b were predicted to be the major metabolites (54 and 100% docking orientations) without evidence for the formation of cis-diols 5a and 5b.[8]

Based on these predictive in silico docking (3EN1M model) studies of TDO (Table 1), experimental evidence was sought, for TDO-catalysed cis-dihydroxylation of meta-anilines 9a-d, by LC-TOFMS analysis of the crude biotransformed culture medium. Aniline substrates 9a-d were added, to P. putida UV4 cultures, under conditions similar to those reported for the corresponding meta-phenols 3a-d.[5a-c] The cyclohex-2-en-1-one cis-diols 6a-d, previously reported[5a-c] as phenol metabolites (Scheme 1), were also detected as aniline metabolites (Scheme 2), in accord with the predictions from in silico studies. The documented high cytotoxicity of anilines[6a-d] required a ten-fold reduction of the substrate concentration (0.05 mg/mL), for total conversion. Phenols 3a-d and corresponding anilines 9a-d, applied in the same low concentrations, produced comparable yields of cyclohexenone cis-diols 6a-d, which were identified by comparison (LC/TOFMS and GC-MS) with authentic samples. A sample of cis-diol 6a (ca. 6 mg) was also isolated by PLC, from the partial biotransformation using a higher concentration of aniline 9a; its structure, absolute configuration (4S,5S) and enantiopurity (>98% ee) was found to be identical with the metabolite derived from phenol 3a. From this result, combined with the in silico docking studies (Figs. 1A-D), it was predicted that the (S)-absolute configuration at the C-5 position and ee value (>98%) of cis-diol metabolites 6b-d, derived from the corresponding aniline substrates 9b-d, would be identical to those obtained from phenols 3b-d.

The first objective of the study was to provide experimental evidence for TDO-catalysed cis-diol formation from aniline substrates, but the presence of other metabolites extended our interest into exploring the complete metabolic profile of anilines with the P. putida biocatalyst. A CRED enzyme, present in P putida UV4, was previously found to catalyse the reduction of the ketone group of metabolite 3c, to yield (1R,2S,4R)-6-(trifluoromethyl)cyclohex-5-ene-1,2,4-triol 12c as a major bioproduct.[5c] A similar result was obtained with aniline substrate 9c when metabolites cis-diol 6c and triol 12c were identified by LC-TOFMS analysis.

Catechols were identified as aniline metabolites[6a-i] and molecular docking (3EN1M and 3EN1M-O2 models) experiments of TDO also suggested their formation from anilines 9a-d (Table 1). LC-TOFMS analysis did not show direct evidence of catechol metabolites 13a-d, but indirect evidence, for the formation of catechol metabolite 9a, was observed by the formation of a carboxylic acid metabolite, whose molecular weight was consistent with structure 15a, formed by catechol dioxygenase-catalysed ring opening and reductase-catalysed reduction (Fig.2). Similar ring-opened metabolites 15 (R = Me, CF3) were previously reported from the corresponding phenols 3b and 3c) and catechols (13b and 13c).[5c] GC-MS analysis of trimethylsilylated samples, prepared from freeze-dried aliquots collected during TDO-catalysed dihydroxylation of anilines 9a and 9c, showed the presence of disilylated cis-diol 6a and 6c and derived hydroquinones 16a and 16c respectively, but no catechols were detected. The difficulty encountered in the detection of catechol metabolites 13a-d was probably due to: (i) the activity of a catechol dioxygenase enzyme present in P. putida UV4 and (ii) the low yields of all metabolites resulting from the cytotoxicity of aniline substrates.

Biotransformations of anilines 9a-d, with the recombinant strain, E. coli pCL-4t (expressing TDO), and LC-TOFMS analysis of the biotransformed aqueous material, again showed the presence of

![Figure 2. Structures of metabolites 15-18 and 25.](image-url)
cyclohexanone cis-diol metabolites 6a-d. The reduced tolerance of E. coli cells, to the toxic aniline substrates, gave lower yields, compared with those found using P. putida UV4 cells. However, the E. coli pCL-4t biotransformation studies did provide evidence that TDO, rather than other types of dioxygenase, was responsible for the formation of cis-diols 6a-d.

The biotransformation and molecular docking results, recorded for aniline substrates 9a-d, were consistent with a metabolic pathway via TDO-catalysed formation of enamine cis-dihydrodiols 10a-d, tautomerisation to the preferred NH-imine cis-diols 11a-d, and rapid hydrolysis to yield cyclohex-2-en-1-one cis-diols 6a-d (Scheme 2). Thus, the family of cyclohex-2-en-1-one cis-diol metabolites is formed, from both substituted phenols and anilines, by TDO-catalysed cis-dihydroxylation. The molecular docking results can also be used to rationalize the reported formation of catechol and hydroquinonine metabolites of aniline substrates,[6a] via ring hydroxylating dioxygenase catalysis.

(ii) Biotransformations of phenol substrates, to yield 4-hydroxycyclohex-2-en-1-ones

Earlier larger scale biotransformations of meta- and ortho-phenols 3 and 4, showed a wide range of metabolite types, including cyclohexanone cis-diols 6 and 8, cyclohexene cis-triols 12, catechols 13, α-hydroxycarboxylic acids 15, hydroquinones 16, cyclohexanone cis-diol isomers 17cis and 17trans and 1,2,4-trihydroxycyclohexanes 18cis-d (Scheme 1 and Fig. 2). Several minor metabolites of methoxyphenols 3a and 4a, however, remained unidentified;[5c] their structures, absolute configurations and metabolic pathways for their formation are presented in this section.

Recrystallization of the crude mixture of metabolites, obtained from an earlier biotransformation of 3-methoxyphenol 3a (96 g), with glucose as carbon source, yielded cis-diol 6a as the major component (38% isolated yield, Scheme 1).[5c] The mother liquors from this recrystallization contained a mixture of unidentified minor metabolites, which were examined further during this study. Column chromatography of the mixture yielded a new minor metabolite (1% isolated yield), which was structurally identified as 4-hydroxy-3-methoxycyclohex-2-en-1-one 19a ([α]D -31.9).

A chemoenzymatic synthesis of metabolite 19a, starting from 2-bromo-2-cyclohexen-1-one 20 established (4S) as its absolute configuration (Scheme 3). Step (i) employed a CRED-catalysed reduction of the ketone group of 2-bromo-2-cyclohexen-1-one 20, to give the enantiopure synthetic precursor cyclohexenol 21s ([α]D -80.3, 88%).[5b] Further chemical steps involved, hydroxyl group protection (ii, 21s → 22s, 94%), allylic oxidation (iii, 22s → 23s, 29%), nucleophilic substitution (iv, 23s → 24s, 69%) and deprotection (v, 24s → 19as, 62%, Scheme 3). This synthetic sample of compound 19as (>98% ee) had a higher optical rotation ([α]D -48.8), compared with the corresponding metabolite (19as) derived from phenol 3a. The lower enantiopurity (65% ee) of the minor metabolite, 4-hydroxy-3-methoxycyclohex-2-en-1-one 19as, compared to the major metabolite, (4S,5S)-3-methoxycyclohex-2-ene-1-one 6a (>98% ee), was of mechanistic relevance, in the context of biosynthetic pathways from 3-methoxyphenol 3a (Scheme 4), that will be discussed in Section (iii).

A previous biotransformation (glucose as carbon source) of 2-methoxyphenol 4a (96 g), followed by column chromatography, resulted in a separable mixture of isomeric cyclohexanone cis-diols, (2S,3S,4S)-17cis (13% isolated yield) and (2R,3S,4S) 17trans (1% isolated yield).[5c] Metabolites 17cis and 17trans were formed via an ene reductase (ERED)-catalysed reduction of the initial bioprotected cyclohex-2-en-1-one cis diol 8a (Scheme 1). Using LC-TOFMS and GC-MS analyses, the relative ratios of metabolites from guaiacol 4a were found to vary widely, during time course studies of the biotransformations, depending on the choice of carbon

![Scheme 3](image)

Scheme 3. Chemoenzymatic synthesis of 4-hydroxy-3-methoxycyclohex-2-en-1-one 19as from 2-bromo-2-cyclohexen-1-one 20.
source (glucose or pyruvate) and TDO source (P. putida or E. coli). Other metabolites from phenol 4a were identified as cyclohex-2-en-1-one cis diol 8a, hydroquinone 16a, catechol 13a and its α-hydroxycarboxylic acid derivative 15a.

Column chromatography fractions, from the earlier study,[5] that appeared to be an inseparable mixture of two unidentified isomeric metabolites of phenol 4a were retained for further examination. During the current study, this mixture, was finally separated by careful multiple elution PLC. The minor isomer (2% isolated yield) was indistinguishable from 4-hydroxy-3-methoxycyclohex-2-en-1-one 19a, derived from 3-methoxyphenol 3a. The structure and absolute configuration of the major isomer (18% isolated yield, [α]D -29), was identified as (4S)-4-hydroxy-2-methoxycyclohex-2-en-1-one 25a. This metabolite was also isolated as a dehydration product of (2S,3R,4S)-3,4-dihydroxy-2-methoxycyclohexanone 17a, (Scheme 4).

A repeat biotransformation of guaiacol 4a again resulted in the formation of chiral metabolites 17a, 25a and 19a, but in a different ratio based on isolated yields, i.e. 11%, 3%, <1% respectively. This prompted a time course biotransformation study of methoxyphenol substrates 3a and 4a, which showed increases in the relative yields of 4-hydroxy-3-methoxycyclohex-2-en-1-ones 19a and 25a, respectively, with glucose, rather than pyruvate, as a carbon source and during the later stages (>8 h) of the biotransformations.

The unexpected discovery of 4-hydroxymethoxycyclohex-2-en-1-ones 19a and 25a, metabolites of phenol substrates 3a and 4a, allied to the earlier isolation of compound 19e, as a minor metabolite of 3-iodophenol 3d,[5] raised the possibility that compounds 19a, 25a and 19e could be the first members of a new family of phenol metabolites. To investigate the possible metabolic pathways, leading to the formation of 4-hydroxycyclohex-2-en-1-ones 19a, 19e and 25a, repeat biotransformations of 3-iodophenol 3d and ortho-cresol 4b were conducted (Scheme 4).

Iodocyclohex-2-en-1-one cis-diol 6d, a major metabolite of 3-iodophenol 3d,[5ac] was isolated in variable yields (30-70%), along with other metabolites including 4-hydroxycyclohex-2-en-1-one 19e. LC-TOFMS analysis of the biotransformed aqueous culture medium, detected the presence of cyclohexanone cis-diol 26e and GC-MS analysis of the EtOAc concentrate, after trimethylsilylation with N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), showed that iodocyclohexene cis-triol 12d, cyclohexane cis-triol 18e, iodocatechol 13d, iodohydroquinone 16d and cyclohexene cis-triol 12 (R=H) were also present as minor metabolites (Scheme 2, 4 and Fig 2).

A preliminary biotransformation of ortho-cresol 4b, resulted in the isolation of (4S,5R,6S)-4,5-dihydroxy-6-methycyclohex-2-en-1-one 8b, as the only identified metabolite (1% yield).[5c] Repeated metabolism studies of phenol 4b, revealed that in addition to cis-diol 8b, four other minor metabolites were present. Catechol 13b and hydroquinone 16b were identified by trimethylsilylation of a small portion of the crude freeze-dried extract, followed by GC-MS analysis of the products and comparison with authentic samples.

Time course LC-TOFMS analysis, of the crude culture medium from a biotransformation of ortho-cresol 4b, indicated that, in addition to 8b, a very minor bioproduct was formed, which rapidly metabolized further. This early eluting bioproduct, was tentatively identified as cyclohexanone cis-diol 17b, (Scheme 4). Its identity was confirmed by catalytic hydrogenation (Pd/C, MeOH) of metabolite 8b, to yield an identical sample of (2S,3R,4S)-3,4-dihydroxy-2-methycyclohexanone 17b. Work up of the biotransformed material[5a] and separation of the crude mixture, by column chromatography followed by PLC of early eluting fractions, gave a new metabolite, which was identified as 4-hydroxy-2-methycyclohex-2-en-1-one 25b ([α]D - 49.0). The opposite enantiomer, 25b, ([α]D + 46.7), had been synthesised earlier by an alternative route, involving manganese acetate-mediated acetoxylation and lipase-catalysed ester hydrolysis.[7]

(iii) Biosynthetic pathways for the formation of 4-hydroxycyclohex-2-en-1-one - metabolites from phenols

The metabolic sequences, to account for the formation of the 4-hydroxycyclohex-2-en-1-one metabolites 19e, 19a, 25a and 25b, as minor products from the corresponding phenol substrates (3d, 3a, 4a and 4b), via the initial cyclohexanone cis-diol metabolites 6d, 6e, 6a, 8a and 8b, are presented in Scheme 4.

It is proposed that, during the P. putida UV4 biotransformation of 3-iodophenol 3d, an ERED-catalysed ene reduction of cyclohexanone cis-diol 6d, followed by a dehydrohalogenation of the resulting cyclohexanone cis-diol could occur, to give the transient parent cyclohex-2-en-1-one cis-diol 6e (Scheme 4). Intermediate 6e was not detected, possibly due to its further rapid ERED-catalysed ene reduction to form the detected transient metabolite 26e. ERED-catalysed reduction of metabolite 26e yielded the cis-triol metabolite 18e (Fig. 2, R = H), while its facile
dehydration also gave 4-cyclohex-2-ene-1-one 19e. A precedent for this type of reductive dehalogenation mechanism, (6d → 6e) by the ERED-catalysed reduction-spontaneous β-elimination of β-halo-α,β-unsaturated carboxylic esters, has been reported.\[8\] Other examples of ERED-catalysed ene reductions, of α,β-unsaturated ketones, e.g. metabolites 8a→17a, and 8b→17b, have been found during biotransformations\[5c\] (Scheme 4).

Further evidence of the metabolic sequence (3d→6d→6e→26e→19e), involving both TDO and ERED enzymes, was found when cyclohex-2-en-1-one cis-diol 6e ([α]_D - 217), obtained by hydrogenolysis of metabolite 6d, was added as substrate. 4-Hydroxycyclohex-2-en-1-one 19e and triol 18e were the only identified metabolites. Enantiopure 4-hydroxycyclohex-2-en-1-one 19e, ([α]_D +110) synthesised by an alternative chemoenzymatic route using lipase enzymes, has been utilized as a chiral precursor in synthesis.\[9a,b\]

Biosynthetic sequences, involving TDO-catalysed cis-dihydroxylation of phenols 4a and 4b, to yield enantiopure cyclohex-2-en-1-one cis-diols followed by an ERED-catalysed reduction / β-elimination mechanism, are shown in Scheme 4. The metabolic pathway proposed for the formation of 4-hydroxycyclohex-2-en-1-one 25a from phenol 4a, (4a→8a→17a,→25a), was supported by results obtained using (25,35,45)-cyclohexanone cis-diol 17a as substrate; compound 25a was the only bioproduct formed. It was presumed that the biotransformation of phenol 4b, to yield 4-hydroxycyclohex-2-en-1-one 25b, would also occur via a similar metabolic pathway (4b→8b→17b,→25b).

While the formation of three 4-hydroxycyclohex-2-en-1-ones (19e, 25a and 25b), from the corresponding phenol substrates (3d, 4a and 4b), resulted from a common biosynthetic sequence, this pathway would not result in the formation of metabolite 19a from phenol 3a. Furthermore, the higher enantiopurity (>98% ee), of bioproducts 19e, 25a and 25b from phenols 3d, 4a and 4b, contrasted with the lower ee value of metabolite 19a (ca. 65% ee, from phenol 3a) found earlier. This indicates the probability of a different mechanism being involved in the formation of 4-hydroxycyclohex-2-en-1-one 19a from phenols 3a and 4a.

With cyclohexenone cis-diol 6a as substrate, 4-hydroxycyclohexenone 19a was identified as the main metabolite with hydroquinone 16a as a minor product resulting from the dehydration. Since hydroquinone 16a also being formed by the dehydration of cyclohexenone cis-diol 8a, its possible role as an intermediate during formation of 4-hydroxycyclohex-2-en-1-one 19a was examined. The biotransformation pathways of phenols 3a and 4a, with hydroquinone 16a as an intermediate, were postulated to proceed in five steps (3a→6a→16a→27a→28a→19a, and 4a→8a→16a→27a→28a→19a) as shown in Scheme 4. Further confirmation of these metabolic sequences was obtained by the biotransformation of hydroquinone 16a as substrate to yield metabolite 4-hydroxycyclohex-2-en-1-one 19a.

It is postulated that the oxidation of hydroquinone 16a, to benzoquinone intermediate 27a, could have resulted from either a non-enzymatic autoxidation or peroxidase activity in P. putida UV4 cells, followed by an ERED-catalysed reduction of benzoquinone 27a (Scheme 4). The transient intermediate, cyclohex-2-ene-1,4-dione 28a, could either tautomerize back to hydroquinone 16a or undergo an asymmetric CRED-catalysed ketone reduction. It could account for metabolite 19a having a lower enantiopurity (65% ee) compared with cis-diol precursors 6a and 8a (>98% ee).

Fungal metabolism of benzoquinone 29, with cultures of *Phanerochaete chrysosporium*,\[10\] to form 4-hydroxycyclohex-2-enone 30 (Fig.3) provides a precedent for the metabolic sequence (27a→28a→19a). Dehydration of cyclohexenone cis-diols 6a and 8a, to give hydroquinone 16a, and of cyclohexanone cis-diols 17a, 17b and 26a, to form 4-hydroxycyclohex-2-en-1-ones 25a, 25b and 19a respectively, could occur during biotransformations, via chemocatalysis or a dehydratase-catalysed process.

(iv) Biotransformations of monocyclic arenes, to yield arene hydrates

The hydration of conjugated and non-conjugated alkene bonds, catalysed by hydratase or hydrolyase enzymes, e.g. aconitase, fumarase and crotonase, is a common step in primary metabolism.\[11a\] There are very few reported examples of enzymatic hydrations of arenes, to form the corresponding arene hydrates. The formation of arene hydrate metabolite 31 from acetophenone substrate 1 (R = COMe) using *P. putida* UV4 cultures, is among the few reports of arene to arene hydrate biotransformations (Scheme 1, Fig. 3).\[11b\]

Metabolite 31, a highly unstable compound, with a propensity to rapidly dehydrate back to substrate 1 (R = COMe), was only identified as an iron tricarbonyl complex.\[11b\] To study the stability of monocyclic arene hydrates, racemic samples of compounds 32 (R = Me, Et, Ph, Pr, Bu), and 33 (R = CO₂Me and Ph), were synthesised from 3-substituted
Scheme 4. Metabolic pathways for the formation of 4-hydroxycyclohex-2-en-1-ones \(19e\), \(19a\), \(25a\) and \(25b\), from phenols \(3d\), \(3a\), \(4a\) and \(4b\) respectively.

Figure 3. Structures of compounds 29-36.

1,4-cyclohexadienes,[11c] and enantiopure arene hydrates \(33\) (\(R = F, Cl, Br, CF_3\)) and \(34\) (\(R = Br\)) from the corresponding cis-dihydrodiol metabolites \(2\).[11d] Kinetic studies of the acid-catalysed dehydration of these arene hydrates showed that they aromatized much faster (3.7x10\(^2\) - 6.9x10\(^4\) fold) than cis-dihydrodiols \(2\).[11c]

The monocyclic arene hydrate \(35\), an unstable intermediate, formed during the biosynthesis of the antibiotic bacilysin, was obtained by the enzymatic decarboxylation of prephenate, using an \(E.\ coli\) recombinant strain, expressing phenate decarboxylase.[11a-c] Arene hydrate \(35\) was found to undergo a slow non-enzymatic, or a rapid enzyme-catalysed isomerization, to yield the more stable vinylogous 4-hydroxycyclohexenone metabolite \(36\). Intermediate \(35\) appears to be among the very few, isolated and fully characterized, monocyclic arene hydrate metabolites.

Phenol metabolites, 4-hydroxycyclohex-2-en-1-one \(19e\), \(19a\), \(25a\) and \(25b\), identified during the study could, in principle, equilibrate with the corresponding phenol hydrate tautomers \(19e'\), \(19a'\), \(25a'\) and \(25b'\); in practice only the keto tautomers were observed by NMR spectroscopy. Similarly, the methoxycyclohexenone cis-diol \(6a\), metabolite of 3-methoxyphenol \(3a\), showed no evidence of enol tautomer \(5a\), from proton NMR analysis (Scheme 1). Trimethylsilylation of cis-diol \(6a\) with MSTFA, and GC-MS analysis of the product, showed a major peak (95%) with \([\text{M}]^+ m/z 302\), indicating formation of the diTMS derivative.[5e] The molecular ion \([\text{M}]^+ m/z 374\), corresponding to the minor peak (5%), was from a triTMS derivative and provided indirect evidence of
the elusive enol tautomer 5a. Similar treatment of 4-hydroxycyclohex-2-en-1-ones 19e and 19a with MSTFA, and GC-MS analyses of the products showed the formation of monoTMS derivatives 19e and 19a (major peaks). The minor peaks were attributed to the diTMS derivatives of the undetected phenol hydrate tautomers 19e and 19a.

The lack of evidence, for monocyclic arene hydrates of similar structure to metabolite 31 during the biotransformations of substituted benzene substrates by P. putida UV4 or other microbial systems could be due to the absence of suitable hydrolase / hydratase enzymes or rapid dehydration and reformulation of the arene substrates. Under similar biotransformation conditions, the TDO-catalysed cis-dihydroxylation of arene hydrate 33 (R = CF3) yielded the opposite enantiomer of cyclohexene triol 12c and thus revealed an alternative metabolic pathway for monocyclic arene hydrates. The results presented herein demonstrate that the more stable keto tautomers of phenols can be obtained from phenols using P. putida UV4.

Conclusion

Molecular docking results, of four aniline substrates with TDO, led to the prediction that in common with phenols, cis-dihydroxylation of anilines could occur, to yield cis-diols and catechols. The premise was confirmed by the detection and isolation, of identical cyclohex-2-en-1-one cis-diol metabolites, from the corresponding meta-phenols and meta-anilines. The initial formation of enamines and the NH-imine tautomers, followed by their rapid hydrolysis, could account for the formation of cyclohex-2-en-1-one cis-diols in low yields from the anilines. Although catechols had been found earlier as aniline metabolites, no direct evidence was found in the study, possibly due to further metabolism by catechol dioxygenase.

Biotransformation of cyclohex-2-en-1-one cis-diols, resulted in the formation of a new range of minor metabolites, which were identified as 4-hydroxycyclohex-2-en-1-ones. Their structures and absolute configurations were determined by chemoenzymatic synthesis and stereochemical correlation and have considerable synthetic potential.

Although single step hydratase or hydrolase activity has been reported for alkenes, we are unaware of similar activity with arene substrates. Multistep metabolic pathways, involving cyclohexeneone cis-diol, cyclohexanone cis-diol and hydroquinone intermediates, are now proposed to explain the formation of 4-hydroxycyclohex-2-en-1-ones as a new type of phenol metabolite. Although direct evidence for the presence of enol tautomers (arene hydrates) of 4-hydroxycyclohexanones was not found, indirect evidence was obtained following trimethylsilylation and GC-MS analyses.

Experimental Section

Experimental Details

NMR spectra were recorded on Bruker Avance-400, DPX-300 and DRX-500 instruments. Chemical shifts (δ) are reported in ppm relative to SiMe4, and coupling constants (J) are given in Hz. IR spectra were recorded on a Perkin-Elmer 983G spectrometer. Optical rotation ([α]D) measurements were carried out on a Perkin-Elmer 214 polarimeter. LC-TOFMS analyses were conducted using an Agilent 1100 series HPLC coupled to an Agilent 6510 Q-TOF (Agilent Technologies, USA) and a reverse phase column (Agilent Eclipse Plus C18, 5 mm, 150 x 2.1 mm) under reported conditions. GC-MS analysis of metabolites was carried after silylation using MSTFA and an Agilent Technologies 6890N gas chromatograph linked to a 5973 mass selective detector and Agilent Technologies HP Ultra 2 column as reported. For TLC analysis, Merck Kieselgel 60F254 analytical plates were used and PL separation of metabolites was carried out using glass plates (20 cm x 20 cm) coated with Merck Kieselgel PF254/366 silica gel. Column chromatography was performed on Merck Kieselgel type 60 (250-400 mesh).

Phenols 3a-3d, 4a, 4b, anilines 9a-d, catechols 13a, 13b, hydroquinones 15a, 15b and methoxybenzoquinone 27a were purchased from Sigma-Aldrich Co. Authentic samples of cyclohex-2-en-1-one cis-diols 6a-d and cyclohexene cis-triol 12c were available from earlier studies. Commercially obtained anilines 9a-d showed no detectable traces of phenols 4a-d on GC-MS analysis, prior to their use as substrates. Biotransformations were carried out using P. putida UV4 cells, unless mentioned otherwise.

Molecular modelling

Substrate docking studies were performed according to an earlier procedure. The required in silico models of substrates were created in .pdb format with UCSF Chimera 1.10.2 (https://www.cgl.ucsf.edu/chimera/). In silico dockings were performed with AutoDock suite 4.2 (autodock4, autogrid4). The Graphical User Interface (GUI), including python scripts for ligand and receptor preparation, was part of AutoDock Tools 1.5.6. AutoDock suite and AutoDock tools (ADT) are provided by the Scripps Research Institute (http://autodock.scripps.edu/).

The TDO crystal structure was accessed from the Protein Data Bank (PDB code 3en1, resolution of 3.2 Å). The raw 3en1 crystal structure .pdb-file of TDO includes a docked toluene structure in the active site, which was removed with UCSF chimera 1.10.2 prior to docking. The resulting model was called 3EN1M. Ligand and receptor were then prepared in accordance with the ADT tutorial.
(http://autodock.scripps.edu/faqs-help/tutorial/using-autodock-4-with-autodocktools/2012_ADṬTut.pdf) utilising the 'prepare_receptor4.py' python script included in ADT, missing atoms were repaired, hydrogens and Gasteiger charges added, and non-polar hydrogens merged. The resulting .pdbqt-file of the crystal structure was used in all docking calculations. .pdb-files of substrates were automatically converted to the required .pdbqt-format by ADT. The docking grid was adjusted to include all amino acids within 5 Å of toluene in the crystal structure: Gln215, Phe216, Asp219, Met220, His222, Ala223, His228, Val309, His311, Leu321, Ile324, Phe366, Phe376.

PyMol was used to incorporate dioxygen into the 3EN1M model, by superimposing the iron complex (Fe, His222, His228, and Asp376) of TDO (pdb code: 3en1) with that of NDO (pdb code: 1o7m), and copying the dioxygen positions to 3EN1M. The resulting model was called 3EN1M-O2.

Docking resolution = 0.247 Å; xsize = 40; xoffset = 12.833; ysize = 60; yoffset = 4.472; zsize = 72; zoffset = 3.917. The grid parameter file (.gpf) was used to create the grid map files (.glg) using autogrid4. The search protocol for docking used the internal default docking parameters of AutoDock 4.2, starting the ligand at a random location. The docking was set to be performed as 100 runs of the Lamarckian genetic algorithm with a population size of 150 each, terminating after 2 500 000 energy evaluations or 27 000 generations (whichever occurs first; standard settings). The docking results were analysed with ADT, to obtain docking coordinates, calculated binding energy (in kJ) and calculated dissociation constant Kd (in μM). All docking studies were performed as rigid docking, keeping all positions, orientations and protonation states of all amino acid atoms locked in place. The 100 docking orientations were automatically analysed by ADT and divided into orientationally and energetically similar groups. The orientation with the highest binding energy of each group was saved as representative for each group. The conformation of toluene, in the crystal structure, was used as a reference, to establish and confirm the viability of the docking procedure and parameters, as a similar conformation was obtained from docking with ADT.

3D visualization

The amino acids of the TDO active site were visualized with PyMol 1.7.4.5, from the Protein Data Bank (PDB) 3EN1 file coordinates. The docking results were imported into PyMol, from the respective .pdb files created with AutoDock4 and AutoDock Tools. Measurements between atoms were calculated with PyMol’s incorporated measurement tool.

Biotransformation of anilines 9a-d

Initial small scale biotransformations of anilines 9a-d and LC-TOFMS analyses of metabolites were conducted, under conditions similar to those reported for phenol substrates.\cite{5a-5d} However, due to the increased toxicity of anilines 9a-d, compared with phenol substrates 3a-d, a significant proportion of residual aniline substrates were consistently present along with cyclohexenone cis-diol metabolites 6a-d. To achieve complete conversions, lower aniline concentrations were used (ca. 0.05 mg/ml). Cyclohexenone cis-diol metabolites 6a-d and cis-triol 12c were readily identified as aniline metabolites, by comparison of LC-TOFMS data with the authentic samples. As cyclohexenone cis-diols 6a-d and cis-triol 12c had been fully characterised\cite{5a-5d}, only the LC-TOFMS retention times (min.) and accurate mass values were recorded for aniline substrates 9a-d.

3-Methoxytoclohex-2-en-1-one cis-diol 6a.\cite{5b} 3.43 min., HRMS: [M+H]+ 159.0646, calcd. for C7H7O1: 159.0652.

3-Methycyclohex-2-en-1-one cis-diol 6b.\cite{5a} 4.85 min., HRMS: [M+H]+ 143.0703.

3-Trifluoromethylcyclohex-2-en-1-one cis-diol 6c.\cite{5a} 10.93 min., HRMS: [M+H]+ 197.0417, calcd. for C7H7F3O3: 197.0420.


Cyclohex-2-en-1-one cis-diol metabolite 6a (ca. 6 mg) was isolated from a biotransformation of aniline 9a (1.25 g in 4 L culture medium). Purification, by column chromatography (hexane → EtOAc), of the crude product obtained after usual work up followed by PLC (50% EtOAc in hexane), of selected combined fractions, gave metabolite 6a. The metabolite was found to have identical spectroscopic (NMR) and chiroptical ([(α)D]3) properties to an authentic sample derived from phenol 3a. No catechol metabolites (6a-6e) were detected (GC-MS analysis) in the crude bio-extracts of aniline substrates 9a-d.

Biotransformation of phenols 3a, 3d, 4a and 4b and synthesis of (1R,3S)-4-hydroxy-3-methoxytoclohex-2-en-1-one 19a:

Large scale biotransformations of phenols 3a and 4a, using whole cell cultures of P. putida UV4 with glucose as a carbon source, were reported.\cite{5c} Pooled culture fractions, from that investigation containing unidentified metabolites, were re-examined during the current study. Time course study of small scale biotransformations of phenols 3a, 3d, 4a and 4b and anilines 9a-d, were conducted under similar conditions, using glucose or pyruvate as carbon sources.\cite{5a-5d} Chiral metabolites, 4-hydroxytoclohex-2-en-1-ones 19a, 19b, 25a and 25b, cyclohexenone cis-diols 6a and 6d, cyclohexanone cis-diols 17a, 17b and 26c, and cis-triol 12c were detected directly in the crude aqueous culture medium by LC-TOFMS analyses, prior to their isolation. Catechols 13a-d, hydroquinones 16a-d were identified by GC-MS analyses of the crude bio-extracts, after EtOAc extraction and trimethylsilylation (MSTFA) of the dried concentrates.

(a) 4-Hydroxy-3-methoxytoclohex-2-en-1-one 19a-a new metabolite of 3-methoxyphenol 3a

In the large scale biotransformation of phenol 3a, the major metabolite, cyclohex-2-en-1-one cis-diol 6a (45 g, 38%
yield), was isolated by crystallization from the crude extract. GC-MS analysis of the retained combined fractions showed that catechol 13a and hydroquinone 14a were also present among a mixture of unidentified metabolites. One very minor metabolite was identified as 2-hydroxy-6-methoxy-6-oxohexaoxonic acid 15a by LC-TOFMS analysis: [M+H]+ 177.0758, calcd. for C9H9O5 177.0758; [M+Na]+ 199.0577, calcd. for C10H9O4Na 199.0582; [M+NH2]+ 194.1018, calcd. for C9H8N2O3 194.1028. LC-TOFMS analysis of the combined fractions indicated that another unidentified metabolite was present; its molecular weight was consistent with structure 19a. Separation of the combined fractions by careful column chromatography (hexane → 50% EtOAc in hexane) gave a pure sample of metabolite 19a.

(4S)-4-Hydroxy-3-methoxy cyclohex-2-en-1-one 19a. Colorless oil (1.2 g, 1%); Rf 0.15 (50% EtOAc in hexane); [α]D -31.9 (c 1.0, CHCl3); HRMS: (TOF-LCMS) [M+H]+ 143.0709, calcd. for C6H10O 143.0708; 1H NMR (400 MHz) δ = 2.01 (1 H, dddd, J = 12.5, 10.1, 7.9, 4.3, 0.6 Hz, H-5), 2.25-2.37 (2 H, m, H-4, H-6), 2.59 (1 H, t, J = 7.7 Hz, H-2), 2.65 (1 H, br s, OH), 3.76 (3 H, s, SiMe), 4.47 (1 H, dd, J = 8.4, 5.0 Hz, H-4), 5.34 (1 H, s, H-2). 13C NMR (100 MHz) δ = 29.5, 34.0, 56.2, 65.8, 102.1, 176.2, 198.6; IR (film) νmax/cm⁻¹ 3389, 2945, 1630, 1609, 1231.

(b) Chemoenzymatic synthesis of (S)-4-hydroxy-3-methoxy cyclohex-2-en-1-one 19a. To a solution of enone 23a (40 mg, 0.13 mmol) in MeOH (2 mL) was added K2CO3 (35 mg, 0.26 mmol), and the mixture kept at room temperature (2 h) without stirring. The reaction mixture was filtered, the filtrate carefully concentrated in vacuo, and the light yellow volatile oil obtained was purified by PLC (10% EtOAc in pentane) to furnish methoxy compound 24a as a colourless oil (23 mg, 69%); Rf 0.2 (5% EtOAc in pentane); HRMS: (ES) [M+H]+ 257.1571, calcd. for C12H20O5Si 257.1573; 1H NMR (400 MHz) δ = 0.08 (3 H, s, SiMe), 0.11 (3 H, s, SiMe), 0.89 (9 H, s, CMe3), 1.95-2.14 (2 H, m, H-5, H-5), 2.29 (1 H, dd, J = 16.7, 5.8, 4.4 Hz, H-6), 2.67 (1 H, dd, J = 16.7, 10.1, 5.0 Hz, H-6), 3.70 (3 H, s, OMe), 4.32 (1 H, dd, J = 5.0, 4.1 Hz, H-4), 5.27 (1 H, s, H-2); 13C NMR (100 MHz) δ = -50.0, -46.6, 18.3, 25.8 (3C), 31.0, 32.9, 55.8, 67.2, 102.2, 176.9, 199.3; LRMS: (EI): 217 (100), 257 (5), 304 (60), 445 (50), 1065 (15); IR (film) νmax/cm⁻¹ 2955, 2930, 1670, 1618, 1226, 834.

(S)-4-(tert-Butyldimethylsiloxy)-3-methoxy cyclohex-2-en-1-one 24a. To a solution of enone 23a (40 mg, 0.13 mmol) in MeOH (2 mL) was added K2CO3 (35 mg, 0.26 mmol), and the mixture kept at room temperature (2 h) without stirring. The reaction mixture was filtered, the filtrate carefully concentrated in vacuo, and the light yellow volatile oil obtained was purified by PLC (10% EtOAc in pentane) to furnish methoxy compound 24a as a colourless oil (23 mg, 69%); Rf 0.2 (5% EtOAc in pentane); HRMS: (ES) [M+H]+ 257.1571, calcd. for C12H20O5Si 257.1573; 1H NMR (400 MHz) δ = 0.08 (3 H, s, SiMe), 0.11 (3 H, s, SiMe), 0.89 (9 H, s, CMe3), 1.95-2.14 (2 H, m, H-5, H-5), 2.29 (1 H, dd, J = 16.7, 5.8, 4.4 Hz, H-6), 2.67 (1 H, dd, J = 16.7, 10.1, 5.0 Hz, H-6), 3.70 (3 H, s, OMe), 4.32 (1 H, dd, J = 5.0, 4.1 Hz, H-4), 5.27 (1 H, s, H-2); 13C NMR (100 MHz) δ = -50.0, -46.6, 18.3, 25.8 (3C), 31.0, 32.9, 55.8, 67.2, 102.2, 176.9, 199.3; LRMS: (EI): 217 (100), 257 (5), 304 (60), 445 (50), 1065 (15); IR (film) νmax/cm⁻¹ 2955, 2930, 1670, 1618, 1226, 834.

(c) New metabolites of 2-methylyphenol 4a. LC-TOFMS analysis of the aqueous bio-extract, obtained from the biotransformation of 2-methylyphenol substrate 4a, showed the presence of following metabolites: cyclohexanone cis-diols 17a-cis and 17a-trans, cyclohex-2-en-1-one cis diol 8a, hydroquinone 16a, catechol 13a and its hydroxycarboxylic acid derivative 15a, and some unidentified bioproducts. The large-scale biotransformation of 2-methylyphenol 4a and purification by column chromatography (hexane → EtOAc) had yielded (2S, 3S, 4S)-cis-diol 17a-cis (2.5 g) and (2R, 3S, 4S)-trans-diol 17a-trans (0.25 g). LC-TOFMS analysis of unidentified pooled chromatography fractions (2.6 g, eluent 50% EtOAc
in hexane), indicated it to be a mixture (9:1) of two isomeric compounds ([M+H]+ 143), which could not be separated. This retained concentrated mixture was re-examined during the study. A pure sample of the major isomer, separated by multiple elution PLC (2.5% MeOH in CHCl₃), was identified as 4-hydroxy-2-methoxycyclohex-2-en-1-one 25a. The minor isomer of the mixture was found to be indistinguishable from metabolite 4-hydroxy-3-methoxycyclohex-2-en-1-one 19a, which was derived from 3-methoxyphenol 3a.

(4S)-4-Hydroxy-2-methoxycyclohex-2-en-1-one 25a. Light yellow oil, Rf 0.26 (5% MeOH in CHCl₃); [α]D -29.0 (c 1.1, CHCl₃). HRMS (LC-TOFMS) [M+H]+ 143.0703, calcd. for C₇H₁₀O₃ 143.0708; [M+Na]+ 165.05216, calcd. for C₈H₁₄O₂Na 165.0528; [M+K]+ 181.0621, calcd. for C₈H₁₄O₂K 181.0627; [M+H - H₂O]0 125.0523, calcd. for C₅H₇O₂ 125.0524; 1H NMR (400 MHz) δ = 1.94 (1 H, ddd, J = 12.6, 11.0, 8.1, 4.5 Hz, H-5), 2.28 (1 H, m, H-5), 2.40 (1 H, ddd, J = 17.0, 11.1, 4.6 Hz, H-6), 2.56 (1 H, bs, OH), 2.68 (1 H, ddd, J = 17.0, 6.3, 4.5 Hz, H-6), 3.60 (3 H, s, OMe), 4.68 (1 H, m, H-4), 5.82 (1 H, dd, J = 3.5 Hz, 0.6, H-3); 13C NMR (100 MHz) δ = 32.6, 35.2, 55.5, 66.5, 118.8, 151.4, 194.0. Addition of (2S, 3S, 4S)-3,4-dihydroxy-2-methoxycyclohexanone 17a or as substrate to P. putida UV4, resulted in the formation of metabolite 4-hydroxy-2-methoxycyclohex-2-en-1-one 25a and provided confirmation of its (4S) absolute configuration by stereochemical correlation.

(d) New metabolites of 2-methylphenol (o-cresol) 4b

Biotransformation (P. putida UV4) of o-cresol 4b was found to yield (4S,5R,6S)-4,5-dihydroxy-6-methylocyclohex-2-en-1-one 8b as the only identified metabolite.²⁵a Repeat biotransformation of phenol 4b, under similar conditions, and GC-MS analysis of a small portion of the crude concentrate, after extraction with EtOAc and silylation, showed that catechol 13b and hydroquinone 16b were present among the minor metabolites. LC-TOFMS analysis (4.31 min.) and comparison with an authentic sample, confirmed that metabolite 8b was the main component: [M+H]+ 143.0702, calcd. for C₇H₁₀O₂ 143.0708 and [M+Na]+ 165.0516, calcd. for C₈H₁₄O₂Na 165.0528. LC-TOFMS analysis also indicated the presence of 4-hydroxy-3-methylocyclohex-2-en-1-one 25b (11.98 min) and cyclohexanone cis-diol 17b (2.92 min) as minor metabolites. PLC purification (50% EtOAc in hexane) yielded 4-hydroxy-3-methylocyclohex-2-en-1-one 25b; the structure and absolute configuration were established by comparison with the literature data of its opposite enantiomer.

(4S)-Hydroxy-2-methylocyclohex-2-en-1-one 25b. Light yellow oil (23 mg, 0.56% yield); Rf 0.20 (50% EtOAc in hexane); [α]D -49.0 (c 0.7, CHCl₃). Lit.¹⁷Ent. [α]D +46.7, CHCl₃); HRMS: (EI) M+ 126.0685, calcd. for C₈H₁₄O₂ 126.0681; 1H NMR (400 MHz) δ = 1.79 (3 H, s, Me), 1.95 (1 H, m, H-5), 2.37 (2 H, m, H-4, H-5), 2.62 (1 H, m, H-6), 2.8 (1 H, br s, OH), 4.55 (1 H, m, H-4), 6.73 (1 H, m, H-3); 13C NMR (100 MHz) δ = 15.9, 33.1, 35.9, 66.9, 135.9, 148.4, 199.9.

(2S,3R,4S)-3,4-Dihydroxy-2-methylcyclohexanone 17b. A solution of 2-methylcyclohex-2-en-1-one cis-diol 8b (5 mg), in methanol (1 mL) containing 10% Pd/C (ca. 1 mg), was stirred overnight at room temperature, under hydrogen atmosphere and normal pressure. The catalyst was filtered off, the filtrate concentrated and the product purified by PLC (80% EtOAc in hexane), to give the hydrogenated cyclohexanone cis-diol 17b (4.2 mg) as a colourless oil; Rf 0.32 (75% EtOAc in hexane); [α]D +1.2 (c 0.35, MeOH); LC-TOFMS: 3.9 min, [M+H]+ 145.0859, calcd. for C₇H₁₄O₂ 145.0865; [M+Na]+ 162.1125, calcd. for C₈H₁₄O₃Na 162.1130; [M+Na]+ 167.0679, calcd. for C₈H₁₄O₃ 167.0684; 1H NMR (400 MHz) δ = 1.14 (1 H, d, J = 6.9 Hz, Me), 1.63 (2 H, br s, 2 OH), 2.03-2.20 (2 H, m, H-5, H-5), 2.33-2.41 (2 H, m, H-6, H-6), 2.57 (1 H, q, J = 6.9, 2.6 Hz, H-2), 4.14 (1 H, m, H-4), 4.17 (1 H, ddd, J = 11.0, 5.3, 2.6 Hz, H-3); 13C NMR (100 MHz) δ = 10.8, 28.4, 38.0, 47.0, 70.8, 76.9, 209.8. The sample of compound 17b was obtained by hydrogenation showed identical LC-TOFMS data to that of metabolite 17b.

(e) GC-MS analysis of diTMS derivatives of 4-hydroxycyclohex-2-en-1-ones 19e and 19a and triTMS derivatives of phenol hydrates (cyclohexa-1,5-diene-1,4-diols) 19e and 19a's

Trimethylsilylation of metabolites 19e and 19a with MSTFA, and GC-MS analyses of the silyl derivatives, showed two peaks in each case. The major peaks were due to the monoTMS derivatives of the keto tautomers 19e (5.05 min., 88%, [M+H]+, m/z = 184) and 19a (8.61 min., 81%, [M+H]+, m/z = 214). The minor peaks (6.40 min., 12%, [M+H]+, m/z = 256) and (8.69 min., 19%, [M+H]+, m/z = 286) were consistent with triTMS derivatives of the corresponding enol tautomers 19e and 19a.'s

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References


Enzyme-catalysed synthesis of cyclohex-2-en-1-one cis-diols from substituted phenols, anilines and derived 4-hydroxycyclohex-2-en-1-ones
