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


Published in:
Advanced Synthesis and Catalysis

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
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Introduction

Enzyme-catalysed formation of phenol metabolites, from aromatic substrates, can proceed directly or indirectly, via transient intermediates. Phenols have been obtained indirectly by: (a) monoxygenase- or peroxygenase-catalysed arené epoxidation and spontaneous isomerization of arené oxide-oxepine intermediates or (b) dioxygenase-catalysed cis-dihydroxylation of arenés and dehydration of cis-dihydrodiol intermediates. Since their discovery by Gibson et al in 1968, 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. Synthetic applications of enantiopure substituted benzene cis-dihydrodiols, obtained as toluene dioxygenase-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 trialkylsilyl ether derivatives.

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

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.201###. (Please delete if not appropriate)
Since phenols are widely distributed in the environment, as natural products, arene metabolites and environmental pollutants, their biodegradation pathways have been extensively studied.[5a-e] 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.[5a-e]

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.[6a-j] Many aniline derivatives are known to be genotoxic and cytotoxic, severely inhibiting cell growth in soil bacteria and slowing their mineralization.[6a-g]

Similar to the metabolism of electron-rich phenols,[5a-e] 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.[6a-j] Aniline-, biphenyl-, diphenylamine- and toluene-dioxygenases, have been reported to catalyse the oxidation of aniline substrates.[64-68] Although cis-diol metabolites were often postulated as intermediates leading to the formation of catechol, hydroquinone and phenol metabolites of anilines,.[6c-j] 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.[6c] Biotransformations using other substituted anilines and bacterial strains, were also found to yield catechol and hydroquinone metabolites,[6a-c] 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,[5b] were based on a comparison with an X-ray crystal structure of TDO and docked toluene substrate.[5g] These studies of TDO, without dioxygen incorporation (3EN1M model), provided preferred orientations of phenol substrates 3a and 3b (Scheme 1).
1) required for production of the corresponding enol cis-dihydriodols 5a (12% docking iterations) and 5b (66% docking iterations). Catechols 13a and 13b (Scheme 2) were also predicted (54% docking iterations from phenol 3a and 34% from 3b).

Docking of phenols 3a and 3b, with dioxygen incorporated TDO (3EN1M-O2 model), led to the predicted formation of catechols (54% docking iterations from 3a, 34% from 3b), but not of cis-dihydriodols 5a and 5b. Further biotransformation (P. putida UV4) of catechols 13, by a catechol dioxygenase-catalysed ring-opening process and other enzyme-catalysed reactions, gave a range of carboxylic acid metabolites. The formation of catechols, as arene metabolites, was also reported to inhibit the TDO activity, therefore reducing cis-dihydriodiol yields.66

The predicted, and isolated, 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.

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. An earlier in silico molecular binding model for diphenylamine 1 (R=NHPk, Scheme 1), at the active site of biphenyl dioxygenase (BPDO), led to the prediction that cis-dihydrioxyls would yield aniline cis-dihydriodiol intermediate 2 (R=NHPk).66

Although BPDO-catalysed cis-dihydrioxylation of substituted aniline 1 (R=NHPk) did not result in the detection of cis-diol 2 (R=NHPk), it was postulated that formation of the major metabolite, phenol 4 (R=NHPk), had resulted from dehydration of this transient intermediate [56].

The qualitative nature of docking results recorded may not quantitatively reflect the experimental results (using P. putida UV4 whole cells), due to further metabolism by the co-induced enzymes. Apart from this caveat, the 3EN1M and 3EN1M-O2 models, employed for TDO docking studies of phenol substrates 3, were found to be useful predictors of the preferred regiochemistry and stereochemistry of cis-diol metabolites. These models have now been applied to cis-dihydrioxylation of anilines 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 cis-diols 6a-d and (ii) as observed, [55] the formation of cis-triols 12, via carbonyl reductase (CRE) catalysed reduction of the ketone group in cis-diols 6, would occur using P. putida UV4 whole cells. The 3EN1M and 3EN1M-O2 model docking studies of anilines 9a-d with TDO were also expected to provide evidence of preferred substrate orientations, leading to the formation of catechols 13a-d, via alternative types of transient aniline cis-diol intermediates 14a-d, resulting from cis-dihydrioxylation at the 1,2- (ipsa-ortho-) bond, as was proposed [6c, 6e-1].

cis-Dihydrioxylation, at the 4,5-bond and % formation of enamine cis-diols 10a-d, was predicted (Table 1), from TDO docking orientations (using 3EN1M model), for 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>Predicteda</th>
<th>Productb</th>
<th>%c</th>
<th>ΔGd</th>
<th>Ke</th>
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<tbody>
<tr>
<td>9a 3-Methoxyaniline</td>
<td>1A</td>
<td>11a</td>
<td>6a</td>
<td>47</td>
<td>-5.08</td>
<td>190.24</td>
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<tr>
<td></td>
<td></td>
<td>13a</td>
<td></td>
<td>26</td>
<td>-5.00</td>
<td>216.49</td>
</tr>
<tr>
<td>9b 3-Methylaniline</td>
<td>1B</td>
<td>11b</td>
<td>6b</td>
<td>90</td>
<td>-5.27</td>
<td>137.66</td>
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<tr>
<td></td>
<td></td>
<td>13b</td>
<td></td>
<td>4</td>
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<td>169.39</td>
</tr>
<tr>
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<tr>
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<td>1D</td>
<td>11d</td>
<td>6d</td>
<td>100</td>
<td>-6.13</td>
<td>31.88</td>
</tr>
</tbody>
</table>

a 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.[50]

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-e]

The cyclohex-2-en-1-one cis-diols 6a-d, previously reported[5a-d] 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[5a,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)
cyclohexenone 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-dihydriodols 10a-d, tautomisation 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 hydroquinonemethanometabolites 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 cyclohexenone cis-diols 6 and 8, cyclohexene cis-triols 12, catechols 13, α-hydroxy carboxylic acids 15, hydroquinones 16, cyclohexanone cis-diol isomers 17cis and 17trans and 1,2,4-trihydroxycyclohexanes 18cised (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%).[58] 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-en-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, (25,3S,4S)-17cis (13% isolated yield) and (2R,3S,4S)-17trans (1% isolated yield).[58] Metabolites 17cis and 17trans were formed via an ene reductase (ERED)-catalysed reduction of the initial bioprocess, 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.** 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,[5a] 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,b (Scheme 4).

A repeat biotransformation of guaiacol 4a again resulted in the formation of chiral metabolites 17a,b, 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 methoxynaphthal 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,[a] as a minor metabolite of 3-iodophenol 3d,[5a] 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.[b] 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 iodocyclohexane 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-methylycyclohex-2-en-1-one 8b, as the only identified metabolite (1% yield).[a] 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,a (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-methylycyclohexanone 17b,a. 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-methylycyclohex-2-en-1-one 25b, ([α]D -49.0). The opposite enantiomer, 25bR, ([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-hydroxyhex-2-en-1-one - metabolites from phenols

The metabolic sequences, to account for the formation of the 4-hydroxyhex-2-en-1-one metabolites 19e,[a] 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. CRED-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 19eR. 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 cis, and 8b→17b cis, have been found during biotransformations[5e] (Scheme 4).

Further evidence of the metabolic sequence (3d→6d→6e→26e→19eR), involving both TDO and ERED enzymes, was found when cyclohex-2-en-1-one cis-diol 6e ([α]₀ - 217), obtained by hydrogenolysis of metabolite 6d, was added as substrate. 4-Hydroxycyclohex-2-en-1-one 19eR and triol 18e were the only identified metabolites. Enantiopure 4-hydroxycyclohex-2-en-1-one 19eR, ([α]₀ +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 25aS from phenol 4a, (4a→8a→17a cis→25aS), was supported by results obtained using (25,35,45)-cyclohexanone cis-diol 17a as substrate; compound 25aS was the only bioproduct formed. It was presumed that the biotransformation of phenol 4b, to yield 4-hydroxycyclohex-2-en-1-one 25bS, would also occur via a similar metabolic pathway (4b→8b→17b cis→25bS).

While the formation of three 4-hydroxycyclohex-2-en-1-ones (19eR, 25aS and 25bS), 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 19aS from phenol 3a. Furthermore, the higher enantiopurity (>98% ee), of bioproducts 19eR, 25aS and 25bS from phenols 3d, 4a and 4b, contrasted with the lower ee value of metabolite 19aS (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 19aS from phenols 3a and 4a. With cyclohexenone cis-diol 6a as substrate, 4-hydroxycyclohexenone 19aS 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 19aS 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→19aS and 4a→8a→16a→27a→28a→19aS) 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 19aS.

It is postulated that the oxidation of hydroquinone 16a, to benzoquinone intermediate 27a, could have resulted from either a non-enzymatic autooxidation 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 tautomerase back to hydroquinone 16a or undergo an asymmetric CRED-catalysed ketone reduction. It could account for metabolite 19aS 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→19aS) Dehydration of cyclohexenone cis-diols 6a and 8a, to give hydroquinone 16a, and of cyclohexanone cis-diols 17a, 17b and 26e, to form 4-hydroxycyclohex-2-en-1-ones 25aS, 25bS and 19eR 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, Pr, Bu), and 33 (R = CO₂Me and Ph), were synthesised from 3-substituted
1,4-cyclohexadienes,\cite{11c} and enantiopure arene hydrates 33 (R = F, Cl, Br, CF₃) and 34 (R = Br) from the corresponding cis-dihydrodiol metabolites 2.\cite{11d}

Kinetic studies of the acid-catalysed dehydration of these arene hydrates showed that they aromatized much faster (3.7x10² - 6.9x10⁴ fold) than cis-dihydrodiols 2.\cite{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.\cite{12a-12c} 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-ones 19e₁₉, 19a₁₉, 2₅a₁₉ and 2₅b₁₉, identified during the study could, in principle, equilibrate with the corresponding phenol hydrate tautomers 19e'₁₉, 19a'₁₉, 2₅a'₁₉ and 2₅b'₁₉; 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 ([M]+ m/z 302), indicating formation of the diTMS derivative.\cite{5e} The molecular ion ([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 $31$,[11b] 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 dehydromination and reformulation of the arene substrates.[11c] Under similar biotransformation conditions, the TDO-catalysed cis-dihydroxylation of arene hydrate $33$ ($R = CF_3$) yielded the opposite enantiomer of cyclohexanone triol $12c$,[11d] and thus revealed an alternative metabolic pathway for monocyclic arene hydrates. The results presented herein demonstrate that the more stable keto tautomers of arene hydrates 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 hydrolase or hydrolyase activity has been reported for alkenes, we are unaware of similar activity with arene substrates. Multistep metabolic pathways, involving cyclohexenone 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-hydroxycyclohexenones 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.[56c] 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.[56] For TLC analysis, Merck Kieselgel 60F₂₅₄ analytical plates were used and PLC separation of metabolites was carried out using glass plates (20 cm x 20 cm) covered with Merck Kieselgel PF₂₅₄₅₆ 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.[56c] 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.[56c] 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/).[13] 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.
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 AutoDock4.2, 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 selected as representative for each group. The positioning 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. 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 only the LC-TOFMS retention times (min.) and accurate mass values were recorded for aniline substrates 9a-d.

3-Methoxy cyclohex-2-en-1-one cis-diol 6a.[5d] 3.43 min., HRMS: [M+H]+ 159.0646, calcd. for C7H10O2 159.0652.

3-Methyl cyclohex-2-en-1-one cis-diol 6b.[5a] 4.85 min., HRMS: [M+H]+ 143.0703, calcd. for C7H10O2 143.0628.


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) properties to an authentic sample derived from phenol 3a. No catechol metabolites (6a–d) 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 (–)-(S)-4-hydroxy-3-methoxycyclohex-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.[5b] Pooled column 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.[5a, 5g] Chiral metabolites, 4-hydroxy cyclohex-2-en-1-ones 19a, 19b, 25a and 25b, cyclohexanone 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-methoxycyclohex-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-oxohexanoic acid 15a by LC-TOFMS analysis: [M+H]+ 177.0758, calcd. for C11H13O3 177.0758; [M+Na]+ 199.0577, calcd. for C12H15O4Na 199.0582; [M+NH4]+ 194.1018, calcd. for C12H14NOS 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-methoxycyclohex-2-en-1-one 19a[14]
Coloured oil (1.2 g, 1%); R\text{f} 0.15 (50% EtOAc in hexane); [\text{\alpha}]_D^\circ = 31.9 (c 1.0, CHCl3); HRMS: (TOF-LCMS) [M+H]+ 143.0709, calcd. for C7H14O2 143.0708; 1H NMR (400 MHz) \( \delta = 2.01 \) (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, ddd, J = 13.7, 7.8, 6.0, 4.6 Hz, H-5), 2.25 (1 H, dddd, J = 13.7, 9.1, 4.6, 4.0 Hz, H-5\text{\textprime}); 2.38 (1 H, ddd, J = 16.9, 7.8, 4.6 Hz, H-6\text{\textprime}); 2.67 (1 H, ddd, J = 16.9, 9.2, 4.6 Hz, H-6\text{\textprime}); 4.50 (1 H, ddd, J = 6.0, 4.0 Hz, H-4), 6.44 (1 H, s, H-2); 13C NMR (100 MHz) \( \delta = -4.6, -4.4, 18.3, 25.8 \) (3C), 32.0, 33.4, 71.0, 132.8, 152.6, 196.0; IR (film) \( \nu_{\text{max}}/\text{cm}^{-1} = 2954, 1659, 1610, 1471 \).

(b) Chemoenzymatic synthesis of (S)-4-hydroxy-3-methoxycyclohex-2-en-1-one 19a[14]

(2S)-2-Bromo-2-cyclohexen-1-ol 21a. This compound was available as a colourless oil from an earlier biotransformation[5b] of 2-bromo-2-cyclohexene-2-en-1-one 20. [\text{\alpha}]_D^\circ = 80.3 (c 1.77, CHCl3); ee 99.8% (chiral GC analysis).

(S)-(2-Bromocyclohex-2-enyloxy)- tert-butylmethyisilane 22a[18] tert-Butylmethyisilyl trifluoromethanesulfonate (280 \mu L, 1.21 mmol) was added to a solution of alcohol 21a (200 mg, 1.10 mmol), maintained at 0°C in dry CH2Cl2 (10 mL) containing triethylamine (240 \mu L, 1.69 mmol). The reaction mixture was stirred (2 h) at 0°C, allowed to warm to room temperature and then ice (20 g) and CH2Cl2 (20 mL) were added to it. After thoroughly mixing the reaction mixture by shaking, the organic layer was separated, washed with brine (15 mL), dried (Na2SO4), and concentrated to give a yellow oil. It was purified by flash chromatography (hexane) to yield the TBS ether 22a as a colourless oil (310 mg, 94%); [\text{\alpha}]_D^\circ = -82.9 (c 0.8, CHCl3); 1H NMR (400 MHz) \( \delta = 0.11 \) (3 H, s, SiMe), 0.17 (3 H, s, SiMe), 0.92 (9 H, s, CMe3), 1.40 (1 H, m, H-5), 1.52-1.68 (3 H, m, H-5\text{\textprime}, H-6, H-6\text{\textprime}), 1.83 (1 H, m, H-4), 1.95 (1 H, m, H-4\text{\textprime}), 4.02 (1 H, m, H-1), 5.97 (1 H, ddd, J = 4.7, 3.7 Hz, H-3); 13C NMR (100 MHz) \( \delta = -4.5, -4.3, 17.4, 18.3, 26.0 \) (3C), 27.9, 33.9, 70.8, 126.0, 132.1; IR (film): \( \nu_{\text{max}}/\text{cm}^{-1} = 2949, 2930, 1644, 1252, 1093 \).

(S)-3-Bromo-(4-tert-butylmethyisilyl)cyclohex-2-en-1-one 23a. A solution of tert-butyl hydroperoxide in water (6 M, 0.63 mL, 3.78 mmol) was added dropwise into a mixture of TBS ether 22a (200 mg, 0.68 mmol), K2CO3 (47 mg, 0.34 mmol), diacetoxyiodobenzene (670 mg, 2.1 mmol) and butyl butyrate (1.5 mL) maintained at 0°C. The reaction mixture was stirred (0°C, 8 h), diluted with a mixture of 20% ether in hexane (10 mL), filtered (diatomaceous earth), and the filtrate concentrated to give a crude yellow oil. It was purified by column chromatography (5% Et2O in hexane) to give enone 23a as a colourless oil (60 mg, 29%); R\text{f} 0.28 (5% Et2O in hexane); [\text{\alpha}]_D^\circ + 41.5, (c 0.5, CHCl3); HRMS: (LC-TOFMS) [M+H]+ 305.0560, calcd. for C12H12O2SiBr 305.0570; 1H NMR (400 MHz) \( \delta = 0.15 \) (3 H, s, SiMe), 0.20 (3 H, s, SiMe), 0.93 (9 H, s, CMe3), 2.07 (1 H, ddd, J = 13.7, 7.8, 6.0, 4.6 Hz, H-5), 2.25 (1 H, dddd, J = 13.7, 9.1, 4.6, 4.0 Hz, H-5\text{\textprime}); 2.38 (1 H, ddd, J = 16.9, 7.8, 4.6 Hz, H-6), 2.67 (1 H, ddd, J = 16.9, 9.2, 4.6 Hz, H-6\text{\textprime}); 4.50 (1 H, ddd, J = 6.0, 4.0 Hz, H-4), 6.44 (1 H, s, H-2); 13C NMR (100 MHz) \( \delta = -4.6, -4.4, 18.3, 25.8 \) (3C), 32.0, 33.4, 71.0, 132.8, 152.6, 196.0; IR (film) \( \nu_{\text{max}}/\text{cm}^{-1} = 2954, 1659, 1610, 1471 \).
(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 C8H10O3Na 165.0528; [M+K]+ 181.0261, calcd. for C8H10O3K 181.0267; [M+H - H2O]+ 125.0523, calcd. for C6H8O2 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, 3.3 Hz, H-6a), 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-methoxycyclohexane (17a) 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.9a 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 C7H10O3 143.0708 and [M+Na]+ 165.0516, calcd. for C8H10O3Na 165.0528. LC-TOFMS analysis also indicated the presence of 4-hydroxy-3-methylocyclohex-2-en-1-one (25b) (11.89 min.) and cyclohexane cis-diol (17b) (3.9 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-methoxycyclohex-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.[7] Ent. [α]D + 46.7, CHCl3); HRMS: (El) M+ 126.0685, calcd. for C7H10O2 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 C7H14O2Na 162.1130; [M+H]- 167.0679, calcd. for C7H14O2Na 167.0684; 1H NMR (400 MHz) δ = 1.14 (1 H, d, J = 6.9 Hz, Me), 1.63 (2 H, br s, 2x 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, dd, 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) obtained by hydrogenation showed identical LC-TOFMS data to that of metabolite (17b).

(e) GC-MS analysis of diTMS derivatives of 4-hydroxyoxycyclohex-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.

Acknowledgements

We thank the Department of Education and Learning, Northern Ireland (to PABMcI) and the Leverhume Trust (to PH) for postgraduate Studentships.

References


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


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