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cis-Dihydroxylation of Tricyclic Arenes and Heteroarenes Catalyzed by Toluene Dioxygenase: A Molecular Docking Study and Experimental Validation

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Abstract: Molecular docking studies of toluene dioxygenase led to the prediction that angular and lateral *cis*-dihydroxylation of tricyclic arene and heteroarene substrates could occur. Biotransformations of biphenylene, dibenzofuran, carbazole and dibenzothiophene, using *Pseudomonas putida* UV4 whole cells expressing toluene dioxygenase, confirmed that both angular and lateral *cis*-dihydroxylation had occurred in the predicted regioselective and stereoselective manner.

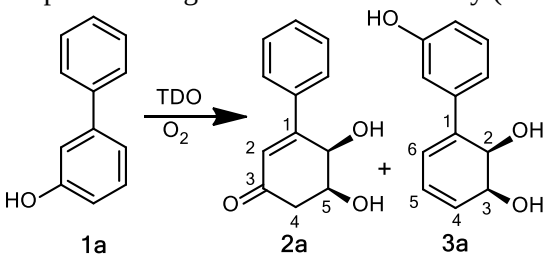
The toluene dioxygenase-catalysed (*Pseudomonas putida* UV4) biotransformation of dibenzofuran was optimized, to produce 1,2-dihydrodibenzofuran-1,2-diol as the major metabolite in excellent yield. 2-Hydroxydibenzofuran, resulting from dehydration of 1,2-dihydrodibenzofuran-1,2-diol, was also found to undergo *cis*- dihydroxylation to give a very minor *cis*-dihydrodiol metabolite. The enantiopurity (>98% *ee*) and (1*R*,2*S*) absolute configuration of the major dibenzofuran *cis* -dihydrodiol was rigorously established by catalytic hydrogenation and formation of dimethoxy phenyl trifluoromethyl acetate derivatives and by X-ray crystallography of an epoxide derivative. Biotransformation of carbazole yielded anthranilic acid as the major metabolite and was consistent with angular *cis*-dihydroxylation. Synthesis of a *cis*- diol epoxide derivative showed that the main *cis*-dihydrodiol metabolite of dibenzofuran has potential in the chemoenzymatic synthesis of natural products.

Keywords: Tricyclic heteroarene biotransformations; molecular docking; toluene dioxygenase; lateral/angular *cis*-dihydroxylation; dibenzofuran *cis*-dihydrodiol; chemoenzymatic synthesis

Introduction

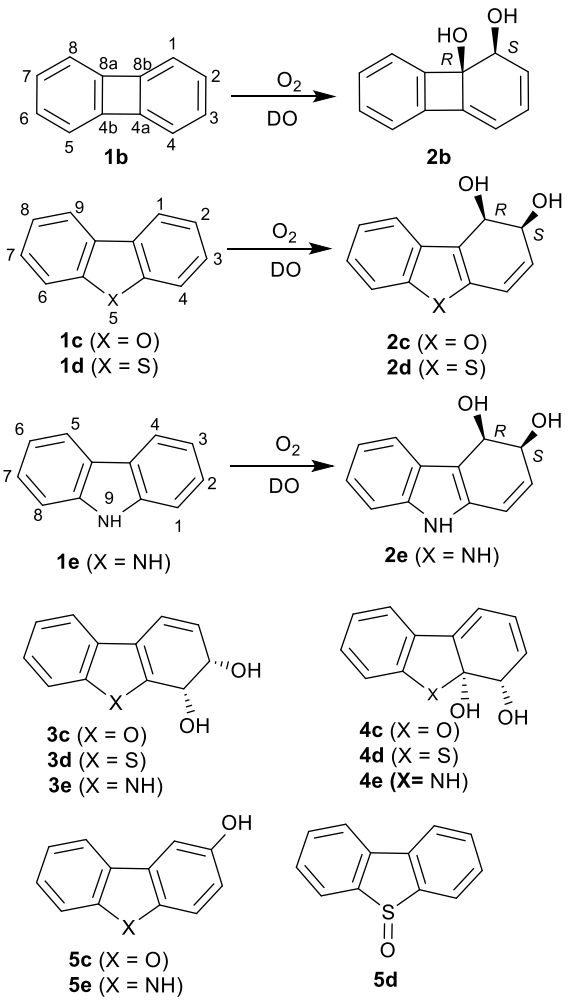
Ring hydroxylating dioxygenase enzymes, having the ability to catalyse arene *cis*-dihydroxylations, are available with a range of binding site capacities, able to accommodate both mono- and poly-cyclic arenes.[1a-f] Among dioxygenases with smaller active sites are benzoate dioxygenase (BZDO) which accepts benzoic acid substrates, and toluene dioxygenase (TDO), which generally accommodate only monocyclic and bicyclic arenes. Larger binding sites are found in naphthalene dioxygenase (NDO), which mostly accept only bicyclic and tricyclic arenes, and biphenyl dioxygenase (BPDO) which catalyse *cis*-dihydroxylation of polycyclic arenes containing up to five fused rings. NDO and BPDO are largely unable to catalyse *cis*-dihydroxylation of monocyclic arenes, exceptions being larger biaryl substrates, *e.g.* 3-phenylphenol **1a**.[2] Dioxygenase enzymes BZDO, TDO, NDO and BPDO have all been employed in chemoenzymatic synthesis, but to date most arene *cis*-dihydrodiol metabolites, used as chiral synthons, have been formed with monocyclic substrates and TDO as biocatalyst. [1a-f]

Several molecular docking studies of TDO with monocyclic aromatic substrates, have recently been used to successfully match the resulting *in silico* predictions with reported benzylic monohydroxylation and arene *cis*-dihydroxylation results. [3a-e] Thus, AutoDock 4.2 molecular docking studies, of substituted phenol and aniline substrates with TDO, led to predictions about: (a) their preferred orientations during *cis*-dihydroxylation and (b) the resulting regioselectivity and stereoselectivity. These expectations were later confirmed by experimental results.[3c,3e] This approach was applied to the TDO docking results obtained using 3-hydroxybiphenyl **1a** as substrate, where the corresponding metabolites **2a** and **3a** were predicted to be the major and minor products respectively, resulting from lateral *cis*-dihydroxylation.[2] Biotransformation (*P. putida* UV4 mutant strain expressing TDO) of substrate **1a**, yielded metabolites (5*S*,6*R*)-**2a** and (2*R*,3*S*)-**3a** with the predicted regio- and stereo-chemistry (Scheme 1).



Scheme 1. *cis*-Dihydroxylation of 3-hydroxybiphenyl **1a** to give *cis*-diols **2a** and **3a**.

TDO-catalyzed regio- and stereo-selective *cis*-dihydroxylations of bicyclic arenes, (*e.g.* naphthalene) and heteroarenes (*e.g.* quinoline), predicted by AutoDock Vina molecular docking (unpublished data), matched with earlier experimental results.[1a-c,4] Similar TDO-catalyzed *cis*-dihydroxylations of tricyclic arenes and heteroarenes, to our knowledge, have not been reported. Thus, the current study, using the AutoDock Vina program, was undertaken to find out if: (i) the larger tricyclic arene substrates, *e.g*. biphenylene **1b**, dibenzofuran **1c**, carbazole **1e** and dibenzothiophene **1d** would be accommodated by the TDO active site, (ii) the experimental *cis*-dihydroxylation results, obtained using TDO and NDO enzymes expressed by mutant *Pseudomonas* strains, would match those predicted by the molecular docking results (Scheme 2) and (iii) supporting evidence could be obtained for the postulated role of transient metabolites leading to the formation of phenols and other metabolites from tricylic arenes and heteroarenes.



Scheme 2. Dioxygenase (DO)-catalysed *cis*-dihydroxylation of arenes **1b-1e** to give *cis*-dihydrodiols **2b-2e, 3c-3e, 4c-4e**, phenols **5c, 5e** and sulfoxide **5d**.

Results and Discussion

The biotransformation (*P. putida* UV4 whole cells, expressing TDO) of biphenylene **1b** had resulted in *cis*-dihydroxylation at a ring junction (angular) bond to give only *cis*-dihydrodiol **2b**.[5a,5b] Conversely, the biotransformation of tricyclic heteroarenes **1c-1e** by mutant strains of *P. putida* 9816/11 and *P. fluorescens* TTC1(NCIMB 40605), both expressing NDO, resulted in lateral *cis*-dihydroxylations to yield *cis*-diols **2c-2e**.[6a-d] Molecular docking studies were conducted to compare with these reported biotransformation results and to predict if similar results would be found using TDO with potential substrates **1c-1e**.

TDO docking of biphenylene 1b: Prior to the earlier reports, using biphenylene and 1,2-dihydrobenzocyclobutene as substrates, TDO-catalysed *cis*-hydroxylation at an angular position, in a substituted monocyclic or bicyclic arene substrate was unprecedented.[5a,5b] The *cis*-dihydroxylation (*P.*

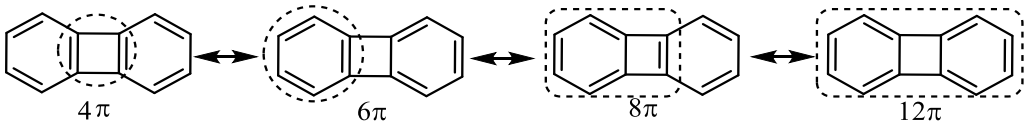


Figure 1. Resonance structures of biphenylene **1b**.

putida UV4) of biphenylene **1b** was thus unusual, since the attack occurred exclusively at an angular bond (1,8b), to give (1*S*,8b*R*)-dihydrodiol **2b** (66% isolated yield) as the only isolated metabolite (Scheme 2).

Biphenylene **1b**, having two fused benzene rings and with four possible canonical (resonance) structures (4π, 6π, 8 π, 12 π), was classified as a tricyclic arene substrate with some anti-aromatic character (Figure 1).[7] Electron density is more localized within the biphenylene ring and the 4 π (cyclobutadiene) resonance structure being disfavoured. Thus, its aromaticity was markedly reduced compared with the tricyclic arenes **1c**-**1e** and was estimated as only 1.59 times that of benzene.[7] TDO-catalysed angular *cis*-dihydroxylation, to yield *cis*-dihydrodiol **2b**, would therefore be more favoured by the 6 π, 8 π and 12 π canonical structures.

Using Autodock Vina, the energetically most favourable binding orientation of biphenylene **1b** (-6.6 kcal·mol^{-1}, **1b$_1$**) within the active site of TDO is shown in Figure 2. This orientation appeared to be mainly due to: (i) attractive interactions of the substrate with the orthogonal phenyl group of Phe-216 and with the imidazole group of His-222 (edge-to-face bonding) and (ii) the proximate hydrophobic amino acids Ile-276, Leu-272, Ile-324, Val-309, Leu-272 Phe-352 (van der Waals interactions). It would also result in an exclusive preference for angular rather than lateral *cis*-dihydroxylation and is matched by the isolation of the remarkably stable enantiopure *cis*-diol (1*S*, 8b*R*)-**2b** (> 98% *ee*), as the only detected metabolite using *P. putida* UV4.[5a,5b]

The opposite enantiomer (1*R*,8b*S*)-**2b** was isolated later when biphenylene **1b** was used as substrate for a different *Pseudomonas* strain (C250), expressing carbazole 1,9a-dioxygenase (CARD).[6b] These reports provide a very rare example of enantiocomplementarity during dioxygenase-catalysed formation of arene *cis*-dihydrodiols. The good agreement between the biotransformation result and *in silico* TDO binding orientation of biphenylene **1b** provided an incentive to extend the docking of tricyclic heteroarenes **1c**-**1e**, in the quest for further evidence of lateral or angular *cis*-dihydroxylation.

1b$_1$

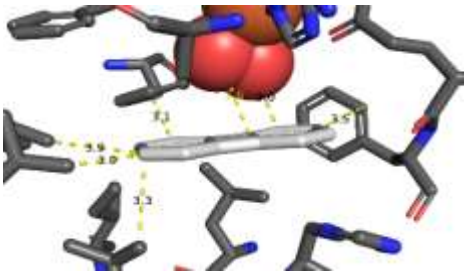
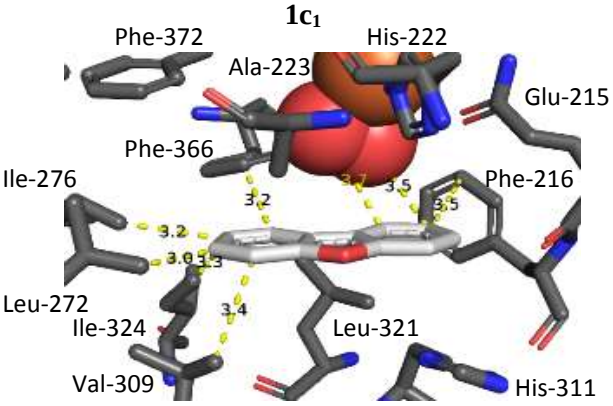


Figure 2: *In silico* docking orientation of biphenylene **1b** with TDO. **1b$_1$:** -6.6 kcal·mol^{-1}.

(ii) TDO docking of dibenzofuran 1c: Molecular docking of dibenzofuran **1c** with TDO (Figure 3) led to the prediction of two preferred orientations (**1c$_1$** and **1c$_2$**), which appeared to be very similar to the preferred orientation of biphenylene **1b** (Figure 2).

1c$_1$



1c$_2$

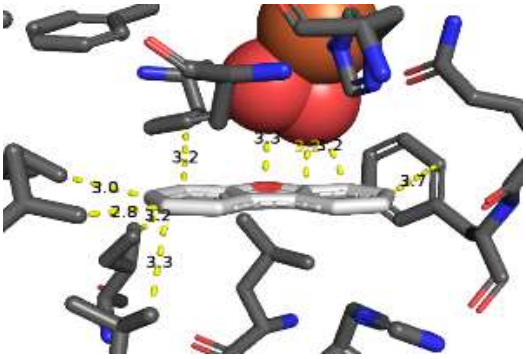


Figure 3. *In silico* docking orientations of dibenzofuran **1c** with TDO. **1c$_1$:** -5.7 kcal·mol^{-1}; **1c$_2$:** -5.0 kcal·mol^{-1}.

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Lateral *cis*-dihydroxylation was predicted to occur preferentially at the 1,2-bond, to yield the (1*R*,2*S*) enantiomer **2c**, based on the higher binding energy (-5.7 kcal·mol^{-1}, **1c$_1$**). This docking study of dibenzofuran **1c** also predicted a less favoured alternative orientation, based on the lower binding energy (-5.0 kcal·mol^{-1}, **1c$_2$**). However, with closer proximity between the 4,4a-bond of substrate and dioxygen (3.2 Å) could result in angular docking, leading to the formation of a transient *cis*-dihydrodiol metabolite **4c**, ring opening to a catechol and further biodegradation products. No docking evidence was found to predict lateral *cis*-dihydroxylation (using TDO) at the 3,4-bond of substrate **1c**, to yield *cis*-dihydrodiol **3c**. This was presumed to be due to the larger distance of the 3,4-bond from the bound dioxygen molecule.

Biotransformations of dibenzofuran **1c** (catalyzed by ring hydroxylating dioxygenases with larger active sites compared to TDO) yielded both *cis*-dihydrodiols **2c** and **3c** along with other metabolites, which were consistent with the initial formation of transient angular *cis*-dihydrodiol **4c**.[8a-8c] Both NDO-catalysed biotransformations (*P. putida* 9816-11 and *P. fluorescens* TTC1 mutant strains) of dibenzofuran **1c** gave metabolites *cis*-dihydrodiol **2c** (60%) and *cis*-dihydrodiol **3c** (40%).[6c,6d]

Recent computational modelling studies of NDO with arene substrates have been reported.[9a,9b] The current docking study of dibenzofuran **1c** with NDO, using Autodock Vina, produced two orientations leading to the prediction that *cis*-dihydroxylation and higher binding energies would result in the formation of metabolites (1*R*,2*S*)-**2c** (Figure 4, **1c$_3$**, -8.8 kcal·mol^{-1}) and (3*S*,4*S*)-**3c** (Figure 4, **1c$_4$**, -8.5 kcal·mol^{-1}). The predicted relative ratio and absolute configurations of these major and minor metabolites were in accord with the isolated bioproducts **2c** and **3c**.[6c,6d]

The primary interactions leading to this orientation of substrate **1c** (Figure 3, **1c$_1$** and **1c$_2$**) were very similar to those found with biphenylene **1b** (Figure 2). Thus, one benzene ring was observed to interact with the proximate hydrophobic amino acids (Ile-276, Leu-272, Ile-324, Val-309, Leu-272 Phe-352). Edge-to face interactions between phenyl rings have been widely reported in reviews, but much less information is available on similar interactions between phenyl groups and five membered heterocyclic rings.[10a] Recently, evidence for the edge- to-face interactions between benzene and furan or thiophene rings has been reported. [10b, 10c] To date little evidence for edge-to-face bonding between benzene and imidazole rings appears to have been been reported. However, the preferred binding orientations of dibenzofuran **1c** with TDO shown in Figure 3, are consistent with edge-to-face interactions between the benzene ring, closer to dioxygen and the phenyl ring of Phe-216, and also with the imidazole ring of His-222.

The differences in stereo- and regio-selectivity between TDO and NDO can be explained by the differing volumes of their active sites. The overall number and distances between amino acids is larger in NDO (*ca*. 20 amino acids, 11-15 Å) than in TDO (17 amino acids, *ca*. 8-13 Å) (Figure SI 1). Furthermore, CASTp calculations suggested that the volume of the NDO active site (92.987) is 130% larger than the TDO active site (40.140).[9c] The larger capacity of the NDO active site, would allow both binding orientations to facilitate formation of the isolated *cis*-dihydrodiols **2c** and **3c** while the smaller dimensions of the TDO active site, would favour only the formation of *cis*-dihydrodiol **2c**.

(iii) TDO-docking of carbazole 1e: The hydrophobic and edge-to-face interactions and preferred TDO binding orientation of carbazole **1e** (Figure 5, **1e$_1$**, -5.3 kcal·mol^{-1}) are very similar to that shown for the predicted lateral *cis*-dihydroxylation of dibenzofuran **1c** (Figure 3, **1c$_1$**, -5.7 kcal·mol^{-1}) and would lead to the formation of (4*R*,3*S*)-*cis*-dihydrodiol **2e**. NDO-catalyzed biotransformation of carbazole **1e** had been reported to give phenol **5e** as the only isolated metabolite.[6a] NDO-docking orientations are shown in Figure SI 2. It was postulated that phenol **5e** could result from dehydration of the unstable metabolite *cis*-dihydrodiol **2e**. Although a lower binding energy was found for this TDO docking orientation (**1e$_2$**, -4.6 kcal·mol^{-1}), the shorter distances between proximate dioxygen and 3,4-bond of substrate (3.1 Å) could facilitate the angular *cis*-dihydroxylation of carbazole **1e**, yielding the transient metabolite **4e** with a (1*S*,9a*R*) configuration. The formation of metabolite **4e** was proposed during the CARDO-catalysed biotransformation of carbazole **1e**; it was assumed to undergo further metabolism and ring opening, to yield a range of products including anthranilic acid.[8a, 8d]

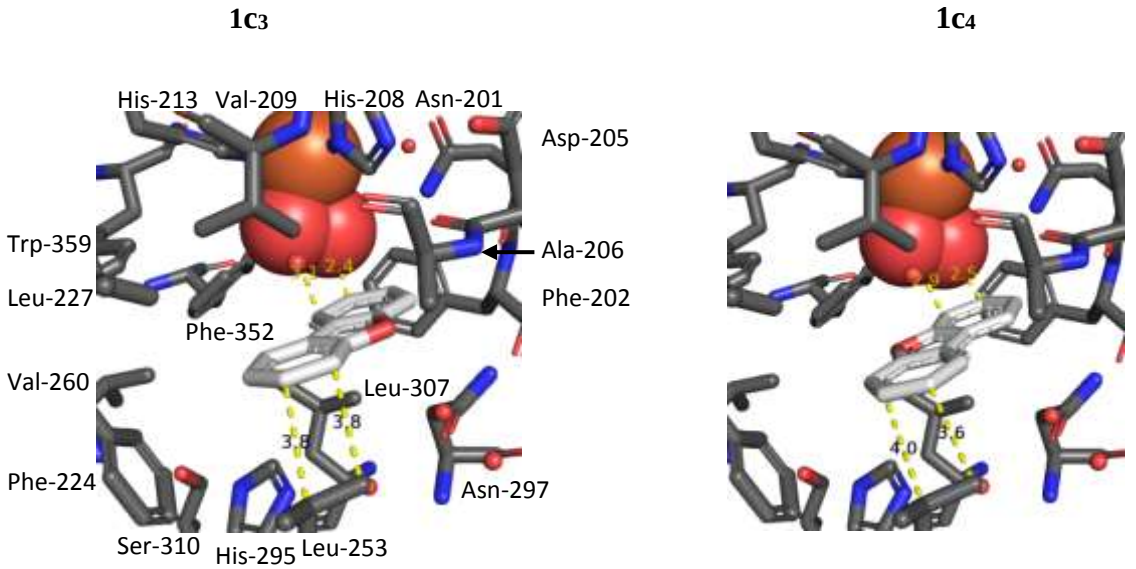

Figure 4: In *silico* docking orientations of dibenzofuran **1c** within the active site of NDO. **1c$_3$:** -8.8 kcal·mol^{-1}; **1c$_4$:** -8.5 kcal·mol^{-1}.

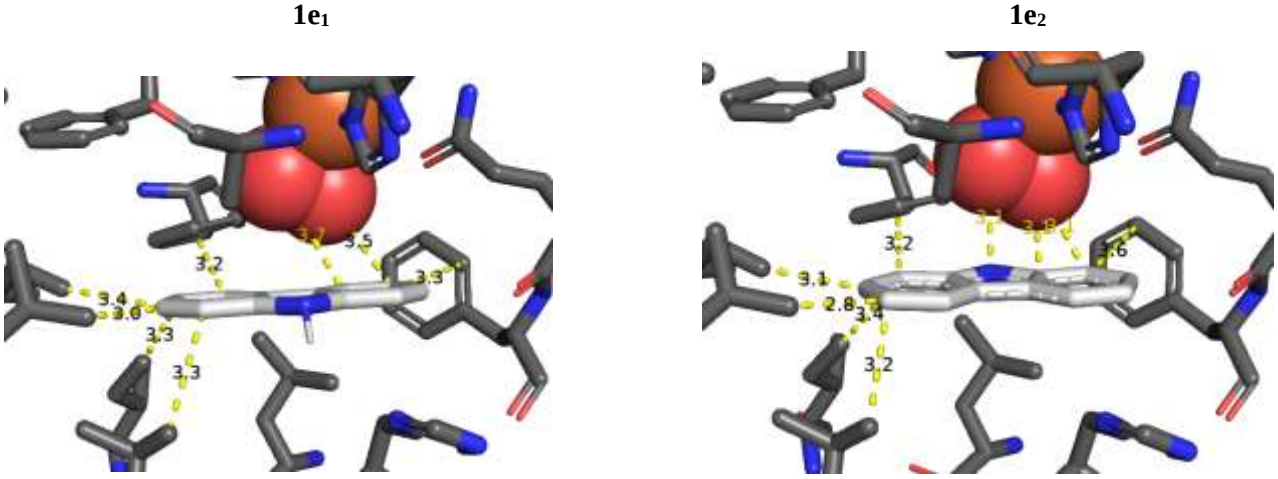

Figure 5. *In silico* docking orientations of carbazole within the active site of TDO. **1e$_1$:** -5.3 kcal·mol^{-1}; **1e$_2$:** -4.6 kcal·mol^{-1}.

(iv) TDO docking of dibenzothiophene 1d: Docking of dibenzothiophene **1d** with TDO (Figure 6) showed one favoured orientation (**1d$_1$**, -5.0 kcal·mol^{-1}) that was similar to those found using biphenylene **1b** (Figure 2), dibenzofuran **1c** (Figure 3) and carbazole **1e** (Figure 5). These orientations were consistent with lateral *cis*-dihydroxylation occurring, preferentially, at the 1,2-bond to form the major metabolite (1*R*,2*S*)-**2d** enantiomer. The sulfur atom in dibenzothiophene **1d** was closer to the nearest oxygen atom (3.3 Å) within the TDO active site (Figure 6, **1d$_2$**) compared with NDO (4.0 Å, Figure SI 3, **1d$_3$**) leading to the prediction that sulfoxidation might occur more readily with TDO. However, the low free binding energy (**1d$_2$**, -2.9 kcal·mol^{-1}) associated with this orientation would not facilitate the formation of sulfoxide **5d** or the transient angular *cis*-dihydrodiol **4d**. It is noteworthy that while transient angular *cis*-diols **4c** and **4e** were postulated as initially formed intermediates during CARDO-catalysed dihydroxylation of dibenzofuran **1c** and carbazole **1e**, formation of the corresponding angular *cis*-diol **4d** of dibenzothiophene **1d** was not proposed. [8a] NDO docking of dibenzothiophene **1d** (Figure SI 3) led to the prediction that *cis*-dihydrodiols **2d** and **3d** would be equally preferred metabolites based on binding energies (**1d$_3$**, -8.5 kcal·mol^{-1}; **1d$_4$**, -8.5 kcal·mol^{-1}). In practice, *cis*-

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dihydrodiol **2d** was the main metabolite (85-95%), along with *cis*-dihydrodiol **3d** (<5%) and sulfoxide **5d** (<15%) as minor products from the NDO-catalysed oxidation of dibenzothiophene **1**.[6c,6d]

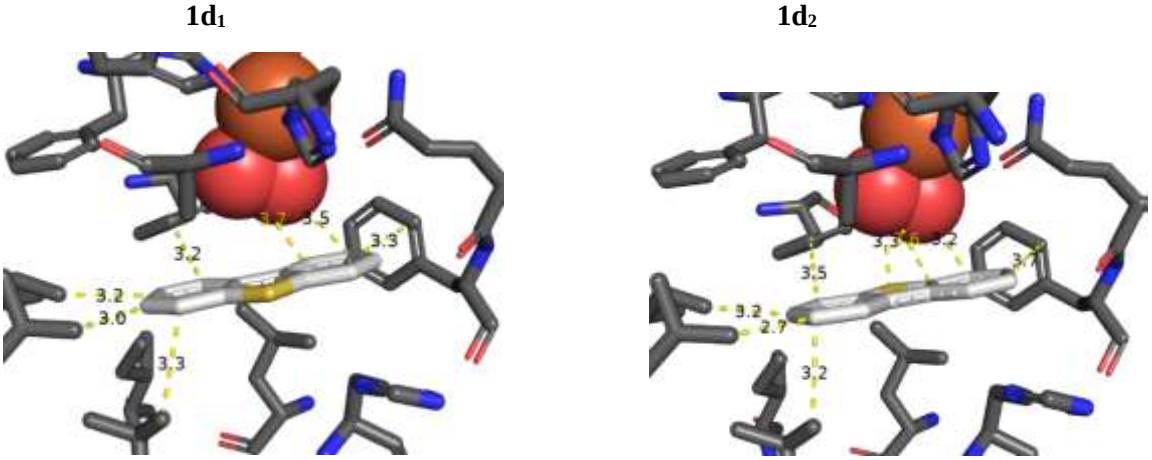
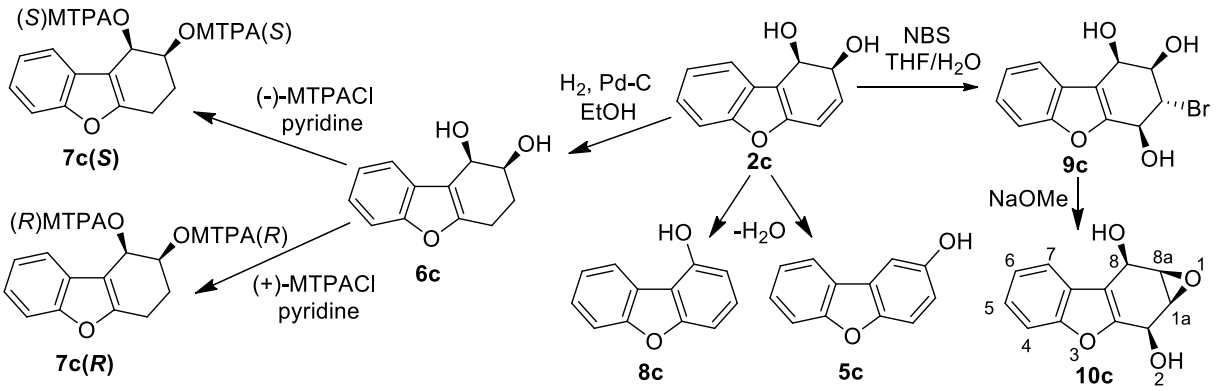


Figure 6: In *silico* docking orientations of dibenzothiophene **1d** with TDO. **1d$_1$:** -5.0 kcal·mol^{-1}; **1d$_2$:** -2.9 kcal·mol^{-1}.

(v) TDO-catalyzed biotransformation of dibenzofuran 1c: Medium-scale biotransformations (*P.putida* UV4) of dibenzofuran **1c** (400-500 mg/L) were conducted in 2 litre baffled flasks, employing a slight modification of reported conditions.[2,3c,5b] At pH below 7.5, very little evidence of *cis*-dihydrodiol **2c** formation was observed (Scheme 3). Maintaining the pH within the range 7.5-8.5 significantly reduced the dehydration of metabolite **2c**, to yield mainly phenol **5c** and enabled yields of up to 90% (LC-MS analysis). Metabolite **2c** was initially isolated in an average yield of *ca*. 40%, but using an extended biotransformation period (48 h) at pH 8.0 and a careful workup procedure an optimal isolated yield of 85% was achieved.

During earlier NDO-catalysed biotransformations of dibenzofuran **1c** at pH 7.0-7.3, a range of metabolites was formed.[6b,6d] Among the mixture, that proved difficult to separate, were *cis*-dihydrodiols **2c** and **3c** and the corresponding phenols, resulting from their partial dehydration. In contrast to the very stable *cis*-diol metabolite **2b**, formed from biphenylene **1b**, *cis*-dihydrodiol **2c** was found to be more susceptible to dehydration at *ca*. pH 7, yielding mainly 2-hydroxydibenzofuran **5c** and 1-hydroxydibenzofuran **8c** as a very minor product (Scheme 3). *cis*-Dihydrodiol **2c** was the only metabolite detected using *E. coli* TOP10109 (pCL-41) and *E. coli* 1/210LK recombinant cells, both expressing TDO. While lower yields were obtained with these recombinant strains, they provided further evidence that TDO was the biocatalyst responsible for the *cis*-dihydroxylation of this tricyclic arene.

Neither enantiopurity values nor absolute configurations were established for metabolites isolated during the NDO-catalysed (*P. fluorescens* TTC1) biotransformation studies of dibenzofuran **1c**.[6d] *cis*-Dihydrodiols **2c** and **3c** obtained using *P. putida* 9816-11 were found to have >95% *ee*, by treatment with (*S*)- and (*R*)-2(1-methoxyethyl)phenylboronic acid (MPBA) and ^{1}H-NMR analysis of the resulting diastereomeric boronate derivatives. A consistent trend in directional chemical shifts of Me and MeO signals for MPBA derivatives, of other *cis*-diol boronate diastereoisomers, of known absolute configurations, was also observed for *cis*- diols **2c** and **3c**; on this basis, metabolite **2c** was assigned a (1*R*,2*S*) configuration, although no optical rotation values ($[\alpha]_D$) were reported for metabolites **2c** and **3c**.[6b] The MPBA method was generally found to



Scheme 3. Reactions of *cis*-dihydrodiol **2c** to yield *cis*-tetrahydrodiol **6c**, diMTPA esters **7c(*S*)**, **7c(*R*)**, bromohydrin **9c** and diol epoxide **10c**.

be very useful in assigning both *ee* values and absolute configurations to most arene *cis*-dihydrodiols.[11a]

cis-Dihydrodiol metabolite **2c** isolated during the current study, was hydrogenated (Pd/C, MeOH) to yield *cis*-tetrahydrodiol **6c** (90 % yield). Reaction with (+) and (-)-MTPA chloride gave the corresponding diastereomeric (+)-**7c (*R*)** diMTPA ester- and (-)-**7c (*S*)** - diMTPA ester (Scheme 3). NMR analysis of the diMTPA esters showed that the metabolite was enantiopure. This method had been used earlier to determine both *ee* values and absolute configurations of other *cis*-diol metabolites, derived from polycyclic arenes. [11b] The diMTPA method showed that the *cis*-dihydrodiol **2c** ($[\alpha]_D$ +217) was enantiopure (>98% *ee*) and was assigned a (1*R*,2*S*) configuration.

As part of a preliminary study of the potential of *cis*-dihydrodiol **2c** as a chiral synthon in natural product synthesis, it was considered appropriate to establish its absolute configuration by X-ray crystallography. *cis*-Diol epoxide derivative **10c** was synthesized in two steps from *cis*-dihydrodiol **2c**, *via trans* bromohydrin intermediate **9c** (Scheme 3). X-ray crystallographic analysis of a suitable crystal (from Et_2O-hexane) of epoxide **10c** established its absolute configuration as (1a*S*,2*R*,8*R*,8a*R*) (Figure 7). The (1*R*,2*S*) configuration for *cis*-dihydrodiol **2c**, presumed by the consistent trends in chemical shifts of specific signals in NMR spectra of the MPBA and diMTPA derivatives, was therefore confirmed.[6c,11a,11b]

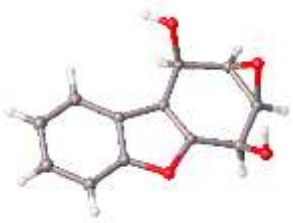
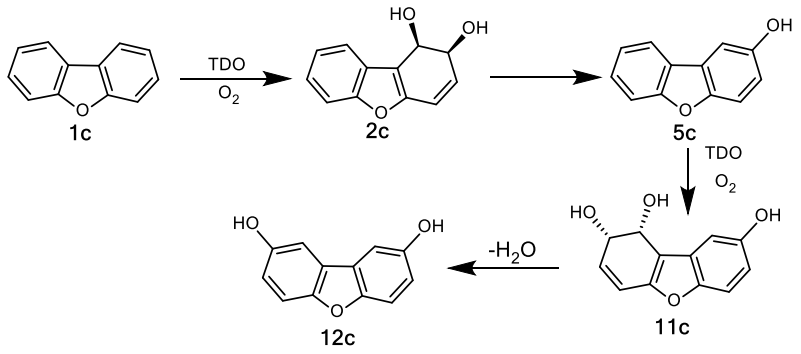


Figure 7. X-ray crystal structure of diol epoxide **10c**.

cis-Dihydrodiol **2c** was initially detected by reverse phase LC-MS analysis of the biotransformation supernatant aqueous solution and was identified by a large peak eluting after 18 min. In some of the repeat biotransformations of dibenzofuran **1c**, evidence of a very minor metabolite eluting after 12 min., with a mass of $[M+NH_4^+]$ = 236.09150, was also observed (SI: Figure 17). Since 2-hydroxydibenzofuran **5c** is readily produced by partial decomposition of *cis*-dihydrodiol **2c**, during the biotransformation, the possibility that this very minor bioproduct could be 8-hydroxydibenzofuran-1,2-diol **11c** (calculated mass: 236.09173), was considered (Scheme 4). Biotransformation of phenol **5c**, to yield the possible metabolite **11c**, resulted in a significant proportion of the substrate being recovered, however, LC-MS analysis of the supernatant again detected the presence of this metabolite in very low yield.

The ^{1}H NMR (400 MHz) spectrum of a late eluting impure fraction, collected during the purification (column chromatography) of the crude biotransformed material obtained from dibenzofuran **1c**, showed most of the characteristic *cis*-dihydrodiol signals: δ = 4.88 (1H, d, J = 6.9, 2.5 Hz, H-1), 5.96 (1H, ddd, J = 10.3, 2.5, 1.3 Hz, H-4), 6.43 (1H, dd, J = 10.0, 2.8 Hz, H-3), which were close to but distinct from those observed for the major *cis*-dihydrodiol **2c**. Attempts to purify it further by PLC resulted in its decomposition. In an effort to obtain more evidence for the formation of hydroxydiol **11c**, molecular docking of phenol **5c** with TDO was conducted **(**Figure SI 4). The results indicated a preferred orientation that would lead to the formation of phenolic *cis*-dihydrodiol **11c** with an identical absolute configuration to that found in dibenzofuran *cis*-dihydrodiol **2c**, but with a low free binding energy (-3.1 kcal·mol^{-1}). Based on the limited LC-MS and NMR data available an molecular docking



Scheme 4: Possible metabolic pathways during TDO - catalyzed biotransformations of dibenzofuran **1c** using *P. putida* UV4.

evidence, the possibility that biotransformation of dibenzofuran **1c** can occasionally yield both *cis*-dihydrodiols **2c** and **11c**, *via* the metabolic pathway shown in Scheme 4, cannot be excluded. The TDO-catalysed formation of *cis*-dihydrodiols from monocyclic phenols, *e.g.* compounds **2a** and **3a** from substrate **1a** was established earlier.[2, 3c, 3e]

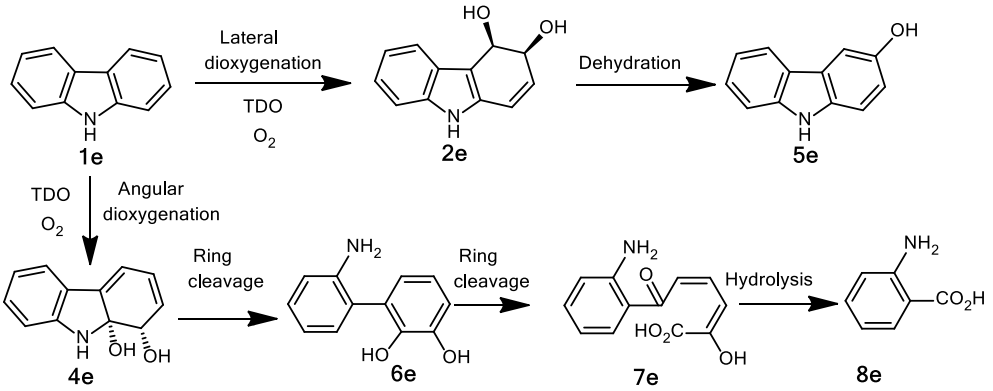
(vi) TDO-catalyzed biotransformation of carbazole 1e: Biotransformations of carbazole **1e** had been reported using, *P. putida* 9816/11 (NDO source), *E. coli* JM109(pKK223-3) (NDO source) and *Sphingobium yaniokuyae* B8/36 (BPDO source).[6a] No metabolites were identified from the attempted biotransformation of carbazole **1e** using *P. putida* F39/D (TDO source), but NDO- and BPDO-catalysed oxidations resulted in the formation of a single metabolite, which was identified as 3-hydroxycarbazole **5e**.[6a] While the possibility of a direct enzymatic monooxygenation was not excluded, an alternative lateral *cis*-dihydroxylation mechanism was proposed, through the formation of an unstable *cis*-dihydrodiol **2e**, followed by its spontaneous dehydration (Scheme 5). A transient angular *cis*-dihydrodiol metabolite **4e**, resulting from a CARDO-catalysed (*Pseudomonas* sp.CA10) *cis*-dihydroxylation of carbazole **1e** was postulated to account for the formation of anthranilic acid **8e** as a metabolite formed *via* catechol **6e** and ketocarboxylic acid **7e** intermediates.[8a]

As expected, repeat biotransformations (*P. putida* UV4) of carbazole **1e** showed no evidence of the predicted transient *cis*-dihydrodiol **2e**. GC-MS analysis of the crude extract, did however indicate that anthranilic acid **8e** was the major identifiable metabolite. 3-Hydroxycarbazole **5e** was also detected but only as a very minor dehydration product (SI: Figure 16), presumably derived from the unstable *cis*-dihydrodiol **2e**. Extraction (EtOAc) of bioproducts of carbazole **1e**, followed by PLC purification, yielded a crystalline product whose NMR spectrum confirmed that the major metabolite was anthranilic acid **8e**. The isolation of bioproduct **8e** and detection of 3-hydroxycarbazole **5e** (Scheme 5), were consistent with the predicted formation of the corresponding transient *cis*-diols **2e** and **4e**, *via* the other undetected intermediates **6e** and **7e** (SI: Figure 2, **1e$_1$**, **2e$_2$**). The isolation of *cis*-diol **2b** and anthranilic acid **8e** from biotransformations of biphenylene **1b** and carbazole **1e** respectively, provided support for *in silico*-predicted angular *cis*-dihydroxylations being catalysed by TDO.

(vii) TDO-catalyzed biotransformation of dibenzothiophene 1d: *cis*-Dihydrodiols, **2d**, **3d** and sulfoxide **5d** were identified as the major NDO-catalysed metabolites of dibenzothiophene **1d**, using *P. putida* 9816-11, *E. coli* JM109(DE3)(pDTG141) and *P. fluorescens* TTC1 cells. The enantiopurity (>95%) of *cis*-diol **2d** was determined by formation of chiral boronate derivatives. [6c,6d]

While neither *ee* value nor absolute configuration of the minor *cis*-dihydrodiol metabolite **3d** (>5% yield) was reported, results from the NDO docking study of dibenzothiophene **1d** (SI Figure: 3 **1d$_3$**, **1d$_4$**) suggest that metabolite **3d** should be enantiopure and have a (3*S*,4*S*) configuration.[6c,6d]

A small-scale (50 mg) biotransformation (*P. putida* UV4) of dibenzothiophene **1d**, under the optimized conditions developed for dibenzofuran **1c**, was carried out. LC-MS analysis of the aqueous supernatant showed the presence of a single metabolite, which was identified as *cis*-dihydrodiol **2d** at a retention time of 19.1 min. The limited quantity of metabolite **2d** available, following PLC purification, was just sufficient to record ^{1}H NMR and ^{13}C NMR spectra, high resolution MS data and a crude optical rotation value. Comparison with the literature data, based on the consistent trend in chemical shifts observed for chiral



Scheme 5: Possible metabolic pathways during TDO - catalyzed biotransformations of carbazole **1e** using *P. putida* UV4.

boronates formed using (*R*) and (*S*) MPBA, was used to assign the (1*R*,2*S*) absolute configuration to metabolite (+)-*cis*-dihydrodiol **2d**.[6c] As this method, provided the correct absolute configuration to the dibenzofuran *cis*-dihydrodiol (+)-**2c** during the current study, the (+)-(1*R*,2*S*)-dihydrodiol **2d** assignment it was again presumed to be correct.

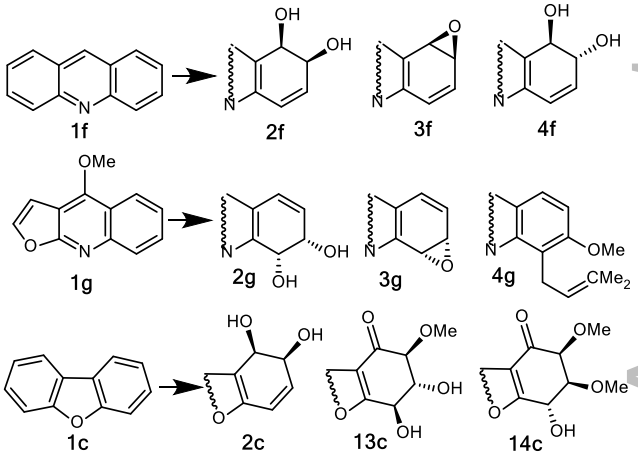
cis-Dihydrodiol **2d** was isolated in an unoptimized yield of 16% using TDO while a yield of 44% was reported using NDO.[6c] It was noteworthy that a much higher yield of *cis*-dihydrodiol **2c** (>85%) was obtained during the current programme. The increased water solubility of the dibenzofuran **1c** (3.1 mg L^{-1}) compared with dibenzothiophene **1d** (1.5 mg L^{-1}) could be an important factor in the higher yield of metabolite **2c**. Furthermore, as *cis*-dihydrodiol **2d** was the only detected metabolite, the task of separation of metabolites encountered using NDO was not required.[6c,6d] The predicted stereochemistry from molecular docking of dibenzothiophene **1d** with TDO was in agreement with the assigned absolute configuration of the *cis*-dihydrodiol **2d** (Figure 5).

Dioxygenase-catalysed sulfoxidation of thiophenes was reported using both TDO and NDO enzymes.[12] Sulfoxide **5d** was also found as a minor metabolite of dibenzothiophene **1d** in one of the studies using NDO.[6c,6d] The very low free binding energy associated with the preferred orientation of dibenzothiophene **1d** with TDO (Figure 6, **1d**$_2$), may be an important factor in sulfoxide **5d** not being detected during the TDO-catalyzed biotransformation.

Several reasons could account for *in silico*-predicted metabolites not being observed experimentally. These include product instability due to decomposition, further metabolisation, alternative oxidation pathways, toxicity and water-solubility. In the case of TDO, the structure and physical properties of the substrate, its free binding energy and proximity to dioxygen within the active site can affect both the binding and arene *cis*-dihydroxylation, rendering the lateral or angular bond more reactive.

Conclusion

Application of the molecular docking program to the TDO-catalysed *cis*-dihydroxylation of the tricyclic arene substrates (**1b**-**1e**), led to the prediction of preferred structures and absolute configurations of isolated lateral (**2c**, **2d**) and angular (**2b**) *cis*-dihydrodiols and transient *cis*-dihydrodiols (**2e**, **4e**). *In silico* predicted results from TDO- and NDO-catalysed *cis*-dihydroxylation of substrates **1c**-**1e**, were compared with literature results obtained using NDO and new experimental results obtained using TDO. The *P. putida* UV4 strain expressing TDO, was selected for biotransformations to confirm the



Scheme 6: Structures of secondary metabolites **3f**, **4f**, **3g**, **4g**, **13c** and **14c** obtained by chemoenzymatic synthesis from *cis*-dihydrodiol metabolites **2f**, **2g**, **2c** and tricyclic arene substrates **1f**, **1g** and **1c**.

Autodock Vina docking predictions. This resulted in the isolation of the relatively stable *cis*-dihydrodiols **2b**, **2c** and **2d**, and identification of metabolites **5e** and **8e**, derived from the unstable carbazole *cis*-diols

2e and **4e** respectively. The direct formation of stable angular *cis*-diol **2b**, and anthranilic acid **8e**, *via* the unstable angular *cis*-diol **4e**, were in accord with *in silico* predictions. These results also provide support for the view that TDO could be responsible for both lateral and angular *cis*-dihydroxylation of tricyclic arenes.

The TDO-catalysed biotransformation of dibenzofuran **1c** was optimized, to obtain *cis*-dihydrodiol **2c** as the main metabolite in enantiopure form and good yield. The (1*R*,2*S*) absolute configuration of *cis*-dihydrodiol **2c** was confirmed by a combination of chemoenzymatic synthesis with NMR analysis of chiral boronate and diMTPA esters and X-ray crystallographic analysis of the derived tricyclic (1a*S*,2*R*,8*R*,8a*R*)-diol epoxide **9c**.

To date nearly all *cis*-dihydrodiols used in the chemoenzymatic synthesis of natural products have been derived from monocyclic arenes.[1f] The BPDO-catalysed *cis*-dihydroxylation of acridine (**1f**) and dictamnine (**1g**) was among the few examples where tricyclic arene *cis*-dihydrodiols (**2f** and **2g**) were used in the chemoenzymatic synthesis of secondary metabolites including alkaloids (Scheme 6).[13] The bacterial *cis*-dihydrodiol metabolites **2f** and **2g** were used in the chemoenzymatic synthesis of (i) mammalian metabolites, *e.g.* arene oxide intermediate **3f** and isolated *trans*-dihydrodiol **4f** and (ii) plant metabolites, *e.g.* intermediate arene oxide **3g** and isolated furoquinoline alkaloid **4g**. The chemoenzymatic synthesis of the tricyclic fungal metabolites, *e.g.* Ribisin A **13c** and Ribisin B **14c** from a monocyclic bacterial *cis*-dihydrodiol metabolite of bromobenzene was reported.[14a-c] Utilizing tricyclic *cis*-dihydrodiol **2c** as a chiral synthon, the chemoenzymatic synthesis of Ribisins **13c** and **14c**, is currently in progress.

Experimental Section

NMR spectra were recorded on Bruker Avance 400, General Electric QE-500, and Bruker AV-600 instruments. Chemical shifts (δ) are reported in ppm relative to $SiMe_4$ and coupling constants (J) are given in Hertz (Hz). Optical rotations ($[\alpha]_D$) measurements were carried out on a Perkin-Elmer 214 polarimeter. LC-TOF-MS analyses were conducted on an Agilent 1100 series HPLC coupled to an Agilent 6510 Q-TOF and a reverse phase column (Agilent Eclipse Plus C18, 5 mm, 150 x 2.1 mm). Mass spectra (ES) were recorded on an LCT Premier Mass Spectrometer. Accurate molecular weights were obtained by the peak matching method, using heptacosafluorotributylamine as the standard reference and were accurate to within $\pm 5 \times 10^{-6}$ a.m.u. Melting points were recorded in degrees Celsius using a Stuart SMP10 melting point apparatus. Merck Kieselgel $60F_{254}$ analytical plates were used for TLC analyses. Preparative layer chromatography (PLC) separations of metabolites were carried out on glass plates (20 cm x 20 cm) coated with Merck Keiselgel $PF_{254/366}$ silica gel (21 g silica gel in 62 mL water).

Molecular Docking and *in silico* analysis

The protein models were obtained from the protein data bank. TDO pdb id: 3en1; NDO pdb id: 1o7n. The crystal structure of TDO does not contain bound dioxygen. Dioxygen was inserted into the 3en1 model by superimposing the iron centre of NDO, including the aspartate and the two histidine residues, onto the iron centre of TDO, using the "super" function of PyMOL (The PyMOL Molecular Graphics System, Version 2.2.0 Schrödinger, LLC.). The dioxygen was extracted from the superposition and combined with the 3en1 model.

Molecular docking was performed using the AutoDock Vina suite, version 1.1.2.[4] The docking site was set to include all amino acids within 6 Å of the substrate.
TDO: center_x = 46.46, center_y = 120.49, center_z = 201.877; size_x = site_y = size_z = 16.
NDO: center_x = 13.375, center_y = 49.781, center_z = 82.071; size_x = 20, size_y = 18, size_z = 18.
All docking was performed with an exhaustiveness of 100. Autodock Vina automatically grouped similar orientations and produced each result with a respective calculated binding energy. Each docking yielded several possible orientations, which were examined critically. The orientations were then examined for their viability as a substrate based on their calculated binding energy, orientation and interaction/distance to the nearest residues.

The volume of the active site was calculated using the Computed Atlas of Surface Topography of proteins (CASTp) 3.0.[9c] Submission of the whole proteins resulted in calculated binding pockets larger than intended. Therefore, the amino acids and iron centre of the active site were submitted as a separate file.

Job IDs:
NDO full: j_5c76b7f48839b (active site: pocket 3)
NDO active site with water: j_5c76becff050b
NDO active site without water: j_5c76bea6a2319
TDO full: j_5c6d8d0808b18 (active site: pocker 6)
TDO active site: j_5c77a83860d0a

Microbiological Methods

All biotransformations were performed using *P. putida* UV4 whole cells, unless mentioned otherwise. The *P. putida* UV4 bacteria were initially streaked on Luria-Bertani medium (LB) plates and incubated for 24 h at 30ºC. Individual colonies were then transferred to MSM plates containing indole (0.0012% w/v 1 mM). Single colonies were transferred to two minimal salt medium (MSM) agar plates with 0.2% sodium pyruvate, one containing 1 mM indole. Colonies on the indole containing plate were visually selected for activity after 24 h of incubation at 30ºC, based on indigo (blue colour) being produced.

Cultures grown on the MSM plates were consistently checked for TDO activity and absence of dehydrogenase activity, using a 100 mL MSM culture, and 50 µL of chlorobenzene. Formation of the *cis*-dihydrodiol metabolite from chlorobenzene was followed by measuring the UV absorbance at 295 nm.

Biotransformations were carried out using MSM cultures (500 mL, pH 7.5), containing 10 g/L glucose, and were inoculated in 2000 mL baffled Erlenmeyer flasks with an active colony from the MSM plate not containing indole. The cultures were grown overnight by shaking at 140 rpm, 30ºC. The tricyclic arenes (500 mg/L) and carbon sources (4 g/L) were added to each 500 mL of culture medium in 2 L baffled shake flasks, which were incubated at 30ºC, on an orbital shaker (140 rpm) for 24 - 48 h and maintaining the pH at 8. Samples were taken every 24 h and immediately frozen for LC-MS analysis. The crude bio-mixture was centrifuged (7000 rpm, 15 min, small scale) and the aqueous supernatant solution was then decanted off. The aqueous solution was concentrated *in vacuo*, maintaining the water bath temperature at 40ºC. The residue was extracted with ethyl acetate (3 x 100 mL) and concentrated *in vacuo* to yield the crude products.

Biotransformation of dibenzofuran 1c to give (1*R*,2*S*)-1,2-dihydrodibenzofuran-1,2-diol 2c [6c,6d]

cis-Dihydrodiol **2c** was isolated as a metabolite from a biotransformation of dibenzofuran **1c** (250 mg, 1.49 mmol) by extraction (3 x 100 mL EtOAc). The extract was concentrated *in vacuo* to give metabolite **2c** as a white solid (250 mg, 84%); m. p. 96-98°C (EtOAc / hexane); R_f = 0.25 (30% EtOAc in hexane); $[\alpha]_D$: +217 (*c* 0.6, $CHCl_3$); (Found: C, 71.8, H 5.2%; $C_{12}H_{10}O_3$ requires C, 71.3; H, 5.0%); HRMS (ES): $(M+Na)^+$: 225.05222, calcd. for $C_{12}H_{10}O_3Na$: 225.052058; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.92 (1H, br s, OH), 2.78 (1H, br s, OH), 4.67 (1H, b m, 2-H), 4.96 (1H, d, *J* = 6.3 Hz, 1-H), 6.05 (1H, ddd, *J* = 10.0, 2.5, 0.7 Hz, 4-H), 6.51 (1H, dd, *J* = 10.0, 2.3 Hz, 3-H), 7.27 (2H, m, 7-H and 8-H), 7.62 (1H, m, 9-H), 7.45 (1H, m, 6-H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 155.1, 152.0, 134.3, 126.5, 124.6, 123.6, 119.2, 117.4, 113.5, 111.7, 70.8, 65.1; LRMS (EI): *m/z* = 202 (26),184 (100).

(1*R*,2*S*)-1,2-Dihydroxy-1,2,3,4-tetrahydrodibenzofuran 6c

A solution of *cis*-dihydrodiol metabolite **2c** (100 mg, 5 mmol), in MeOH (15 mL) containing 10% Pd/C (5 mg) was stirred (3 h) in an atmosphere of hydrogen under normal pressure. The catalyst was filtered off, the filtrate concentrated under reduced pressure, and the crude product crystallized from $CHCl_3$ to furnish the hydrogenated tetrahydrodiol **6c** as a white powder (91 mg, 90%); m. p. 96-97°C ($CHCl_3$/hexane); $[\alpha]_D$: +37.3 (*c* 0.7, $CHCl_3$); (Found: C, 70.8, H, 6.3%; $C_{12}H_{12}O_3$ requires C, 70.6, H, 5.9%); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.05 (1H, m, 3-H), 2.17 (1H, m, 3´-H), 2.80 (1H, m, 4-H), 2.94 (1H, dt *J* = 17.0, 5.2 Hz, 4´-H), 4.07 (1H, dt, *J* = 9.9, 3.4 Hz, 2-H), 4.98 (1H, d, *J* = 3.9 Hz, 1-H), 7.27-7.34 (2H, m, ArH), 7.43 (1H, m, ArH), 7.62 (1H, m, ArH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 155.4, 155.2, 127.3, 123.9, 122.9, 118.9, 113.7, 111.1, 69.4, 68.8, 26.2, 21.2. LRMS (EI): *m/z* = 204 (M^+, 10), 186 (56), 121 (100).

(1*R*,2*S*)-1,2-Di-[(2*R*)-2-methoxy-2-phenyl-2-trifluoromethylacetoxyl]-1,2,3,4-tetrahydrodibenzofuran 7c(*R*)

Diesterification of *cis*-tetrahydrodiol **6c** with (+)-MTPA chloride/pyridine yielded the diMTPA ester **7c(*R*)** as a colourless oil, which was purified by PLC (15% ether in hexane, $[\alpha]_D$: +39.5 (*c* 0.7, $CHCl_3$); (Found: M^+ 636.15813, $C_{32}H_{26}O_7F_6$ calcd. for 636.15827); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.27 (1H, m, 3-H), 2.46 (1H, m, 3-H), 2.99 (2H, m, 4-H), 3.27 (3H, s, OMe), 3.54 (3H, s, OMe), 5.57 (1H, m, 2-H), 6.50 (1H, d, *J* = 2.8 Hz, 1-H), 7.11 (2H, m, ArH), 7.22-7.31 (7H, m, ArH), 7.39-7.45 (3H, m, ArH), 7.55 (1H, m, ArH) and 7.62 (1H, m, ArH); LRMS (EI): *m/z* = 636 (M^+, 35), 403 (56), 170 (100).

(1*R*,2*S*)-1,2-Di-[(2*S*)-2-methoxy-2-phenyl-2-trifluoromethylacetoxyl]-1,2,3,4-tetrahydrodibenzofuran 7c(*S*)

Colourless oil; $[\alpha]_D$: -33.7 (*c* 0.9, $CHCl_3$); (Found: M^+ 636.15854, $C_{32}H_{26}O_7F_6$ calc. for 636.15827); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.15-2.26 (2H, m, 3-H), 2.98 (2H, m, 4-H), 3.28 (3H, s, OMe), 3.57 (3H, s, OMe), 5.59 (1H, m, 2-H), 6.68 (1H, d, *J* = 3.4 Hz, 1-H), 7.04 (2H, m, ArH), 7.21-7.46 (10H, m, ArH) and 7.51 (2H, m, ArH); LRMS (EI): *m/z* = 636 (M^+, 48), 403 (64), 170 (100).

Biotransformation of dibenzothiophene 1d to give (1*R*,2*S*)-1,2-Dihydrodibenzothiophene-1,2-diol 2d [6c,6d]

cis-Diol **2d** was isolated as a metabolite from a small-scale biotransformation of dibenzothiophene **1d** (50 mg, 0.27 mmol) by extraction (3 x 100 mL EtOAc). The extract was concentrated *in vacuo* and the concentrate purified by PLC to give *cis*-diol **2d** as a white solid (9.4 mg, 16%). R_f = 0.25 (30% EtOAc in hexane); $[\alpha]_D$: +51 (*c* 0.2, MeOH); HRMS (ES): $[M+Na]^+$: 241.0296, $C_{12}H_{10}O_2SNa$ calcd. for 241.0299; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 4.68 (1H, dt, *J* = 5.4, 2.6 Hz, 2-H), 4.99 (1H, dd, *J* = 5.9, 0.7 Hz, 1-H), 6.06 (1H, ddd, *J* = 9.5, 2.7, 0.7 Hz, 4-H), 6.53 (1H, dd, *J* = 9.7, 2.5 Hz, 3-H), 7.33 (1H, dt, *J* = 7.2, 1.2 Hz, 8-H), 7.42 (1H, dt, *J* = 7.2, 1.0 Hz, 7-H), 7.82 (1H, d, *J* = 8.1 Hz, 6-H), 7.91 (1H, d, *J* = 8.1 Hz, 9-H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 139.7, 137.9, 135.8, 132.2, 129.2, 125.0, 124.7, 122.8, 121.4, 120.7, 70.6, 65.5.

Biotransformation of carbazole 1e to give anthranilic acid 8e and 2-hydroxycarbazole 5e[6a]

A small scale biotransformation of carbazole **1e** (50 mg, 0.30 mmol) followed by EtOAc extraction of the aqueous supernatant on concentration gave the crude bioproduct mixture. GC-MS analysis (Agilent Technologies Ultra 2 12 m x 0.2 mm i.d. x 0.33 µm column) of the crude extract showed the presence of a major peak (9.5 min.) which was identified as that of anthranilic acid **8e** (MW 137), by comparison with an authentic standard and also formation

of the trimethylsilyl derivative. A very minor peak (16.6 min.) was identified as 3-hydroxy carbazole **5e** (MW 183: *m/z* 183, 154, 127, 92, 77); its mass fragmentation pattern was identical to that reported in the literature. PLC purification (35% EtOAc in hexane, R_f = 0.25) of the crude mixture yielded (6.4 mg) pure crystalline anthranilic acid **8e**.

(1*R*,2*R*,3*S*,4*S*)-3-Bromo-1,2,3,4-tetrahydrodibenzo[b,d]furan-1,2,4-triol 9c

To a solution *cis*-dihydrodiol **2c** (27 mg, 0.13 mmol) in THF-H_2O (4:1, 3 mL) was added NBS (40 mg, 0.22 mmol) and the mixture was stirred overnight at room temperature. After addition of $Na_2S_2O_5$ (10 mg), the mixture was stirred for 10 min., diluted with EtOAc (10 mL) and washed with brine (5 mL). The organic phase was separated, dried ($MgSO_4$), and concentrated *in vacuo*. The crude material, was purified by flash column chromatography (silica gel, 30% EtOAc in hexane) to give bromohydrin **9c** as a purple solid (27 mg, 68%); R_f = 0.22 (40% EtOAc in hexane); $[\alpha]_D$: -33 (*c* 0.83, MeOH); ^{1}H NMR (400 MHz, CD_3OD): δ = 3.91 (1H, ddd, *J* = 10.3, 3.9, 0.9 Hz, 2-H), 4.35 (1H, ddd, *J* = 10.3, 7.6, 0.7 Hz, 3-H), 4.96 (1H, d, *J* = 7.6 Hz, 4-H), 5.05 (1H, d, *J* = 3.9 Hz, 1-H), 7.26 (1H, dt, *J* = 7.6, 0.9 Hz, 8-H), 7.32 (1H, dt, *J* = 7.5, 1.0 Hz, 7-H), 7.48 (1H, dm, *J* = 8.0 Hz, 6-H), 7.65 (1H, dm, *J* = 7.5 Hz, 9-H); ^{13}C NMR (150 MHz, CD_3OD): δ = 157.3, 154.3, 128.1, 126.2, 124.4, 121.2, 116.7, 112.6, 73.5, 71.6, 65.2, 59.1.

(1a*S*,2*R*,8*R*,8a*R*)-1a,2,8,8a-Tetrahydro-benzo[b]oxireno[2,3-f]benzofuran-2,8-diol 10c

Sodium methoxide (10 mg, 185 µmol) was added to a solution of bromohydrin **9c** (20 mg, 67 µmol) in THF (3 mL) and the reaction mixture was stirred (3 h) at room temperature. The salts were removed by gravity filtration and the filtrate was concentrated *in vacuo*. Purification of the residue by flash column chromatography (silica gel, 50% EtOAc in hexane) gave epoxide **10c** as a white crystalline solid (8 mg, 55%); R_f = 0.2 (60% EtOAc in hexane); HRMS (ES): $[M+NH_4]^+$: 236.0868, $C_{12}H_{14}NO_4$ calcd. for 236.0923; ^{1}H NMR (400 MHz, CD_3OD): δ = 3.68 (2H, m, 1a-H, 8a-H), 5.02 (1H, m, 2-H), 5.15 (1H, m, 8-H), 7.21 (1H, dt, *J* = 7.6, 1.3 Hz, 6-H), 7.27 (1H, dt, *J* = 7.5, 1.5 Hz, 5-H), 7.45 (1H, d, *J* = 8.0 Hz, 4-H), 7.74(1H, dd, *J* = 7.4, 1.2 Hz, 7-H); ^{13}C NMR (100 MHz, CD_3OD): δ = 157.0, 150.8, 128.4, 125.4, 123.8, 122.0, 114.3, 112.1, 64.9, 63.5, 57.6, 56.9; IR (KBr) ν_{max}/cm^{-1} 3995, 2956, 2927, 2853, 1781, 1596, 1354, 1181, 1228, 1051.

X-ray crystallographical analysis for 10c[15]

Crystal data collection was performed on a Rigaku Single Crystal X-ray diffractometer, equipped with cryostat. Measurements were carried out at 100 K using a Cu-Kα, monochromator, λ = 1.54184 Å. Using Olex2, the structure was solved with the ShelXT structure solution program using Intrinsic Phasing and further refined with the ShelXL refinement package using least squares minimisation.

Crystal Data: $C_{12}H_{10}O_4$, M = 218.2: orthorhombic, space group $P2_12_12_1$ (no.19), *a* =5.56920(10), *b* = 7.35790(10), *c* = 23.3201(5) Å, *V* = 955.60(3) Å^3, *Z* = 4, *T* = 100.0(2) K, *Dcalc* = 1.361 g/cm^3, 10979 reflections measured (12.616° ≤ 2Θ ≤ 144.426°), 1878 unique (R_{int} = 0.0236, R_{sigma} = 0.0138) which were used in all calculations. The final R_1 was 0.0395 (I > 2σ(I)) and wR_2 was 0.0962 (all data).

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[15] CCDC 1894810 contains the supplementary crystallographic data for this compound that can be obtained free of charge from the Cambridge Crystallographic Data Centre *via* www.ccde.cam.ac.uk/data.

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FULL PAPER

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