An aryl dioxygenase shows remarkable double dioxygenation capacity for diverse bis-aryl compounds, provided they are carbocyclic

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Abstract

The bacterial dioxygenation of mono- or polycyclic aromatic compounds is an intensely studied field. However, only in a few cases has the repeated dioxygenation of a substrate possessing more than a single aromatic ring been described. We previously characterized the aryl-hydroxylating dioxygenase BphA-B4h, an artificial hybrid of the dioxygenases of the biphenyl degraders *Burkholderia xenovorans* LB400 and *Pseudomonas* sp. strain B4-Magdeburg, which contains the active site of the latter enzyme, as an exceptionally powerful enzyme. We now show that this dioxygenase possesses a remarkable capacity for the double dioxygenation of various bicyclic aromatic compounds, provided that they are carbocyclic. Two groups of biphenyl analogues were examined: series A compounds containing one heterocyclic aromatic ring and series B compounds containing two homocyclic aromatic rings. Whereas all of the seven partially heterocyclic biphenyl analogues were solely dioxygenated in the homocyclic ring, four of the six carbocyclic bis-aryls were converted into *ortho,meta*-hydroxylated bis-dihydrodiols. Potential reasons for failure of heterocyclic dioxygenations are discussed. The obtained bis-dihydrodiols may, as we also show here, be enzymatically re-aromatized to yield the corresponding tetraphenols. This opens a way to a range of new polyphenolic products, a class of compounds known to exert multiple biological activities. Several of the obtained compounds are novel molecules.

Keywords Dioxygenation, Dihydrodiol, Dehydrogenation, Heteroaryl, Homoaryl, Polyphenol

Introduction

The dioxygenation of the aromatic system constitutes the first step in the aerobic bacterial catabolism of aromatic compounds (Furukawa 2000). It destroys the aromatic nucleus and functionalizes the molecule for further degradation. This reaction is catalyzed by aryl-hydroxylating dioxygenases (ARHDOs). Typically, these initial pathway enzymes mainly determine the substrate range for the metabolic route (Cámara et al. 2007; Furukawa et al. 1993; Seeger et al., 1999; Zielinski et al. 2006), which sometimes enables the microorganism to utilize the respective aromatic compound as growth substrate.

The enzymatic dioxygenation of aromatic compounds is also of considerable synthetic interest (Boyd and Sheldrake 1998; Hudlicky et al. 1999). Generally, de-aromatization reactions are chemically difficult due to the stability of the aromatic system. The enzyme-catalyzed dioxygenation, however, is carried out under mild conditions, although it uses molecular dioxygen as oxidizing agent (Kobayashi et al. 1964). The conversion of the aromatic nucleus into a ring possessing vicinal hydroxy groups as well as a conjugated diene substructure (Fig. 1) yields a molecule which represents a versatile chiral building block, as it is able to undergo a variety of different follow-up reactions (Hudlicky et al. 1999).

An *in-vitro*-constructed hybrid enzyme (BphA-B4h) which contains the active site of an ARHDO from the soil bacterium *Pseudomonas* sp. strain B4-Magdeburg (Kahl and Hofer 2003) has been identified as a powerful dioxygenating biocatalyst (Cámara et al. 2007; Kahl and Hofer 2003; Overwin et al. 2012, 2015b). These investigations *inter alia* demonstrated that this enzyme is able to hydroxylate homo-aromatic compounds with different steric requirements (Overwin et al. 2015b). They also showed that it was able to further transform the biphenyl dioxygenation product by a dioxygenation in the second ring (Overwin et al. 2012). Such a bis-dihydrodiol (bis-DHD) formation has only been described for a few other aromatic substrates (Boyd et al. 1999, 2006; Haddock et al. 1995).

These results prompted us to particularly further examine the double dioxygenation potential of BphA-B4h. This was done with two series of biphenyl analogues. Series A (Fig. 2a) consisted of biphenyl analogues that are sterically very similar to the parent compound, but which contain one heterocyclic aromatic ring. Two types of the latter were selected, six-membered rings with electron-poor carbons that are less prone to electrophilic attack, but are more prone to nucleophilic attack than a homoaromatic ring, and five-membered rings with electron-rich carbons, for which the opposite reactivities apply. Series B (Fig. 2b) consisted of biphenyl analogues that contain two homocyclic aromatic rings, which are linked by different types of spacers. The formed metabolites were purified and structurally characterized. Moreover, it was investigated whether the doubly dioxygenated products obtained could be enzymatically further converted into the corresponding polyphenols.

Materials and methods

Chemicals

Compounds used as substrates were of the highest purity available. Biphenyl, diphenylmethane, 1,2-diphenylethane (bibenzyl), *cis*- and *trans*-stilbene, diphenylacetylene and biphenylene (96 - 99 %) were obtained from Sigma-Aldrich (Munich, Germany), Fluka AG (Buchs, Switzerland) or Merck AG (Darmstadt, Germany), respectively. All heteroaromatic substrates (96 - 98 %) were from Sigma-Aldrich.

Bacterial strains, plasmids and culture conditions

The *E. coli* strains used in this study were all derivatives of the host BL21(DE3)[pLysS] (Studier 1991). They harboured one of the following plasmids that are based on the phage T7

expression vector pT7-6. Plasmid pAIA1104 contains a modified *bphA1A2A3A4* gene cluster from *B. xenovorans* LB400 in which part of *bphA1* has been replaced by the respective segment from an ARHDO gene from *Pseudomonas* sp. strain B4. Its construction has previously been described (Cámara et al. 2007; Overwin et al. 2015a). Plasmid pAIA20 (Overwin et al. 2015b) carries the *bphB* gene of strain LB400. These *E. coli* strains were grown in LB medium (Sambrook and Russel, 2001), supplemented with 150 µg/ml ampicillin and 25 µg/ml chloramphenicol, on a gyratory shaker at 150 rpm and 37 °C.

Preparation of resting cells

Preparation of resting cells was essentially carried out as previously described (Overwin et al. 2015b). In brief, strains were grown in LB medium at 30 °C. At an optical density at 600 nm (OD₆₀₀) of about 1.0, 0.4 mM isopropyl-β-D-thiogalactopyranoside was added, and the incubation was continued for 14 h. Cells were harvested, washed with one volume of 50 mM sodium phosphate buffer (pH 7.5), resuspended in the same buffer and supplemented with glucose to 0.2 % (w/v). The volume was adjusted to give the desired cell density.

Analytical transformations of aromatic compounds by BphA-B4h and of bis-DHDs by BphB-LB400

Incubations were essentially carried out as previously described (Overwin et al. 2015b). Briefly, for dioxygenations resting cell suspensions (OD₆₀₀ of 2.0) of *E. coli* BL21(DE3) [pLysS] harbouring pAIA1104 were incubated with a nominal concentration of 0.25 mM of the respective compound on a gyratory shaker at 30 °C for up to 120 h. At various time points, aliquots were analysed by high performance liquid chromatography (HPLC, see below) after removal of cells by centrifugation. For dehydrogenations, resting cell suspensions of *E. coli* BL21(DE3)[pLysS] harbouring pAIA20 were combined with resting cell supernatants of dioxygenations to give a final cell density of 2.0 OD₆₀₀ and were incubated as above. Aliquots were analysed as described above.

Preparative transformations of aromatic compounds by BphA-B4h

Transformations were carried out under somewhat varying conditions. In the following, typical parameters are given and their ranges are indicated in parentheses. Substrates at final nominal concentrations of 2 (1 – 5) mM were incubated with resting cell suspensions of *E. coli* BL21(DE3)[pLysS] harbouring pAIA1104 at a cell density of 6.0 (4.5 – 9.0) OD₆₀₀ units for up to 120 h. Reaction progress was monitored by HPLC (see below) and incubations were terminated when maximal end product concentrations were reached.

Purification of heteroaryl dioxygenation products

The volumes of resting cell supernatants were reduced by evaporation under reduced pressure. Unless stated otherwise (see below), they were extracted 3 – 5 times with 1 vol. of ethyl acetate after saturation of the aqueous phase with sodiumsulphate decahydrate. For the more hydrophilic product from 3-phenylpyrazole, 8 extractions were used. In case of the product from 1-phenylimidazole, the supernatant was evaporated to dryness and the residue was extracted with ethanol. Extracts were briefly centrifuged and passed through a 0.2 µm Minisart RC25 filter (Sartorius, Göttingen, Germany). They were further purified by chromatography on a Gemini C18 10 µm column (10 × 250 mm or 21.2 × 250 mm), protected by a C18 Security Guard cartridge (10 x10 mm) (Phenomenex, Aschaffenburg, Germany). Elutions (3 or 5 ml/min) of the different products were done with aqueous methanol as follows. Isocratic conditions were applied for the DHDs of 2-phenylpyridine (20 % methanol), 3-phenylpyridine and 4-phenylpyridine (30 % methanol). A gradient with a linear increase of 5 – 55 % methanol over 50 min was applied for the DHDs of 4-phenylpyrimidine, 1-phenylimidazole and 3-phenylpyrazole. A gradient with 5 – 40 % methanol over 50 min and

40 – 55 % methanol over 30 min was applied for the DHD from 1-benzylpyrrole.

Purification of homoaryl dioxygenation products (bis-DHDs)

These products were purified from resting cell supernatants by passage through a C₁₈ cartouche No. 7020-06 (J. T. Baker, Griesheim, Germany). The bound metabolites were eluted with methanol and further purified on the C₁₈ column described above. In case of bibenzyl-bis-DHD a linear gradient of aqueous methanol (0 – 50 % over 55 min) was used, in the other cases elution was done isocratically with 30 % of aqueous methanol. With diphenylacetylene-bis-DHD, the eluate of the C₁₈ cartouche was simply further purified by two-fold extraction of residual mono-DHD with 1 vol. of ethylacetate.

Analytical HPLC

Analyses were done with a Shimadzu LC10AD instrument (Shimadzu Corp., Kyoto, Japan) equipped with a diode array detector and an SC Lichrosphere 100 RP8 5 µm column (4.6×125 mm; Bischoff, Leonberg, Germany). Elution was done at a flow rate of 1 ml/min with aqueous methanol as indicated in the footnotes of the respective Tables.

Mass spectrometry

Liquid chromatography-mass spectrometry (LC-MS) and high resolution mass spectrometry (HRMS) analyses were carried out as previously described (Overwin et al. 2015b).

Nuclear magnetic resonance (NMR) analyses

Analyses were carried out with purified products, dissolved in deuterated methanol, water or acetone, respectively. 1D and 2D ¹H and ¹³C NMR spectra (COSY, HMBC, occasionally HSQC, DEPT and 1D NOE) were recorded at 300 K on Bruker DPX-300, DMX-600, Avance III 500 and Avance III HD 700 NMR spectrometers.

Results

Formation and characterization of dioxygenation end-products obtained from two series of biphenyl analogues

Structural formulas and carbon numbering of the two series of biphenyl analogues studied are shown in Fig. 2. Extensive incubations with recombinant resting cells containing the dioxygenase BphA-B4h revealed that series A biphenyl analogues, which are sterically almost identical with the parental substrate, but possess one N-heterocyclic aromatic ring, yielded, in contrast to the turnover of biphenyl (Overwin et al. 2012), only a single dioxygenation product. This was found for both compound subtypes, those with electron-poor and those with electron-rich heterocycles. HPLC retention times and absorption maxima of these dioxygenation products are shown in Table 1. Typically, substrate conversions were incomplete, suggesting inhibiting effects by substrates and/or products. After biotransformations on preparative scale, all products were further characterized by high-resolution mass and NMR spectrometry. Molecular masses (Table 1) were in agreement with singly dioxygenated molecules. 1D and 2D (COSY, HMBC) ¹H and ¹³C NMR spectra confirmed these results (Table S1). They additionally showed that dioxygenation invariably took place at the carbocyclic ring and that the hydroxy groups were always attached at *ortho* and *meta* positions. The NMR data clearly indicated a de-aromatization of the carbocyclic ring with aliphatic signals from C2'/H2' and C3'/H3' and olefinic signals from C1' and C4'/H4' through C6'/H6'. NMR data for DHDs of 2-, 3-, 4-phenylpyridines and of 4-phenylpyrimidine have previously been published (Boyd et al. 2008, Misawa et al. 2002). However, no full assignments were given for ¹H and ¹³C signals. As far as these and our data

can be compared, they appear to be in satisfactory agreement, taking into account differences in solvents and possibly temperature. The structures of all products are shown in Fig. S1. According to recent searches in the SciFinder database (Chemical Abstracts Services 2015), the DHDs derived from compounds 5, 6 and 7 (Fig. 2) have previously not been described.

The results of dioxygenations were remarkably different with series B of biphenyl analogues, namely diphenylmethane, bibenzyl, *cis*- and *trans*-stilbene, diphenylacetylene and biphenylene. These molecules harbour two differently spaced aromatic rings, which, however, are both carbocyclic. In long-term incubations, BphA-B4h converted the previously described single-ring dioxygenation products (Overwin et al. 2015b) of four of the six substrates into follow-up products. The exceptions were *cis*-stilbene and biphenylene. All of the follow-up products showed the following characteristics relative to the primary metabolites: a bathochroic shift of the electronic absorption maximum (two or three adjacent maxima were observed when the benzene ring spacers provided conjugated double or triple bonds) and a much shorter retention time on the reversed phase column, indicating a decreased hydrophobicity. In accordance with this finding, we also observed that during extractions of the transformation supernatants with ethylacetate, the late products remained in the aqueous phase. All of these features suggest that the later metabolites were formed by oxidation of the second benzene ring. HPLC retention times and absorption maxima of these late dioxygenation products are given in Table 2. The data for the biphenyl product (Overwin et al. 2012) are shown for comparison. In contrast to incubations with heterocyclic substrates, most of the homocyclic compounds were completely converted into their final dioxygenation products. With *trans*-stilbene and diphenylacetylene, these conversions were very slow and still incomplete, but progressing at an almost linear rate, at the longest assayed incubation times (70 h). Molecular masses of the final products (Table 2) were in agreement with doubly dioxygenated molecules, bis-DHDs. NMR data confirmed these results (Table S2). They clearly indicated symmetrical dioxygenations of both rings. All aromatic signals were lost and only signals of a single dihydrodiol ring, but with duplicated intensity, were observed. This shows that, in the second dioxygenation, the hydroxy groups were also attached with the typical regiospecificity, *i. e.*, at *ortho* and *meta* carbons. The structures of all products are shown in Fig. S2. The capability of the parental dioxygenase from strain LB400 (BphA-LB400) to convert mono-DHDs into bis-DHDs was clearly much lower than that of BphA-B4h. As previously reported, BphA-LB400 was able to catalyze formation of the bis-DD from biphenyl, but with a yield of only 20 % of that of BphA-B4h (Overwin et al. 2012). With the biphenyl analogues examined here, no bis-DHDs were found except for marginal amounts (ca. 3 % of the yield with BphA-B4h) of the product from *trans*-stilbene. All of the bis-DHDs described, except the one from biphenyl, are novel compounds (Chemical Abstracts Services 2015).

Conversion of the bis-DHDs into bis-catechols

In order to investigate the possibility to convert the bis-DHDs into bis-catechols, we used recombinant cells synthesizing the short-chain alcohol dehydrogenase BphB of *B. xenovorans* LB400, which is part of the microbe's pathway for the catabolism of biphenylic compounds and has already successfully been used for the respective transformation of the bis-DHD derived from biphenyl (Overwin et al. 2012) and of the mono-DHDs derived from the homo-aromatic biphenyl analogues (Overwin et al. 2015b). Within a few hours, complete depletion of the bis-DHDs was observed with concomitant formation of a single major product with a significantly enhanced retention time in reversed-phase chromatography, as expected (Table 3). In all cases, except diphenylmethane and bibenzyl, where the bis-DHDs possess only short systems of conjugated double bonds, a large hypsochromic shift of the characteristic absorption maximum was found (Table 3). The formation of bis-catechols was confirmed by LC-MS data of positively and negatively charged ions (Table 3).

Discussion

Direct and indirect evidences for double dioxygenations

A large number of reports have been published describing the turnover of primarily aromatic substrates by bacterial ARHDOs (Hudlicky et al. 1999; Johnson 2004). Many of these compounds possess more than a single aromatic ring. Therefore, in principle, their single-ring dioxygenation products may be substrates for a second round of dioxygenation. However, only in a few cases has direct or indirect evidence for such a reaction been obtained. Haddock et al. (1995), working with BphA-LB400, reported, on the grounds of GC-MS data, the likely 3,4,3',4'-bis-DHD formation exclusively from 2,5,2',5'-tetrachlorobiphenyl among a range of chlorobiphenyls investigated. Kimura et al. (1996), studying biotransformations by the mutant strain *Pseudomonas* sp. KF712C1, described the formation of probably catecholic tetrahydroxy compounds from biphenyl, diphenylmethane, bibenzyl and diphenylether, also inferred from GC-MS analyses. Boyd et al. (1999, 2006) reported the double dioxygenation of polycyclic (hetero)arenes and the further dioxygenation of acetonide derivatives of DHDs derived from biphenyl and phenanthrene by *Sphingomonas yanoikuyae* B8/36. Shindo et al. (2007) described the transformation of biphenyl and 2-phenyl-benzoxazole into bis-catechols by a recombinant strain expressing artificial BphA variant and BphB dehydrogenase genes. We recently characterized a bis-DHD of biphenyl, the symmetrical 1-(1'-cyclohexyl-4',6'-diene-2',3'-diol)-cyclohexa-4,6-diene-2,3-diol (Overwin et al. 2012). It was formed by the well-known BphA-LB400 and, in significantly higher amount, by the hybrid dioxygenase BphA-B4h, constructed in this laboratory (Cámara et al. 2007; Kahl and Hofer 2003). The present study now demonstrates an exceptional ability of the latter enzyme for double dioxygenation that is by far not limited to biphenyl itself. The primary dioxygenation products of several compounds, in which two benzene rings were linked in different ways, were further dioxygenated upon prolonged incubations. However, it became also clear, that not all biphenyl analogues were prone to double dioxygenation.

Potential reasons for failures of double dioxygenations

With respect to the compounds that were recalcitrant to double dioxygenation, it seems fairly clear that the reasons are different for the heteroaryl series A and the homoaryl series B compounds.

In the aerobic bacterial metabolism of N-heteroaromatic compounds, the typical initial reaction appears to be a monohydroxylation through a nucleophilic attack by a water molecule at a carbon in *ortho* or *para* position to a nitrogen. This is catalyzed by molybdenum-containing hydroxylases (Fetzner 2000; Hille 2005). In contrast, ARHDO-catalyzed hydroxylations of heteroaryls in the heterocycle appear to be rare reactions. They have only occasionally been reported (Boyd et al. 1987, 1993, 1996, 1998; Ensley et al. 1983; Garrett et al. 2006; Misawa et al. 2002), and it is not even sure if all described metabolites originate from dioxygenations. Often only monohydroxylated products were found and experiments were carried out directly with the dioxygenase source strains, which may also harbour monooxygenases responsible for these conversions.

The partly heteroaromatic series A compounds are sterically almost identical to biphenyl. At first glance, the reason for failure of attack of either electron-rich or electron-poor heteroaryl rings appears to be predominantly an unfavourable electronic effect on the reaction. Although the nature of the activated oxygen species attacking the aromatic substrate and, consequently, the reaction mechanism, is not yet entirely clear, most available evidence favours a radical mechanism with consecutive formation of the two novel C-O bonds (Bugg and Ramaswamy 2008; Barry and Challis 2013). In spite of the radical character of the reaction, increased electron density at the primarily attacked carbon appears to enhance reactivity (Barry and Challis 2013; Rivard et al. 2015). In agreement with this notion, it was

expected that electron-poor aryls are poor and electron-rich aryls are good substrates for ARHDOs (Boyd et al. 2008). However, experiments showed only in some cases agreement with this assumption. Taken together, previous data indicate that fused heteroaryls are typically more prone to ARHDO attack than linked ones. But no clear picture emerges for the effects of electron-rich and electron-poor aryls. While the expected behaviour was observed in some instances, good dioxygenations of 2-(p-tolyl)-pyridine (Misawa et al. 2002) and quinazoline (Boyd et al. 1993) and no dioxygenation of furan or poor dioxygenation of thiophene (Boyd et al. 1996) contradict the expectations. The same holds for the effects of electron-donating and -withdrawing substituents, as halo-pyridines gave higher yields than pyridine (Garrett et al. 2006). This suggests that in these cases the influence of heteroatomic ring constituents and/or of (missing) substituents, respectively, of the substrates on their binding within the active site, resulting in more or less favourable orientations with respect to the activated oxygen species and a longer or shorter lifetime of the complex, significantly modulates the mentioned electronic effects on the reaction itself. As this modulation critically depends on the active site architecture, it should be enzyme-specific. With the ARHDO catalytic mechanism outlined above, observations like the preferred dioxygenations of 2,2'-dichlorobiphenyl at carbons 2 and 3 (Haddock et al. 1995; Seeger et al. 1995) and the double dioxygenation of 2,5,2',5'-tetrachlorobiphenyl (Haddock et al. 1995) appear difficult to be interpreted alone by electronic influences on the reaction mechanism. This is underpinned by the findings that these specific dioxygenations are shown only by some ARHDOs, but not by others that accept these substrates. On the grounds of these considerations, it seems that the failure of heterocyclic dioxygenation of all of the series A substrates is not exclusively due to electronic influences, but also has steric reasons, *i. e.*, an unfavourable positioning of the substrate within the active site due to the smaller ring size of some compounds and to interactions between the compound nitrogens and the substrate-lining amino acids of the enzyme.

As the aromatic systems of series B substrates are electronically almost identical, the reason for a very slow rate or failure of the second dioxygenation for some of the compounds appears to be predominantly steric. In some cases, these results are largely explained by the low rates of the first dioxygenation (Overwin et al. 2015b). The typically much slower second dioxygenation (Overwin et al. 2012) would then only yield trace amounts of bis-DHDs. This explanation does not apply, however, to the very slow bis-DHD formation from diphenylacetylene, where the rate of the first dioxygenation was comparatively high. For the second dioxygenation, the molecule has to re-dock to the active site in a turned-around orientation. This may be prevented if the now dihydroxylated and non-planar ring cannot be accommodated in a cavity where this could be achieved for the non-dioxygenated ring during the first dioxygenation. Alternatively, the dihydroxylated ring may be accommodated, but may engage in interactions with the protein that favour an orientation which is non-productive for the second dioxygenation. Attempts to rationalize rate differences for the first dioxygenation by docking calculations with the BphA-LB400 structure and a BphA-B4h homology model were in most cases not successful (Overwin et al. 2015b). Similarly, in most cases no correlations were found between docking results for the mono-DHDs and their turnover rates. Thus, for the critical second dioxygenation of diphenylacetylene by BphA-B4h, dockings carried out as described (Overwin et al. 2015b) did not explain the very slow reaction. The energetically most favourable dockings were indeed found for a non-productive flipped orientation of the diphenylacetylene-mono-DHD, *i. e.*, where the DHD ring was positioned in the cavity at the mononuclear iron centre. However, similar results were obtained for the much faster second dioxygenations of the mono-DHDs of biphenyl, diphenylmethane and bibenzyl.

Applications of bis-DHDs

Double dioxygenation generates two dienediol substructures in the substrate molecules. These are, as already shown for mono-DHDs, quite useful as precursors in synthetic applications due to two different features, the vicinal hydroxy groups and the conjugated diene substructure (Boyd and Sheldrake 1998; Hudlicky et al. 1999). Thus, the bis-DHDs may serve as scaffolds for the syntheses of numerous symmetric bicyclic entities. For example, they may be decorated via esterification of the hydroxy groups. The vicinal diols may also be used to fuse 1,3-dioxolane or 1,4-dioxane rings (Boyd et al. 2006), thereby converting the bis-DHD core into a tetracyclic structure. Further decorations may be carried out through the many possibilities of additions to the carbon-carbon double bonds of the diene substructures. This includes [4+2] cycloadditions to the dienes, which lead to bridged bicycles (Pazos et al. 2015), molecules with a rigid three-dimensional core that have recently been shown to be interesting candidates for bioactivity screens (Birudukota et al. 2016). As the double dioxygenation generates four new chiral centres, these metabolites are of particular interest for enantioselective syntheses (Hudlicky and Reed 2009). Dioxygenations of both aromatic rings may also lead to the generation of asymmetric bicyclic entities. ARHDOs in general and BphA-B4h in particular are able to tolerate substrate substituents at several positions (Cámara et al. 2007). It therefore will be interesting to investigate which asymmetrically substituted substrates can be transformed into the respective bis-DHDs. Asymmetric products may also be obtained in a different way. As usually the dioxygenation of the first ring is faster than that of the second (Overwin et al. 2012), mono-DHDs may be isolated and modified before subjection to the second dioxygenation. Thus, it has been shown that catechols or acetonides obtained from mono-DHDs may serve as substrates for dioxygenation of the second ring (Boyd et al. 1999, 2006; Overwin et al. 2012), which, after DHD formation, may be derivatized in a way different from that of the first ring. Simple conversions of bis-DHDs include their acid-catalyzed dehydrations to yield different phenols as well as their enzyme-catalyzed dehydrogenations to provide bis-catechols. As we showed in the current work, a prototype dehydrogenase of a biphenyl catabolic pathway is able to carry out such transformations. This opens a new way to a range of new polyphenolic products, a class of compounds known to exert multiple biological activities (Mushtaq and Wani 2013; Pasinetti et al. 2015). Such a synthetic route will be of particular interest for already hydroxylated substrates, that would significantly increase their degree of hydroxylation, and for skeletons that frequently attain bioactivity when multiply hydroxylated, such as chalcones and flavonoids. For double dioxygenation the latter substrates, a powerful ARHDO of the PAH type may be required.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical Statement

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figure Legends

Fig. 1 Typical dioxygenation product. The metabolite was derived from a benzene ring with a single substituent (R). Hydroxy groups are typically attached at *ortho* and *meta* carbons, thereby creating two stereocentres. They are always found in a *cis* configuration, typically with 2R, 3S stereochemistry. The two conformers of this configuration are depicted.

Fig. 2 Structural formulas of compounds examined. A. Heteroaryls: 1, 2-phenylpyridine; 2, 3-phenylpyridine; 3, 4-phenylpyridine; 4, 4-phenylpyrimidine; 5, 1-benzylpyrrole; 6, 1-phenylimidazole; 7, 3-phenylpyrazole. B. Homoaryls: 8, biphenyl; 9, diphenylmethane; 10, bibenzyl; 11, *cis*-stilbene; 12, *trans*-stilbene; 13, diphenylacetylene; 14, biphenylene. Examples explaining the carbon numbering as applied in this study are given at representative compounds.

Table 1 HPLC retention times, absorption maxima and HRMS data of final products formed by dioxygenase BphA-B4h from heteroaromatic biphenyl analogues.

Substrate		HPLC data of product		HRMS data of product		
No.	Name	t _r [min] ^a	λ _{max} [nm] ^b	Formula	<i>m/z</i> values ^c	
					Calculated	Observed
1	2-Phenylpyridine	3.20	311	C ₁₁ H ₁₁ NO ₂	190.0863	190.0862
2	3-Phenylpyridine	3.26	302	C ₁₁ H ₁₁ NO ₂	190.0863	190.0862
3	4-Phenylpyridine	3.22	308	C ₁₁ H ₁₁ NO ₂	190.0863	190.0861
4	4-Phenylpyrimidine	2.25	322	C ₁₀ H ₁₀ N ₂ O ₂	191.0815	191.0816
5	1-Benzylpyrrole	5.33	265	C ₁₁ H ₁₃ NO ₂	192.1019	192.1026
6	1-Phenylimidazole	2.20	280	C ₉ H ₁₀ N ₂ O ₂	179.0815	179.0813
7	3-Phenylpyrazole	2.34	303	C ₉ H ₁₀ N ₂ O ₂	179.0815	179.0824

^a RP8 column. Eluent: 20 - 100 % of aqueous methanol, acidified with 0.085 % phosphoric acid. For details see “Materials and methods”.

^b Determined by diode array detector.

^c For the hydrogen cation.

Table 2 HPLC retention times, absorption maxima and HRMS data of final products formed by dioxygenase BphA-B4h from homoaromatic biphenyl analogues.

Substrate		HPLC data of product		HRMS data of product		
No.	Name	t_r [min] ^a	λ_{max} [nm] ^b	Formula	m/z values ^c	
					Calculated	Observed
8	Biphenyl	4.10	339	C ₁₂ H ₁₄ O ₄	245.0784	245.0783 ^d
9	Diphenylmethane	6.74	274	C ₁₃ H ₁₆ O ₄	259.0941	259.0939
10	Bibenzyl	7.87	269	C ₁₄ H ₁₈ O ₄	273.1097	273.1097
12	<i>trans</i> -Stilbene	7.62	341, 357, 377	C ₁₄ H ₁₆ O ₄	271.0941	271.0941
13	Diphenylacetylene	7.52	348, 368	C ₁₄ H ₁₄ O ₄	269.0784	269.0785

^a RP8 column. Eluent: 20 - 100 % of aqueous methanol, acidified with 0.085 % phosphoric acid. For details see "Materials and methods".

^b Determined by diode array detector.

^c For the sodium cation.

^d Data taken from Overwin et al. 2012.

Table 3 LC-MS data of dehydrogenation products of bis-dihydrodiols of biphenyl and its homoaromatic analogues.

Substrate		HPLC data of product		LC-MS data of product		
No.	Name	t_r [min] ^a	λ_{max} [nm] ^b	Mass _{exp.} ^c	m/z ⁺ _{obs.} ^d	m/z ⁻ _{obs.} ^e
8	Biphenyl	3.60	247	218	219	217
9	Diphenylmethane	4.38	275	232	233	231
10	Bibenzyl	5.40	275	246	247	245
12	<i>trans</i> -Stilbene	4.38	305	244	245	243
13	Diphenylacetylene	7.00	289	242	243	241

^a RP8 column. Eluent: 50 % of acidified aqueous methanol. For details see “Materials and methods”.

^b Determined by diode array detector.

^c Molecular mass expected for the neutral bis-catechol.

^d Molecular mass observed for the protonated compound.

^e Molecular mass observed for the de-protonated compound.

Fig. 1

Fig. 2

a

b

Supplementary Material

Table S1 Nuclear magnetic resonance data of final dioxygenation products of heteroaromatic biphenyl derivatives formed by BphA-B4h.^a

Dioxygenation product of	Atom position	No. of ¹ H	¹ H Chemical shift, multiplicity and coupling constant(s)	¹³ C Chemical shift
2-Phenylpyridine ^b	C-1'	0		136.2
	C-2'	1	4.81 d 5.7	66.4
	C-3'	1	4.74 ddd 5.6, 2.8, 2.6	70.7
	C-4'	1	6.08 ddd 9.6, 2.5, 1.2	132.6
	C-5'	1	6.29 ddd 9.6, 5.6, 2.8	124.2
	C-6'	1	6.91 d 5.6	125.8
	C-2	0		155.5
	C-3	1	7.76 d 8.1	121.1
	C-4	1	7.93 ddd 8.0, 7.8, 1.8	138.4
	C-5	1	7.42 dd 7.6, 4.9	123.2
	C-6	1	8.57 bd 4.7	148.8
	C-1'	0		136.4
3- Phenylpyridine ^c	C-2'	1	4.48 dd 5.9, 1.1	69.4
	C-3'	1	4.57 ddd 5.7, 2.8, 2.6	71.8
	C-4'	1	5.99 dddd 9.6, 2.4, 1.2, 1.1	133.7
	C-5'	1	6.16 ddd 9.6, 5.6, 2.6	124.6
	C-6'	1	6.55 d 5.6	124.9
	C-2	1	8.78 d 2.3	147.3
	C-3	0		137.4
	C-4	1	8.07 ddd 8.1, 2.3, 1.6	135.1
	C-5	1	7.46 ddd 8.1, 4.8, 0.8	125.1
	C-6	1	8.45 dd 4.8, 1.4	148.5
	C-1'	0		134.2
	C-2'	1	4.65 d 5.7	66.9
4- Phenylpyridine ^b	C-3'	1	4.74 "qui" 5.6, 2.7, 2.5	70.6
	C-4'	1	6.12 d 9.4	133.6
	C-5'	1	6.29 "sep" 9.3, 5.9, 2.8	124.0
	C-6'	1	6.90 d 5.7	127.2
	C-2	1	8.57 d 6.4	147.7
	C-3	1	7.73 d 6.2	120.7
	C-4	0		148.9
	C-5	1	7.73 d 6.2	120.7
	C-6	1	8.57 d 6.4	147.7
	C-1'	0		136.5
	C-2'	1	4.77 dd 6.1, 1.2	66.6
	C-3'	1	4.58 ddd 6.1, 2.7, 2.5	72.1
4-Phenylpyrimidine ^c	C-4'	1	6.17 dddd 9.6, 2.3, 1.1, 1.1	138.3
	C-5'	1	6.23 ddd 9.6, 5.6, 2.8	124.1
	C-6'	1	7.32 d 5.5	130.0
	C-2	1	9.11 d 1.4	159.2
	C-4	0		165.1
	C-5	1	7.77 dd 5.5, 1.5	117.9
	C-6	1	8.72 d 5.5	158.1
	C-1'	0		136.5
	C-2'	1	4.77 dd 6.1, 1.2	66.6
	C-3'	1	4.58 ddd 6.1, 2.7, 2.5	72.1

Dioxygenation product of	Atom position	No. of ¹ H	¹ H Chemical shift, multiplicity and coupling constant(s)	¹³ C Chemical shift
1-Benzylpyrrole ^d	C-1'	0		139.6
	C-2'	1	3.89 d 6.2	68.2
	O-2'		3.73 bs	
	C-3'	1	4.17 bs	69.4
	O-3'		3.81 bs	
	C-4'	1	5.78 dd 9.6, 3.2	131.2
	C-5'	1	5.85 ddd 9.6, 5.4, 1.9	123.9
	C-6'	1	5.58 d 5.4	121.0
	C-2	1	6.69 "t" ~2.1, ~2.1	121.8
	C-3	1	6.02 "t" ~2.1, ~2.1	108.6
	C-4	1	6.02 "t" ~2.1, ~2.1	108.6
	C-5	1	6.69 "t" ~2.1, ~2.1	121.8
	C-6	2	4.74 d 16.2	52.3
			4.62 d 16.2	
1-Phenylimidazole ^c	C-1'	0		136.8
	C-2'	1	4.48 d 6.3	69.2
	C-3'	1	4.62 ddd 6.4, 3.0, 2.6	71.6
	C-4'	1	5.93 dd 9.7, 2.6	132.3
	C-5'	1	6.06 ddd 9.7, 5.9, 2.8	122.7
	C-6'	1	6.28 d 5.9	115.4
	C-2	1	8.03 s	136.8
	C-4	1	7.10 bs	129.7
	C-5	1	7.48 dd 1.4, 1.4	119.1
3-Phenylpyrazole ^c	C-1'	0		122.1
	C-2'	1	4.52 dd 5.4, 2.6	68.7
	C-3'	1	4.52 dd 5.5, 2.7	71.8
	C-4'	1	5.91 d 9.5	133.0
	C-5'	1	6.10 ddd 9.6, 5.6, 2.7	124.5
	C-6'	1	6.57 d 5.5	122.1
	C-3	0		103.7
	C-4	1	6.55 d 2.1	103.7
	C-5	1	7.61 s ^e	124.5

^a Chemical shifts (in ppm) are normally given relative to the residual signal of the solvent (¹H: 4.80 ppm for water-d₂; ¹H: 3.35 ppm, ¹³C: 49.0 ppm for methanol-d₄; ¹H: 2.05 ppm, ¹³C: 29.8 ppm for acetone-d₆). ¹³C shifts in water-d₂ are given relative to the signal of DSS.

^b Solvent: water-d₂.

^c Solvent: methanol-d₄.

^d Solvent: acetone-d₆.

^e COSY showed coupling with H-4.

Table S2 Nuclear magnetic resonance data of final dioxygenation products of homoaromatic biphenyl derivatives formed by BphA-B4h.^a

Bis-dihydrodiol derived from	Position of ¹ H	No. of ¹ H	Chemical shift, multiplicity and coupling constant(s)
Diphenylmethane ^b	5/5'	2	5.96 ddd 9.5, 5.3, 2.3
	6/6'	2	5.88 dd 5.3, 1.3
	4/4'	2	5.77 dd 9.4, 3.0
	3/3'	2	4.30 ddd 5.8, 2.8, 2.5
	2/2'	2	3.97 d 5.9 Hz
	7	2	3.16 s
Bibenzyl ^b	5/5'	2	5.94 ddd 9.6, 5.4, 2.1
	6/6'	2	5.79 d 5.3
	4/4'	2	5.76 dd 9.6, 3.3
	3/3'	2	4.26 ddd 5.8, 3.3, 2.2
	2/2'	2	4.02 d 5.9
	7/7'A	2	2.55 m
	7/7'B	2	2.43 m
<i>trans</i> -Stilbene ^c	7/7'	2	6.70 s
	6/6'	2	6.25 d 5.6
	5/5'	2	6.18 ddd 9.6, 5.6, 2.9
	4/4'	2	5.92 bd 9.6
	3/3'	2	4.65 "qui" 5.4, ~2.8, ~2.7
	2/2'	2	4.54 d 5.5
Diphenylacetylene ^b	6/6'	2	6.34 d 5.6
	5/5'	2	6.08 ddd 9.7, 5.6, 2.0
	4/4'	2	6.00 dd 9.4, 3.6
	3/3'	2	4.34 ddd 5.9, 3.5, 1.9
	2/2'	2	4.12 d 5.9

^a Chemical shifts (in ppm) are given relative to the residual signal of methanol (3.35 ppm) or, in case of water, relative to the signal of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), set to 0 ppm. Coupling constants in Hz.

^b Solvent: methanol-d₄.

^c Solvent: water-d₂.

Fig. S1 Structural formulas of the 2',3'-mono-dihydrodiols derived from examined heteroaryls. Substrate names are indicated.

Fig. S2 Structural formulas of the 2,3,2',3'-bis-dihydrodiols derived from examined homoaryls. Substrate names are indicated.