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FULL PAPER

Bacterial Catabolism of Biphenyls. Synthesis and Evaluation of Analogues

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Dedication

Abstract: A series of alkylated 2,3-dihydroxybiphenyls (DHBs) have been prepared on gram scale using an effective Directed *ortho* Metalation (DoM) – Suzuki-Miyaura cross-coupling strategy. These compounds have been used to investigate the substrate specificity of the *meta*-cleavage dioxygenase BphC, a key enzyme in the microbial catabolism of biphenyl. Isolation and characterization of the *meta*-cleavage products allows the study of related processes, including the catabolism of lignin-derived biphenyls.

Introduction

Understanding the bacterial catabolism of aromatic compounds is critical for developing effective biocatalysts for various technologies, including bioremediation[1] and the upgrading of lignin to commodity chemicals.[2] For example, the catabolism of biphenyl, a paradigm for the microbial catabolism of aromatic

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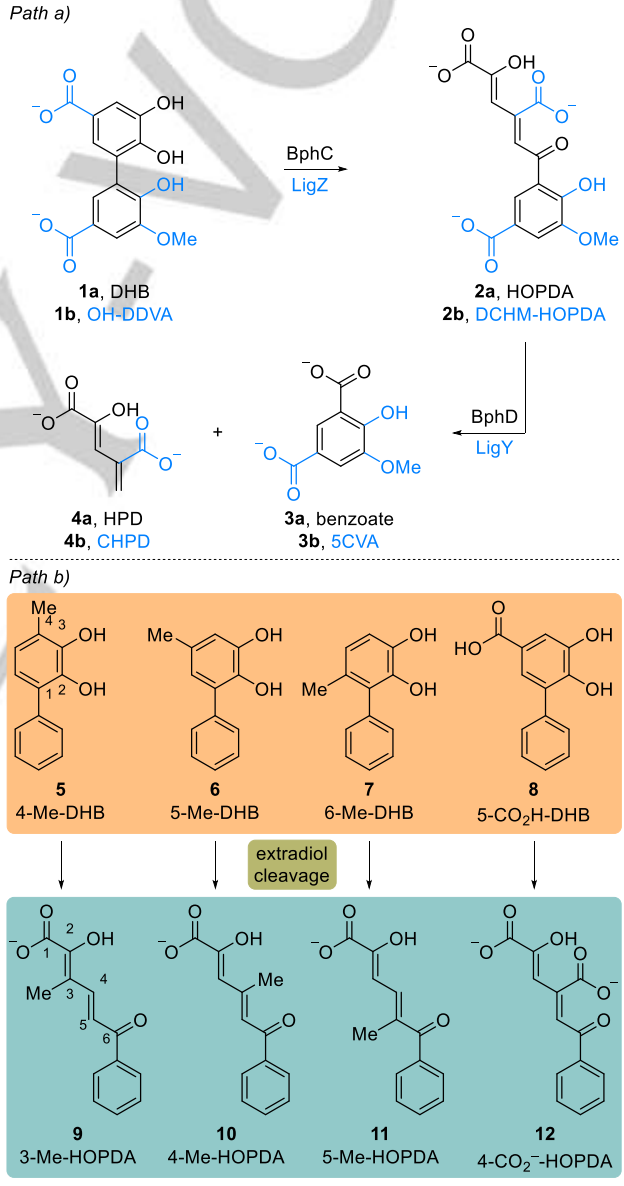


Figure 1. *a*) The transformation of dihydroxybiphenyls (DHBs) and *meta*-cleavage products (MCPs) in the bacterial catabolism of DDVA (enzyme names in blue) and biphenyl (enzyme names in black), respectively. The substituents in light blue only occur in DDVA catabolism. *b*) Synthetic analogues **5**-**8** and their *meta*-cleavage products. The carboxylate of **8** is considered to be the enzyme's substrate.

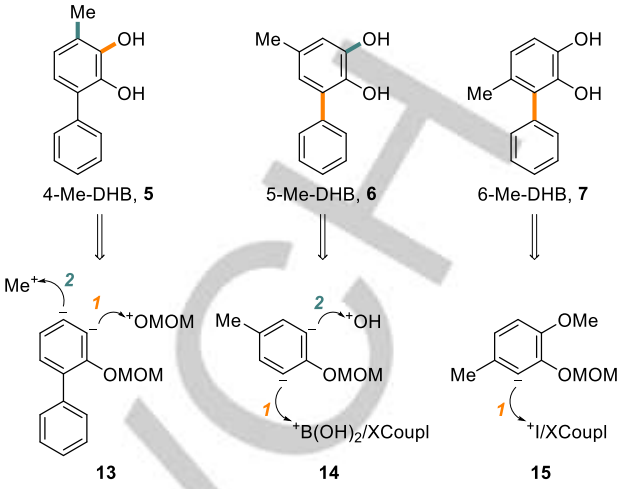
compounds, has been studied and engineered to degrade PCBs.[3] More recently, the recognition that bacteria partially deconstruct lignin has stimulated studies of enzymes involved in depolymerizing lignin and catabolizing the resulting products.[4]*Sphingobium* sp. strain SYK-6 is among the best characterized bacterial strains that grow on lignin-derived aromatic compounds. The strain contains pathways responsible for catabolizing a number of lignin-derived aromatics, including 2,2'-dihydroxy-3,3'-dimethoxy-5,5'-dicarboxybiphenyl (DDVA), β-aryl ethers, phenylcoumaran-type biaryls, pinoresinols and diaryl propanes.[5]

The catabolism of DDVA[5b] is similar to that of biphenyl in that both proceed via a 2,3-dihydroxybiphenyl (DHB, **1**) that is subject to *meta*-cleavage by an extradiol dioxygenase to 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA, **2**). A *meta*-cleavage product (MCP) hydrolase then catalyzes the C_5-C_6 bond cleavage of HOPDA through a vinylogous retro-Claisen reaction. In biphenyl catabolism, these two reactions are catalyzed by BphC (2,3-dihydroxybiphenyl 1,2-dioxygenase) and BphD, respectively. In DDVA catabolism, the corresponding enzymes are LigZ and LigY, whose substrates are 2,2',3-trihydroxy-3'-methoxy-5,5'-dicarboxybiphenyl (OH-DDVA, **1b**) and 4,11-dicarboxy-8-hydroxy-9-methoxy (DCHM-HOPDA, **2b**), respectively (Figure 1, catabolism pathway). BphD is a serine-dependent member of the α/β-hydrolase superfamily,[6] typical of all but one of the MCP hydrolases characterized to date. The lone MCP hydrolase that differs is LigY; it is a Zn^{2+}-dependent member of the amidohydrolase superfamily.[7] It is unclear how these two enzymes use such different catalytic machinery to catalyze similar reactions.

To facilitate the further study of bacterial pathways that catabolize aromatic compounds, we now report the synthesis of a series of alkyl substituted DHBs (**5-8**, Figure 1). To illustrate the utility of these compounds in characterizing PCB- and lignin-degrading enzymes, we used them to investigate the specificity of BphC. The corresponding alkylated MCPs **9-12** were characterized for studies of BphD and LigY.

Results and Discussion

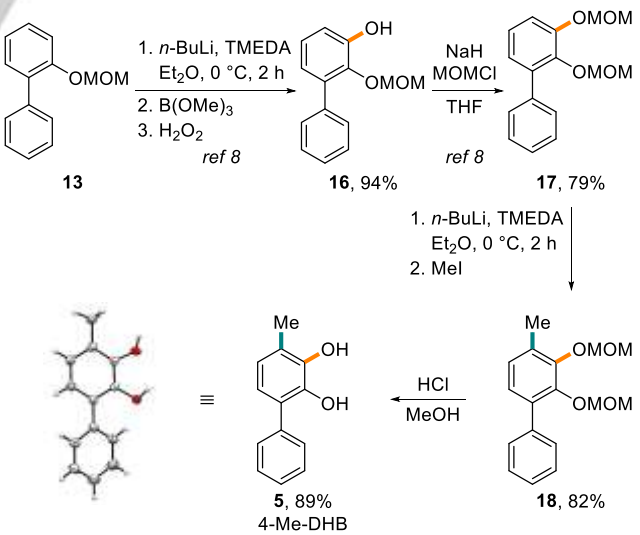
The retrosynthetic analysis of Me-DHBs **5-7** conceptualized in Scheme 1 follows a unified Directed *ortho* Metalation (DoM) – Suzuki-Miyaura cross-coupling strategy analogous to that documented previously.[8] Thus, 4-Me-DHB (**5**) is considered to be synthesized by a 'walk around the ring' tactic[9] from the methoxymethyl-protected (OMOM) derivative **13** of commercially available 2-phenylphenol. The first OMOM directed metalation group (DMG) serves to introduce the second OMOM DMG (step 1) which itself is then used to introduce the methyl group (step 2). 5-Me-DHB (**6**) is derived from MOM-protected *p*-cresol **14** via two successive DoM reactions without concern of methyl group deprotonation.[10] In step 1, boronation is followed by Suzuki-Miyaura cross-coupling, while in step 2 the boronation is followed by hydroxylation. The order of steps (1 before 2) is dictated by the requirement of protection-deprotection steps of the introduced phenolic hydroxyl group (step 2). 6-Me-DHB (**7**) is derived from



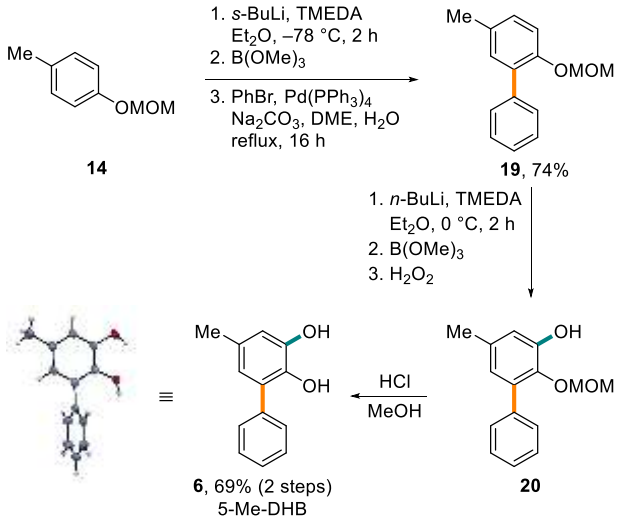
Scheme 1. Retrosynthetic analysis of model substrates 4-Me-DHB **5**, 5-Me-DHB **6** and 6-Me-DHB **7**.

the OMOM derivative **15** of commercially available 2-methoxy-5-methylphenol. Site selectivity (step 1) for the more hindered position is expected based on the greater directing power of the OMOM DMG compared to the weaker OMe group.[11] Subsequent Suzuki-Miyaura cross-coupling delivers 6-Me DHB (**7**).

Synthesis of 4-Me-DHB. The synthesis of 4-Me-DHB (**5**) (Scheme 2) was initiated by the preparation of the di-MOM phenyl catechol **17** according to our previously reported procedure.[8] Treatment of **17** with *n*-BuLi/TMEDA followed by quenching with iodomethane afforded **18**, a reaction which was carried out on 7 gram scale (see SI). Global deprotection using methanolic HCl delivered 3 grams of 4-Me-DHB (**5**).



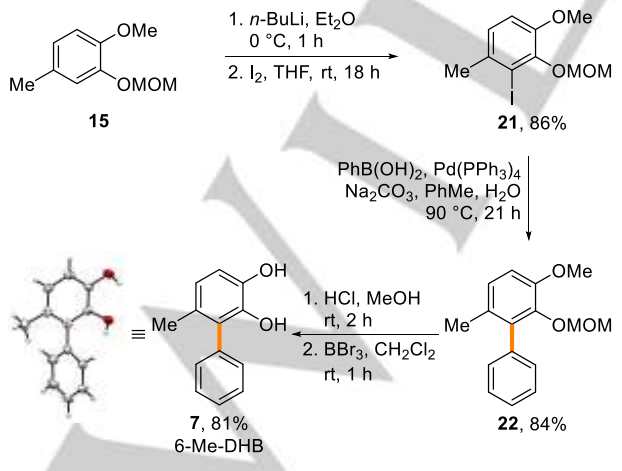
Scheme 2. Synthesis of 4-methyl-2,3-dihydroxybiphenyl (4-Me-DHB). *[x-ray structure added]*



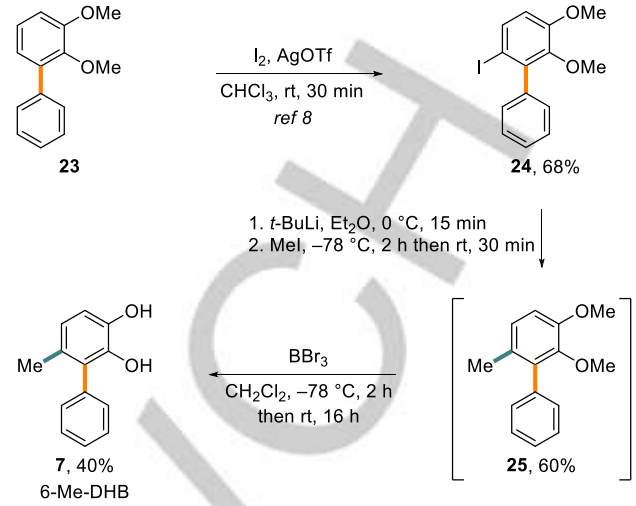
Scheme 3. Synthesis of 5-methyl-2,3-dihydroxybiphenyl (5-Me-DHB). *[x-ray structure added]*

Synthesis of 5-Me-DHB. The preparation of 5-Me-DHB (**6**) (Scheme 3) starts from the symmetrical MOM-protected *p*-cresol **14** which, in a three-step sequence involving no purification of intermediates, was subjected to metalation (*s*-BuLi/TMEDA/–78 °C) followed by trapping of the resulting anion with freshly distilled $B(OMe)_3$ to give the corresponding boronic acid which, upon direct Suzuki-Miyaura cross-coupling, afforded biphenyl **19** (4 gram scale reaction, see SI). Subsequent hydroxylation was then achieved through a second metalation/boronation sequence followed by oxidation to give phenol **20** which, on final deprotection, furnished 2.5 grams of 5-Me-DHB (**6**).

Synthesis of 6-Me-DHB. The synthesis of 6-Me-DHB (**7**) (Scheme 4) begins with the OMOM protected phenol **15** which provides a DMG hierarchy (OMOM > OMe) advantage irrespective of the methyl group steric effect to give, upon metalation and iodine quench, compound **21** in high yield.[12]



Scheme 4. Synthesis of 6-methyl-2,3-dihydroxybiphenyl (6-Me-DHB).

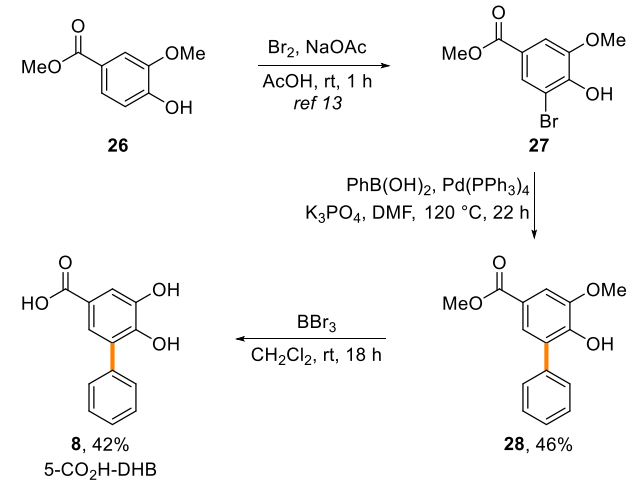


Scheme 5. Alternate synthesis of 6-methyl-2,3-dihydroxybiphenyl (6-Me-DHB).

Suzuki-Miyaura coupling gave the biphenyl **22** in 84% yield, which upon deprotection of the MOM group with HCl/MeOH followed by demethylation with BBr_3 afforded 6-Me-DHB (**7**), whose structure was confirmed unambiguously by x-ray crystallography.

In an alternate approach to 6-Me-DHB (Scheme 5), site selective iodination[8] of 2,3-dimethoxybiphenyl **23** furnished the contiguously substituted product **24**, which underwent facile lithium-halogen exchange on treatment with *t*-BuLi in Et_2O. Trapping of the resulting lithiated species with iodomethane followed by final deprotection with BBr_3 delivered 6-Me-DHB (**7**).

Synthesis of 5-CO_2H-DHB. The synthesis of the final target, 5-CO_2H-DHB (**8**) (Scheme 6), was initiated by bromination of phenol **26** as described.[13] Suzuki-Miyaura coupling delivered 3 grams of biphenyl **28** which, upon treatment with BBr_3, led to concomitant deprotection of both the methoxy and ester groups to afford 5-CO_2H-DHB (**8**).



Scheme 6. Synthesis of 5-carboxy-2,3-dihydroxybiphenyl (5-CO_2H-DHB).

Table 1. Apparent steady-state kinetic and inactivation parameters of BphC.

substrate	K_M^{app} (μM)	k_{cat}^{app} (s^{-1})	k_{cat}^{app}/K_M^{app} (x 10^6 M^{-1} s^{-1})	partition ratio (x 10^3)	k_{inact}^{app} (x 10^3 s^{-1})	$k_{inact}^{app}/K_M^{app}$ (x 10^3 M^{-1} s^{-1})
DHB[a]	12 (1)	251 (6)	21 (1)	84.9 (1.4)	3.0 (0.1)	0.25 (0.02)
4-Me-DHB	36 (4)	116 (4)	3.2 (0.2)	1.7 (0.1)	68 (8)	1.9 (0.3)
5-Me-DHB	33 (3)	506 (20)	16 (1)	1.6 (0.1)	320 (20)	10 (1)
6-Me-DHB	6 (1)	53 (3)	8.5 (0.8)	9.2 (0.4)	5.9 (0.7)	1.0 (0.2)

Experiments were performed using air-saturated 40 mM HEPES (I = 0.1 M), pH 7.5 at 25 °C. The values in parentheses represent standard errors. [a] Kinetic parameters for DHB were obtained from reference 15. [b] k_{inact}^{app} was calculated by dividing k_{cat}^{app} by the partition ratio.

Table 2. Molar extinction coefficients and pKa_2 of Me-HOPDAs.

DHB	Corresponding HOPDA	pKa_2 of HOPDA[a]	Wavelength (nm)	Extinction coefficient[b] (mM^{-1} cm^{-1})
DHB[a]	HOPDA	7.3	434	26.6
4-Me-DHB	3-Me-HOPDA	7.5	428	39.7
5-Me-DHB	4-Me-HOPDA	10	446	42.4
6-Me-DHB	5-Me-HOPDA	7.7	402	45.5

[a] pK_a values were determined in potassium phosphate buffer titrated with either acid or base. [b] Molar extinction coefficients determined in 0.1 M KOH, 25°C, with freshly generated Me-HOPDAs.

Specificity of BphC. We used an oxygraph assay[14] to investigate the ability of BphC from *Burkholderia xenovorans* LB400 to catalyze the *meta*-cleavage of the alkylated DHBs. Because the steady-state cleavage of the DHBs exhibited some substrate inhibition, the kinetic parameters were determined using DHB concentrations only up to ~5-times the estimated K_M^{app}. As summarized in Table 1, BphC catalyzed the cleavage of the Me-DHBs with apparent specificities (k_{cat}^{app}/K_M^{app}) that were 20–70% those of unsubstituted DHB in the following order: DHB > 5-Me > 6-Me > 4-Me-DHB. This is similar to what was observed with chlorinated DHBs although the order of apparent specificity was slightly different: DHB > 6-Cl > 4-Cl > 5-Cl-DHB.[15] Indeed, the steady-state kinetic parameters for methylated DHBs were similar to those reported for the corresponding chlorinated DHBs with the exception of 5-Me-DHB, for which BphC had ~7-fold higher apparent specificity than 5-Cl-DHB. Interestingly, BphC did not detectably cleave 5-CO_2H-DHB. Moreover, 5-CO_2H-DHB at concentrations up to 200 μM did not detectably inhibit the BphC-catalyzed cleavage of DHB. This suggests that BphC does not cleave the carboxylated substrate because it has low affinity for it. Consistent with this analysis, inspection of the BphC:DHB complex[14] revealed that the 5-position is closely bounded by three residues (Phe187, Ile173 and Asn243), two of which are hydrophobic. By contrast, protocatechuate 4,5-dioxygenase accommodates the carboxylate substituent of its substrate with hydrogen bond-forming residues.[16]

We also evaluated the susceptibility of BphC to inactivation during the cleavage of methylated DHBs. This inactivation occurs through the adventitious oxidation of the active site iron during the catalytic cycle.[17] The susceptibility to inactivation, $k_{inact}^{app}/K_M^{app}$, was calculated from the partition ratio, which is the average number of times the enzyme turns over before inactivation. Based on $k_{inact}^{app}/K_M^{app}$, BphC was up to 40-fold more susceptible to inactivation by Me-DHBs in the following order: 5-Me > 4-Me > 6-Me > DHB. Indeed, 5-Me-DHB inactivated BphC 100-times more efficiently (k_{inact}^{app}) than DHB. These results contrast with those for the corresponding chlorinated DHBs, which did not inactivate BphC more potently than DHB.[15] Indeed, BphC was slightly less susceptible to inactivation by 5-Cl DHB than DHB.

Properties of Me-HOPDAs. BphC catalyzes the 1,2-cleavage of DHB to HOPDA,[14] which exists predominantly as the yellow-colored dienolate anion (λ_{max} = 434 nm) under the reaction conditions used here (potassium phosphate (*I* = 0.1 M), pH 7.5, 25 °C).[18] Under these conditions, reaction of BphC with each Me-DHB (**5** to **8**) also yielded a yellow-colored product, consistent with 1,2-cleavage to the corresponding Me-HOPDA and its occurrence as a dienolate, as reported for Cl-DHBs.[18] 4-Me-HOPDA rapidly converted to a colorless species ($t_{½}$ ~4 s) under these conditions. The dienolate form of 4-Me-HOPDA could be regenerated under alkaline pH, as reported for DCHM-HOPDA.[19]

The pK_a values of the Me-HOPDAs together with their molar extinction coefficients in 0.1 M KOH are summarized in Table 2. Overall, methyl substitution of the dienol moiety increased the pK_a value of HOPDA. The corresponding chlorinated HOPDAs had lower pK_a values, consistent with the greater electronegativity of the chloro substituent.[19]

The identity of Me-HOPDAs arising from the cleavage of respective Me-DHBs was confirmed by mass spectrometry and the results are summarized in Table 3. Unlike the 3-Me- and 5-Me-HOPDAs, the parent-ion for 4-Me-HOPDA was not detected using our standard mass spectrometry conditions. Subsequently, the 4-Me-HOPDA was derivatized with ammonia to produce the more stable pyridinal derivative whose mass was used to infer the identity of the 4-Me-HOPDA, similar to the strategy used to identify the MCP resulting from the cleavage of DCHM-HOPDA by LigZ.[18]

Table 3. Mass determination of Me-HOPDAs from BphC catalyzed Me-DHB cleavage.

DHB	corresponding HOPDA	Charge state	Calculated m/z	Observed m/z
4-Me-DHB (**5**)	3-Me-HOPDA (**9**)	+1	233.0813	233.0849
5-Me-DHB (**6**)	4-Me-HOPDA[a] (**10**)	+1	214.0868	214.0844
6-Me-DHB (**7**)	5-Me-HOPDA (**11**)	+1	233.0813	233.0849

[a] 4-Me-HOPDA was derivatized with ammonia as discussed in text.

Conclusions

In summary, we have reported the synthesis of a series of alkylated 2,3-dihydroxybiphenyls (DHBs), and their use in the evaluation of the substrate specificity of the extradiol dioxygenase BphC. The use of an effective Directed *ortho* Metalation (DoM) – Suzuki-Miyaura cross-coupling strategy proved crucial in providing rapid access to the requisite gram scale quantities of the isomeric highly substituted arenes needed for this study. BphC catalyzed the cleavage of the methylated DHBs with substrate specificities similar to those reported for the corresponding chlorinated DHBs. However, the Me-DHBs inactivated BphC more potently than the Cl-DHBs, particularly 5-Me-DHB. The substituted DHBs and HOPDAs will facilitate studies of related catabolic processes, including homologous enzymes and downstream catabolism. For example, 5-carboxy DHB could be a useful probe for extradiol dioxygenases whose cognate substrates have 5-carboxy substituents. Similarly, the 4-substituted HOPDAs are of interest as analogs of DCHM-HOPDA, the substrate of LigY.

Experimental Section

Synthesis

General Information: All reactions were carried out in oven-dried glassware under an atmosphere of argon unless otherwise stated. Flash chromatography was carried out using Silicycle Silicaflash P60 silica gel. Melting points were determined on an Electrothermal IA9100 melting point apparatus from chromatographically homogenous compounds without further recrystallization. IR spectra were recorded on a Bruker Alpha FTIR spectrometer. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker Avance-400 or Avance-300 spectrometer operating at 400 MHz and 300 MHz respectively (^{1}H frequency, corresponding ^{13}C frequencies are 100 MHz and 75 MHz). In the ^{13}C NMR spectra, signals corresponding to CH, CH_2, or Me groups are assigned from DEPT. Mass spectra were recorded on a Micromass 70-250S double focusing mass spectrometer (EI) or Waters ZQ Single Quad mass spectrometer (ESI). X-Ray crystallographic analyses for 4-Me-DHB and 5-Me-DHB were measured with a Bruker D8 Quest diffractometer equipped with a Photon 100 CMOS detector and molybdenum radiation (λ = 0.710713 Å) from an Incoatec microfocus tube. For the multiscan absorption correction of the intensity data, Sadabs 2014/5 was used, and the structure solutions and parameter refinement was performed with the Shelxs/l-2013.[20] X-Ray crystallographic analysis for 6-Me-DHB was measured with a Rigaku Oxford Diffraction Gemini-R Ultra diffractometer using graphite monochromatized Mo-Ka (l=0.71073 Å) radiation. Data reduction including intensity integration, background corrections as well as Lorentz and polarization correction was performed with the CrysAlis^{PRO} software package. The structure was solved by direct methods using the SIR2004 program suite.[21] All non-hydrogen atoms of the molecule were obtained from the E-map with the highest combined figure of merit. Full-matrix least-squares refinement on F^2 was carried out using the SHELXL97software.[20] All hydrogen atoms (except those belonging to the hydroxyl groups) were geometrically fixed and allowed to ride on the corresponding carrier atoms with C–H = 0.98 – 0.99 Å and U_{iso}(H)= 1.2 x U_{eq}(C) of the attached C atom. Hydrogen atoms of the hydroxyl groups were located in a difference Fourier map and refined isotropically using the same numerical coupling for U_{iso} but without any restraints concerning the O-H bond distances. CCDC 1842733 (4-Me-DHB), 1844073 (5-Me-DHB) and 1839038 (6-Me-DHB) contain the supplementary crystallographic data for this paper. These data are provided free of charge by the Cambridge Crystallographic Data Centre.

2,3-Bis(methoxymethoxy)-4-methyl-1,1'-biphenyl (18): To a solution of 2,3-bis(methoxymethoxy)-1,1'-biphenyl[8] **17** (7.00 g, 25.5 mmol) in Et_2O (120 mL) was added TMEDA (4.44 mL, 30.6 mmol, 1.2 equiv). The solution was stirred for 5 min then cooled to 0 °C before the dropwise addition of *n*-BuLi (1.52 M solution in hexane, 20.1 mL, 30.6 mmol, 1.2 equiv). The reaction mixture was stirred for 2.5 h at 0 °C, then cooled to –78 °C and quenched by the rapid addition of iodomethane (6.35 mL, 102 mmol, 4.0 equiv). After warming to rt, stirring was continued for a further 1 h, then sat. brine solution was added (50 mL), and the layers were separated. The aqueous layer was extracted once with Et_2O (50 mL). The combined organic layers were washed with brine (50 mL), dried over $MgSO_4$, subjected to filtration, and the solvents were removed in vacuo. The crude product was purified by flash chromatography on silica gel (1:9 EtOAc/hexanes) to give the title compound **18** (5.73 g, 82%) as a pale yellow oil. IR (ATR): $\tilde{\nu}$ = 3056, 2990, 2927, 2826, 1477, 1421, 1387, 1302, 1268, 1155, 1073, 1033, 958, 919, 818, 759, 698; ^{1}H NMR (400 MHz; $CDCl_3$): δ = 7.52-7.50 (m, 2 H), 7.41-7.37 (m, 2 H), 7.31 (tt, *J* = 7.3 and 1.4 Hz, 1 H), 7.02 (d, *J* = 7.8 Hz, 1 H), 6.99 (d, *J* = 7.8 Hz, 1 H), 5.20 (s, 2 H), 4.80 (s, 2 H), 3.63 (s, 3 H), 3.01 (s, 3 H), 2.36 (s, 3 H) ppm; ^{13}C NMR (100 MHz; $CDCl_3$): δ = 149.1 (C), 146.9 (C), 138.6 (C), 135.0 (C), 132.1 (C), 129.6 (CH), 128.1 (CH), 126.9 (CH), 126.4 (CH), 125.7 (CH), 99.4 (CH_2), 98.9 (CH_2), 57.6 (Me), 57.0 (Me), 16.6 (Me) ppm; EI HRMS: cald for $C_{17}H_{20}O_4^+$ 288.1362, found 288.1355.

4-Methyl-2,3-dihydroxybiphenyl (4-Me-DHB, 5): To a solution of 2,3-bis(methoxymethoxy)-4-methyl-1,1'-biphenyl **18** (5.00 g, 17.3 mmol) MeOH (30 mL) was added 3 N HCl (15 mL). The reaction mixture was stirred at rt for 16 h, then diluted with CH_2Cl_2 (100 ml) and the phases were separated. The aqueous layer was extracted with CH_2Cl_2 (100 mL) and the combined organics were washed with sat. brine solution (100 mL), dried over $MgSO_4$, subjected to filtration, and the solvents were removed in vacuo. The crude product was purified by flash chromatography on silica gel (1:3 EtOAc/hexanes) followed by recrystallization (EtOAc/hexane) to give the title compound 4-Me-DHB **5** (3.08 g, 89%) as pale purple crystals. M.p. 63.5–65 °C; IR (ATR): $\tilde{\nu}$ = 3396, 3299, 3058, 2920, 1628, 1569, 1485, 14770, 1431, 1352, 1324, 1288, 1270, 1189, 1160, 1092, 1031, 944, 888, 805, 763, 748, 724, 693; 1H NMR (400 MHz; $CDCl_3$): δ = 7.52-7.48 (m, 4 H), 7.42-7.38 (m, 1 H), 6.80 (d, *J* = 7.9 Hz, 1 H), 6.75 (d, *J* = 7.9 Hz, 1 H), 5.50 (s, 1 H), 5.31 (s, 1 H), 2.32 (s, 3 H) ppm; 13C NMR (100 MHz; $CDCl_3$): δ = 142.5 (C), 139.4 (C), 137.1 (C), 129.3 (CH), 128.8 (CH), 127.7 (CH), 125.9 (C), 124.0 (C), 122.6 (CH), 120.5 (CH), 15.5 (Me) ppm; EI HRMS: cald for $C_{13}H_{12}O_2^+$ 200.0837, found 200.0832.

2-(Methoxymethoxy)-5-methyl-1,1'-biphenyl (19): To a solution of 4-(methoxymethoxy)toluene **14** (6.00 g, 39.4 mmol) in Et_2O (120 mL) was added TMEDA (7.09 mL, 47.3 mmol, 1.2 equiv). The solution was stirred for 5 min then cooled to –78 °C before the dropwise addition of *s*-BuLi (1.53 M solution in cyclohexane, 30.9 mL, 47.3 mmol, 1.2 equiv). The reaction mixture was stirred for 2.5 h at –78 °C, then quenched by the rapid addition of freshly distilled $B(OMe)_3$ (17.6 mL, 158 mmol, 4.0 equiv). Stirring was continued for 2 h at –78 °C, then the reaction mixture was allowed to warm to rt, quenched by the addition of H_2O (60 mL), and then acidified with 2 N HCl to pH 6. The aqueous layer was extracted with CH_2Cl_2 (2 × 150 mL). The combined organics were washed with H_2O (50 mL) and sat. brine (50 mL), dried over $MgSO_4$, subjected to filtration, and the solvents evaporated in vacuo. The crude boronic acid was used in the next step without further purification.

A solution of bromobenzene (4.04 g, 25.7 mmol) in 1,2-DME (140 mL) was degassed by bubbling with argon for 15 min. $Pd(PPh_3)_4$ (2.97 g, 2.57 mmol, 10 mol%) was added and stirring continued for 15 min. A degassed 2 N

aqueous Na_2CO_3 solution (80 mL) was added and the mixture stirred for a further 30 min at rt, whereupon the reaction mixture became cloudy. A solution of the arylboronic acid (7.55 g, 38.6 mmol, 1.5 equiv) in degassed 1,2-DME (75 mL) was then added dropwise, and the reaction mixture stirred at reflux for 16 h. The solvent was removed in vacuo and the residue was extracted with Et_2O (2 × 75 mL). The combined organics were washed with H_2O (75 mL), 2 N NaOH (75 mL), and sat. brine solution (75 mL), dried over $MgSO_4$, subjected to filtration, and the solvents removed in vacuo. The crude product was purified by flash chromatography on silica gel (1:4 EtOAc/hexanes) to give compound **19** (4.33 g, 74%) as pale yellow crystals. IR (ATR): $\tilde{\nu}$ = 3026, 2951, 2923, 2898, 2824, 1601, 1503, 1488, 1443, 1397, 1309, 1264, 1227, 1188, 1154, 1138, 1076, 1045, 983, 920, 884, 810, 770, 735, 697; ^{1}H NMR (400 MHz; $CDCl_3$): δ = 7.59-7.57 (m, 2 H), 7.48-7.44 (m, 2 H), 7.39-7.35 (m, 1 H), 7.21-7.14 (m, 3 H), 5.12 (s, 2 H), 3.42 (s, 3 H), 2.40 (s, 3 H) ppm; ^{13}C NMR (100 MHz; $CDCl_3$): δ = 152.0 (C), 138.7 (C), 131.8 (C), 131.6, 131.5, 129.5, 129.0, 127.9, 126.8, 116.0 (CH), 95.3 (CH_2), 56.0 (Me), 20.6 (Me) ppm; EI HRMS: cald for $C_{15}H_{16}O_2^+$ 228.1146, found 228.1150.

5-Methyl-2,3-dihydroxybiphenyl (5-Me-DHB, 6): *Method A*: To a solution of 2-(methoxymethoxy)-5-methyl-1,1'-biphenyl **19** (4.20 g, 18.4 mmol) in Et_2O (100 mL) was added TMEDA (3.31 mL, 22.1 mmol, 1.2 equiv). The solution was stirred for 5 min then cooled to –78 °C before the dropwise addition of *n*-BuLi (1.53 M solution in cyclohexane, 30.9 mL, 47.3 mmol, 1.2 equiv). The reaction mixture was stirred for 2.5 h at –30 °C, then quenched by the rapid addition of freshly distilled $B(OMe)_3$ (17.6 mL, 158 mmol, 4.0 equiv). The reaction mixture was allowed to warm to rt and stirred for 3.5 h. H_2O (50 mL) was added rapidly, followed by 30% H_2O_2 (16.2 mL) and stirring was continued for a further 2 h. The layers were separated, and the aqueous layer was extracted with Et_2O (50 mL). The combined organic layers were washed with sat. brine solution (50 mL), dried over $MgSO_4$, subjected to filtration, and concentrated in vacuo. The crude product was dissolved in MeOH (20 mL) and 3 N HCl (10 mL) was added. The reaction mixture was stirred at rt for 16 h, then diluted with CH_2Cl_2 (100 mL) and the layers were separated. The aqueous phase was extracted with CH_2Cl_2 (100 mL) and the combined organics washed with sat. brine solution (100 mL), dried over $MgSO_4$, subjected to filtration, and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel (1:3 EtOAc/hexanes) followed by recrystallization (EtOAc/hexane) to give the title compound 5-Me-DHB **6** (2.54 g, 69%) as pale purple crystals.

Method B: To a solution of 2-(methoxymethoxy)-5-methyl-1,1'-biphenyl **19** (2.30 g, 10.1 mmol) in Et_2O (50 mL) at –7 °C was added TMEDA (1.81 mL, 12.1 mmol, 1.2 equiv) followed by *n*-BuLi (1.94 M solution in cyclohexane, 6.25 mL, 12.1 mmol, 1.2 equiv) by dropwise addition so that the internal temperature did not rise above –2 °C. The reaction mixture was stirred for 2 h at –5 °C, then quenched by the rapid addition of freshly distilled $B(OMe)_3$ (4.50 mL, 40.4 mmol, 4.0 equiv). The reaction mixture was allowed to warm to rt and stirred for 1 h. H_2O (17 mL) was added rapidly, followed by 30% H_2O_2 (10 mL) and stirring was continued for a further 2 h. The layers were separated, and the aqueous layer was extracted with Et_2O (50 mL). The combined organic layers were washed with sat. brine solution (50 mL), dried over $MgSO_4$, subjected to filtration, and concentrated in vacuo. The crude product **20** was dissolved in MeOH (10 mL) and 3 N HCl (5 mL) was added. The mixture was stirred at rt for 16 h, then diluted with CH_2Cl_2 (50 mL) and the layers separated. The aqueous phase was extracted with CH_2Cl_2 (50 mL) and the combined organics washed with sat. brine solution (50 mL), dried over $MgSO_4$, subjected to filtration, and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel (1:3 EtOAc/hexanes) to give 5-Me-DHB **6** (1.23 g, 61%) as a colourless solid. M.p. 117–119 °C; IR (ATR): $\tilde{\nu}$ = 3486, 3448, 3319, 3029, 2914, 1595, 1524, 1506, 1488, 1444, 1414, 1383, 1327, 1204, 1156, 1103, 1070, 1031, 1003, 977, 914, 867, 835, 787, 756, 738, 694; ^{1}H NMR (400 MHz; $CDCl_3$): δ = 7.49-7.47 (m, 4 H), 7.42-7.37 (m, 1 H), 6.75 (d, *J* = 1.6 Hz, 1 H), 6.65 (d, *J* = 1.6 Hz, 1 H), 5.48 (br s, 1 H), 5.18 (br s, 1 H), 2.30 (s, 3 H) ppm; ^{13}C NMR (100 MHz; $CDCl_3$): δ = 144.0 (C), 137.6 (C), 137.1 (C), 130.6 (C), 129.2 (CH), 128.9 (CH), 128.1 (C), 127.8 (CH), 121.9 (CH), 115.3 (CH), 20.8 (Me) ppm; EI HRMS: cald for $C_{13}H_{12}O_2^+$ 200.0837, found 200.0843.

2-Iodo-4-methoxy-3-(methoxymethoxy)-1-methylbenzene (21): A solution of 1-methoxy-2-(methoxymethoxy)-4-methylbenzene[22] **15** (4.56 g, 25.0 mmol) in Et_2O (50 mL) at 0 °C was treated with a solution of *n*-BuLi (2.25 M in hexane, 16.7 mmol, 1.5 equiv) by dropwise addition over 15 min so that the internal temperature did not rise above 4 °C. The reaction mixture was stirred at 0 °C for 1.5 h, then a solution of I_2 (12.7 g, 50.0 mmol, 2 equiv) in THF (50 mL) was added dropwise over 30 min such that the internal temperature did not rise above 10 °C. The reaction mixture was allowed to warm to rt, stirred for 16 h, then quenched by the addition of sat. $Na_2S_2O_3$ solution (150 mL). The aqueous phase was extracted with EtOAc (3 × 150 mL), and the combined organics washed with sat. $Na_2S_2O_3$ solution (150 mL), dried over $MgSO_4$, subjected to filtration, and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel (0:1 to 1:19 EtOAc/hexanes) to give the title compound **21** (6.60 g, 86%) as a pale yellow oil. IR (ATR): $\tilde{\nu}$ = 2938, 2831, 1589, 1477, 1436, 1381, 1287, 1252, 1211, 1156, 1080, 1021, 967, 903, 796; ^{1}H NMR (400 MHz; $CDCl_3$): δ = 6.97 (d, *J* = 8.3 Hz, 1 H), 6.80 (d, *J* = 8.3 Hz, 1 H), 5.16 (s, 2 H), 3.82 (s, 3 H), 3.69 (s, 3 H), 2.41 (s, 3 H) ppm; ^{13}C NMR (100 MHz; $CDCl_3$): δ = 149.8 (C), 145.7 (C), 134.7 (C), 124.8 (CH), 112.4 (CH), 99.9 (C), 98.7 (CH_2), 58.5 (Me), 56.2 (Me). 28.0 (Me) ppm; EI HRMS: cald for $C_{10}H_{13}IO_3^+$ 307.9909, found 307.9905.

3-Methoxy-2-(methoxymethoxy)-6-methyl-1,1'-biphenyl (22): A mixture of 2-iodo-4-methoxy-3-(methoxymethoxy)-1-methylbenzene **21** (0.200 g, 0.649 mmol), $PhB(OH)_2$ (0.159 g, 1.30 mmol, 2 equiv), $Pd(PPh_3)_4$ (0.075 g, 0.065 mmol, 10 mol%) and Na_2CO_3 (0.138 g, 1.30 mmol, 2 equiv) in toluene (3.25 mL) and H_2O (3.25 mL) was heated at 90 °C for 12 h. Further $PhB(OH)_2$ (0.138 g, 1.13 mmol, 1.75 equiv) was added and heating continued at 110 °C for 8 h. After cooling to rt, H_2O (5 mL) was added and the aqueous phase extracted with EtOAc (3 × 20 mL). The combined organics were dried over $MgSO_4$, subjected to filtration, and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel (1:19 EtOAc/hexanes) to give compound **22** (0.141 g, 84%) as a colourless oil. IR (ATR): $\tilde{\nu}$ = 2935, 2835, 1598, 1573, 1481, 1439, 1396, 1296, 1252, 1209, 1154, 1124, 1079, 1032, 1017, 976, 921, 845, 801, 761, 724; ^{1}H NMR (400 MHz; $CDCl_3$): δ = 7.42-7.39 (m, 2 H), 7.33-7.26 (m, 3 H), 7.00 (d, *J* = 8.4 Hz, 1 H), 6.85 (d, *J* = 8.4 Hz, 1 H), 4.80 (s, 2 H), 3.86 (s, 3 H), 2.89 (s, 3 H), 2.05 (s, 3 H) ppm; ^{13}C NMR (100 MHz; $CDCl_3$): δ = 150.6 (C), 143.3 (C), 137.7 (C), 137.0 (C), 130.3 (CH), 129.2 (C), 127.9 (CH), 126.7 (CH), 125.4 (CH), 111.3 (CH), 98.4 (CH_2), 56.6 (Me), 55.9 (Me), 20.0 (Me) ppm; EI HRMS: cald for $C_{16}H_{18}O_3^+$ 258.1256, found 258.1262.

6-Methyl-2,3-dihydroxybiphenyl (6-Me-DHB, 7): *Method A*: A solution of 3-methoxy-2-(methoxymethoxy)-6-methyl-1,1'-biphenyl **22** (0.120 g, 0.466 mmol) in a mixture of MeOH (2 mL) and 2 N HCl (0.62 mL, 1.85 mmol, 4.0 equiv) was stirred at rt for 2 h. The solvent was removed in vacuo, and the residue partitioned between H_2O (10 mL) and EtOAc (3 × 15 mL). The combined organics were dried over $MgSO_4$, subjected to filtration, and concentrated in vacuo to give the crude phenol which was used in the next step without further purification.

A solution of the phenol in CH_2Cl_2 (5 mL) and cooled to –78 °C was treated with a solution of BBr_3 (1 M in CH_2Cl_2, 0.7 mL, 0.700 mmol) by dropwise addition over 2 min, and stirring was continued at –78 °C for 15 min. The reaction mixture was allowed to warm to rt, stirred for 1 h, and then quenched by the addition of 1 N HCl (10 mL). The aqueous phase was

extracted with EtOAc (3 × 15 mL), and the combined organics were dried over $MgSO_4$, subjected to filtration, and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel (1:19 EtOAc/hexanes) to give 6-Me-DHB (**7**) (0.075 g, 81%) as a colourless solid.

Method B: A solution of 6-iodo-2,3-dimethoxybiphenyl[8] (0.205 g, 0.600 mmol) in dry Et_2O (50 mL) at 2 °C was treated dropwise with a solution of *t*-BuLi (1.7 M in pentane, 0.42 mL, 0.710 mmol, 1.2 equiv). The reaction mixture was stirred at 2 °C for 15 min, then cooled to –78 °C and quenched by the rapid addition of iodomethane (0.149 mL, 2.40 mmol, 4.0 equiv). Stirring was continued at –78 °C for 2 h, then the reaction mixture was warmed to rt and stirred for a further 30 min, evaporated to dryness in vacuo, and the resulting solid was dissolved in EtOAc (50 mL). The organic layer was washed with H_2O (2 × 30 mL) and sat. brine solution (30 mL), dried over Na_2SO_4, subjected to filtration, and the solvents were removed in vacuo. The crude product was purified by flash column chromatography on silica gel (1.5:8.5 EtOAc/*n*-heptane) to give compound **25** (93 mg, 68%, approx. 88% purity) which was used directly in the next step without further purification. ^{1}H NMR (300 MHz; CD_2Cl_2): δ = 7.33-7.45 (m, 3H), 7.19-7.23 (m, 2 H), 6.97 (d, *J* = 8.5 Hz, 1 H), 6.85 (d, *J* = 8.5 Hz, 1 H), 3.86 (s, 3H), 3.53 (s, 3 H), 1.99 (s, 3 H).

To a solution of compound **25** (28 mg, 0.120 mmol) in CH_2Cl_2 (30 mL) at –78 °C was added BBr_3 (1 M in CH_2Cl_2, 0.54 mL, 0.540 mmol). Stirring was continued at –78 °C for 2 h, then the reaction mixture was warmed to rt and stirred for a further 15.5 h. After cooling to 2 °C, the reaction was quenched by the dropwise addition of H_2O (1.8 mL). The mixture was then warmed to rt and stirring continued for 30 min. The aqueous layer was separated and extracted with CH_2Cl_2 (50 mL). The combined organics were washed with H_2O (50 mL) and sat. brine solution (30 mL), dried over Na_2SO_4, subjected to filtration, and the solvents were removed in vacuo. The crude product was purified by flash column chromatography on silica gel (1:33 to 1:9 EtOAc/*n*-heptane) to give 6-Me-DHB (**7**) as a colourless solid (9.6 mg, 40%). M.p. 118.5–119.5 °C; IR (ATR): $\tilde{\nu}$ = 3506, 3310, 3057, 3036, 2953, 2926, 2858, 1728, 1623, 1597, 1575, 1490, 1460, 1434, 1370, 1320, 1283, 1235, 1202, 1157, 1143, 1091, 1071, 1021, 953, 916, 880, 807, 765, 739, 699, 680 cm^{-1}; ^{1}H NMR (400 MHz; $CDCl_3$): δ = 7.53-7.48 (m, 3 H), 7.43 (tt, *J* = 7.5 and 1.3 Hz, 1 H), 7.31-7.29 (m, 2 H), 6.85 (d, *J* = 8.2 Hz, 1 H), 6.76 (d, *J* = 8.2 Hz, 1 H), 5.23 (br s, 1 H), 4.80 (br s, 1 H), 2.02 (s, 3 H) ppm; ^{13}C NMR (100 MHz; $CDCl_3$): δ = 141.9 (C), 140.0 (C), 135.3 (C), 130.1 (CH), 129.4 (CH), 128.2 (CH), 128.0 (C), 121.9 (CH), 114.1 (CH), 19.7 (Me) ppm; EI HRMS: cald for $C_{13}H_{12}O_2^+$ 200.0837, found 200.0843.

Methyl 6-hydroxy-5-methoxy-[1,1'-biphenyl]-3-carboxylate (28): A solution of methyl 3-bromo-4-hydroxy-5-methoxybenzoate[13] **27** (6.53 g, 25.0 mmol), $PhB(OH)_2$ (6.10 g, 50.0 mmol, 2.0 equiv), $Pd(PPh_3)_4$ (1.44 g, 1.25 mmol, 5 mol%), and K_3PO_4 (21.2 g, 100 mmol, 4.0 equiv) in degassed DMF (100 mL) was heated at 120 °C for 22 h. After cooling to rt, the reaction mixture was partitioned between 2 N HCl (100 mL) and EtOAc (3 × 100 mL EtOAc). The combined organics were dried over $MgSO_4$, subjected to filtration, and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel (1:9 EtOAc/hexanes) to give the title compound **28** (2.97 g, 46%) as colourless solid. M.p. 93.5–94.5 °C; IR (ATR): $\tilde{\nu}$ = 3512, 3473, 3064, 2998, 2944, 2847, 1708, 1597, 1576, 1500, 1488, 1464, 1446, 1419, 1376, 1315, 1284, 1266, 1232, 1205, 1188, 1112, 1044, 1028, 1002, 985, 901, 868, 846, 806, 774, 759, 732, 700, 641; ^{1}H NMR (400 MHz; $CDCl_3$): δ = 7.76 (d, *J* = 1.9 Hz, 1 H), 7.63-7.71 (m, 2 H), 7.55 (d, *J* = 1.9 Hz, 1 H), 7.47-7.43 (m, 2 H), 7.36 (tt, *J* = 7.4 and 1.2 Hz, 1 H), 6.27 (br s, 1 H), 4.00 (s, 3 H), 3.91 (s, 3 H) ppm; ^{13}C NMR (100 MHz; $CDCl_3$): δ = 166.9 (C), 147.1 (C), 146.5 (C), 136.8 (C), 129.2 (CH), 128.3 (CH), 127.6 (CH), 127.3 (C), 125.3 (CH), 121.8 (C), 110.4 (CH), 56.4 (Me), 52.1 (Me) ppm; EI HRMS: cald for $C_{15}H_{14}O_4^+$ 258.0892, found 258.0902.

5-Carboxy-2,3-dihydroxybiphenyl (5-CO_2H-DHB, 8): To a solution of methyl 6-hydroxy-5-methoxy-[1,1'-biphenyl]-3-carboxylate **28** (1.29 g, 5.00 mmol) in CH_2Cl_2 (25 mL) at 0 °C was added a solution of BBr_3 (1 M in CH_2Cl_2, 25 mL, 25.0 mmol) dropwise over 20 min. Stirring was continued at 0 °C for 30 min, then at rt for 17 h. The reaction mixture was cooled to 0 °C and quenched by the addition of 2 N HCl (50 mL, containing ice). The aqueous phase was extracted with EtOAc (4 × 50 mL) and the combined organics washed with sat. brine solution (50 mL), dried over $MgSO_4$, subjected to filtration, and concentrated in vacuo. The crude product was purified by flash column chromatography on silica gel (1:48 to 1:19 MeOH/CH_2Cl_2) to give 5-CO_2H-DHB (**8**) (0.485 g, 42%) as a colourless solid. M.p. 216–218 °C; IR (ATR): $\tilde{\nu}$ = 3515, 3506, 3334, 3056, 2954, 2817, 2642, 1648, 1610, 1516, 1440, 1418, 1338, 1293, 1263, 1171, 1106, 1032, 968, 897, 865, 763, 715, 700, 625; ^{1}H NMR (400 MHz; DMSO): δ = 12.44 (br s, 1 H), 9.98 (br s, 1 H), 9.20 (br s, 1 H), 7.53-7.51 (m, 2 H), 7.43-7.40 (m, 1 H), 7.39 (d, *J* = 2.0 Hz, 1 H), 7.37 (d, *J* = 2.0 Hz, 1 H), 7.31 (tt, *J* = 7.3 and 1.2 Hz, 1 H) ppm; ^{1}H NMR (400 MHz; MeOD): δ = 7.57 (d, *J* = 7.5 Hz, 2 H), 7.53 (d, *J* = 1.9 Hz, 2 H), 7.46 (d, *J* = 1.9 Hz, 2 H), 7.39 (t, *J* = 7.5 Hz, 2 H), 7.30 (d, *J* = 7.5 Hz, 2 H) ppm; ^{13}C NMR (100 MHz; MeOD): δ = 170.2 (C), 148.8 (C), 146.3 (C), 139.3 (C), 130.3 (CH), 129.5 (C), 129.1 (CH), 128.0 (CH), 125.0 (CH), 122.6 (C), 115.9 (CH) ppm; EI HRMS: cald for $C_{13}H_{10}O_4^+$ 230.0579, found 230.0571.

Enzymatic Studies

Chemicals and reagents. All reagents were of at least analytical grade unless otherwise noted. Water for buffers was purified using a Barnstead Nanopure DiamondTM system to a resistance of at least 18 megaohms. BphC was produced as described elsewhere.[14]

Steady-state kinetics. Kinetic assays were performed by monitoring the consumption of O_2 using a Clark-type polarographic O_2 electrode OXYG1 (Hansatech, Pentney, UK) connected to a circulating water bath. Assays were performed in 1 mL of air-saturated 40 mM HEPES (I = 0.1 M, pH 7.5) at 25 °C, and initiated by the addition of BphC. The amount of enzyme used in the assay was determined from the initial velocity of a standard reaction using 80 µM of DHB and the reported value [14]. Reaction velocities were corrected for the background reading prior to enzyme addition. The electrode was calibrated daily by a two-point calibration using air-saturated water and O_2-depleted water via addition of sodium hydrosulfite, according to the manufacturer's instructions. Stock solutions were prepared fresh daily. Enzyme stock solution was prepared anaerobically, stored in a sealed vial on ice, and aliquoted using a gas-tight syringe for use. The apparent steady-state kinetic parameters were determined using substrate range up to approximately 5x estimated K_M value. Steady-state kinetic parameters were evaluated by fitting the Michaelis–Menten equation to the data using the least-squares fitting of LEONORA.

pK determination for Me-HOPDAs. The Me-HOPDAs were prepared in potassium phosphate solution (*I* = 0.1 M), pH 7.5, by treatment of the corresponding DHB with BphC. Aliquots of enzyme-free filtrate were titrated with sodium hydroxide or hydrochloric acid, as measured with a pH electrode, while keeping the concentration of Me-HOPDA constant at ~20 µM. Absorbance spectra were recorded using a Cary 60 UV-vis spectrophotometer (Agilent). The nonenzymatic transformation of Me-HOPDA as a function of pH was monitored spectrophotometrically at or near its respective λ_{max} and fitted using Hill equation in Origin 8.1 software (Northampton, MA).

Mass determination for Me-HOPDAs. The Me-HOPDAs, prepared as described above, were treated with 1% (v/v) glacial acetic acid and the heterogeneous solutions were subjected to filtration through 0.22 µm syringe-driven filter prior to MS analysis. 4-Me-HOPDA was treated with

0.1 M ammonium carbonate prior to acidification. The samples were resolved on an Agilent 1100 series HPLC unit equipped with an ACE Excel 2 C18-PFP (50 x 2.1 mm) column using a gradient of formic acid and acetonitrile. The mass spectra were measured using an Applied Biosystem Qstar mass spectrometer.

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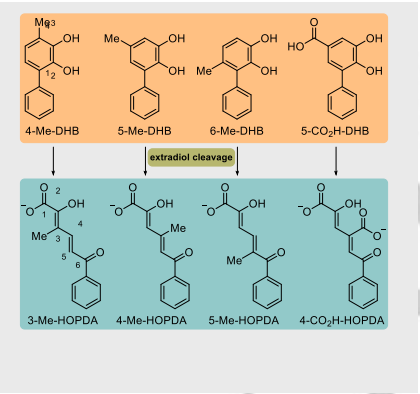
Entry for the Table of Contents

Layout 1:

FULL PAPER

A series of alkylated 2,3-dihydroxybiphenyls (DHBs) have been prepared on gram scale using an effective Directed *ortho* Metalation (DoM) – Suzuki-Miyaura coupling strategy. These compounds have been used to investigate the substrate specificity of the *meta*-cleavage dioxygenase BphC, a key enzyme in the microbial catabolism of biphenyl. Isolation of the *meta*-cleavage products will allow further study of the catabolism of lignin-derived biphenyls.



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