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Synthetic 6-aryl-2-hydroxy-6-ketohexa-2,4-dienoic acid substrates for C–C hydrolase BphD: investigation of a general base catalytic mechanism

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A chemical synthesis of the 2-hydroxy-6-ketohexa-2,4-dienoic acid intermediates on bacterial *meta*-cleavage pathways has been established, using a Heck coupling strategy. Coupling of ethyl 3-bromo-2-acetoxyacrylate with 1-aryl vinyl ketals or 1-aryl allylic alcohols proceeded in 70–90% yield. Heck coupling with an alkyl vinyl ketal was also successful, allowing the synthesis of an alkyl-substituted ring fission intermediate. The synthetic ring fission intermediates were used to investigate the enzymatic reaction catalysed by C–C hydrolase BphD from *Pseudomonas* LB400. A reduced substrate analogue 2,6-dihydroxy-6-phenylhexa-2,4-dienoic acid was processed enzymatically to benzaldehyde by C–C hydrolase BphD, consistent with a catalytic mechanism involving general base-catalysed attack of water to give a *gem*-diol intermediate, and not consistent with a nucleophilic mechanism. A series of *para*-substituted 2-hydroxy-6-keto-6-phenylhexa-2,4-dienoic acid substrates were assayed against BphD, and the derived Hammett plot ($\rho = -0.71$) is consistent with a departing carbanion in the transition state for C–C cleavage.

Introduction

The bacterial degradation of aromatic compounds by soil bacteria such as *Pseudomonas* and *Acinetobacter* commonly proceeds *via* aerobic pathways, involving the oxidative cleavage of catechol intermediates.[1] Intradiol oxidative cleavage of catechol, catalysed by non-haem iron(III) dependent catechol 1,2-dioxygenase, yields *cis*,*cis*-muconic acid ring cleavage products; whereas extradiol oxidative cleavage, catalysed by non-haem iron(II) dependent catechol 2,3-dioxygenase, yields 2-hydroxymuconic acid semialdehyde.[1] Extradiol cleavage of 3-substituted catechols likewise gives 6-substituted 2-hydroxy-6-ketohexa-2,4-dienoic acids, which have been produced enzymatically, but for which no synthetic route has been reported (see Fig. 1).

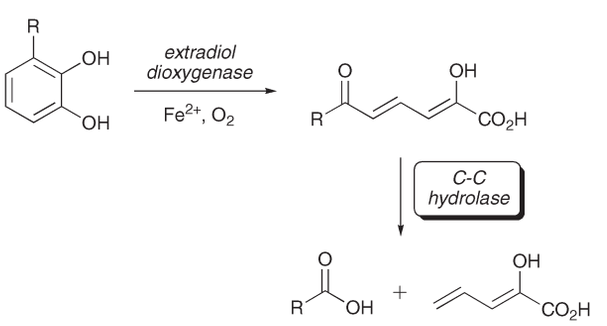

Fig. 1 Bacterial *meta*-cleavage pathway for degradation of 3-substituted catechols (R = H, CH_3, $CH_2CH_2CO_2H$, Ph), *via* extradiol catechol oxidative cleavage, to give a 6-substituted 2-hydroxy-6-ketohexa-2,4-dienoic acid, followed by C–C hydrolytic cleavage.

We have previously reported the isolation and chemical characterisation of 2-hydroxy-6-ketonona-2,4-dienoic acid, the *meta*-ring fission product on the phenylpropionate catabolic pathway of *Escherichia coli*, produced *via* enzymatic ring cleavage.[2] This compound was found to exist in the *trans*,*transoid* conformation, and was observed only as the dienol tautomer.[2] We have also recently reported a model reaction for extradiol oxidative cleavage, involving an iron(II) complex of 1,4,7-triazacyclononane, which yields the methyl ester of the *meta*-cleavage product.[3]

This family of *meta*-ring fission products are substrates for a hydrolytic C–C cleavage reaction, catalysed by C–C hydrolases that belong to the αβ-hydrolase family (see Fig. 1).[4] Crystallographic studies on *Rhodococcus* sp. RHA1 C–C hydrolase BphD have revealed that this family of enzymes contain a serine–histidine–aspartate triad at their active site.[5] The existence of a keto-intermediate, formed by the initial keto/enol tautomerisation of the dienol substrate (see Fig. 2), has been verified by pre-steady state kinetics and by isotope exchange studies on hydrolase MhpC from *Escherichia coli*.[6,7] Cleavage of the C–C bond could proceed either by nucleophilic attack of the active site serine, followed by C–C fragmentation to give an acyl enzyme intermediate, or by base-catalysed attack of water to give a *gem*-diol intermediate, as shown in Fig. 2. Despite the presence of the serine triad at the active site of these enzymes, repeated attempts to trap a covalent acyl enzyme intermediate using a ^{14}C-labelled substrate yielded very low stoichiometries (<1%) of enzyme-bound ^{14}C label.[8] In contrast, ^{18}O incorporation experiments have demonstrated 4–6% incorporation of 2 atoms of ^{18}O from $H_2{}^{18}O$ in the enzyme-catalysed reaction, and have shown that MhpC catalyses the exchange of ^{18}O into the C-4 ketone of a non-cleavable substrate analogue.[8] These data are consistent with the formation of a *gem*-diol intermediate in the MhpC-catalysed reaction, arising from a general base mechanism.

The dienol substrates for these enzymes have previously been available only by enzymatic[2] or biomimetic[3] catechol oxidative cleavage. In this paper we report the investigation of two retrosynthetic disconnections of this target molecule (see Fig. 3), and the first chemical synthesis of this family of compounds. Disconnection of the 5,6-bond (A) requires the γ-alkylation of a silyl dienol ether with an acylium or oxonium ion equivalent, while disconnection of the 3,4-bond (B) requires the Heck coupling of a bromoenol acetate with an α,β-unsaturated ketone. We then describe the application of these synthetic compounds to investigate the catalytic mechanism of *Pseudomonas* sp. LB400 C–C hydrolase BphD, found on the biphenyl degradative pathway. A communication describing a part of this work has previously been published.[9]

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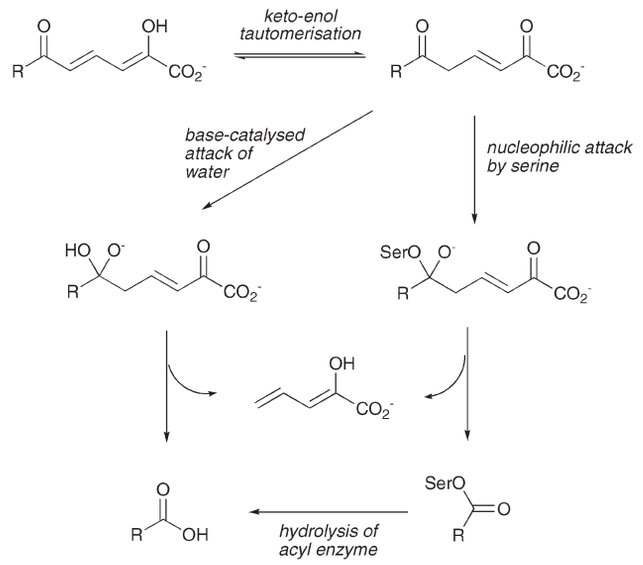


Fig. 2 Nucleophilic and general base mechanisms for C–C hydrolases BphD (R = Ph) and MhpC (R = $CH_2CH_2CO_2H$).

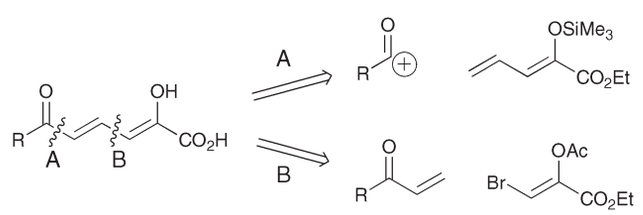


Fig. 3 Retrosynthetic disconnections A and B for *meta*-ring fission product.

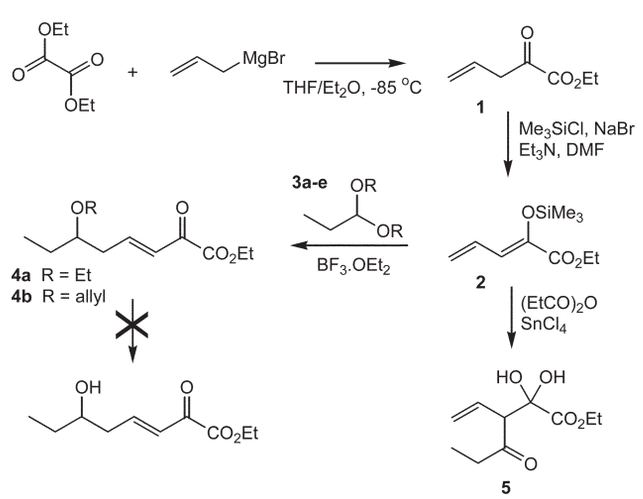
Results

Lewis acid-catalysed coupling of a silyl dienol ether (disconnection A)

Disconnection A (see Fig. 3) requires the γ-alkylation of a dienol ether substrate by an acylium ion equivalent. The selective γ-alkylation of trimethylsilyl dienol ethers, catalysed by Lewis acids, has previously been reported by Fleming and Lee.[10] Thus, silyl dienol ether **2** was prepared, by reaction of diethyl oxalate with allyl magnesium bromide at −90 °C in diethyl ether/tetrahydrofuran (1 : 1),[11] to give ester **1**, followed by treatment with trimethylsilyl bromide and triethylamine in anhydrous dimethylformamide, in 66% overall yield (see Scheme 1).

Reaction of silyl dienol ether **2** with propionaldehyde diethyl acetal (**3a**) in the presence of $BF_3.OEt_2$ was found to give the γ-alkylation product **4a**, in 33% yield, with none of the α-alkylation product observed. Reaction of **2** with acylium ion equivalents was found to give none of the desired γ-acylation product, but in two cases the α-acylation product **5**, containing a hydrated ketone (δ_C 137.5 ppm), was isolated: by reaction of **2** with propionic anhydride in the presence of $SnCl_4$ (34% yield), or by reaction with propionyl chloride in the presence of $FeCl_3$ (30% yield).

The γ-alkylation of **2** in the presence of $BF_3{\cdot}OEt_2$ was investigated further using several additional acetals of propionaldehyde (**3b–e**), which could be subjected to subsequent deprotection. Only in the case of the diallyl acetal **3b** was the γ-alkylation product **4b** obtained, in 26% yield, in the presence of $BF_3{\cdot}OEt_2$. Unfortunately, deprotection of the allyl protecting group of **4b** proved unsuccessful using a range of Pd^0, Rh^I, or Ir^I catalysts, giving either no reaction, or decomposition of **4b**.

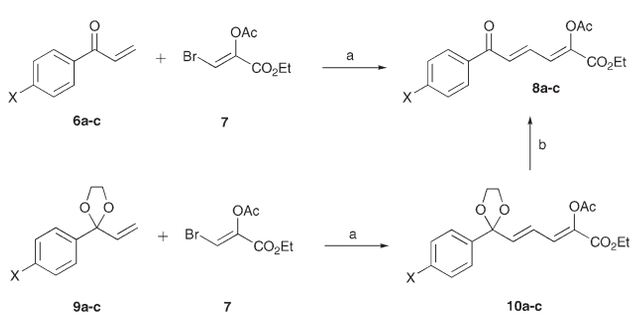


Scheme 1 Synthetic route based on disconnection A, *via* γ-alkylation of silyl enol ether **2**. Acetals **3a–e**: **3a**, R = Et; **3b**, R = allyl; **3c**, R = *p*-nitrobenzyl; **3d**, R = CH_2CCl_3; **3e**, R = $COCH_3$.

Heck coupling of bromoenol acetate to α,β-unsaturated ketone equivalents (disconnection B)

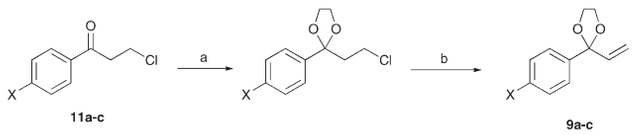
The palladium-catalysed Heck coupling of alkenyl halides with a range of alkene acceptors, including α,β-unsaturated ketones, has been used to synthesise a wide range of dienes.[12] Disconnection of the diene portion of the target molecule, namely the 3,4 bond, requires the coupling of phenyl vinyl ketone (**6**) with a bromoenol acetate (**7**).

Bromoalkene **7** was synthesised from ethyl 3-bromopyruvate by reaction with acetic anhydride.[13] Palladium-catalysed couplings of **7** with tolyl vinyl ketone **6b** were attempted under a variety of conditions, however the optimum yield of the coupled product **8b** using palladium(II) acetate was only 10% (see Scheme 2). Therefore, alternative equivalents for the alkene acceptor were tested. Tolyl vinyl ketone **6b** could be converted into the corresponding ketal **9b** upon treatment with ethylene glycol and pyridinium tosylate,[14] in 67% yield. Treatment of bromide **7** with ketal **9b**, in the presence of palladium(II) acetate (0.2 equiv.) and silver(I) carbonate (1 equiv.), in refluxing THF for 16 h, cleanly gave the coupled ketal **10b** in 78% yield. Ketal **10b** was converted quantitatively to the desired ketone **8b** by treatment with pyridinium tosylate in acetone/HCl. ^{1}H NMR signals for the diene portion of **8b** were observed at 7.47, 7.24, and 7.06 ppm. The Heck coupling reaction appeared to take place selectively at the terminal β-carbon of alkene **9b**, since none of the regio-isomer arising from attack at the α-carbon was detected.



Scheme 2 Synthetic route based on disconnection B, *via* Heck diene coupling. X = H (**a**), CH_3 (**b**), OCH_3 (**c**). Reaction conditions: a. $Pd(OAc)_2$ (0.1 equiv.), Ag_2CO_3, toluene, 80 °C; b. 2 M HCl/H_2O. Yields described in text.

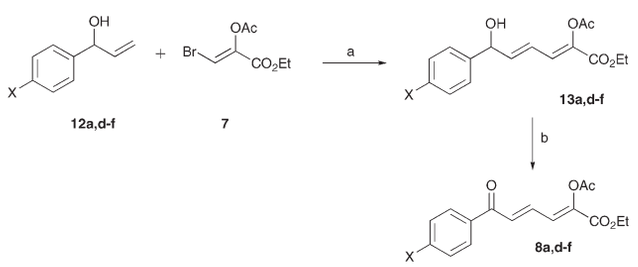
Synthesis of other *para*-substituted ketals **9a–f** directly from vinyl ketones **6a–f** was found to be problematic, therefore an alternative route was developed, as shown in Scheme 3. Friedel–Crafts acylation of benzene with 3-chloropropionyl chloride, in the presence of $AlCl_3$, gave aryl ketone **11a**. Ketal formation with ethylene glycol and pyridinium tosylate, followed by elimination by treatment with triethylamine, gave ketal **9a** in 79% yield. Ketals **9b** (X = CH_3) and **9c** (X = OCH_3) were also synthesised as shown in Scheme 3, and were tested in



Scheme 3 Synthetic route for preparation of ketals **9a–c**. X = H (**a**), CH_3 (**b**), OCH_3 (**c**). Reaction conditions: a. ethylene glycol, pyridinium tosylate; b. Et_3N, 1,2-dichloroethane, reflux, 2 h. Yields described in text.

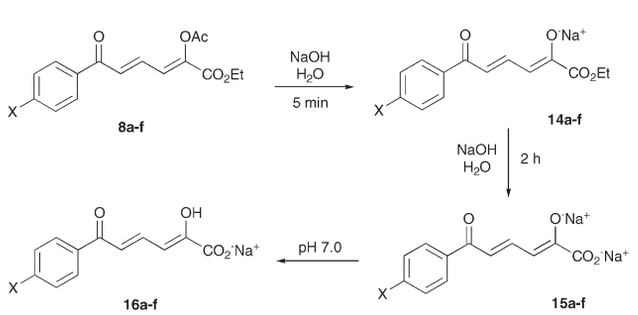
the Heck coupling. Reaction of ketals **9a** and **9c** with bromide **7** proceeded in 78% and 81% yield respectively.

Ketal formation as shown in Scheme 3 was unsuccessful for aryl ketones bearing electron-withdrawing substituents, hence the corresponding allylic alcohols **12** were also synthesised and tested under the Heck reaction conditions. Alcohols **12a** (X = H), **12d** (X = Cl), **12e** (X = CN) and **12f** (X = CF_3) were synthesised by reaction of the *para*-substituted benzaldehyde with allyl magnesium bromide, in 90–95% yield. Reaction of **12a** with bromide **7**, in the presence of palladium(II) acetate (0.1 equiv.) and silver(I) carbonate (2.5 equiv.), was found to give the desired alcohol **13a** in 87% yield, as shown in Scheme 4. Alcohol **13a** was oxidised to ketone **8a** using CrO_3/H_2SO_4 in 91% yield. Heck couplings of alcohols **12d–f** with bromide **7** also gave the desired diene as the major product, although in these cases other alkene by-products were visible in the crude reaction product by ^{1}H NMR spectroscopy. Oxidation of the coupled alcohol product to the corresponding ketone, followed by column chromatography, gave the desired ketones **8d** (X = Cl, 74%), **8e** (X = CN, 65%), and **8f** (X = CF_3, 69%) in good overall yield.



Scheme 4 Heck coupling of allylic alcohols **12a,d–f** with bromoalkene **7**. X = H (**a**), Cl (**d**), CN (**e**), CF_3 (**f**). Reaction conditions: a. $Pd(OAc)_2$ (0.1 equiv.), Ag_2CO_3, toluene, 80 °C; b. CrO_3, HCl. Yields described in text.

Hydrolysis of diester **8a** to the corresponding acid was carried out under aqueous alkaline conditions. Treatment of **8a** with aqueous sodium hydroxide gave within minutes the dienolate **14a** (λ_{max} 435 nm) arising from acetyl ester cleavage. Hydrolysis of the ethyl ester proceeded more slowly, but was complete after 2 h at room temperature, giving the disodium salt **15a** (λ_{max} 420 nm), which was neutralised to pH 7 and freeze-dried as the monosodium salt **16a**, as shown in Scheme 5. The ^{1}H NMR spectrum of **16a** showed signals for the diene portion at 7.07 (d, J = 11 Hz, H-3), 7.47 (dd, J = 15, 11 Hz) and



Scheme 5 Ester hydrolysis of **8a–f**. X = H (**a**), CH_3 (**b**), OCH_3 (**c**), Cl (**d**), CN (**e**), CF_3 (**f**).

7.23 ppm, indicating that **16a** exists in the *trans*,*transoid* conformation, as found previously for an enzymatically-generated ring fission intermediate.[2] Diesters **8b–f** were similarly deprotected by alkaline hydrolysis, and were stored as their mono-sodium salts **16b–f**.

BphD-catalysed conversion of *para*-substituted 2-hydroxy-6-keto-6-phenylhexa-2,4-dienoic acids

C–C hydrolase enzyme BphD from *Pseudomonas* sp. LB400 was overexpressed from *E. coli* BL21(DE3)[pLysS]/pAIA51 containing the *bphD* gene,[15] and was purified to >90% homogeneity (specific activity 6.4 units mg^{-1}). Treatment of a solution of **16a** in 50 mM potassium phosphate buffer pH 7.0 with BphD gave a linear decrease in absorbance at 430 nm, and an appearance of the 2-hydroxypentadienoic acid product at 270 nm, consistent with the BphD reaction (see Fig. 4).

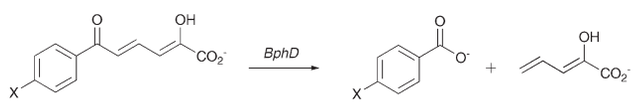


Fig. 4 Hydrolytic cleavage of substrates **16a–f** catalysed by C–C hydrolase BphD.

The series of *para*-substituted 2-hydroxy-6-keto-6-phenylhexa-2,4-dienoic acids (X = H, CH_3, OCH_3, Cl, CF_3, CN) was used to investigate of the effect of introducing electron-withdrawing or electron-donating substituents on the rate of the reaction catalysed by BphD.[15,16] Each of the substrates was processed by purified BphD, as judged by the disappearance of the substrate chromophore at 430 nm. In each case, the reaction products were monitored by HPLC, and the appropiate *para*-substituted benzoic acid was detected in each case. The extinction coefficient for each substrate was calculated by calibration of the *para*-substituted benzoic acid by HPLC upon 100% conversion, by comparison with stock solutions of authentic materials (see Table 1). The steady-state parameters for conversion of each substrate were then measured, using Eadie–Hofstee plots. The K_m and k_{cat} values are shown in Table 2. A plot of log(k_{cat}) *versus* Hammett coefficient σ, shown in Fig. 5, gives a slope ρ of −0.71.[17] The k_{cat} parameter was used for this plot (rather than k_{cat}/K_m), because the K_m values for BphD are extremely low (<1 μM),[16] giving rise to more scatter in plots involving k_{cat}/K_m.

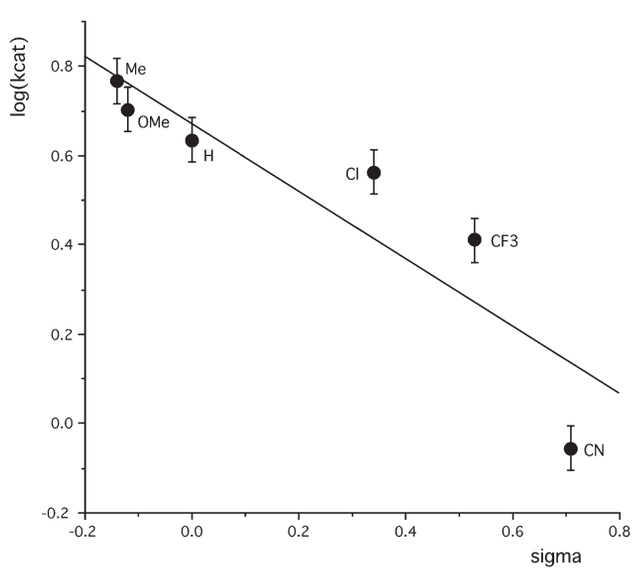


Fig. 5 Hammett plot of log(k_{cat}) *vs.* substituent coefficient σ for processing of substrates **16a–f** by C–C hydrolase BphD. Gradient of least squares fit line ρ = −0.71 ± 0.1.

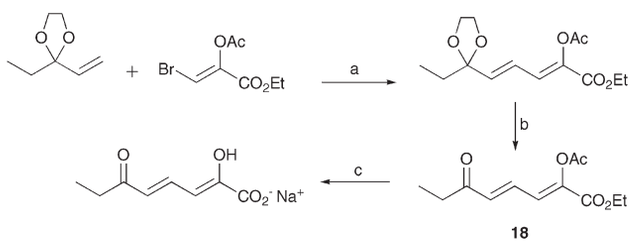
Table 1 Conversion of *para*-substituted BphD substrates **16a–f** to *para*-substituted benzoic acids by BphD, and calculation of molar extinction coefficients for **16a–f**

Substituent (X)	Retention time of product/min	Molar extinction coefficient/M^{-1} cm^{-1}
H	3.73	26,300
CH_3	3.12	25,900
OCH_3	3.03	25,500
Cl	4.05	26,700
CN	2.70	27,100
CF_3	3.08	26,700

Synthesis of 6-ethyl ring fission product using Heck coupling

Since most aromatic degradation pathways involve alkyl-substituted catechol intermediates,[1] it was of wider interest to examine whether this synthetic route could be used to prepare an alkyl-substituted ring fission product.

Heck reaction of bromoalkene **7** with the alkyl-substituted allylic alcohol 3-hydroxyhex-1-ene under the above reaction conditions gave none of the desired reaction product. Therefore, an alkyl-substituted vinyl ketal, 3-(ethylenedioxy)pent-1-ene (shown in Scheme 6), was prepared using a literature method,[18] and was reacted with **7** under the standard Heck reaction conditions. The reaction proceeded successfully to give the coupled product **17**, which was isolated in 42% yield, and was deprotected to give ketone **18** in 80% yield. The identity of the product was confirmed by alkaline hydrolysis to the corresponding acid, which showed identical UV/vis spectra to material generated enzymatically from 3-ethyl catechol (λ_{max} 390 nm), and which was processed efficiently as a substrate by C–C hydrolase MhpC from *Escherichia coli*.[8] This synthetic sequence indicates that the Heck coupling of **7** can be performed successfully using alkyl vinyl ketals.



Scheme 6 Heck coupling of an alkyl vinyl ketal. Reaction conditions: a. $Pd(OAc)_2$ (0.1 equiv.), Ag_2CO_3, toluene, 80 °C; b. 2 M HCl/H_2O; c. $NaOH/H_2O$. Yields described in text.

Assay of reduced substrate 2,6-dihydroxy-6-phenylhexa-2,4-dienoic acid

In view of our previous studies on C–C hydrolase MhpC,[6–8] it was of interest to examine whether C–C hydrolase BphD would accept a reduced substrate containing an alcohol at C-6, in place of a ketone (see Fig. 6). Enzymatic processing of **19** would be consistent with a general base catalytic mechanism, but not consistent with a nucleophilic mechanism (see Discussion).

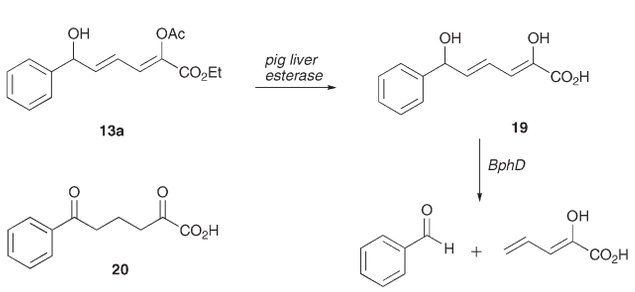


Fig. 6 Hydrolytic cleavage of reduced substrate **19** catalysed by C–C hydrolase BphD.

Table 2 Kinetic parameters measured for processing of substrates **16a–f** by C–C hydrolase BphD

Substrate (substituent X)	Substituent constant σ	K_m/μM	v_{max}/ΔA min^{-1}	k_{cat}/s^{-1}
16a (H)	0.00	0.19 ± 0.10	0.0745	4.31 ± 0.4
16b (CH_3)	−0.14	0.39 ± 0.2	0.0996	5.85 ± 0.5
16c (OCH_3)	−0.12	1.06 ± 0.5	0.0857	5.04 ± 0.5
16d (Cl)	+0.34	0.13 ± 0.07	0.0621	3.65 ± 0.4
16e (CN)	+0.71	0.71 ± 0.3	0.0149	0.88 ± 0.1
16f (CF_3)	+0.53	0.23 ± 0.1	0.0438	2.57 ± 0.3

Attempts to deprotect ethyl 2-acetoxy-6-hydroxy-6-phenylhexa-2,4-dienoate (**13a**) using aqueous sodium hydroxide or potassium carbonate gave none of the desired product **19**, the only characterised reaction product being 2,6-diketo-6-phenylhexanoic acid (**20**). The formation of **20** under basic conditions can be rationalised by deprotonation at C-6, followed by re-protonation at C-4, and tautomerisation to the diketo tautomer. In order to avoid alkaline conditions, enzymatic hydrolysis of **13a** was attempted. Treatment of **13a** with 5 units of pig liver esterase in 50 mM potassium phosphate buffer pH 7.0, followed by acidification and extraction, gave a product containing **19** as judged by ^1H NMR spectroscopic analysis (δ_H 7.29, 6.97, 6.72, 6.41, 5.19 ppm), but attempts to purify **19** were unsuccessful.

Therefore, the enzymatic processing of **19** by C–C hydrolase BphD was tested by addition of 1 unit of BphD to the PLE-catalysed hydrolysis of **13a** to **19**. Aliquots of the reaction mixture were removed during the reaction, the products extracted into chloroform, and the chloroform extract analysed by GC-MS for the appearance of the cleavage product, benzaldehyde (see Fig. 6). A new peak of *m/z* 106 was observed from incubations containing BphD, which matched authentic samples of benzaldehyde. The benzaldehyde peak increased in size with increasing incubation time, and control incubations lacking either pig liver esterase or BphD showed no formation of benzaldehyde by GC-MS. By calibration against authentic benzaldehyde, the k_{cat} for production of benzaldehyde was calculated to be 0.043 s^{-1}.

Discussion

In this paper we describe the first chemical synthesis of the ring fission intermediates on bacterial *meta*-cleavage pathways, using a Heck coupling strategy. It is noteworthy that the Heck coupling of bromide with the α,β-unsaturated ketone **6** proceeded in <10% yield, whereas much higher yields (70–90%) were obtained using the ketals **9a–f** or the allylic alcohols **12a–f**. Higher regio-selectivity in coupling was observed using the ketals **9a–f**, perhaps due to the steric bulk of the ketal substituent. Coupling of alkyl-substituted reagents was successful using a ketal alkene substrate, but not using an allylic alcohol substrate. This synthetic route is therefore amenable to the synthesis of aryl- and alkyl-containing *meta*-ring fission intermediates. This route could also be used to synthesise intermediates found on the degradation pathways of xenobiotics such as polychlorinated biphenyls, and could be used to access isotopically labelled derivatives.

Previous investigations of the catalytic mechanism of the C–C hydrolase family of enzymes have focussed upon nucleophilic *vs.* general base mechanisms of catalysis.[6–8] The amino acid sequence and structure of these enzymes clearly demonstrates that they are members of the αβ-hydrolase family, containing a serine–histidine–aspartate triad at their active sites. The normal function of Ser-110 in such a catalytic triad would be to act as a nucleophile, forming an acyl enzyme intermediate. Surprisingly, previous studies have failed to implicate such an intermediate; moreover, evidence has been obtained from ^{18}O isotope

exchange experiments, consistent with a *gem*-diol intermediate arising from base catalysed attack of water.[8]

In this paper we have shown that hydrolase BphD catalyses the C–C cleavage of reduced substrate **19** to give benzaldehyde, in a time-dependent fashion. The k_{cat} for benzaldehyde production is approximately 1% of k_{cat} for turnover of the natural substrate by BphD. The processing of a reduced substrate is consistent with a general base mechanism of catalysis, illustrated in Fig. 7, since the intermediate *gem*-diol would be replaced with an alcohol functional group, which is able to undergo retro-aldol cleavage at a reduced rate. This observation is not, however, consistent with a nucleophilic mechanism, since the reduced substrate **19** contains no incipient carbonyl group for attack by Ser-110, and would therefore not be able to access the oxyanion intermediate required for reaction. The enzyme-catalysed processing of analogue **19** therefore provides a further piece of experimental evidence in favour of a general base mechanism for C–C cleavage. We note that there may be enantioselectivity (at C-6) in the PLE-catalysed hydrolysis of **13a** to **19**, and especially in the BphD-catalysed processing of **19**, but that the chemical instability of **19** has precluded an analysis of this detail.

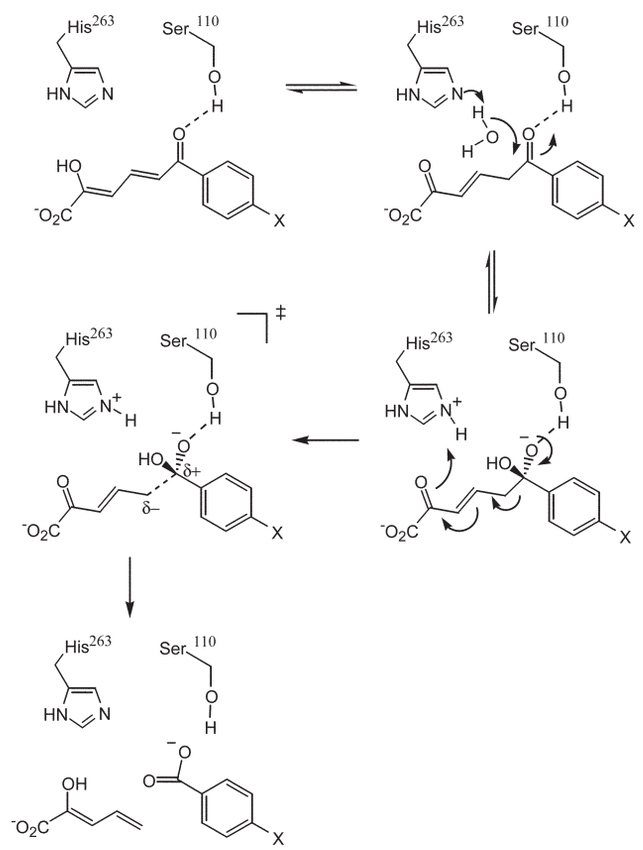


Fig. 7 Proposed non-nucleophilic mechanism for C–C hydrolase BphD, illustrating a dissociative transition state for C–C bond cleavage.

The availability of a synthetic route to this class of compounds has enabled us to synthesise a range of *para*-substituted derivatives, in order to carry out a Hammett analysis of the enzyme-catalysed reaction. A plot of $\log(k_{cat})$ *versus* Hammett coefficient σ (see Fig. 5) gives a reaction coeffcient ρ of −0.71, indicating that the reaction is favoured by electron-donating substituents. The two larger substituents OCH_3 and CN both give relatively low values of k_{cat}, probably due to steric effects upon substrate binding. A negaitive value of ρ is consistent with a mechanism involving C–C cleavage of a *gem*-diol intermediate, where the departing carbon atom (C-5) bears a δ− charge (stabilised by the adjacent α,β-unsaturated ketone), and C-6 bears a δ+ charge. Hammett coefficients of +1.8 and +0.9 have been measured for serine hydrolases α-chymotrypsin[19] and subtilisin,[20] which proceed *via* a nucleophilic mechanism, using *p*-substituted phenyl acetates as substrates. The opposite sign of ρ is further confirmation of a difference in mechanism from the serine hydrolases. For comparison, ρ values for base-catalysed and acid-catalysed ester hydrolysis are +2.55 and −0.57, respectively.[17]

Several experimental data now favour a non-nucleophilic mechanism for this class of C–C hydrolases: 1) the very low stoichiometry of trapping of a covalent intermediate in the MhpC-catalysed reaction; 2) the catalytic activity of MhpC at pH 4–5; 3) ^{18}O exchange from $H_2{}^{18}O$ catalysed by MhpC; 4) processing of a reduced substrate analogue by BphD; 5) observation of a negative ρ value for processing of *p*-substituted substrates by BphD. The implication is therefore that the role of the active site serine residue is not to act as a nucleophile in these C–C hydrolases: a fundamental difference in mechanism from the serine hydrolases. There are other reports in the literature that indicate alternative roles for the active site serine in other αβ-hydrolase enzymes. A general base mechanism has been proposed for hydroxynitrile lyase from *Hevea brasiliensis*, based on high resolution crystal structures.[21] Aldolase activity has been reported for a S105A mutant of lipase CALB from *Candida antarctica*.[22] Structure determination of *E. coli* MhpC is currently in progress, and preliminary analysis reveals a longer distance between catalytic residues His-263 and Ser-110, consistent with the proposed mechanism;[23] details will be published in due course.

Experimental

Materials

Propionaldehyde diallyl acetal (**3b**) was prepared from propionaldehyde, allyl alcohol and $CaCl_2$, as described by Mutterer *et al.*[24] Propionaldehyde di(nitrobenzyl) acetal (**3c**) and propionaldehyde di(trichloroethyl) acetal (**3d**) were prepared from propionaldehyde, the requisite alcohol, and toluenesulfonic acid, using the method of Schilling *et al.*[25] 1,1-Diacetoxypropane (**3e**) was prepared from propionaldehyde, acetic anhydride, and $FeCl_3$, using the method of Kochbar *et al.*[26] Ethyl 2-acetyloxy-3-bromo-2-propenonate (**7**) was prepared by the method of Fryzuk *et al.*[13] 3-Chloro-1-phenylpropan-1-ones (**11a–c**) were prepared by reaction of 3-chloropropionyl chloride and aluminium trichloride with the appropriate monosubstituted benzene, and were isolated in 75–82% yield. Phenyl vinyl ketone (**6**) was prepared by elimination of 3-chloro-1-phenylpropan-1-one (**11a**) in the presence of triethylamine, in 92% yield. 1-Phenylprop-2-en-1-ols (**12a,d–f**) were prepared by reaction of vinyl magnesium bromide with the appropriate *p*-substituted benzaldehyde, in tetrahydrofuran, and were isolated in 75–95% yield. *E. coli* strain BL21(DE3)[pLysS]/pAIA51 was a gift from Dr Bernd Hofer (GBF, Braunschweig). All other chemicals and biochemicals were purchased from Sigma-Aldrich.

Preparation of ethyl 2-ketopent-4-enoate (1)

A solution of diethyl oxalate (3.0 mL, 60 mmol) in anhydrous tetrahydrofuran/diethyl ether (1 : 1, 240 mL), under a nitrogen atmosphere, was cooled to −85 °C. Allyl magnesium bromide (60 mL of 1.0 M solution in diethyl ether) was added dropwise, and the reaction was stirred for a further 30 min at −85 °C. A further aliquot of allyl magnesium bromide (15 mL) was added, and the reaction stirred for a further 10 min. The reaction was then warmed to −60 °C, and quenched by addition of 1 M H_2SO_4 (100 mL). The product was extracted into diethyl ether (2 × 100 mL), and the combined extracts washed with brine (2 × 100 mL), dried ($MgSO_4$), and evaporated under reduced pressure to give the product as a yellow oil (6.53 g, 97%). R_f 0.37 (85 : 15 petroleum ether/ethyl acetate); IR (liquid film) 3081(w), 2982(m), 1751(s), 1730(s), 1643(w) cm^{-1}; δ_H (300 MHz, $CDCl_3$) 6.77 (1H, dt, J = 17.4, 10.3, 10.3 Hz, H-4), 6.20 (1H, d, J = 10.3 Hz, H-3), 5.39 (1H, dq, J = 17.4, 2.1 Hz, H-5_Z), 5.23 (1H, dd, J = 10.3, 2.1, H-5_E), 4.30 (2H, q, J = 7.0 Hz, OCH_2CH_3), 1.33 (3H, t, J = 7.0 Hz, OCH_2CH_3) ppm; δ_C (75 MHz, $CDCl_3$)

165.7, 139.6, 129.9, 120.2, 112.4, 62.4, 13.9 ppm; *m/z* (EI) 142 (M^+, 5%).

Preparation of ethyl 2-(trimethylsiloxy)penta-2,4-dienoate (2)

To a solution of sodium bromide (2.4 g, 27 mmol) in distilled dimethylformamide (12 mL) was added distilled trimethylsilyl chloride (5.5 mL, 27 mmol), and the mixture stirred for 20 min at room temperature, forming a precipitate of sodium chloride. Ethyl 2-ketopent-4-enoate (**1**, 3.6 g, 25 mmol) and distilled triethylamine (3.8 mL, 27 mmol) were added, and the reaction stirred for 18 h at room temperature. The reaction mixture was added to pentane (90 mL), and the pentane layer washed with sat. sodium bicarbonate solution (3 × 40 mL), brine (40 mL), dried (Na_2SO_4), and evaporated under reduced pressure to give a yellow oil (3.62 g, 68%); IR (liquid film) 3087(w), 2979(s), 2957(s), 1720(s), 1629(s) cm^{-1}; δ_H (300 MHz, $CDCl_3$) 6.64 (2H, m, H-3 and H-4), 5.44 (1H, dd, J = 16.5, 2.0 Hz, H-5_E), 5.26 (1H, dd, J = 10.0, 2.0 Hz, H-5_Z), 4.24 (2H, q, J = 7.5 Hz, OCH_2CH_3), 1.33 (3H, t, J = 7.5 Hz, OCH_2CH_3), 0.25 (9H, s, $SiMe_3$) ppm; δ_C (75 MHz, $CDCl_3$) 165.1, 140.5, 130.5, 121.2, 120.8, 61.3, 14.4, 0.6 ppm; *m/z* (EI) 214 (M^+, 57%); HRMS calc. ($C_{10}H_{18}O_3Si$) 214.1025, obs. 214.1013.

Preparation of ethyl 2-keto-6-ethoxyocta-3-enoate (4a)

To a stirred solution of silyl dienol ether **2** (128 mg, 0.6 mmol) and propionaldehyde diethyl acetal (**3a**, 0.1 mL, 0.6 mmol) in anhydrous dichloromethane (5 mL) was added boron trifluoride etherate (2 drops). The mixture was stirred for 4 h at room temperature, then water (5 mL) was added. The dichloromethane layer was washed with water (3 × 5 mL), brine (5 mL), dried (Na_2SO_4), and evaporated under reduced pressure. The product was purified by silica column chromatography, eluting with 9 : 1 petroleum ether/ethyl acetate, to give **4a** as a yellow oil (46 mg, 33%). R_f 0.15 (9 : 1 petroleum ether/ethyl acetate); IR (liquid film) 2973(s), 2933(s), 2876(s), 1737(s), 1701(m), 1678(m), 1624(m) cm^{-1}; δ_H (300 MHz, $CDCl_3$) 7.19 (1H, dt, J = 16.0, 7.5 Hz, H-4), 6.69 (1H, dt, J = 16.0, 1.5 Hz, H-3), 4.33 (2H, q, J = 7.0 Hz, $COOCH_2CH_3$), 3.49 (2H, q, J = 7.0 Hz, OCH_2CH_3), 3.35 (1H, qui, J = 6.0 Hz, H-6), 2.47 (2H, td, J = 7.5, 1.5 Hz, H-5), 1.51 (2H, qd, J = 7.5, 2.0 Hz, H-7), 1.36 (3H, t, J = 7.0 Hz, $COOCH_2CH_3$), 1.17 (3H, t, J = 7.0 Hz, OCH_2CH_3), 0.91 (3H, t, J = 7.5 Hz, H-8) ppm; δ_C (75 MHz, $CDCl_3$) 183.2, 162.3, 151.6, 126.8, 79.1, 62.3, 37.5, 27.0, 15.5, 14.0, 9.5 ppm; *m/z* (CI) 246 ($M + NH_4^+$, 6%), 229 (MH^+, 5%); HRMS calc. (MH^+, $C_{12}H_{21}O_4$) 229.1440, obs. 229.1408.

Preparation of ethyl 6-allyloxy-2-ketoocta-3-enoate (4b)

The same method was used to prepare **4b** from silyl dienol ether **2** and propionaldehyde diallyl acetal (**3b**). **4b** was isolated as a yellow oil after Kugelrohr distillation, in 26% yield. R_f 0.18 (85 : 15 petroleum ether/ethyl acetate); IR (liquid film) 3078(w), 2967(s), 2936(s), 2876(s), 1731(s), 1701(m), 1678(m), 1625(m) cm^{-1}; δ_H (300 MHz, $CDCl_3$) 7.20 (1H, dt, J = 16.0, 7.5 Hz, H-4), 6.70 (1H, td, J = 16.0, 1.5 Hz, H-3), 5.90 (1H, ddt, J = 16.5, 10.5, 5.5 Hz, allyl C-2), 5.27 (1H, ddd, J = 16.5, 3.0, 1.5 Hz, allyl C-3), 5.16 (1H, ddd, J = 10.5, 3.0, 1.5 Hz, allyl C-3), 4.35 (2H, q, J = 7.0 Hz, $COOCH_2CH_3$), 4.00 (2H, dt, J = 5.5, 1.5 Hz, allyl C-1), 3.44 (1H, qui, J = 6.0 Hz, H-6), 2.51 (2H, ddd, J = 7.5, 6.0, 1.5 Hz, H-5), 1.55 (2H, m, H-7), 1.38 (3H, t, J = 7.0 Hz, $COOCH_2CH_3$), 0.92 (3H, t, J = 7.5 Hz, H-8) ppm; δ_C (75 MHz, $CDCl_3$) 183.3, 162.4, 151.6, 135.0, 127.0, 117.1, 78.8, 70.4, 62.5, 37.5, 27.0, 14.2, 9.6 ppm; *m/z* (EI) 240 (M^+, 1%), 167 ($M - CO_2Et$, 5%); HRMS (CI) calc. (MH^+, $C_{13}H_{21}O_4$) 241.1440, obs. 241.1441.

Preparation of ethyl 2,2-dihydroxy-3-vinyl-4-ketohexanoate (5)

To a stirred solution of silyl dienol ether **2** (242 mg, 1.13 mmol) and propionic anhydride (0.15 mL, 1.13 mmol) in anhydrous dichloromethane (5 mL) was added tin(IV) tetrachloride (2 drops). The mixture was stirred for 2 h at room temperature, then water (10 mL) was added. The dichloromethane layer was washed with water (3 × 10 mL), brine (10 mL), dried (Na_2SO_4), and evaporated under reduced pressure. The product was purified by silica column chromatography, eluting with 85 : 15 petroleum ether/ethyl acetate, to give **5** as a yellow oil (81 mg, 34%). R_f 0.32 (85 : 15 petroleum ether/ethyl acetate); IR (liquid film) 2982(m), 2942(w), 1767(s), 1726(s), 1649(m) cm^{-1}; δ_H (300 MHz, $CDCl_3$) 6.91 (1H, d, J = 11.5 Hz, H-3), 6.51 (1H, ddd, J = 17.5, 11.5, 10.5 Hz, $CHCH{=}CH_2$), 5.63 (1H, ddd, J = 17.5, 1.5, 0.5 Hz, $CHCH{=}CH_2$), 5.47 (1H, ddd, J = 10.5, 1.5, 0.5 Hz, $CHCH{=}CH_2$), 4.25 (2H, q, J = 7.0 Hz, $COOCH_2CH_3$), 2.58 (2H, q, J = 7.5 Hz, H-5), 1.31 (3H, t, J = 7.0 Hz, $COOCH_2CH_3$), 1.23 (3H, t, J = 7.5 Hz, H-6) ppm; δ_C (75 MHz, $CDCl_3$) 172.4, 162.3, 137.5, 128.2, 128.2, 125.6, 61.7, 27.3, 14.3, 9.2 ppm; *m/z* (CI) 216 ($M - H_2O + NH_4^+$, 24%), 199 ($M - H_2O + H^+$, 100%); HRMS calc. ($[M - H_2O]^+$, $C_{10}H_{14}O_4$) 198.0892, obs. 198.0887.

General procedure for 2-phenyl-2-vinyl-[1,3]dioxolanes (9a–c)

To the ketone (**11a–c**) (10 mmol), dissolved in toluene (25 mL), was added ethylene glycol (2 mL, excess) and pyridinium tosylate (0.1 g, cat.). The solution was then refluxed under Dean–Stark conditions overnight. The solvent was removed under reduced pressure, and diethyl ether (20 mL) added. The solution was then washed with sat. sodium hydrogen carbonate solution (20 mL), and brine (20 mL). The organic layer was dried ($MgSO_4$), and the solvent removed under reduced pressure to give the crude ketal as a pale yellow oil. The ketal was dissolved in 1,2-dichloroethane (30 mL), and triethylamine (3 mL) was added dropwise. The reaction was then refluxed for 2 h before cooling to room temperature and quenching with water (25 mL). The aqueous layer was separated, extracted with dichloromethane (2 × 20 mL) and the organic layers were combined, dried ($MgSO_4$) and evaporated under reduced pressure. Purification by silica column chromatography (eluent 9 : 1 Et_2O/petroleum ether) gave **9** as a dark yellow oil.

2-Phenyl-2-vinyl-[1,3]dioxolane (9a, X = H). Yield 0.70 g (65%). δ_H (300 MHz, $CDCl_3$) 7.50 (2H, d, 2H, J = 8.0 Hz), 7.34 (3H, m), 6.01 (1H, dd, J = 16.5, 10.5 Hz), 5.33 (1H, dd, J = 16.5, 1.5 Hz), 5.21 (1H, dd, J = 10.5, 1.5 Hz), 3.99 (4H, m); δ_C (75 MHz, $CDCl_3$) 141.5, 138.5, 129.1, 128.5, 126.4, 116.9, 108.0, 65.1; *m/z* (EI) 176 (M^+); HRMS calc. ($C_{11}H_{12}O_2$) 176.0834, obs. 176.0837.

2-*p*-Tolyl-2-vinyl-[1,3]dioxolane (9b, X = CH_3). Yield 0.83 g (73%). δ_H (300 MHz, $CDCl_3$) 7.29 (2H, d, J = 8.2 Hz), 7.09 (2H, d, J = 8.2 Hz), 5.95 (1H, dd, J = 16.5, 10.5 Hz), 5.25 (1H, dd, J = 16.5, 1.5 Hz), 5.14 (1H, dd, J = 10.5, 1.5 Hz), 3.95 (4H, m), 2.27 (3H, s); δ_C (75 MHz, $CDCl_3$) 141.5, 138.5, 132.7, 129.3, 128.7, 116.8, 108.1, 65.1, 20.1; *m/z* (CI) 191 (MH^+); HRMS calc. ($C_{12}H_{15}O_2$) 191.1068 obs. 191.1070.

2-*p*-Methoxyphenyl-2-vinyl-[1,3]dioxolane (9c, X = OCH_3). Yield 0.84 g (68%). δ_H (300 MHz, $CDCl_3$) 7.38 (2H, d, J = 8.0 Hz), 6.83 (2H, d, J = 8.0 Hz), 6.01 (1H, dd, J = 16.0, 11.0 Hz), 5.32 (1H, dd, J = 16.0, 1.5 Hz), 5.23 (1H, dd, J = 11.0, 1.5 Hz), 3.99 (4H, m), 3.78 (3H, s); δ_C (75 MHz, $CDCl_3$) 162.8, 138.7, 138.5, 129.7, 116.8, 113.5, 108.0, 65.2, 64.0; *m/z* (EI) 206 (M^+); HRMS calc. ($C_{12}H_{14}O_3$) 206.0939, obs. 206.0943.

General procedures for ethyl 2-acetoxy-6-oxo-6-phenylhexa-2,4-dienoates (8a–f)

Method A (for 8a–c, by Heck reaction of vinyl ketal 9)

To ethyl 2-acetoxy-3-bromo-2-propenonate (**7**) (50 mg, 0.25 mmol) under nitrogen was added dropwise a solution containing palladium acetate (10 mg, 0.2 equiv.) and silver(I)

carbonate (78 mg, 1 equiv.) in freshly distilled THF (10 mL). To this was then added the vinyl ketal (**9a–c**) (0.2 mmol) in THF (5 mL). The reaction was stirred at room temperature for 1 h, before refluxing overnight. The solution was then cooled to room temperature, the solution diluted with diethyl ether and filtered through Celite. The organic layer was washed with brine (20 mL) and the products extracted into ethyl acetate (3 × 20 mL). The combined extracts were dried ($MgSO_4$), and concentrated under reduced pressure, to give **10** as a dark orange oil. Data for **10a**: δ_H (300 MHz, $CDCl_3$) 7.49 (2H, dd, J = 8.5, 1.3 Hz), 7.35 (3H, m), 6.95 (1H, d, J = 11.0 Hz, H-5), 6.54 (1H, dd, J = 16.5, 11.0 Hz, H-4), 6.20 (1H, d, J = 16.5 Hz, H-3), 4.22 (2H, q, J = 8.2 Hz), 4.01 (4H, m), 2.26 (3H, s), 1.28 (3H, t, J = 8.2 Hz); *m/z* (EI) 332 (M^+); HRMS calc. ($C_{18}H_{20}O_6$) 332.1254, obs. 332.1260. To the ketal (**10a–c**) (0.1 mmol) in acetone (5 mL) was added pyridinium tosylate (0.01 g, cat) and conc. sulfuric acid (0.1 mL) and the reaction stirred vigorously for 1 h. The reaction was quenched by the addition of water (15 mL). The aqueous layer was separated, extracted with diethyl ether (2 × 5 mL) and the organic layers combined, further washed with water (2 × 15 mL), dried ($MgSO_4$) and the solvent removed under reduced pressure. Purification by silica column chromatography (eluent 20% EtOAc/petroleum ether) gave **8a–c** as dark orange oils.

Method B (for 8a,d–f, by Heck reaction of allylic alcohol 12)

Ethyl 2-acetoxy-3-bromo-2-propenonate (**7**) (50 mg, 0.25 mmol) was dissolved in THF (5 mL) to which was added palladium acetate (22 mg, 0.06 mmol), allylic alcohol (**12**) (0.2 mmol) and silver(I) carbonate (276 mg, 1 mmol). The reaction was then stirred for 1 h at room temperature and then at reflux overnight. The reaction was then diluted with ether (10 mL) and filtered through a Celite pad. The products were washed with water (2 × 20 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure to give ethyl 2-acetoxy-6-hydroxy-6-phenylhexa-2,4-dienoate **13** as a dark yellow/orange oil. Data for **13a**: δ_H (300 MHz, $CDCl_3$) 7.32 (5H, s), 6.95 (1H, d, J = 11.1 Hz, H-3), 6.53 (1H, ddd, J = 15.3, 11.3, 1.5 Hz, H-4), 6.22 (1H, dd, J = 15.2, 5.6 Hz, H-5), 5.27 (1H, dd, J = 5.5, 1.5 Hz, H-6), 4.21 (2H, q, J = 7.1 Hz), 2.25 (3H, s), 1.27 (3H, t, J = 7.1 Hz); δ_C (75 MHz, $CDCl_3$) 168.8, 161.9, 143.6, 141.5, 136.9, 128.5, 127.9, 127.3, 126.3, 120.9, 74.0, 61.4, 20.1, 13.9; *m/z* (EI) 290 (M^+); HRMS calc. ($C_{16}H_{18}O_5$) 290.1150, obs. 290.1155. To the alcohol (**13a,d–f**) (0.1 mmol) dissolved in diethyl ether/acetone (1 : 1, 5 mL) was added water (5 mL) then chromium trioxide (0.2 g, excess) and conc. sulfuric acid (0.1 mL). The reaction was then stirred at room temperature for 1 h. The reaction was then extracted into ether (20 mL) and washed with brine (2 × 20 mL). The organic layers were combined, dried ($MgSO_4$), and concentrated under reduced pressure. Purification by silica column chromatography (eluent 20% EtOAc/petroleum ether) gave **8a,d–f** as dark orange oils.

Ethyl 2-acetoxy-6-oxo-6-phenylhexa-2,4-dienoate (8a, X = H). Yield 26.8 mg (73%, method A). δ_H (300 MHz, $CDCl_3$) 7.97 (2H, d, J = 7.2 Hz, Ar *ortho*), 7.62 (1H, t, J = 7.2 Hz, Ar *para*), 7.56 (1H, dd, J = 15.2, 11.5 Hz, H-4), 7.49 (2H, t, J = 7.2 Hz, Ar *meta*), 7.30 (1H, d, J = 15.2 Hz, H-5), 7.14 (1H, d, J = 11.5 Hz, H-3), 4.20 (2H, q, J = 7.2 Hz), 2.25 (3H, s), 1.23 (3H, t, J = 7.2 Hz); δ_C (75 MHz, $CDCl_3$) 189.2, 168.4, 161.2, 142.5, 137.1, 134.2, 133.2, 130.8, 128.6, 128.3, 124.9, 61.4, 20.1, 13.9; *m/z* (CI) 289 (MH^+); HRMS calc. ($C_{16}H_{17}O_5$) 289.1071, obs. 289.1078.

Ethyl 2-acetoxy-6-oxo-6-(*p*-tolyl)hexa-2,4-dienoate (8b, X = CH_3). Yield 27.6 mg (72%, method A). δ_H (300 MHz, $CDCl_3$) 7.81 (2H, d, J = 7.2 Hz), 7.47 (1H, dd, J = 15.2, 11.5 Hz, H-4), 7.24 (2H, d, J = 7.2 Hz), 7.22 (1H, d, J = 15.2 Hz, H-5), 7.07 (1H, d, J = 11.5 Hz, H-3), 4.21 (2H, q, J = 7.2 Hz), 2.56 (3H, s), 2.26 (3H, s), 1.27 (3H, t, J = 7.2 Hz); δ_C (75 MHz, $CDCl_3$) 188.4, 168.4, 161.2, 144.0, 142.5, 137.1, 134.7, 130.8, 129.3, 128.7, 125.0, 61.4, 20.3, 19.8, 14.0; *m/z* (CI) 303 (MH^+); HRMS calc. ($C_{17}H_{19}O_5$) 303.1227, obs. 303.1233.

Ethyl 2-acetoxy-6-oxo-6-(*p*-methoxyphenyl)hexa-2,4-dienoate (8c, X = OCH_3). Yield 26.6 mg (68%, method A). δ_H (300 MHz, $CDCl_3$) 7.98 (2H, d, J = 7.2 Hz), 7.56 (1H, dd, J = 15.2, 11.5 Hz, H-4), 7.31 (2H, d, J = 15.2 Hz, H-5), 7.28 (1H, d, J = 11.5 Hz, H-3), 7.02 (2H, d, J = 7.2 Hz), 4.21 (2H, q, 2H, J = 7.2 Hz), 3.84 (3H, s), 2.29 (3H, s), 1.28 (3H, t, J = 7.2 Hz); δ_C (75 MHz, $CDCl_3$) 189.4, 168.4, 162.9, 160.9, 142.4, 137.6, 132.0, 130.7, 129.7, 124.8, 107.8, 65.2, 61.5, 20.2, 14.0; *m/z* (EI) 318 (M^+); HRMS calc. ($C_{17}H_{18}O_6$) 318.1098, obs. 318.1105.

Ethyl 2-acetoxy-6-oxo-6-(*p*-chlorophenyl)hexa-2,4-dienoate (8d, X = Cl). Yield 29.2 mg (74%, method B). δ_H (300 MHz, $CDCl_3$) 7.68 (2H, d, J = 7.2 Hz), 7.52 (3H, m), 7.28 (2H, d, J = 15.1 Hz, H-5), 7.14 (1H, d, J = 11.5 Hz, H-3), 4.20 (2H, q, J = 7.2 Hz), 2.28 (3H, s), 1.27 (3H, t, J = 7.2 Hz); δ_C (75 MHz, $CDCl_3$) 189.3, 168.5, 161.3, 143.6, 142.5, 135.8, 133.8, 130.6, 129.7, 128.9, 125.0, 61.5, 20.2, 14.0; *m/z* (EI) 322 (M^+); HRMS calc. ($C_{16}H_{15}ClO_5$) 322.0603, obs. 322.0621.

Ethyl2-acetoxy-6-oxo-6-(*p*-cyanophenyl)hexa-2,4-dienoate (8e, X = CN). Yield 25.6 mg (65%, method B). δ_H (300 MHz, $CDCl_3$) 7.98 (2H, d, J = 7.2 Hz), 7.64 (1H, dd, J = 15.0, 11.5 Hz, H-4), 7.53 (2H, d, J = 7.2 Hz), 7.36 (1H, d, J = 15.0 Hz, H-5), 7.23 (1H, d, J = 11.5 Hz, H-3), 4.22 (2H, q, J = 7.2 Hz), 2.26 (3H, s), 1.28 (3H, t, J = 7.2 Hz); δ_C (75 MHz, $CDCl_3$) 189.5, 168.6, 161.4, 143.5, 142.3, 136.5, 132.2, 130.5, 129.4, 128.5, 117.1, 61.5, 20.2, 14.0; *m/z* (EI) 313 (M^+); HRMS calc. ($C_{17}H_{15}NO_5$) 313.0946, obs. 313.0972.

Ethyl 2-acetoxy-6-oxo-6-(*p*-trifluoromethylphenyl)hexa-2,4-dienoate (8f, X = CF_3). Yield 30.6 mg (69%, method B). δ_H (300 MHz, $CDCl_3$) 8.12 (2H, d, J = 7.2 Hz), 7.61 (2H, d, J = 7.2 Hz), 7.41 (1H, dd, J = 15.0, 11.5 Hz, H-4), 7.31 (1H, d, J = 15.0 Hz, H-5), 7.08 (1H, d, J = 11.5 Hz, H-3), 4.22 (2H, q, J = 7.2 Hz), 2.25 (3H, s), 1.26 (3H, t, J = 7.2 Hz); δ_C (75 MHz, $CDCl_3$) 189.5, 168.6, 161.3, 142.5, 137.2, 135.8, 132.5, 130.5, 129.5, 129.3, 128.7, 119.3, 61.5, 20.3, 14.0; *m/z* (EI) 356 (M^+); HRMS calc. ($C_{17}H_{15}F_3O_5$) 356.0867, obs. 356.0874.

Ethyl 2-acetoxy-6-oxoocta-2,4-dienoate (18). This was prepared from 3-ethylenedioxypent-1-ene[18] using method A, in 34% overall yield. δ_H (300 MHz, $CDCl_3$) 7.28 (1H, dd, J = 15.0, 13.5 Hz, H-4), 7.02 (1H, d, J = 13.8 Hz, H-3), 6.48 (1H, d, J = 15.4 Hz, H-5), 4.31 (2H, q, J = 7.2 Hz), 2.66 (2H, t, J = 7.2 Hz), 2.35 (3H, s), 1.35 (3H, t, J = 7.2 Hz), 1.15 (3H, t, J = 7.2 Hz); δ_C (75 MHz, $CDCl_3$) 142.1, 134.4, 131.8, 124.7, 61.6, 30.0, 20.2, 13.9, 7.6 (carbonyls not seen); *m/z* (CI) 214 (MH^+); HRMS calc. ($C_{12}H_{17}O_5$) 214.1076, obs. 214.1074.

2-Hydroxy-6-oxo-6-arylhexa-2,4-dienoic acids (16a–f)

To the ethyl 2-acetoxy-6-oxo-6-(*p*-substituted phenyl)hexa-2,4-dienoate (**8a–f**) (0.05 mmol) dissolved in methanol (2 mL) was added 2 M sodium hydroxide (2 mL). The solution immediately turned orange and was rapidly stirred for 2 h. The organic layer was washed with sat. sodium bicarbonate (10 mL) and the aqueous layers acidified to pH 3 with 2 M HCl. The product were then extracted into ethyl acetate (2 × 5 mL), dried ($MgSO_4$) and concentrated under reduced pressure to give the acid **16a–f** as a yellow or orange solid.

2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (16a, X = H). Yield 26.8 mg (93%). δ_H (300 MHz, d_6-acetone) 8.05 (2H, d, J = 7.1 Hz), 7.90 (1H, dd, J = 15.2, 12.0 Hz, H-4), 7.65 (1H, t, J = 7.1 Hz), 7.55 (2H, t, J = 7.1 Hz), 7.40 (1H, d, J = 15.2 Hz, H-5), 6.55 (1H, d, J = 12.0 Hz, H-3); δ_C (75 MHz, d_6-acetone)

206.6, 172.5, 139.5, 138.3, 135.6, 134.0, 129.9, 129.8, 126.9, 110.2; *m/z* (CI) 218 (M^+); HRMS calc. ($C_{12}H_{10}O_4$) 218.0576, obs. 218.0572.

2-Hydroxy-6-oxo-6-(*p*-tolyl)hexa-2,4-dienoic acid (16b, X = CH_3). Yield 27.6 mg (91%). δ_H (300 MHz, d_6-acetone) 7.89 (2H, d, *J* = 7.0 Hz), 7.84 (1H, dd, *J* = 15.0, 12.0 Hz, H-4), 7.40 (1H, d, *J* = 15.0 Hz, H-5), 7.25 (2H, d, *J* = 7.0 Hz), 6.53 (1H, d, *J* = 12.0 Hz, H-3), 2.43 (3H, s); *m/z* (CI) 232 (M^+); HRMS calc. ($C_{13}H_{12}O_4$) 232.0736, obs. 232.0721.

2-Hydroxy-6-oxo-6-(*p*-methoxyphenyl)hexa-2,4-dienoic acid (16c, X = OCH_3). Yield 26.6 mg (84%). δ_H (300 MHz, d_6-acetone) 7.92 (2H, d, *J* = 7.0 Hz), 7.83 (1H, dd, *J* = 15.0, 12.0 Hz, H-4), 7.34 (1H, d, *J* = 15.0 Hz, H-5), 7.12 (2H, d, *J* = 7.0 Hz), 6.62 (1H, d, *J* = 12.0 Hz, H-3), 3.73 (3H, s); *m/z* (EI) 248 (M^+); HRMS calc. ($C_{13}H_{12}O_5$) 248.0681, obs. 248.0679.

2-Hydroxy-6-oxo-6-(*p*-chlorophenyl)hexa-2,4-dienoic acid (16d, X = Cl). Yield 29.2 mg (91%). δ_H (300 MHz, d_6-acetone) 7.83 (2H, d, *J* = 7.0 Hz), 7.74 (1H, dd, *J* = 15.0, 12.0 Hz, H-4), 7.49 (2H, d, *J* = 7.0 Hz), 7.34 (1H, d, *J* = 15.0 Hz, H-5), 6.91 (1H, d, *J* = 12.0 Hz, H-3); *m/z* (EI) 252/254 (M^+); HRMS calc. ($C_{12}H_9ClO_4$) 252.0186, obs. 252.0201.

2-Hydroxy-6-oxo-6-(*p*-cyanophenyl)hexa-2,4-dienoic acid (16e, X = CN). Yield 25.6 mg (82%). δ_H (300 MHz, d_6-acetone) 8.11 (2H, d, *J* = 7.0 Hz), 7.89 (1H, dd, *J* = 15.0, 12.0 Hz, H-4), 7.65 (2H, d, *J* = 7.0 Hz), 7.42 (1H, d, *J* = 15.0 Hz, H-5), 6.58 (1H, d, *J* = 12.0 Hz, H-3); *m/z* (EI) 243 (M^+).

2-Hydroxy-6-oxo-6-(*p*-trifluoromethylphenyl)hexa-2,4-dienoic acid (16f, X = CF_3). Yield 30.6 mg (86%). δ_H (300 MHz, d_6-acetone) 7.92 (2H, d, *J* = 7.0 Hz), 7.79 (1H, dd, *J* = 15.0, 12.0 Hz, H-4), 7.68 (2H, d, *J* = 7.0 Hz), 7.38 (1H, d, *J* = 15.0 Hz, H-5), 6.71 (1H, d, *J* = 12.0 Hz, H-3); *m/z* (EI) 286 (M^+); HRMS calc. ($C_{13}H_9F_3O_4$) 286.0450, obs. 286.0458.

Partial purification of BphD

E. coli BL21(DE3)[pLysS]/pAIA51 was grown in Luria Broth media (3.5 L) at 37 °C. Cells were induced with IPTG (0.12 mg mL^{-1}, 0.5 mM) at A600 = 0.7, then grown for 3 h at 37 °C. The cells were harvested by centrifugation for 8 min at 6000*g*, resuspended in 50 mM phosphate buffer pH 8.0 (100 mL), harvested by centrifugation for 8 min at 6000*g*. The cell pellet was resuspended in 50 mM phosphate buffer pH 8.0 (150 mL). Cell lysis was carried out by sonication (10 × 15 s), and cell debris was removed by centrifugation for 45 min at 100,000*g*. Powdered ammonium sulfate was slowly added to the crude extract to 35% saturation (197 g L^{-1}), the suspension stirred for 1 h at 5 °C and centrifuged for 30 min at 12,000*g*. To the supernatant was slowly added ammonium sulfate (221 g L^{-1}) to 75% saturation, the mixture stirred for 1 h at 4 °C and centrifuged for 30 min at 12,000*g*. The resulting pellet was resuspended in 50 mM phosphate buffer pH 8.0 (12 mL) and centrifuged for 5 min at 20,000*g* to remove any particulates before being loaded onto an FPLC Sephacryl S-200 gel-filtration column. The column was eluted at 0.5 mL min^{-1} with 50 mM phosphate buffer pH 8.0 and fractions containing hydrolase BphD activity were pooled and stored at 4 °C. The purified enzyme (total 89 units, 14 mg protein) had specific activity 6.4 units mg^{-1}.

Continuous UV assays of BphD

BphD was assayed by monitoring the decrease in absorbance at 434 nm due to the consumption of the ring fission product (ε_{max} = 26300 M^{-1} cm^{-1} for X = H (**16a**)[17]) in 50 mM potassium phosphate buffer (pH 8.0). Extinction coefficients of **16a–f** were determined by conversion with excess BphD to the corresponding *para*-substituted benzoic acid (see Table 1). K_m and k_{cat} values were determined using Eadie–Hofstee plots (see Table 2).

HPLC analysis of benzoic acid products

Benzoic acid (2.46 mg) was dissolved in methanol (10 mL) and analysed on a Phenomenex ODS semi-prep C_{18} reverse-phase HPLC column eluted with a gradient of 100% methanol to 100% water over 30 min (flow rate 0.5 mL min^{-1}). The retention time was 3.045 min. A standard curve for peak height *vs.* concentration was determined. 3.98 mg of substrate (**16a**) was then added to a solution containing BhpD (1 unit) and the reaction allowed to go to completion, as observed by the total loss of UV absorbance at 434 nm. The products of the enzyme assay were passed through a 0.2 μ filter. A 50 mL aliquot was analysed on the reverse-phase HPLC column under the same elution conditions as for the standard.

BphD-catalysed conversion of reduced substrate 19

A solution of allylic alcohol **13a** (5 mg) in methanol (2 mL) was added to 50 mM potassium phosphate buffer (pH 8.0; 8 mL), to which was added pig liver esterase (20 units). The solution was incubated for 2 h at 4 °C, generating the reduced substrate **19** (λ_{max} 340 nm). BphD (5 units) was added, and the solution incubated at 4 °C. Aliquots (1.0 mL) were withdrawn at 1 h intervals. Each aliquot was acidified to pH 3.0, and the product extracted with chloroform. A control experiment was performed in parallel, with no added BphD. The samples were analysed on a GC-17A gas chromatograph, linked to a QP-5000 mass spectrometer, using a temperature gradient of 50 °C to 250 °C over 25 min.

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