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Hammett analysis of a C–C hydrolase-catalysed reaction using synthetic 6-aryl-2-hydroxy-6-ketohexa-2,4-dienoic acid substrates

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A Hammett plot (ρ = −0.71) has been measured for C–C hydrolase enzyme BphD from *Pseudomonas LB400*, using six 6-aryl-2-hydroxy-6-ketohexa-2,4-dienoic acids synthesised by a Heck coupling strategy.

The bacterial degradation of aromatic compounds by soil bacteria such as *Pseudomonas spp.* commonly proceeds *via* the oxidative cleavage of catechol intermediates.[1] Extradiol catechol cleavage of 3-substituted catechols, catalysed by non-haem iron(II) dependent catechol 2,3-dioxygenase, yields 6-substituted 2-hydroxy-6-ketohexa-2,4-dienoic acids (see Fig. 1). NMR spectroscopic analysis has shown that 2-hydroxy-6-ketonona-2,4-dioic acid, the *meta* ring fission product on the phenylpropionate catabolic pathway of *Escherichia coli*, exists as the dienol tautomer in the *trans,transoid* conformation.[2]

This family of *meta*-ring fission products are substrates for a hydrolytic C–C cleavage reaction, catalysed by C–C hydrolases which belong to the α/β-hydrolase family (see Fig. 1).[3] Crystallographic studies on C–C hydrolase BphD have revealed that this family of enzymes contain a serine–histidine–aspartate triad at their active site,[4] yet mechanistic studies to date are not consistent with a nucleophilic mechanism, but instead favour a general base mechanism involving a *gem*-diol intermediate.[5–7] Studies on this unusual family of enzymes are hampered by the lack of a synthetic route for the dienol substrates. In this paper we report the first total synthesis of these biological intermediates, and the kinetic evaluation *via* a Hammett plot of a series of synthetic substrates for C–C hydrolase BphD.

Synthesis of the diene portion of the target molecule was attempted using the palladium-catalysed Heck coupling[8] of bromo-enol acetate **1**, prepared from ethyl 3-bromopyruvate.[9] Palladium-catalysed couplings of **1** with phenyl vinyl ketone gave < 10% yield of the coupled diene product. However, reaction of **1** with the corresponding ketal **2a** in the presence of palladium(II) acetate (0.1 equiv.), silver(I) acetate and triphenylphosphine at 80 °C cleanly gave the coupled ketal **3a** in 78% yield. Treatment with aqueous 2 M HCl gave the desired ketone **4a** in 93% yield. No other regioisomer was detected by NMR spectroscopy, thus reaction occurred selectively at the terminal β-carbon of alkene **2a**. Ketals **2b** (X = CH_3) and **2c** (X = OCH_3) were also coupled to give **4b** and **4c** in 76 and 68% yield, respectively, as shown in Scheme 1.

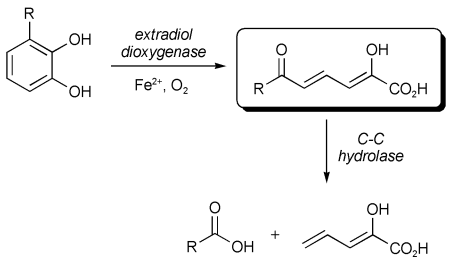


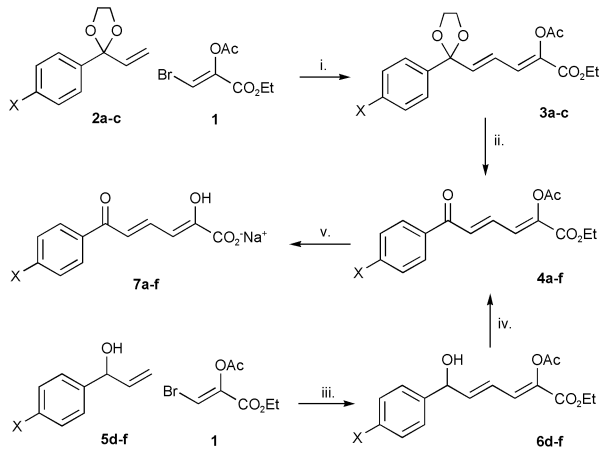
Fig. 1 Bacterial *meta*-cleavage pathway for degradation of 3-substituted catechols (R = H, CH_3, $CH_2CH_2CO_2H$, Ph), *via* extradiol catechol oxidative cleavage, to give a 6-substituted 2-hydroxy-6-ketohexa-2,4-dienoic acid, followed by C–C hydrolytic cleavage.

Aryl ketones containing electron-withdrawing substituents gave low yields of the corresponding ketal **2**. In these cases, Heck coupling was carried out using the corresponding allylic alcohols **5a** (X = H), **5d** (X = Cl), **5e** (X = NO_2) and **5f** (X = CN), synthesised by reaction of the *p*-substituted benzaldehyde with allyl magnesium bromide. Reaction of **5a** with bromide **1**, in the presence of $Pd(OAc)_2$/AgOAc/PPh_3, was found to give the desired alcohol **6a** as the major product, although other alkene by-products were also visible in the crude reaction product by ^{1}H NMR spectroscopy. The labile alcohol **6a** was oxidised to ketone **4a** using CrO_3/H_2SO_4, and isolated after chromatography in 69% overall yield. Alcohols **5d–f** were similarly coupled by this method, yielding the ketones **4d–f** in 70–80% yield.

Deprotection of di-esters **4a–f** was carried out by alkaline hydrolysis in aqueous sodium hydroxide for 2 h at room temperature, followed by neutralisation to pH 7 to give the products **7a–f** as their sodium salts in 80–90% yields. The ^{1}H NMR spectrum of **7b** showed signals for the diene portion at 7.07 (d, *J* 11 Hz, H-3), 7.47 (dd, *J* 15, 11 Hz, H-4) and 7.23 ppm, indicating that **7** exists in the *trans,transoid* (2-*Z*, 4-*E*) diene conformation, as found previously for an enzymatically-generated ring fission intermediate.[2]

C–C hydrolase BphD from the biphenyl degradation pathway of *Pseudomonas spp.* LB400[10,11] was purified from an overexpressing strain of *E. coli*, to specific activity 3.6 u mg^{-1}. Treatment of a solution of **7a** in 50 mM potassium phosphate buffer pH 7.0 with an aliquot of BphD gave a linear decrease in absorbance at 434 nm, confirming the identity of **7a**.

Steady-state kinetic parameters were measured for the processing of synthetic substrates **7a–f** by hydrolase BphD. A plot of log(k_{cat}) *vs.* substituent parameter σ (Fig. 2) shows a decreasing rate for electron-withdrawing substituents, with reaction constant ρ = −0.71 ± 0.1.† The two larger substituents



Scheme 1 Synthetic route for 6-aryl-2-hydroxy-6-ketohexa-2,4-dienoic acids. X = H (**a**), CH_3 (**b**), OCH_3 (**c**), Cl (**d**), CN (**e**), CF_3 (**f**). *Reagents and conditions*: i. $Pd(OAc)_2$ (0.1 equiv.), AgOAc, PPh_3, toluene, 80 °C; ii. 2 M HCl/H_2O; iii. $Pd(OAc)_2$ (0.1 equiv.), AgOAc, PPh_3, toluene, 80 °C; iv. CrO_3, H_2SO_4; v. NaOH, H_2O, 2 h. Yields described in text.

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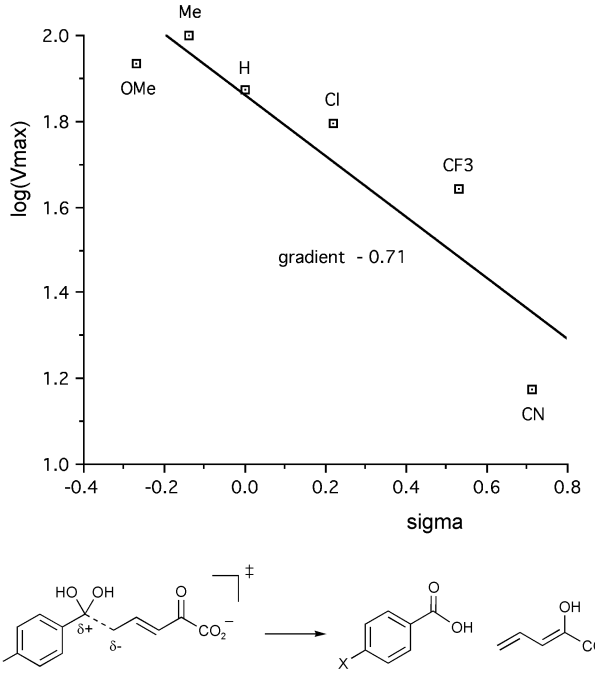

Fig. 2 Hammett plot of log(k_{cat}) *vs.* substituent coefficient σ for processing of substrates **7a**–**f** by C–C hydrolase BphD. Gradient of least squares fit line $\rho = -0.71 \pm 0.1$. The scheme shows likely transition state for C–C cleavage of a *gem*-diol reaction intermediate.

OCH_3 and CN both give relatively low values of k_{cat}, probably due to steric effects upon substrate binding, but comparison of isosteric CH_3 *vs.* CF_3 and OCH_3 *vs.* CN gives a consistent pattern. These data give some insight into the transition state for C–C bond cleavage during the enzymatic reaction, implying an accumulation of δ+ charge adjacent to the aryl ring. This is consistent with heterolytic cleavage of the C–C bond, and delocalisation of the departing δ− charge by the adjacent α,β-unsaturated ketone. This value is opposite in sign to the ρ values of +1.8 and +0.9 reported for serine proteases α-chymotrypsin[12] and subtilisin[13] respectively (using substituted phenyl acetates as substrates), which proceed *via* a nucleophilic mechanism. For comparison, ρ values for base-catalysed and acid-catalysed ester hydrolysis are +2.55 and −0.57, respectively.[14]

The availability of a synthetic route to this class of biological intermediates has made possible the synthesis of a range of substrates for C–C hydrolase BphD, and could be used to synthesise ring fission intermediates found on other aromatic *meta*-cleavage pathways.

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Notes and references

† The k_{cat} kinetic parameter was used, since values of K_m for this enzyme are extremely low (< 1 μM),[11] generating more experimental error in k_{cat}/K_m data.

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