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Biosynthesis of hydroxydiphenylacetylene by regiospecific monooxygenation†

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Bacterial monooxygenase enzymes catalyze a regiospecific single-step hydroxylation of diphenylacetylene to yield *meta*- and *para*-hydroxydiphenylacetylene.

Synthesis of high performance polymers and composites often requires feedstocks that are difficult to synthesize with traditional chemistry. Regio and stereospecific functionalization of aromatic compounds can be particularly challenging. Here we describe a biocatalytic approach to the production of two isomers of the endcapping agent, hydroxydiphenylacetylene (HDPA)[1] *via* single-step regioselective monooxygenation (Fig. 1). This type of endcap can be used in the formation of phenylethynyl terminated reactive oligomers, which have received much attention over the past decade due to their high thermal stability and excellent mechanical properties.[2] The biocatalytic route provides an alternative single-step method for synthesis and has the added advantage of being environmentally benign, in contrast to the harsh conditions and reagents used in conventional chemical synthesis.

Multicomponent dioxygenases can catalyze the conversion of aromatic substrates to *cis*-dihydrodiol intermediates which undergo dehydration to phenolic products.[3] For example, a mutant derived from a toluene-degrading organism accumulated a *cis*-dihydrodiol derivative of diphenylacetylene (DPA) which could be converted to *meta*-HDPA or *ortho*-HDPA by treatment with acid or base.[4] The *para*-HDPA isomer cannot be formed by the above method because of the 2,3-position of the initial hydroxylation.

Bacterial multicomponent monooxygenases are a family of nonheme, di-iron enzymes capable of using molecular oxygen to hydroxylate a variety of organic compounds that allow bacteria to use the compounds as the sole source of carbon and energy. Monooxygenase enzymes catalyze a single-step direct hydroxylation of aromatic rings, often in a highly regiospecific manner. The substrate range of many of the monooxygenases is broad and the enzymes will oxidize a wide range of aromatic and aliphatic compounds that do not support growth.[5]

We tested a variety of bacterial monooxygenase enzymes for the ability to catalyze the direct monooxygenation of DPA. The biotransformation of DPA was investigated with recombinant strains expressing various toluene monooxygenases; all are capable of transforming toluene to cresol, and the isomer obtained is enzyme-specific (Table 1). All of the recombinant strains tested catalyzed the transformation of DPA to HDPA with varying degrees of efficiency and regioselectivity. The isomer of HDPA could be correlated to the type of monooxygenase (Table 1). Toluene-4-monooxygenases catalyzed the formation of predominantly *meta*-HDPA. In contrast, toluene-2-monooxygenases formed predominantly *para*-HDPA. Initial experiments with recombinant organisms indicated that toluene monooxygenase enzymes can catalyze the hydroxylation of DPA, but the overall yields were low.

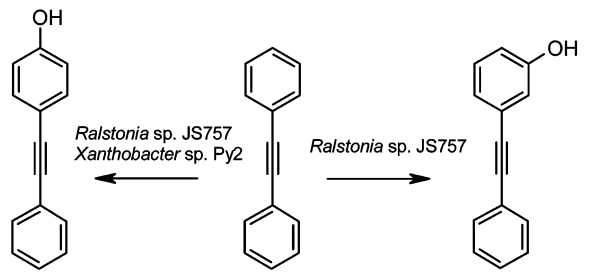

Fig. 1 Biosynthesis of *meta* and *para*-hydroxydiphenylacetylene.

A second strategy was pursued to obtain bacterial strains that catalyze higher DPA conversion rates and product yields. A collection of 226 toluene-degrading isolates[6] was screened for the ability to transform DPA; 39 produced HDPA. *Ralstonia pickettii* JS757[7] produced the highest yield. When grown with toluene as the sole carbon source, it converted 1 mM DPA to 0.41 (±0.022) mM *meta*-HDPA and 0.29 (±0.021) mM *para*-HDPA (Fig. 2A).†‡ The intial product formation rates (first 2.5 h) were 1.25 and 0.86 nmol min^{-1} g^{-1} cell dry weight for the *meta*- and *para*-isomers respectively. The turnover of substrate was not complete and some residual DPA was detected at the end of the reaction, traces of uncharacterized polar products also accumulated during the transformations.

We speculated that a monooxygenase enzyme catalyzed the monohydroxylation of DPA in strain JS757 and tested toluene-grown cells for the ability to oxidize intermediates of various toluene assimilation pathways. Oxygen uptake studies showed that strain JS757 rapidly oxidised intermediates of a toluene monooxygenase pathway (all cresol isomers) but did not oxidise intermediates of a dioxygenase pathway (toluene or benzene *cis*-dihydrodiol).[8]

The alkene monooxygenase from *Xanthobacter* strain Py2 is a soluble di-iron monooxygenase known to catalyze the oxidation of

Table 1 Screening of recombinant monooxygenase strains for conversion of diphenylacetylene

Enzyme function	Recombinant strain[9]	Product of toluene transformation	Product of DPA[b] transformation
Toluene-2-monooxygenase	*E. coli* TG1 (pTKW [TOM])	*o*-cresol	*para*-HDPA[b] (77%)[c]
	E. coli Top10F′ (pJS407) Tol-2-mo[a]	*o*-cresol	*para*-HDPA (52%)
Toluene-4-monooxygenase	*E. coli* TG1 (pTKW [T4MO])	*p*-cresol	*meta*-HDPA (64%)
	E. coli Top10F′ (pJS409) Tol-4-mo[a]	*p*-cresol	*meta*-HDPA (69%)
Tol/xylene monooxygenase	*E. coli* TG1 (pTKW [TOMO])	*o*-, *m*- and *p*-cresol	*para*-HDPA (69%)

[a] This study. See ESI [b] HDPA: hydroxydiphenylacetylene, DPA: diphenylacetylene. [c] Major product isomer shown, residual percentage is alternate isomer, either *meta*- or *para*- in all cases; *ortho*-HDPA was not detected.

† Electronic supplementary information (ESI) available: Fig. S1: ^{1}H and ^{13}C NMR data, Fig. S2: construction of expression plasmids pJS407 and pJS409. See http://www.rsc.org/suppdata/cc/b4/b408766f/

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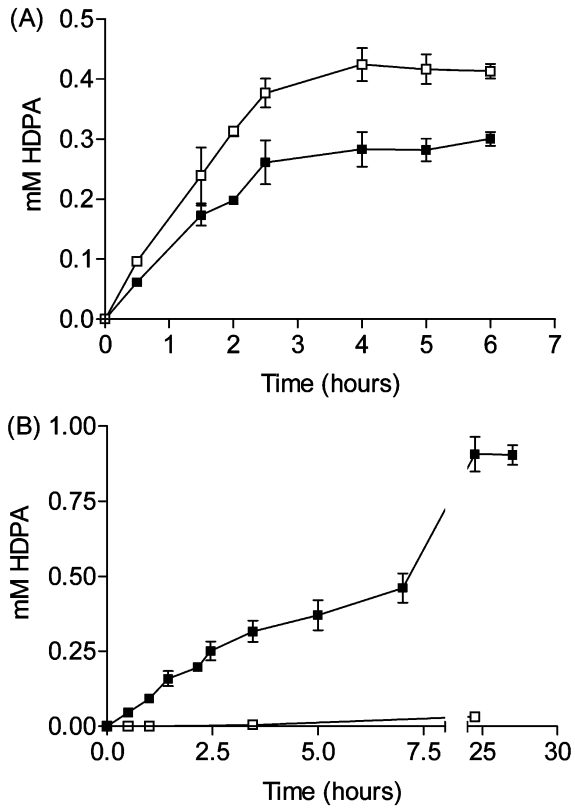

Fig. 2 Formation of hydroxydiphenylacetylene (HDPA) from diphenylacetylene by *Ralstonia pickettii* JS757 (A) and *Xanthobacter* strain Py2 (B), (□: *meta*-HDPA, ■: *para*-HDPA).

aromatic compounds including benzene, toluene and phenol,[10] but the transformation of more structurally complex substrates has not been reported. Propylene grown cells of *Xanthobacter* strain Py2 expressing the alkene monooxygenase catalyzed the formation of 0.9 mM (±0.054) of *para*-HDPA from 1 mM DPA (Fig. 2B).†‡ The initial product formation rate (first 2.5 h) was 1.90 nmol min^{-1} g^{-1} cell dry weight for the *para*- isomer. *meta*-HDPA was detected as a minor metabolite (less than 2% total).

In all experiments with toluene-degrading bacteria, DPA was converted at a considerable rate initially, but the activity declined rapidly. Alkynes are mechanism-based inactivators of a number of oxygenase enzymes, including several bacterial monooxygenases, and substrates with interior triple bonds are more potent inhibitors of monooxygenase enzymes than substrates with terminal triple bonds.[11a] Such inhibition was not observed however, during the transformation of DPA by *Xanthobacter* strain Py2. It is reasonable to assume that because *Xanthobacter* strain Py2 can grow on propylene, the alkene monooxygenase enzyme system is not inhibited by alkene and alkyne substrates. Practical application of the reactions will require optimisation to increase productivity and overcome the limitations due to low solubility of the substrate and toxicity of the products.

Although *Xanthobacter* strain Py2 converts toluene to all isomers of cresol[10] the reaction with DPA is highly regiospecific. Many of the toluene-degrading bacterial strains investigated produced both *meta*- and *para*-HDPA but the primary isomer produced was dependent upon the type of monooxygenase. The results are consistent with a transient epoxide intermediate formed from DPA that undergoes an active-site directed regiospecific rearrangement to *meta*- or *para*-HDPA. The involvement of an epoxide intermediate in a monooxygenation reaction has previously been proposed,[11b,c] and further study to identify the intermediate would help to elucidate the reaction mechanism.

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Notes and references

‡ Recombinant strains were cultured at 37 °C in Luria–Bertani medium (Difco) containing glycerol (1%) and ampicillin (100 mg l^{-1}) or kanamycin (50 mg l^{-1}) to maintain plasmid selection. All of the strains were induced with 1 mM IPTG (isopropyl-β-D-thiogalacto pyranoside) during exponential growth. *R. pickettii* strain JS757 was grown in minimal salts medium at 30 °C, pH 7.0 with toluene in the headspace. *Xanthobacter* strain Py2 was cultured as described previously.[10] In a typical biotransformation reaction, cells were harvested in late exponential phase by centrifugation and then washed and suspended in fresh medium to give a 10-fold concentration. DPA was added to a final concentration of 1 mM and pyruvate (2 mM) was added as a carbon and energy source. The concentration of products and reactants were monitored by HPLC (Hewlett Packard 1100). Compounds were separated on a Chromolink RP-18 column (100–4.6 mm, Merck) or a Supelcosil LC-ABZ + PLUS column with an acetonitrile–water gradient. For product purification, the reaction mixture was filtered through glass wool to remove any residual DPA crystals and then passed through a C18 solid phase extraction column (Waters, 12 g). Products were eluted in acetonitrile and the excess solvent removed in a rotary evaporator under reduced pressure. The two HDPA isomers were separated by preparative scale HPLC with a Supelcosil LC-ABZ column (25 cm × 10 mm, 5 μm, Supelco) with a mobile phase as above. DPA is insoluble in water and was therefore difficult to quantify during the reaction. Conversion calculations were based on product concentration and initial DPA concentration. NMR analysis: samples were dissolved in acetone-*d6*. NMR spectra were recorded on a Varian Inova spectrometer equipped with a 5 mm indirect detection probe, operating at 500 MHz for ^{1}H and at 125 MHz for ^{13}C. Chemical shifts were consistent with proposed structures and previous literature reports.[2a,b,12]

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