Cis−*Trans* Isomerization of a Cyclopropyl Radical Trap Catalyzed by Extradiol Catechol Dioxygenases: Evidence for a Semiquinone Intermediate

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Abstract: Substrate analogues *cis*- and *trans*-2-(2,3-dihydroxyphenyl)cyclopropane-1-carboxylic acid were synthesized as probes for a semiquinone radical intermediate in the (2,3-dihydroxyphenyl)propionate 1,2-dioxygenase reaction. These analogues were found to be substrates for oxidative cleavage by extradiol dioxygenases from *Escherichia coli* and *Alcaligenes eutrophus*. The stereochemistry of the ring fission products was analyzed by conversion to cyclopropane-1,2-dicarboxylic acids using the ensuing hydrolase enzyme MhpC, followed by GCMS analysis. This analysis revealed 85−94% *trans* product and 6−15% *cis* products, implying that *cis/trans* isomerization of the cyclopropyl ring substituents had taken place during the enzymatic conversion. These results are consistent with a reversible opening of the cyclopropyl ring, and hence consistent with the intermediacy of a semiquinone radical intermediate in the extradiol catechol dioxygenase reaction.

Introduction

The rapid ring opening of cyclopropylmethyl radicals has been employed in the design of cyclopropyl radical traps to study the existence of radical intermediates in a number of enzyme-catalyzed reactions.[1] Evidence for radical intermediates in the reactions of several iron-dependent oxygenase enzymes has been obtained by the observation of products in which a cyclopropyl ring has been opened.[2] In other cases enzyme inactivation has been observed by cyclopropyl substrates, via covalent enzyme modification by a ring-opened butenyl radical.[3]

The catechol dioxygenases comprise two families (intradiol and extradiol) of non-heme iron-dependent enzymes which catalyze the oxidative cleavage of the aromatic ring of catechol substrates. These enzymes play an important role in the biodegradation of a wide range of man-made industrial pollutants containing aromatic rings.[4] High-resolution structural information is now available for both the iron(III)-dependent intradiol dioxygenases[5] and the iron(II)-dependent extradiol dioxygenases.[6]

There is considerable evidence from ESR spectroscopic studies on the catechol dioxygenases that the non-heme iron cofactor in both families of enzyme binds the catecholate oxygens and also binds molecular oxygen.[7,8] Mechanistic proposals for the subsequent steps of the extradiol catechol dioxygenase mechanism have invoked a Criegee rearrangement of a peroxy intermediate.[8−10] Experimental evidence for such a rearrangement has been obtained from ^{18}O-labeling studies on the extradiol enzyme (2,3-dihydroxyphenyl)propionate 1,2-dioxygenase (MhpB) from *Escherichia coli*, for which the mechanistic scheme shown in Figure 1 has been proposed.[10] Coordination of substrate **1** to the non-heme iron(II) cofactor is followed by electron transfer to form a semiquinone intermediate (**2**). Recombination with superoxide could form one of two possible peroxy intermediates, either of which could undergo a Criegee rearrangement to give an unsaturated lactone intermediate (**3**). The hydrolysis of lactone **3** by enzyme-bound iron(II) hydroxide is supported experimentally by the MhpB-catalyzed hydrolysis of a saturated lactone analogue.[10]

Precedent for the involvement of semiquinone intermediates in these reactions is provided by model catecholate complexes of redox-active metal ions. Exposure of a rhodium(III) catecholate complex to oxygen gave a semiquinone−metal−superoxide complex (**5**),[11] while the corresponding iridium(III) complex gave a peroxy adduct (**6**) presumably formed by recombination of semiquinone with superoxide.[12]

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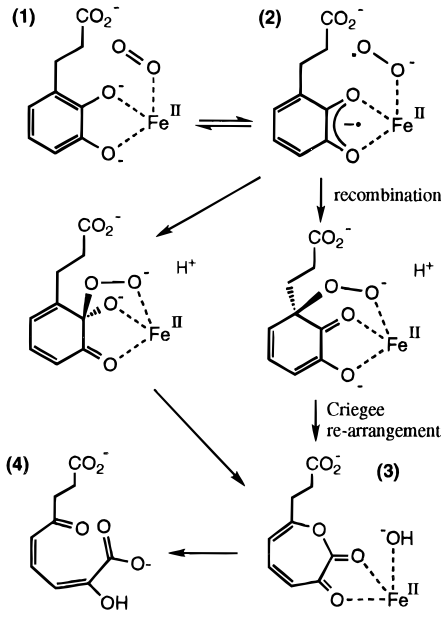


Figure 1. Proposed mechanistic scheme for (2,3-dihydroxyphenyl)-propionate 1,2-dioxygenase (see ref 10).

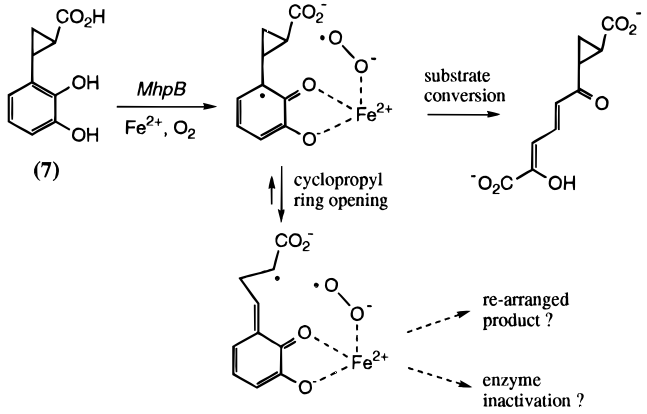
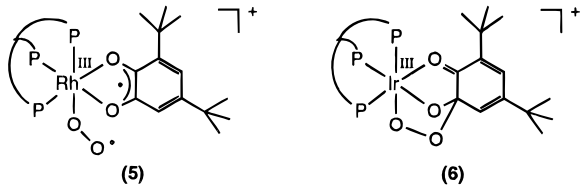


Figure 2. Anticipated outcomes for reaction of cyclopropyl analogue **7** with dioxygenase MhpB.



In order to probe the existence of semiquinone **2**, we anticipated that analogues containing a cyclopropyl group in the propionate side chain might act as radical traps for this enzyme. Analogue **7** was designed, in which the introduction of a cyclopropyl group is achieved with only minor modification of the propionate side chain. Upon formation of the semiquinone radical intermediate, we anticipated that the cyclopropyl ring of **7** would open, leading either to a novel enzymatic product or to enzyme inactivation (Figure 2). We report the preparation of *cis* and *trans* diastereoisomers of **7** and analysis of their reaction with the *Escherichia coli* dioxygenase and with a related extradiol dioxygenase from *Alcaligenes eutrophus.*

Results

Preparation of Cyclopropyl Analogues. *trans*-2-(2,3-Dihydroxyphenyl)cyclopropane-1-carboxylic acid (**7a**) was prepared as shown in Scheme 1. 2,3-Dimethoxycinnamic acid was demethylated using boron tribromide, which gave the desired 2,3-dihydroxycinnamic acid and a 15−40% yield of 8-hydroxycoumarin (**8**). The methoxymethyl (MOM) ether was found to be the most suitable protecting group for the phenolic hydroxyl groups in this route, with bulkier protecting groups at C-2 hindering the cyclopropanation reaction. The 2,3-MOM-protected cinnamate ethyl ester (**9**) was reacted with trimethyloxosulfonium ylide,[13] affording selectively the *trans*-cyclopropyl ester (**10**) in 52% yield. Alkaline hydrolysis of the ethyl ester, followed by acidic removal of the MOM groups, gave the *trans*-cyclopropyl analogue (**7a**) in 62% yield.

The *cis*-cyclopropyl analogue was prepared in similar fashion (see Scheme 1) from 8-hydroxycoumarin (**8**). MOM protection of **8** proceeded smoothly in 97% yield to give the MOM-protected lactone (**11**). However, in this case cyclopropanation of **11** did not proceed to completion, and at no stage could the *cis*-cyclopropyl lactone (**12**) be separated from the lactone precursor (**11**).[29] Thus the *cis*-cyclopropyl analogue (**7b**) was isolated as a 4:1 mixture with 8-hydroxycoumarin (**10**), which was shown to be neither a substrate nor an inhibitor for dioxygenase MhpB.

^{1}H-NMR spectroscopic analysis of **7a** and **7b** revealed characteristic signals for the C*H*-Ar proton at 2.71 and 2.55 ppm, respectively. Close inspection revealed that both **7a** and **7b** were diastereomerically pure, to the limits of detection (>99%).

Processing of Cyclopropyl Analogues by Extradiol Dioxygenases. Both *cis*- and *trans*-cyclopropyl analogues (**7a** and **7b**) were tested as substrates for *Escherichia coli* (2,3-dihydroxyphenyl)propionate 1,2-dioxygenase (MhpB), purified as previously described.[14] The *trans*-analogue (**7a**) was subsequently assayed as a substrate for *Alcaligenes eutrophus* dioxygenase MpcI, purified to >50% homogeneity from over-expressing strain JM109/pAE166.[15] The *A. eutrophus* enzyme shares significant sequence similarity with the *E. coli* enzyme, and processes a similar range of substrates with comparable catalytic efficiency.[14,15]

In each case the rapid formation of a UV absorption at 400−405 nm was observed corresponding to the *meta*-ring-fission product, indicating that these analogues were substrates for oxidative *meta* cleavage. Apparent K_m and k_{cat} values of 100 μM and 18 s^{-1} were measured for the processing of **7a** by MhpB, which upon comparison with the natural substrate (**1**) reveal that **7a** is processed at 16% of the catalytic efficiency of **1** (see Table 1).[29] **7a** is also processed efficiently by *A. eutrophus* MpcI (see Table 1), suggesting that these substrates adopt similar orientations to the natural substrates at the respective active sites. Extended incubation with **7a** or **7b** gave no detectable enzyme inactivation, compared with control experiments.

Attempts were made to isolate and characterize the ring-fission products **13a** and **13b** from enzymatic conversion of **7a** and **7b**. The isolated products showed no characterizable signals by ^{1}H NMR spectroscopy other than **7a** and **7b**, in contrast to the natural ring fission product (**4**) which has been previously characterized.[16] It appears that the presence of the cyclopropyl substituent in the side chain renders the ring-fission products **13a** and **13b** chemically unstable. No additional signals were detected which might correspond to products in which the cyclopropyl ring has been opened.

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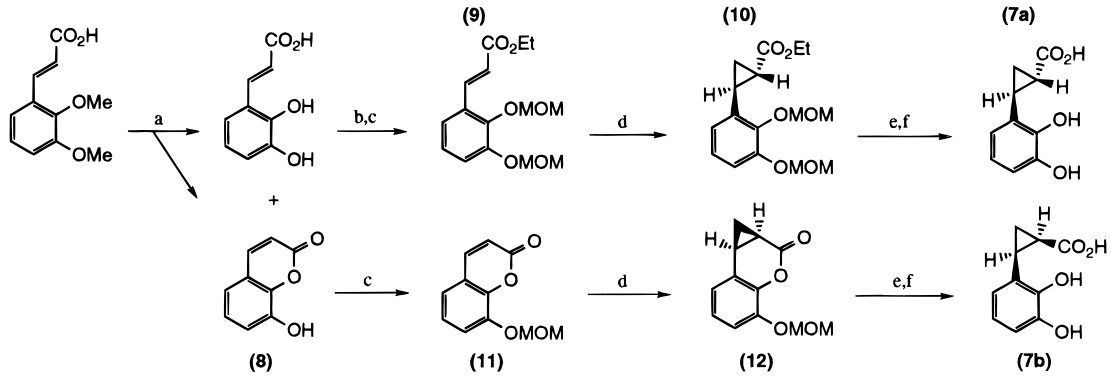
Scheme 1. Synthetic Route for Cyclopropyl Analogues[a]



[a] (a) BBr_3, CH_2Cl_2, −78 °C. (b) H_2SO_4, EtOH, reflux. (c) CH_3OCH_2Cl, $^iPr_2NEt$, CH_2Cl_2. (d) $Me_3SO^+I^-$, NaH, DMSO. (e) 0.5 M NaOH/H_2O. (f) 2 M HCl/H_2O. Yields given in the Experimental Section.

Table 1. Steady State Kinetic Parameters for *E. coli* MhpB and *A. eutrophus* MpcI[a]

		E. coli MhpB			*A. eutrophus* MpcI	
substrate	λ_{max}(RFP) (nm)	$K_{m(app)}$ (μM)	k_{cat} (s^{-1})	$k_{cat}/K_{m(app)}$ (M^{-1} s^{-1})	$K_{m(app)}$ (μM)	v_{rel}
(2,3-dihydroxyphenyl)propionic acid (**1**)	394	26	29	1.1×10^6	7.6	1.0
trans-cyclopropyl analogue **7a**	405	100	18	1.8×10^5	40	0.85
2,3-dihydroxycinnamic acid	452	36	19	5.3×10^5	6.9	0.67
2,3-dihydroxyphenoxyacetic acid		300	6	2.0×10^4	ND	0.55

[a] K_{mapp} values measured under saturating concentrations of oxygen. v_{rel} values measured for *A. eutrophus* MpcI since the enzyme was not homogeneous and is prone to time-dependent inactivation.[15] ND = not determined.

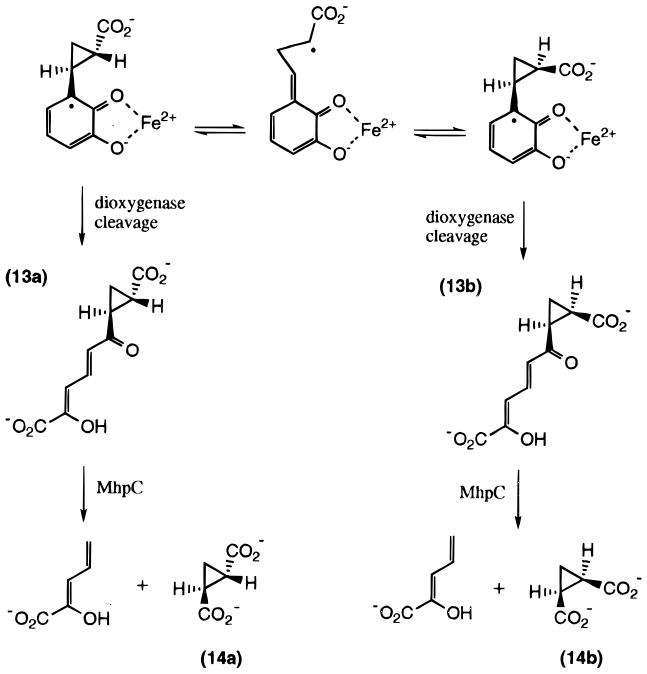


Figure 3. Mechanism for *cis*−*trans* isomerization of cyclopropyl ring via reversible ring opening of a semiquinone radical intermediate.

In order to confirm the identity of the cyclopropyl-containing ring-fission products **13a** and **13b**, they were further treated with hydrolase enzyme MhpC. This enzyme catalyzes the next step of the phenylpropionate pathway, namely the hydrolytic cleavage of the C-5/C-6 bond (see Figure 3).[16] Treatment with *E. coli* MhpC, purified as described elsewhere,[17] led to the disappearance of the UV absorptions corresponding to **13a** and **13b**, verifying that they correspond to authentic *meta*-ring-fission products. No additional UV absorbent materials were observed after MhpB/MhpC treatment, again suggesting that no stable rearrangement product was formed from cyclopropyl ring opening.

Stereochemical Analysis of Enzymatic Products. We hypothesized that upon formation of the semiquinone intermediate in the MhpB reaction, a reversible opening of the cyclopropyl ring might be taking place, leading to *cis/trans* isomerization of the cyclopropyl ring substituents and the production of *cis* (**14b**) and *trans* (**14a**) isomers of cyclopropane-1,2-dicarboxylic acid (see Figure 3). In order to test this hypothesis authentic standards of *cis*- and *trans*-dimethylcyclopropane-1,2-dicarboxylate were synthesized by the method of Saal *et al.*[18] Analysis of the standards by GC revealed that these diastereoisomers could be clearly resolved, and GCMS analyis under electron impact conditions gave distinct retention times and distinct fragmentation patterns for the two isomers (see Figure 4). The *trans* isomer **15a** at retention time 1071 s showed a fragment ion at *m/z* 127 (100%) and a major fragment ion (72%) at *m/z* 98, whereas the *cis* isomer **15b** at retention time 1105 s showed a fragment ion at *m/z* 127 (100%) and a minor fragment ion (32%) at *m/z* 99.

Incubation of *trans*-cyclopropyl analogue **7a** with MhpB and MhpC was then carried out, and the acidic products were derivatized with diazomethane (see Scheme 2). Analysis by GCMS revealed the presence of *trans*-product **15a** and a small peak corresponding to *cis* product **15b**, both with identical fragmentation patterns to authentic standards. The ratio of **15a:15b** was estimated at 94:6. Observation of 6% *cis* product implied that *cis*−*trans* isomerization was taking place during the enzymatic conversion of **7a**.

Processing of *cis*-cyclopropyl analogue **7b** with MhpB and MhpC followed by methylation and GCMS analysis also gave a mixture of *trans* **15a** and *cis* **15b**, with the *trans* product again the major product. The ratio of **15a:15b** was 90:10 in this case, indicating that substantial *cis*−*trans* isomerization is taking place. The similar ratios of **15a:15b** obtained starting from

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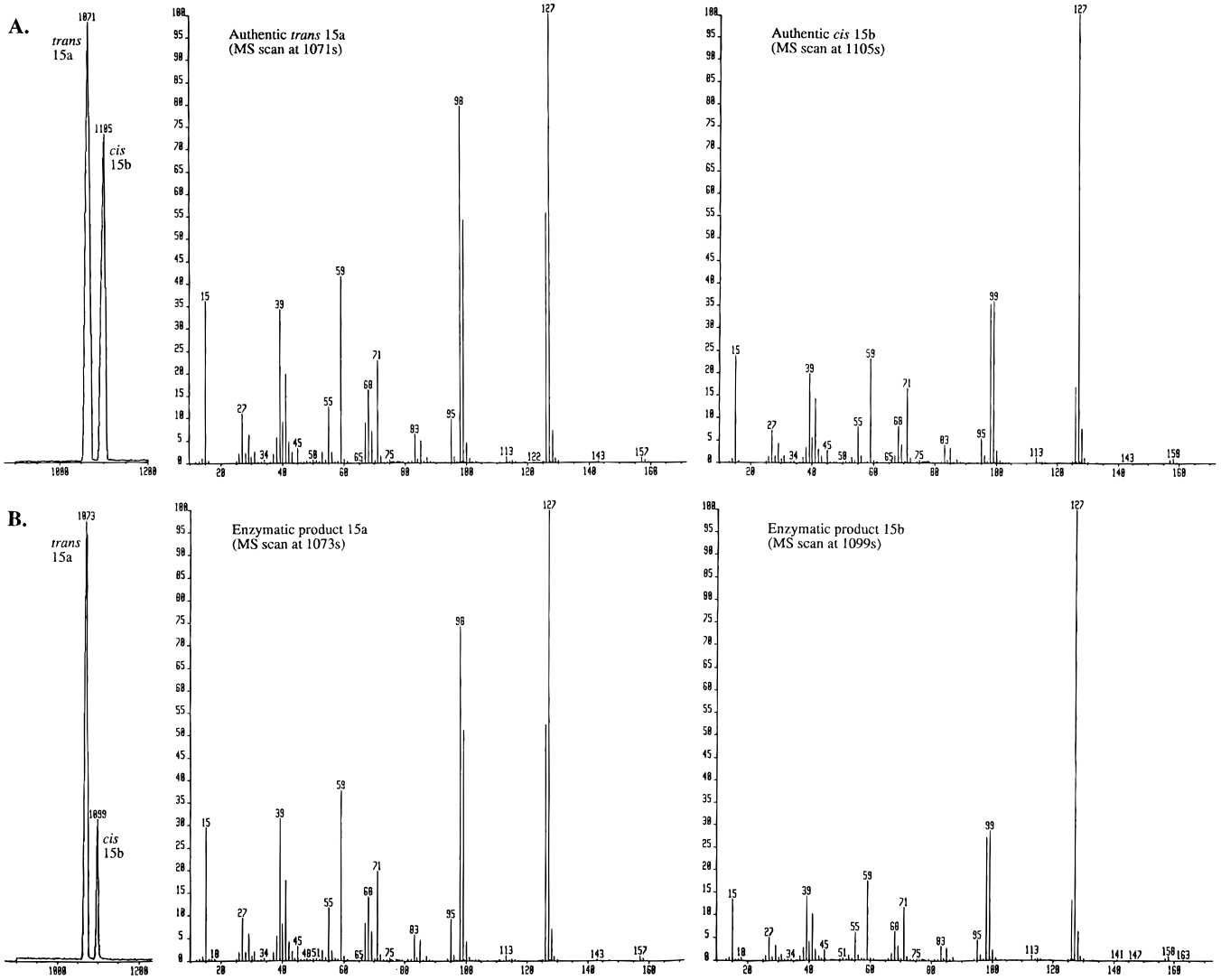
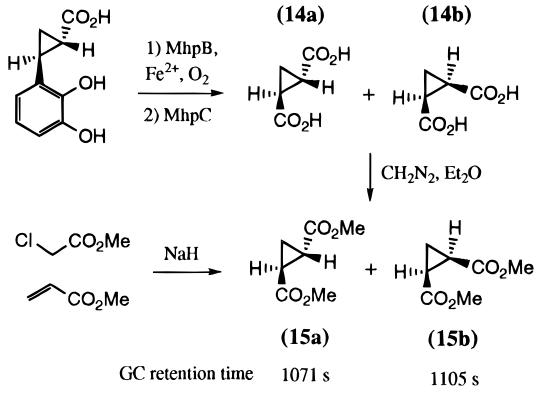


Figure 4. GCMS data for **15a** and **15b**. (A) Authentic standards, synthesized as described in the Experimental Section. (B) Enzymatic products, generated from treatment of **7a** with *Alcaligenes eutrophus* MpcI and *E. coli* MhpC, followed by treatment with diazomethane.

Scheme 2. *Cis* and *Trans* Isomers of Dimethyl Cyclopropane-1,2-dicarboxylate Produced Enzymatically and Synthetically



either **7a** or **7b** suggest that the rate of isomerization of the cyclopropyl substituents is substantially faster than the rate of the enzymatic reaction.

Processing of *trans*-analogue **7a** with *Alcaligenes eutrophus* dioxygenase MpcI, followed by treatment with *E. coli* MhpC and derivatization as before, also gave both **15a** and **15b** by GCMS analysis (see Figure 4), this time with a ratio of **15a:15b** of 85:15. The increased percentage of **15b** obtained in this case suggests that the ratio of *cis* to *trans* products is dependent on the structure of the enzyme active site, presumably being affected by the relative fit of *cis* vs *trans* isomers and the freedom of rotation of the ring-opened radical. A summary of the data obtained is given in Table 2, and the GCMS data obtained from the *A. eutrophus* MpcI conversion are illustrated in Figure 4.

Table 2. Ratios of *trans*-**15a**:*cis*-**15b** Products Obtained from Enzymatic Conversion of *trans*-**7a** and *cis*-**7b** Substrate Analogues[a]

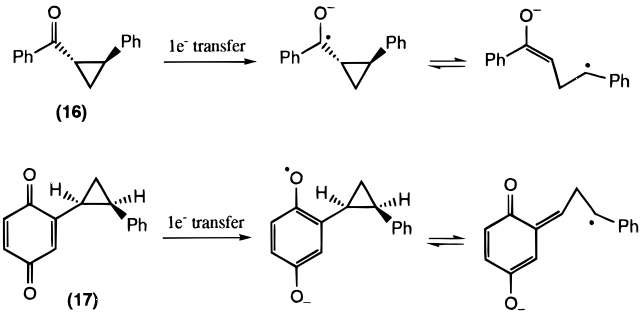
substrate	*E. coli* MhpB + MhpC **15a:15b**	*A. eutrophus* MpcI + MhpC **15a:15b**
7a	94:6	85:15
7b	90:10	ND

[a] Ratios determined by weighing of peaks obtained from GCMS analysis. ND = not determined.

Further Control Experiments. The observed *cis*−*trans* isomerization could in theory be explained by other epimerization mechanisms, in particular deprotonation/reprotonation of the cyclopropyl protons of **7**, **13**, or **14**. *trans*-Cyclopropyl substrate **7a** and *cis*-cyclopropane-1,2-dicarboxylic acid **14b** were incubated in NaOD/D_2O, and examined directly by ^1H NMR spectroscopy. No ^2H exchange or epimerization was observed after 24 h, implying that the protons attached to the cyclopropyl ring are not appreciably acidic.

However, there is a possibility that hydrogen atom abstraction is occurring at the stage of the *meta*-ring-fission products **13a**

Scheme 3. Other Examples of Cyclopropane Isomerization Reactions Occurring via Free Radical-Induced Reversible Ring Openings (See ref 21 and 22)



and **13b** or during the MhpB-catalyzed reaction. Since the rate of isomerization is apparently fast compared with the rate of enzymatic processing, hydrogen atom abstraction should lead to significant deuterium exchange from D_2O into **15a** and **15b**. Accordingly, the enzymatic processing of **7a** by MhpB/MhpC was carried out 98% D_2O/2% H_2O, and the derivatized products examined by GCMS.[29] The observed mass spectra of **15a** and **15b** again showed peaks at *m/z* 127, but in neither case was any increase in intensity observed at *m/z* 128 corresponding to deuterium incorporation. This experiment implies that there is no significant hydrogen atom exchange on the cyclopropyl ring during the MhpB and MhpC reactions. The only reasonable explanation for the *cis/trans* isomerization is therefore a reversible opening of the cyclopropyl ring.

Cis/trans isomerization could also occur prior to conversion by dioxygenase MhpB. In order to test this **7a** was treated on a larger scale (10 mg) with MhpB (10 units), and the residual substrate isolated and analyzed by ^{1}H NMR spectroscopy. Signals corresponding to residual **7a** were clearly visible, but no trace (<1%) of the clearly resolvable α proton of **7b** was observed, implying that the 6% *trans*-to-*cis* isomerization occurs after an irreversible step in the enzymatic reaction.

Generation of a free semiquinone species from **7a** was attempted chemically and enzymatically, in order to examine whether *cis/trans* isomerization could take place in solution. Air oxidation of catechols under basic conditions and oxidation by commercially available tyrosinase are both precedented to involve semiquinone generation,[19,20] and in both cases treatment of catechol gave a characteristic EPR spectrum of a semiquinone radical species. However, upon incubation of **7a** in either system, re-isolation and ^{1}H-NMR spectroscopic analysis revealed that no isomerization to **7b** had taken place. Thus it appears that the isomerization of **7a** occurs only at the dioxygenase active site: perhaps bidentate iron coordination by the non-heme cofactor is also required.

Discussion

Cis−*trans* isomerization of a cyclopropyl radical trap has to our knowledge not been observed in an enzymatic reaction. However, at least two non-enzymatic examples have been reported, as shown in Scheme 3. Tanner *et al*. observed upon single electron reduction of ketone **16** *cis/trans* isomerization of the cyclopropyl substituents, via a reversible opening of a highly stabilized cyclopropylmethyl radical.[21] Recently Zeng *et al*. have reported that upon single electron reduction of *cis*-substituted cyclopropylbenzoquinone **17** 70% of the *trans*-hydroquinone was isolated.[22] The latter example also involves the reversible opening of a cyclopropyl ring adjacent to a semiquinone radical, similar to our observations.

We have established that there is no exchange of the protons attached to the cyclopropyl ring during the MhpB- and MhpC-catalyzed reactions, nor does exchange occur non-enzymatically. Therefore, the only reasonable explanation for the observed *cis*−*trans* isomerization is that an adjacent radical is formed during the enzymatic processing of **7**. Since the MhpC reaction involves neither cofactors nor redox chemistry the radical intermediate must be in the extradiol dioxygenase reaction. Hence this behaviour is consistent with the existence of a transient semiquinone intermediate (**2**) in the MhpB reaction.

The reversibility of radical-induced cyclopropyl ring openings in chemical reactions has been demonstrated in several instances.[23−25] In these cases the equilibrium constant for cyclopropyl ring opening is governed by the relative stability of the ring-closed and ring-opened radicals. Thus, for example, Bowry *et al*. have reported that a reversible opening of the α-cyclopropylbenzyl radical can take place, although none of the ring-opened radical could be detected, due to the high stability of a benzylic radical relative to an alkyl radical.[24] The reversibility of MhpB-catalyzed opening of **7a** and **7b** can therefore be explained in this case by the high stability of the initial semiquinone radical.[26] Since no re-arranged product in which the cyclopropyl ring was opened was detected by NMR spectroscopy or HPLC the equilibrium constant for the cyclopropyl ring opening would appear to lie strongly in favor of the semiquinone radical. The similar ratios of **15a:15b** obtained above from enzymatic processing of **7a** or **7b** imply that the rates of opening and reclosing of the cyclopropyl ring system are fast compared with the rate of reaction of the semiquinone radical with superoxide in the enzymatic reaction. However, we have at present no insight into the absolute values of these rate constants.

The existence of a semiquinone radical supports the proposed mechanism for the MhpB reaction, involving binding of the catechol substrate and dioxygen to iron(II), followed by electron transfer to generate the semiquinone−iron(II)−superoxide intermediate.[10] However, the observation of radical character at C-1 does not allow us to differentiate between subsequent recombination of the semiquinone with superoxide at C-1 vs C-2, since radical character can be found on every carbon center in the semiquinone system via either electron delocalization or iron−oxygen electron transfer (see Figure 5).

The enzymatic reaction could thence proceed either via formation of a C-1 peroxy adduct followed by acyl migration to give lactone **3** or by formation of a C-2 peroxy adduct followed by alkenyl migration to give **3** (see Figure 5).[10] Further studies are required in order to distinguish between these possibilities; however, we have found that 2,3-dihydroxycinnamic acid (side chain $-CH{=}CHCO_2H$) and 2,3-dihydroxy-

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(29) Conversions with MpcI, kinetic analysis, and further control experiments were not carried out with **7b** due to its instability toward lactonization and problems experienced in its re-synthesis. Attempts to re-synthesize **7b** gave lower conversions for the cyclopropanation of **11**, yielding impure samples of **7b**.

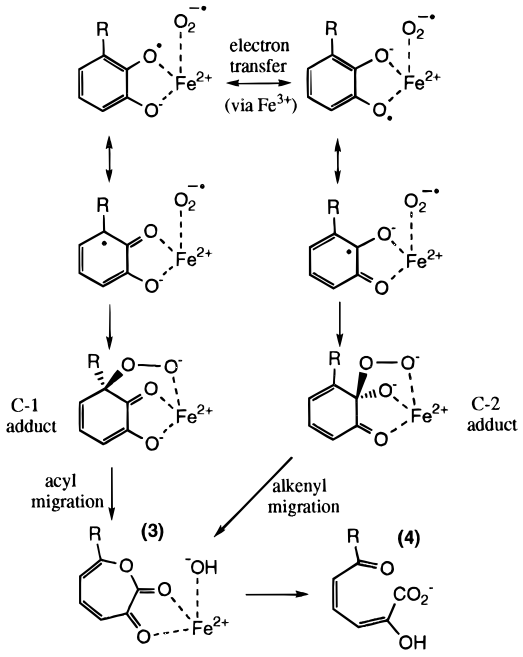

Figure 5. Delocalization of radical character in the semiquinone intermediate **2** and possible subsequent enzymatic reactions at C-1 or C-2.

phenoxyacetic acid (side chain $-OCH_2CO_2H$) are both efficient substrates for oxidative cleavage by MhpB (see Table 1). If a C-1 peroxy intermediate were formed, then one might expect the introduction of electron-withdrawing and electron-donating groups into the side chain to have a significant effect on the rate of the Criegee rearrangement, and hence on k_{cat} and K_m. The absence of such an effect appears more consistent with a C-2 adduct, whose structure is precedented by the iridium(III) model complex (**6**) mentioned above.[12]

The reversible ring opening of **7a** and **7b** provides further evidence for the existence of stabilized radical intermediates in iron-dependent oxygenase-catalyzed reactions.[2] It is interesting to speculate whether other cyclopropyl radical trap studies of enzymatic reactions might also have involved reversible ring openings which have escaped detection.

Experimental Methods

General. ^{1}H and ^{13}C NMR spectra were recorded on a Joel 300-MHz spectrometer in deuteriated solvents as described below. Mass spectra and GCMS were recorded on a VG 70-250 mass spectrometer. IR spectra were recorded on a 1600 series Perkin-Elmer FTIR infrared spectrometer in NaCl discs. UV/visible spectra were recorded on a Cary 1 UV/visible spectrophotometer using 1 mL quartz cells. ESR spectra were recorded on a Bruker ECS106 ESR spectrometer by S. Broadbridge (University of Southampton). GC was carried out using a 25 m × 0.22 mm BP1 column with 25 μM film thickness and a temperature gradient of 50 °C for 4 min followed by a 5 °C/min to 150 °C. HPLC was carried out on a Waters Associates chromatograph using a Bio-Rad HPX-87H Organic Acids column (eluent 0.005 M H_2SO_4 at 0.6 mL/min).

Chemicals. 2,3-Dihydroxyphenoxyacetic acid was synthesized from pyrogallol according to the method of Christiansen.[27] *cis*- and *trans*-dimethylcyclopropane-1,2-dicarboxylates were synthesized according to the method of van der Saal.[18]

***trans*-2,3-Dihydroxycinnamic Acid and 8-Hydroxycoumarin (8).** A solution of BBr_3 (20 g, 80 mmol) in CH_2Cl_2 (50 mL) was maintained at −78 °C under an atmosphere of N_2. 2,3-Dimethoxycinnamic acid (7.55 g, 34 mmol) in CH_2Cl_2 (50 mL) was added over 1 h with stirring. The resultant mixture was allowed to warm to room temperature and was stirred for a further 24 h. Careful addition of water (100 mL) to the mixture was followed by extraction into ether (6 × 40 mL). The combined organic portions were initially extracted with 5% $NaHCO_3$ solution (6 × 40 mL) to separate *trans*-2,3-dihydroxycinnamic acid from its corresponding *cis* lactone 8-hydroxycoumarin. The remaining organic layer was extracted further with K_2CO_3 (3 × 40 mL) in order to obtain the *cis* lactone. Both of the aqueous layers were acidified to pH 1−2 and re-extracted into ether (6 × 30 mL). Drying over $MgSO_4$ and evaporation of the ether portions furnished an orange solid (3.8 g, 62%), identified as *trans*-2,3-dihydroxycinnamic acid and a cream solid (1.7 g, 31%) which was identified as 8-hydroxycoumarin. The crude products were recrystallized (ether/petroleum ether) to afford both *trans*-2,3-dihydroxycinnamic acid and 8-hydroxycoumarin as white crystalline solids.

***trans*-2,3-Dihydroxycinnamic acid:** mp 201−203 °C [lit.[28] mp 202 °C]; IR (Nujol) 3460, 3283, 3104, 1681, 1625, 1587, 1485, 1273, 1255 cm^{-1}; ^{1}H NMR (300 MHz, d_6-acetone) 8.00 (1H, d, J = 15.8 Hz, C**H**-Ar), 7.06 (1H, dd, J = 7.8, 1.4 Hz, *o*-Ph), 6.91 (1H, dd, J = 7.7, 1.5 Hz, *p*-Ph), 6.69 (1H, t, J = 7.9 Hz, *m*-Ph), 6.55 (1H, d, J = 16.2 Hz, C**H**-CO_2H) ppm; ^{13}C NMR (75.5 MHz, d_6-acetone) 168.20, 145.30, 145.27, 140.43, 121.49, 119.50, 119.25, 118.05, 116.55 ppm; *m/z* 180 (M^+, 58%), 162 (M − H_2O, 99%), 134 (M − CO_2H − H, 100%), 78 (31%).

8-Hydroxycoumarin (8): mp 161−162 °C [lit.[28] mp 160 °C]; IR (Nujol mull) 3384, 1711, 1603, 1578, 1273 cm^{-1}; ^{1}H NMR (300 MHz, d_6-acetone) 7.92 (1H, d, J = 9.6 Hz, C**H**−Ar), 7.13 (3H, m, Ph), 6.39 (1H, d, J = 9.6 Hz, C**H**−CO_2R) ppm; ^{13}C NMR (75.5 MHz, d_6-acetone) 160.13, 145.19, 144.80, 143.22, 125.11, 120.47, 119.44, 119.00, 116.91 ppm; *m/z* 162 (M^+, 100%), 134 (M − CO, 66%); HRMS M^+ 162.0326, $C_9H_6O_3$ requires 162.0317.

Ethyl 2,3-Dihydroxycinnamate. To a solution of 2,3-dihydroxycinnamic acid (2.0 g, 11.1 mmol) in ethanol (50 mL) was added concentrated H_2SO_4 (5 mL) and the resultant mixture was refluxed over night. The mixture was then diluted with water (50 mL), and the product extracted into ether (3 × 40 mL), washed with saturated 5% $NaHCO_3$ solution (2 × 30 mL), dried over $MgSO_4$, and concentrated *in vacuo* to give an orange solid. Recrystallization (ether/petroleum ether) furnished ethyl 2,3-dihydroxycinnamate as white crystals (2.04 g, 88%). Mp 137−139 °C; IR (Nujol mull) 3593, 3280, 1686, 1632, 1590, 1506, 1323, 1195 cm^{-1}; ^{1}H NMR (300 MHz, d_6-acetone) 7.99 (1H, d, J = 16.2 Hz, C**H**-Ar), 7.10 (1H, dd, J = 8.1, 1.5 Hz, *o*-Ph), 6.91 (1H, dd, J = 8.1, 1.5 Hz, *p*-Ph), 6.72 (1H, t, J = 7.7 Hz, *m*-Ph), 6.60 (1H, d, J = 16.2 Hz, C**H**-CO_2Et), 4.20 (2H, q, J = 7.4 Hz, C**H**$_2$CH$_3$), 1.29 (3H, t, J = 7.3 Hz, CH$_2$C**H**$_3$) ppm; ^{13}C NMR (75.5 MHz, d_6-acetone) 166.76, 145.41, 145.04, 139.85, 121.64, 119.66, 119.57, 118.20, 116.53, 59.73, 13.82 ppm; *m/z* 208 (M^+, 35%), 162 (M − C_2H_5OH, 100%), 134 (M − HCO_2Et, 60%); HRMS M^+ 208.0725, $C_{11}H_{12}O_4$ requires 208.0736.

Ethyl 2,3-Bis(methoxymethyleneoxy)cinnamate (9). To an ice-cooled solution of ethyl 2,3-dihydroxycinnamic acid (0.5 g, 2.41 mmol) in CH_2Cl_2 (25 mL) and *N*,*N*-diisopropylethylamine (dried over CaH_2) (1.17 g, 1.57 mL, 9.02 mmol) was added, dropwise, MOMCl (0.73 g, 0.70 mL, 9.02 mmol). The reaction mixture was stirred on ice for 1 h before it was warmed to room temperature and stirred for a further 20 h. The resulting mixture was diluted with water (50 mL), extracted into ether (3 × 30 mL), washed (10% $NaHCO_3$, saturated NaCl), dried over $MgSO_4$, and concentrated *in vacuo* to afford **9** as a yellow oil (0.65 g, 90%). IR (liquid) 2980, 1711, 1635, 1578, 1477, 1441, 1262 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) 8.11 (1H, d, J = 16.2 Hz, C**H**-Ar), 7.24 (1H, dd, J = 7.4, 1.5 Hz, *o*-Ph), 7.18 (1H, dd, J = 8.1, 1.5 Hz, *p*-Ph), 7.04 (1H, t, J = 8.1 Hz, *m*-Ph), 6.43 (1H, d, J = 16.2 Hz, C**H**-CO_2Et), 5.20 (2H, s, C**H**$_2$OCH$_3$), 5.16 (2H, s, C**H**$_2$OCH$_3$), 4.25 (2H, q, J = 7.3 Hz, C**H**$_2$CH$_3$), 3.62 (3H, s, CH$_2$OC**H**$_3$), 3.59 (3H, s, CH$_2$OC**H**$_3$), 1.32 (3H, t, J = 7.4 Hz, CH$_2$C**H**$_3$) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$) 167.01, 150.23, 145.92, 139.63, 129.59, 124.55, 120.23, 119.33, 118.03, 99.41, 95.12, 60.41, 57.82, 56.28, 14.30 ppm; *m/z* 296 (M^+, 21%), 251 (M − CH_2OCH_3, 10%), 206 (30%), 45 ($CH_2OCH_3^+$, 100%); HRMS M^+ 296.1271, $C_{15}H_{20}O_6$ requires 296.1260.

Ethyl *trans*-2-(2,3-Bis(methoxymethyleneoxy)phenyl)cyclopropane-1-carboxylate (10). NaH (60% mineral oil dispersion, 0.18 g, 4.5 mmol) was placed in a flask and washed with petroleum ether at 40−60 °C (3 × 5 mL) by swirling and decanting. The system was evacuated to remove all traces of petroleum ether. Powdered trimethoxysulfonium iodide (0.99 g, 4.5 mmol) was then added to the

flask and the vessel filled with nitrogen. DMSO was added carefully until the evolution of hydrogen ceased (~20 min) to produced a milky solution of the ylide. A solution of **9** (1.0 g, 3.38 mmol) in DMSO (30 mL) was added dropwise to the ylide. The resulting mixture was heated at 50 °C for 2 h and allowed to stir at room temperature for a further 12 h. The resulting mixture was concentrated on a rotary evaporator and diluted with water (100 mL). The product was extracted into ether (4 × 50 mL), washed with water (5 × 50 mL), and dried over $MgSO_4$ and the ether was removed *in vacuo* to yield a brown oil. The crude material was chromatographed (silica gel, 50% EtOAc in light petroleum) to give **10** as a pale yellow oil (0.55 g, 52%). IR (liquid film) 3040, 2956, 1722, 1602, 1583, 1475, 1439, 1271 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) 6.97−6.89 (2H, m, *o*-, *m*-Ph), 6.46 (1H, dd, J = 7.4, 1.5 Hz, *p*-Ph), 5.14 (2H, s, C**H**$_2$OCH$_3$), 5.11 (2H, s, C**H**$_2$OCH$_3$), 4.12 (2H, q, J = 7.4 Hz, C**H**$_2$CH$_3$), 3.54 (3H, s, CH$_2$OC**H**$_3$), 3.45 (3H, s, CH$_2$OC**H**$_3$), 2.85 (1H, ddd, J = 9.6, 6.6, 4.4 Hz, C**H**-Ar), 1.81 (1H, ddd, J = 7.3, 4.8, 4.8 Hz, C**H**CO$_2$Et), 1.56 (1H, ddd, J = 8.3, 4.4, 4.4 Hz, C**H**$_2$), 1.28 (1H, m, C**H**$_2$), 1.23 (3H, t, J = 7.4 Hz, CH$_2$C**H**$_3$) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$) 173.36, 149.83, 145.92, 134.42, 124.34, 118.15, 114.63, 99.07, 95.03, 60.56, 57.42, 56.12, 23.77, 20.87, 16.25, 14.22 ppm; *m/z* 310 (M^+, 11%), 234 (34%), 45 ($CH_2OCH_3^+$, 100%); HRMS M^+ 310.1410, $C_{16}H_{22}O_6$ requires 310.1416.

***trans*-2-(2,3-Dihydroxyphenyl)cyclopropane-1-carboxylic Acid (7a).** To a solution of **10** (0.90 g, 2.90 mmol) in methanol (1 mL) was added a solution of NaOH (0.5 M, 20 mL). The reaction was stirred at room temperature for 3 h. On completion the mixture was acidified (2 M, HCl), extracted into ether (3 × 50 mL), washed with brine (2 × 30 mL), dried over $MgSO_4$, and concentrated *in vacuo* to give a brown carboxylic acid product (0.69 g, 85%). The carboxylic acid (0.6 g, 2.13 mmol) was taken up in methanol (1 mL) and placed under an atmosphere of N_2. To this solution was added 2 M HCl (30 mL) and the resulting mixture was stirred at room temperature for 12 h. The product was then extracted into ether (3 × 30 mL), washed with brine (30 mL), dried over $MgSO_4$, and evaporated to dryness to furnish an orange solid. Recrystallization (ether/light petroleum) afforded **7a** as white crystals (0.30 g, 73%). A small amount of this material (~1.0 mg) was further purified by HPLC on an organic acid column eluted with 0.005 M H_2SO_4 which gave rise to a peak at 160 min. Mp 142−144 °C; IR (solution) 3399, 3169, 1690, 1592, 1476, 1283 cm^{-1}; ^1H NMR (300 MHz, d_6-acetone) 6.72 (1H, dd, J = 7.4, 1.5 Hz, *o*-Ph), 6.62 (1H, t, J = 7.4 Hz, *m*-Ph), 6.42 (1H, dd, J = 7.4, 1.5 Hz, *p*-Ph), 2.71 (1H, ddd, J = 8.8, 6.6, 4.4 Hz, C**H**-Ar), 1.82 (1H, ddd, J = 8.1, 4.4, 4.4 Hz, C**H**$_2$), 1.45 (1H, ddd, J = 8.1, 4.4, 4.4 Hz, C**H**$_2$), 1.36 (1H, ddd, J = 8.8, 7.4, 4.4 Hz, C**H**$_2$) ppm; ^{13}C NMR (75.5 MHz, d_6-acetone) 174.75, 145.11, 144.82, 127.30, 120.98, 117.30, 113.83, 22.85, 21.31, 15.86 ppm; *m/z* 194 (M^+, 61%), 176 (M − H_2O, 100%), 147 (M − HCO_2H − H, 99%), 77 ($C_6H_5^+$, 52%), 51 ($C_4H_3^+$, 30%); HRMS M^+ 194.0574, $C_{10}H_{10}O_4$ requires 194.0579.

8-(Methoxymethyleneoxy)coumarin (11). To an ice-cooled solution of **8** (0.30 g, 1.85 mmol) in CH_2Cl_2 (20 mL) and *N*,*N*-diisopropylethylamine (dried over CaH_2) (0.47 g, 0.64 mL; 3.70 mmol) was added, dropwise, MOMCl (0.30 g, 0.28 mL, 3.70 mmol). The reaction mixture was stirred on ice for 1 h before it was warmed to room temperature and stirred for a further 20 h. The resulting mixture was diluted with water (30 mL), extracted into ether (3 × 30 mL), washed (10% $NaHCO_3$, saturated NaCl), dried over $MgSO_4$, and concentrated *in vacuo* to afford the desired product as a yellow oil (0.37 g, 97%). IR (liquid film) 2962, 1718, 1609, 1567, 1463, 1153, 1048 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) 7.68 (1H, d, J = 9.6 Hz, C**H**-Ar), 7.31 (1H, dd, J = 7.3, 1.5 Hz, *o*-Ph), 7.15 (1H, t, J = 8.1 Hz, *m*-Ph), 7.10 (1H, dd, J = 8.1, 1.5 Hz, *p*-Ph), 6.39 (1H, d, J = 9.6 Hz, C**H**-CO$_2$R), 5.26 (2H, s, C**H**$_2$OCH$_3$), 3.50 (3H, s, CH$_2$OC**H**$_3$) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$) 160.22, 144.59, 144.43, 143.72, 124.34, 120.94, 119.74, 118.60, 116.74, 95.51, 56.48 ppm; *m/z* 206 (M^+, 48%), 176 (M − CH_2O, 82%), 77 ($C_6H_5^+$, 19%), 45 ($CH_2OCH_3^+$, 100%); HRMS M^+ 206.0580, $C_{11}H_{10}O_4$ requires 206.0579.

***cis*-2-(2-Hydroxy-3-(methoxymethylene)phenyl)cyclopropane-1-carboxylic Acid Lactone (12).** NaH (60% mineral oil dispersion, 0.21 g, 5.35 mmol) was placed in a flask and washed with light petroleum (3 × 5 mL) by swirling and decanting. The system was evacuated to remove all traces of light petroleum. Powdered trimethyloxosulfonium iodide (1.15 g, 5.35 mmol) was then added to the flask and the vessel filled with nitrogen. DMSO was added carefully until the evolution of hydrogen ceased (~20 min) to produce a milky solution of the ylide. A solution of **11** (0.63 g, 3.06 mmol) in DMSO (15 mL) was added dropwise to the ylide. The resulting mixture was heated at 65 °C for 12 h and allowed to stir at room temperature for a further 24 h. The resulting mixture was concentrated on a rotary evaporator and diluted with water (100 mL). The product was extracted into ether (4 × 50 mL), washed with water (5 × 30 mL), and dried over $MgSO_4$, and the ether was removed under *vacuo* to yield a brown oil. The crude material was chromatographed (silica gel, 50% EtOAc in light petroleum) to give a pale brown oil containing a 2:3 mixture of **12**:**11** (overall yield 0.55 g, 52%) which was not further separable.[29] ^1H NMR (300 MHz, $CDCl_3$) 7.07−6.98 (3H, m, Ph), 5.19 (2H, s, C**H**$_2$OCH$_3$), 3.49 (3H, s, CH$_2$OC**H**$_3$), 2.57 (1H, td, J = 8.8, 5.1 Hz, C**H**-Ar), 2.32 (1H, ddd, J = 9.6, 7.4, 5.1 Hz, C**H**CO$_2$R), 1.73 (1H, ddd, J = 9.6, 8.1, 4.4 Hz, C**H**$_2$), 0.98 (1H, dt, J = 10.3, 5.1 Hz, C**H**$_2$) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$) 166.69, 144.45, 139.93, 124.11, 123.26, 120.82, 115.23, 95.48, 56.35, 20.13, 17.53, 14.48 ppm; *m/z* 220 (M^+, 13%), 190 (M^+ − CH_2O, 17%), 45 ($CH_2OCH_3^+$, 100%); HRMS M^+ 220.0733, $C_{12}H_{12}O_4$ requires 220.0736.

***cis*-2-(2,3-Dihydroxyphenyl)cyclopropane-1-carboxylic Acid (7b).** To a 3:2 mixture of **11** and **12** (0.30 g), dissolved in methanol (1 mL), was added a solution of NaOH (0.5 M, 20 mL). The reaction was stirred at room temperature for 3 h. On completion (by TLC) the mixture was acidified to pH 1 and stirred at room temperature for a further 4 h. After this time the products were extracted into ether (3 × 50 mL), washed with brine (2 × 30 mL), dried over $MgSO_4$, and concentrated *in vacuo* to give a brown solid containing both **7b** and **8** (overall recovery 0.25 g). The crude mixture was dissolved in EtOAc (20 mL) and extracted in portions of 50 mM potassium phosphate buffer at pH 7.0 (5 × 10 mL). The combined aqueous layers were acidified to pH 2.0, re-extracted into EtOAc, and dried over $MgSO_4$ and the solvent was removed *in vacuo* to afford 70 mg of an orange solid, shown by ^1H NMR spectroscopy to contain a 4:1 mixture of **7b** and **8**, and small amounts of the lactone form of **7b**.[29] IR (solution) 3422, 2400, 1696, 1590 cm^{-1}; ^1H NMR (300 MHz, d_6-acetone) 6.64 (1H, dd, J = 7.4, 1.5 Hz, *o*-Ph), 6.62 (1H, dd, J = 7.4, 1.5 Hz, *p*-Ph), 6.55 (1H, t, J = 7.4 Hz, *m*-Ph), 2.55 (1H, q, J = 8.4 Hz, C**H**-Ar), 2.10 (1H, ddd, J = 8.4, 7.2, 5.1 Hz, C**H**CO$_2$H), 1.53 (1H, ddd, J = 8.1, 5.1, 4.5 Hz, C**H**$_2$), 1.32 (1H, ddd, J = 8.0, 7.2, 4.5 Hz, C**H**$_2$) ppm; *m/z* 194 (M^+, 43%), 176 (M − H_2O, 97%), 147 (M − HCO_2H − H, 100%); HRMS M^+ 194.0585, $C_{10}H_{10}O_4$ requires 194.0579.

Enzyme Assays. *Escherichia coli* (2,3-dihydroxyphenyl)propionate 1,2-dioxygenase (MhpB) was purified as previously described.[14] *Alcaligenes eutrophus* dioxygenase MpcI was purified to 50% homogeneity from an overexpressing strain of *E. coli* JM109/pAE166, as described elsewhere.[15] *Escherichia coli* 2-hydroxy-2-ketonona-2,4-diene-1,9-dioic acid 5,6-hydrolase (MhpC) was purified from an overexpressing strain of W3110/pTB9 as described elsewhere.[17]

MhpB and MpcI were activated prior to use by addition of 100 mM ammonium iron(II) sulfate (5 μl) and 100 mM sodium ascorbate (5 μL) to apoenzyme (100 μL) at 0 °C, as previously described.[14] MhpB and MpcI were assayed by addition of re-activated enzyme to a solution of substrate in 50 mM Tris at pH 8.0 and 20 °C. Appearance of ring-fission products were observed for **1** at 394 nm (ϵ = 15 600 M^{-1} cm^{-1}),[14] for **7a** at 405 nm, and for 2,3-dihydroxycinnamic acid at 460 nm. For 2,3-dihydroxyphenoxyacetic acid, where no strong absorbance change was observed, O_2 consumption was measured electrochemically using a Clark-type oxygen electrode (Rank Bros.). MhpC was assayed by monitoring the disappearance of the ring-fission products by UV spectroscopy.[17]

Substrate analogues were assayed in triplicate in the concentration range 0.5−5 K_m. Apparent K_m values were calculated from Lineweaver/Burk and Eadie/Hofstee plots. k_{cat} values were obtained using the oxygen electrode: maximal rate values were compared with the maximal rate of the natural substrate **1**, whose k_{cat} is previously established.[14] Large-scale conversions using MhpB were carried out in 50 mM Tris buffer (pH 8.0), followed by acidification to pH 1 and extraction of products into ethyl acetate.

GC-MS Experiments. To a solution of **7a** (~2 mg) in H_2O (30 mL) was added Tris buffer (pH 8.0, 1 mL, 1.0 M). An aliquot of

reactivated MhpB (200 μL, 70 units/mL) was added and the resulting solution was stirred slowly at room temperature. Subsequent aliquots of reactivated MhpB (100 μL) were added at 2, 4, and 6 min. After 10 min, aliquots of MhpC (20 × 30 μL, 25 units/mL) were added over a further 10 min. The solution was acidified (concentrated HCl) to pH 2 and the organic products were extracted into EtOAc (6 × 25 mL) and dried over $MgSO_4$ and the solvent was removed *in vacuo*. The resulting residue was dissolved in ether (∼3 mL) and treated with excess diazomethane. The excess diazomethane was removed by bubbling nitrogen through the ethereal solution and the resulting solution was concentrated *in vacuo* to generate the derivatized enzyme products as an orange oil. The oil was submitted for GC-MS along with a sample of chemically synthesized standards. **7b** was converted enzymatically exactly as described for **7a**. Conversion of **7a** by MpcI was carried out using reactivated MpcI (total volume 3 mL, ∼5 units/mL).

Conversion of **7a** in D_2O was carried out as above, except that the Tris buffer had been lyophilized and re-suspended in D_2O (30 mL). **7a** (2 mg) was stirred in the deuteriated buffer for 30 min prior to addition of re-activated MhpB (3 × 100 μL in H_2O, ∼70 units/mL) over 10 min. MhpC (20 × 10 μL in H_2O, ∼80 units/mL) was added over 10 min, and the products isolated and derivatized as described above. The final proportion of D_2O was approximately 98%.

Semiquinone Generation. Generation of the catechol semiquinone under alkaline conditions was carried out by the method of Beck and Nibler.[19] In order to determine whether any isomerization of **7a** occurs as a result of semiquinone formation a 0.5 M solution of **7a** in methanol (1 mL) was mixed with 1 M sodium hydroxide (100 μL) until the solution became yellow. The mixture was stirred for a further 10 min, diluted with water, and acidified to pH 4. The product was extracted into ethyl acetate, dried ($MgSO_4$), and evaporated to dryness. Analysis by ^{1}H NMR spectroscopy revealed the presence of only **7a**.

Enzyme-catalyzed formation of catechol semiquinone was carried out by addition of mushroom tyrosinase (Sigma, 10 μg) to a solution of catechol (0.2 mg) in 50 mM potassium phosphate buffer (pH 8.0), total volume 1.0 mL. Semiquinone formation was observed by UV spectroscopy (λ_{max} 410 nm) and ESR spectroscopy.[20] Semiquinone formed from **7a** was also observed by UV spectroscopy (λ_{max} 425 nm). To determine if isomerization of **7a** occurs as a result of semiquinone formation a sample of **7a** (15 mg) was dissolved in 50 mM deuteriated potassium phosphate buffer (pH 8.0) and examined by ^{1}H NMR spectroscopy. This sample was treated with tyrosinase (20 μg) of stirred for 20 min, forming a yellow solution. The sample was examined by ^{1}H NMR spectroscopy after 3 and 24 h. Only signals corresponding to **7a** were observed.

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Supporting Information Available: ^{1}H NMR spectra of **7a** and **7b** and GCMS data for **7a** and **7b** with MhpB/MhpC (8 pages). See any current masthead page for ordering and Internet access instructions.

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