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Supporting Information

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The New Diagnostic Substrate Bicyclohexane Reveals a Radical Mechanism for Bacterial Cytochrome P450 in Whole Cells

Rachel N. Austin, Dayi Deng, Yongying Jiang, Kate Luddy, Jan B. van Beilen, Paul R. Ortiz de Montellano, John T. Groves**

Syntheses.

Bicyclo[3.1.0]hexane,[1] cyclopenten-2-enylmethanol,[2] cyclopent-2-ene-1-carboxaldehyde,[3] cyclohex-2-en-1-ol [4] , bicyclo[3.1.0]hex-2-ene[5-7] and *trans*-phenylmethylcyclopropane[8] were prepared according to literature procedures. Bicyclo[3.1.0]hexan-2-ols were synthesized by performing a Simmons-Smith reaction[1] with cylohex-2-en-1-one and reducing the resulting ketone with $LiAlH_4$. Bicyclo[3.1.0]hexan-3-ols were synthesized by performing a Simmons-Smith reaction[1] with cyclopent-2-en-1-ol. Bicyclo[3.1.0]hexan-3-one was synthesized by oxidizing the 3-alcohol with PCC by slight modification of a published procedure.[3] Cyclohex-3-en-1-one was synthesized by oxidizing cyclohex-3-en-1-ol with PCC following a literature procedure.[3] Barton esters were prepared according to the literature using 2-norcaranyl carboxylic acid and 2-bicyclohexanyl carboxylic acid as starting materials.[9]

Whole cell, cell free extract and isolated P450 experiments

For whole cell experiments, cells were grown in MSB medium with octane (provided to the cells via a glass bulb hanging over the medium that allows the vapors to partition into the liquid phase) as the sole carbon source. Cells were grown until they reached an optical density of 1 (600 nm). At that point, two different approaches were used. In the *growing cell* approach, diagnostic substrate, bicyclohexane or *trans*-phenylmethylcyclopropane, was simply provided in lieu of octane and cells allowed to grow for another 2-3 hr. Then, the samples were centrifuged at 8,000 x g and the supernatant extracted three times with ethyl acetate, dried through anhydrous sodium sulfate and concentrated on a rotary evaporator. The *resting cell* approach requires that the cells be centrifuged after they reach an optical density of 1 at 600 nm and resuspended in pH 7.2 50 mM phosphate buffer. The substrate is then provided and the cells incubated for two to three hours. The workup is the same for the resting cell approach as the growing cell approach. Both results yielded comparable results. Total product yields were generally higher with the growing cell approach while the resting cell approach provided a less complex baseline and less over oxidation of products.

Cell free extract oxygenations were done by growing cells in MSB medium with octane until an optical density between 1 and 1.5 at 600 nm. Cells were centrifuged. The cell pellet was resuspended in 50 mM potassium-phosphate buffer (pH=7.4) with 5% glycerol, 1 mM DTT, 200 μM PMSF (phenylmethylsulfonyl fluoride) at a concentration of 20 g cell dry weight/L (100 g cell wet weight/L). The cells were sonicated on ice water for four 20 s pulses with 1 min rests between pulses. After sonication, the cells were centrifuged at 100,000 x g for 20 min. The supernate was decanted. 1 mL of supernate was used for each assay. To that 1 mL of supernate, 2 μL of substrate and DTT (1 mM) was added and stirred for 2 min. NADH (12.5 mM) was added and the reaction shaken in a 37 ^{o}C incubator for two hr. The reaction was quenched with the addition of 1 mL CH_2Cl_2, and then vortexed and centrifuged. The CH_2Cl_2 layer was removed and dried through anhydrous sodium sulfate.

Oxidations of bicychexane and norcarane by CYP101 were performed as we have previously described.[10] The results for norcarane oxidation clearly confirmed the conclusions drawn in the published work. In addition, small amounts of secondary oxidation products derived from norcarene and epoxides of the radical alcohol **2** were detected.

Mass spectral data collection and analysis:

Product analyses were performed on a high-sensitivity GC-MS and quantified with by digital integration of the peaks in the total ion current. Areas under each peak were taken to be proportional to the concentrations and were checked as appropriate with concentration standards. Data for bicyclohexane oxidations is presented in Table 1. In Figure 2, characteristic ions are plotted. Ion intensity ratios were compared to those of authentic standards. For *trans*-phenylmethylcyclopropane the relative amounts of *trans*-hydroxymethyl-phenylcyclopropane to 4-hydroxy-4-phenyl-1-butene found from culture with CYP153A1 and CYP153A6 in *P. Putida* GPo12 were found to be 96.8:3.2 and 96.3:3.7, respectively.

Lifetimes for the substrate-based radical created in the initial step of the rebound mechanism can be calculated:

$$\text{radical lifetime} = \left(\frac{1}{k_{\text{rearrangement}} \frac{[\text{ring closed}]}{[\text{ring opened}]}} \right).$$

For bicyclohexane as substrate relatively small amounts of the corresponding 2-bicyclohexanone that were detected in some cases were included in the total concentration of ring-closed products.

Additional details on bicyclohexane chemistry and comparison to norcarane chemistry

For experiments in which the norcaranyl radical is generated there is a 49:1 bifurcation between ring opening to produce cyclohexenyl products and ring opening to afford cycloheptenyl products. For bicyclohexane, the ratio is 12:1. Accordingly, ring-opening of the 2-bicyclohexyl radical is expected to produce 8% cylohex-2-en-1-ol (**3**) relative to hydroxymethyl cyclohexene (**2**).

For solvolytic experiments in aqueous acetone in which the norcaranyl cation is generated, 10% of the cationic intermediate is trapped as the ring-expanded cycloheptenyl compound and 90% is trapped as the ring-closed norcaranyl compound. For bicyclohexane, 27% of the cationic intermediate is trapped as ring-expanded compounds and 73% is trapped as the ring-closed compound.[10]

Control experiments
3-Hydroxymethylcyclopentene was provided to the P450-containing cells as a substrate and incubated for 2-6 hr. In these experiments, the corresponding carboxylic acid was detected, but this product was not observed in the bicyclohexane oxygenation experiments.

Sterile control experiments were performed for all whole cell experiments where all components were added to sterilized media and incubated at the same temperature and for the same length of time as the live experiments.

Control experiments were done for the cell free experiments by adding all substrate and NADH to buffer and incubating the vial at the same temperature and for the same length of time as the live experiments.

Control experiments were done for the purified enzyme experiments by adding all components except NADH to the vial and incubating the vial at the same temperature and for the same length of time as the live experiments.

Additional details about clocking reaction and analytical methods

A 1.5 mL GC/MS vial was equipped with a mini stir bar, sealed with septum and flushed with argon. The vial was then shielded from light with Al foil and cooled to -78^{0}C. Stock solutions of both norcaranyl and bicyclohexyl Barton esters with a concentration of 0.1M were prepared separately in degassed ether. A sample of each PTOC ester containing 0.01 mmol was introduced into the vial. PhSH was added to a final concentration of 0.5 M. The final volume was adjusted to 1 mL with addition degassed diethyl ether. The vial was then allowed to equilibrate at RT for ca. 2 min. The reactions were irradiated for 45 min with a 125 W tungsten filament lamp. Products were analyzed by GC/MS (Shimadzu GCMS-QP2010) equipped with a capillary column (0.25mmX30.0m, Supelco SPB-624). Retention times and fragmentation patterns of the product are compared with authentic standards.

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