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Directed evolution of the dioxygenase complex for the synthesis of furanone flavor compounds☆

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Abstract—Herein we report a new preparation of 4-hydroxy-2,5-dimethyl-2,3-dihydrofuran-3-one, the flavor compound strawberry furanone, based on a 'green' approach with a minimum number of steps. The first step is an enzymatic dioxygenation of *p*-xylene to form cyclohexadiene-*cis*-diol, followed by ring opening via ozonolysis, and ring closure to form the furanone. In efforts to improve the efficiency of the enzymatic step, a directed evolution approach was taken to increase the substrate specificity and selectivity of the toluene dioxygenase enzyme system.

1. Introduction

The flavor compound strawberry furanone (**1**)[1,2] is a high value fine chemical commercially available as Furaneol® from Firmenich.[3] It is found naturally in pineapples, strawberries, mangoes, as well as in roasted or cooked foods such as malt, sesame seeds, roasted beef, and soy sauce, where it is formed via the Maillard reaction.[4] Despite this natural occurrence of **1** in fruits and cooked foods, most of the commercially available flavor compound is a non-natural, synthetic product manufactured from relatively expensive 2,5-hex-3-ynediol.[5] Herein, we report a new preparation of **1** based on a chemoenzymatic approach with a minimum number of steps from inexpensive *p*-xylene using bacterial dioxygenase enzymes for enabling the strategy and the execution of the synthesis (Fig. 1). In addition, we explore the directed evolution of these bacterial dioxygenase enzymes for increased efficiency and potential for commercial applications of the synthesis of **1** and similar flavor compounds.

Microorganisms contain aryl dioxygenases that in a single step convert aromatic hydrocarbons to corresponding diene-*cis*-diols. Substituted cyclohexadiene-*cis*-diols are interesting synthons for the manufacture of a range of fine chemicals.[6] The various functionalities and stereospecific centers of these molecules can be exploited chemically, for example through oxidations, cycloadditions, ring opening and ring closure reactions, to generate precursors for synthetic applications. Economic application of microbial dioxygenases for the production of substituted cyclohexadiene-*cis*-diols has been hampered by limited volumetric productivity.

Directed evolution is a technology that allows for the diversification of enzymes into biocatalyst platforms by

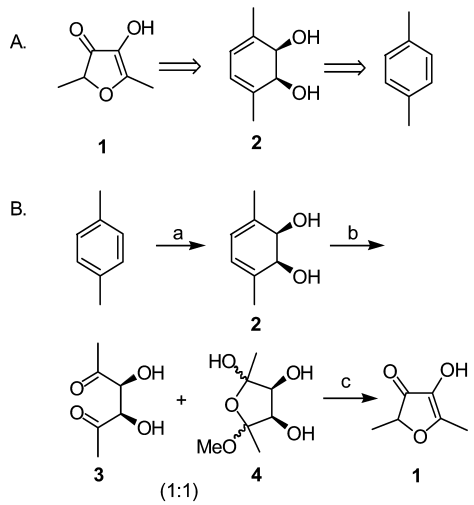

Figure 1. A novel route to strawberry furanone was envisioned in which the *p*-xylene derived *cis*-1,2-dihydroxy-3,6-dimethyl-cyclohexa-3,5-diene is converted in a short reaction sequence to the desired product. B. The strategy and execution of the synthesis. Conditions (a) *E. coli* JM109 (pTrctodNK1); (b) O_3, MeOH; then aq. $Na_2S_2O_3$, $NaHCO_3$ (84–92%); (c) *n*BuOAc, H_2O, phosphate buffer, 95 °C, 5 h (39–40%).

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recombination of homologous gene sequences that encode similar enzymes.[7] These platforms can be screened for desired biocatalyst characteristics such as substrate specificity, specific activity, enantioselectivity, regioselectivity, substrate tolerance (to allow conversions at economically relevant substrate concentrations), product tolerance (to prevent inhibition of conversion as the conversion approaches completion), and half-life time of the enzyme. The technology is applicable to single genes, encoding homomeric enzymes, multiple genes, encoding heteromeric enzymes or enzyme pathways, and even whole genomes.[8]

Aryl dioxygenases are multi-subunit, heteromeric, iron containing enzyme complexes that catalyze the incorporation of both atoms of molecular oxygen into aromatic hydrocarbons.[9] Toluene and biphenyl dioxygenase oxidize their substrates toluene and biphenyl to *cis*-1,2-dihydroxy-3-methyl-cyclohexa-3,5-diene and *cis*-1,2-dihydroxy-3-phenyl-cyclohexa-3,5-diene, respectively.[9,10] Directed evolution of toluene dioxygenase has resulted in improved substrate specificity,[11] while biphenyl dioxygenase has been evolved for remediation purposes by extending its substrate range.[12,13]

Aryl dioxygenases are encoded by four genes that constitute a gene cluster or operon. The four genes encode each of the two subunits of the oxygenase component, ferredoxin and ferredoxin reductase[14] (Fig. 2A). In all reported aryl dioxygenase enzyme improvement studies to date, only the large subunit of the oxygenase was subjected to directed evolution.[11–16] While this subunit is generally regarded as the main contributor to substrate specificity,[17–19] all components of the enzyme complex can in principle represent the rate-limiting step in substrate oxidation. In this report, we present the first directed evolution studies on a multi enzyme complex in which all components were subjected to recombination of genetic diversity. It is expected that the improved biocatalysts that are discovered in this program will enable novel chemical processes at commercial scale. One such new application is the synthesis of 3(2*H*)-furanone flavor compounds. Here we describe the application of dioxygenase to a novel route for strawberry furanone synthesis starting from *p*-xylene.

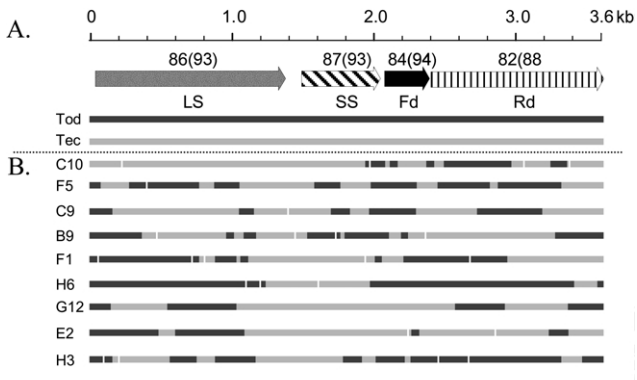

Figure 2. A. Genetic maps of the toluene (*todC1C2BA*) and tetrachlorobenzene dioxygenase (*tecA1A2A3A4*) gene clusters. The operons are approximately 3.6 kb in length and encode the oxygenase large subunit (LS, encoded by *todC1* and *tecA1*, respectively), the oxygenase small subunit (SS, *todC2* and *tecA2*), a ferredoxin (Fd, *todB* and *tecA3*) and reductase (Rd, *todA* and *tecA4*). Homology between the genes and encoded enzymes is given in percentages at the nucleotide sequence level and amino acid sequence level (in parentheses) above the gene arrows. B. Composition of the chimeric dioxygenase clusters for nine improved dioxygenase clones. Sequence derived from *todC1C2BA* is in dark grey, sequence derived from *tecA1A2A3A4* is in light grey, point mutations due to the PCR process are indicated as white lines.

2. Results and discussions

2.1. Directed evolution of aryl dioxygenase

Multigene DNA shuffling was applied to the complete toluene dioxygenase and tetrachloro-benzene dioxygenase operons to improve the biotransformation of a range of aromatics. The four gene operons encoding toluene and tetrachlorobenzene dioxygenase,[20] *todC1C2BA* and *tecA1A2A3A4*, were shuffled (Fig. 2A). The shuffled library was pre-screened according to the tier 1 assay (see Section 4) in *E. coli* DH5α for active clones using the indole oxidation assay[21] and was 70% active. Restriction enzyme digests of 24 clones from the library indicated that 23 of the randomly picked clones were chimeras of the parental genes (results not shown).

The diene-*cis*-diol resulting from *p*-xylene oxidation is of interest as a starting material for a novel synthetic route to strawberry furanone as described below. Active clones from the first tier assay were therefore, evaluated for the ability to oxidize *p*-xylene to *cis*-1,2-dihydroxy-3,6-dimethyl-3,5-cyclohexadiene. A high throughput screen was developed in which *p*-xylene toxicity to the bacterial host was circumvented by supplying the substrate in a slow-release system (Fig. 3). The amount of diol produced was compared to the amount of product produced by the parental clones. In total, 1044 different dioxygenases were screened and nine exhibited 2 to 3.5-fold higher activity when compared to the best parent.

The best nine clones were retested in multiples of eight (Table 1). The greatest improvement in activity was that of clone C10, which had a 4.4-fold higher activity. It is known that the details of a screening procedure may have an effect

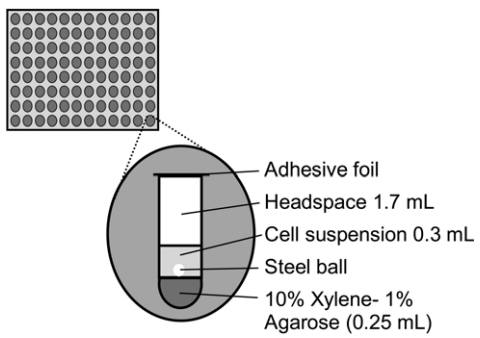

Figure 3. High throughput screen for *p*-xylene oxidation. A 96-well system was developed in which toxicity of high *p*-xylene concentrations was prevented by supplying the substrate from a slow-release formulation. Cell suspensions were added on top of the semi-solid substrate and efficient mixing was achieved by shaking with a 1 mm steel ball. Evaporation of substrate was prevented by sealing the plates with an adhesive aluminum foil. Head space over the reaction mixture was sufficient to provide oxygen for the bioconversion. Upon termination of the reaction after 1 h of incubation, the presence of diene-*cis*-diol was measured spectrophotometrically.

Table 1. Apparent activity[a] of diol formation in parental and family shuffled clones

	DH5α clones 2×YT			LS5218 clones minimal media		
	Average activity	sd[b]	Fold improvement	Average activity	sd	Fold improvement
tod	0.830	0.134	1.0	1.64	0.292	1.00
tec	0.663	0.070		1.24	0.550	
B9	1.59	0.259	1.9	1.10	0.242	(0.7)
C9	3.06	0.970	3.7	1.45	0.178	(0.9)
C10	3.67	0.894	4.4	4.12	0.683	2.5
E2	2.18	0.402	2.6	2.46	0.761	1.5
F1	3.03	0.451	3.7	3.40	0.743	2.1
F5	2.85	0.404	3.4	2.16	0.389	1.3
H3	2.99	0.902	3.6	3.59	1.13	2.2
H6	2.38	0.481	2.9	3.51	1.10	2.1
G12	2.63	0.438	3.2	2.88	0.706	1.8

[a] Apparent activity=μmol/min mL whole cells. Activities are normalized to optical density of cell suspensions.
[b] Standard deviation.

on the outcome of such screen. To test the influence of the host cell background and the growth medium on the activity of the shuffled dioxygenases, plasmid purified from the nine clones and the parental clones were transformed into *E. coli* LS5218. The transformants were tested for activity on *p*-xylene after growth in minimal media with fructose as carbon source. Improved activity was reconfirmed for seven of the nine shuffled clones, although the extent of such improvements were reduced to 1.3- to 2.5-fold. This indicates that either the host strain or the growth conditions or both have a direct impact on overall dioxygenase activity. The exact cause of this effect remains to be determined.

The same nine clones were examined for improved activity on a range of substituted benzene derivatives (Fig. 4). Depending on the substrate, among the nine clones improvements ranged from 2.7-fold (on *m*-xylene) to 16-fold on cumene. In addition to *p*-xylene, clone C10 exhibited the highest activity on *m*-xylene, 1,2,3-trimethylbenzene and naphthalene, while clone F1 showed the highest activity on cumene and toluene. DNA sequence analysis of these nine clones indicated that significant diversification had occurred (Fig. 2B). Clone C10 consisted primarily of the *tec* sequence, with significant amounts of *tod* sequence in the reductase gene only. Surprisingly, the large dioxygenase subunit in clone F1 consisted primarily of *tod* sequence where the *tod* operon was shown to have less activity on toluene than the *tec* operon. This could indicate that for oxidation of toluene, the large subunit is not necessarily the limiting factor. On this substrate, activity does not seem to be limited by the large subunit of the complex.

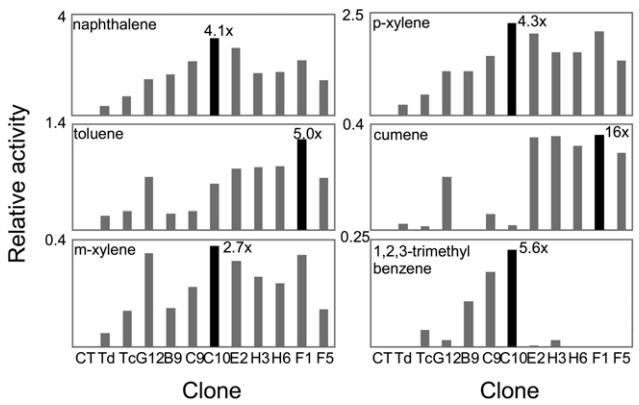

Figure 4. Substrate specificity of nine clones that were improved for oxidation of *p*-xylene. Clones were examined individually for activity on the indicated substrates. Relative activity is measured as the UV absorbance of the supernatant of cultures of each clone at the λ_{max} for each product diene-*cis*-diol. Note that the absolute activity varies per substrate. The clone with highest activity, with respect to the best parent, is indicated by the black bar with the fold improvement noted.

The availability of a wide range of highly active dioxygenase enzymes may enable the commercial application of these catalysts. One novel application would be in the manufacture of 3(2*H*)-furanone flavor compounds.

2.2. Dioxygenase enabled route to strawberry furanone

A novel route to strawberry furanone was envisioned in which the *p*-xylene derived diene-*cis*-diol is converted in a short reaction sequence to the desired product (Fig. 1A). Our chemoenzymatic approach as well as the execution of the synthesis are outlined in Figure 1B.

Biotransformation of *p*-xylene by whole cells of *E. coli* strains JM109 (pTrctodNK1) or LS5218 (pTrctodNK1) expressing toluene dioxygenase produced **2**. Cultures were grown in minimal media with fructose as the carbon source and *p*-xylene was fed as a vapor introduced into the air stream. Fed-batch cultures of 1.0 and 5.0 L volume yielded 20 and 88 g of **2**, respectively, and which was purified from cell-free culture broth by extraction. Synthesis of **2** up to 0.2 g/L had been reported for *Pseudomonas* mutants that were unable to convert *p*-xylene beyond the diol.[10]

The *meso* diol was subjected to ozonolysis in MeOH at −78 °C. When the solution turned a light blue color, it was quenched with aqueous thiosulfate/bicarbonate, and the mixture was extracted with ethylacetate. A semisolid was obtained in ~90% yield. NMR analysis of this material indicated the desired molecule as a 1:1 mixture of the diketone **3** and its diastereomeric methoxyketal–hemiketal **4**.[22] As similar mixtures were obtained when this sequence was performed on the acetonide derived from **2**, protection of the diol was not required. The crude ozonolysis products were dissolved in *n*-butylacetate and added to phosphate buffer. The mixture was heated at 95 °C for 5 h, then allowed to stand for 12 h. Furanone **1** was isolated by extraction with ethylacetate or by steam distillation. Its

physical and spectral properties were identical to those of a commercial sample. Consistent yields of ca. 40% were attained with *n*-butylacetate, even with varying cosolvents/buffers and temperature regimes. The entire sequence can be successfully performed on a 5 g scale without purification of intermediates. Further optimization at the process level will provide for a potential commercial process for this important flavor additive.

3. Conclusions

Improved dioxygenase biocatalysts were generated through multigene DNA shuffling of the entire dioxygenase gene cluster. Because such improvements were generated by recombining different fragments of the dioxygenase operons, we expect that further improvement of these catalysts is possible. Such improvements are desirable to further lower the cost of these diene-*cis*-diols. It is our estimate that with a product titer in the range of 60–80 g/L in the biotransformation, the novel route to strawberry furanone will be competitive with the current process. Analogous routes to other commercial flavor compounds such as 4-hydroxy-5-methyl-3(2*H*)-furanone and 5-ethyl-2-methyl-4-hydroxy-3(2*H*)-furanone can be developed based on toluene and *p*-ethyl-toluene, respectively and in fact, a range of new flavor compounds may be available through dioxygenase-mediated supply of diene-*cis*-diols (Fig. 5). As directed evolution of enzymes is applicable to provide efficient catalysts for any desired substrate, such applications are coming closer to reality.

4. Experimental

4.1. Biotransformation; purification of the cyclohexadiene-*cis*-diol; directed evolution

For biotransformation studies at the 1 and 5 L scale, clones containing pTrctodNK1 were preferred over pTrctecNK1. *E. coli* LS5218 (pTrctodNK1) was grown in a 2 L fermentor (New Brunswick BioFlo 2000, Edison, NJ), on E2[23] with R2 trace elements,[24] 0.2% yeast extract, 5 mM $MgSO_4$ and 0.5% fructose at 37 °C. Ampicillin was added to a final concentration of 100 μg/mL. Aeration was at 1.8 L/min and pH was maintained at 7.0 with KOH. The culture was grown to an OD_{600} of 3.5, at which time a 50% fructose, 6% ammonium chloride and 2% magnesium sulfate feed was initiated to maintain the dissolved oxygen at 30%. After the culture reached an OD_{600} 15, the temperature was reduced to 34 °C and 200 μM ferrous ammonium sulfate was added. The expression of toluene dioxygenase was induced by the addition of 250 μM of IPTG. The culture was grown for another 2 h, after which *p*-xylene was fed as vapor to the culture through the air stream at a flow rate of 3 L/min. The formation of *cis*-1,2-dihydroxy-3,6-dimethyl-hexa-3,5-diene, was monitored by measuring the absorbance of cell-free supernatant at a wavelength of 280 nm and estimating the concentration using an extinction coefficient of ε=6500 mol^{-1} cm^{-1}.[10]

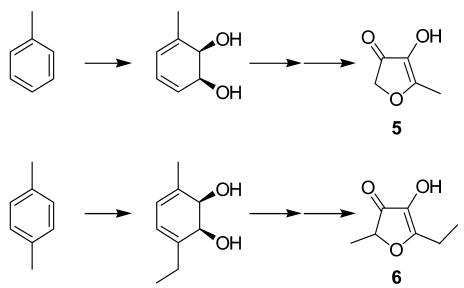

Figure 5. Analogous routes to other flavor compounds such as 4-hydroxy-5-methyl-3(2*H*)-furanone (**5**) and 5-ethyl-2-methyl-4-hydroxy-3(2*H*)-furanone (**6**) can be developed based on toluene and *p*-ethyl-toluene, respectively and in fact, a range of new flavor compounds may be available through dioxygenase-mediated supply of diene-*cis*-diols.

After the formation of the product was complete, the culture was harvested and the cells removed by centrifugation at 5,000 rpm, 20 min, at 4 °C. NaCl (280 g) was dissolved in the supernatant and the product phase extracted three times with approximately 0.6 L of ethyl acetate. The organic layers were combined and the solvent evaporated under reduced pressure to yield 20 g of nearly colorless crystals of the desired compound. The extracted samples were homogenous by TLC analysis after development in ethyl acetate giving an UV absorbance at 254 nm and staining positive with iodine vapor. The biotransformation procedure was scaled to a 5.0 L vessel (BioFlo 3000, New Brunswick, Edison, NJ) using the same medium and process conditions as described above. The 5.0 L of cell culture yielded approximately 88 g or 17.6 g/L of the desired product.

4.2. Bacterial strains and plasmid construction

P. putida F1 (ATCC 700007) was obtained from the American Type Culture Collection. The toluene dioxygenase operon *todC1C2BA* (GenBank accession number J04996) were PCR amplified using the forward primer CCATG GCTTGAAAAGTGAGAAGAC and the reverse primer TCTAGAGCGCCACCGCC TGTCACC. pSTE7 containing the tetra-chlorobenzoate dioxygenase genes from *Burkholderia* sp. PS12, was a kind gift from Dr Deitmar H. Pieper, Division of Microbiology, GBF-National Research Centre for Biotechnology, Braunschweig, Germany. The *tec* operon *tecA1A2A3A4* (GenBank accession number U78099) was PCR amplified from plasmid pSTE7 using the forward primer CCATGGCAT GAAACG TGAGGAGAC and reverse primer TCTA GAGCG CCTCCGCCCGTCACC. The PCR product was digested with *NcoI* and *XbaI*, gel purified, ligated into similarly digested expression vector pTrc99a (Amersham Pharmacia Biotech, Piscataway, NJ), and transformed into *E. coli* DH5α (Invitrogen Corp., Carlsbad, CA). Recombinant strains were maintained on LB agar plates or liquid media containing 100 μg/mL ampicillin. Strains were stored at −80 °C as 20% glycerol stocks. DNA sequences of the corresponding pTrctodNK1 and pTrctecNK1 inserts were verified by sequencing both DNA strands on an ABI 377 sequencer (Perkin–Elmer, Foster City, CA). PCR reagents were obtained from Qiagen (Valencia, CA) and restriction enzymes from New England Biolabs (Beverly, MA).

4.3. Shuffling

The two multigene operons from parent plasmids pTrctodNK1 and pTrctecNK1, were shuffled as previously

described.[25] The PCR product was digested with *NcoI* and *XbaI*, gel purified, ligated into similarly digested expression vector pTrc99a and transformed into *E. coli* DH5α. PCR product of insert DNA from randomly selected clones was digested with restriction enzyme *Hinc*II, and the restriction digest pattern compared to that of similarly digested parental PCR product to confirm that the shuffling reaction produced chimeric sequences. DNA sequences of improved shuffled clones were determined by sequencing both DNA strands on an ABI 377 sequencer (Perkin–Elmer, Foster City, CA). The sequences were compared to the parental gene sequences to confirm the formation of chimeric sequences. DNA sequences were analyzed using Sequencher version 4.0.5 (Gene Codes Corp., Ann Arbor, MI) and Vector NTI 6 (InforMax Inc., Bethesda, MD).

4.4. Screening. First tier assay

Shuffled dioxygenase libraries were initially screened for activity in vivo using the ability of the dioxygenase to transform endogenous indole to indigo.[21] Colonies containing active dioxygenase protein turn blue from the production of indigo. The shuffled libraries were transformed into *E. coli* DH5α and plated onto LB agar containing 100 μg/mL ampicillin. Plates were incubated for 20 h at 37 °C and then moved to room temperature for 24 h. The plates were checked for blue colored colonies indicating the presence of active dioxygenase. The blue color of the colonies varied from light to dark among the active shuffled clones. These active clones were picked to 96 well plates containing LB media, 100 μg/mL ampicillin, 0.2% glucose, and 20% glycerol. These master plates were incubated 20 h at 37 °C and stored at ~20 °C and used to inoculate cultures for substrate activity assays.

4.5. Second tier assay

Active shuffled clones were screened for the conversion of *p*-xylene to *cis*-1,2-dihydroxy-3,6-dimethylcyclohexa-3,5-diene in 96-well-plate format. Clones were grown in 200 μL of 2×YT media containing 100 μg/mL ampicillin and 0.2% glucose. Plate wells were inoculated with 3 μL/well of inoculum from the master plate and incubated for 20 h at 37 °C (250 rpm and 85% relative humidity (RH)). The 20-h cultures were subcultured into 2.2-mL-deep well plates containing 500 μL/well of 2×YT media containing 100 μg/mL ampicillin and 1 mM IPTG. These plates were incubated under the same conditions for 6 h. The plates were centrifuged using an Allegra 6KR centrifuge (Beckman Instruments, Fullerton, CA) (3000 rpm, 10 min, 10 °C) to pellet cells, washed once in 40 mM phosphate buffer, pH 7.0, and resuspended in 0.5 mL of 40 mM potassium phosphate buffer, pH 7.0.

The washed cell suspensions (300 μL) were transferred to a 96-deep well assay plate that contained 250 μL of solid agarose/10% *p*-xylene emulsion on the bottom of each well. The solidified agarose/substrate emulsion served as the source of volatile substrate for the biotransformation reaction. The deep well plates were sealed with Biomek Seal and Sample foil seals (Beckman Instruments, Fullerton, CA) and shaken at room temperature for 1 h. The cell suspensions were then transferred to a 96 shallow well plate, the cells pelleted (3000 rpm, 10 min, 10 °C) after which 200 μL of the supernatants were transferred to a Corning UV plate (VWR, So. Plainfield, NJ) and the absorbance of the cell-free supernatants was read at 280 and 320 nm on a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA).

4.6. Analytical methods

TLC analysis of cyclohexadiene-*cis*-diols was performed on Merck silica gel 60 F_{254} plates (2.5×7.5 cm, Aldrich, Milwaukee, WI). Aliquots of *p*-xylene transformation assays were extracted three times with an equal volume of ethyl acetate, the organic layers combined and dried under nitrogen. The extracted samples were dissolved in 20 μL ethyl acetate and developed in ethyl acetate vapor giving an UV absorbance at 254 nm and staining positive with iodine vapor. The samples were compared to cyclohexadiene-*cis*-diol standard prepared from the wild type toluene dioxygenase clone.

4.7. Oxidation by ozonolysis of an unprotected diol–diene to form a diol–dione

The *cis*-1,2-dihydroxy-3,6-dimethylhexa-3,5-diene **2** was not purified prior to ozonolysis. All reagents were purchased from Fischer Scientific (Pittsburgh, PA) and used without further purification. Sodium sesquicarbonate solution A was prepared by dissolving 84 g of $NaHCO_3$ and 53 g of Na_2CO_3 in 1 L of water. The final pH of the solution was about 10–11. 5.07 g of 1,2-dihydroxy-3,6-dimethylhexa-3,5-diene **2** (vacuum dried) was dissolved in 50 mL of MeOH and ozonized at −78 °C for 3 h (until blue color persisted) (65 kV, 3 lpm). The resulting solution was transferred via insulated canula to a solution of 21 mL of 1 M $Na_2S_2O_3$ and 13 mL of 1.5 M sesquicarbonate solution A and stirred at 0 °C. The transfer took 5 min. The resulting solution quickly became peroxide negative. Stirring was continued for 30 min at 0 °C and for another 30 min at room temperature. Methanol was distilled off under reduced pressure at 40 °C, and the resulting solution (with white precipitate) was extracted 4 times with 125 mL ethyl acetate. Combined extracts were dried over anhydrous sodium sulfate and evaporated to give colorless and practically odorless oil, which after vacuum drying partially crystallized on standing (4.92 g). This material was the desired product as a 1:1 mixture of the free 2,5-diketo-3,4-dihydroxyhexane **3** and it's methoxyketal–hemiketal **4**, with combined yields in 84–92% range.

4.8. Cyclization of an unprotected diol–dione

The following condition was used after modification of the procedure published in US Patent 5,149,840,[26] which describes an optimized cyclization of rhamnose and other 6-deoxyhexoses to **1**. Crude ozonolysis product, 800 mg, was added to buffer containing: 340 mg of $NaH_2PO_4{\times}1H_2O$, 80 mg $NaHCO_3$, 0.260 mL of H_2O, 0.100 mL of 40% NaOH. The semi-solid mixture was added to *n*-butylacetate or ethylacetate and the two-phase system was, after degassing with argon, heated in a closed vial (see Table 2). The solvent was separated from the dark-reddish-brown aqueous phase, the aqueous layer was extracted with 3×5 mL EtOAc, and the organic layer and extracts were combined, dried over anhydrous $MgSO_4$ and the solvent evaporated. All

Table 2. Summary of two-phase cyclization reactions for 4-hydroxy-3[2*H*]-furanone **1** synthesis

Mass of starting material	Solvent (volume)	Buffer, amounts of $NaH_2PO_4 \cdot H_2O$ and $NaHCO_3$	Temp. (°C)	Time (h)	Weight recovered	Ratio of **1**:**3** (by NMR)
800 mg	*n*-BuOAc (3 mL)	340 mg+80 mg	95, then rt	4–16	280 mg	4:1
1.3 g	EtOAc (3 mL)	510 mg+120 mg	Reflux ~80	22	630 mg	1:1
1 g	*n*-BuOAc (5 mL)	425 mg+100 mg	95, then rt	8–15	390 mg	3.5:1
2.6 g	*n*-BuOAc (10 mL)	170 mg+40 mg	110	22	1.3 g	3:1

experiments were repeated at least 3 times. The experiments were reproducible up to 2–6 g. The material was analyzed by TLC, ^{13}C NMR, and ^{1}H NMR with 4-hydroxy-2,5-dimethyl-2,3-dihydrofuran-3-one **1** as comparative standard.[22] Deuterochloroform was used as the solvent and D_2O exchange was also performed. The final 4-hydroxy-2,5-dimethyl-2,3-dihydrofuran-3-one **1** product is very volatile and oxidizes readily in the presence of air, as evidenced by appearance of additional spots on subsequent TLC analysis.

4.8.1. 4-Hydroxy-3,5-dimethyl-3(2*H*)-furanone-1. ^{1}H NMR (400 MHz, $CDCl_3$): 7.6 (1H), 4.5 (dq, J=7, 1 Hz, 1H), 2.26 (d, J=1 Hz, 3H), 1.44 (d, J=7 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 198.84, 174.89, 133.95, 80.02, 16.27, 13.37.

4.8.2. *cis*-1,2-Dihydroxy-3,6-dimethyl-hexa-3,5-diene-2. ^{1}H NMR (400 MHz, $CDCl_3$): 5.63 (2H), 4.06 (2H), 2.55 (2H), 1.88 (6H). ^{13}C NMR (100 MHz, $CDCl_3$): 135.14, 120.25, 72.05, 19.48. Compound **2** is unstable and dehydrates readily to 2,5-dimethylphenol.[7] This degradation product was detected in the spectra of compound **2**: ^{1}H NMR (400 MHz, $CDCl_3$): 6.97 (d, J=7.6 Hz, 1H), 6.63 (d, J=7.6 Hz, 1H), 6.59 (1H), 2.25 (3H), 2.19 (3H).

^{13}C NMR (100 MHz, $CDCl_3$): 163.8, 136.8, 130.66, 121.04, 120.68, 115.62, 20.88, 15.3.

4.8.3. 2,5-Diketo-3,4-dihydroxyhexane-3/methoxyketal–hemiketal-4 mixture: 3. ^{1}H NMR (400 MHz, $CDCl_3$): 4.38 (2H), 2.31 (6H). ^{13}C NMR (100 MHz, $CDCl_3$): 207.87, 78.53, 16.31. **4**: ^{1}H NMR (400 MHz, $CDCl_3$): 3.83 (d, J=3.5 Hz, 1H), 3.69 (d, J=3.5 Hz, 1H), 3.36 (3H), 1.4 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): 105.56, 101.8, 70.89, 66.88, 48.88, 27.03, 21.46.

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