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Investigation of the Mechanism of Action of Pyrogallol–Phloroglucinol Transhydroxylase by Using Putative Intermediates

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Dedicated to Professor Bernhard Kräutler on the occasion of his 60th birthday

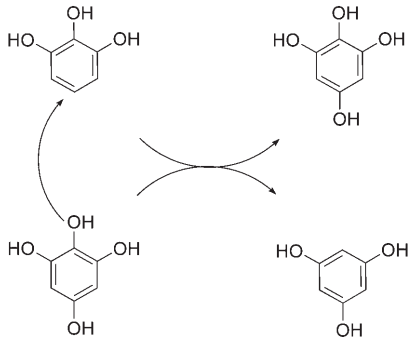
Abstract: Pyrogallol–phloroglucinol transhydroxylase from *Pelobacter acidigallici*, a molybdopterin-containing enzyme, catalyzes a key reaction in the anaerobic degradation of aromatic compounds. In vitro, the enzymatic reaction requires 1,2,3,5-tetrahydroxybenzene as a cocatalyst and the transhydroxylation occurs without exchange with hydroxy groups from water. To test our previous proposal that the transfer of the hydroxy group occurs via 2,4,6,3′,4′,5′-hexahydroxydiphenyl ether as an intermediate, we synthesized this compound and investigated its properties. We also describe the synthesis and characterization of 3,4,5,3′,4′,5′-hexahydroxydiphenyl ether. Both compounds could substitute for the cocatalyst in vitro. This indicates that the diphenyl ethers can intrude into the active site and initiate the catalytic cycle. Recently, the X-ray crystal structure of the transhydroxylase (TH) was published[16] and it supports the proposed mechanism of hydroxy-group transfer.

Keywords: enzymes · ethers · molybdopterin (Moco) · OH transfer · reaction mechanisms

Introduction

Pyrogallol–phloroglucinol transhydroxylase (TH) from *Pelobacter acidigallici* is one of the key enzymes in the anaerobic degradation of aromatic compounds like gallic acid and various phenols.[1–3] All these compounds are converted into phloroglucinol, which is then reductively dearomatized and degraded to three acetyl-coenzyme A (acetyl-CoA) molecules via 3-hydroxy-5-oxohexanoate. This pathway leads not only to important building blocks but can also be used for adenosine triphosphate (ATP) synthesis.

Schink and co-workers showed that TH contains a molybdopterin cofactor (Moco) and iron sulphur clusters [4Fe–4S] and needs 1,2,3,5-tetrahydroxybenzene as a cosubstrate, or rather as a cocatalyst because it is regenerated in the reaction cycle.[1–6] Furthermore, as shown by ^{18}O labeling, no hydroxy groups are incorporated into the product from water.[5] On the basis of these results, the reaction was formulated[4,5] as depicted in Scheme 1. The 2-OH group from 1,2,3,5-tetrahydroxybenzene is transferred to pyrogallol, whereby phloroglucinol and a new molecule of the cocatalyst are produced. Such ingenious use of a cocatalyst already has precedence in enzyme chemistry; for example, the glycolytic enzymes, phosphoglucomutase and phosphoglycerate mutase, take advantage of their cocatalysts, that is, glucose-1,6-bisphosphate and glycerate-2,3-bisphosphate, respective-



Scheme 1. The role of 1,2,3,5-tetrahydroxybenzene in the mechanism of action of the Mo-cofactor-containing pyrogallol–phloroglucinol transhydroxylase as postulated by Schink and co-workers.[4,5]

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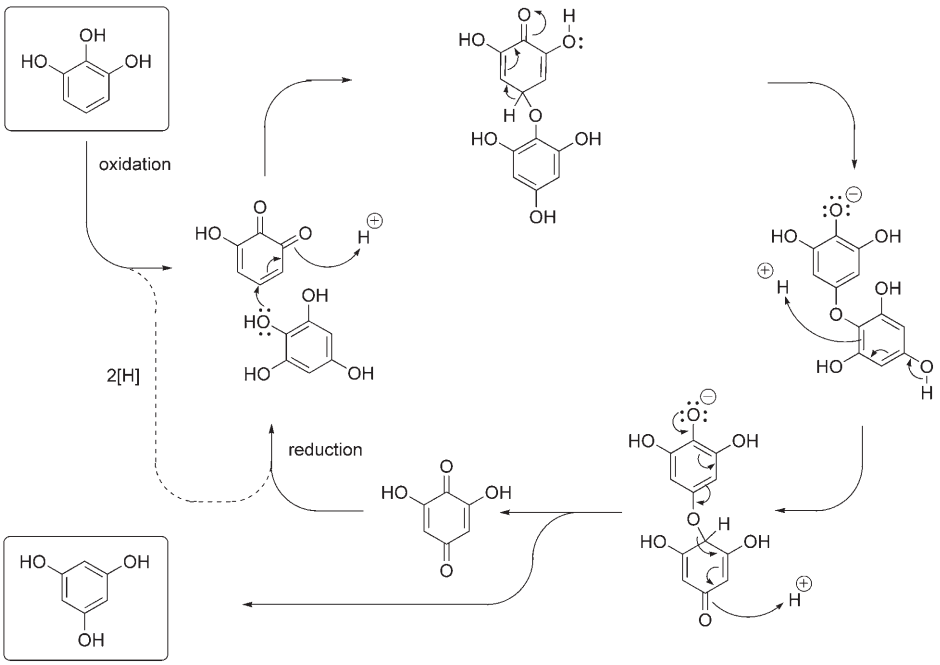


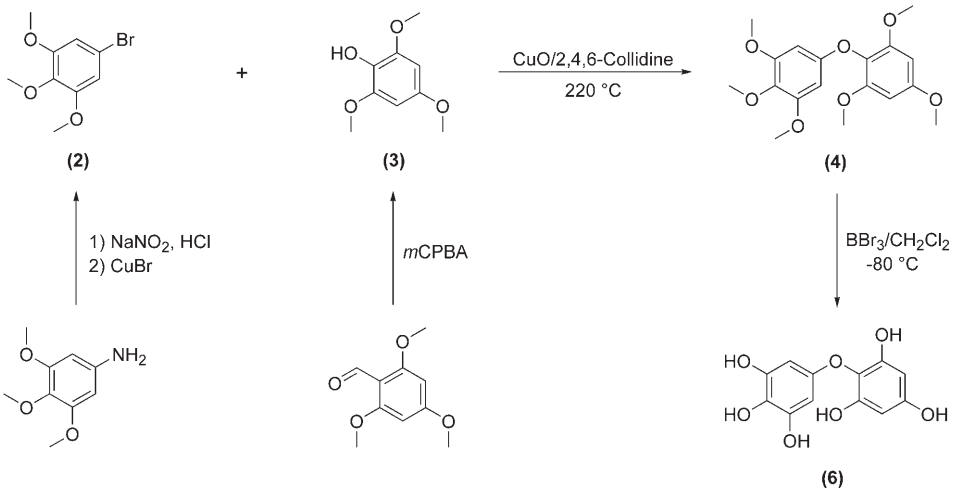
Scheme 2. Proposed mechanism for the action of the pyrogallol–phloroglucinol transhydroxylase. Oxidation of pyrogallol to the corresponding orthoquinone allows nucleophilic attack by the 2-OH group of the tetrahydroxybenzene cocatalyst.[9]

ly. In these cases, the substrates are converted into the cocatalysts by phosphate transfer, while the original cocatalyst becomes the product.[7] In the formulation of a detailed mechanism for the TH reaction, the mode of hydroxy transfer is a matter of debate. It is believed that in Moco-containing hydroxylases which use water as the hydroxy group source, the OH group is first ligated to the molybdenum and then transferred to the substrate; a similar mechanism was also discussed for the TH reaction.[8]

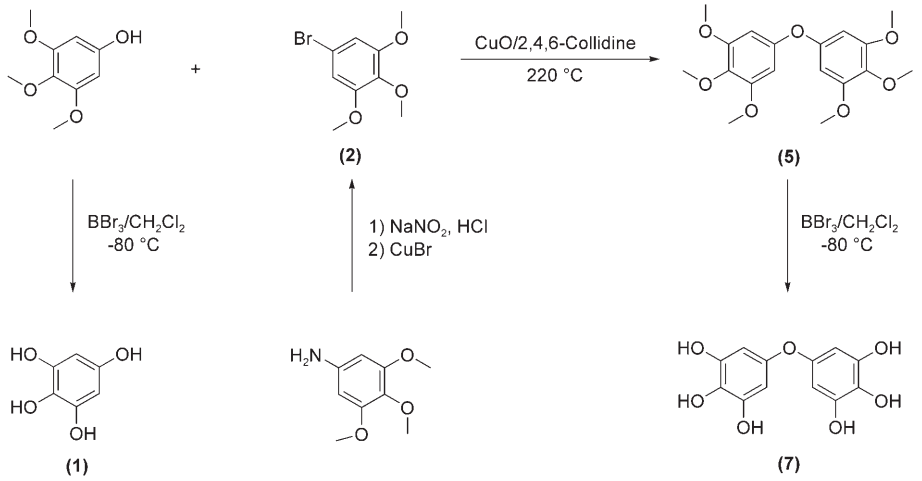
A second mechanistic proposal by Rétey and co-workers[9] (see also reference [10]) requires direct hydroxy transfer from the cocatalyst 1,2,3,5-tetrahydroxybenzene to the orthoquinone form of pyrogallol. Here, the main function of Moco is the oxidation of pyrogallol to quinone. Along the postulated pathway, an enzyme-bound 2,4,6,3′,4′,5′-hexahydroxydiphenyl ether is generated as an intermediate (Scheme 2). Fragmentation of this diphenyl ether into phloroglucinol and the orthoquinone form of tetrahydroxybenzene is followed by reduction of the latter to regenerate the cocatalyst. Here we describe the synthesis of 2,4,6,3′,4′,5′-hexahydroxy- and 3,4,5,3′,4′,5′-hexahydroxydiphenyl ether (**6** and **7**, respectively) and their interaction with the transhydroxylase.

Results and Discussion

Synthesis of hexahydroxydiphenyl ethers: 2,4,6,3′,4′,5′- and 3,4,5,3′,4′,5′-Hexahydroxydiphenyl ethers (**6** and **7**, respectively) were found as components of the ethyl acetate soluble mixture of polyhydroxyphenyl esters occurring in various kinds of algae, such as *Bifurcaria bifurcate* and *Caprophyllum maschalocarpumas*.[11,12] Only the hexaacetylated form of the diphenyl ether was isolated; the unprotected compound could not be obtained in pure form due to its oxygen sensitivity. Both **6** and **7** have previously been synthesized from the corresponding hexamethoxydiphenyl ethers by using boron tribromide as a demethylation agent,[13] but the products were not isolated and were transformed into dibenzofurans.[14] The syntheses of **6**, **7** and 1,2,3,5-tetrahydroxybenzene (**1**) are presented in Schemes 3 and 4. Using modern HPLC methods, we successfully purified these compounds. The hexahydroxydiphenyl ethers were characterized for the first time by spectroscopic methods (^{1}H and ^{13}C NMR spectroscopy and mass spectrometry). They had to be handled under an inert gas atmosphere.



Scheme 3. Synthesis of the proposed intermediate, 2,4,5,3′,4′,6′-hexahydroxydiphenyl ether (**6**). *m*CPBA = *meta*-chloroperbenzoic acid.



Scheme 4. Synthesis of 1,2,3,5-tetrahydroxybenzene and symmetrical 3,4,5,3′,4′,5′-hexahydroxydiphenyl ether (**7**).

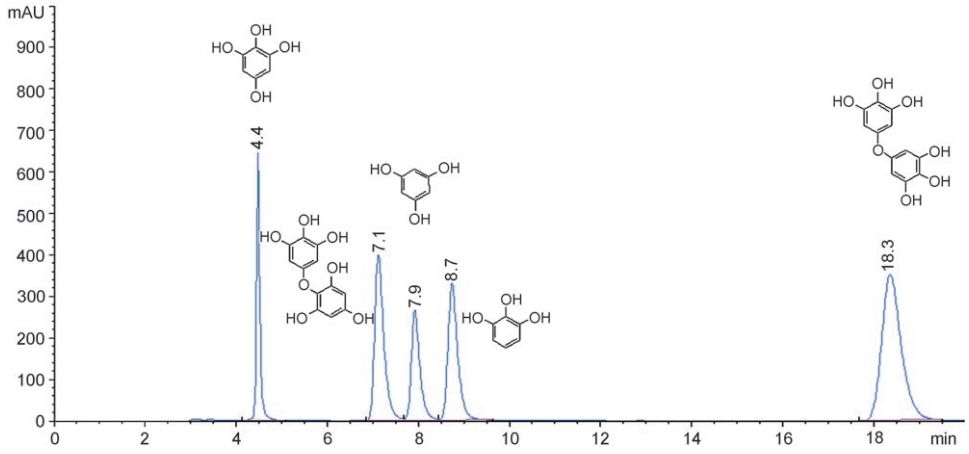


Figure 1. Elution diagram of 1,2,3,5-tetrahydroxybenzene, pyrogallol, phloroglucinol, and the unsymmetrical and symmetrical hexahydroxydiphenyl ethers for reference. 1 μmole of each compound was applied to the column.

Interaction of the hexahydroxydiphenyl ethers with pyrogallol–phloroglucinol transhydroxylase: TH is a heterodimeric enzyme consisting of a large α and a small β subunit with 875 and 275 amino acid residues, respectively. On the basis of its amino acid sequence, TH was considered to be a member of the dimethylsulfoxide reductase family.[15] More recently, the X-ray crystal structure of TH has been published.[16] Since recombinant TH could not be expressed in the active form,[15] the enzyme isolated directly from *Pelobacter acidigallici* was used in all experiments.

Since there is no measurable difference in the UV absorption of pyrogallol and phloroglucinol, an enzyme assay was developed that relied on HPLC analysis. Under the conditions used, the retention times for authentic **1**, **6**, phloroglucinol, pyrogallol, and **7** were approximately 4.4, 7.1, 7.9, 8.7, and 19.3 min, respectively (Figure 1). In some cases, small deviations from these values were observed.

When pure transhydroxylase, isolated as described by Schink and co-workers,[5] was incubated with pyrogallol only, no reaction occurred as monitored by HPLC. In the presence of **1**, phloroglucinol was produced; after a longer incubation (15 min) at 30 °C, the concentration of **1** was only slightly decreased (Figure 2), as it was regenerated in the reaction cycle. This observation is in good agreement with the proposed role of **1** as the cocatalyst. The enzyme assay is a modification of that described by Schink and co-workers.[4–6] Based on the areas under the HPLC traces and the amounts of the pyrogallol (10 μmol) and enzyme (0.1 mg) used in the reaction, the enzyme activity can be estimated to be 2 $\mu mol\,min^{-1}\,mg^{-1}$ enzyme. As some oxidation of the extremely oxygen-sensitive polyhydroxyphenols was unavoidable, the limit of error is estimated to be about 10%. To investigate whether the transhydroxylase will accept **6** as a cocatalyst or intermediate, the latter compound and pyrogallol were added to the enzymatic reaction mixture (see the Experimental Section and Figure 3). Under these conditions, as predicted, phloroglucinol and **1** were formed. After 60 min of reaction time, about 90% of the pyrogallol and **6** had been converted into phloroglucinol and the postulated paraquinone form of 1,2,3,5-tetrahydroxybenzene. The latter is supposed to remain enzyme bound and will be reduced by pyrogallol and serve as the cocatalyst, as depicted in Scheme 2 (Note that usually no intermediates are seen in the HPLC analyses, except when they are added to the reaction mixture; however, **1** can leak out from the active site of the enzyme). On the basis of the areas under the HPLC traces and the amounts of the pyrogallol, hexahydroxydiphenyl ether, and enzyme, it is estimated that in the enzymatic reaction about 2 μmol of **6** and pyrogallol were consumed per minute. In other words, the kinetic competence of this hexahydroxydiphenyl ether as an intermediate in the reaction is shown.

In a further experiment, **6** was incubated with transhydroxylase. In this case no reaction occurred, but when an equimolar amount of glutathione was introduced into the

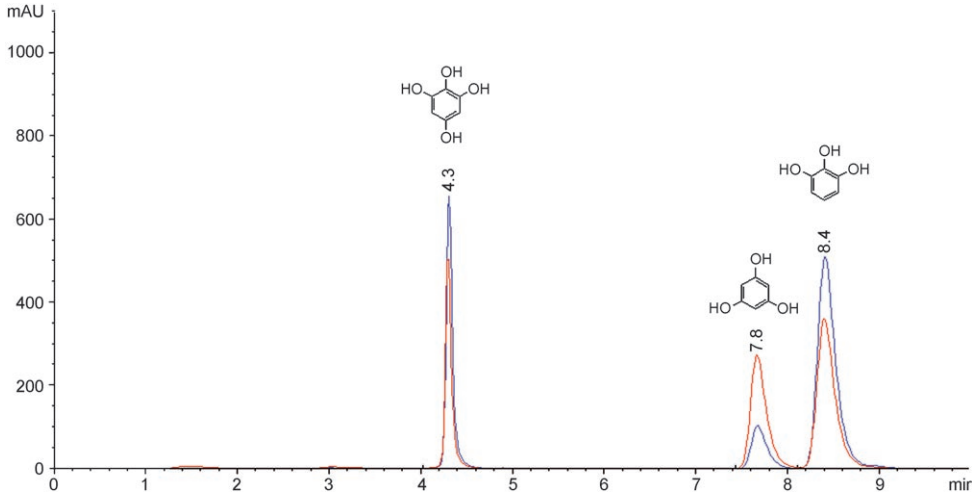


Figure 2. Elution diagram of the enzyme assay with pyrogallol and 1,2,3,5-tetrahydroxybenzene; after 5 min = blue trace, after 15 min = red trace. For the conditions of the assay, see the Experimental Section.

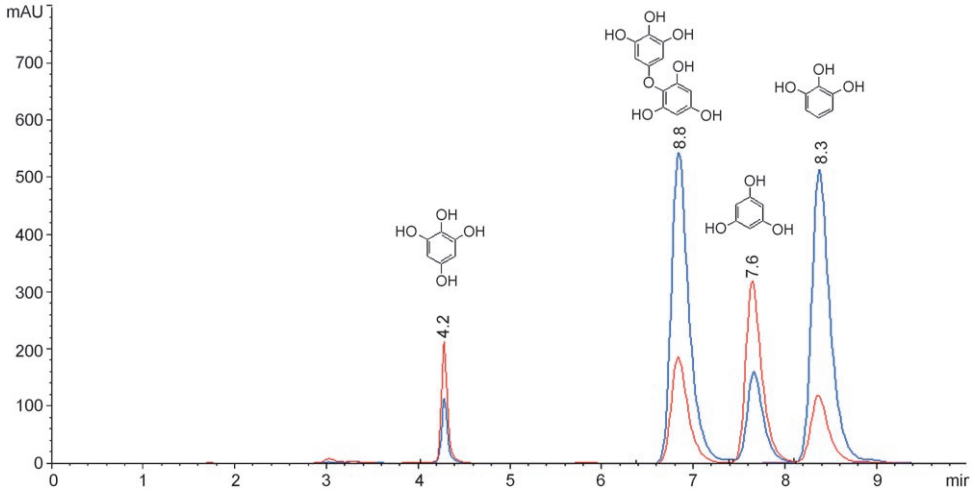


Figure 3. Elution diagram showing the conversion of pyrogallol into phloroglucinol and 1,2,3,5-tetrahydroxybenzene by transhydroxylase in the presence of 2,4,6,3′,4′,5′-hexahydroxydiphenylether; after 15 min = blue trace, after 1 h = red trace.

After 4 h, more phloroglucinol was formed and the amount of pyrogallol was reduced by about 90 % (Figure 5), while only a relatively small portion of **7** was consumed. Accordingly, only a very small amount of **1** can be seen in Figure 5 (peaks between 4–5 min). The low concentration of the cocatalyst may be the reason for the sluggish reaction in this experiment. The reactions illustrated in Figures 4 and 5 were also performed on a preparative scale; the isolated products showed the same analytical data as the reference **1** and phloroglucinol. These results suggest that the symmetrical hexahydroxydiphenyl ether can also intrude into the active site of the enzyme and is cleaved into pyrogallol and the paraquinone form of **1**, although much more slowly than its unsymmetrical counterpart (Scheme 5). In other words, the symmetrical hexahydroxydiphenyl ether does not have the kinetic competence to be a genuine intermediate in the reaction.

Thus, our results support the intermediacy of **6** in the TH reaction and, consequently, the mechanism previously proposed by us (Scheme 2). At least in this case, the formerly postulated role of Moco as an OH-transfer agent must be revised. Therefore, the two alternative mechanisms considered by Hille et al.[9] are practically ruled out.

enzymatic reaction mixture, phloroglucinol and **1** were formed (Figure 4). These results show that a reducing agent is necessary to keep the reaction going, as according to Scheme 2 the cleavage of the hexahydroxydiphenyl ether leads, in addition to phloroglucinol, to the enzyme-bound paraquinone form of **1**. In the absence of pyrogallol, glutathione can reduce the enzyme-bound quinone to provide the cocatalyst.

The symmetrical **7** could also play the role of a cocatalyst for TH. Its retention time (around 18 min) was, however, completely different from that of its unsymmetrical counterpart (Figures 1 and 5). Ether **7** alone was completely inert with transhydroxylase and no detectable amount of phloroglucinol was found, even when an equimolar amount of glutathione was added to the reaction mixture. Addition of pyrogallol initiated a reaction, which produced phloroglucinol.

The recently published X-ray crystal structure of the TH–1,2,4-trihydroxybenzene complex[16] is consistent with the mechanism discussed here (Scheme 2). It is now possible to speculate about the catalytic roles of some amino acid residues found at the active site (Asp174, His144, Tyr404; Scheme 6).[16] The 1-OH group of pyrogallol coordinates to the Mo^{VI} atom of Moco (stage a) and is oxidized to the orthoquinone form, while His144 accepts the phenolic proton (stage b). This oxidation may take place in two steps with Mo^{V} and the semiquinone as intermediates. In the next step, the 2-OH group of **1** adds to the quinone in a Michael-type reaction, which is assisted by proton transfer from Asp174 (stage **c**). Subsequently the substrate–cocatalyst adduct is tautomerized with the assistance of Tyr404, whereby the

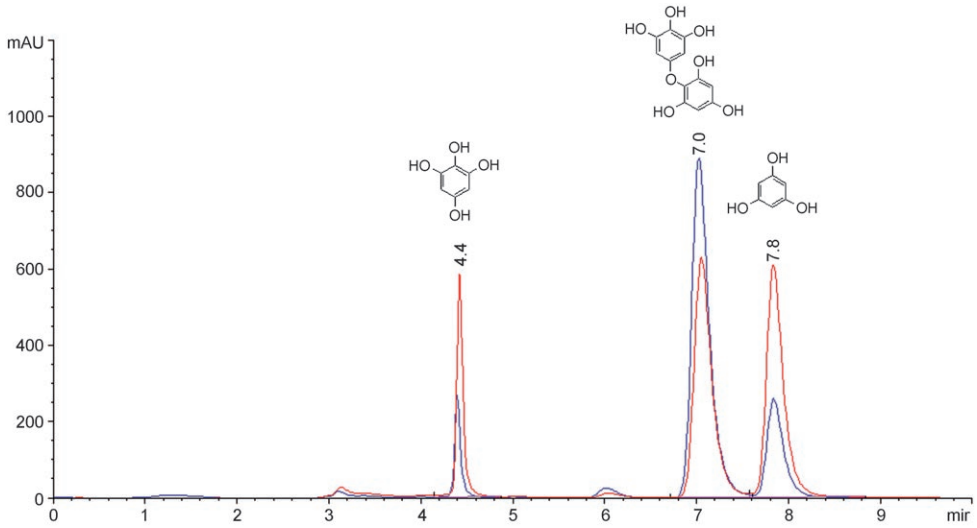


Figure 4. Elution diagram showing the cleavage of 2,4,6,3′,4′,5′-hexahydroxydiphenyl ether catalyzed by transhydroxylase in the presence of glutathione as a reducing agent; after 15 min = blue trace, after 1 h = red trace. The produced phloroglucinol and 1,2,3,5-tetrahydroxybenzene were isolated and identified by mass spectrometry and ^{1}H and ^{13}C NMR spectroscopy.

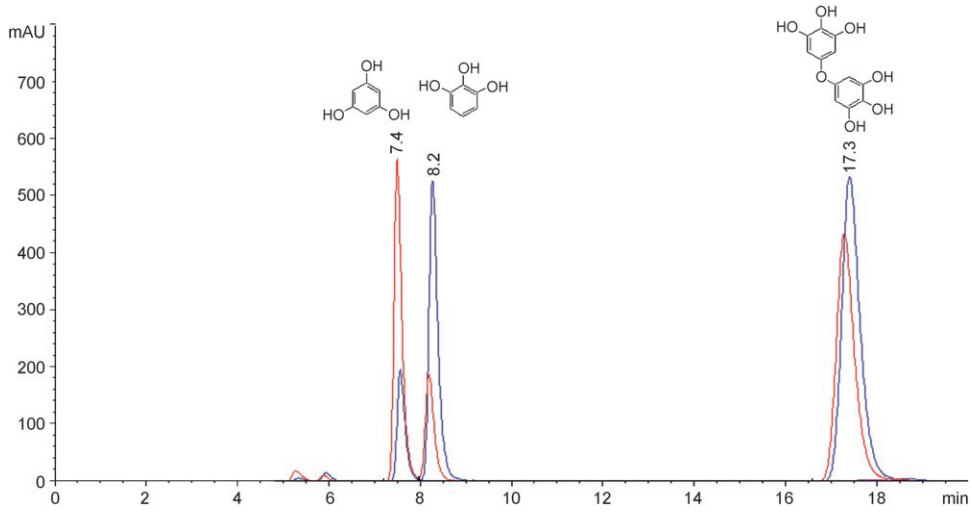
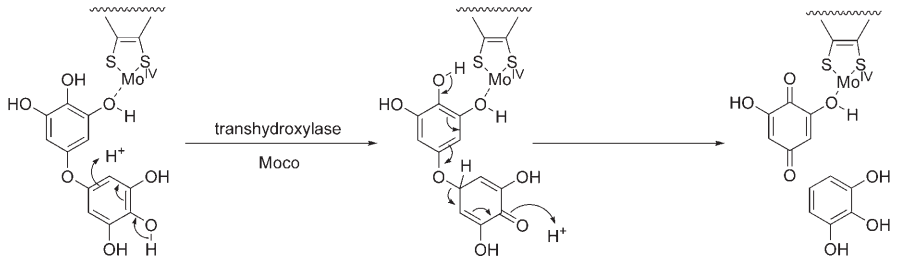


Figure 5. Elution diagram showing the conversion of pyrogallol by transhydroxylase in the presence of 3,4,5,3′,4′,5′-hexahydroxydiphenyl ether; after 1 h = blue trace, after 4 h = red trace. The latter is consumed according to the mechanism outlined in Scheme 5. The produced phloroglucinol was isolated and identified by mass spectrometry and ^{1}H and ^{13}C NMR spectroscopy.



Scheme 5. Proposed mechanism of the cleavage of the 3,4,5,3′,4′,5′-hexahydroxydiphenyl ether (**7**) to 2,6-dihydroxyparaquinone and pyrogallol catalyzed by the transhydroxylase.

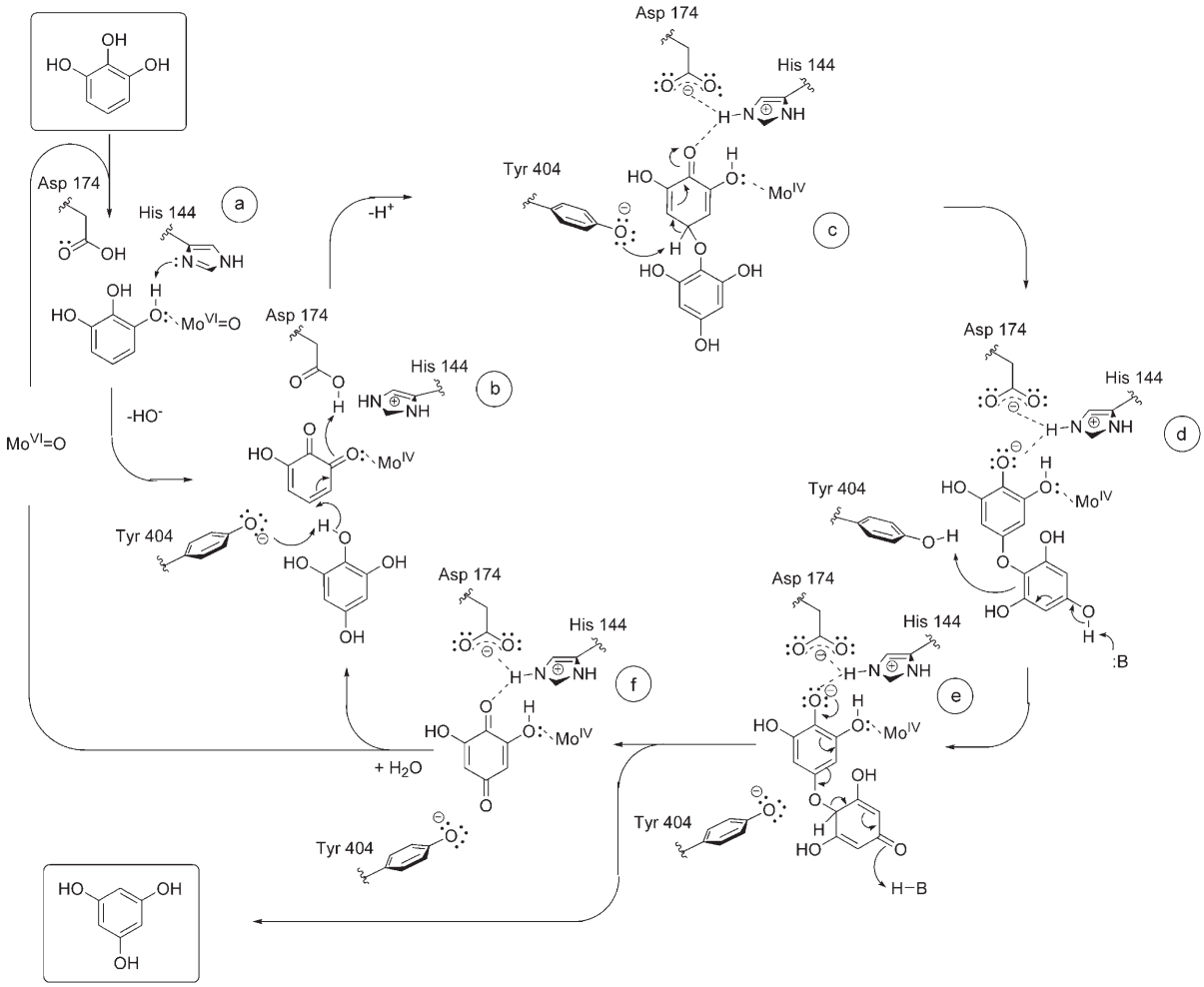
proposed intermediate **6** is formed (stage d). The 3(5)-OH group is still coordinated to the Mo^{IV} atom. The next step involves the tautomerization of the "lower" portion of the intermediate (originating from tetrahydroxybenzene) with the assistance of Tyr404 as a proton donor and an undefined enzymatic base as a proton acceptor (stage e). The adduct is now prepared for cleavage to phloroglucinol and the paraquinone form of the tetrahydroxybenzene. In this step, the previously implied undefined enzymatic base returns the proton to form the product. In the last stage (f) of the catalytic cycle, the paraquinone form of the tetrahydroxybenzene is reduced by Mo^{IV}, thus returning the reaction to stage b.

In conclusion, the roles assigned to the three active-site amino acids are plausible but remain uncertain until confirmed by mutation analysis.

Experimental Section

Enzymatic experiments: Transhydroxylase was isolated as previously described.[6] The enzyme assay was carried out under anoxic conditions at 30 °C in a discontinuous fashion by HPLC analysis of the products. All ingredients were stored and transferred under argon.

The concentrations of compounds in the assay mixture were as follows: 100 mM potassium phosphate buffer (pH 7.2), 10 μmol pyrogallol,



Scheme 6. Proposed mechanism of action of the pyrogallol–phloroglucinol transhydroxylase[9] and the supposed role of certain active-site amino acid residues.[16]

10 μmol 1,2,3,5-tetrahydroxybenzene, and 0.1 mg of the enzyme. The total volume of the enzymatic assay was 1 mL. Samples (20 μL) were withdrawn with a unimetric pipette, the reaction was terminated by adding 0.1 M H_3PO_4 (5 μL), and the samples were diluted 10 times with double-distilled water before injection. The withdrawals occurred soon after the start of the reaction and then at regular intervals. All enzymatic reactions were carried out under anaerobic conditions. In the case of reactions in which pyrogallol was incubated with transhydroxylase, no reducing agent was required in the enzymatic assays. Conditions for the HPLC analyses: Nucleodur 100–5 C8 ec column with 20 mM HCl in water/acetonitrile (95:5 v/v) as the eluent in an isocratic manner at a 1 mL min^{-1} flow rate. Chromatograms were recorded at 218 nm. The assays with hexahydroxydiphenyl ethers were similar to the enzyme assay described above.

For preparative purposes, an enzymatic assay with a volume of 10 mL was used and, after the reaction was stopped with 0.1 M H_3PO_4, the mixture was heated under argon to 80 °C for 5 min, cooled, and centrifuged to separate the precipitated protein. The supernatant was injected in 2-mL portions on a preparative RP-18 column and eluted with 20 mM HCl in water/acetonitrile (95:5 v/v) in an isocratic manner at a 5 mL min^{-1} flow rate. Fractions of subsequent separations with the same retention time (9–12 min for 1,2,3,5-tetrahydroxybenzene and 16–19 min for phloroglucinol) were combined and the products were obtained after freeze drying.

Analytical methods: The ^1H and ^{13}C NMR spectra were recorded on a Bruker spectrometer operating at 400 and 100 MHz, respectively. Mass spectra were recorded on a VG 7070E mass spectrometer. HPLC analyses were conducted with an HP 1050 instrument.

Reagents and solvents: 2,4,6-Trimethoxybenzaldehyde, 3,4,5-trimethoxyaniline, 3,4,5-trimethoxyphenol, all inorganic reagents, and solvents were from Aldrich or Fluka. All solvents were purified and dried by standard methods as required.

Synthesis of 3,4,5-trimethoxybromobenzene (2): A solution of $Na_2SO_3 \cdot 7H_2O$ (8.57 g, 34 mmol) in water (20 mL) was added dropwise to a stirred solution of $CuSO_4 \cdot 5H_2O$ (16.48 g, 66 mmol) and NaBr (10.3 g, 100 mmol) in water (60 mL). For better precipitation of CuBr, the suspension was cooled to 0 °C. After 30 min, the supernatant was decanted and the precipitate was washed with cold water (2 × 30 mL). The suspension was rendered clear by addition of concentrated HBr (26 mL) and was stored under N_2. 3,4,5-Trimethoxyaniline (9.16 g, 50 mmol) was added to a half-concentrated HBr water solution (160 mL) in one portion at room temperature. The suspension was heated to 70 °C to obtain a clear solution, which was then cooled to 0 °C. Subsequently, a solution of $NaNO_2$ (3.45 g, 50 mmol) in water (30 mL) was added dropwise while the temperature was kept below 5 °C. The reaction went to completion in 30 min and then a small portion of urea was added to destroy the unreacted nitrous acid. The previously prepared CuBr solution was added to the solution of the diazonium salt at 0 °C under argon. The reaction mixture was allowed to warm to room temperature and was then heated to 100 °C. The cooled mixture was extracted with diethyl ether (3 × 200 mL) and the combined organic layers were washed with HCl solution (20 %, 3 × 100 mL), then with water (3 × 100 mL), and finally with KOH

solution (20%, 3×100 mL). The organic phase was dried over anhydrous Na_2SO_4 and filtered, then the solvent was removed under vacuum. The crude product was purified by column chromatography on silica gel by using dichloromethane as the eluent; this yielded pure 3,4,5-trimethoxybromobenzene (9.25 g, 75%).

Synthesis of 2,4,6-trimethoxyphenol (3): A solution of 3-chloroperoxybenzoic acid (*m*CPBA, 8.6 g, 50 mmol) in dichloromethane (140 mL) was added dropwise to a cooled (ice-bath) solution of 2,4,6-trimethoxybenzaldehyde (5 g, 25.5 mmol) in dry dichloromethane (30 mL) over approximately 90 min. The reaction was complete after 3 h at room temperature. The reaction mixture was washed with saturated $NaHCO_3$ solution (3× 25 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The crude product was dissolved in methanol (50 mL) and cooled to 0°C, then KOH solution (5 g in 25 mL water) was added within 20 min. The reaction was complete in 45 min at 0°C. The pH value of the solution was adjusted to 2 by addition of HCl solution (2 M). The reaction mixture was extracted with diethyl ether (3×200 mL), the etheric phase was washed with water (3×100 mL), and the organic layer was dried over anhydrous Na_2SO_4. The solvent was removed by distillation under reduced pressure and the crude product was purified by column chromatography on silica gel by using a dichloromethane:acetone mixture (95:5 v/v) as the eluent. The pure 2,4,6-trimethoxyphenol (2.34 g, 50%) was crystallized in 2–3 d.

Preparation of hexamethoxydiphenyl ethers 4 and 5: 2,4,6-Trimethoxyphenol (**3**) or 3,4,5-trimethoxyphenol (2.46 g, 13.4 mmol), 1-bromo-3,4,5-trimethoxybenzene (**2**; 6.62 g, 26.8 mmol), and CuO (4.3 g, 30 mmol) were refluxed in 2,4,6-collidine (35 mL) for 63 h under argon. The cooled reaction mixture was treated with half-concentrated HCl (concentrated HCl/water 1:1, 150 mL) and extracted with diethyl ether (4×200 mL). The combined organic layers were washed with half-concentrated HCl (500 mL), then with KOH solution (20%, 750 mL), and finally with water. The dried organic layer was concentrated under reduced pressure and the crude product was purified by column chromatography on silica gel (dichloromethane/acetone (90:10 v/v)) to yield **4** or **5** (according to the starting material).

*2,4,6,3′,4′,5′-Hexamethoxydiphenyl ether (**4**)*: Amorphous white powder; yield 41%; ^{1}H NMR (CD_3OD): δ=3.75 (s, 6H), 3.78 (s, 3H), 3.79 (s, 6H), 3.84 (s, 3H), 6.12 (s, 2H), 6.23 ppm (s, 2H); ^{13}C NMR (CD_3OD): δ=55.5, 55.9, 56.3, 60.9, 91.8, 92.2, 125.9, 132.6, 153.6, 153.9, 155.3, 157.6 ppm; HRMS: *m*/*z*: calcd for $C_{18}H_{23}O_7$: 351.1444 $[M+1]^+$; found: 351.1450.

*3,4,5,3′,4′,5′-Hexamethoxydiphenyl ether (**5**)*: Amorphous white powder; yield 48%; ^{1}H NMR (CD_3OD): δ=3.67 (s, 12H), 3.81 (s, 6H), 6.28 ppm (s, 4H); ^{13}C NMR (CD_3OD): δ=56.2, 61.0, 96.3, 134, 153.4, 153.9 ppm; HRMS: *m*/*z*: calcd for $C_{18}H_{23}O_7$: 351.1444 $[M+1]$; found: 351.1446.

Preparation of 1,2,3,5-tetrahydroxybenzene (1) and of the hexahydroxydiphenyl ethers 6 and 7: Boron tribromide solution in dichloromethane (1 M, 18.8 mL) was added to a solution of 3,4,5-trimethoxyphenol and hexamethoxydiphenyl ether **4** or **5** (1.88 mmol) in dichloromethane (10 mL) at −80°C under argon. After being stirred for 1 h at −80°C, the reaction mixture was left to warm to room temperature. After 10 h, the solution was cooled to 0°C and water (5 mL) was added. After removal of the dichloromethane under reduced pressure, the remaining mixture was extracted with ethyl acetate (3×50 mL). The combined organic phases were dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The crude product was treated with water (20 mL) and the suspension was centrifuged. The supernatant was decanted and purged with argon. A portion (2 mL) of the mixture was purified on a preparative RP-18 column by using HCl (20 mM) in water/acetonitrile (95:5 v/v) as the eluent at a 5 mL min^{-1} flow rate. Fractions with retention times between 9–14 min for **1**, 30–45 min for **6**, and 20–35 min for **7** were collected and the pure products were obtained after freeze drying.

*1,2,3,5-Tetrahydroxybenzene (**1**)*: Yield 52%; ^{1}H NMR (D_2O): δ= 5.93 ppm (s, 2H); ^{13}C NMR (D_2O): δ=95.5, 125.4, 145.3, 146.4 ppm; HRMS: *m*/*z*: calcd for $C_6H_6O_4$: 142.0247 $[M+1]^+$; found: 142.0266.

*2,4,6,3′,4′,5′-Hexahydroxydiphenyl ether (**6**)*: Amorphous white powder; yield 58%; ^{1}H NMR (D_2O): δ=5.92 (s, 2H), 5.98 ppm (s, 2H); ^{13}C NMR (D_2O): δ=94.2, 94.8, 123.9, 127.1, 146, 151.1, 152.1, 154.6 ppm; HRMS: *m*/*z*: calcd for $C_{12}H_{11}O_7$: 267.0505 $[M+1]$; found: 267.0505.

*3,4,5,3′,4′,5′-Hexahydroxydiphenyl ether (**7**)*: Yield 78%; HRMS: *m*/*z*: calcd for $C_{12}H_{11}O_7$: 267.0501 $[M+1]^+$; found: 267.0505; ^{1}H NMR (D_2O): δ=5.97 ppm (s, 4H); ^{13}C NMR (D_2O): δ=97.7, 128.3, 146.1, 150.5 ppm.

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