Biocatalysis

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Biocatalytic Properties and Structural Analysis of Phloroglucinol Reductases

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Dedicated to Professor Georg Fuchs

Abstract: *Phloroglucinol reductases (PGRs) are involved in anaerobic degradation in bacteria, in which they catalyze the dearomatization of phloroglucinol into dihydrophloroglucinol. We identified three PGRs, from different bacterial species, that are members of the family of NAD(P)H-dependent short-chain dehydrogenases/reductases (SDRs). In addition to catalyzing the reduction of the physiological substrate, the three enzymes exhibit activity towards 2,4,6-trihydroxybenzaldehyde, 2,4,6-trihydroxyacetophenone, and methyl 2,4,6-trihydroxybenzoate. Structural elucidation of PGRcl and comparison to known SDRs revealed a high degree of conservation. Several amino acid positions were identified as being conserved within the PGR subfamily and might be involved in substrate differentiation. The results enable the enzymatic dearomatization of monoaromatic phenol derivatives and provide insight into the functional diversity that may be found in families of enzymes displaying a high degree of structural homology.*

Phloroglucinol (**1**) mainly occurs as a structural element in natural products such as flavonoids and phlorotannins. It is also found as a discrete molecule synthesized by a type III polyketide synthase in *Pseudomonas protegens* Pf-5 (Scheme 1).[1] Monocyclic compound **1** is an intermediate in the degradation of many flavonoids and tannins and is used by several anaerobic fermenting bacteria as a sole carbon source.[2–5] The anaerobic degradation of **1** into dihydrophloroglucinol (**2**) requires an initial NADPH-dependent reduction, which is followed by a hydrolytic ring cleavage (Scheme 1). The dearomatization reaction is enabled by the 1,3,5-arrangement of the three hydroxy groups of **1**, which results in a tendency towards distinct tautomerization and thus favors nucleophilic attack on the oxo carbon groups.[6] Indeed, Lohrie and Knoche reported up to 10 tautomeric forms of **1** in aqueous solution.[7] Such biocatalytic dearomatization reactions are frequently found in the biosynthesis of secondary metabolites as a tool for deoxygenation, which in this case would result in resorcinol (**3**; Scheme 2).[8] Patel et al. have shown the NADPH-dependent reduction of **1** into **2** by a partially purified phloroglucinol reductase (PGR) from *Coprococcus* sp., which has a molecular weight of about 130 kDa. The supposed partial enzyme activity was also measured in the presence of menadione.[9] Fermentative degradation of gallic acid (**4**), pyrogallol (**5**), 2,4,6-trihydroxybenzoic acid (**6**), and **1** was reported in a pure culture of *Pelobacter acidigallici* gen. nov. sp. nov.[10,11] Here too, the pathway was assumed to proceed via **2**.[10] Analogous anaerobic catabolism of **1** and **4**–**6** was observed with *Eubacterium oxidoreducens* (Scheme 1).[3,12,13] Haddock and Ferry have described the purification of a PGR from the cell extract of *E. oxidoreducens*. The described enzyme, which has a native molecular weight of 78 kDa (homodimer), was specific for **1** and NADPH. Moreover, a short N-terminal amino acid motif (NCBI reference: P57793.1) has been determined.[14]

We have previously reported short-chain dehydrogenase/reductase (SDR)-catalyzed reductions of (polyhydroxylated) naphthalene and anthracene derivatives.[8,15–18] The enzymatic dearomatization of polyhydroxylated benzenes should be possible in a similar manner. Hence, we focused on the identification and characterization of PGRs, which catalyze the dearomatization of phenolic, monoaromatic compound **1**. For the identification of putative PGRs, we applied a biogenetic approach based on information from previous studies.[9,10,13,14,19,20] The published genomes of *Clostridium* sp. and *Eubacterium* sp. were used as a template. A putative SDR from *Clostridium* sp. ATCC BAA-442 (NCBI reference sequence: WP_021630531.1; denoted here as PGRcl) was conspicuous due to its proximity to a putative cyclase (WP_007491232.1) and a putative 3-hydroxybutyryl-CoA dehydrogenase (WP_021630532.1), both of which are potentially involved in the degradation of **1**.[12] The putative cyclase probably corresponds to the dihydrophloroglucinol hydrolase annotated by Krumholz et al., which is supposed to be involved in the ring cleavage of **2** (Scheme 1).[12] *Clostridium* sp. ATCC BAA-442 was isolated by Hur et al. from a human fecal sample and is known for degradation of the isoflavonoid daidzein.[21]

The codon-optimized gene *PGRcl* was cloned into the pET19b expression vector, which carries an N-terminal His-tag, and overexpressed in *Escherichia coli* BL21(DE3). The His-tagged protein (PGRcl-his) was then purified by Ni-NTA affinity chromatography (see the Supporting Information). To check for activity, **1** was incubated on an analytical scale with

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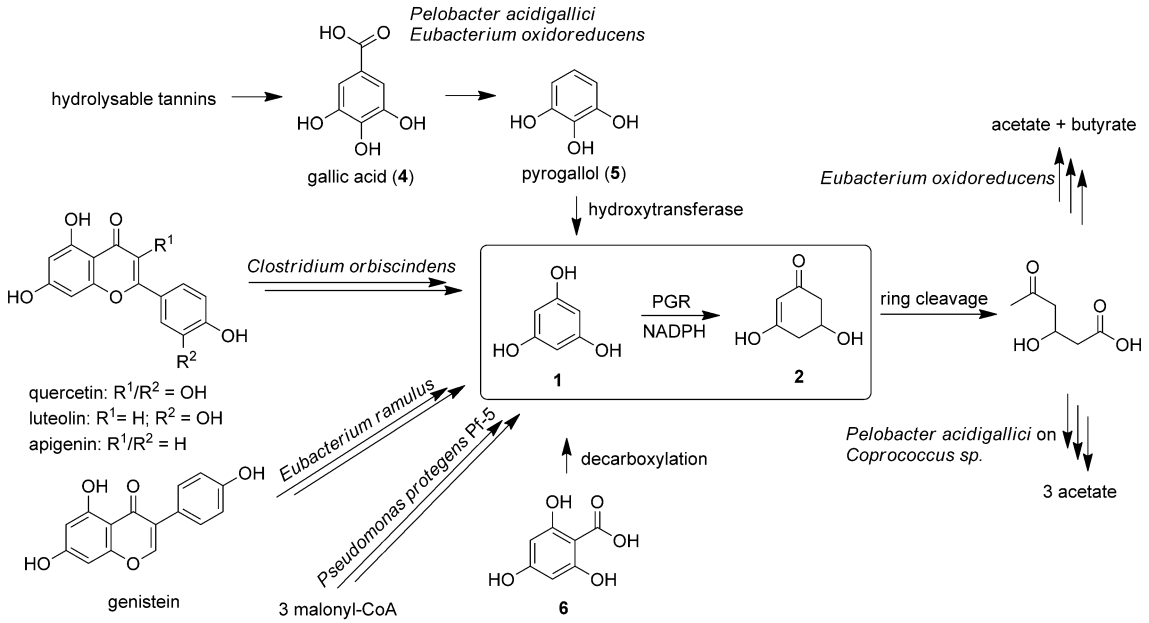
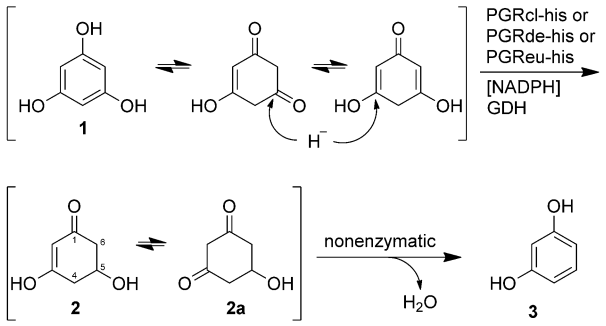


Scheme 1. Phloroglucinol (**1**) is a biosynthetic product from *P. protegens* Pf-5 and a central intermediate in the degradation of flavonoids and tannins. It is further degraded to acetate and butyrate units.[1,3,4,9,10,12–14,19,20,22–24]



Scheme 2. Enzymatic reduction of phloroglucinol (**1**) with PGRcl-his, PGReu-his, or PGRde-his, using glucose dehydrogenase (GDH)/D-glucose as an NADPH regeneration system. Resorcinol (**3**) arises through nonenzymatic water elimination from dihydrophloroglucinol (**2**).

PGRcl-his and $NADP^+$, using glucose dehydrogenase/D-glucose as an NADPH regeneration system (Scheme 2). After 18 hours, the reaction was stopped with phosphoric acid and the mixture extracted with ethyl acetate.

A conversion of 37% of **1** into dihydrophloroglucinol (**2**) was determined by ^{1}H NMR analysis. Transformation of **1** on a preparative scale (2.4 mmol) resulted in 90% conversion (see the Supporting Information). Enol **2** and its tautomer 5-hydroxycyclohexane-1,3-dione (**2a**) were observed in the ^{1}H NMR spectrum ([D_6]acetone) in a ratio of 5:1 (**2**/**2a**), which is in accordance with reported values.[25] Resorcinol (**3**) was observed in the ^{1}H NMR spectrum in trace amounts (<5%), probably as a result of nonenzymatic water elimination and rearomatization.

We assumed that **2** was achiral owing to rapid tautomerism, which leads to a coincidence of H-4 and H-6 in the ^{1}H NMR spectrum (Scheme 2 and the Supporting Information). Experiments using NADPD showed the incorporation of deuterium at C-5 of **2** (see the Supporting Information). Therefore, the reduction mechanism is proposed to be a common carbonyl reduction of a keto tautomer of **1** or a Michael-addition-like β-reduction of an α,β-unsaturated ketone, but it is as yet impossible to differentiate these two mechanisms.

In order to identify further PGRs, PGRcl homologues were sought by using the parental amino acid sequence and the genetic environment within the genome sequence of different organisms. Putative dehydrogenases of unknown function from *Eubacterium* sp. AB3007 (WP_027868985.1, PGReu) and *Desulfosporosinus orientis* (WP_014184752.1, PGRde), with pairwise sequence identities of 83% and 57% to PGRcl, respectively, and a similar genetic environment (see Scheme S1 in the Supporting Information) were cloned, heterologously expressed, and purified in the same way as described for PGRcl-his. Phloroglucinol (**1**) was accepted as a substrate by both enzymes, yielding **2** with conversions of 36% for PGReu-his and 30% for PGRde-his (Scheme 2).

This functional description of three PGRs allows the designation of further PGRs from bacteria that are known to degrade natural products with **1** as a structural element. The quercetin-degrading bacterium *Clostridium orbiscindens* (*syn. Flavonifractor plautii*) and the genistein-degrading bacterium *Eubacterium ramulus* (Scheme 1) possess promising dehydrogenases (WP_007489454.1, WP_021737919.1) with 99% and 88% amino acid sequence identity to PGRcl, respectively, again in the same genetic environment as described above.[20,22]

Partially purified PGRs from *E. oxidoreducens* and *Coprococcus* sp. have been described as being highly specific for **1**.[9,14] Nevertheless, menadione also seems to be converted

by the enzyme from *Coprococcus* sp.[9] PGRcl-his, PGReu-his, and PGRde-his were individually applied to 26 different compounds, including bicyclic compounds such as flaviolin and 2-tetralone, and several aromatic and nonaromatic monocyclic compounds, to check for possible reductase activity. Surprisingly, none of the compounds were converted by any of the three enzymes (Figure S1 in the Supporting Information). Menadione and 1,4-naphthoquinone showed false-positive results because they were converted nonenzymatically in the presence of the reduced cofactor NADPH. Regarding the compounds tested so far, we propose that a 1,3,5-arrangement of the hydroxy groups is essential for being accepted by PGRs. Therefore, the phloroglucinol derivatives **7**–**9**[26] were tested and were found to be converted in the same fashion as **1** (Table 1). Nevertheless, the

Table 1: Enzymatic reduction of phloroglucinol derivatives **7**–**9** by PGRcl-his, PGReu-his, and PGRde-his.

Substrate	Product	Enzyme		Conversion[a] [%]
		PGRcl-his	**7a**	29
			8a	8
			9a	11
		PGReu-his	**7a**	64
			8a	n.c.
			9a	14
		PGRde-his	**7a**	82
			8a	11
			9a	40
7: R = H **8:** R = CH_3 **9:** R = OCH_3	**7a:** R = H **8a:** R = CH_3 **9a:** R = OCH_3			

[a] Conversions were determined by ^{1}H NMR analysis of the crude product; n.c. = no conversion.

conversion of acetophenone **8** into the corresponding dihydro product **8a** was significantly diminished. PGReu-his did not convert **8** under the tested conditions. Product **8a**, a rare natural product from the fungal endophyte *Nodulisporium* sp., induces chlorosis in Japanese barnyard millet.[27]

The above-noted deuterium transfer onto **1** was repeated with substrate **7** using PGRde-his, and the deuterium was incorporated accordingly at C-4 of **7a** (see the Supporting Information). The formation of enantiomerically enriched **7a**–**9a** might be expected owing to a hydrogen bond between the carbonyl and the enolic hydroxy group, which probably hinders tautomerization (five ^{1}H NMR signals for the aliphatic hydrogens of **7a**–**9a** relative to three signals for **2**). The transformation of **7** with PGRde-his was performed on a preparative scale (71% conversion), with subsequent purification and circular dichroism (CD) measurement of product **7a**. The resulting spectrum shows no CD effect, thus the enzymatic products **7a**–**9a** are probably racemic (see the Supporting Information). Formation of the racemic products **7a**–**9a** might be explained by variable orientation of the substrates in the active site, or by a nonselective tautomerization step in the case of a Michael-type addition.

The identification and heterologous production of the three phloroglucinol reductases enabled substrate characterization with purified enzymes, which broadened the substrate range (**1**, **7**–**9**) and allowed menadione to be excluded as a substrate. Nevertheless, PGRs possess an unusually narrow substrate range in comparison to other SDRs, such as the tri- and tetrahydroxynaphthalene reductases (T_3HNR, T_4HNR). The latter are known for the reduction of a variety of compounds (Figure S1 in the Supporting Information); however, they do not share any common substrates with the PGRs.[8,15] Although PGRs and T_3HNR/T_4HNR share the common catalytic function of participating in dearomatization reactions, they have crucially different biological functions. T_3HNR and T_4HNR are involved in the secondary metabolism of fungi, and therefore display high substrate promiscuity.[28,29] In contrast, PGRs are involved in the primary metabolism of bacteria, where they participate in the anaerobic degradation of **1** as a carbon source. Moreover, and in regard to structural comparison within the SDR family, substrate promiscuity and substrate specificity are probably determined by variations in the flexible C-terminal segment.[30,31]

Despite the low pairwise sequence identities between SDRs (about 10–30%) in general, they still share a highly similar three-dimensional structure within the α/β-folding pattern known as the Rossmann fold for cofactor binding.[32–34] To check for differences in the active site between PGRs (monocyclic substrates) and T_3HNR/T_4HNR (bicyclic substrates), especially in the C-terminal segment, the three-dimensional structures of PGRcl in the apo form and with bound NADPH were determined to 1.7 Å and 1.8 Å resolution, respectively. The enzyme crystallizes as a homotetramer (125 kDa), with two monomers in the asymmetric unit. Its NADPH binding motif (G_{24}-X-X-G_{27}-X-X-G_{30}) differs slightly from that of T_3HNR/T_4HNR (G-X-X-X-G-X-G), but shares the Y_{167}-X-X-X-K_{171} motif typical for SDRs, which is complemented by S_{154} and D_{125} to form the catalytic tetrad that mediates catalysis.[33] The C-terminal substrate binding loop spans 33 amino acids (T_{199}–E_{231}) and is thus considerably shorter than that of T_3HNR/T_4HNR (43 amino acids). In the apo form, the loop attains a highly flexible open-state conformation, leaving a wide cleft open for the substrate. Upon cofactor binding, it adopts a semi-closed state, fixing the nucleotide in place but still leaving the substrate binding site open, as seen in the NADPH-bound structure, where multiple loop conformations could be modelled. Owing to a lack of suitable inhibitors, PGRcl could not be crystallized in the closed state. Nevertheless, sequence and structure alignments of T_3HNR and PGRcl suggest the amino acids W_{208}–E_{213} in the C-terminal loop and Y_{164} on the opposite site as prime candidates for examination in further studies.

In summary, we have identified and characterized three different PGRs from anaerobic fermenting bacteria that exhibit a narrow substrate range comprising **1** and its derivatives **7**–**9**. Accordingly, substrates for these PGRs need to consist of a monoaromatic compound with a 1,3,5-arrangement of unsubstituted hydroxy groups. In a patent application, Frost et al. have reported the sequence of a PGR from *E. oxidoreducens*.[35] Our rational approach is validated by the protein sequence identified by Frost et al., which shows 51–76% amino acid sequence identity to PGRcl, PGReu, and PGRde.

Our results broaden the scope of enzymatic dearomatization reactions and provide access to the biocatalytic synthesis of industrially useful compounds, such as the phenolic compound **3**, which is a parent substance for several dyes and polymers.[36–38] In particular, our structural elucidation of PGRcl contributes to a more profound understanding of related SDRs and now allows further investigation of the underlying structure–activity relationships within SDRs. Moreover, our results provide the basis for the identification of other SDRs that could be applicable to the reduction of monocyclic aromatic compounds and enable the identification of gene clusters in organisms that are considered to be involved in the degradation of **1** or its derivatives.

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Keywords: dearomatization · enzyme catalysis · flavonoids · redox enzymes · reductases

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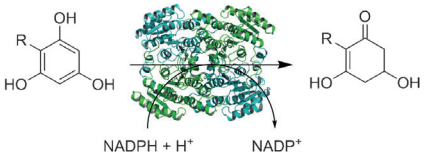
Biocatalytic Properties and Structural Analysis of Phloroglucinol Reductases



R = H; $COOCH_3$; CHO; $COCH_3$

United in diversity: Three phloroglucinol reductases (SDRs) were discovered that show distinct and narrow substrate specificity compared to other structurally highly homologous SDRs, with a 1,3,5-arrangement of hydroxy groups being essential. The results enable the enzymatic dearomatization of monoaromatic phenol derivatives and provide insight into the functional diversity that may be found in families of enzymes displaying high structural homology.