# Antioxidative galloyl esters as enzyme inhibitors of *p*-hydroxybenzoate hydroxylase

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**Abstract Gallic acid and its esters were evaluated as enzyme inhibitors of recombinant *p*-hydroxybenzoate hydroxylase (PHBH), a NADPH-dependent flavin monooxygenase from *Pseudomonas aeruginosa*. *n*-Dodecyl gallate (DG) ($IC_{50}$ = 16 μM) and (−)-epigallocatechin-3-*O*-gallate (EGCG) ($IC_{50}$ = 16 μM), a major component of green tea polyphenols, showed the most potent inhibition, while product-like gallic acid did not inhibit the enzyme significantly ($IC_{50}$ > 250 μM). Inhibition kinetics revealed that both DG and EGCG inhibited PHBH in a non-competitive manner ($K_I$ = 18.1 and 14.0 μM, respectively). The enzyme inhibition was caused by specific binding of the antioxidative gallate to the enzyme, and by scavenging reactive oxygen species required for the monooxygenase reaction. Molecular modeling predicted that EGCG binds to the enzyme in the proximity of the FAD binding site via formation of three hydrogen bonds.** 

*Key words:* *p*-Hydroxybenzoate hydroxylase; Flavin monooxygenase; Enzyme inhibition; Galloyl ester; (−)-Epigallocatechin-3-*O*-gallate; Molecular modeling

## 1. Introduction

*p*-Hydroxybenzoate hydroxylase (PHBH) (EC 1.14.13.2) from *Pseudomonas* sp. is a well-characterized flavin monooxygenase that catalyzes the conversion of *p*-hydroxybenzoate (PHB) (**1**) to 3,4-dihydroxybenzoate (**2**) [1]. PHBH is a homodimer of two identical subunits with a molecular mass of 45 kDa, each containing one FAD molecule. The flavoprotein-mediated aromatic hydroxylation is initiated by a random sequential binding of the two substrates, PHB and NADPH, to the flavoenzyme, and proceeds via formation of a flavin C(4a)-hydroperoxide intermediate [2] (Fig. 1). Mechanistic studies on enzyme kinetics, chemical transients in catalysis, substrate and flavin analogs have been extensively performed [1]. Further, X-ray crystallography [3,4] and site-directed mutagenesis [5,6] have revealed structural details of the monooxygenase reaction.

In the course of our studies on squalene epoxidase (SE) (EC 1.14.99.7), another flavoprotein monooxygenase catalyzing a rate-limiting step of cholesterol biogenesis [7], we found that esters of gallic acid (3,4,5-trihydroxybenzoate) (**8**), such as naturally occurring (−)-epigallocatechin-3-*O*-gallate (EGCG) (**3**) ($IC_{50}$ = 0.69 μM), a major component of green tea polyphenol, and synthetic *n*-dodecyl gallate (DG) (**5**) ($IC_{50}$ = 0.061 μM), were potent enzyme inhibitors of recombinant rat SE [8,9]. The SE inhibition was highly selective; they did not show significant enzyme inhibition toward other enzymes involved in cholesterol biosynthesis including a cytochrome P-450 monooxygenase, lanosterol 14α-demethylase (CYP51) ($IC_{50}$ > 100 μM). From these observations, we postulated that the potent SE inhibition was caused by specific binding of galloyl esters to the flavoenzyme, possibly in the proximity of the FAD binding domain, and by scavenging reactive oxygen species required for the monooxygenase reaction.

In this paper, we describe that the galloyl esters also showed good inhibition toward recombinant PHBH from *Pseudomonas aeruginosa* [10]. The amino acid sequence of *P. aeruginosa* PHBH shows ca. 20% identity to that of rat SE. In particular, the two flavoenzymes share significant sequence similarities around the so-called GxGxxG motif (β1-sheet–α-helix–β2-sheet folding) and GD motif, both involved in the FAD binding (Fig. 2) [11]. Further, in order to test our hypothesis, a molecular modeling study with the published 3D crystal structure of the enzyme was also carried out.

## 2. Materials and methods

### 2.1. Cloning and expression of recombinant PHBH

The PHBH used in this study was cloned by polymerase chain reaction (PCR) from *P. aeruginosa* (strain PAO1C; ATCC 15692). The PCR was performed on the chromosomal DNA in the presence of 5% dimethyl sulfoxide, primed by specific N-terminal and C-terminal primers; 5′-AGAGTTGGATCCGCAATGAAGACTCAA-3′ and 5′-GAATTCCTACTCGAGTTCCTCGTA-3′, respectively, containing the *Bam*HI and the *Xho*I sites, designed on the basis of the reported DNA sequence (GenBank, accession number M23173) [10]. Thus, a recombinant PHBH with an additional hexahistidine tag at the C-terminal was subcloned into the *Bam*HI–*Xho*I large fragment of expression vector pET-22b(+) (Novagen). After confirmation of the sequence, the vector plasmid was transformed into *Escherichia coli* BL21(DE3)pLysS. The recombinant enzyme was then expressed by isopropyl β-D-thiogalactopyranoside induction and purified by Ni-chelate affinity chromatography according to the manufacturer's protocol.

### 2.2. Enzyme inhibition assay

Inhibitors were dissolved in 10 μl of water (EGCG) or 20% ethanol in water (alkyl gallates), and preincubated at 25°C for 5 min. The assay mixture contained in a total volume of 1 ml, 50 mM Tris–HCl, pH 8.0, the recombinant PHBH (0.02 U), 100 μM PHB, and 200 μM NADPH. The enzyme reaction was started by addition of NADPH, and incubated at 25°C for 10 min, which was monitored by measuring UV absorption at 340 nm. For the inhibition kinetics, the $K_I$ values were determined using the same conditions as described above. The experiments were carried out in duplicate using three concentrations of inhibitor (0, 1/2×[$IC_{50}$] and 1×[$IC_{50}$]). For each inhibitor concentration, substrate was added to give four substrate concentrations: 25, 33, 50 and 100 μM.

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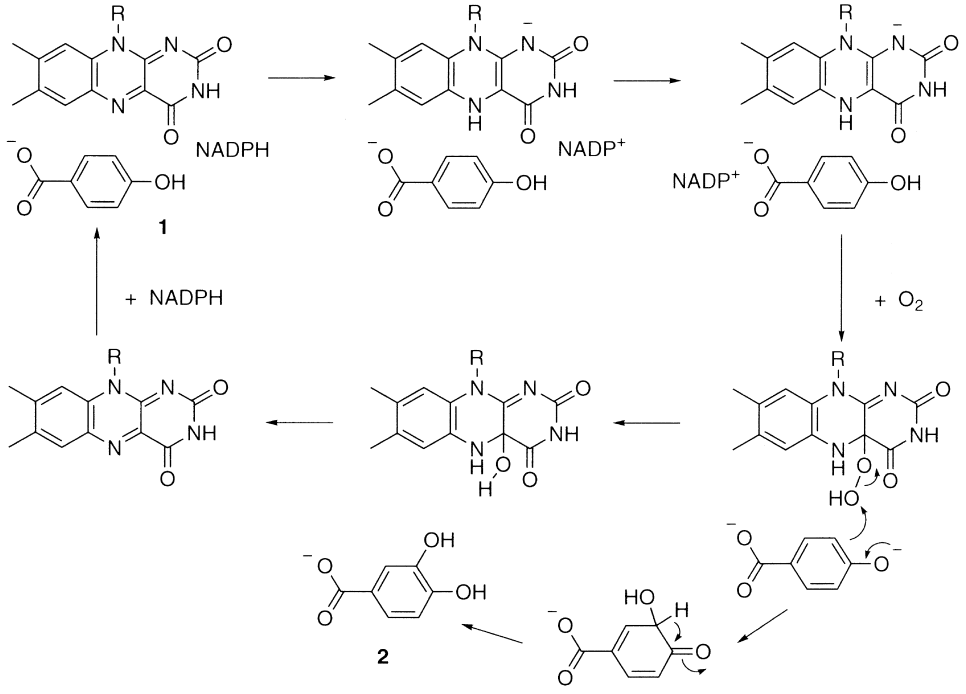


Fig. 1. Proposed mechanism of hydroxylation of PHB (**1**) to 3,4-dihydroxybenzoate (**2**).

*2.3. Molecular modeling*

The structure of PHBH was downloaded from Protein Data Bank (identification code 1PBE) into X-PLOR (ver. 3.851). The substrate (PHB) used for crystallization and crystallographic water molecules were removed during modeling. The modeling methods follow standard protocols, and include minimization of the protein structure prior to docking to accommodate hydrogen atoms not included in the crystal structure coordinates. The ligand (EGCG) was built by MacroModel (ver. 6.5), energy minimized using the Monte Carlo method (MM3* force field) to energy derivatives of less than 0.1 kcal/Å, the structure was then optimized for the CHARMM force field using parameters obtained by the calculation. The ligand was manually docked into PHBH within 5 Å of the C(4a) flavin isoalloxazine ring, while monitoring docking energy to achieve a minimum value. Energy minimization (CHARMM force field) was run for 1000 steps, followed by minimizing to the energy derivatives of less than 0.1 kcal/Å using the conjugate gradient method (Powell algorithm). Molecular dynamics ran for 8000 iterations of 0.5 fs after 1000 steps of equilibration (Varlet algorithm), while the distance between the galloyl 3′hydroxyl group and the C(4a) flavin isoalloxazine ring, was constrained to be within 5 Å. All energy minimization and molecular dynamics were carried out at 300 K.

## 3. Results and discussion

Gallic acid and its esters were evaluated as enzyme inhibitors of recombinant *P. aeruginosa* PHBH whose amino acid sequences show ca. 20% identity to that of rat SE, the flavin monooxygenase catalyzing the rate-limiting step of the cholesterol biosynthesis. DG (**5**) ($IC_{50}$ = 16 μM) and EGCG (**3**) ($IC_{50}$ = 16 μM) showed the most potent inhibition. *n*-Cetyl ($C_{16}$) gallate (**4**) ($IC_{50}$ = 25 μM) and *n*-octyl ($C_8$) gallate (**6**) ($IC_{50}$ = 51 μM) were also good inhibitors, however, ethyl gallate (**7**) ($IC_{50}$ > 250 μM) and gallic acid (**8**) ($IC_{50}$ > 250 μM) showed poor inhibition. Furthermore, as a control, *N,N*-dimethyl-*n*-dodecyl amine ($IC_{50}$ > 250 μM) did not show significant inhibition. These results were in good accordance with those obtained for recombinant rat SE, although the latter enzyme suffered more potent inhibition; the reported $IC_{50}$ values for EGCG and DG were 0.69 and 0.061 μM, respectively [8,9]. Thus, it was again demonstrated that the hydro-

**(a) GxGxxG motif**

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Human SE     125 EVIIVGAGVLGSALAAVLSRDGRKVTVIERDLKEPD
Rat SE       124 EVIIIGSGVLGSALATVLSRDGRTVTVIERDLKEPD
Mouse SE     123 EVIIVGSGVLGSALAAVLSRDGRKVTVIERDLKEPD
PHBH           4 QVAIIGAGPSGLLLGQLLHKAGIDNVILERQT--PD
                 * *:* *  *  *. :*   *    ::**    **
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**(b) GD motif**

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Human SE     390 ASFLPP-SSVKK---RG-VLLLGDAYNMRHPLTGGGMTVAFKDIK-LWRKLLKD
Rat SE       389 ASFLPP-SSVNK---RG-VLLLGDAYNLRHPLTGGGMTVALKDIK-IWRQLLKD
Mouse SE     388 ASFLPP-SSVNK---RG-VLILGDAYNLRHPLTGGGMTVALKDIK-LWRQLLKG
PHBH         263 KSIAPLRSFVVEPMQHGRLFLAGDAAHIVPPTGAKGLNLAASDVSTLYRLLLKA
                  *: *  * * :   :* ::: *** ::  *  . *:.:* .*:. ::* ***
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Fig. 2. Comparison of FAD binding sequence (a) GxGxxG motif and (b) GD motif of *P. aeruginosa* PHBH with vertebrate (human, rat and mouse) SE enzymes. Pro267, Arg269 and Pro293 residues, predicted to be involved in the hydrogen bond formation with EGCG, are underlined.

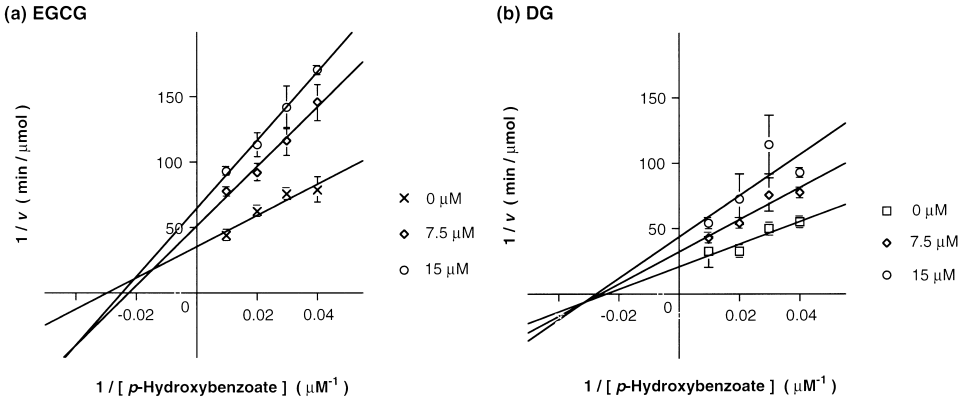


Fig. 3. Lineweaver–Burk blot analysis for PHBH inhibition by (a) EGCG and (b) DG.

phobicity of the alkyl side chain of the gallate molecule plays an important role in the flavoenzyme inhibition. Inhibition kinetics revealed that both EGCG and DG inhibited PHBH in a non-competitive manner with $K_I$ values of 14.0 and 18.1 μM, respectively (Fig. 3), showing a large increase in affinity of the inhibitors compared to the normal substrate PHB ($K_M = 41.6$ μM). Further, this indicated that the binding sites of both inhibitors are different from the PHB binding pocket. Indeed, according to the crystal structure of *Pseudomonas fluorescens* PHBH [3,4], the substrate binding pocket (143 Å$^3$) is too small to fully accommodate the inhibitor molecules, i.e. EGCG (350 Å$^3$) and DG (328 Å$^3$). Here it should be noted that *P. aeruginosa* PHBH is almost identical in its catalytic properties to *P. fluorescens* PHBH; there are only two amino acid residue differences, and these are both on the surface of the enzyme [1]. Interestingly, *P. aeruginosa* PHBH was not significantly inhibited by substrate-like (or product-like) gallic acid. On the other hand, 2,4-dihydroxybenzoate has been reported to be well accepted as a substrate and converted to a mixture of 2,3,4- and 2,4,5-trihydroxybenzoate [12]. In contrast, gallic acid no longer has high affinity to the substrate binding site of the enzyme.

As in the case of SE inhibition, we postulate that the inhibition of PHBH would be caused by specific binding of gallate to the enzyme, presumably, in close proximity to the FAD binding domain. The enzyme reaction is thought to proceed via formation of a flavin C(4a)-hydroperoxide intermediate, which would be trapped by the antioxidative gallate [8,9]. In order to test this hypothesis, we used molecular modeling in conjunction with the X-ray structure coordinates for the published 3D structure of *P. fluorescens* PHBH. The modeling methods followed standard protocols, and EGCG was manually docked into PHBH so that the hydroxyl group of the gallate was located within 5 Å of the C(4a) isoalloxazine ring of FAD where formation of the hydroperoxide takes place. The obtained energy-minimized structure predicted that EGCG indeed binds in close proximity to the FAD binding site via the formation of three hydrogen bonds with the active site residues between (i) the galloyl 3″-hydroxyl group and carbonyl oxygen of Pro293 (distance of 3.42 Å), (ii) the galloyl 5″-hydroxyl group and carbonyl oxygen of Pro267 (distance of 4.99 Å) and (iii) the 4′-hydroxyl group of the B-ring and the side chain amino group of Arg269 (distance of 2.97 Å) (Fig. 4). Furthermore, the distance between the galloyl 3″-hydroxyl group and C(4a) of the flavin isoalloxazine ring was predicted to be 4.50 Å. During the molecular dynamics simulation, EGCG remained at approximately the same location as that determined in the energy-minimized structure, therefore implicating the formation of a stable complex with the flavin monooxygenase.

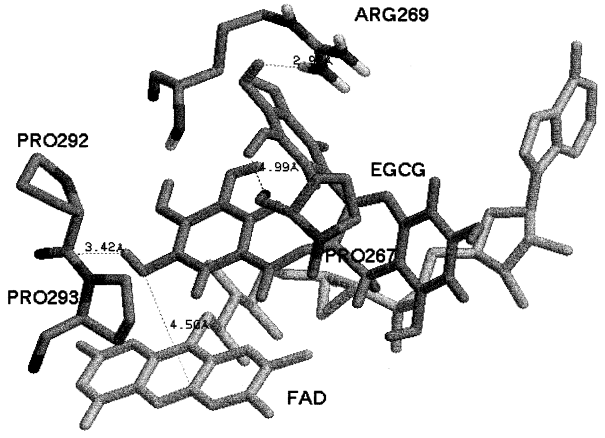


Fig. 4. Energy minimized structure of PHBH–FAD–EGCG complex.

It has been reported that the flavin ring structure moves substantially in the active site of the enzyme to enable translocation of substrate/product and to regulate the reduction of the flavin by NADPH [13]. Besides scavenging the reactive oxygen species required for the enzyme reaction, formation of the stable complex with EGCG would also interrupt such movement and block the interactions with the substrate and NADPH, leading to the inactivation of the enzyme. Further, it should be also noted that Pro267 and Pro293, the residues thought to be involved in the hydrogen bond formation with EGCG, are also well conserved in SE enzymes (Fig. 2), suggesting that the formation of a similar complex with EGCG may be possible. Finally, crystallization of the PHBH–EGCG complex, which is now in progress in our laboratory, will provide us with further detailed structural information on the inhibition mechanism.

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