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Running Title: Enzymatic carboxylation of hydroxystilbenes

Enzymatic carboxylation of hydroxystilbenes by the γ -resorcylic acid decarboxylase from *Rhizobium radiobacter* WU-0108 under reverse reaction conditions

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1 **Abstract**

2 We examined 66 aromatics for carboxylation by the γ -resorcylic acid decarboxylase
3 from *Rhizobium radiobacter* WU-0108 under reverse reaction conditions. The enzyme
4 carboxylated resorcinol, catechol, 5-methylresorcinol and three hydroxystilbenes
5 (resveratrol, gnetol, and piceatannol) with high yields. Except for catechol, the
6 structures of these substrates include a 1,3-dihydroxybenzene moiety. Other compounds
7 gave no reaction products. The reaction products from resveratrol and gnetol were
8 2,6-dihydroxy-4-[(*E*)-2-(4-hydroxyphenyl)ethenyl]benzoic acid and
9 2,6-dihydroxy-4-[(*E*)-2-(2,6-dihydroxyphenyl)ethenyl]benzoic acid, respectively, as
10 determined by mass spectrometry and nuclear magnetic resonance analyses. Kinetic
11 analyses of the carboxylation reactions indicated that resveratrol and gnetol are better
12 substrates than resorcinol or catechol.

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15 **Key words:** enzymatic carboxylation; γ -resorcylic acid decarboxylase; hydroxystilbene;
16 resveratrol; gnetol.

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18 **Abbreviations:** HPLC, high-performance liquid chromatography; LC-MS, liquid
19 chromatography-mass spectrometry; Rdc, γ -resorcylic acid decarboxylase isolated from
20 *R. radiobacter* WU-0108

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1. Introduction

Carboxylation of phenolic compounds is a basal reaction for producing various starting materials for pharmaceuticals and polymers, and the Kolbe-Schmitt reaction has been used as a standard industrial process to produce aromatic hydroxyl carboxylic acids from phenolic compounds and CO₂ [1]. However, the reaction requires high CO₂ pressure (up to 100 atm) and high temperature (100 to 400°C) and produces byproducts that must be separated prior to industrial use. In contrast, enzymatic carboxylation of phenolic compounds proceeds under ambient pressure and temperature and produces fewer byproducts. Another advantage of enzymatic reactions is their high specificity for particular types of chemical bonds or functional groups on complex molecules, which is referred to as “substrate specificity”, in contrast with the low reaction selectivity of conventional organic chemistry processes. Thus, particular bonds can be formed via enzymatic reactions without the need to block functional groups, thereby simplifying reaction processes when synthesizing complex molecules such as natural products for medicinal use. Therefore, much attention has been focused on enzymatic carboxylation in the past few decades [2], as well as on other enzymatic reactions.

Several microbial decarboxylases are known to carboxylate phenolic and heterocyclic compounds under reverse reaction conditions, with relatively high conversion rates. A salicylic acid decarboxylase from the yeast *Trichosporon moniliiforme* WU-0401 produces salicylic acid from phenol with a 27% (mol/mol) yield [3] and 4-aminosalicylic acid from 3-aminophenol with a 70% (mol/mol) yield [4]. A 3,4-dihydroxybenzoate decarboxylase from *Enterobacter cloacae* P241 carboxylates catechol to 3,4-dihydroxybenzoic acid with a molar conversion ratio of 28% [5]. A γ-resorcylic acid decarboxylase isolated from *Rhizobium radiobacter* WU-0108 [6] that has 98% similarity to a decarboxylase isolated from *Rhizobium* sp. MTP-10005 [7] converts resorcinol (1,3-dihydroxybenzene) to γ-resorcylic acid (1,3-dihydroxybenzoic acid) with a molar conversion yield of 30% (mol/mol). An indole-3-carboxylate

1 decarboxylase converts indole to indole-3-carboxylic acid with a molar conversion yield
2 of 34% (mol/mol) [8]. A pyrrole-2-carboxylate decarboxylase from *Bacillus*
3 *megaterium* PYR2910 catalyzes the carboxylation of pyrrole in the reverse reaction [9].
4 In these cases, a limited number of aromatics were examined for carboxylation.
5 Wuensch et al. [10] examined carboxylation of phenol, 1-naphthol, 3-aminophenol,
6 catechol, resorcinol, olivetol, 4-vinylphenol, 2-methoxy-4-vinylphenol, and
7 2,6-dimethoxy-4-vinylphenol by five decarboxylases isolated from the microorganisms
8 *Aspergillus oryzae* (2,3-DHBD_Ao), *Rhizobium* sp. (2,6-DHBD_Rs), *Trichosporon*
9 *moniliiforme* (SAD_Tm), *Lactobacillus plantarum* (PAD_Lp) and *Bacillus*
10 *amyloliquefaciens* (PAD_Bs), respectively, and found varying substrate specificity and
11 yield of carboxylated product in the respective reverse reactions of these enzymes. The
12 substrate specificity of enzymes 2,3-DHBD_Ao, 2,6-DHBD_Rs and SAD_Tm toward
13 32 small aromatics, including some of the previously examined compounds, was
14 recently reported [11]. In these previous studies, small aromatics have been examined as
15 substrates for carboxylation by decarboxylases. Although there are several inexpensive
16 industrial processes for producing aromatic hydroxyl carboxylic acids, information is
17 lacking regarding enzymatic carboxylation of bulky or complex substrates that may be
18 useful for producing valuable chemicals for applications in fields such as medicine.

19 In this study, we examined the carboxylation of various aromatics by the
20 γ -resorcylic acid decarboxylase isolated from *R. radiobacter* WU-0108, which is known
21 to carboxylate resorcinol and catechol [6]. Our results demonstrate that the enzyme
22 carboxylates hydroxystilbenes such as resveratrol, gnetol and piceatannol, that are bulky
23 molecules, as well as resorcinol, catechol and 5-methylresorcinol in a regio-specific
24 manner. Interestingly, the hydroxystilbenes were shown to be better substrates for the
25 enzyme than resorcinol and catechol in terms of K_m and k_{cat}/K_m values. To our
26 knowledge, this is the first report demonstrating decarboxylase-catalyzed introduction
27 of a carboxyl group into hydroxystilbenes.

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2. Materials and methods

2.1. Chemicals

The substrates for carboxylation (1 to 66 in Supplementary FigS1) and other chemicals were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Sigma-Aldrich (St. Louis, MO, USA).

2.2. Enzyme preparation

The recombinant decarboxylase isolated from *Rhizobium radiobacter* WU-0108 was prepared as reported elsewhere [6], with some modifications. Briefly, *Escherichia coli* BL21(DE3) (Merck, Darmstadt, Germany) with the pET21-d(+) vector (Merck) harboring the gene encoding the decarboxylase fused with a C-terminal hexahistidine-tag was cultivated for 16 h at 30°C in 100 mL of Luria-Bertani medium containing 50 µg/mL of carbenicillin and 0.1 mM isopropylthiogalactopyranoside, with shaking at 180 rpm. The enzyme was purified from the cell lysate by Ni²⁺ affinity chromatography using a His GraviTrap column (GE Healthcare, Little Chalfont, UK) and desalted by gel permeation chromatography using a PD-10 column (GE Healthcare). Protein concentration was determined using a Coomassie blue (Bradford) Protein Assay Reagent kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Enzymatic reaction

The reaction mixture (1 mL) containing 100 µg of the purified decarboxylase, 10 mM substrate, 3.0 M KHCO₃ and 2% methanol was incubated at 30°C with vigorous shaking for 20 h and then acidified by adding 1 mL of 5 N HCl. The reaction mixture was extracted twice with the same volume of ethyl acetate. Kinetic parameters were determined by fitting the quantification data to a Lineweaver-Burk plot. All kinetic parameters are shown as the mean of at least triplicate experiments.

2.4. High-performance liquid chromatography (HPLC)

A LaChrom Elite HPLC system (Hitachi High-Technologies, Tokyo, Japan) equipped with a ZORBAX Eclipse XDB-C18 column (Agilent Technologies, Santa Clara, CA, USA) was used for detection and quantification of substrates and reaction products. The elution profile was configured as 50 mM KH₂PO₄ (pH 2.4, adjusted with phosphoric acid):methanol (95:5 [v/v]) from 0 to 2 min, followed by a linear gradient to 5:95 (v/v) at the endpoint of 20 min. The column was kept at 40°C, and the absorbance of the eluate was monitored at 254 nm.

2.5. Liquid chromatography-mass spectrometry (LC-MS)

LC-MS analyses were carried out using an Agilent 1100 series HPLC (Agilent Technologies) configured to an LTQ-FT mass spectrometer (Thermo Fisher). Compounds were separated using an Innertsil ODS-4 column (GL Science, Tokyo, Japan) and ionized using the atmospheric pressure chemical ionization method. The mobile phases consisted of water/0.1% formic acid (A) and acetonitrile/0.1% formic acid (B). The gradient elution profile was configured as A:B = 75:25 at 0 min, followed by a linear gradient to 10:90 at the endpoint of 40 min. At the end of each analysis, the column was washed with mobile phase A:B = 5:95 and re-equilibrated with the initial mobile phase composition. The column temperature and flow rate were kept at 40°C and 500 µL/min, respectively.

2.6. Mass spectrometry and NMR analyses

Reaction products from hydroxystilbenes were purified by precipitation with methanol and 1 M HCl and analyzed using the LTQ-FT mass spectrometer and an AVANCE600 NMR (Bruker, Billerica, MA, USA). The reaction products were dissolved in methanol containing 0.1% formic acid and then directly introduced into the mass spectrometer using electrospray ionization and negative-ion mode. In NMR analyses, chemical structures were determined from the ¹H- and ¹³C-NMR and HMBC

and ^{13}C -DEPT, as indicated below.

2,6-dihydroxy-4-[(E)-2-(4-hydroxyphenyl)ethenyl]benzoic acid (product 1).

HR-ESIMS m/z $[\text{M}-\text{H}]^-$: calcd. for $\text{C}_{15}\text{H}_{11}\text{O}_5$, 271.0606; found, 271.0610; NMR δ_{H} (600 MHz, CH_3OD): 6.58 (2H, s, C2, C6-H), 6.79 (2H, d, $J=6$ Hz, C3', C5'-H), 6.83 (1H, d, $J=12$ Hz, olefin-H), 7.15 (1H, d, $J=18$ Hz, olefin-H), and 7.41 (2H, d, $J=12$ Hz, C2', C6'-H); NMR δ_{C} (150 MHz, CH_3OD): 100.1 (C4), 106.3 (C2, C6), 116.8 (C3', C5'), 125.4 (olefinic C), 129.7 (C2', C6'), 129.8 (C1'), 133.5 (olefinic C), 147.1 (C1), 159.4 (C4'), 162.5 (C3, C5), and 170.0 (carboxyl C).

2,6-dihydroxy-4-[(E)-2-(2,6-dihydroxyphenyl)ethenyl]benzoic acid (product

2). HR-ESIMS m/z $[\text{M}-\text{H}]^-$: calcd. for $\text{C}_{15}\text{H}_{11}\text{O}_6$, 287.0556; found, 287.0559; NMR δ_{H} (600 MHz, CH_3OD): 6.32 (2H, d, $J=12$ Hz, C3', C5'-H), 6.56 (2H, s, C2', C6'-H), 6.88 (1H, t, $J=6$ Hz, C4'-H), 7.50 (1H, d, $J=18$ Hz, olefin-H), and 7.61 (1H, d, $J=18$ Hz, olefin-H); NMR δ_{C} (150 MHz, CH_3OD): 99.8 (C4), 106.2 (C2, C6), 108.0 (C3', C5'), 112.8 (C1'), 125.9 (olefinic C), 130.1 (olefinic C), 130.3 (C4'), 149.2 (C1), 158.8 (C2', C6'), 162.5 (C3, C5), 173.1 (carboxyl C).

Products **1** and **2** were further purified for kinetics analyses by liquid-liquid extraction using diethyl ether and 0.1 M sodium hydrogen bicarbonate (NaHCO_3) and then precipitated with methanol and 1 M HCl. As these compounds were unstable in ambient air, the purification was carried out under nitrogen atmosphere.

2.7. Docking simulation

The PDB data for a subunit of γ -resorcyate decarboxylase (PDB ID: 2DVT chain A) was pre-processed by the "Dock Prep" function of UCSF Chimera, and submitted to SwissDock web server for ligand-docking simulation with resveratrol. The all processes were done with default parameters of the software. Molecular graphics and analyses were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).

3. Results

3.1. Carboxylation of aromatics by the decarboxylase

To determine the substrate specificity of the *R. radiobacter* WU-0108 decarboxylase, we examined 66 aromatics, including bulky compounds (50 to 66), for the reverse reaction (Supplementary Fig. S1). As it is known that resorcinol (19; 1,3-dihydroxybenzene), catechol (9; 1,2-dihydroxybenzene), olivetol (5-pentyl-1,3-dihydroxybenzene) and 5-methylresorcinol (36; 5-methyl-1,3-dihydroxybenzene) are substrates for the carboxylation reaction [6, 10, 11], we chose various aromatics for the enzymatic reverse reaction. Those included mono- and di-benzenes with -OH, -SH, -SO₃, -NH₂, -NH₂CO, -CN, -CH₃, -C₂H₄, -C₃H₆, -OCH₃, -COH, -CH₃CO, -4-tert-butylphenyl, -benzaldehyde or -2-phenylthiophene substitution groups, as well as hydroxystilbenes (50 to 52) and flavonoids (53 to 66). The enzyme was expressed in *E. coli* harboring the plasmid vector with the decarboxylase gene, purified by affinity chromatography and subjected to the reverse reaction on the aromatics. The reaction was carried out for 20 h using 3 M KHCO₃ as the CO₂ source. The reaction temperature was set at 30°C, as we observed more byproduct formation when the reaction of hydroxystilbenes was carried out at 50°C compared with 30°C, although Yoshida *et al.* [7] carried out the carboxylation of resorcinol and catechol at 50°C. The reaction products were analyzed by HPLC. A new peak, with a signal to noise ratio >10, appeared on the HPLC chromatogram and was defined as a reaction product.

Out of the 66 aromatics, only 6 compounds (catechol, resorcinol, 5-methylresorcinol and the hydroxystilbenes resveratrol (50), gnetol (51) and piceatannol (52)) gave reaction products (Fig. 1). With the exception of catechol, the structures of the compounds were found to have a 1,3-dihydroxybenzene moiety. Although the flavonoids butein (53), isoliquiritigenin (54), kaempferol (56), morin (55), myricetin (60), quercetin (58), 3',4',5,7-tetrahydroxyflavone (59), apigenin (57) and naringenin (62) also contain this moiety, they did not give any reaction products. With

the exception of catechol, compounds having the 1,2-dihydroxybenzene moiety in the structures (4-methylcatechol (37), butein, quercetin, fisetin (66), 3',4',5,7-tetrahydroxyflavone, 7,8-dihydroxyflavone (65) and catechin (63)) gave no reaction products. The other aromatics did not give any reaction products. As phloroglucinol (38; 1,3,5-trihydroxybenzene) was carboxylated under alkaline reaction conditions without the enzyme, it was not clear whether the enzyme utilized it as a substrate, while the rest of the substrates showed no spontaneous carboxylation when compared with the reaction mixture without the enzyme.

3.2. Identification of the reaction products

The reaction products from resorcinol and catechol were identified by HPLC using the authentic compounds 2,6-dihydroxybenzoic acid and 2,3-dihydroxybenzoic acid, as previously reported [6]. The carboxylation product of 5-methylresorcinol was identified as 2,6-dihydroxy-4-methylbenzoic acid using the authentic compound, the HPLC retention time of which differed from that of the regio-isomer 2,4-dihydroxy-6-methylbenzoic acid (Supplementary Fig. S2).

The reaction mixtures from the hydroxystilbenes were subjected to LC-MS analysis (Fig. 1), which showed that each reaction product was carboxylated by introduction of a single CO₂ molecule. The products from resveratrol, gnetol and piceatannol had single-carboxylated phenols, which were confirmed based on their mass values.

The reaction products from resveratrol, gnetol and piceatannol were purified by liquid extraction and precipitation to remove the substrate and were then subjected to NMR analysis. The purity of the reaction products from resveratrol, gnetol and piceatannol was estimated as 98, 91, and 79%, respectively. Due to low purity, NMR spectra of the products from piceatannol were not clear enough to assign the chemical shifts. The structures of the reaction products from resveratrol and gnetol were determined as 2,6-dihydroxy-4-[(*E*)-2-(4-hydroxyphenyl)ethenyl]benzoic acid (product

1 **1**) and 2,6-dihydroxy-4-[(*E*)-2-(2,6-dihydroxyphenyl)ethenyl]benzoic acid (product **2**),
2 respectively. These results indicate that the enzyme introduces a carboxyl group
3 preferentially at the 2 position of the 1,3-dihydroxybenzene moiety, with the exception
4 of catechol, in which the 3 position of 1,2-dihydroxybenzene is carboxylated (Fig. 2).

6 3.3. Kinetic parameters and yield of phenol carboxylation products

7 The k_{cat}/K_m values for the carboxylation of resveratrol and gnetol were
8 determined as 1.62×10^{-1} and 1.24×10^{-1} mM•s⁻¹, respectively, higher than the values
9 for resorcinol (2.47×10^{-2} mM•s⁻¹) and catechol (7.25×10^{-3} mM•s⁻¹) (Table 1). The
10 Lineweaver-Burk plots for each reaction were shown in supplementary Fig. S3. These
11 results indicate that hydroxystilbenes are better substrates than resorcinol and catechol
12 in terms of K_m and k_{cat}/K_m values.

13 The yield of carboxylation carried out in 3 M KHCO₃ at 30°C for 20 h was
14 25% (mol/mol) for γ -resorcylic acid from resorcinol, 11% for 2,3-dihydroxybenzoic
15 acid from catechol and 47% for 2,6-dihydroxy-4-methylbenzoic acid from
16 5-methylresocinol. The yield of γ -resorcylic acid (25%) was comparable to the 30%
17 (mol/mol) reported for a previous study [6], in which the reaction was carried out in 1 M
18 KHCO₃ at 40°C. The yields of carboxylation of resveratrol and gnetol were 26 and 27%,
19 respectively, when the reaction was carried out at 30°C for 1 h but decreased drastically
20 upon more prolonged reaction. The decrease was ascribed to further reaction of the
21 carboxylated products (data not shown).

23 4. Discussion

24 To assess the carboxylation specificity of the *R. radiobacter* WU-0108
25 γ -resorcylic acid decarboxylase under reverse reaction conditions, we examined the
26 carboxylation of a total of 66 aromatics, including resorcinol and catechol, which are
27 known substrates of this enzyme [6] (Supplementary Figs. S1 and S4). We found that
28 the enzyme introduces CO₂ into the bulky compounds (hydroxystilbenes), resveratrol,

1 gnetol and piceatannol as well as the small ones resorcinol, catechol and
2 5-methylresorcinol (Fig. 2). To our knowledge, this is the first report demonstrating the
3 carboxylation of bulky substrates by a decarboxylase. Interestingly, the bulky substrates
4 appear to be better than resorcinol and catechol. The preference for bulky substrates
5 may lead to application of enzymatic carboxylation to complex chemicals such as
6 medicinal compound intermediates and functional building blocks.

7 With the exception of catechol (containing a 1,2-dihydroxybenzene moiety), a
8 common feature of the substrates carboxylated by the enzyme is the presence of a
9 1,3-dihydroxybenzene moiety. Structural analyses of the reaction products from
10 resorcinol, 5-methylresorcinol, resveratrol and gnetol showed that CO₂ was introduced
11 at the 2 position of the 1,3-dihydroxybenzene group (Fig. 2). These results suggest that
12 the γ -resorcylic acid decarboxylase recognizes the 1,3-dihydroxybenzene moieties and
13 carboxylates the 2 position of the moiety. However, the flavonoids butein,
14 isoliquiritigenin, kaempferol, morin, myricetin, quercetin, 3',4',5,7-tetrahydroxyflavone,
15 apigenin and naringenin, which also have a 1,3-dihydroxybenzene moiety, were not
16 carboxylated by the enzyme. This result indicates that structural features in addition to
17 the 1,3-dihydroxybenzene moieties are critical for recognition as a substrate for the
18 reverse reaction.

19 Of the aromatics examined that have a 1,2-dihydroxybenzene moiety in the
20 structure (Fig. 2), only catechol was carboxylated. The k_{cat}/K_m value for the
21 carboxylation was much lower for catechol than for resorcinol, resveratrol and gnetol
22 (Table 1), indicating that catechol is a poor substrate for the enzymatic reaction.

23 Crystal structures of the γ -resorcyate decarboxylase from *Rhizobium* sp.
24 MTP-10005 [12], which has 98% amino acid sequence similarity with the enzyme
25 examined in the present study, show that the active site cavity accepts
26 2,3-dihydroxybenzoate and 2,3-dihydroxybenzaldehyde (a 2,3-dihydroxybenzoate
27 analogue), suggesting that catechol is accepted in the active site cavity as a substrate,
28 similar to resorcinol. The structural requirement for accepting substrates in the cavity

1 seems to be stricter for substrates with a 1,2-dihydroxybenzene moiety than for those
2 with a 1,3-dihydroxybenzene moiety. In addition, the crystal structures likely accept
3 bulkier compounds such as resveratrol, gnetol and piceatannol at the active site cavity.
4 To confirm such binding at the cavity, we carried out a computational simulation using a
5 program UCSF Chimera and SwissDock [13, 14, 15] (Supplementary Fig. S5, A). One
6 of the possible docking models suggested that the orientation of resveratrol (Fig. S5, B)
7 was similar to that of resorcinol reported by Goto, et al. [12]. Hence, it is likely that the
8 carboxylation of resveratrol and resorcinol occurs in the same manner at the active site.

10 Conclusion

11 Several microbial decarboxylase were known to show carboxylation activity
12 toward phenolic and heterocyclic compounds. In this study, the γ -resorcylic acid
13 decarboxylase isolated from *R. radiobactor* WU-0108 also exhibited the carboxylation
14 activity toward hydroxystilbenes possessing 1,3-dihydroxybenzene moiety. These
15 finding provides broad applicability of enzymatic carboxylation, and may lead to simple
16 and low-cost production of complex aromatic carboxylic acids.

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Figure captions

Fig. 1. LC-MS identification of carboxylation products. Left panels: total ion

1 chromatograms of the reaction mixture containing resveratrol (A), gnetol (B) and
2 piceatannol (C). Reaction products are indicated by asterisks. Right panels: mass spectra
3 of the reaction products.

4
5 **Fig. 2.** Chemical structures of the substrates carboxylated by the γ -resorcilic acid
6 decarboxylase from *R. radiobacter* WU-0108. The numbers shown below the chemical
7 names are corresponding to those shown in **Supplementary Fig. S1.**

8

Table 1. Kinetic Parameters in Carboxylations by the γ -Resorcylic Acid Decarboxylase from *R. radiobacter* WU-0108.

Substrate	Product	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ •s ⁻¹)
Resorcinol	γ -Resorcylic acid	23.1	0.571	2.47×10^{-2}
Catechol	2,3-Dihydroxybenzoic acid	19.4	0.141	7.25×10^{-3}
5-Methylresorcinol	2,6-Dihydroxy-4-methylbenzoic acid	32.79	4.98	1.52×10^{-1}
Resveratrol	Product 1	4.46	0.722	1.62×10^{-1}
Gnetol	Product 2	2.67	0.331	1.24×10^{-1}

Fig. 1. Sato *et al.*

Fig. 2. Sato, *et al.*