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Kinetic study of mushroom tyrosinase inhibition using naturally occurring bromophenols and comparison with related phenolic compounds

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Abstract

Naturally occurring bromophenols and commercially available halogenated phenols were investigated kinetic inhibition study and a comparison of inhibitory activity against mushroom tyrosinase. All four bromophenols investigated showed noncompetitive inhibition from the results of Lineweaver-Burk plots. The inhibitors may bind another site of the active site and combine with either free enzyme or enzyme-substrate complex. Among them, inhibition constants of symmetric bromophenol dimers, bis(2,3-dibromo-4,5-dihydroxyphenyl)methane and bis(2,3-dibromo-4,5-dihydroxybenzyl)ether, exhibited low values compared to ones of asymmetric dimers. Additionally, 2,3-dibromo-6-(2,3-dibromo-4,5-dihydroxybenzyl)-4,5-dihydroxybenzyl methyl ether showed the most potent inhibition among the compounds investigated. Chlorinated phenols, 2,4-dichlorophenol and 2,2'-methylenebis-(4-chlorophenol), exhibited moderate inhibition against mushroom tyrosinase among commercially available phenols. These results suggest that inhibition of halogenated phenols against mushroom tyrosinase requires a catechol moiety and/or halogen substitution in the one *ortho*-position of phenolic hydroxy group and vacant substitution in the other *ortho*-position.

Keywords: bromophenols, kinetic study, tyrosinase, marine algae

Introduction

Many halogenated compounds have been isolated from marine red algae of the family Rhodomelaceae. These halogenated compounds represent unique secondary metabolite in terms of structural and biological diversity [1]. Most halogenated compounds of Rhodomelaceae origin are brominated or chlorinated. On the basis of structural characteristics halogenated terpenoids, nonterpenoidal C₁₅-acetogenins, and indoles were isolated from genus *Laurencia*, while halogenated phenols were isolated from genera *Polysiphonia*, *Rhodomela*, *Symphocladia*, and *Odonthalia* (Rhodomelaceae). Usually most of halogenated phenols consist of 4,5-dihydroxybenzyl moiety substituted by one to three bromine atoms per moiety. Halogenated phenols have been reported to showed wide range of functionalities [2, 3]. These functionalities are relying on number and position of phenolic hydroxy groups and/or bromine atoms. Ten bromophenols 1-10 were isolated from the marine red alga *Odonthalia corymbifera* [4-7]. These bromophenols had displayed the functionality of tyrosinase inhibition, and few members of these bromophenols showed stronger inhibition than positive control kojic acid. Tyrosinase is a copper-containing enzymes which responsible for forming melanin through polymerization of phenolic compounds [8]. Excess production of melanin creates aesthetic problem in human and enzymatic browning in agricultural commodities decreasing market value [9]. Researchers are searching naturally occurring inhibitors rather synthetic kojic acid as its association with toxicity. Tyrosinase inhibition mechanism by marine algae derived bromophenol was the core interest of this research. To unveil this inhibition mechanism, kinetic study of mushroom tyrosinase inhibition using the bromophenols 1-4, of which sufficient amount were obtained, and comparison of inhibitory activity between naturally occurring bromophenols 1-10 (Fig 1) and commercially available phenolic compounds 11-23 (Fig 2).

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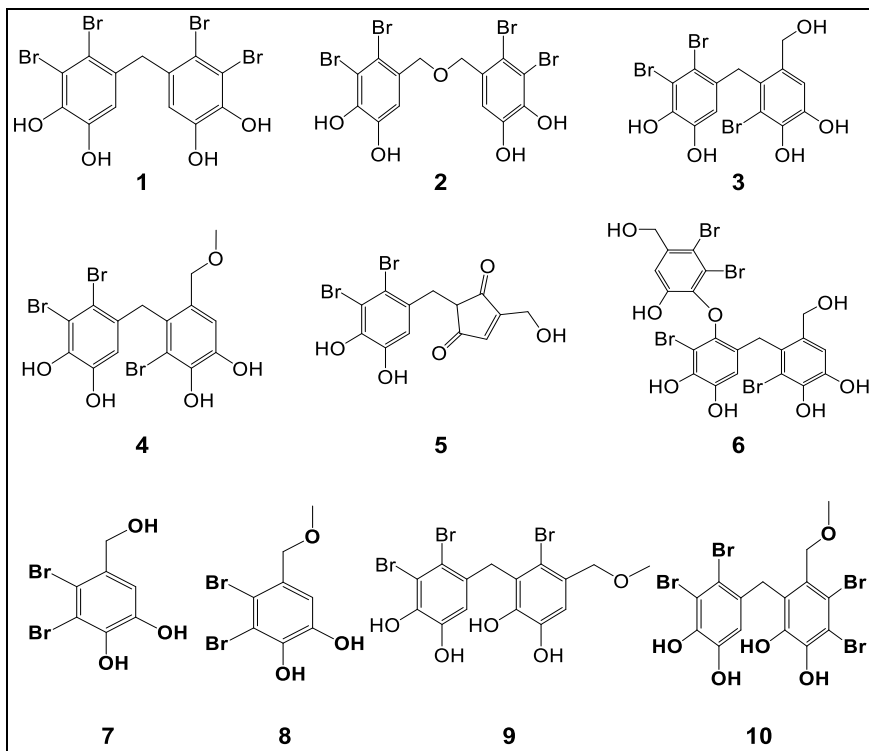


Fig 1: Bromophenols from *Odonthalia corymbifera*.

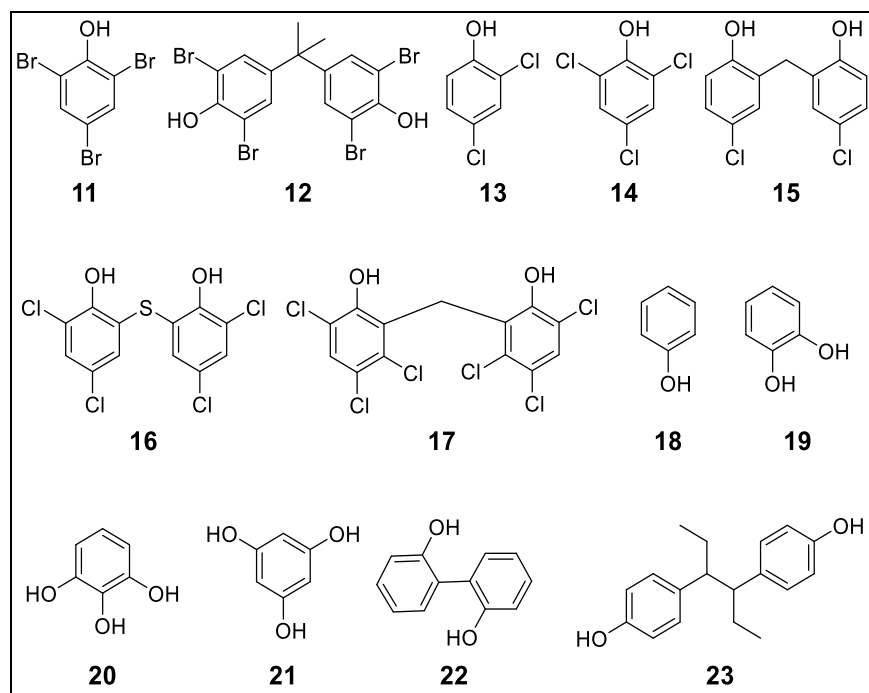


Fig 2: Related available compounds in this study.

2. Materials and Methods

2.1. Reagents

2,4,6-Tribromophenol (11), tetrabromobisphenol A (12), 2,4-dichlorophenol (13), 2,4,6-trichlorophenol (14), 2,2'-methylenebis-(4-chlorophenol) (15), 2,2'-thiobis(4,6-dichlorophenol) (16), hexachlorophene (17), 2,2'-biphenol (22), hexestrol (23), and kojic acid were purchased from Tokyo Chemical Industry Co. LTD. (Tokyo, Japan). Phenol crystal (18) was obtained from Kanto Chemical Co. LTD. (Tokyo, Japan). Catechol (19), pyrogallol (20), phloroglucinol (21), and L-tyrosine were available from Wako Pure Chemical Industries LTD. (Osaka, Japan). Mushroom tyrosinase (EC 1.14.18.1) was purchased from Sigma-Aldrich

(St. Louis, MO, USA). All other reagents were analytical grade.

2.2. Extraction and isolation

The marine red alga *O. corymbifera* was collected at the coast of Hakodate city, Japan in May, 2016. The species identification was done by one of the author (H. Kurihara), supervised by Professor H. Mizuta (Faculty of Fisheries Sciences, Hokkaido University, Japan), and a voucher specimen was deposited at the laboratory. Isolation and purification of the bromophenols (1-10) were according to the methods described by authors [4-7].

2.3. Tyrosinase inhibition assay

Bromophenols 1-10 and related phenolic compounds 11-13 were dissolved in methanol and added (15 μ l) into 50 mM sodium phosphate buffer (780 μ l, pH 6.8) in test tube, followed by addition of 0.1 mg/ml L-tyrosine (0.5 ml) as substrate [4, 10]. Enzymatic reaction was started after adding 200 U/ml mushroom tyrosinase solution (205 μ l). Reaction solution was incubated at 25 $^{\circ}$ C for 30 min and absorbance was measured at 490 nm. Kojic acid was used as a positive control. The IC_{50} value was expressed as sample concentration which showed 50% inhibition of tyrosinase reaction. The percent inhibition of tyrosinase activity was calculated as follows:

$$\% \text{ Inhibition} = [(A_2 - A_1) - (B_2 - B_1)] / (A_2 - A_1) \times 100$$

Where A_1 is the absorbance at 490 nm without test compounds (control) at 0 min, A_2 is the absorbance at 490 nm without test compounds (control) at 30 min, B_1 is the absorbance at 490 nm with test compounds at 0 min, and B_2 is the absorbance at 490 nm with test compounds at 30 min.

Determination of inhibition constant and inhibition type:

The inhibition type and inhibition constant (K_i) was assayed by Lineweaver-Burk (double reciprocal) plot and Dixon plot analysis, respectively. In Lineweaver-Burk plot, different concentrations of L-tyrosine solution (0.05, 0.1 and 0.2 mg/ml) were used as substrate and their reciprocal $1/[S]$ plotted against $1/v$ was a straight line, while different bromophenol concentration was plotted instead of $1/[S]$ in Dixon plot.

3. Results and Discussions

3.1. Kinetic study of tyrosinase inhibition using naturally occurring bromophenols

The inhibition types and constants of bromophenols 1-4, which were obtained sufficient amount for the kinetic study, were determined from Lineweaver-Burk and Dixon plots which shown in Figs 3-6. All the tested compounds 1-4 produced intersection points of lines on horizontal axes. Thus, all the bromophenols examined are noncompetitive inhibitors, while they show competitive or mixed inhibition against yeast α -glucosidase [7]. 7-Phloroecol [11], a phlorotannin, derived from brown algae and prenylated flavonoid [12] derived from terrestrial plant also exhibit noncompetitive inhibition against mushroom tyrosinase. This suggests that phenolic tyrosinase inhibitors might bind other site of the active site in either free enzyme or enzyme-substrate complex and change the structure and shape of enzyme to be incapable of binding correctly with the substrate [13]. Inhibitor constant (K_i), an indication of inhibitor binding affinity toward enzyme or enzyme-substrate complex [14], values were enlisted in Table 1. Bromophenol type tyrosinase inhibitors could be ranked as compounds $2 > 1 > 4 > 3$ among the bromophenols examined. Symmetric dimers 1 and 2 also inhibit α -glucosidase much strongly compared with asymmetric dimers [7]. Although the reason why symmetric dimers inhibit enzyme strongly is unclear, this evidence is important for development of enzyme inhibitors.

Table 1: Inhibitor constant (K_i) value and inhibition type of isolated bromophenols against tyrosinase activity.

Bromophenol	K_i value (μ M)	Inhibition Type
1	8.0	Non-competitive
2	2.4	Non-competitive
3	40.0	Non-competitive
4	24.7	Non-competitive

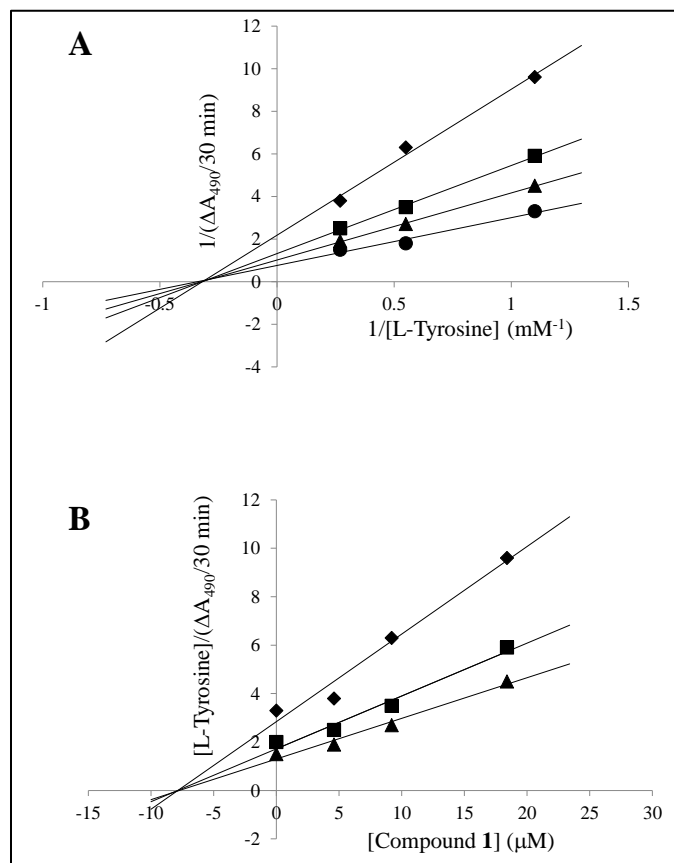


Fig 3: Kinetic analysis of compound 1 against tyrosinase activity. A, Lineweaver-Burk plot. B, Dixon plot.

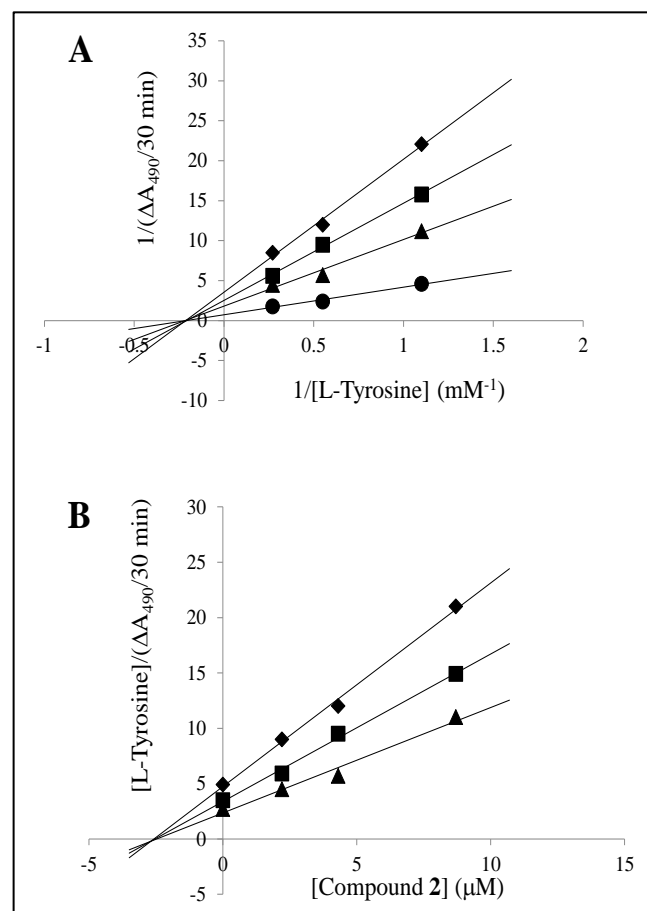


Fig 4: Kinetic analysis of compound 2 against tyrosinase activity. A, Lineweaver-Burk plot. B, Dixon plot.

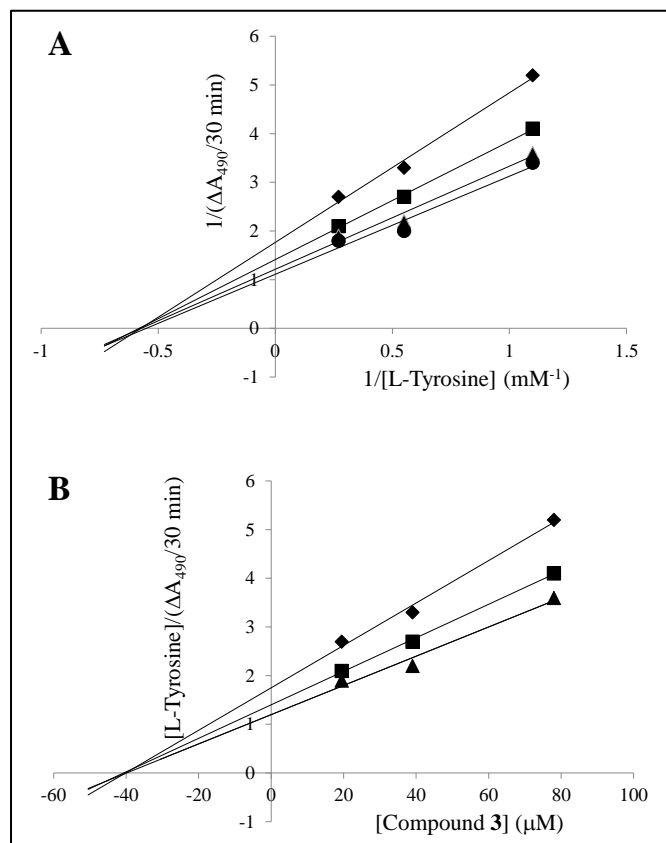


Fig 5: Kinetic analysis of compound 3 against tyrosinase activity. A, Lineweaver-Burk plot. B, Dixon plot.

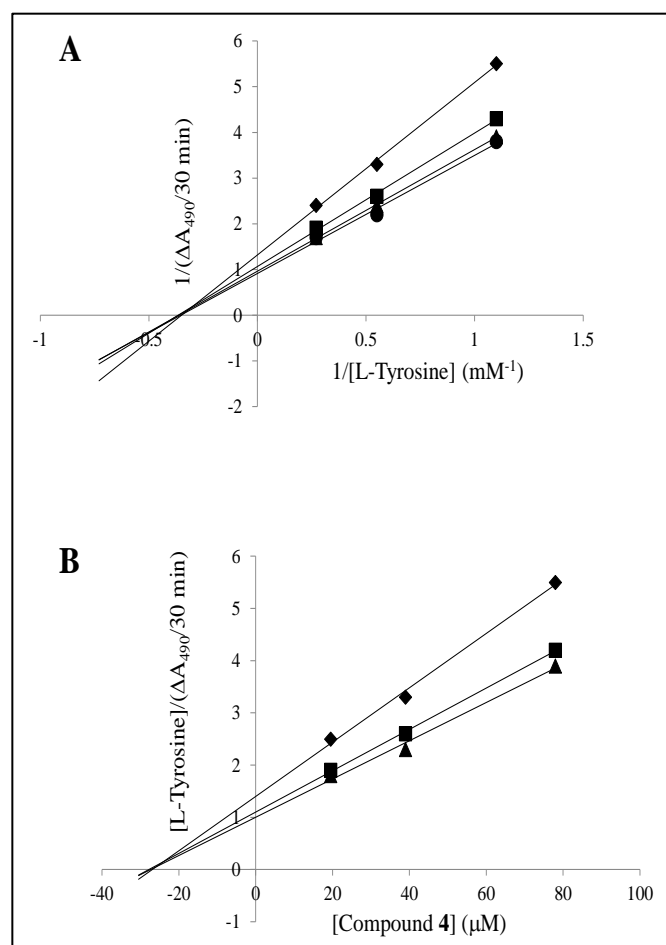


Fig 6: Kinetic analysis of compound 4 against tyrosinase activity. A, Lineweaver-Burk plot. B, Dixon plot.

3.2. Comparison of inhibitory activity against mushroom tyrosinase with naturally occurring bromophenols and related phenolic compounds

In vitro results of tyrosinase inhibition of naturally occurring bromophenols 1-10 and commercially available phenolic compounds 11-23 were listed in Table 2.

Table 2: Tyrosinase inhibition of naturally occurring bromophenols and commercially available phenolic compounds.

Compound	Tyrosinase Inhibition IC ₅₀ (μM) ^a	Compound	Tyrosinase inhibition, IC ₅₀ (μM) ^a
1	11.0±0.1 ^b	11	>1000
2	5.2±0.0 ^b	12	>1000
3	50.0±0.1 ^b	13	61.3±1.9
4	39.0±0.0 ^b	14	>1000
5	17.3±0.1 ^c	15	37.2±0.5
6	31.0±0.1 ^c	16	>1000
7	67.5±0.2	17	>1000
8	96.8±0.1	18	>1000
9	39.2±0.0 ^b	19	136.2±0.2
10	1.0±0.1 ^b	20	>1000
		21	462.5±0.5
Kojic acid	35.0±0.0 ^b	22	>1000
		23	>1000

^aMean±standard error (n=3).

^bData from the literature [4].

^cData from the literature [5].

Key factors influencing enzyme inhibition with halogenated phenols are substituting number and position of hydroxy groups and halogen atoms per benzene ring, number of benzene ring, and symmetry of structure. Phenolic compounds 11 and 12 which possess dibrominated and tribrominated phenol moieties exhibited no inhibitory activity, while naturally occurring compound 7 which possesses a dibrominated catechol moiety showed an IC₅₀ value of 67.5 μM. It is unclear whether inhibitory potency relies on phenolic and catechol structures or position an number of bromine substitution. Among the commercially available halogenated phenols 11-17 tested, chlorinated phenols 13 and 15 showed moderate inhibition while compounds 14, 16 and 17 showed no inhibition. Both the compounds 13 and 15 possess at least one vacant substituting group in *ortho*-position adjacent to hydroxy group, while the other phenols possess substituting halogen groups in the both *ortho*-positions. Monophenol monooxygenase activity of tyrosinase is mediated incorporation of one oxygen atom in the *ortho*-position adjacent to phenolic hydroxy group of L-tyrosine [8]. Compounds 13 and 15 might enter important site of tyrosinase easily because of vacancy in the *ortho*-position of hydroxy group.

Most of the tyrosinase inhibitors are phenolic compounds derived from terrestrial plants. Catechol moiety of inhibitors play significant role as a copper-chelator of tyrosinase enzyme [15]. Therefore, inhibitory potency of simple phenolic compounds 18-23 were investigated. This was found true as catechol 19 displayed inhibition. Phloroglucinol 21 exhibited weak inhibition, nevertheless, pyrogallol 20 and compounds 18 and 22 exhibited no inhibition. Phenolic tyrosinase inhibitors would require at least no substitution in one *ortho*-position adjacent to phenolic hydroxy group other than copper-chelating activity.

Number and position of hydroxy group are important for antioxidant activity [16] but halogen substitution directly

related with anticancer [17, 18], enzyme inhibition [19], antimicrobial and anti-diabetic activity [17]. Specifically, higher number of bromine substitution is gradually increased compound bioactive potency [16]. Symmetric chlorinated compounds 16 and 17 did not exhibit any inhibition but only compound 15 showed moderate tyrosinase inhibition. Compound 15 was reported to show glucose 6-phosphate dehydrogenase inhibitory activity [20]. Naturally occurring symmetric brominated compounds 1 and 10 displayed high tyrosinase inhibition as well.

Naturally occurring bromophenols 1-10 and commercially available compounds 11-23 tyrosinase inhibitory activity were examined and try to understand structure-inhibitory based mechanisms of these phenolic compounds. From this study and previous reports [4, 5], it might be stated that hydroxy group, halogen substitution, catechol moiety and symmetric structure of phenolic compounds could influence tyrosinase inhibitory activity. Among commercially available phenolic compounds, only compounds 13 and 15, phenol-type, showed moderate tyrosinase inhibition. Although the exact reason behind enzyme inhibition of the compounds are not clearly understood, tyrosinase inhibition of halogenated phenolic compounds would require the phenol or catechol moiety that is substituted with halogen in the one *ortho*-position of phenolic hydroxy group and vacant substitution in the other *ortho*-position.

Conclusion

Naturally occurring bromophenols and commercially available phenolic compounds were investigated structure-activity relationship against tyrosinase. All the bromophenols were noncompetitive inhibitors from kinetic study. The tyrosinase inhibitors in this study were assigned the halogenated catechol or phenol that possesses halogen substitution in the one *ortho*-position of phenolic hydroxy group and vacant substitution in the other *ortho*-position.

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