Research Article

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Synthesis and evaluation of anti-tyrosinase activity of phenyl benzyl ether derivatives: Effects of functional groups and their positions

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ABSTRACT

Thirteen phenyl benzyl ethers were synthesized and their *in vitro* inhibitory activity towards tyrosinase, rate-determining enzyme in melanogenesis, was evaluated. The results showed that *p*-substituted phenyl benzyl ethers, especially *p*-chlorophenyl ones (**23**), exhibited significantly higher inhibition percentage to the ethers substituted at *meta*- and *ortho*-positions at 500 μM. At the same concentration, polysubstituted phenyl benzyl ethers **31** and **32** exhibited comparable inhibition percentage to kojic acid. Furthermore, *p*-chlorophenyl (**23**) and tribrominated phenyl analogues (**32**) were proven to have similar and even higher inhibition potency compared to kojic acid (IC_{50} = 106 μM) with IC_{50} of 55.7 and 93.8 μM, respectively. This study suggested that phenyl benzyl ethers **23** and **32** might be promising candidates for skin whitening agents for pharmacological and cosmetical products.

Keywords: monobenzone; phenyl benzyl ether analogue; melanogenesis; anti-tyrosinase agent; whitening agent

1. INTRODUCTION

Melanin is a natural heterogeneous polymeric pigment found commonly in bacteria, fungi, plants and animals (Kim and Uyama, 2005). It is responsible for skin colors (Seo et al., 2003; Nesterov et al., 2008), accumulation of dark spots on the skin in animals, and browning process in plants (Cooksey et al., 1997). The production of melanin is induced by exposure to sunlight. It helps to protect cells and tissues from damage and photocarcinogenesis (Lindquist, 1973). Melanin is synthesized through a biological process called melanogenesis in melanocytes located in the basal layer of the dermis (Spritz and Hearing, 1994). Melanogenesis includes the oxidation of L-tyrosine to L-DOPA, then L-dopaquinone catalyzed by tyrosinase, and followed by polymerization to the pigment (Cooksey et al., 1997). However, biosynthesis of melanin is the key role for enzymatic browning in raw fruits, vegetable, crustaceans, which is a major problem in food industry (Loizzo et al., 2012). Moreover, in human and animals, overexposure to sunlight can induce hyperpigmentation (Solano et al.,

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2006: Briganti et al., 2003) such as melasma, freckles, senile lentigines and actinic damages which causes serious esthetic problems (Harmon, 1964; Curto et al., 1999).

Hyperpigmentation is usually caused by tyrosinase-catalyzed reactions. Thus, inhibition of tyrosinase is an approach to suppress melanogenesis, which may reduce or stop browning reaction and melanin accumulation (Loizzo et al., 2012; Parvez et al., 2006). Tyrosinase inhibitors can be used as whitening agents, depigmentating agents and for prevention and treatment of pigmentation disorders (Zhang et al., 2009). Therefore, discovering effective anti-tyrosinase agents is crucial in food, cosmetic and pharmaceutical industries (Loizzo et al., 2012; Mishima et al., 1988; Qiu et al., 2009; Maeda and Fukuda, 1991). Many organic and medicinal chemists are focusing on searching appropriate tyrosinase inhibitors from both natural and synthetic origin (Loizzo et al., 2012; Park et al., 2006; Chen and Kubo, 2002; Jones et al., 2002; Kubo and Kinst-Hori, 1999).

Hydroquinone (**1**) (Fitzpatrick et al., 1966; Kligman and Willis, 1975), kojic acid (**2**) (Ohyama, 1990) and arbutin (**3**) (Tokiwa et al., 2007; Hori et al., 2004) are well-known tyrosinase inhibiting agents used in many whitening cosmetics. Among them, hydroquinone (**1**) was found to cause skin irritation, be mutagenic to mammalian cells and toxic to melanocytes. (Curto et al., 1999; Parvez et al., 2006) Thus, kojic acid (**2**) and arbutin (**3**) are alternative agents used. However, the use of kojic acid (**2**) and arbutin (**3**) are still limited due to adverse side effects, low formulation stability and poor skin penetration (Hermanns et al., 2000). Bibenzyl compounds (**4**) showed anti-tyrosinase activity up to almost 20-fold higher inhibitory activity towards tyrosinase than that of kojic acid (Oozeki et al., 2008), whereas monobenzone analogues (**5**) and 3,5-dihydroxyphenoxy benzyl ethers (**6**) exhibited superior activity to arbutin (Sapkota et al., 2011). However, it was proven that 4-hydroxyphenyl benzyl ether analogues failed to inhibit tyrosinase function (Sapkota et al., 2011). Recently, it was reported that bis (4-hydroxybenzyl) sulfide (**7**) inhibited tyrosinase *in vitro* about 80 times better than that of kojic acid (Chen et al., 2015). The chemical structures of aforementioned tyrosinase inhibitors are illustrated in Figure 1.

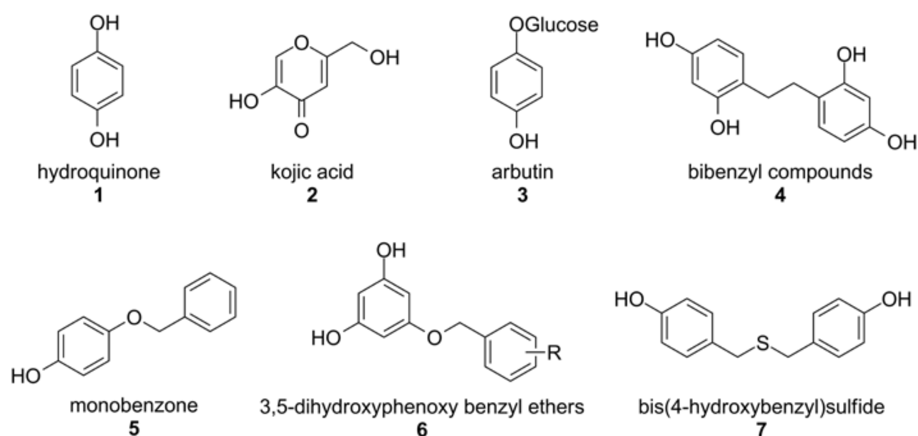


Figure 1 The chemical structures of anti-tyrosinase agents; hydroquinone (**1**) (Fitzpatrick et al., 1966; Kligman and Willis, 1975), kojic acid (**2**) (Ohyama, 1990), arbutin (**3**) (Tokiwa et al., 2007; Hori et al., 2004), bibenzyl compounds (**4**) (Oozeki et al., 2008), monobenzone (**5**) (Sapkota et al., 2011), 3,5-dihydroxyphenoxy benzyl ether (**6**) (Sapkota et al., 2011) and bis (4-hydroxybenzyl) sulfide (**7**) (Chen et al., 2015)

We are interested in improving their tyrosinase inhibitory activity of monobenzone and phenyl benzyl ethers. Although it was reported that 4-hydroxyphenyl benzyl ethers were unable to inhibit tyrosinase activity (Sapkota et al., 2011), effects of other functional groups and their locations on the phenyl ring have not been yet studied. Herein, various substituted phenyl benzyl ethers were prepared and their anti-tyrosinase activity *in vitro* was investigated. The influences of substituents and their positions at the phenyl ring on their activity were studied.

2. MATERIALS AND METHODS

2.1 Materials

Chemicals and reagents used were purchased from Acros Organics, Sigma-Aldrich, Tokyo Chemical Industry (TCI), Carlo Erba and Fisher Scientific. All reagents received were analytical grade and used as received without purification, unless stated otherwise. Deionized water was used in this experiment. Preparative chromatographic separations were performed on silica gel 63-200 μm purchased from Merck. All reactions were followed by TLC analysis using precoated silica gel 60 TLC sheets (Merck) with fluorescent indicator (254 nm) and visualized with a UV lamp (254 and 365 nm).

2.2 Instrumentation

Melting points were measured on a Büchi Melting Point B-545 apparatus. Infrared (IR) spectra were recorded on a Thermo Scientific Nicolet 6700 FT-IR or Perkin Elmer Spectrum 100 FT-IR spectrophotometer. ^{1}H and ^{13}C NMR (complete proton decoupling) spectra were recorded on either a Bruker AVANCE 300 or Bruker AVANCE III 400 or Bruker AV-500 in Fourier transform mode at the field strength specified on either a 300, 400 or 500 MHz spectrometer. Spectra were obtained on $CDCl_3$ and DMSO-d_6 solutions on 5 mm diameter tubes, and chemical shifts in ppm (part per million) are quoted relative to either internal standard with TMS (δ_H 0.00 ppm) or the residual signals of either $CDCl_3$ (δ_H 7.26 ppm, or δ_C 77.22 ppm) or DMSO-d_6 (δ_H 2.50 ppm, or δ_C 39.51 ppm). Data are reported as follows: chemical shifts, multiplicity, coupling constant. Multiplicities in the ^{1}H NMR spectra are described as: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad; coupling constants (J) are reported in Hz. High resolution mass spectra (HRMS) are recorded using Bruker micrOTOF mass spectrometer with ESI-TOF mode and reported with ion mass/charge (*m/z*) ratios as values in atomic mass units.

2.3 Synthesis of phenyl benzyl ethers

2.3.1 General Procedure: preparation of phenyl benzyl ethers

To a stirred mixture of substituted phenol and potassium carbonate in acetonitrile at room temperature was added benzyl bromide dropwise. The obtained mixture was heated to reflux with stirring for a given reaction time. The reaction mixture was cooled down to room temperature and worked up. The aqueous phase was extracted with EtOAc and the combined organic phases were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, EtOAc and hexane) to give the titled product.

*2.3.2 Phenyl benzyl ether (**21**)* (Huang and Kang, 2017)

As described in the general procedure 4.3.1, phenol (**8**) (1.58 g, 16.8 mmol), potassium carbonate (4.42 g, 32.0 mmol), acetonitrile (50 mL) and benzyl bromide (4.04 g, 23.6 mmol) were used in the reaction. The resulting mixture was refluxed for 20 h. After the reaction was cooled down and concentrated, the residue was diluted with dichloromethane (40 mL) and washed with 10% aqueous NaOH (3 x 40 mL), water (1 x 40 mL) and brine (1 x 40 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated

in vacuo. The crude product was purified by flash column chromatography (silica gel, 1% EtOAc in hexane) to furnish phenyl benzyl ether (**21**) as white crystals (2.81 g, 15.3 mmol, 91%). Mp. 45-47°C; IR (Nujol) ν (cm^{-1}) 2925, 2855, 1600, 1588, 1496, 1460, 1377, 1242, 1170, 1077, 1030, 973; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 5.01 (s, 2H), 6.85-6.95 (m, 3H), 7.23-7.45 (m, 7H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 70.2 (CH_2), 113.1 (CH), 116.7 (CH), 127.4 (2xCH), 128.1 (2xCH), 128.6 (2xCH), 132.3 (2xCH), 136.5 (C), 157.8 (C); HRMS (ESI) calcd for $C_{13}H_{12}ONa$ $[M+Na]^+$ *m/z* 207.0786, found *m/z* 207.0780.

*2.3.3 4-Hydroxyphenyl benzyl ether or monobenzone (**5**)* (Schmidt and Riemer, 2016)

As described in the general procedure 4.3.1, 4-acetoxyphenol (**9**) (0.53 g, 3.5 mmol), potassium carbonate (0.92 g, 6.7 mmol), acetonitrile (25 mL) and benzyl bromide (0.5 mL, 4.2 mmol) were used in the reaction. The resulting mixture was refluxed for 19 h. The reaction was cooled down, quenched with water (30 mL) and extracted with EtOAc (3 x 30 mL). The combined organic phase was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, 3% EtOAc in hexane) to furnish 4-acetoxyphenyl benzyl ether as white powder (0.64 g, 2.7 mmol, 77%). Mp. 108-110°C; IR (ATR) ν (cm^{-1}) 3476 (br), 2815, 1601, 1506, 1448, 1367, 1170, 1097, 1046, 1015; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 2.30 (s, 3H), 5.05 (s, 2H), 6.98 (d, *J*=6.0 Hz, 4H), 7.29-7.44 (m, 5H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm) 21.1 (CH_3), 70.4 (CH_2), 115.4 (2xCH), 122.32 (2xCH), 127.5 (2xCH), 128.1 (2xCH), 128.6 (CH), 136.8 (C), 144.4 (C), 156.5 (C), 169.9 (C); HRMS (ESI) calcd for $C_{15}H_{14}O_3Na$ $[M+Na]^+$ *m/z* 265.0841, found *m/z* 265.0846. It was used in the next step.

A solution of 4-acetoxyphenyl benzyl ether (15.8 mg, 0.07 mmol) in 1 M aqueous NaOH in EtOH (1:1, 15 mL) was heated to reflux at 80°C for 2 h. The reaction mixture was cooled down to room temperature and quenched with 1 M aqueous HCl (7 mL). The aqueous phase was extracted with dichloromethane (3 x 30 mL). The combined organic phase was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash column chromatography (silica gel, 10% EtOAc in hexane) to give 4-hydroxyphenyl benzyl ether (**5**) as a yellow solid (12.6 mg, 0.068 mmol, 97%). Mp. 114-116°C; IR (ATR) ν (cm^{-1}) 3388, 3055, 2987, 1602, 1509, 1454, 1422, 1439, 1266, 1230, 1018, 986; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 4.71 (s, 1H), 5.04 (s, 2H), 6.76 (d, *J* = 9.0 Hz, 2H), 6.87 (d, *J* = 9.0 Hz, 2H), 7.29-7.41 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 70.9 (CH_2), 116.1 (CH), 127.2 (CH), 127.6 (CH), 127.8 (CH), 128.0 (CH), 128.1 (CH), 128.6 (2xCH), 128.7 (CH), 137.2 (C), 149.9 (C), 152.8 (C); HRMS (ESI) calcd for $C_{13}H_{12}O_2Na$ $[M+Na]^+$ *m/z* 223.0735, found *m/z* 223.0730.

*2.3.4 4-Fluorophenyl benzyl ether (**22**)* (Gamache et al., 2016)

As described in the general procedure 4.3.1, 4-fluorophenol (**10**) (0.30 g, 2.9 mmol), potassium carbonate (0.77 g, 5.6 mmol), acetonitrile (15 mL) and benzyl bromide (0.4 mL, 3.3 mmol) were used in the reaction. The resulting mixture was refluxed for 16 h. The reaction was cooled down and washed with 10% aqueous NaOH (2 x 20 mL), water (1 x 20 mL) and brine (1 x 20 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, hexane) to afford 4-fluorophenyl benzyl ether (**22**) as a colorless liquid (0.4485 g, 2.2 mmol, 76%). IR (neat) ν (cm^{-1}) 3034, 2886, 2855, 1642, 1598, 1501, 1450, 1380, 1295, 1248, 1197, 1097, 1039, 1026, 905; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 5.00 (s, 2H), 6.88-7.00 (m, 4H), 7.30-7.44 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 70.7 (CH_2), 114.9 (CH), 115.7 (CH), 115.8 (CH),

115.9 (CH), 116.0 (CH), 127.5 (CH), 128.0 (CH), 128.6 (CH), 136.9 (CH), 154.9 (C), 155.8 (C), 159.9 (C); HRMS (ESI) calcd for $C_{13}H_{11}FONa$ $[M+Na]^+$ *m/z* 225.0692, found *m/z* 225.0686.

*2.3.5 4-Chlorophenyl benzyl ether (**23**)* (Singh et al., 2014)

As described in the general procedure 4.3.1, 4-chlorophenol (**11**) (0.53 g, 4.1 mmol), potassium carbonate (1.07 g, 7.7 mmol), acetonitrile (25 mL) and benzyl bromide (0.6 mL, 0.5 mmol) were used in the reaction. The resulting mixture was refluxed for 17 h. The reaction was cooled down and washed with 10% aqueous NaOH (4 x 20 mL), water (1 x 30 mL) and brine (1 x 30 mL). The combined organic phases were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, hexane) to afford 4-chlorophenyl benzyl ether (**23**) as yellow needles (0.785 g, 3.6 mmol, 88%). Mp. 40-42°C; IR (Nujol) ν (cm^{-1}) 2934, 2725, 2671, 1642, 1601, 1582, 1491, 1460, 1377, 1310, 1286, 1241, 1169, 1096, 1040, 1026, 1007, 972; ^1H NMR (300 MHz,$CDCl_3$) δ (ppm) 5.05 (s, 2H), 6.89 (d, *J* = 9.0 Hz, 2H), 7.22 (d, *J* = 9.0 Hz, 2H), 7.32-7.44 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 70.3 (CH_2), 115.0 (CH), 115.7 (CH), 115.9 (CH), 116.2 (CH), 125.8 (CH), 127.4 (CH), 128.1 (CH), 128.6 (CH), 129.4 (CH), 136.8 (C), 154.9 (C), 157.4 (C); HRMS (ESI) calcd for $C_{13}H_{11}ClONa$ $[M+Na]^+$ *m/z* 241.0396, found *m/z* 241.0391.

*2.3.6 4-Bromophenyl benzyl ether (**24**)* (Xiong et al., 2017)

As described in the general procedure 4.3.1, 4-bromophenol (**12**) (0.3 g, 1.3 mmol), potassium carbonate (0.39 g, 2.82 mmol), acetonitrile (30 mL) and benzyl bromide (0.3 mL, 2.51 mmol) were used in the reaction. The resulting mixture was refluxed for 20 h. After the reaction was cooled down, quenched with water (30 mL) and extracted with EtOAc (3 x 30 mL), the combined organic phases were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, hexane) to afford 4-bromophenyl benzyl ether (**24**) as a white powder (0.211 g, 0.9 mmol, 69%): Mp. 62-64°C; IR (ATR) ν (cm^{-1}) 3032, 2907, 2862, 1589, 1576, 1489, 1455, 1383, 1287, 1247, 1116, 1103, 1016, 999; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 5.00 (s, 2H), 6.84 (d, *J* = 9.0 Hz, 2H), 7.36 (d, *J* = 9.0 Hz, 2H), 7.29-7.42 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 70.2 (CH_2), 113.1 (C), 115.2 (CH), 115.8 (CH), 116.1 (CH), 116.7 (CH), 127.4 (CH), 128.0 (CH), 128.6 (CH), 128.8 (CH), 132.3 (CH), 136.5 (C), 157.8 (C); HRMS (ESI) calcd for $C_{13}H_{11}BrONa$ $[M+Na]^+$ *m/z* 284.9891, found *m/z* 284.9885.

*2.3.7 3-Hydroxyphenyl benzyl ether (**25**)* (Taniguchi et al., 2015)

As described in the general procedure 4.3.1, resorcinol (**13**) (0.419 g, 3.80 mmol), potassium carbonate (0.357 g, 2.58 mmol), acetonitrile (50 mL) and benzyl bromide (0.637 g, 3.72 mmol) were used in the reaction. The resulting mixture was refluxed for 18 h. The reaction was cooled down, quenched with water (60 mL) and extracted with EtOAc (3 x 60 mL). The combined organic phases were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, 10% EtOAc in hexane) to afford 3-hydroxyphenyl benzyl ether (**25**) as a yellow oil (0.302g, 1.51 mmol, 41%). IR (neat) ν (cm^{-1}) 3369 (br), 3064, 3033, 2940, 2874, 1597, 1491, 1455, 1382, 1329, 1284, 1216, 1173, 1148, 1080; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 4.97 (s, 2H), 6.40-6.47 (m, 2H), 6.57 (d, *J* = 2.4 Hz, 1H), 7.11 (t, 1H, *J* = 8.1 Hz), 7.28-7.41 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 70.2, 102.7, 107.5, 108.1, 127.4, 127.9, 128.6, 130.2, 137.1, 156.8, 160.3; HRMS (ESI) calcd for $C_{13}H_{12}O_2Na$ $[M+Na]^+$ *m/z* 223.0735, found *m/z* 223.0730.

*2.3.8 2-Hydroxyphenyl benzyl ether (**26**)* (Pilkington et al., 2015)

As described in the general procedure 4.3.1, pyrocatechol (**14**) (0.453 g, 5.14 mmol), potassium carbonate (0.406 g, 2.94 mmol), acetonitrile (50 mL) and benzyl bromide (0.879 g, 5.14 mmol) were used in the reaction. The resulting mixture was refluxed for 6 h. The reaction was cooled down. Water (50 mL) was added to the mixture and extracted with EtOAc (3 x 50 mL). The combined organic phases were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, 5% EtOAc in hexane) to afford 2-hydroxyphenyl benzyl ether (**26**) as a colorless oil (0.544 g, 2.72 mmol, 66%). IR (Nujol) ν (cm^{-1}) 3453 (br), 3064, 3033, 2939, 2874, 1597, 1503, 1466, 1455, 1385, 1356, 1262, 1220, 1108, 1037, 1010, 916, 856; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm)5.06 (s, 2H), 5.73 (br s, 1H), 6.78-6.96 (m, 4H), 7.30-7.39 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 71.3 (CH_2), 112.6 (CH), 114.9 (CH), 120.1 (CH), 122.0 (CH), 127.7 (CH), 128.3 (CH), 128.7 (CH), 136.5 (C), 145.9 (C), 146.1 (C); HRMS (ESI) calcd for $C_{13}H_{12}O_2Na$ $[M+Na]^+$ *m/z* 223.0735, found *m/z* 223.0730.

*2.3.9 2-Fluorophenyl benzyl ether (**27**)* (Dubbaka et al., 2015)

As described in the general procedure 4.3.1, 2-fluorophenol (**15**) (0.502 g, 4.50 mmol), potassium carbonate (1.40 g, 10.0 mmol), acetonitrile (15 mL) and benzyl bromide (0.70 mL, 5.7 mmol) were used in the reaction. The resulting mixture was refluxed for 16 h. The reaction was cooled down. Water (20 mL) was added to the mixture and extracted with EtOAc (3 x 20 mL). The combined organic phases were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, hexane) to afford 2-fluorophenyl benzyl ether (**27**) as white crystals (0.906 g, 4.5 mmol, 100%). Mp. 67-70°C; IR (Nujol) ν (cm^{-1}) 3069, 2924, 2855, 1615, 1594, 1507, 1457, 1377, 1316, 1279, 1260, 1208, 1157, 1110, 1079, 1038, 1025, 916; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 5.13 (s, 2H), 6.85-7.15 (m, 4H), 7.30-7.51 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 71.4 (CH_2), 116.3 (CH), 116.5 (CH), 120.4 (CH), 121.5 (CH), 121.6 (CH), 124.4 (CH), 125.4 (CH), 126.5 (CH), 127.1 (CH), 136.8 (C), 151.5 (C), 154.8 (C); HRMS (ESI) calcd for $C_{13}H_{11}FONa$ $[M+Na]^+$ *m/z* 225.0692, found *m/z* 225.0686.

*2.3.10 2-Chlorophenyl benzyl ether (**28**)* (Morin et al., 2013)

As described in the general procedure 4.3.1, 2-chlorophenol (**16**) (0.50 g, 3.9 mmol), potassium carbonate (1.05 g, 7.6 mmol), acetonitrile (20 mL) and benzyl bromide (0.60 mL, 0.5 mmol) were used in the reaction. The resulting mixture was refluxed for 17 h. The reaction was cooled down and washed with 10% aqueous NaOH (2 x 20 mL), water (1 x 20 mL) and brine (1 x 20 mL). The combined organic phases were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, hexane) to afford 2-chlorophenyl benzyl ether (**28**) as a colorless liquid (0.664 g, 3.0 mmol, 77%). IR (ATR) ν (cm^{-1}) 3066, 3032, 2873, 1777, 1749, 1630, 1590, 1485, 1446, 1380, 1294, 1247; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 5.15 (s, 2H), 6.95-7.05 (m, 4H), 7.32-7.43 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 70.7 (CH_2), 114.9 (CH), 115.7 (CH), 115.9 (CH), 115.9 (CH), 116.0 (CH), 127.5 (CH), 128.0 (CH), 128.6 (CH), 129.6 (CH), 136.9 (C), 155.8 (C), 159.0 (C); HRMS (ESI) calcd for $C_{13}H_{11}ClNaO$ $[M+Na]^+$ *m/z* 241.0396, found *m/z* 241.0391.

*2.3.11 2,4-Dibromophenyl benzyl ether (**29**)* (Parra et al., 2011)

As described in the general procedure 4.3.1, 2,4-dibromophenol (**17**) (0.501 g, 2.0 mmol), potassium

carbonate (0.55 g, 4.0 mmol), acetonitrile (25 mL) and benzyl bromide (0.30 mL, 2.5 mmol) were used in the reaction. The resulting mixture was refluxed for 24 h. The reaction was cooled down and quenched with water (25 mL) and extracted with EtOAc (3 x 25 mL). The combined organic phases were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, hexane) to afford 2,4-dibromophenyl benzyl ether (**29**) as white crystals (0.706 g, 2.0 mmol, 100%). Mp. 64-66°C; IR (Nujol) ν (cm^{-1}) 2923, 2725, 1578, 1566, 1497, 1460, 1377, 1309, 1288, 1264, 1246, 1150, 1079, 1051, 1022, 974; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 5.15 (s, 2H), 6.75 (m, 1H), 7.30-7.50 (m, 6H), 7.70 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 71.0 (CH_2), 113.4 (C), 115.0 (CH), 127.1 (CH), 128.2 (CH), 128.7 (CH), 129.2 (CH), 129.7 (CH), 131.2 (CH), 135.6 (CH), 136.1 (C), 154.4 (C); HRMS (ESI) calcd for $C_{13}H_{10}Br_2ONa$ $[M+Na]^+$ *m/z* 364.8976, found *m/z* 362.8991.

*2.3.12 2,6-dibromo-4-methylphenyl benzyl ether (**30**)* (Cram et al., 1984)

As described in the general procedure 4.3.1, 2, 6-dibromo-4-methylphenol (**18**) (0.50 g, 1.9 mmol), potassium carbonate (0.41 g, 3.0 mmol), acetonitrile (15 mL) and benzyl bromide (0.35 g, 2.0 mmol) were used in the reaction. The resulting mixture was refluxed for 24 h. The reaction was cooled down and quenched with water (20 mL) and extracted with EtOAc (3 x 20 mL). The combined organic phases were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, hexane) to afford 2,6-dibromo-4-methylphenyl benzyl ether (**30**) as a white solid (0.6584 g, 1.8 mmol, 95%). Mp. 72-74°C; IR (ATR) ν (cm^{-1}) 3068, 3031, 2948, 2892, 1589, 1541, 1497, 1454, 1373, 1254, 1217; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 2.30 (s, 3H), 5.00 (s, 2H), 7.35 (s, 2H) 7.37-7.45 (m, 3H), 7.60 (d, *J* = 9.0 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 20.4 (CH_3), 74.7 (CH_2), 118.1 (C), 127.4 (2 x CH), 128.4 (2 x CH), 128.5 (2 x CH), 132.8 (CH), 136.5 (2 x C), 136.8 (C), 150.6 (C); HRMS (ESI) calcd for $C_{14}H_{12}Br_2NaO$ $[M+Na]^+$ *m/z* 376.9153, found *m/z* 376.9147.

*2.3.13 2,4,6-Tribromophenyl benzyl ether (**31**) (Johnson et al., 2008)*

As described in the general procedure 4.3.1, 2,4,6-tribromophenol (**19**) (1.03 g, 3.1 mmol) and potassium carbonate (0.85 g, 6.2 mmol), acetonitrile (30 mL) and benzyl bromide (0.40 mL, 3.3 mmol) were used in the reaction. The resulting mixture was refluxed for 16 h. The reaction was cooled down and quenched with water (30 mL) and extracted with EtOAc (3 x 30 mL). The combined organic phases were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, hexane) to deliver 2,4,6-tribromophenyl benzyl ether (**31**) as white crystals (1.3560 g, 3.1 mmol, 100%). Mp. 84-86°C; IR (Nujol) ν (cm^{-1}) 3069, 2924, 2854, 1560, 1538, 1463, 1376, 1306, 1246, 1170, 1065, 969; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 5.02 (s, 2H), 7.34-7.45 (m, 3H), 7.58 (dd, *J* = 7.8, 1.8 Hz, 2H), 7.67 (s, 2H) ; ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 74.8 (CH_2), 117.7 (CH), 119.3 (2 x CH), 127.7 (C), 128.5 (2 x CH), 129.5 (2 x C), 135.1 (2 x CH), 135.9 (C), 152.4 (C); HRMS (ESI) calcd for $C_{13}H_9Br_3ONa$ $[M+Na]^+$ *m/z* 440.8101, found *m/z* 440.8113.

*2.3.14 2,4,6-Tribromo-3-methylphenyl benzyl ether (**32**)*

As described in the general procedure 4.3.1, 2,4,6-tribromo-3-methylphenol (**20**) (0.552 g, 1.6 mmol) and potassium carbonate (0.43 g, 3.1 mmol), acetonitrile (15 mL) and benzyl bromide (0.32 g, 1.9 mmol) were used in the reaction. The resulting mixture was refluxed for 22 h. The reaction was cooled down and quenched with water (20 mL) and extracted with EtOAc (3 x 20 mL). The combined organic phases

were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, hexane) to deliver 2,4,6-tribromo-3-methylphenyl benzyl ether (**32**) as a white powder (0.666 g, 1.5 mmol, 94%). Mp. 96-98°C; IR (Nujol) ν (cm^{-1}) 2925, 2725, 1463, 1377, 1346, 1257, 1201, 1155, 1077, 1037, 946; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 2.60 (s, 3H), 5.00 (s, 2H), 7.35-7.45 (m, 3H), 7.60 (d, *J* = 6.6 Hz, 2H), 7.78 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 24.3 (CH_3), 74.5 (CH_2), 115.6 (C), 120.1 (C), 122.4 (C), 127.3 (2 x CH), 128.5 (2 x CH), 134.9 (CH), 136.1 (C), 138.7 (C), 152.4 (C); HRMS (ESI) calcd for $C_{14}H_{11}Br_3ONa$ $[M+Na]^+$ *m/z* 454.8258, found *m/z* 454.8252.

2.4 *In vitro* tyrosinase inhibitory activity assay

Tyrosinase activity inhibition was determined by the method as described previously (Chompoo et al., 2012) by measuring the DOPAchrome formed due to the action of tyrosinase enzyme on DOPA substrates. In brief, the 96-well plate was set up in the following order; 140 μl of 50 mM phosphate buffer (pH 6.8), 20 μl of sample and 20 μl of 400 units/ml mushroom tyrosinase in 50 mM phosphate buffer (pH 6.8). After incubation at 25°C for 10 min, reaction was initiated by adding 20 μl of 3.0 mM DOPA substrate solutions to each well and incubated further at 25°C for 10 min. The enzyme activity was determined by measuring the absorbance at 492 nm using microplate reader spectrophotometer. Kojic acid was used as a positive control. Negative control without extracts was set up in parallel. The percentage of tyrosinase inhibition was calculated as follows:

$$\text{Tyrosinase inhibition (\%)} = \frac{[(A - B) - (C - D)]}{(A - B)} \times 100$$

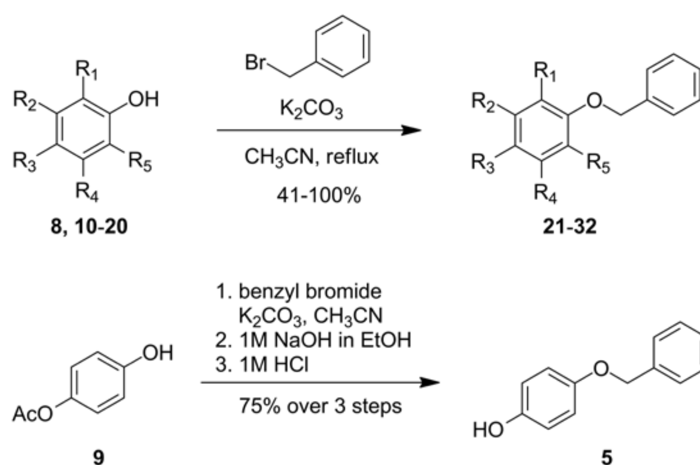
where A is the absorbance of the control with the enzyme, B is the absorbance of the control without the enzyme, C is the absorbance of the test sample with the enzyme and D is the absorbance of the test sample without the enzyme.

3. RESULTS AND DISCUSSION

3.1 Chemistry: Synthesis of phenyl benzyl ether analogues

Monobenzone consists of two benzene rings; phenyl and benzyl rings. According to the substituents used in the phenyl benzyl analogues in the previous literature (Sapkota et al., 2011), hydroxyl group with ability to form hydrogen bonding, halogens with different size (fluorine, chlorine and bromine) and nonpolar methyl group were selected to be introduced to the phenyl moiety at different positions, in order to study the effects of substituents and their positions at the phenyl ring on their anti-tyrosinase activity *in vitro*. In this work, thirteen phenyl benzyl ether analogues including monobenzone were prepared and identified mainly by NMR and MS.

The syntheses of phenyl benzyl ethers **21**-**32** were performed by bimolecular nucleophilic substitution (S_N2) of the corresponding substituted phenols **8**, **10**-**20** and benzyl bromide under basic conditions with moderate to excellent yields as depicted in Scheme 1. Preparation of monobenzone (**5**) was successful with a good yield *via* nucleophilic substitution of 4-acetoxyphenol (**9**) and benzyl bromide, followed by hydrolysis (Scheme 1). The structures of starting materials and products, and yield percentages of the reaction were shown in Table 1. The structures of the synthetic inhibitors were confirmed mainly by infrared spectroscopy (IR), nuclear magnetic resonance (NMR) and mass spectrometry (MS), which spectra of all known compounds (**5**, **21**-**31**) agreed with the literature (see the experimental section).



Scheme 1 The nucleophilic substitution reaction between phenols **8**-**20** and benzyl bromide to monobenzone (**5**) phenyl benzyl ether analogues **21**-**32**

Table 1 The chemical structures of starting materials **8**-**20**, synthetic inhibitors **5**, **21**-**32**, yield of reactions, inhibition percentages at 500 μM and IC_{50} values

Entry	Phenol	Product	Substituents					Yield	% Inhibition at 500 μM	IC_{50} (μM)
			R_1	R_2	R_3	R_4	R_5			
1	**8**	**21**	H	H	H	H	H	91%	23.4 ± 0.20	-
2	**9**	**5**	H	H	OH	H	H	75%	44.9 ± 0.40	-
3	**10**	**22**	H	H	F	H	H	76%	27.7 ± 0.18	-
4	**11**	**23**	H	H	Cl	H	H	88%	66.0 ± 0.18	55.7 ± 0.29
5	**12**	**24**	H	H	Br	H	H	69%	17.6 ± 0.24	-
6	**13**	**25**	H	OH	H	H	H	41%	19.3 ± 0.27	-
7	**14**	**26**	OH	H	H	H	H	66%	0.29 ± 0.10	-
8	**15**	**27**	F	H	H	H	H	100%	11.0 ± 0.18	-
9	**16**	**28**	Cl	H	H	H	H	77%	29.2 ± 0.09	-
10	**17**	**29**	Br	H	Br	H	H	100%	25.6 ± 0.50	-
11	**18**	**30**	Br	H	CH_3	H	Br	95%	56.0 ± 0.24	216 ± 1.8
12	**19**	**31**	Br	H	Br	H	Br	100%	79.1 ± 0.18	164 ± 0.8
13	**20**	**32**	Br	CH_3	Br	H	Br	94%	84.8 ± 0.24	93.8 ± 0.33
14	Kojic acid (**2**)								78.0 ± 0.25	106 ± 0.3

3.2 Relationship of structure and *in vitro* anti-tyrosinase activity

After obtaining the synthetic inhibitors, their anti-tyrosinase activity was investigated *in vitro* (Chompoo et al., 2012). Preliminarily, inhibition percentage at 500 μM of all synthetic inhibitors was determined. For the potent agents, the IC_{50} values were determined compared to that of kojic acid (**2**). All results are indicated in Table 1.

It was revealed that substitution at different positions on the phenyl part of the ethers influences significantly on their activity. *p*-Hydroxy- **5**, *p*-fluoro- **22** and *p*-chlorophenyl analogues **23** showed 44.9%, 27.7% and 66.0% inhibition, respectively, which were significantly higher tyrosinase inhibition compared to those of unsubstituted (**21**), *meta*- (**25**) and *ortho*-substituted version (**26-28**) at the same concentration. *p*-Hydroxy compound **5** was able to inhibit tyrosinase better than *m*-hydroxy **25** and *o*-hydroxy ones **26** at 500 μM, respectively. In the same way, *p*-fluoro **22** and *p*-chloro analogues **23** exhibited obviously higher inhibition percentage than *o*-fluoro **27** and *o*-chloro **28** compounds at the same concentration. Having a substituent, such as halogen (F and Cl atom) and OH group, which can form hydrogen bond, at *para*-position help to improve the inhibition percentage compared with unsubstituted analogue. Nevertheless, *p*-bromophenyl benzyl ether (**24**) possessed the lowest inhibition percentage in the group. Among monosubstituted phenyl series, the *p*-chlorinated analogue **23** had the most effective tyrosinase inhibitory activity, even better than monobenzone (**5**). Unfortunately, the monosubstituted analogues still inhibited tyrosinase less efficiently than kojic acid (**2**).

Thus, multi-substituted phenyl benzyl ethers **29-32** were considered to be prepared and tested. Tribrominated analogues **31** and **32** exhibited with 79.1% and 84.8% inhibition at 500 μM, comparable to kojic acid (78.0% inhibition), whereas dibrominated **29** and **30**, and monobrominated inhibitors **24** showed significantly lower inhibition percentages. Interestingly, it was observed that the more substituents on the phenyl moiety, the more anti-tyrosinase activity they possessed. Most of polysubstituted ethers were more potent than monosubstituted ones, except in the case of *p*-chlorophenyl benzyl ether **23**. Dibrominated ether **30** with methyl group had higher inhibition ability than dibromo analogue **29** without methyl one. Especially, the tribrominated ether **32** with an additional methyl group inhibited tyrosinase stronger than tribromo inhibitor **31** without a methyl substituent. Substituents on phenyl ring of the polysubstituted phenyl inhibitors might help to interact more efficiently with the active site of the enzyme than those of mono- and di-substituted analogues. Comparison of 2,6-dibromo-4-methylphenyl benzyl ether (**30**) and 2,4,6-tribromophenyl benzyl ether (**31**) revealed that replacing a methyl group with a bromine increased the inhibition percentage from 56% to 79% at 500 μM, and decreased IC_{50} from 216 μM to 164 μM.

To confirm their inhibitory property, we evaluated the IC_{50} of the selected compounds that could potentially be highly effective inhibitors to tyrosinase (% inhibition at 500 μM > 50%). The IC_{50} values confirmed efficient inhibitory property of chlorinated and brominated analogues **23** and **32** with IC_{50} = 55.7 μM and 93.8 μM, respectively. Ultimately, *p*-chlorinated ether **23** showed almost two-fold more potent than kojic acid (IC_{50} = 106 μM). In comparison with phenyl benzyl compounds in the literature (Sapkota et al., 2011), *p*-chlorophenyl benzyl ether (**23**) exhibited similar inhibition potency against tyrosinase to 5-(3-chlorobenzyloxy) benzene-1, 3-diol (IC_{50} = 54.7 μM) and 5-(3,4-dichlorobenzyloxy) benzene-1,3-diol (IC_{50} = 66.4 μM) (Sapkota et al., 2011), while the structure of the ether **23** is much simpler. Thus, analogue **23** can be a suitable core structure for further development of phenyl benzyl ethers as tyrosinase inhibitors. In addition, the IC_{50} value of tribrominated analogue with a methyl group

32 (IC_{50} = 93.8 μM) was obviously lower than that of **31** (without methyl function, IC_{50} = 164 μM). These findings suggested that proper type and number of substituents, and their locations on the phenyl ring are necessary effects for inhibiting tyrosinase function.

4. CONCLUSION

Thirteen phenyl benzyl ether analogues with various functional groups at different positions on the phenyl ring were successfully prepared for evaluation of their anti-tyrosinase activity. Our results revealed that having a functional group at *para*-position led to higher inhibition than having none and substituent at *ortho*- and *meta*-positions. Among monosubstituted inhibitors, *p*-chlorophenyl benzyl ether **23** exhibited highest inhibition percentage at the same concentration. In addition, the IC_{50} value of *p*-chloro inhibitor **23** was almost two-fold superior to kojic acid, and comparable to those of phenyl benzyl ethers in the previous study (Sapkota et al., 2011). Furthermore, we demonstrated that most synthetic polysubstituted phenyl analogues inhibited tyrosinase more efficiently than monosubstituted ones, for examples, analogue **32**, which possessed similar inhibitory potency to kojic acid. Thus, the synthesized inhibitors **23** and **32** were identified to be potential candidates for anti-browning and whitening agents. In particular, the structure of **23** is very simple, which can be an appropriate lead structure for further development. However, tyrosinase inhibition of di- and trichlorophenyl benzyl analogues and intensive studies, such as binding affinity by molecular modeling and inhibition of α-MSH-induced melanin synthesis using mouse melanoma B-16 cells as well as toxicity test, are considered to be carried out in our future work.

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