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Synthesis and Tyrosinase Inhibitory Activity of (E)-5-Benzyl-7-(3-Bromobenzylidene)-3-(3-Bromophenyl)-2-Phenyl-3,3a,4,5,6,7-Hexahydro-2H-Pyrazolo[4,3-c]Pyridine

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Abstract

The tyrosinase enzyme plays an essential role in the pigmentation of human skin, fruits, and vegetables. It has been tied with several human skin diseases and post-harvest problems. Hence, the tyrosinase enzyme becomes an excellent therapeutic target to overcome these issues. This study aimed to screen tyrosinase inhibitors by synthesizing halogen-substituted pyrazolopyridine derivatives. The pyrazolopyridine compound was obtained through two stages of synthesis. First, the intermediate compound, a derivative of 3,5-bis(arylidene)-4-piperidone, was synthesized through the Cleisen-Schmidt condensation reaction of 4-piperidone and benzaldehyde derivatives. Furthermore, the intermediate compound was reacted with phenylhydrazine through a cyclocondensation reaction to produce the titled compound with an 11% yield. The chemical structure of the target compound was identified through the interpretation of UV, FTIR, NMR, and HRMS spectra. Then an *in vitro* assay was conducted on the tyrosinase enzyme of the fungus *Agaricus bisporus* by detecting the presence of dopachrome at a wavelength of 492 nm. As a result, the *in vitro* assay showed that the titled compound had a weak inhibitory activity, and the IC_{50} value was > 500 µM. Thus, the synthesized compound is considered inactive.

1. Introduction

Tyrosinase is the rate-limiting enzyme of the melanogenic pathway, a copper-containing glycoprotein. The tyrosinase enzyme is the most common target for treating hyperpigmentation [1, 2]. The biosynthesis of the two primary forms of melanin, black/brown eumelanin and yellow/red pheomelanin, is catalyzed by tyrosinase [3]. The formation of melanin can lead to unwanted things such as the browning of fruits, fungi, vegetables, and hyperpigmentation on human skin [4, 5, 6]. Excessive melanin formation can cause human skin diseases such as hyperpigmentation, lentigo, vitiligo, and skin cancer [7, 8]. Furthermore, the role of the tyrosinase enzyme in the browning of fruits and vegetables can also cause post-harvest losses [9, 10].

Kojic acid, a fungal metabolite, is the most widely used tyrosinase inhibitor today. However, animal experiments have shown that kojic acid has weak carcinogenicity; thus, its usage in humans is limited up to a concentration of 1% [11]. Hydroquinone is also a tyrosinase inhibitor that has been used clinically in the treatment of hyperpigmentation in leading cosmetics [12]. However, it has also been found to cause several problems by generating reactive oxygen species (ROS), which causes oxidative lipid damage and permanent loss of melanocytes. Furthermore, hydroquinone has been banned for general use by the European Committee and can only be prescribed by a dermatologist. This has urged researchers and scientists to focus on identifying, isolating, synthesizing, and characterizing new safe tyrosinase inhibitors for various applications in the food,

cosmetic and pharmaceutical industries [13, 14, 15]. However, very few inhibitors qualify for clinical use.

One group of compounds that have received great attention in the search for tyrosinase inhibitor compounds is pyrazole derivatives. Among them are the pyrazole derivatives 3-benzofuran-2-yl-5-(4-dimethylamino-naphthalene-1-yl)-4,5-dihydro-pyrazole-1-carboxylate-(4-chloro-phenyl)-amide has been reported, it exhibits high inhibitory activity against the tyrosinase enzyme, with an IC_{50} value of 5.13 µM [16]. In this matter, the pyrazolo[4,3c]pyridine compound is also a pyrazole derivative compound and hence potentially has similar activity [17]. This compound has various bioactivities, including analgesic, anticancer, anti-inflammatory, antioxidant, antituberculosis, antiviral, and antimicrobial [18, 19, 20, 21, 22]. However, no literature on the tyrosinase inhibitory activity of this pyrazolo[4,3c]pyridine compound has been reported. Moreover, this compound could be obtained by using a biologically active compound containing α,β-unsaturated ketone as starting materials. For instance, curcumin derivatives have been reported as anticancer, antiinflammation, and antioxidant, including SARS-CoV-2 inhibitors [23].

Accordingly, this research conducted the synthesis of (E)-5-Benzyl-7-(3-Bromobenzylidene)-3-(3-Bromophenyl)-2-Phenyl-3,3a,4,5,6,7-Hexahydro-2H-Pyrazolo[4,3-c]Pyridine compound from the reaction of compounds containing unsaturated α,β-keto, a curcumin derivative of 4-piperidone then reacted with phenylhydrazine. The synthesized compound was studied for its bioactivity through *in vitro* assay on fungal tyrosinase enzymes using spectrophotometric methods.

2. Methodology

2.1. Materials

Reagent grade materials of ≥ 95% purity were purchased from Sigma-Aldrich and Merck without further purification. The materials were *N*-benzyl piperidone (Sigma-Aldrich), 3-Bromobenzaldehyde (Sigma-Aldrich), phenylhydrazine (Sigma-Aldrich), hydrochloric acid (Merck), sodium hydroxide (Merck), TLC plate GF_{254} (Merck), universal pH indicator, silica gel 60 (0.063–200 mm) (Merck), and organic solvents.

2.2. Instrument

The instrument used in this study were Monowave50 reactor (Anton-Paar Inc., Austria), UV lamp for TLC (Cole Parmer UV Lamp 254 and 366 nm), Fisher John's melting point device (SMP 11-Stuart®), UV-Visible spectrophotometer (Genesys 10S UV-VIS v4.002 2L9N175013), HPLC (UFLC Prominence-Shimadzu LC Solution, UV detector SPD 20AD), FTIR spectrophotometer (FTIR Shimadzu, IR Prestige-21), mass spectrometer (Water LCT premiere XE positive mode), NMR spectrometer (Agilent 500 MHz with DD2 console system), microplate reader, as well as glassware commonly used in a laboratory.

2.3. Synthesis Procedure of Curcumin Derivate (3)

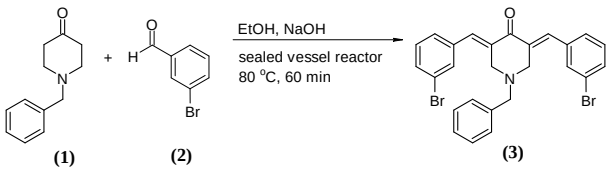


Figure 1. Synthesis of curcumin derivate **(3)**

A mixture of 3-Bromobenzaldehyde **(1)** (3 mmol) and *N*-benzyl piperidone **(2)** (3 mmol) was dissolved in 5 mL of alcohol absolute in a pressure tube equipped with a stir bar. Then 1 mL of sodium hydroxide 3 N was added to the mixture dropwise. The mixture was reacted in a sealed vessel reactor at 80℃ for 60 minutes. After the reaction was completed, the mixture was neutralized using hydrochloric acid 1 N. The mixture was then poured into crushed ice in a beaker glass. The resulting precipitate was filtered, then washed with water and dried. Crystallization of crude product in methanol resulted in desired intermediate compound **(3)** with 88% yield, m.p 212-213℃, UV (EtOH) λmax of 365 nm, FT-IR (KBr) $\bar{\upsilon}$ (cm^{-1}): 3024, 2969, 1675, 1608, 1351, and 697.

2.4. Synthesis Procedure of Pyrazolo[4,3c]pyridine (5)

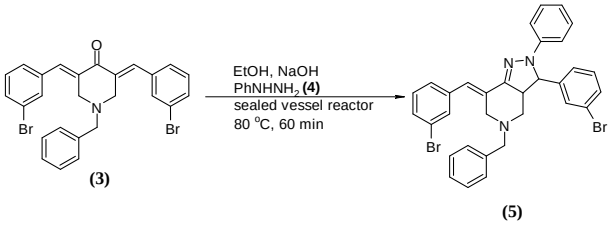


Figure 2. Synthesis of pyrazolo[4,3c]pyridine **(5)**

A mixture of curcumin derivate **(3)** (1 mmol) and phenylhydrazine **(4)** (2 mmol) was dissolved in 5 mL of absolute ethanol in a pressure tube. The experiment was performed in a sealed-vessel reactor in the presence of 1 mL of 3 N sodium hydroxide solution. The mixture was reacted at 80℃ for 60 minutes. After the reaction was completed, the reaction mixture was neutralized and then poured into a beaker glass containing crushed ice. The mixture was allowed to stand in the refrigerator until a maximum precipitate was formed. The solid obtained was filtered, washed with cold water, and then dried at room temperature.

Furthermore, crude product was purified by column chromatography in *n*-hexane:ethylacetate (7:3) to obtain pyrazolo[4,3c]pyridine compounds with 11% yield, m.p 106-107℃. UV (EtOH) λmax of 257 and 365 nm. FT-IR (KBr) $\bar{\upsilon}$ (cm^{-1}): 3083, 2948, 1597, 1566, 1499, 1425, 1278, and 690. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 (s, 1H), 7.45 (d, J = 7.9 Hz, 1H), 7.37 (s, 2H), 7.31 (t, J = 6.9 Hz, 2H), 7.26 – 7.15 (m, 9H), 7.13 (d, J = 7.7 Hz, 1H), 7.02 (d, J = 8.1 Hz, 2H), 6.87 (t, J = 7.3 Hz, 1H), 4.58 (d, J = 12.2 Hz, 1H), 3.97 (d, J = 14.1 Hz, 1H), 3.67 (s, 2H), 3.37–3.23 (m, 2H), 3.13 (d, J = 13.5 Hz, 1H), 2.51 (t, J = 10.3 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 151.02, 146.33, 144.05, 138.05, 137.21, 132.12, 131.05, 130.88, 130.36, 129.77, 129.19, 129.12, 128.92, 128.85, 128.42, 127.97, 127.45, 124.82, 123.36, 122.37, 120.72, 115.05, 71.19, 61.90, 55.58, 55.27,

54.00. HR-MS (ESI): m/z 612.0653, $[M+H]^+$ (cal. for $C_{32}H_{28}N_3Br_2$: 612.0650).

2.5. Tyrosinase Inhibitory Activity Assay

The inhibitory activity assay was conducted using a method that has been reported without modification [24]. The enzyme used in this research was tyrosinase from *Agaricus bisporus*, while the substrate was L-tyrosine. A blank solution was made of 30 µL enzyme tyrosinase 333 U/mL mixed with 70 µL phosphate buffer pH 6.5, then incubated at 37℃ for 5 minutes. Subsequently, 110 µL L-tyrosine 2 mM was added and incubated at 37℃ for 30 minutes. Inhibitory activity of the synthesized compound was determined by mixing 30 µL of the enzyme tyrosinase 333 U/mL and 70 µL of the sample (in phosphate buffer), then incubated at 37℃ for 5 minutes. Followed by the addition of 110 µL L-tyrosine 2 mM and incubated at 37℃ for 30 minutes. The absorbance of each solution was observed at 492 mm using a microplate reader. The inhibition for each enzyme assay was calculated as follows:

$$\% \text{ Inhibition} = \frac{ABS_{control} - ABS_{sample}}{ABS_{control}} \times 100$$

Each experiment was carried out in triplicate (n=3). The IC_{50} value was calculated from linear regression of ln concentration vs % inhibition graph.

3. Results and Discussion

A pyrazolo[4,3c]pyridine compound was successfully synthesized through a two-step reaction using a sealed-vessel reactor (Figures 1 and 2). Firstly, curcumin derivate was synthesized through a Claisen-Schmidt condensation reaction of N-benzyl piperidone **(2)** with 3-Bromobenzaldehyde **(1)** in ethanol in the presence of strong base sodium hydroxide [25]. The reaction, namely an aldol condensation initiated by deprotonation of the symmetrical α carbon atom of the 4-piperidone ring to form enolate ions. The enolate ion, which acts as a nucleophile, attacked the carbonyl carbon atom of the aldehyde to create β-hydroxy curcumin derivate. Subsequently, condensation of intermediate formed curcumin derivate **(3)**. The proposed reaction mechanism is shown in Figure 3.

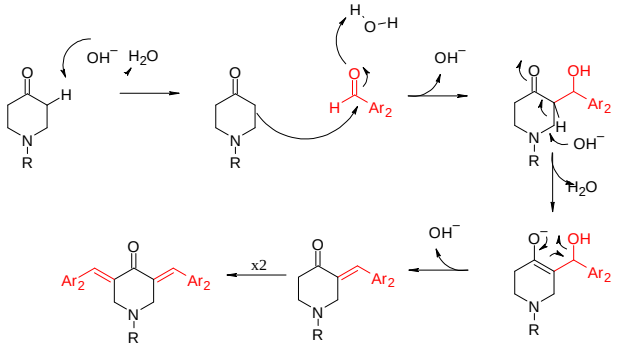


Figure 3. Proposed reaction mechanism of curcumin derivate formation

Secondly, curcumin derivate **(3)** was further reacted with phenylhydrazine **(4)** under similar conditions [18]. The deprotonation of a terminal amine of the hydrazine group increases the nucleophilicity of nitrogen. Hence, attacks on the carbonyl group of curcumin derivate **(3)** are possible. Afterward, the attacks of secondary amine to the alkene group form a cyclic, namely pyrazolo[4,3c] pyridine. The proposed reaction mechanism is shown in Figure 4.

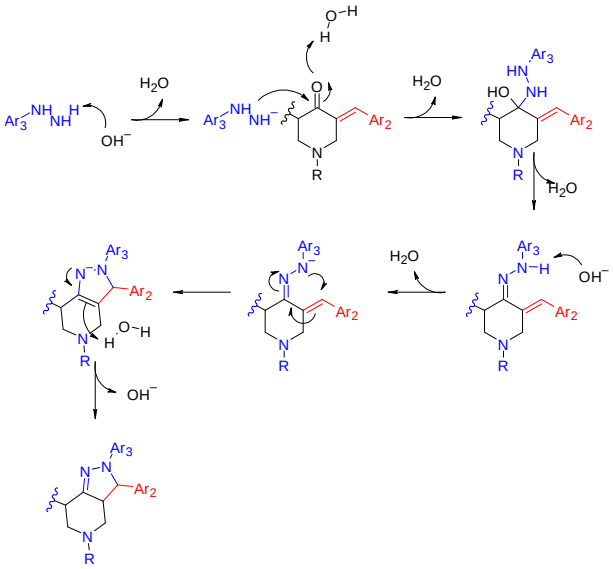


Figure 4. Proposed reaction mechanism of pyrazolo[4,3c]pyridine formation

The molecular structure of the synthesized compound was identified using Fourier Transform Infrared (FTIR), Nuclear Magnetic Resonance (NMR), and High-Resolution Mass Spectroscopy (HRMS) analysis. Many absorption bands were detected in the FTIR spectra, indicating that the pyrazolo[4,3]pyridine compounds had a characteristic bond vibration (Figure 5). Absorption bands $\bar{\upsilon}$ (cm^{-1}) at 3083 shows the vibration of the aromatics C-H bond. The presence of acyclic C-H vibrations is shown at 2948 (cm^{-1}). Absorption bands $\bar{\upsilon}$ (cm^{-1}) at 1597 (cm^{-1}) indicate the presence of a C=C bond vibration. The absorption bands $\bar{\upsilon}$ (cm^{-1}) at 1425 (cm^{-1}) indicate the presence of C-N vibrations of pyrazolo[4,3c]pyridine ring. Furthermore, the absences of C=O absorption (approx. 1650 cm^{-1}) showed a pyrazolo[4,3c]pyridine ring formation.

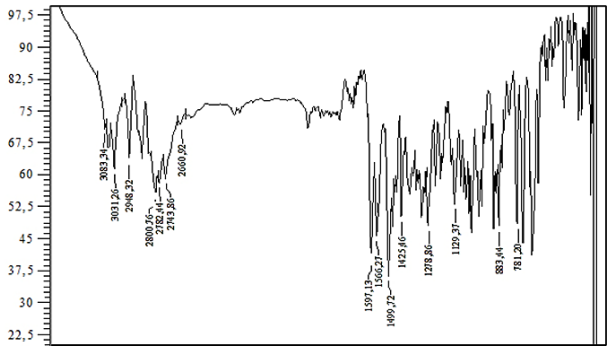


Figure 5. FTIR spectrum of compound **(5)**

The characteristics of the pyrazolopyridine compound are evident in the HNMR spectra of compound **5**, where six proton peaks were found from the sp^3 carbon on the pyrazolopyridine ring, four protons each from the two methylene carbons of the pyridine ring, and two protons on the pyrazole ring (Figure 6). Proton H3a,

which has a characteristically multiplet-oriented peak due to vicinal coupling to protons H3, protons H4a and H4b, was found at δ_H 3.37–3.23 ppm. Meanwhile, the H4a peak was found in the δ_H 2.51 ppm with a triplet peak orientation due to the geminal coupling of the H4b proton ($J_{H4a-H4b}$=10.3 Hz) and the vicinal coupling with the H3a proton ($J_{H4a-H3a}$=10.3 Hz). Furthermore, proton H3 was found at δ_H 4.58 ppm with a doublet peak orientation due to the vicinal coupling to proton H3a with the coupling constant of J_{H3-H3a}=12.2 Hz. The protons H6a and H6b were found at δ_H 3.13 ppm and δ_H 3.97 ppm, respectively. Like the proton at position 4, position 6 also has a different environment, separating the peaks. Each proton peak H6a and H6b is doublet oriented, resulting in a geminal coupling between the protons with a coupling constant, namely $J_{H6a-H6b}$= 14 Hz. Additionally, benzyl substituent's proton of methylene carbon is found at δ_H 3.67 ppm as a singlet.

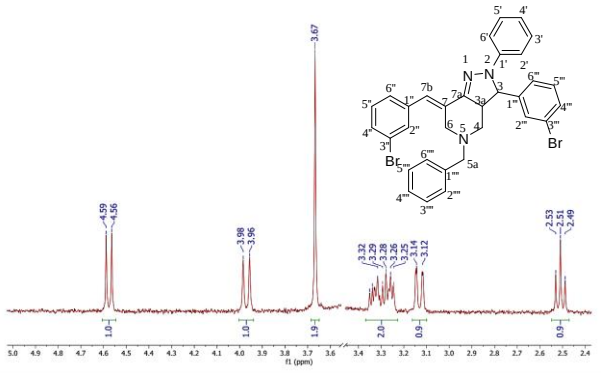
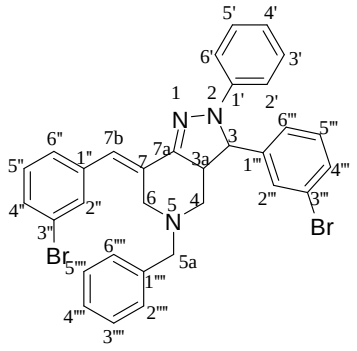


Figure 6. H-NMR upfield region of compound **(5)**

In this regard, to confirm the formation of compound **(5)**, CNMR spectroscopic analysis was carried out. The CNMR spectrum shows a total of 27 peaks. The two phenyl rings are symmetrical, so the total peak corresponds to the target compound. In addition, the presence of the pyrazolopyridine ring was confirmed by the presence of a peak at δ_C 151 ppm indicating the C7a (C=N) carbon of the pyrazolopyridine. The methylene and methine carbons from pyrazolopyridine were also detected at chemical shifts of δ_C 61.9 ppm, δ_C 55.6 ppm, δ_C 55.3 ppm, and δ_C 54.0 ppm, which respectively showed carbons of C3, C3a, C6 and C4 consecutively.

Furthermore, the presence of benzyl substituent was indicated by a peak at δ_C 71.2 ppm, indicating the benzyl C5a methylene carbon. Meanwhile, the presence of a Bromo-substituted ring was confirmed by the appearance of peaks at δ_C 122.4 ppm and δ_C 123.4 ppm, indicating C-Br at positions C3" and C3'", respectively. The interpretation of the NMR spectrum is shown in Table 1.

Table 1. Interpretation of compound **(5)** NMR spectra



Position	^{1}HNMR	^{13}CNMR
	δ_H (ppm) (Multiplisity, *J*)	δ_C (ppm)
3	4.58 (d, *J* = 12.2 Hz, 1H)	61.9
3a	3.37–3.23 (m, 1H)	55.6
4	H4a: 2.51 (t, *J* = 10.3 Hz, 1H) H4b: 3.37–3.23 (m, 1H)	54
5	-	-
5a	3.67 (s, 2H)	71.2
6	H6a: 3.13 (d, *J* = 13.5 Hz, 1H) H6b: 3.97 (d, *J* = 14.1 Hz, 1H)	55.3
7	-	129.2
7a	-	151.0
7b	7.55 (s, 1H)	130.4
1'	-	146.3
2'	7.26–7.15 (m, 1H)	115.1
3'	7.31 (t, *J* = 6.9 Hz, 2H)	128.9
4'	6.87 (t, *J* = 7.3 Hz, 1H)	120.7
5'	7.31 (t, *J* = 6.9 Hz, 2H)	128.9
6'	7.26–7.15 (m, 1H)	115.1
1''	-	137.2
2''	7.37 (s, 2H)	132.1
3''	-	122.4
4''	7.45 (d, *J* = 7.9 Hz, 1H)	129.8
5''	7.26–7.15 (m, 1H)	128.9
6''	7.26–7.15 (m, 1H)	128.0
1'''	-	138.0
2'''	7.37 (s, 2H)	130.9
3'''	-	123.4
4'''	7.13 (d, *J* = 7.7 Hz, 1H)	131.1
5'''	7.26–7.15 (m, 1H)	129.1
6'''	7.26–7.15 (m, 1H)	124.8
1''''	-	144.1
2''''	7.02 (d, *J* = 8.1 Hz, 2H)	128.4
3''''	7.26–7.15 (m, 1H)	128.9
4''''	7.26–7.15 (m, 1H)	127.4
5''''	7.26–7.15 (m, 1H)	128.9
6''''	7.02 (d, *J* = 8.1 Hz, 2H)	128.4

The HRMS spectrum of the synthesized compound **(5)** showed the confirmed molecular particle peak as $[M+H]^+$ of m/z = 612.0653 with 100% abundance (Figure 7). There is only a slight difference with the calculated mass of $C_{32}H_{28}N_3Br_2$, which is 612.0650. Furthermore, the appearance of m/z 616.0692 indicated compound **(5)** with ^{81}Br isotope. In addition, fragmentation of molecule peak that lost both of its Br atoms was identified by the appearance of m/z 448.1496.

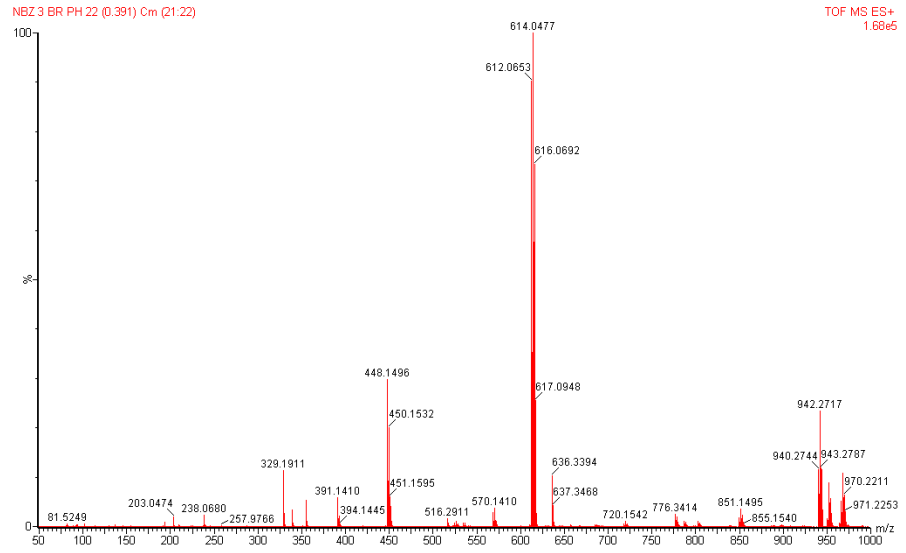


Figure 7. HRMS spectrum of compound **(5)**

According to the methodology reported in a previous publication [24], compound **(5)** was subjected to a tyrosinase inhibition assay with L-tyrosine as a substrate. Kojic acid (5-hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one) was used as a positive control. The assay was carried out using various triplicate concentrations with two-fold dilutions starting from 500 ppm to 7.8125 ppm. The IC_{50} of tested compounds is summarized in Table 2.

Table 2. Inhibitory activity of the synthesized compound and kojic acid

No	Compound	IC_{50} (µM)
1	Pyrazolo[4,3c]pyridine **(5)**	> 500
2	Kojic acid	88.52

The results showed that compound **(5)** showed weak inhibitory activity on fungal tyrosinase with an IC_{50} value greater than 500 µM. This value was significantly higher than the reference kojic acid, with an IC_{50} value was 88.52 µM. The results revealed that, as compared to reported chalcones, pyrazolines showed inferior inhibitory activities [26, 27]. The low solubility of this compound is assumed to be the cause of its weak activity. In addition, it is caused by the lack of polar groups of this compound, so its ability to form hydrogen bonds with the target is reduced. This is also supported by the structure of kojic acid, which contains two hydroxyl groups (-OH), whereas compound **5** does not have these groups.

4. Conclusion

In this study, a pyrazolopyridine compound, (*E*)-5-Benzyl-7-(3-Bromobenzylidene)-3-(3-Bromophenyl)-2-Phenyl-3,3a,4,5,6,7-Hexahydro-2*H*-Pyrazolo[4,3-c] pyridine, has been successfully synthesized with low yields of 11%. The chemical structure of the compound was successfully confirmed by analysis of FTIR, NMR, and HRMS spectroscopic data. An *in vitro* inhibitory activity assay on fungal tyrosinase enzymes showed that compound 5 had significantly low inhibitory activity than positive controls with IC_{50} values of 500 µM and 88.52 µM, respectively. Hence it is considered inactive against the target. The addition of a polar group presumably could increase the solubility and the activity of the compound to the target.

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