Synthesis of Two Schiff Bases Enamine Isomers and Study on Their Tyrosinase Inhibitory Activity

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Abstract: In this study, tyramine and 4-hydroxybenzylamine were used as raw materials, two Schiff bases 3 and 4 were synthesized by condensation reaction with 2,3,4-trihydroxybenzaldehyde respectively. 1H NMR, 13C NMR and MS confirmed that they exist as Z-enamine isomers.Then, L-DOPAwere used as a substrate, the activity and mechanism of tyrosinase inhibition by compounds 3 and 4 were measured using the mushroom tyrosinase dopa rate oxidation method and enzyme inhibition kinetics experiments.The results shows that the compound 4 (2,3,4-trihydroxybenzaldehyde- tyrosamine) has a better tyrosinase inhibitory activity (IC50=32.13 μmol·L-1), which is significantly stronger than the compound 3 (2,3,4-trihydroxybenzaldehyde-4-aminomethylphenol) (IC50=150.80 μmol·L-1), and better than the control drug kojic acid (IC50=67.14 μmol·L-1). The results of the inhibition mechanism studies showthat the inhibitory effect of compound 4 on tyrosinase is reversible inhibition. Z-enamine compound 4 synthesized in this study has good tyrosinase inhibitory activity and deserves further study.

1. Introduction

Schiff base mainly refers to a generic name for a class of compounds containing imine or methylimine characteristic groups (-RC = N-), which has important applications in the fields of medicine, pesticides, catalysis, analytical chemistry, corrosion and photochromism[1-2]. Among them, the ortho-hydroxy aromatic Schiff base has attracted much attention due to the enol-imine (OH) and keto-amine (NH) tautomerism in its structure [3].In addition to these two isomers, another possible structural form is called zwitterion, which is a variant of keto-amine (NH) form [4].

Studies have shown that the ortho-hydroxy aromatic Schiff baseexists mainly in the keto-amine (NH) isomeric form in the gas phase or solution, while solids mainly exist in the resonance hybrid structure of keto-amine (NH) and zwitterion [5]. In this study, 2,3,4-trihydroxybenzaldehyde was used as the raw material, through condensation reaction with tyramine and 4-hydroxybenzylamine respectively, two new 2,3,4-trihydroxyphenyl Schiff base compounds containing a methylimine structure were synthesized (see Figure 1 for the synthetic route).Their structures were characterized by ^{1}H NMR, ^{13}C NMR and MS. The result shows that these two compounds also exist in the form of keto-amine (NH) isomers in solution, which proves that the ortho-hydroxy methylimine Schiff base is in the same form in solution as the ortho-hydroxy aromatic Schiff base. However, in the solid phase, it needs to be further studied through the single crystal structure.

In addition, phenols and their derivatives have been the focus of research due to their wide pharmacological activity [6,7]. Especially in recent years, they have received increasing attention in the screening of tyrosinase (TYR) inhibitors [8]. Tyrosinase (EC 1.14.18.1) is a multifunctional dinuclear copper ion metal enzyme that is widely found in animals, plants, and microorganisms, playing an important role in a variety of physiological and pathological processes [9]. Overexpression of TYR causes melanin accumulation, which causes a series of pigmented skin diseases [10]. High levels of TYR activity are also associated with neurodegenerative diseases such as Parkinson's disease [11]. In addition, enzymatic browning of fruits and vegetables, and insect molt and wound healing are also directly related to TYR activity [12,13]. Finding specific, efficient and safe TYR inhibitors has attracted much attention in the fields of medicine, cosmetics, agriculture

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and food industry. Therefore, this study carried out preliminary research on the TYR inhibitory activity and mechanism of two synthetic phenol-containing Z-enamine compounds 3 and 4, which has provided a certain reference for better research and development of phenolic TYR inhibitors in the future.

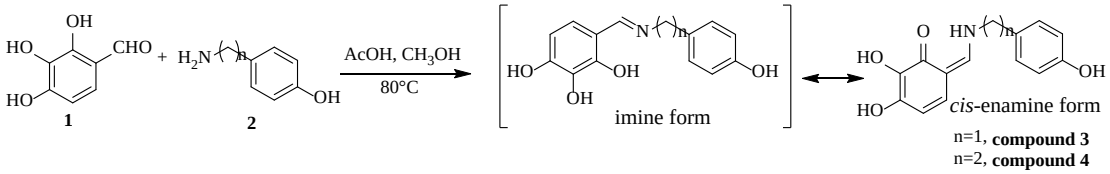

Fig.1 Synthetic Routes of Target Compounds 3 and 4

2. Experiment Part

2.1 Instruments and Reagents

Bruker AVANCE III 400 NMR (Bruker, Switzerland), TMS is the internal standard and DMSO-d_6 is the solvent;Xevo G2 Q-TOF LC-MS (Waters, America);DNM-9602G Enzyme Label Analyzer (Beijing Perlong New Technology Co., Ltd.); BSA124S Electronic Analytical Balance (Sartorius, Germany); N-1100D-WD Rotary Evaporator (Tokyo Physical and Chemical Instruments Co., Ltd.). Tyramine, 4-hydroxybenzylamine, L-DOPA, 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 2,3,4-benzaldehyde were purchased from Energy Chemical, the remaining reagents are commercially available analytical grade.

2.2 Experimental Methods

2.2.1 The Synthesis of Target Compounds 3 and 4

Adding 2,3,4-trihydroxybenzaldehyde 0.154 g (1 mmol), tyramine 0.137 g (1 mmol), 10 mL of dry methanol and 1 drop of acetic acid to a 100 mL round bottom flask, and heating, stirring reflux in an oil bath at 80^{o}C.After about 1 hour of reaction, solids were precipitated on the flask wall.TLC monitoring (developing solvent is petroleum ether: ethyl acetate = 1: 2) until the reaction of the starting materials was complete.After cooling to room temperature, suction filtration was proceeded. After that, cold methanol was washed and dried to obtain pure product.

(*Z*)-2,3-dihydroxy-6-(((4-hydroxybenzyl)amino)methylene)cyclohexa-2,4-dien-1-one(3):Brown solid,yield coefficient 62.4%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 13.72(s, 1H), 12.83(s, 1H), 9.53(s, 1H), 8.32(d, 1H, *J*=12.8 Hz), 7.21(d, 2H, *J*=8.0Hz), 6.94(d, 1H, *J*=8.8Hz), 6.78(d, 2H, *J*=8.1 Hz), 6.03(d, 1H, *J*=8.8 Hz), 4.65(d, 2H, *J*=4.3 Hz), 4.27(s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ:174.62, 163.55, 163.45, 157.27, 132.13, 129.43, 126.77, 124.22, 115.55, 108.52, 107.69, 52.55;ESI-MS (m/z) Calcd. for $C_{14}H_{14}NO_4$[M+H]$^{+}$: 260.0917, Found 260.0914.

(*Z*)-2,3-dihydroxy-6-(((4-hydroxyphenethyl)amino)methylene)cyclohexa-2,4-dien-1-one(4):Bro wn solid,yield coefficient 65.1%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 13.77(s, 1H), 12.64(s, 1H), 9.24(s, 1H), 8.09(d, 1H, *J*=13.1 Hz), 7.06(d, 2H, *J*=8.4Hz), 6.86(d, 1H, *J*=8.9Hz), 6.70(d, 2H, *J*=8.4 Hz), 6.03(d, 2H, *J*=8.8 Hz), 4.28(s, 1H), 3.72(q, 2H, *J*=6.4Hz), 2.85(t, 2H, *J*=7.0Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ:174.59, 163.92, 163.45, 155.94, 131.97, 129.80, 127.93, 124.27, 115.29, 108.41, 107.52, 51.34, 35.12;ESI-MS (m/z) Calcd. for $C_{15}H_{16}NO_4$[M+H]$^{+}$: 274.1074, Found 274.1071.

2.2.2 Tyr Activity Inhibition Experiment

Referring to the method reported in literature[14] to test the inhibitory activity of two target compounds on mushroom TYR, the experiment did slight modifications.The experiment used L-DOPA as substrate and kojic acid as positive control. Compounds 3 and 4 were respectively dissolved in DMSO to prepare a stock solution of 10 mol·L^{-1}, and then diluted with phosphate buffered saline (PBS) at pH 7.4 into 8 solutions of different concentrations.The reaction was performed in a 96-well culture plate with a total reaction system of 300 μL.Next, 111 μL of PBS solution, 6 μL of tyrosinase, 3 μL of tyrosinase inhibitor were added successively to the test system.

After blending thoroughly, 60 μL of 3-methyl-2-benzothiazolinone hydrazone (MBTH) (5 mmol·L-1) solution and 20 μL of L-DOPA (2.5 mmol·L-1) solution were added and mix thoroughly at room temperature for 10 min. Then 300 μL of acetonitrile was added to the system to stop the reaction. In the end, 200 μL of the mixed solution was taken out and its absorbance was measured at 490 nm with a microplate reader to calculate the inhibition rate of TYR.

TYR inhibition rate=[1-(A_1-A_2)/(A_3-A_4)]×100%.

A_1:Absorbance measured with a mixed solution of inhibitor and enzyme; A_2:Absorbance measured with a mixed solution of inhibitor and no enzyme; A_3:Absorbance measured in a mixed solution of enzyme and no inhibitor; A_4:Absorbance measured in a mixed solution of no enzyme and no inhibitor. Each experiment was repeated three times, and the IC50 value of the compound was finally calculated using graphpad prism 6 software.

2.2.3 Judgment Experiment on the Mechanism of Inhibiting Tyr Activity

The concentration of L-DOPA was fixed, TYR was diluted to four concentrations of 2.7, 5.4, 8.1, 10.8 μg·mL^{-1}, and the inhibitor was diluted to solutions with final concentration of10 and 25 μmol· L^{-1}. By measuring the relationship between the speed of the enzymatic reaction and the mass concentration of the enzyme, and the effect of inhibitors with different molar concentrations on the enzymatic reaction (catalytic oxidation of L-DOPA), The figure with the enzyme activity (Y axis) and the mass concentration of enzyme (X-axis) is plotted to determine the type of inhibition mechanism of the inhibitor on the enzyme.

3. Conclusion and Discussion

3.1 The Synthesis of Target Compounds

The synthesis of the target compounds takes the conventional synthetic method of imine compounds. That is, tyramine and 4-hydroxybenzylamine are respectively mixed with the amount of materials such as aromatic aldehyde. After adding acetic acid which is the catalytic amount, they are placed in methanol to have heating reflux reaction. The target compounds can be directly precipitated from the reaction solution, suction filtration after cooling, washed with methanol, and then dried to obtain the pure product.

3.2 Structure Characterization of Target Compounds

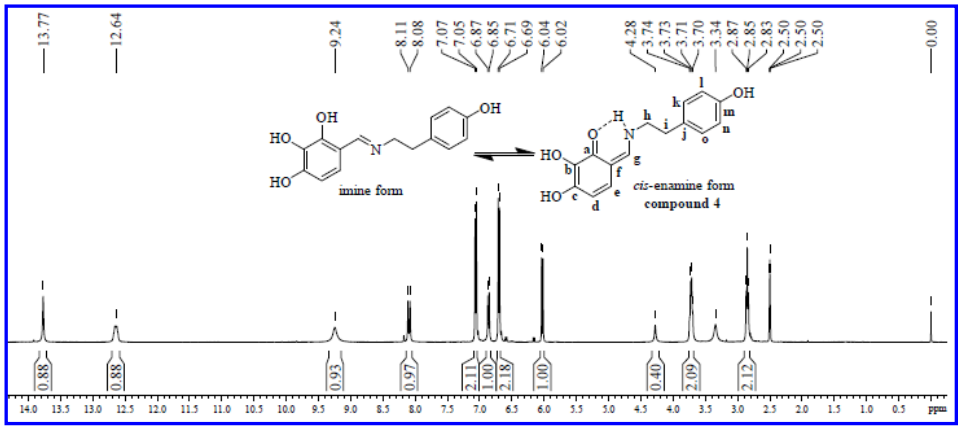

Fig.2 1 h Nmrspectrum of Compound 4 and Its Possible Tautomeric Form

The spectra of ^{1}H NMR and ^{13}C NMR (Figure 2 and Figure 3) show that compounds 3 and 4 obtained by the condensation of 2,3,4-trihydroxybenzaldehyde with 2-hydroxybenzylamine and tyramine mainly exist in the form of (Z) -configuration enamine, which is the tautomer of imines. This is consistent with the spectral data of similar compounds reported in the literature [5]. Taking compound 4 as an example, in the spectrum of ^{1}H NMR, three single peaks appear at the low fields δ 13.77, δ 12.64, and δ 9.24, which correspond to the hydroxyl hydrogen on the carbon at b-position, c- position, and m-position; The carbon at a-position and its attached hydroxyl become

carbonyl, so there is one less hydroxyl hydrogen peak in the low field. The hydrogen atom migrates to a nitrogen atom, and it has a single peak at δ 4.27, and the peak area is relatively small that be caused by the formation of intramolecular hydrogen bonds with the carbonyl at a-position; In addition, due to the coupling of the hydrogen atom on the nitrogen, the adjacent g-position carbon splits into double peaks, and the peak position is at δ 8.32;The hydrogen on the carbon of the h-position is affected by the combined coupling of hydrogen on the nitrogen and hydrogen on the carbon of the i-position, and splits into fourfold peaks, and the peak position is at δ 3.72.In the spectrum of ^{13}C-NMR, the chemical shift of a π-π conjugated carbon atom with hydroxyl usually appears around δ 160. For example, the chemical shifts of the three carbon atoms at b-position, c-position, and m-position are δ 163.92. δ 163.45 and δ 155.94;Moreover, the carbon atom at a-position with hydroxyl at the beginning is changed to a carbonyl carbon due to tautomerization, and its chemical shift shifts significantly to a low field, appearing at δ 174.59.

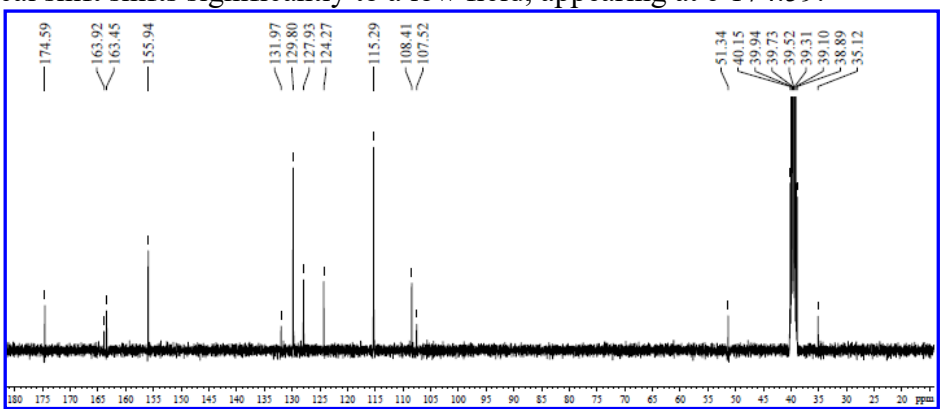

Fig.313 C Nmrspectrum of Compound 4

3.3 Target Compounds Inhibit Tyr Activity

The inhibition rates of TYR activity by compound 3, compound 4 and kojic acid, which is the control drug, at different concentrations are shown in Figure 4A. By curve fitting, the inhibitory concentrations (IC_{50}) of compound 3, compound 4, and Kojic acid that reduced the activity of TYR by half are 150.80, 32.13, and 67.14 μmol·L^{-1}.It is not difficult to find from the results of the activity that the compound 4 (2,3,4-trihydroxybenzaldehyde-tyrosamine) has a better activity of inhibiting TYR, which is 2 times the activity of the control drug kojic acid, and is significantly stronger than the compound 3 (2,3,4-trihydroxybenzaldehyde-4-aminomethylphenol). This shows that the length of the chain between the two rings has a greater effect on the activity of the target compounds to inhibit TYR. The activity of the target compounds can be enhanced significantly by adding one carbon atom.

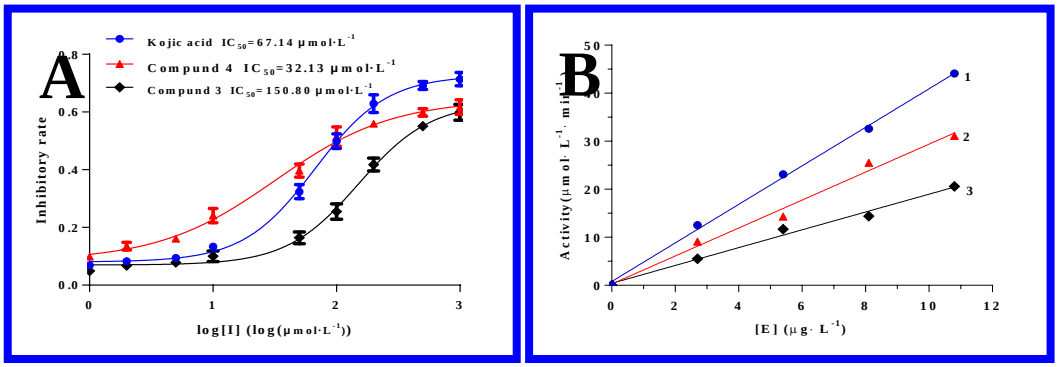

Fig.4 (A) Inhibition Curve of Compound 3, Compound 4 and Kojic Acid on Tyrosinase;(B)Inhibition Mechanism of Compound 4 on Tyrosinase. The concentrations of curves 1 to 3 are 0, 10 and 25 μmol· L^{-1}.

3.4 Determination of the Mechanism of Compound 4 Inhibiting Tyr

In order to determine the mechanism by which the target compound inhibits TYR activity,

Compound 4, which is with better activity, was chosen and its effect on L-DOPA activity under the enzyme-catalyzed oxidation at different concentrations was determined.Enzyme activity was used to plot the concentration of TYR, and a group of lines obtained passed through the origin, as shown in Figure 4B.As the concentration of compound 4 in the system increased, the slope of the straight lines gradually decreased, indicating that compound 4 caused the decrease of catalytic efficiency by binding with tyrosinase in non-covalent bond reversibility. In other words, the decrease of enzyme activity was not caused by the decrease of TYR, but was the result of the inhibition of compound 4 on TYR.

4. Conclusion

In this study, tyramine and 4-hydroxybenzylamine were used as raw materials, two new compounds (compound 3 and compound 4) were synthesized by condensation reaction with 2,3,4-trihydroxybenzaldehyde respectively. ^{1}H NMR, ^{13}C NMR and MS confirmed that they exist as Z-enamine isomers.The preliminary activity test results showed that the compound 4 (2,3,4-trihydroxybenzaldehyde-tyrosamine) has a better inhibitory activity effect on TYR (IC_{50}=32.13 μmol·L^{-1}), which is significantly stronger than the control drug kojic acid (IC_{50}=67.14 μmol·L^{-1}).Preliminary studies on the mechanism of action indicate that the inhibitory effect of compound 4 on TYR is reversible.In summary, Z-enamine compound 4 has a good effect on inhibiting TYR activity, and it can be used as a new TYR inhibitor lead compound for further research.

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