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Original article

Design, synthesis and bioactivity of chalcones and its analogues

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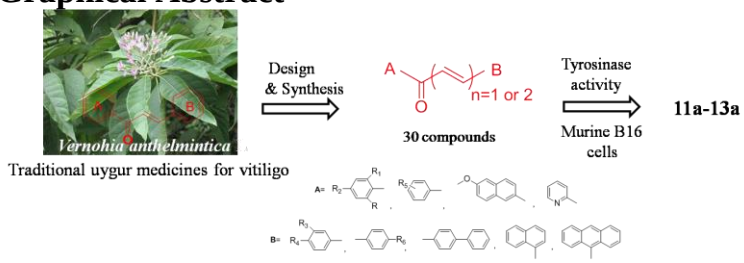
Graphical Abstract



Twenty-one chalcones and nine analogues were synthesized and evaluated for their activity on tyrosinase in cell-free system. Several compounds were more potent than the positive control 8-MOP and **13a** was further biologically investigated in B16 cells.

ABSTRACT

The Vernohia anthelmintica L.'s extract is one of the most popular Uygur medicines used for vitiligo. It is believed that the chalcone compounds of the plant play an important role in the treatment since they may activate tyrosinase and improve melanin production. In this study, twenty-one chalcones and nine analogues were synthesized in view of three different components of chalcone (A, B ring and α, β-unsaturated carbonyl). After biological evaluation of their activity on tyrosinase in cell-free systems, the result showed that most compounds (except polyhydroxy chalcones) possess activator effect on the tyrosinase, especially for 13a-15a, 20a and 1b, which bearing a comparable activity to the positive control 8-MOP. SAR of these tyrosinase activator was summed up for the first time as well. Finally, compound 13a was found to increase melanin contents and tyrosinase activity 1.75 and 1.3 fold, respectively, compared with that of untreated murine B16 cells at the concentration of 40 μg/mL.

Keywords:
Vitiligo
Activator of tyrosinase
Chalcone
B16 cells
SAR

1. Introduction

Vitiligo is an acquired pigmentary disorder of unknown etiology that is clinically characterized by the development of white macules related to the selective loss of melanocytes [1,2]. It was believed that the disease was mainly resulted from destruction of the melanocyte and obstruction of the melanin synthesis [3]. Tyrosinase is the rate-limiting enzyme in melanin biosynthesis and catalyze the reaction of hydroxylation and oxidation in the formation of melanin [4-8].

The fruits extract of *Vernohia anthelmintica* L. (Fig. 1) is one of the most popular Uygur medicines used for vitiligo and initially recorded in *"Yao Yong Zong Ku"* around 300 years ago [9-12]. Some important flavonoids are isolated from the plant [13-16] and it is suggested that these compounds play an important role in this treatments, since they may activate tyrosinase and improve the melanin production [17,18].

Unfortunately, few flavonoids as activator of tyrosinase have been reported [19-22]. Our group have been dedicated on the drug development of the vitiligo for many years [23-26]. And some chalcone derivatives with potential activating effect on tyrosinase and melanin synthesis in B16 cells were discovered as well [27-29]. Based on above research, twenty-one chalcones and nine analogues were prepared in view of three different parts of chalcone (A, B ring and *α, β*-unsaturated carbonyl). After that, the activity of them on tyrosinase were studied and the SAR was summarized as well.

2. Results and discussion

2.1 Chemistry

All the compounds were characterized by ^{1}H NMR, ^{13}C NMR, IR and HRMS (ESI). Firstly, nine mono-, di-, tri- and tetra-hydroxychalcones (**1a-9a)** were synthesized to study the impact of the hydroxyl for the activity on tyrosinase. The synthetic route was described in Scheme 1 (take Butein as example) [30]; Second, with the aim of investigating the significance of different substituents on phenyl ring for the effect on tyrosinase, compounds **10a-21a** substituted in different positions of A ring and *para*-position of B ring were prepared as well (Scheme 2); After that, five chalcone analogues (**1b-2b** and **1c-3c** in Fig. 2) with other aromatic A or B rings were synthesized respectively under the same condition as depicted in Scheme 2. At last, modification on the chain was explored by preparing four chain-prolonged chalcone analogues (**1d-4d** in Fig. 2) as represented in Fig. 2. Additionally, **1d** and **4d** were identified as novel compounds.

As shown in Fig. 3, the crystals of the compounds **13a** and **2c** used for X-ray diffraction analysis were obtained by slow evaporation of acetone-ethanol (1:3) mixed solution at room temperature (**13a**: CCDC No. 1451159; **2c**: CCDC No. 1451160).

2.2 Activity on tyrosinase

The biological activity of polyhydroxy chalcones against tyrosinase was evaluated (Table 1). All the compounds inhibit the tyrosinase. Generally, the higher inhibitory activity can be observed with more hydroxyl in chalcone. For **2a** and **3a**, **5a** and **6a**, the result showed that the most important factor for their efficacy was the location of the hydroxyl on benzene ring, with a significant preference to a 4-substituted B ring rather than a substituted A ring. Moreover, a 2,4-substituted resorcinol with more hydroxyl on ring A improved their activity, such as compounds **7a** and **9a**.

It was not hard to find that hydroxyl in chalcone skeleton seriously abolished the activator effect on tyrosinase according to the result above. Therefore, some other groups were introduced to the chalcone to improve the activity. As shown in Table 2, chalcones substituted with -X (**14a**, **15a**) and -$N(CH_3)_2$ (**13a**) were more potent than ones with -H, -OH, -CH_3 and -OCH_3, suggesting that the electron-withdrawing group (EWG) may be favorable to enhance the activity. In the view of best activity of compound **13a**, further modification was performed on this active molecule. Neither changing the location of the -OCH_3 on A ring nor replacement of -OCH_3 with -NO_2, -NH_2 or -NHAc enhanced the activator effect, which emphasizes the importance of the *para*-position of the substituted group on A ring and the -$N(CH_3)_2$ on B ring.

With few exceptions, when A ring was replaced with naphthalene or pyridine ring, similar effect was found with compound **1b** and **2b**, proving that the benzene for the A ring was not necessary for this effect. However, the activity dramatically decreased once other aromatic rings were introduced to B ring (compounds **1c**-**3c**), which means that the benzene for B ring was essential for maintaining the activity (Table 3). For the linker group between A and B ring, the *α, β*-unsaturated carbonyl was prolonged to give compound **1d**-**2d**, based on the active compounds synthesized above. Unfortunately, in Table 3, the extended distance between the two groups failed to retain the biological activity.

2.3 Activity on tyrosinase and melanin synthesis in B16 cells

The viability of **13a** on B16 melanoma cells was examined using the MTT assay. The cells were treated with various concentrations of **13a** (6.25-200 μg/mL). As shown in Fig. 4, there was no significant difference between the control and treated group at concentrations of 6.25-200 μg/mL. **13a** showed very small cytotoxic effects on B16 cells at concentrations of 200 μg/mL.

The effect of **13a** on tyrosinase in cells was measured by L-DOPA oxidation (Fig. 5). Compared with treatment with medium only (untreated condition), treatment with **13a** at 0-40μg/mL resulted in a dose-dependent increase in tyrosinase activity in B16 cells. In the melanin content assay, to exclude the possibility that a rise in melanin content may be induced by cell-proliferating effect of **13a**, the absorbance of the same number of cells across **13a** concentrations (0-40 μg/mL) was measured as well. We found that melanin levels increased in a dose-dependent manner by **13a** treatment in B16 cells (Fig. 6). At 40 μg/mL of **13a**, the melanin content only slightly increased, so 20 μg/mL was chosen as an effective concentration of **13a** for further experiments.

3. Conclusion

This work showed that good activator effect can be obtained with small chalcone molecules and the SAR was summed up for the first time as follows. (1) The hydroxyl on either benzene of chalcone may cause an inhibitory activity, especially for the B ring. (2).The A ring of the chalcone can be substituted with other conjugate aromatic rings to retain the activator effect while the B ring cannot. It was speculated that the planar molecule was essential for the activity. (3) The linker should not be prolonged for maintaining the activity. In addition, **13a** increased both activity of the tyrosinase and the contents of the melanin in a dose-dependent manner in murine B16 cells.

4. Experimental

4.1 Chemistry

Reagents and solvents were purchased from Sigma, Sodipro or VMR, and used without further purification. Dimethylsulfoxide (DMSO), mushroom tyrosinase, *L*-3, 4-dihydroxyphenylalanine (L-DOPA), 3,4-dihydroxyphenylalanine and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA).

*4.2 General method for preparation of chalcone and analogues (**1a**-**21a**, **1b**-**2b**, **1c**-**3c**, **1d**-**4d**)*

The corresponding aldehyde (1.0 equiv) and ketone (1.0 equiv) were dissolved in ethanol at 0 °C and 10% NaOH (1.0 equiv.) solution was slowly added. After that, the mixture was allowed to warm up to room temperature and stirred for 48 h. The resulting mixture was evaporated to dryness and the residue was purified by chromatography on silica gel eluted with petroleum ether-ethyl acetate to afford the final compound. The characterization data of the synthesized compounds are in the Supporting information.

4.3 Biological methods

Preparation of **13a**: Compound **13a** was obtained and then dissolved in DMSO. A stock solution of **13a** (5 mg/mL) was prepared in DMSO for further applications.

Cell culture: The murine B16 melanoma cell line was obtained from the CAS (Chinese Academy of Sciences, China). B16 cells were grown in DMEM medium (Gibco, Life Technologies, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Hyclone, USA) in a humidified atmosphere with 5% CO_2 at 37 °C.

Cell viability assay: Cell viability was determined using the MTT assay. B16 cells were plated in 96-well dishes at a density of 5×10^3 cells per well. After 24 h, different concentrations of **13a** were added and the cells were incubated for 48 h. Then, 10 μL of MTT (5 mg/mL in PBS) solution was added into each well and the cells were incubated at 37 °C for another 4 h. Following medium removal, 150 μL of DMSO was added to each well and plates were gently shaken for 10 min. Optical absorbance was determined at 570 nm with a Spectra Max M5 (Molecular Devices, USA). Absorbance of cells without treatment was regarded as 100% cell survival. Each treatment was performed in quintuplicate and each experiment was repeated three times.

4.3.1 Activator effect of tyrosinase in cell-free assay

The activities on tyrosinase of synthesized compounds were performed according to the modified method **[**31], with 8-MOP [32-35] and Kojic acid [36-38] as positive control at the Xinjiang Technical Institute of Physics and Chemistry, Chinese Academy of Sciences. Potassium phosphate buffer (0.06 mL, 50 mmol/L) at pH 6.5, 0.04 mL tyrosinase (250 U/mL) and 2 μL of the test compounds (0.5-300 μmol/L), dissolved in DMSO were inserted into 96-well plates. After 5 min incubation at 25-30 °C, 0.1 mL of L-tyrosine (2 mmol/L) was added and incubated for additional 30 min. After that, the optical density of the systems at 490 nm was measured on ELASA and the rate of activation (RA) was calculated according the formula: RA=[(C-D)-(A-B)]/(A-B)*l00, The A was optical density of the system at 490 nm with only tyrosinase; B was the one with neither compounds nor tyrosinase; C was the one with both compounds and tyrosinase; And the D was the one with only compounds. Finally, the activity of the compounds was expressed as the compounds concentration that gave a 50% activator effect (when RA=50%) in the enzyme activity (EC_{50}).

4.3.2 Tyrosinase activity and melanin content assay in B16 cells

Tyrosinase activity was estimated by measuring the rate of L-DOPA oxidation as previously reported. B16 cells were seeded in a 12-well plate at a density of 2×10^5 cells per well and allowed to attach for 24 h. Then, cells were treated with **13a** for 48 h, washed with ice-cold PBS twice, trypsinized with 0.25% trypsin (Hyclone) and collected in an Ep tube. After being centrifuged at 3,000 rpm for 5 min the cells were washed once with PBS, and then 200 μL of Tris-0.1% Triton X-100 (pH 6.8) were added to each tube. All tubes were incubated at -20 °C for 30 min, and then the lysates were centrifuged at 12,000 rpm for 15 min to obtain the supernatant for the tyrosinase activity assay. Protein concentrations were determined by the Bradford method with bovine serum albumin (BSA) as a standard. 100 mL of supernatant containing 10 μg total protein was added to each well in a 96-well plate and this was mixed with 100 μL of 0.1% L-DOPA in PBS (pH 6.8). After incubation at 37 °C for 1 h, the dopachrome was monitored by measuring the absorbance at 475 nm. The total melanin in the cell pellet was dissolved in 100 mL of 1 mol/L NaOH/10% DMSO for 1h at 80 °C and solubilized melanin was measured at 470 nm.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data (experimental procedures, spectroscopic characterizations) associated with this article can be found.

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Fig. 1. The plant of the *Vernohia anthelmintica* L.

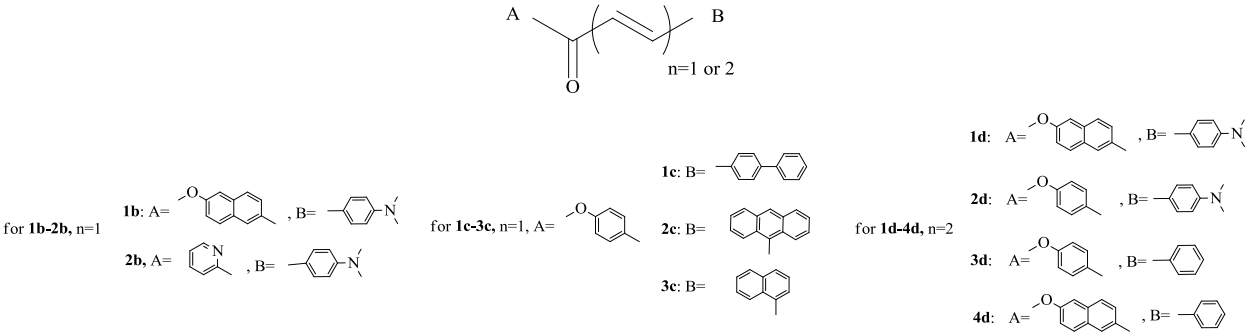
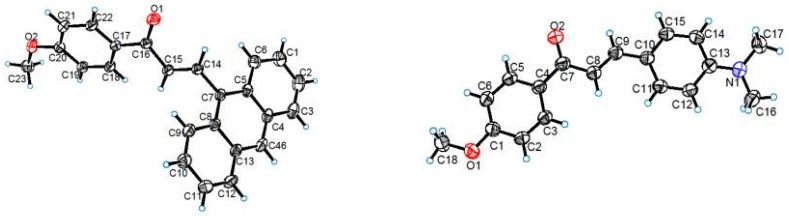


Fig. 2. Structures of synthesized analogues



2c **13a**

Fig. 3. Chemical structures of the crystals in **2c** and **13a**

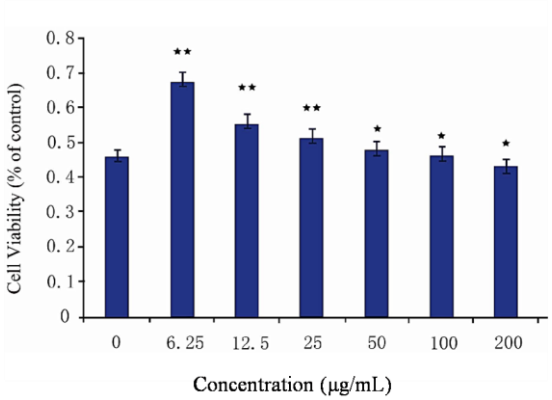


Fig. 4. The Cell viability of **13a** determined using the MTT assay with different concentrations in B16 cells.

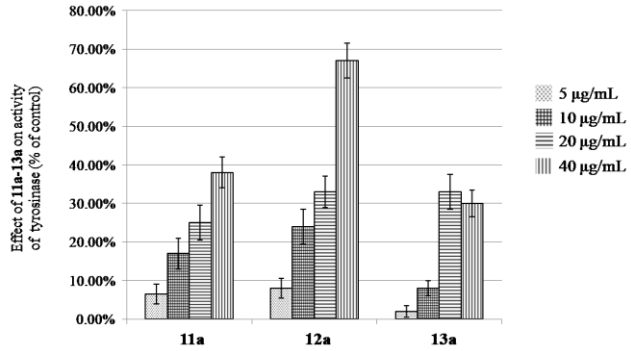


Fig. 5. Effect of **11a-13a** on tyrosinase in B16 cells (expressed as the percentage of untreated control).

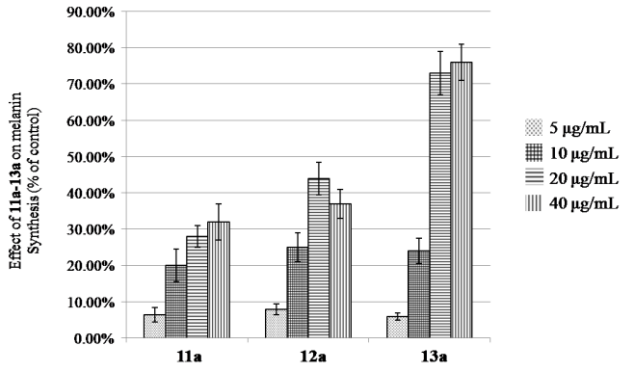
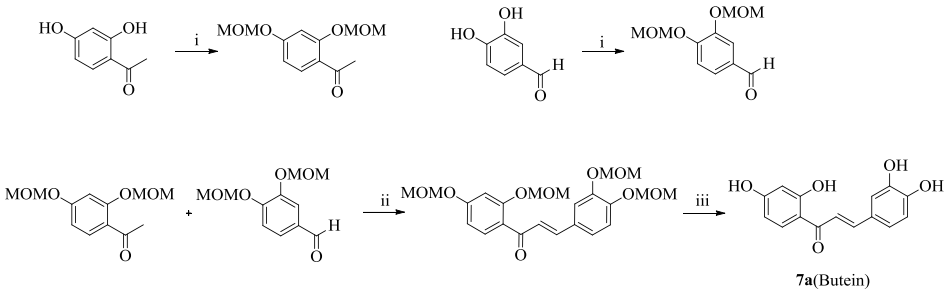
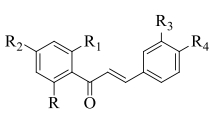


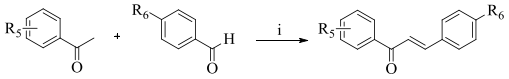
Fig. 6. Effect of **11a-13a** on melanin content in B16 cells (expressed as the percentage of untreated control).





R_1=R_2=R_3=R_4=R=H	**1a**
R_1=R_2=R_3=R=H,R_4=OH	**2a**
R_1=R_3=R_4=R=H,R_2=OH	**3a**
R_1=R_3=R=H,R_3=R_4=OH	4**a**
R_1=R_2=OH,R_3=R_4=R=H	5**a**
R_1=R_2=R_4=OH,R_3=R=H	**6a**
R_1=R_2=R_3=R_4=OH,R=H	**7a**
R_1=R=H,R_2=R_3=R_4=OH	**8a**
R_1=R_2=R_3=R_4=R=OH	**9a**

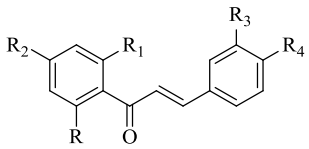
Scheme 1. Synthesis of polyhydroxychalcone. Regents and condition: (i) $ClCH_2OCH_3$, K_2CO_3, Acetone, reflux (ii) 10% NaOH, ethanol, rt (iii) 3 mol/L HCl, ethanol, reflux



R_5=4-OCH_3, R_6=H	**10a**
R_5=4-OCH_3, R_6=OH	**11a**
R_5=4-OCH_3, R_6=CH_3	**12a**
R_5=4-OCH_3, R_6=$N(CH_3)_2$	**13a**
R_5=4-OCH_3, R_6=Cl	**14a**
R_5=4-OCH_3, R_6=F	**15a**
R_5=4-OCH_3, R_6=OCH_3	**16a**
R_5=2-OCH_3, R_6=$N(CH_3)_2$	**17a**
R_5=3-OCH_3, R_6=$N(CH_3)_2$	**18a**
R_5=4-NO_2, R_6=$N(CH_3)_2$	**19a**
R_5=4-NH_2, R_6=$N(CH_3)_2$	**20a**
R_5=4-NHAc, R_6=$N(CH_3)_2$	**21a**

Scheme 2. Synthesis of substituted chalcones. Regents and condition: (i) 10% NaOH, ethanol, rt

Table 1. The inhibitory effect of polyhydroxy chalcones on tyrosinase

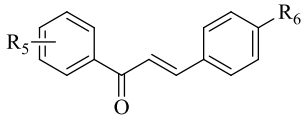


1a-9a

Compound	R_1	R_2	R_3	R_4	R	Substrate	IC_{50}[a] (μmol/L)
1a	H	H	H	H	H	L-tyrosine	NE[b]
2a	H	H	H	OH	H	L-tyrosine	35.4±2.02
3a	H	OH	H	H	H	L-tyrosine	NI[c]
4a	H	H	OH	OH	H	L-tyrosine	25.1±1.57
5a	OH	OH	H	H	H	L-tyrosine	NI
6a	OH	OH	H	OH	H	L-tyrosine	31.0±0.91
7a	OH	OH	OH	OH	H	L-tyrosine	19.6±1.42
8a	H	OH	OH	OH	H	L-tyrosine	27.3±2.64
9a	OH	OH	OH	OH	OH	L-tyrosine	11.4±1.03
Kojic acid[d]	-	-	-	-	-		10.70

[a] 50% inhibitory concentration, result were the means±SD from three independent experiment; [b] not effect; [c] NI means slightly inhibited and IC_{50}>200 μmol/L; [d] Kojic acid as positive control

Table 2. The activator effect of substituted chalcones on tyrosinase



10a-21a

Compound	R_5	R_6	Substrate	EC_{50}[a] (μmol/L)
10a	4-OCH_3	H	L-tyrosine	NA[b]
11a	4-OCH_3	OH	L-tyrosine	NA
12a	4-OCH_3	CH_3	L-tyrosine	58.4±6.54
13a	4-OCH_3	$N(CH_3)_2$	L-tyrosine	17.1±2.18
14a	4-OCH_3	Cl	L-tyrosine	26.3±1.67
15a	4-OCH_3	F	L-tyrosine	20.6±3.58
16a	4-OCH_3	OCH_3	L-tyrosine	126.0±8.21
17a	2-OCH_3	$N(CH_3)_2$	L-tyrosine	NA
18a	3-OCH_3	$N(CH_3)_2$	L-tyrosine	46.3±4.60
19a	4-NO_2	$N(CH_3)_2$	L-tyrosine	NA
20a	4-NH_2	$N(CH_3)_2$	L-tyrosine	35.5±0.87
21a	4-NHAc	$N(CH_3)_2$	L-tyrosine	NA
8-MOP[c]			L-tyrosine	14.6

[a] 50% effective concentration, result were the means±SD from three independent experiment; [b] NA means slightly activated and EC_{50}>200 μmol/L; [c] 8-MOP as positive control

Table 3. The activator effect of chalcone analogues on tyrosinase

Compound	Substrate	EC_{50}[a] (μmol/L)
1b	L-tyrosine	25.8±1.73
2b	L-tyrosine	41.7±2.25
1c	L-tyrosine	NA[b]
2c	L-tyrosine	NA
3c	L-tyrosine	NA
1d	L-tyrosine	84.0±7.51
2d	L-tyrosine	73.6±5.36
3d	L-tyrosine	NA[b]
4d	L-tyrosine	NA
8-MOP[c]	L-tyrosine	14.6

[a] 50% effective concentration, result were the means±SD from three independent experiment; [b] NA means slightly activated and EC_{50}>200 μmol/L; [c] 8-MOP as positive control