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Original article

In vitro and *in vivo* evaluation of the antidiabetic activity of ursolic acid derivatives

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ABSTRACT

In this study, a series of ursolic acid derivatives were synthesized, and their structures were confirmed. The activity of the synthesized compounds against α-glucosidase was determined *in vitro*. The results suggested that all compounds have significant inhibitory activity, especially compounds **3**–**5** and **8**, the IC_{50} values of which were 2.66 ± 0.84, 1.01 ± 0.44, 3.26 ± 0.22, and 3.24 ± 0.21 μM. These compounds were more potent than acarbose (positive control) against α-glucosidase. Kinetic studies were performed to determine the mechanism of inhibition by compounds **3**–**5** and **8**. The kinetic inhibition studies indicated that compound **3** was a non-competitive inhibitor, and the inhibition constant K_i was calculated to be 2.67 ± 0.19 μM. Moreover, the kinetic inhibition studies of compounds **4**, **5** and **8** demonstrated that they were mixed-type inhibitors. Furthermore, the actual pharmacological potentials of synthesized compounds **3** and **4** were demonstrated by the reduction of postprandial blood glucose levels in normal Kunming mice. The hypoglycemic effects of these compounds were more evident 30 and 60 min after maltose ingestion ($P < 0.05$), which was similar to the effect displayed by the positive control, acarbose.

1. Introduction

Diabetes mellitus is a chronic disease that occurs when the pancreas does not produce sufficient insulin or when the body cannot use it effectively, thereby leading to an increased blood glucose concentration [1]. Over the last century, the ever-changing lifestyle of the population has resulted in a dramatic increase in the incidence of diabetes. The chief form of the disease, type 2 diabetes, is associated with 'diabesity' and 'metabolic syndrome'. In conjunction with genetic susceptibility in certain ethnic groups, type 2 diabetes is influenced by environmental and other factors such as a sedentary lifestyle, overly rich nutrition and obesity [2]. Treatment of diabetes involves lowering blood glucose through different mechanisms, including insulin secretion, glucose absorption, and metabolism adjustment [3,4].

Pentacyclic triterpenes and their derivatives are ubiquitous in the plant kingdom and possess interesting bioactivities, such as antitumor [5–8], anti-HIV [9], antibacterial [10], antimalarial [11], protein tyrosine phosphatase 1B inhibition [4,12], etc. Ursolic acid (**UA**, 3β-hydroxy-urs-12-en-28-oic acid, **1**) is a well-known pentacyclic triterpene that has been reported to possess a wide range of biological activities. It serves as one of the major effective components of many traditional Chinese herbal medicines [13]. Studies have shown that **UA** has a positive effect on lowing blood glucose levels and curing diabetic complications in diabetic mice [14–17]. However, **UA** has a very low water solubility, which limits its bioavailability and therapeutic applications in clinical medicine [18]. To improve its activity and bioavailability, chemical modification of **UA** at the 3-OH or 17-COOH positions has recently been widely investigated. It has been reported that **UA** and its derivatives esterified at the C-3 and/or C-28 positions exhibit significant cytotoxicity against several tumor cell lines [5,19,20]. Moreover, a series of **UA** derivatives were synthesized by condensation of **UA** and 1,4-bis(3-aminopropyl) piperazine pharmacophore, resulting in promising *in vitro* anti-malarial activity [12,21]. However, there are few studies focused on the anti-diabetic properties of **UA** derivatives.

In an attempt to explore the activity and bioavailability of **UA** and its derivatives, and study their structure–activity relationships and mechanisms, structural modifications were made to the 3-OH or 17-COOH positions of **UA**. A series of **UA** derivatives, particularly

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new halogen-containing derivatives were synthesized using previously reported methods [5,19,20] with slight modifications and different reactants. The hypoglycemic activity of **UA** and its derivatives was investigated *in vitro* and *in vivo*. In the present study, the results of experiments measuring the activity of **UA** and its derivatives toward inhibiting alpha-glucosidase and lowering blood glucose in a normal mice model were reported.

2. Results and discussion

2.1. Chemistry

To obtain a series of **UA** derivatives, structural modifications were made at the 3-OH or 17-COOH positions, beginning with **UA** as the parent compound. The synthetic routes are shown in Schemes 1 and 2.

Ursolic acid (**1**) was esterified in anhydrous pyridine with acetic anhydride to give its 3-*O*-acetate (**2**). Compounds **3**–**5** were synthesized following the same procedure used to prepare the 3-*O*-acetate, but with different anhydrides (Scheme 1). Compound **2** was treated with oxalyl chloride to give the intermediate 28-acyl chloride [7,20], which is highly reactive and was coupled with small molecules containing reactive amine groups to produce compounds **6**–**12** (Scheme 2). The target compounds were purified by column chromatography using petroleum ether/ethyl acetate and/or chloroform/methanol as the eluent. Their structures were confirmed by electrospray ionization mass spectrometry (ESI-MS), high-resolution mass spectrometry (HRMS), ^{1}H NMR and ^{13}C NMR.

2.2. In vitro α-glucosidase inhibition assay

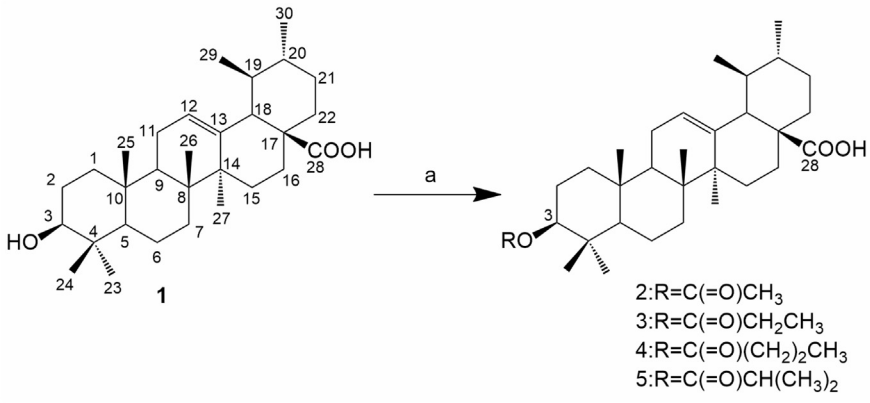
To investigate the inhibitory ability of each synthesized **UA** derivative, an *in vitro* experiment using α-glucosidase from baker's yeast, which is widely used in screens for compounds with antidiabetic activity, was conducted [22]. A stock solution of each derivative (dissolved in DMSO at concentrations of 0.1 μM–1 mM) was diluted with 0.1 M phosphate buffer solution (pH = 6.8) containing an appropriate concentration of enzyme solution (0.1 U/mL). After a 10 min pre-incubation at 37 °C, the reactions were initiated by adding the substrate (1 mM *p*-nitrophenyl-α-D-glucopyranoside) and incubated at 37 °C for 30 min. The reactions were then terminated by adding 1 M Na_2CO_3, and their optical density values were measured using a Multimodel Plate Reader (Infinite 200). Table 1 displays the preliminary results. The inhibited enzyme activity of all compounds was firstly evaluated at two concentrations (5 and 10 μM), which was a guidance for future selection of inhibitor concentrations. Table 1 clearly shows that the inhibition effects were more obvious at higher concentrations.

To determine the IC_{50} value of each derivative, the enzyme activity was measured at a fixed substrate concentration, in which a series of different inhibitor concentrations were tested. The IC_{50} value was calculated based on the curve fit to the series of concentrations versus the corresponding inhibition activities (data not shown). All tested compounds had lower IC_{50} values than both positive control and **UA** against α-glucosidase from baker's yeast except compound **11** which had a low solubility and could not disperse well at higher concentrations.

To obtain insight into the mechanism by which the derivatives inhibited α-glucosidase, kinetic studies was carried out. Lineweaver–Burk plots of the initial velocity versus the enzyme concentrations in the presence of different concentrations of the synthesized compounds gave a series of straight lines. As illustrated in Figs. 1–4, compound **3** was determined to be a noncompetitive inhibitor because increasing substrate concentrations resulted in a series of lines with a common intercept on the − [I] axis, but different slopes (Fig. 1). The equilibrium constant for inhibitor binding, K_i, was obtained from the − [I] value at the intersection of the four straight lines. The family of lines for compounds **4** and **5** intersected in the second quadrant (Figs. 2 and 3), while the family of lines for compound **8** intersected in the third quadrant (Fig. 4). The inhibition constant K_i and K_i' values (Scheme 3) for compounds **3**–**5** and **8** were calculated using the appropriate equations, and the results are presented in Table 2. It was implied that compounds **4**, **5** and **8** inhibit α-glucosidase in two different ways: compounds **4**, **5** and **8** could retard enzyme function by not only directly binding to α-glucosidase (EI) but also interfering the formation of α-glucosidase-PNPG (ES) intermediate through forming an α-glucosidase-PNPG-inhibitor (ESI) complex in a noncompetitive manner [23]. According to the inhibition type of tested compounds, it could not dismiss the possibility that an α-glucosidase inhibitor attaches to a wide region including the binding site of α-glucosidase, or attaches to a different region incurring structural modification [24].

2.3. Hypoglycemic effect in normal mice

To investigate the pharmacological potential of the most active α-glucosidase inhibitors among the synthesized **UA** derivatives as lead compounds for the development of new drugs to treat diabetes, compounds **3** and **4** were synthesized on a larger scale to obtain enough to perform assays of their hypoglycemic activity in normal mouse. With oral administration at a 50 mg/kg



Scheme 1. Synthesis of compounds **2**–**5** from ursolic acid. Reagents and conditions: (a) anhydride/Pyr/DMAP, r.t.