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A hydrophilic conjugate approach toward the design and synthesis of Ursolic acid derivatives as potential antidiabetic agent

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Abstract: In this study, a series of novel ursolic acid (UA) derivatives were designed and synthesized successfully via conjugation of hydrophilic and polar groups at 3-OH and/or 17-COOH position. Molecular docking studies were carried out with the binding of UA and acabose in the active site of α-glucosidase, in order to prove that the hydrophilic/polar moieties can interact with hydrophobic group of catalytic pocket and form hydrogen bonds. The bioactivities of these synthesized compounds against α-glucosidase were determined *in vitro*. Kinetic studies were performed to determine the mechanism of inhibition by compounds **3**, **4**, **10** and **11**. The results indicated that most of the target compounds have significant inhibitory activity, and the compound **3**, **4**, **10** and **11**were potent inhibitors of α-glucosidase, with the IC_{50} values of 0.149 ± 0.007, 0.223 ± 0.023, 0.466 ± 0.016 and 0.298 ± 0.021μM, respectively. These compounds were more potent than parent compound and acarbose. The kinetic inhibition studies revealed that compound **3** and **4** were mix-type inhibitors while compound **10** and **11** were non-competitive inhibitors. Furthermore, the molecular docking studies for these two kinds of compounds suggested that free carboxylic group at either C-3 or C-28 position could remarkably improve inhibitory activity. It is noteworthy that the exploration of relationship between hydrophilic and polar groups of these structures and the hydrophobic group in catalytic pocket is benefited from our rational design of potent α-glucosidase inhibitor.

Keywords: α-glucosidase inhibition; hydrophilic; hydrogen bonds; molecular docking; synthesis; Ursolic acid derivatives

1. INTRODUCTION

Diabetes mellitus, which is characterized as a consequence of genetically based predisposition and dietary indiscretion, is a chronic metabolic disease occurred by multifactorial disordering, resulting in hyperglycemia [1]. Type 2 diabetes (non-insulin dependent DM) is a metabolic disease characterized by insulin resistance and insulin deficiency [2]. Type 2 diabetes (T2D) is induced by a combination of factors, including lifestyle and genetic factors. Today, T2D is a serious health concern for its escalating prevalence worldwide and high morbidity and mortality associated with diabetic complications [3-5]. Postprandial hyperglycemia contributes much to the overall glycemic control in T2D patients. Several therapeutic approaches have been proposed since modulation of postprandial hyperglycemia is shown to play crucial role in the treatment and prevention of diabetes and its complications, including the use of a-glucosidase inhibitors to delay the digestion and absorption of carbohydrates [6, 7], and protein tyrosine phosphatase 1B (PTP1B) inhibitors to modulate insulin receptor phosphorylation, along with insulin therapy to reduce hyperglycemia and maintain normoglycemia [8].

Ursolic acid (UA, 3b-hydroxy-urs-12-en-28-oic acid, **1**) is pentacyclic triterpene acid existing abundantly in plant kingdom. Ursolic acid and its derivatives have been reported to have interesting bioactivity, including anti-HIV [9], antihepatodamage [10], antimalarial [11], antitumor [12-14], protein tyrosine phosphatase 1B inhibition [8, 15]. The bioactivity of UA has attracted the attention of researchers who aim to develop natural biological agents. UA is constituted by a rigid pentacyclic skeleton, which is highly hydrophobic and makes UA poorly water-soluble. The bioavailability and therapeutic application in clinical medicine are limited by this property. As an effective nature compound, considerable structure modifications of UA at the 3-OH or 17-COOH positions have recently been widely investigated. It is expected that incorporation of polar and hydrophilicity moieties onto the C-3 or C-28 position might improve the bioactivity and thus clinical utility. Very

recently, there have been some reports about the evaluation of a series of quaternary ammonium salt derivatives of pentacyclic triterpene acids for their anti-cancer activities which pointed out that the cytotoxic activities of these compounds were correlated with their hydrophilicity [12, 16]. In our lab, Wu has studied and shown that UA has a positive effect on lowing blood glucose levels and curing diabetic complications in diabetic mice [17]. Moreover, the structure activity relationship demonstrated that a hydrogen donor group at either position 3-OH and/or 17-COOH was essential for cytotoxic activity, and a significant improvement in cell growth inhibition [18]. In our studies, we focused on the anti-diabetic properties of UA derivatives by improving its activity and bioavailability through chemical modification of UA at the 3-OH or 17-COOH positions conducted with conjugation with hydrophilicity and polar moieties following our docking studies.

Thus, our strategy has been to design effective α-glucosidase inhibitors as antidiabetic drugs not only showing strong inhibitory effects against α-glucosidase but also bioavailability of UA derivatives by inserting hydrophilicity and polar moieties onto UA so as to make them be potential oral drugs. As part of our research program aimed at developing new potent and selective α-glucosidase inhibitors, we specifically sought to design in a rational way UA derivatives conjugated with hydrophilicity and polar moieties binding with protein of α-glucosidase with a high degree of binding affinity. And we reported that the synthetic compounds **3**, **4**, **10** and **11** were more active inhibitors of α-glucosidase than the natural product UA. In addition, the introduction of a free carboxylic acid group at either C-3 position or C-28 position led to an improved enzyme inhibition. We also studied the kinetic inhibition of active compounds. Furthermore, we highlighted in detail various mechanisms by which ursolic acid interacted with α-glucosidase and the relationship between molecular simulation and *in vitro* inhibitory activity.

2. RESULTS AND DISCUSSION

2.1. Molecular design

As most of the biological testing of α-glucosidase inhibitors results were carried out on Saccharomyces cerevisiae, the 3D structure of protein is demanded for an investigation of the binding mode of ursolic acid derivatives within α-glucosidase structure. Although the 3D structure of protein is not available by now, the homology modeling of the protein has already been reported in several publications. The homology modeling method was used like that of previous researches to conduct the 3D structure of the α-glucosidase. The structure of oligo-1, 6-glucosidase from Saccharomyces cerevisiae (**PDB:1UOK**) was chosen as template and the sequence alignments between the template and α-glucosidase disclosing their sequence identity and similarity were 38.0% and 62.0%, respectively. These sequence alignments indicated that 3D structure of α-glucosidase can be expected in the homology modeling [19].

The rational design of novel α-glucosidase inhibitors should be carried out for an insight into the most conceivable binding conformation of acarbose. As is shown in Figure **1**, acarbose revealed high binding affinity with the protein whose binding free energy was calculated to be -9.13 $kcalmol^{-1}$. This binding mode was probably owing to the large number of hydroxyl groups of acarbose as well as hydrophobic interaction. Indeed, this docking analysis predicted that acarbose with the active site residues afforded important information of catalytic site which was formed into hydrogen bonds with ASP60, GLN167, SER222, GLU255, LYS293, ASP329 and ARG415 residues. These observations suggested that it was possible to improve the binding affinity between ligand and α-glucosidase by inserting the hydrophilic or polar moieties [20, 21].

Therefore, here we selected UA as the parent compound as established blocks to evaluate how the conjugation between UA and hydrophilic groups would bind with the α-glucosidase (**PDB:1UOK**). UA is composed by a strict pentacyclic skeleton, which is highly hydrophobic and makes UA poorly water-soluble. Although UA possesses a hydroxyl group at C-3 position and a carboxy group at C-28 position, a large surface area of hydrophobic environment of UA is less affected by these two hydrophilic moieties. The binding mode of UA with α-glucosidase is depicted in Figure **1**. The analysis of interaction between UA and catalytic pocket is similar to that of acarbose, which showed that 3-OH group of UA interacts with GLU255 and ASP329 through hydrogen bonds, as well as a hydrogen bond interaction between 17-COOH group and SER222. Compared with acarbose, UA whose binding free energy was calculated to be -4.17 $kcalmol^{-1}$ was possessed with lower binding affinity with α-glucosidase than acarbose.

These observations of the binding modes raised our inspiration of forming hydrogen bonds with ligand in the active site of protein as a possible way of improving the binging affinity. With the aim of enhancing hydrophilicity of UA, we have made some modifications by inserting polar or hydrophilic moieties at 3-OH and 17-COOH position of UA to obtain certain long hydrophilic group of UA derivatives which may raise the possibility to afford more potent α-glucosidase inhibitors.

2.2. Chemistry

Based on the above docking studies, we designed and synthesized ursolic acid derivatives **2-20**. For a purpose of achieving a series of UA derivatives conjugated with hydrophilic moieties that coupled with long flexible spacers, structural modifications were made at the 3-OH and 17-COOH position, with the UA as lead compound. The synthetic routes are outlined in scheme 1, 2 and 3.

The synthesis of **2-6** is summarized in scheme 1. Ursolic acid (**1**) was benzylated with benzyl chloride and potassium carbonate in N, N-dimethylformamide gave benzyl ester **2**. The compound **2** was then acylated by use of succinic acid anhydride and glutaric anhydride afforded the corresponding carboxylic acids **3** and **4** respectively. Debenzylation of **3** and **4** by hydrogenolysis over Pd/C in THF yielded **5** and **6**, respectively. Reaction of UA with ethyl bromoacetate in the presence of potassium carbonate in N, N-dimethylformamide was esterified to give ester **7**. Saponification of **7** with sodium hydroxide in a mixture of tetrahydrofuran and methanol gave the corresponding carboxylic acids **8**, which was treated with different anhydrides to afford compounds **9-11** (scheme 2).

According to our previous studies [17], ursolic acid was successively esterified in anhydrous pyridine with Propionic anhydride to form its 3-O-Propionate **12**, which was treated with oxalyl chloride to give the 28-acyl chloride. Condensation of this intermediate with various amino compounds in the presence of triethylamine and dichloromethane afforded compounds **13-20** (scheme 3). The structures and purities of target compounds were characterized by electrospray ionization mass spectrometry (ESI-MS), ^{1}H NMR, ^{13}C NMR, high-resolution mass spectrometry (HRMS) and elemental analysis (EA).

2.3. Biological activity

2.3.1. α-glucosidase inhibitory activity

All the target compounds of ursolic acid derivatives were evaluated *in vitro* against

α-glucosidase from baker's yeast, which was conducted to screen compounds with antidiabetic activity. The α-glucosidase inhibitory assay was performed according to the method described by Worawalai et al [22] with slight modification. The α-glucosidase (0.1 U/mL) and substrate (1 mM p-nitrophenyl-α-D-glucopyranoside) were prepared in 0.1 M phosphate buffer solution (pH=6.8), as a simulation model of intestinal fluid. A stock solution of synthesized target compounds dissolved in DMSO at concentration from 0.05 μM-500 μM containing appropriate concentration of enzyme solution were pre-incubated at 37 ºC for 10 min. The enzymatic reaction was initiated by adding substrate and incubated at 37 ºC for 30 min, and then the catalytic reaction was terminated by addition of a 1 M Na_2CO_3 solution. Enzymatic activity was quantified by measuring the absorbance at 405 nm with a Multi-model Plate Reader (Infinite 200). All the tests were run in duplicate and results were illustrated in Table **1** and Figure **2**. The IC_{50} data demonstrated that the tested compounds inhibited a-glucosidase with IC_{50} values ranging from 0.149 μM-30.374 μM and most of them had lower IC_{50} value than UA and positive control against α-glucosidase, suggesting that the ursolic acid derivatives had significant effects on the inhibitory potencies.

Within this series of target compounds, compound **3** exhibited strong inhibitory activity against yeast α-glucosidase (IC_{50}=0.149 ± 0.007 μM) which bore free carboxylic acid group of succinyl group at C-3 position. And the compound **4** showed potent inhibitory activity against yeast α-glucosidase (IC_{50}=0.223 ± 0.023 μM) which bore free carboxylic acid group of glutaryl group at C-3 position. As a result, compound **3** and **4** were 37 and 25 times more potent than their parent compound UA in enzyme inhibition activity. Interestingly, when compound **5** and **6** were deprotected by debenzylating which gave free carboxyl group at C-28 position, they would decrease potency by about 37-fold and 28-fold, respectively. According to the assay result, compound **9**, **10** and **11**, which bore long flexible carboxylic acid group at C-28 position and were protected by different anhydrides at C-3 position have more potent inhibitory activity against yeast α-glucosidase whose IC_{50} were 2.17 ± 0.055, 0.466 ± 0.016 and 0.298 ± 0.021μM, respectively, implying that bearing certain long hydrophilic group at either of 3-OH or 17-COOH position will increase enzyme inhibition activity. This result suggested compounds containing a free carboxyl at both 3-OH and 17-COOH position might not improve enzyme inhibition activity, while hydrophobic groups at either 3-OH or 17-COOH position and the free carboxylic acid group at either of 3-OH or 17-COOH position will benefit enzyme inhibition activity. Due to hydrophobic pocket in α-glucosidase, the hydrophilic group at one side position of ursolic acid might be increase the binding activity between target compounds and α-glucosidase. As the modification being protected by Propionic anhydride at c and amino groups being introduced at C-28 position of UA, compound **13-15** showed similar inhibitory activity with that of parent compound UA, while the potency of compound **16-20** decreased in enzyme inhibition activity. This result indicated that the hydrophobic groups conjugated with UA led to a pronounced decrease in inhibiting yeast α-glucosidase. On the basis of these findings, it clearly appeared that the free carboxyl group at C-3 position and C-28 position of UA might lead to the production of highly potent and selective inhibitors of α-glucosidase and we found that the inhibitory potency of these compounds against α-glucosidase increased with increasing length of the alkyl chain. For the compounds **5** and **6**, the introduction of both free carboxyl group at C-3 position and C-28 position of UA reduced the inhibition activities compared to that of **3** and **4**. Furthermore, we found that the introduction of hydrophobic groups at both C-3 position and C-28 position of UA were even weaker than that of ursolic acid.

2.3.2. Enzyme Kinetics

Since the results of the inhibitory activities demonstrated that compound **3**, **4**, **10** and **11** exhibited more potent inhibition than parent compound and positive control against α-glucosidase, the inhibition mechanism of the enzyme was further determined by use of enzyme kinetic analysis [23, 24]. Lineweaver-Burk plots of initial velocity versus enzyme concentrations of target compounds gave a series of straight lines. As is shown in Figure **3** and Figure **4**, compound **3** and **4** intersected in second quadrant. The analysis demonstrated that V_{max} decreased with elevated K_m in the presence of increasing concentrations of **3** and **4**. This behavior implied that compound **3** and **4** inhibit α-glucosidase by two distinct ways: competitively forming enzyme-inhibitor (EI) complex and interrupting enzyme-substrate (ES) intermediate by forming enzyme-substrate-inhibitor (ESI) complex in noncompetitive manner. As illustrated in Figure **5** and Figure **6**, the value of horizontal axis intercept ($-1/K_m$) remained unchanged and the value of vertical axis intercept ($1/V_{max}$) increased along with the concentrations of **10** and **11**, indicating that compound **10** and **11** were noncompetitive inhibitors. The inhibition constant *Ki* values for compound **10** and **11** were calculated to be 5.05 μM and 5.14 μM, respectively, with appropriate equations. To gain insights for the binding affinities of EI and ESI complexes, a Dixon plot and secondary replot of compound **3** and **4** were performed, respectively (Figure **7** and Figure **8**). Dixon plot of slope against concentration of **3** and **4** displayed EI dissociation constant *(Ki*) of 3.36 μM and 9.08 μM, respectively, whereas secondary replot of intercept versus inhibitor concentration of **3** and **4** generated ESI dissociation constant (*K'i*) of 6.64 μM and 4.14 μM, respectively. The lower dissociation constant of *Ki* for compound **3** pointed out that stronger binding between α-glucosidase and **3**, suggesting an inhibition mechanism is competitive predominant over noncompetitive.

2.4. Molecular docking mode

In order to expound how these ursolic acid derivatives conjugate with α-glucosidase, as well as to afford some guidance for a reasonable design of α-glucosidase inhibitor in the future, the molecular docking studies was conducted to explore the binding mode of ursolic acid derivatives within the binding pocket of α-glucosidase and to realize their structure activity relationship by use of SYBYL 2.0 as docking software.

All the target compounds were docked into the active pocket of a developed homology model of α-glucosidase (**PDB: 1UOK**) enzyme. It was observed that binding site of ursolic acid derivatives were similar to that of acarbose. The docking studies were performed for two kinds of potent inhibitors, **3** and **10**. As depicted in Figure **9** and Figure **10**, the binding free energy of **3** and **10** was calculated to be -7.55 $kcalmol^{-1}$ and -5.89 $kcalmol^{-1}$, which is much lower than that of UA itself. Compound **3** and **10** were mainly surrounded by the catalytic pocket included in the residues of ARG-197, SER-222, GLU-255, SER-288, ASP-329, ARG-415. Compound **3** was bound to α-glucosidase through H-bonds interaction of ester group at C-28 position with SER-222. The succinyl group at C-3 position formed H-bonds with ARG-197, GLU-255 and ARG-415 located inside the pocket, which have significant inhibitory activity towards α-glucosidase. Compound **10** was bound to α-glucosidase through H-bonds interaction of free carboxylic group at C-28 position with SER-288 and the ester group at C-3 position with ARG-415. MOLCAD lipophilic potential (LP) showed that free carboxylic group at C-3 position of **3** and free carboxylic group at C-28 position of **10** were closed to the hydrophobic region of pocket. That indicated the increased hydrophilic group could improve inhibitory activity. In addition, MOLCAD hydrogen bonding sites of the binding surfaces exhibited the hydrophobic pocket which has presented several hydrogen

bond donors and acceptors while **3** and **10** formed four and two hydrogen bonds just as an acceptor. Compounds of **3** and **10** were interact with the target α-glucosidase, which the hydrophilic group of **3** and **10** located into catalytic site, might improve inhibition activity, presumably via competitively binding the active pocket. Thus, the hydrophilic group conjugated with UA at 3-OH or 17-COOH position to form hydrogen bonds is one crucial factor for improved inhibitory activity. This conclusion raised our inspiration to optimize the design of UA derivatives as antidiabetic agents.

To investigate the relationship between our simulation and in vitro inhibitory activity, the binding free energies calculated by docking procedures were correlated to inhibitory activities of UA derivatives [25]. As was shown in Figure **11**, compound **3**, **4**, **10**, **11** revealed better inhibitory activities with predicted binding free energies lower than -5.5 $kcalmol^{-1}$. For other target compounds, most of them showed lower binding affinity than UA in which binding free energies were calculated from -5.1 $kcalmol^{-1}$ to -2.3 $kcalmol^{-1}$, implying that that UA was conjugated with hydrophobic moieties without hydrogen bonds formation by weakening binding affinity. In all synthesized compounds, **3**, **4**, **10**, **11** displayed strong inhibitory activity against α-glucosidase *in vitro*, and docking results were shown that better binding free energies of compound **3**, **4**, **10**, **11** which compared with other target compounds. This suggested that synthesized of active compounds by choosing the protein of α-glucosidase as docking model has certain guiding significance.

3. CONCLUSION

In summary, we have reported the design of Ursolic derivatives which conjugates polar and hydrophilicity moieties as novel α-glucosidase inhibitors based on molecular docking studies. The homology model of Baker's yeast α-glucosidase used by Docking simulations have provided insights into the binding mechanism at the molecular level, aiming at providing guidance for rational design of potent α-glucosidase inhibitors. Our molecular modeling results indicated that the active pocket mainly consists of residues ASP60, GLN167, SER222, GLU255, LYS293, ASP329 and ARG415, whereas the Hydrogen bonds formation with SER222, ASP329, ARG415 may improve the binding affinity between ligand and protein, and play a key role in enhancing inhibitory activities.

To verify this concept, we have designed and synthesized a novel series of UA derivatives as potential anti-diabetic agents through inhibiting α-glucosidase. The results of half maximal inhibitory concentration showed that all tested target compounds exhibited greater potency than acarbose in α-glucosidase inhibition assay. Especially, compounds **3**, **4**, **10** and **11**, bearing certain long hydrophilic group at either of 3-OH or 17-COOH position, displayed twelvefold to thirty-sevenfold higher inhibitory activity than that of the parent compound UA. However, Compounds containing a free carboxyl at both 3-OH and 17-COOH position might weaken enzyme inhibition activity. The hydrophobic groups conjugated with UA showed that the inhibitory activities of these compounds might decrease in inhibiting Baker's yeast α-glucosidase. Furthermore, mechanism underlying the inhibitory effect of **3** and **4** against α-glucosidase proved to be mix-type inhibition; compound **10** and **11** presented non-competitive inhibition mechanism. Moreover, studies of relationship between IC_{50} and the binding free energy shown that docking result has certain guiding significance. This suggested that UA derivatives conjugated with polar and hydrophilicity moieties could be a new class of promising compounds for further animal studies or clinical trials as potential anti-diabetic agents.

4. EXPERIMENTAL SECTION

4.1 General Experimental procedures

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE III 600 MH_Z or Mercury-Plus 300 MH_Z NMR spectrometers in either $CDCl_3$ or [D_6] DMSO. Chemical shift (δ) were given in parts per million (PPM) with tetramethylsilane (TMS) as an internal standard. The following abbreviations as used: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet. Mass spectra (MS) were measured on an LC-MS-2010A or Thermo Fisher LCQ Fleet Using the Electrospray ionization mode and the results are reported as m/z. Melting point were obtained on Fischer-Johns apparatus and are uncorrected. High-resolution mass spectra (HRMS) of compounds **3-6**, **9-11**, **13-20** were Bruker maXis impact. Elemental analysis was carried out on an Elementar Vario ELCHNS elemental analyzer. The enzyme inhibition activity was measureed with a Multimodel Plate Reader (Infinite 200).

Ursolic acid was purchased from Nanjing Zelang Medical Technology Co., Ltd., with over 98% purity. Purifications of those compounds were made by flash column chromatography with Tsingtao Marine chemistry Co., Ltd., silica gel (100-200, 200-300 mesh). Further purification was carried out by column chromatography with Sephadex column (LH-20). Thin layer chromatography (TLC) plates was performed on precoatedkieselgel F_{254} and supplied by Merck KGaA, Darmstadt, Germany. Unless otherwise noted, all other materials and reagents were purchased from commercial suppliers and without further purification. Compound **2**, **7**, **8**, **12** were prepared according to literature procedures.

4.2 Synthesis

4.2.1 3β-hydroxy-urs-12-en-28-oic acid benzyl ester (2). To a solution of Ursolic acid **1** (3 g, 6.57 mmol) in DMF (90 mL) was added K_2CO_3 (1.81 g, 13.14 mmol) and benzyl chloride (0.92 g, 7.21 mmol). The reaction mixture was stirred for 10 h at room temperature. Next, the reaction mixture was condensed under reduced pressure, and then was added with ice water (200 mL) slowly while being vigorously stirred. The precipitate was collected with a filter, washed with H_2O and dried to give compound **2** as white solid without further purification. Yield: 92%; white powder.

4.2.2 General procedure for the preparation of compounds (3-4). To a solution of compound **2** (1 g, 1.83 mmol) in anhydrous pyridine (20 mL) was added the corresponding anhydride and DMAP (cat.). The reaction mixture was stirred at room temperature for over 24 h. Then it was cooled to 0 ^{o}C, diluted with HCl solution (1 M, 10 mL) and dispersed in 50 ml of distilled water overnight. The precipitate was collected with a filter, then the filtration procedure was repeated several times and dried to obtain solid product. The crude product was purified by column chromatography over silica gel with an eluent (petroleum ether/ethyl acetate, 10:1) to produce compound **3**-**4**.

4.2.2.1 3β-[(3-carboxy)propionyloxy]-urs-12-en-28-oic acid benzyl ester (3). According to the general procedure, compound **2** was treated with succinic anhydride, and then purified on silica gel column by use of petroleum ether/ethyl acetate (v/v 10:1) as eluent to give compound 3 (R_f=0.56). Yield: 62%, white powder; Mp 101-102 ºC; ^{1}H NMR(600 MHz, $CDCl_3$) δ 7.37 – 7.29 (m, 4H), 5.23 (t, J = 3.6 Hz, 1H), 5.13 – 5.07 (m, 1H), 4.97 (dd, J = 12.4, 4.7 Hz, 1H), 4.55 – 4.50 (m, 1H), 2.69 (dd, J = 10.2, 4.3 Hz, 2H), 2.63 (dd, J = 10.2, 4.3 Hz, 2H), 2.26 (d, J = 11.1 Hz, 1H), 2.01 (m, J = 17.9, 12.2, 5.6 Hz, 1H), 1.92 – 1.75 (m, 3H), 1.74 – 1.66 (m, 2H), 1.66 – 1.57 (m, 4H), 1.52 – 1.46 (m, 3H), 1.38 – 1.31 (m, 2H), 1.28 (m, J = 13.2, 6.9, 3.5 Hz, 2H), 1.09 – 1.03 (m, 4H), 1.02 – 0.97 (m, 1H), 0.92 (dd, J = 15.8, 7.9 Hz, 5H), 0.89 – 0.79 (m, 10H), 0.63 (s, J = 9.6 Hz, 3H).); ^{13}C NMR(600 MHz, $CDCl_3$) δ 177.32, 176.44, 171.85, 138.16, 136.40, 128.41, 128.15, 127.94, 125.60, 81.59, 65.98, 55.33, 52.90, 48.14, 47.49, 42.05, 39.55, 39.10, 38.85, 38.27, 37.75, 36.86, 36.64,

32.96, 30.67, 29.36, 28.83, 28.05, 27.96, 24.26, 23.55, 23.50, 23.28, 21.17, 18.19, 17.04, 17.01, 16.76, 15.49.). ESI-MS m/z(647.5). HRMS (ESI) calcd for $C_{41}H_{58}O_6$ $[M+H]^+$=647.4306, found: 647.4312; Anal. Calcd for C, 76.12; H, 9.04. Found: C, 76.04; H, 9.09.

4.2.2.2 3β-[(3-carboxy)n-butyryloxy]-urs-12-en-28-oic acid benzyl ester (4). According to the general procedure, compound **2** was treated with glutaric anhydride, and then purified on silica gel column by use of petroleum ether/ethyl acetate (v/v 10:1) as eluent to give compound **4** (R_f=0.54). Yield: 57%, white powder; Mp 108-110 ºC; ^{1}H NMR600 MHz, $CDCl_3$) δ 7.36 – 7.29 (m, 5H), 5.23 (t, J = 3.6 Hz, 1H), 5.10 (dd, J = 12.0, 5.5 Hz, 1H), 4.98 (dd, J = 12.4, 4.8 Hz, 1H), 4.53 – 4.49 (m, 1H), 2.43 (t, J = 7.4 Hz, 2H), 2.40 (t, J = 7.4 Hz, 2H), 2.26 (d, J = 11.1 Hz, 1H), 2.02 (dd, J = 13.4, 4.4 Hz, 1H), 2.00 – 1.95 (m, 2H), 1.90 – 1.86 (m, 1H), 1.85 – 1.80 (m, 1H), 1.77 (dd, J = 13.6, 4.6 Hz, 1H), 1.71 (m, J = 13.8, 7.5, 2.9 Hz, 2H), 1.62 (m, J = 9.4, 4.2 Hz, 4H), 1.48 (m, J = 15.7, 11.5, 5.2 Hz, 4H), 1.31 (m, J = 17.1, 10.4, 9.4, 5.4 Hz, 5H), 1.10 – 1.03 (m, 5H), 0.99 (m, J = 11.9, 10.0, 3.9 Hz, 1H), 0.95 – 0.91 (m, 6H), 0.87 – 0.84 (m, 10H), 0.83 (dd, J = 5.3, 3.9 Hz, 1H), 0.81 – 0.77 (m, 1H), 0.63 (s, J = 9.6 Hz, 3H).); ^{13}C NMR (600 MHz, CDCl3) δ 177.95, 177.33, 172.63, 138.16, 136.40, 128.41, 128.15, 127.94, 125.61, 81.12, 65.99, 55.30, 52.90, 48.14, 47.49, 42.05, 39.55, 39.10, 38.85, 38.28, 37.73, 36.86, 36.64, 33.65, 32.96, 32.91, 30.67, 28.15, 27.96, 24.26, 23.60, 23.54, 23.28, 21.17, 20.02, 18.20, 17.03, 17.01, 16.81, 15.49.). ESI-MS m/z(662.3). HRMS (ESI) calcd for $C_{42}H_{60}O_6$ $[M+H]^+$=661.4463, found: 661.4461. Anal. Calcd for C, 76.33; H, 9.15. Found: C, 76.28; H, 9.17.

4.2.2.3 3β-[(3-carboxy)propionyloxy]-urs-12-en-28-oic acid (5). To a solution of compound **3** (200 mg, 0.31 mmol) in THF 10 mL was added Pd/C (10%, cat.). The reaction mixture was stirred at room temperature under H_2 atmosphere for over 12 h. After being filtered, the organic solvent was removed by a rotary evaporator in vacuo. The residue was purified on a silica gel column with petroleum ether/ethyl acetate (v/v 3.5:1) as eluent to obtain compound **5** (R_f=0.53). Yield: 75%, white powder; Mp 97-98 ºC; ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.23 (t, J = 3.5 Hz, 1H), 4.59 – 4.48 (m, 1H), 2.66 (dd, J = 12.6, 9.0 Hz, 4H), 2.18 (d, J = 11.3 Hz, 1H), 2.05 – 1.97 (m, 1H), 1.93 – 1.84 (m, 3H), 1.72 (dd, J = 9.5, 3.1 Hz, 1H), 1.70 – 1.61 (m, 5H), 1.52 (dd, J = 14.8, 6.1 Hz, 3H), 1.47 – 1.42 (m, 1H), 1.36 – 1.25 (m, 6H), 1.10 – 1.03 (m, 6H), 0.99 (d, J = 6.1 Hz, 1H), 0.95 (dd, J = 12.5, 6.2 Hz, 6H), 0.86 (t, J = 3.1 Hz, 7H), 0.82 (s, 3H), 0.76 (s, 3H).^{13}C NMR (600 MHz, $CDCl_3$) δ 184.06, 177.94, 171.53, 137.98, 125.74, 81.49, 55.17, 52.41, 47.97, 47.24, 41.76, 39.47, 39.00, 38.82, 38.03, 37.78, 36.94, 36.79, 32.65, 30.55, 29.45, 29.18, 28.19, 28.00, 23.96, 23.65, 23.46, 23.26, 21.18, 18.20, 17.24, 17.02, 16.92, 15.67.ESI-MS m/z(556.19). HRMS (ESI) calcd for $C_{34}H_{52}O_6Na$ $[M+Na]^+$=579.3656, found:579.3658. Anal. Calcd for C, 73.34; H, 9.41. Found: C, 76.27; H, 9.45.

4.2.2.4 3β-[(3-carboxy)n-butyryloxy]-urs-12-en-28-oic acid (6). To a solution of compound **4** (200 mg, 0.30 mmol) in THF 10 mL was added Pd/C (10%, cat.). The reaction mixture was stirred at room temperature under H2 atmosphere for over 12 h. After filtered, the organic solvent was removed by a rotary evaporator in vacuo. The residue was purified on a silica gel column with petroleum ether/ethyl acetate (v/v 4:1) as eluent to obtain compound **6** (R_f=0.55). Yield: 73%, white powder; Mp 105-106 ºC; ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.23 (t, J = 3.5 Hz, 1H), 4.52 (dd, J = 11.1, 4.7 Hz, 1H), 2.49 – 2.37 (m, 5H), 2.17 (d, J = 11.2 Hz, 1H), 2.03 (dd, J = 13.6, 4.3 Hz, 1H), 1.97 (dd, J = 14.7, 7.6 Hz, 2H), 1.92 (dd, J = 8.7, 3.6 Hz, 2H), 1.89 – 1.84 (m, 1H), 1.76 – 1.70 (m, 2H), 1.66 (ddd, J = 32.3, 18.0, 7.8 Hz, 6H), 1.58 – 1.49 (m, 4H), 1.49 – 1.42 (m, 2H), 1.32 (dd, J = 16.5, 6.4 Hz, 4H), 1.12 (d, J = 5.5 Hz, 1H), 1.08 (d, J = 12.8 Hz, 4H), 1.01 (d, J = 6.3 Hz, 1H), 0.98 – 0.93 (m, 6H), 0.88 (d, J = 7.4 Hz, 5H), 0.85 (d, J = 10.6 Hz, 5H), 0.74 (s, 3H).^{13}C NMR (600 MHz, $CDCl_3$) δ 184.53, 179.36, 172.18, 137.99, 125.72, 81.03, 55.14, 52.40, 48.02, 47.89, 47.23, 41.73,

39.47, 38.97, 38.82, 37.96, 37.77, 36.94, 36.64, 33.88, 33.13, 32.63, 30.52, 28.32, 28.00, 23.98, 23.70, 23.58, 23.24, 21.17, 20.25, 18.17, 17.01, 16.99, 16.90, 15.62.ESI-MS m/z(579.9). HRMS (ESI) calcd for $C_{34}H_{52}O_6$ $[M+Na]^+$=593.3813, found:593.3810. Anal. Calcd for C, 73.65; H, 9.54. Found: C, 76.59; H, 9.61.

4.2.3 General procedure for the preparation of compounds (7-11). Compound **7** and **8** were synthesized with the previous method. Ursolic acid **1** (2 g, 4.4 mmol) and K_2CO_3 (1.2 g, 8.7 mmol) were added to 60 mL DMF and stirred at room temperature until the UA completely dissolved. Then the ethyl bromoacetate was slowly added in the mixture reaction. After an overnight stirring t, the mixture solvent was removed by a rotary evaporator in vacuo at 80 ^oC, dispersed in 50 mL distilled water and filtered, then dried to obtain compound **7**. To a solution of compound **7** (1 g, 1.84 mmol) in 40 mL THF/MeOH (v/v=1.5:1) was added 30 mL NaOH solution (4 M), the reaction mixture was stirred at room temperature for 8 h and concentrated under reduced pressure, dried to be the crude product. The crude product was purified by column chromatography over silica gel with an eluent (petroleum ether/ethyl acetate, 5:1) to produce compound **8** (R_f=0.52). To a solution of compound **8** (200 mg, 0.39 mmol) in dry pyridine (10 mL) was added the various anhydride and DMAP (cat.) and stirred at room temperature for 10 h. The mixture solvent concentrated under reduced pressure, dispersed in 20 ml of distilled water and adjusted with HCl solution (1 M) to pH 3-4 and filtered. The crude product was purified on a silica gel column with appropriate eluent to obtain **9**-**11**.

4.2.3.1 Carboxymethyl-3β-Acetoxy-urs-12-en-28-oate (9). According to the general procedure, compound **8** was treated with acetic anhydride, then purified by column chromatography over silica gel with an eluent (petroleum ether/ethyl acetate, 4:1) to produce compound **9** (R_f=0.51). Yield: 53%, white powder; Mp 119-121 ºC; ^{1}H NMR(600 MHz, $CDCl_3$) δ 5.26 (t, J = 3.6 Hz, 1H), 4.62 – 4.54 (m, 2H), 4.50 (dd, J = 10.5, 5.6 Hz, 1H), 2.25 (d, J = 11.2 Hz, 1H), 2.08 – 2.02 (m, 4H), 1.91 (dd, J = 7.4, 3.4 Hz, 2H), 1.82 – 1.78 (m, 1H), 1.77 – 1.73 (m, 2H), 1.73 – 1.70 (m, 1H), 1.70 – 1.59 (m, 4H), 1.57 – 1.46 (m, 4H), 1.43 – 1.24 (m, 6H), 1.23 – 1.12 (m, 1H), 1.07 (d, J = 12.0 Hz, 4H), 0.94 (t, J = 6.5 Hz, 6H), 0.89 – 0.85 (m, 10H), 0.84 (d, J = 1.7 Hz, 1H), 0.82 – 0.78 (m, 1H), 0.74 (s, 3H).); ^{13}C NMR(600 MHz, $CDCl_3$) δ 176.74, 172.11, 171.11, 137.97, 125.76, 80.97, 59.87, 55.31, 52.77, 48.23, 47.50, 42.10, 39.56, 39.09, 38.79, 38.33, 37.69, 36.87, 36.43, 32.97, 30.63, 28.08, 27.97, 24.24, 23.56, 23.45, 23.31, 21.31, 21.16, 18.20, 17.03, 17.00, 16.75, 15.53.); ESI-MS m/z(556.2). HRMS (ESI) calcd for $C_{34}H_{52}O_6$ $[M+H]^+$=579.3656, found:579.3663. Anal. Calcd for C, 73.34; H, 9.41. Found: C, 76.26; H, 9.48.

4.2.3.2 Carboxymethyl-3β-propionyloxy-urs-12-en-28-oate (10). According to the general procedure, compound **8** was treated with propionic anhydride, and then purified by column chromatography over silica gel with an eluent (petroleum ether/ethyl acetate, 4.5:1) to produce compound **10** (R_f=0.56). Yield: 51%, white powder; Mp 125-126 ºC; ^{1}H NMR(600 MHz, $CDCl_3$) δ 5.26 (t, J = 3.4 Hz, 1H), 4.62 – 4.53 (m, 2H), 4.50 (td, J = 10.2, 6.3 Hz, 1H), 2.41 – 2.30 (m, 3H), 2.25 (d, J = 11.2 Hz, 1H), 2.08 – 2.02 (m, 1H), 1.91 (dd, J = 7.2, 3.4 Hz, 2H), 1.83 – 1.76 (m, 2H), 1.76 – 1.73 (m, 1H), 1.70 (dd, J = 13.5, 3.7 Hz, 2H), 1.66 (dd, J = 10.4, 4.0 Hz, 1H), 1.63 (d, J = 5.4 Hz, 2H), 1.58 – 1.46 (m, 5H), 1.41 – 1.29 (m, 5H), 1.29 – 1.24 (m, 1H), 1.17 – 1.13 (m, 4H), 1.11 – 1.06 (m, 5H), 1.04 – 0.99 (m, 1H), 0.97 – 0.92 (m, 7H), 0.87 (d, J = 7.1 Hz, 10H), 0.84 (d, J = 5.1 Hz, 2H), 0.82 – 0.78 (m, 1H), 0.74 (s, 3H).); ^{13}C NMR(600 MHz, $CDCl_3$) δ 176.78, 174.44, 173.10, 137.91, 125.78, 80.69, 59.92, 55.31, 52.78, 48.15, 47.50, 42.10, 39.57, 39.10, 38.80, 38.31, 37.78, 36.88, 36.42, 32.98, 30.64, 28.10, 27.99, 27.27, 24.25, 23.58, 23.47, 23.32, 21.17, 18.21, 17.04, 16.80, 15.53, 9.35, 8.73.); ESI-MS m/z(569.4). HRMS (ESI) calcd for $C_{35}H_{54}O_6$ $[M+H]^+$=571.3993.

found:5771.3993. Anal. Calcd for C, 73.65; H, 9.54. Found: C, 76.56; H, 9.58.

4.2.3.3 Carboxymethyl-3β-n-Butyryloxy-urs-12-en-28-oate (11). According to the general procedure, compound **8** was treated with butyric anhydride, and then purified by column chromatography over silica gel with an eluent (petroleum ether/ethyl acetate, 3:1) to produce compound **11** (R_f=0.55). Yield: 54%, white powder; Mp 128-130 ºC; ^{1}H NMR(600 MHz, $CDCl_3$) δ 5.26 (t, J = 3.3 Hz, 1H), 4.62 – 4.53 (m, 2H), 4.53 – 4.48 (m, 1H), 2.34 (t, *J* = 7.4 Hz, 1H), 2.31 – 2.23 (m, 3H), 2.08 – 2.01 (m, 1H), 1.91 (dd, *J* = 7.3, 3.4 Hz, 2H), 1.80 (dd, *J* = 13.9, 5.0 Hz, 1H), 1.78 – 1.73 (m, 2H), 1.71 (d, *J* = 4.1 Hz, 1H), 1.68 (dd, *J* = 13.0, 5.7 Hz, 2H), 1.63 (m, *J* = 20.9, 12.2, 4.6 Hz, 4H), 1.54 – 1.49 (m, 4H), 1.39 – 1.25 (m, 5H), 1.08 (d, *J* = 2.9 Hz, 4H), 0.98 (d, *J* = 7.4 Hz, 1H), 0.95 (dd, *J* = 9.9, 4.7 Hz, 9H), 0.86 (t, *J* = 4.0 Hz, 9H), 0.85 – 0.81 (m, 2H), 0.73 (s, *J* = 2.9 Hz, 3H).); ^{13}C NMR(600 MHz, $CDCl_3$) δ 176.78, 173.65, 173.19, 137.90, 125.78, 80.65, 59.94, 55.31, 52.78, 48.22, 47.50, 42.10, 39.57, 39.10, 38.80, 38.32, 37.73, 36.88, 36.78, 36.42, 35.79, 32.99, 30.64, 28.10, 27.99, 24.25, 23.61, 23.46, 23.32, 21.17, 18.64, 18.21, 17.04, 16.82, 15.52, 13.73.); ESI-MS m/z(584.6). HRMS (ESI) calcd for $C_{36}H_{56}O_6$ $[M+H]^+$=585.4150. found:585.4146. Anal. Calcd for C, 73.93; H, 9.65. Found: C, 76.87; H, 9.70.

4.2.4 General procedure for the preparation of compounds (13-20). Compound **12** was synthesized from Ursolic acid, whose preparation was described in our previous report. To a solution of compound **12** (0.5 g, 1mmol) in CH_2Cl (50 mL) was added oxalyl chloride (0.6 mL) The reaction mixture was stirred at room temperature for 36 h. The reaction solvent was removed by a rotary evaporator in vacuo to dryness to yield crude 3-O-propionylursolyl chloride. This intermediate was dissolved in CH_2Cl (30 mL), and the mixture was stirred in the presence of triethylamine, and then treated with various desired amine. The reaction mixture was stirred at room temperature for 8 h. The reaction solvent was removed under reduced pressure to yield crude product, which was purified by column chromatography over silica gel with an eluent (petroleum ether/ethyl acetate, 10:1) to produce white powder. Further purification was carried out by column chromatography with Sephadex column (LH-20) by use of trichloromethane and methanol (v/v=1:1) as the eluent to yield compounds **13**-**20**.

4.2.4.1 N-[3β-propionyloxy-urs-12-en-28-oyl]-para-fluoroaniline (13).

According to the general procedure, 3-O-propionylursolyl chloride was treated with p-fluoroaniline, and then purified by column chromatography over silica gel with an eluent (petroleum ether/ethyl acetate, 10:1) to produce compound **13** (R_f=0.58). Yield: 68%, white powder; Mp 181-183 ºC; ^{1}H NMR(400 MHz, $CDCl_3$) δ 7.68 (s, 1H), 7.45 – 7.37 (m, 2H), 6.98 (t, *J* = 8.7 Hz, 2H), 5.48 (t, *J* = 3.3 Hz, 1H), 4.50 (dd, *J* = 10.3, 5.6 Hz, 1H), 2.37 – 2.28 (m, 2H), 2.11 – 1.93 (m, 5H), 1.82 (dd, *J* = 13.6, 1.8 Hz, 1H), 1.78 – 1.68 (m, 2H), 1.67 – 1.60 (m, 3H), 1.57 (t, *J* = 5.2 Hz, 2H), 1.55 – 1.45 (m, 4H), 1.42 – 1.24 (m, 3H), 1.19 – 1.06 (m, 8H), 0.99 (s, 4H), 0.92 (d, *J* = 8.0 Hz, 6H), 0.83 (t, *J* = 8.3 Hz, 7H), 0.69 (s, 2H).^{13}C NMR(400 MHz, $CDCl_3$) δ 176.29, 174.31, 160.37, 157.96, 140.22, 134.21, 126.02, 121.34, 121.26, 115.63, 115.41, 80.48, 55.19, 54.30, 48.55, 47.43, 42.65, 39.89, 39.56, 39.13, 38.32, 37.74, 37.05, 36.81, 32.65, 30.88, 28.06, 27.89, 25.10, 23.57, 23.54, 23.27, 21.19, 18.08, 17.30, 16.87, 16.73, 15.57, 9.34. ESI-MS m/z(605.4). HRMS (ESI) calcd for $C_{39}H_{56}FNaNO_3$ $[M+Na]^+$= 628.4137, found: 628.4136. Anal. Calcd for C, 77.31; H, 9.32. Found: C, 77.24; H, 9.34.

4.2.4.2 N-[3β-propionyloxy-urs-12-en-28-oyl]-para-chloroaniline (14).

According to the general procedure, 3-O-propionylursolyl chloride was treated with p-chloroaniline, and then purified by column chromatography over silica gel with an eluent (petroleum ether/ethyl acetate, 10:1) to produce compound **14** (R_f=0.57). Yield: 70%, white powder;

Mp 184-186 ºC; ^{1}H NMR (600 MHz, DMSO) δ 8.99 (s, 1H), 7.58 (d, J = 8.9 Hz, 2H), 7.31 (d, J = 8.9 Hz, 2H), 5.28 (s, 1H), 4.40 (dd, J = 11.5, 4.6 Hz, 1H), 2.36 (d, J = 11.0 Hz, 1H), 2.31 – 2.24 (m, 2H), 2.07 – 2.00 (m, 1H), 1.92 – 1.85 (m, 1H), 1.84 – 1.73 (m, 3H), 1.70 (d, J = 13.0 Hz, 1H), 1.57 (d, J = 11.0 Hz, 2H), 1.53 (d, J = 11.2 Hz, 2H), 1.50 (d, J = 8.0 Hz, 1H), 1.49 – 1.44 (m, 3H), 1.44 – 1.39 (m, 3H), 1.37 – 1.19 (m, 4H), 1.14 (s, 1H), 1.08 (s, 3H), 1.03 (t, J = 7.5 Hz, 5H), 0.94 (d, J = 6.3 Hz, 3H), 0.89 – 0.83 (m, 8H), 0.80 (t, J = 7.6 Hz, 7H), 0.61 (s, 3H).^{13}C NMR(600 MHz, DMSO) δ 175.81, 173.74, 138.92, 138.77, 128.70, 127.09, 125.06, 122.26, 80.18, 54.96, 52.09, 48.11, 47.24, 42.02, 39.19, 38.78, 38.15, 37.80, 36.86, 36.73, 32.81, 30.72, 30.07, 28.27, 27.86, 27.70, 26.81, 23.89, 23.80, 23.71, 21.52, 18.15, 17.59, 17.14, 17.05, 15.56, 9.66; ESI-MS m/z(621.3). HRMS (ESI) calcd for $C_{39}H_{56}ClNO_3$ $[M+H]^+$= 622.4021, found: 622.4034. Anal. Calcd for C, 75.27; H, 9.07. Found: C, 75.19; H, 9.11.

4.2.4.3 N-[3β-propionyloxy-urs-12-en-28-oyl]-para-bromoaniline (15). According to the general procedure, 3-O-propionylursolyl chloride was treated with p-bromoaniline, and then purified by column chromatography over silica gel with an eluent (petroleum ether/ethyl acetate, 10:1) to produce compound **15** (R_f=0.58). Yield: 74%, white powder; Mp 183-185 ºC; ^{1}H NMR(600 MHz, DMSO) δ 8.99 (s, 1H), 7.54 (d, J = 8.9 Hz, 2H), 7.44 (t, J = 5.9 Hz, 2H), 5.27 (d, J = 3.5 Hz, 1H), 4.40 (dd, J = 11.5, 4.6 Hz, 1H), 2.36 (d, J = 11.0 Hz, 1H), 2.34 – 2.24 (m, 2H), 2.10 (d, J = 18.8 Hz, 1H), 2.07 – 2.00 (m, 1H), 1.91 – 1.85 (m, 1H), 1.84 – 1.78 (m, 2H), 1.77 – 1.73 (m, 1H), 1.70 (d, J = 12.9 Hz, 1H), 1.58 (t, J = 12.0 Hz, 2H), 1.51 (ddd, J = 21.4, 11.9, 5.5 Hz, 4H), 1.46 – 1.39 (m, 4H), 1.37 – 1.26 (m, 2H), 1.21 (td, J = 15.5, 7.3 Hz, 2H), 1.14 (s, 1H), 1.08 (s, 3H), 1.03 (t, J = 7.5 Hz, 5H), 0.94 (d, J = 6.3 Hz, 3H), 0.87 (d, J = 6.4 Hz, 3H), 0.86 – 0.83 (m, 4H), 0.80 (t, J = 7.6 Hz, 6H), 0.61 (s, 3H).^{13}C NMR(600 MHz, DMSO) δ 175.82, 173.74, 139.20, 138.92, 131.61, 125.06, 122.64, 115.12, 80.18, 56.31, 54.96, 52.07, 48.14, 47.24, 42.02, 39.19, 38.78, 38.14, 37.80, 36.85, 36.71, 32.81, 30.71, 30.07, 28.27, 27.85, 27.70, 26.81, 23.89, 23.71, 23.36, 21.52, 17.59, 17.15, 17.05, 15.56, 9.66. ESI-MS m/z(665.3).HRMS (ESI) calcd for $C_{39}H_{56}BrNO_3$ $[M+H]^+$= 666.3516, found: 666.3535. Anal. Calcd for C, 70.25; H, 8.47. Found: C, 70.17; H, 8.51.

4.2.4.4 N-[3β-propionyloxy-urs-12-en-28-oyl]-ortho-fluoroaniline (16).

According to the general procedure, 3-O-propionylursolyl chloride was treated with o-fluoroaniline, and then purified by column chromatography over silica gel with an eluent (petroleum ether/ethyl acetate, 20:1), followed by a Sephadex column (LH-20) with trichloromethane and methanol (v/v=1:1) as the eluent to produce compound **16** (R_f=0.51). Yield: 62%, white powder; Mp 180-182 ºC; ^{1}H NMR(600 MHz, DMSO) δ 8.68 (s, 1H), 7.57 (td, J = 7.7, 2.3 Hz, 1H), 7.25 – 7.19 (m, 1H), 7.17 – 7.10 (m, 2H), 5.30 (s, 1H), 4.41 (dd, J = 11.6, 4.6 Hz, 1H), 2.35 – 2.24 (m, 3H), 2.08 – 2.01 (m, 1H), 1.94 – 1.88 (m, 1H), 1.87 – 1.83 (m, 1H), 1.80 (t, J = 12.2 Hz, 2H), 1.76 – 1.72 (m, 1H), 1.59 (dd, J = 13.4, 3.6 Hz, 3H), 1.56 – 1.53 (m, 1H), 1.53 – 1.39 (m, 6H), 1.33 (dd, J = 23.7, 11.4 Hz, 2H), 1.25 (d, J = 13.1 Hz, 1H), 1.09 (s, 3H), 1.05 – 1.00 (m, 6H), 0.94 (d, J = 6.3 Hz, 3H), 0.87 (dd, J = 10.2, 3.6 Hz, 7H), 0.82 (d, J = 17.5 Hz, 7H), 0.69 (s, 3H).^{13}C NMR(600 MHz, DMSO) δ 175.90, 173.74, 138.74, 126.74, 126.66, 126.32, 126.22, 125.53, 124.57, 115.93, 115.80, 80.17, 54.96, 52.45, 48.25, 47.26, 42.20, 39.30, 38.78, 38.19, 37.81, 37.14, 36.86, 32.95, 30.84, 28.28, 27.87, 27.70, 24.09, 23.73, 23.70, 23.37, 21.53, 18.18, 17.55, 17.16, 17.12, 15.62, 9.67. ESI-MS m/z(605.4). HRMS (ESI) calcd for $C_{39}H_{56}FNaNO_3$$[M+Na]^+$= 628.4136, found: 628.4142. Anal. Calcd for C, 77.31; H, 9.32. Found: C, 77.26; H, 9.36.

4.2.4.5 N-[3β-propionyloxy-urs-12-en-28-oyl]-ortho-chloroaniline (17).

According to the general procedure, 3-O-propionylursolyl chloride was treated with o-chloroaniline, and then purified by column chromatography over silica gel with an eluent

(petroleum ether/ethyl acetate, 20:1), followed by a Sephadex column (LH-20) with trichloromethane and methanol (v/v=1:1) as the eluent to produce compound **17** (R_f=0.55). Yield: 65%, white powder; Mp 183-186 ºC; ^{1}H NMR(600 MHz, DMSO) δ 8.62 (s, 1H), 7.75 – 7.70 (m, 1H), 7.47 (dd, J = 8.0, 1.3 Hz, 1H), 7.33 – 7.27 (m, 1H), 7.15 (td, J = 7.9, 1.5 Hz, 1H), 5.32 (s, 1H), 4.41 (dd, J = 11.6, 4.6 Hz, 1H), 2.34 – 2.24 (m, 3H), 2.08 – 2.04 (m, 1H), 1.94 – 1.88 (m, 1H), 1.87 – 1.83 (m, 1H), 1.78 (dd, J = 23.6, 11.8 Hz, 3H), 1.63 (dd, J = 13.7, 4.1 Hz, 1H), 1.59 (s, 1H), 1.57 (d, J = 3.0 Hz, 1H), 1.56 – 1.53 (m, 1H), 1.52 – 1.43 (m, 5H), 1.38 – 1.29 (m, 2H), 1.29 – 1.21 (m, 2H), 1.14 (s, 1H), 1.10 (s, 3H), 1.03 (t, J = 7.5 Hz, 6H), 0.94 (d, J = 6.3 Hz, 3H), 0.87 (t, J = 7.5 Hz, 6H), 0.80 (d, J = 2.1 Hz, 6H), 0.68 (s, 3H).^{13}C NMR(600 MHz, DMSO) δ 175.82, 173.75, 129.78, 128.62, 127.88, 126.33, 125.96, 80.17, 54.94, 52.70, 48.51, 47.23, 42.17, 39.34, 38.78, 38.36, 38.23, 38.16, 37.81, 37.21, 36.85, 32.87, 30.83, 30.07, 29.60, 28.27, 27.83, 27.70, 24.25, 23.77, 23.73, 23.33, 21.52, 18.17, 17.55, 17.23, 17.15, 15.60, 9.67. ESI-MS m/z(621.4). HRMS (ESI) calcd for $C_{39}H_{56}ClNO_3$ $[M+H]^+$= 622.4021, found: 622.4044. Anal. Calcd for C, 75.27; H, 9.07. Found: C, 75.19; H, 9.10.

4.2.4.6 N-[3β-propionyloxy-urs-12-en-28-oyl]-ortho-bromoaniline (18).

According to the general procedure, 3-O-propionylursolyl chloride was treated with o-bromoaniline, and then purified by column chromatography over silica gel with an eluent (petroleum ether/ethyl acetate, 20:1), followed by a Sephadex column (LH-20) with trichloromethane and methanol (v/v=1:1) as the eluent to produce compound **18** (R_f=0.59). Yield: 76%, white powder; Mp 184-186 ºC; ^{1}H NMR (600 MHz, DMSO) δ 8.57 (s, 1H), 7.71 (d, J = 9.3 Hz, 1H), 7.63 (d, J = 9.2 Hz, 1H), 7.34 (t, J = 7.1 Hz, 1H), 7.08 (t, J = 8.4 Hz, 1H), 5.33 (t, J = 3.2 Hz, 1H), 4.41 (dd, J = 11.6, 4.7 Hz, 1H), 2.30 (ddd, J = 12.0, 11.4, 5.7 Hz, 2H), 2.27 – 2.23 (m, 1H), 2.11 – 2.04 (m, 4H), 1.94 – 1.88 (m, 1H), 1.88 – 1.81 (m, 2H), 1.81 – 1.75 (m, 2H), 1.64 (dd, J = 13.7, 4.1 Hz, 1H), 1.60 (d, J = 10.7 Hz, 1H), 1.55 (dd, J = 11.8, 7.5 Hz, 2H), 1.53 – 1.43 (m, 5H), 1.38 – 1.29 (m, 2H), 1.26 (dd, J = 13.3, 9.8 Hz, 2H), 1.10 (s, 3H), 1.03 (t, J = 7.5 Hz, 6H), 0.95 (d, J = 6.3 Hz, 3H), 0.87 (t, J = 7.9 Hz, 7H), 0.82 (t, J = 10.6 Hz, 7H), 0.69 (s, 3H).^{13}C NMR (400 MHz, $CDCl_3$) δ 176.33, 174.31, 138.09, 136.15, 132.22, 128.31, 127.14, 124.67, 121.83, 113.34, 80.55, 55.22, 53.84, 49.42, 47.46, 42.22, 39.81, 39.57, 39.04, 38.25, 37.75, 37.48, 36.82, 32.60, 30.87, 28.07, 27.82, 25.10, 23.65, 23.54, 23.30, 21.21, 18.11, 17.27, 16.73, 15.49, 9.35; ESI-MS m/z(665.3). HRMS (ESI) calcd for $C_{39}H_{56}BrNO_3$ $[M+H]^+$= 666.3516, found: 666.3527. Anal. Calcd for C, 70.25; H, 8.47. Found: C, 70.14; H, 8.53.

4.2.4.7 N-[3β-propionyloxy-urs-12-en-28-oyl]-aminobenzene (19).

According to the general procedure, 3-O-propionylursolyl chloride was treated with phenylamine, and then purified by column chromatography over silica gel with an eluent (petroleum ether/ethyl acetate, 10:1) to produce compound **19** (R_f=0.53). Yield: 63%, white powder; Mp 168-170 ºC;^{1}H NMR(600 MHz, $CDCl_3$) δ 7.58 (s, 1H), 7.36 (d, J = 9.0 Hz, 2H), 6.83 (d, J = 9.0 Hz, 2H), 5.47 (t, J = 3.4 Hz, 1H), 4.50 (dd, J = 11.2, 4.9 Hz, 1H), 3.78 (s, 3H), 2.32 (qd, J = 7.6, 2.1 Hz, 2H), 2.06 (dd, J = 13.7, 4.1 Hz, 1H), 2.04 – 1.99 (m, 2H), 1.98 (s, 1H), 1.96 (t, J = 3.7 Hz, 1H), 1.86 – 1.80 (m, 1H), 1.74 (td, J = 13.8, 4.3 Hz, 1H), 1.63 (td, J = 10.1, 5.1 Hz, 3H), 1.61 – 1.55 (m, 4H), 1.50 (d, J = 10.8 Hz, 2H), 1.37 – 1.33 (m, 1H), 1.33 – 1.25 (m, 2H), 1.16 (s, 1H), 1.15 – 1.12 (m, 5H), 1.12 – 1.07 (m, 2H), 0.98 (s, 3H), 0.94 – 0.90 (m, 6H), 0.85 (dd, J = 10.3, 5.2 Hz, 7H), 0.72 (s, 3H).^{13}C NMR(600 MHz, $CDCl_3$) δ 176.03, 174.30, 156.16, 140.22, 131.41, 125.95, 121.36, 114.07, 80.53, 55.47, 55.23, 54.35, 48.44, 47.49, 42.67, 39.92, 39.60, 39.16, 38.35, 37.76, 37.10, 36.84, 32.71, 30.94, 28.08, 27.94, 25.11, 23.57, 23.28, 21.21, 18.12, 17.32, 16.97, 16.74, 15.58,

9.34. ESI-MS m/z(587.4). HRMS (ESI) calcd for $C_{39}H_{57}NO_3$ $[M+H]^+$= 588.4411, found: 588.4434. Anal. Calcd for C, 79.68; H, 9.77. Found: C, 79.59.; H, 9.81.

4.2.4.8 N-[3β-propionyloxy-urs-12-en-28-oyl]-para-methoxyaniline (20).

According to the general procedure, 3-O-propionylursolyl chloride was treated with p-methoxyaniline, and then purified by column chromatography over silica gel with an eluent (petroleum ether/ethyl acetate, 12.5:1), followed by a Sephadex column (LH-20) with trichloromethane and methanol (v/v=1:1) as the eluent to produce compound **20** (R_f=0.55). Yield: 78%, white powder; Mp 174-176 ºC; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.58 (s, 1H), 7.36 (d, J = 9.0 Hz, 2H), 6.83 (d, J = 9.0 Hz, 2H), 5.47 (t, J = 3.4 Hz, 1H), 4.50 (dd, J = 11.2, 4.9 Hz, 1H), 3.78 (s, 3H), 2.33 (dd, J = 7.6, 2.2 Hz, 1H), 2.31 (dd, J = 7.6, 2.0 Hz, 1H), 2.06 (dd, J = 13.7, 4.1 Hz, 1H), 2.04 – 1.99 (m, 2H), 1.97 (dd, J = 10.5, 7.5 Hz, 2H), 1.83 (d, J = 13.7 Hz, 1H), 1.74 (td, J = 13.8, 4.3 Hz, 1H), 1.67 – 1.61 (m, 3H), 1.61 – 1.57 (m, 3H), 1.56 (d, J = 4.3 Hz, 1H), 1.55 – 1.52 (m, 1H), 1.50 (d, J = 10.8 Hz, 2H), 1.39 – 1.25 (m, 4H), 1.14 (dd, J = 10.1, 5.0 Hz, 6H), 0.98 (s, 3H), 0.93 – 0.90 (m, 6H), 0.86 – 0.81 (m, 8H), 0.72 (s, 3H).^{13}C NMR (600 MHz, $CDCl_3$) δ 176.03, 174.30, 156.16, 140.22, 131.41, 125.95, 121.36, 114.07, 80.53, 55.47, 55.23, 54.35, 48.44, 47.49, 42.67, 39.92, 39.60, 39.16, 38.35, 37.76, 37.10, 36.84, 32.71, 30.94, 28.08, 27.94, 25.11, 23.57, 23.28, 21.21, 18.12, 17.32, 16.97, 16.74, 15.58, 9.34. ESI-MS m/z(617.4). HRMS (ESI) calcd for $C_{40}H_{59}NO_4$ $[M+H]^+$= 618.4517, found: 618.4539. Anal. Calcd for C, 77.75; H, 9.62. Found: C, 77.63.; H, 9.69.

4.3 α-glucosidase inhibitory activity

Inhibitory activity of tested compounds against a-glucosidase from Baker's yeast was assessed with the method of Wacharasindhu et al with slightly modification. The α-glucosidase (0.1 U/mL) and substrate (1 mM p-nitrophenyl-α-D-glucopyranoside) were dissolved in 0.1 M phosphate buffer, pH 6.8. 10 μL of synthesized compounds (2 mg/mL in DMSO) was pre-incubated with 10 μL of α-glucosidase at 37 ºC for 10 min. A 100 μL substrate solution was then added to the reaction mixture and incubated at 37 ºC for 20 min, and terminated by adding 100 μL of 1 M Na2CO3. Enzymatic activity was quantified by measuring the absorbance at 405 nm with a Multimodel Plate Reader (Infinite 200). The percentage inhibition was calculated by [(A0-A1)/A0] × 100, where A0 is the absorbance without the sample, and A1 is the absorbance with the sample. The IC50 value was determined from a plot of percentage inhibition versus sample concentration. Acarbose was used as the standard control and the experiment was performed in duplicate.

4.4 Kinetic study of a-glucosidase inhibition

For kinetic analyses of maltase by the active compounds, enzyme and active compounds were incubated with increasing concentration of of p-nitrophenyl-α-D-glucopyranoside (0-10 μM). The type of inhibition was investigated by analyzing enzyme kinetic data based on Lineweaver-Burk plots. A series of V_{max} and K_m values were obtained from Y intercepts and calculated by slope × V_{max}, respectively.

4.5 Molecular Modeling

The molecular minimizing of target compounds was built by use of the Sybyl molecular modeling package, version 8.0 (Tripos, Shanghai, China). All structures were minimized with the Tripos force field, and the hydrogen atoms were added. Powel optimized the energy gradient, the maximum times to 1000 times the energy convergence criterion reaching 0.005 kcal mol−1, by use of Gasteiger–Hückle charges. Ligand-protein docking was performed by the Surflex Dock in SYBYL8.0. The crystal structure of α-glucosidase was retrieved from RCSB Protein Data Bank

(PDB: 1UOK). Biopolymer module was then used to repair the crystal structure of the protein termini treatment, to fix side chain amides and residues and to add charges. The potent target compounds docking with α-glucosidase selected catalytic pocket of acarbose as active site. The active pocket was formed through computing, the others being the default settings.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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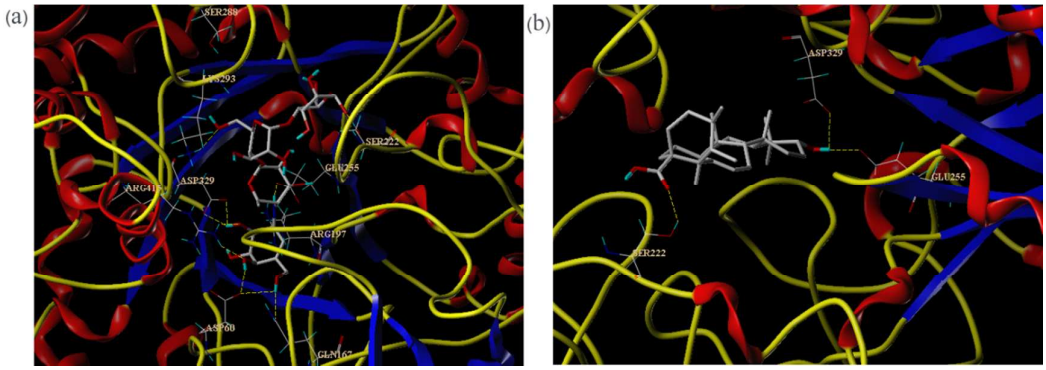


Fig 1. (a) Predicted binding mode of acabose docked with α-glucosidase. (b) Binding mode of Ursolic docked with α-glucosidase in the similar active pocket.

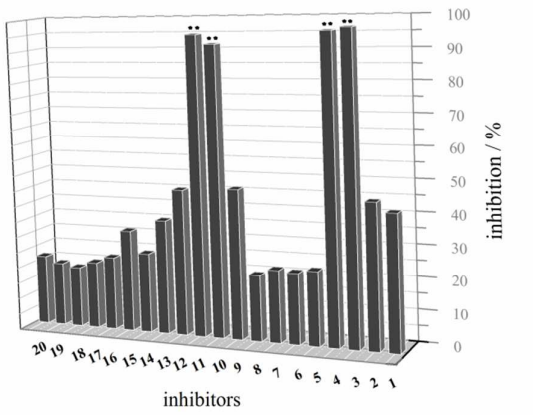


Fig 2. Inhibitory activities of **1** and synthesized compounds (2 μM) against α-glucosidase. **Significant difference compared to Ursolic acid(compound **1**)($P < 0.01$).

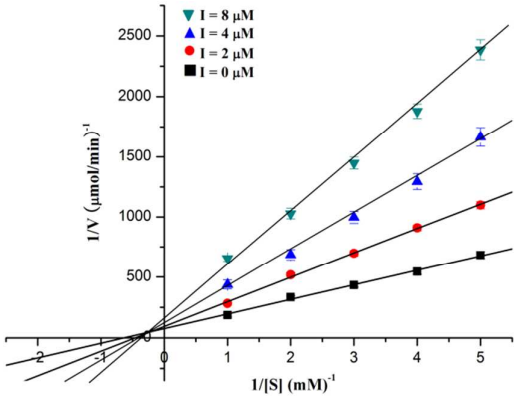


Fig 3. Lineweaver-Burk plots for inhibitory activity of **3** against α-glucosidase.

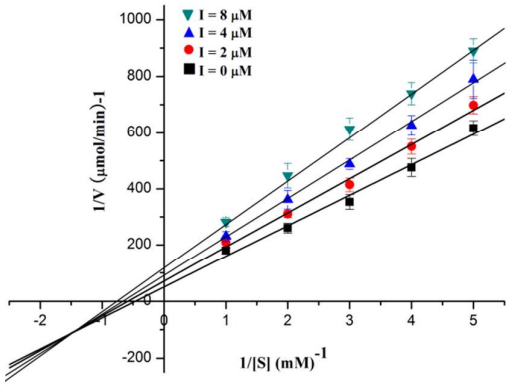


Fig 4. Lineweaver-Burk plots for inhibitory activity of **4** against α-glucosidase.

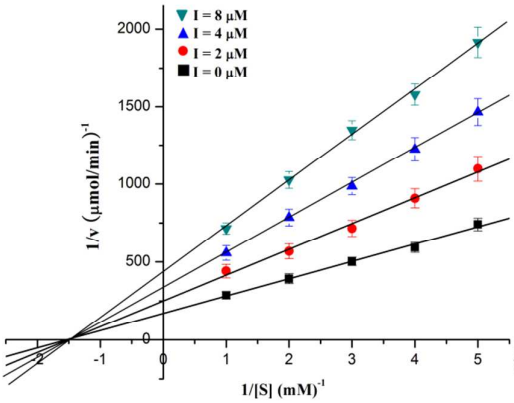


Fig 5. Lineweaver-Burk plots for inhibitory activity of **10** against α-glucosidase.

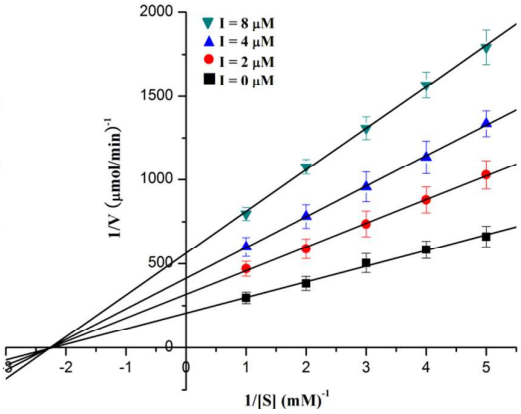


Fig 6. Lineweaver-Burk plots for inhibitory activity of **11** against α-glucosidase.

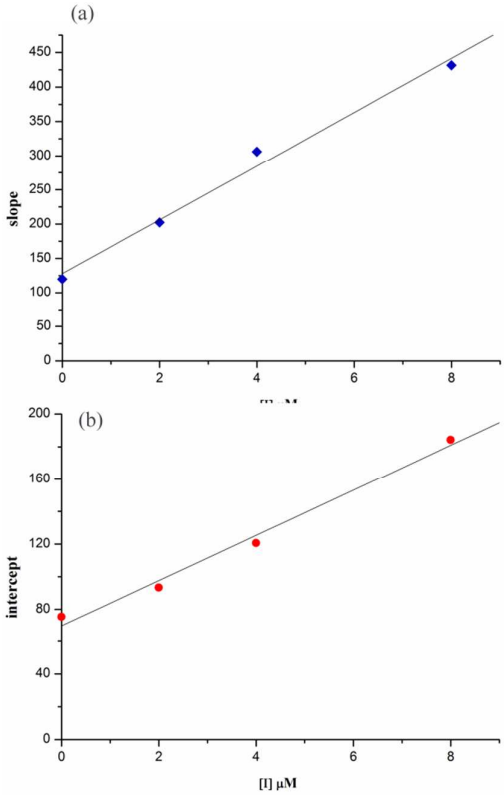


Fig 7. (a) Dixon plot of slope versus concentration of **3**, [**I**], from a Lineweaver–Burk plot for the determination of K_i. (b) secondary plot of intercept versus [**I**] from a Lineweaver–Burk plot for the determination of K'_i.

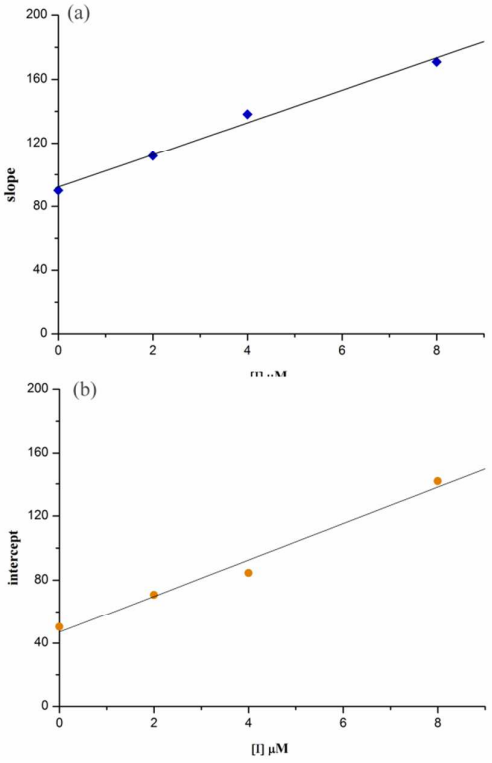


Fig 8. (a) Dixon plot of slope versus concentration of **4**, [**I**], from a Lineweaver-Burk plot for the determination of K_i. (b) secondary plot of intercept versus [**I**] from a Lineweaver–Burk plot for the determination of K'_i .

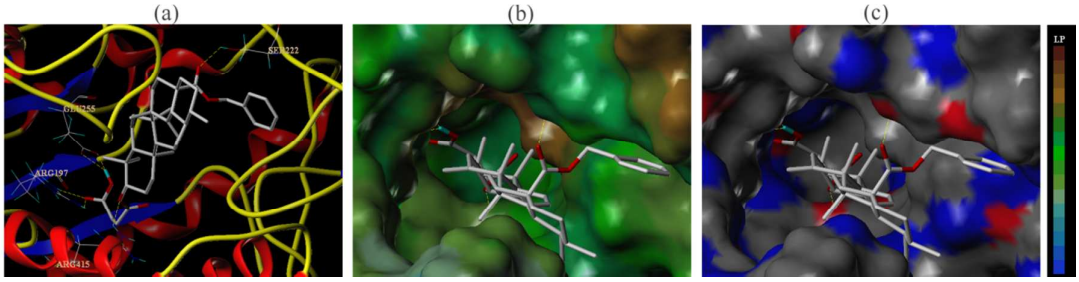


Fig 9. (a) The binding mode between compound **3** with α-glucosidase. (b) Active site MOLCAD surface representation Liphilic potential (c) Active site MOLCAD surface representation Hydrogen Bonding

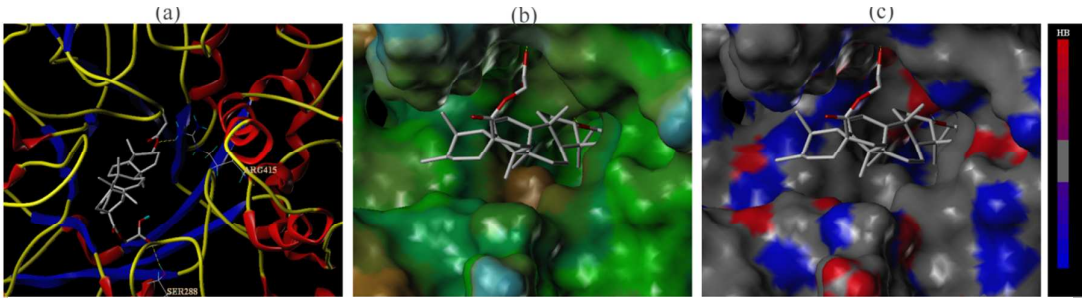


Fig 10. (a) The binding mode between compound **10** with α-glucosidase. (b) Active site MOLCAD surface representation Liphilic potential (c) Active site MOLCAD surface representation Hydrogen Bonding

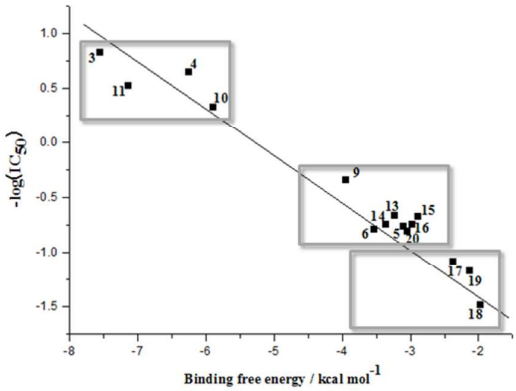


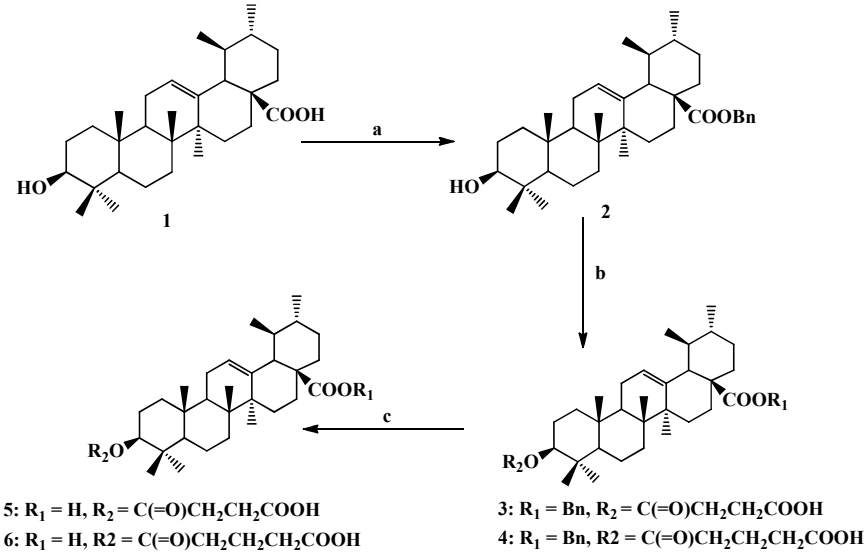
Fig 11. Correlation of binding free energies with inhibitory activities for UA-derivatives conjugates.

Table 1

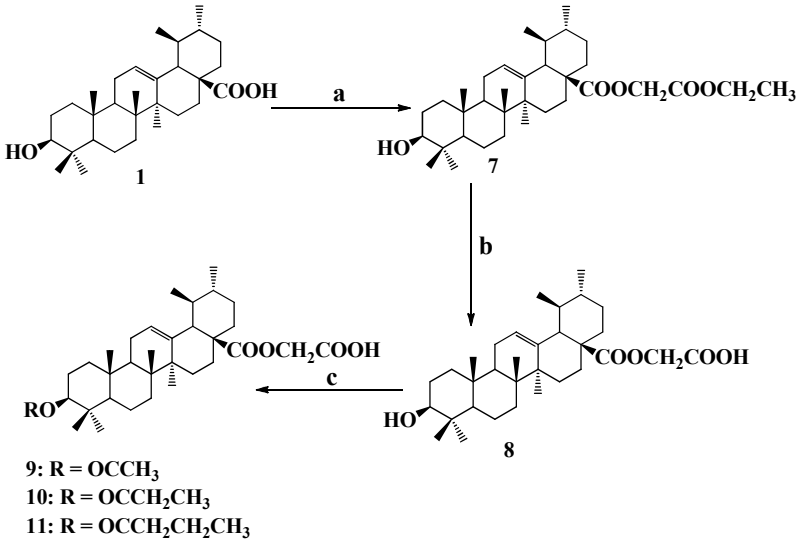
In vitro activity of target compounds against α-glucosidase from baker's yeast

compound	Polar moiety	（IC_{50}μM）[a]
1	1 × OH, 1 × COOH	5.52 ± 0.054
2	1 × OH	3.89 ± 0.076
3	1 × COOH	0.149 ± 0.00729
4	1 × COOH	0.223 ± 0.023
5	2 × COOH	5.72 ± 0.073
6	2 × COOH	6.14 ± 0.096
7	1 × OH	8.24 ± 0.113
8	1 × OH, 1 × COOH	10.62 ± 0.098
9	1 × COOH	2.17 ± 0.055
10	1 × COOH	0.466 ± 0.016
11	1 × COOH	0.298 ± 0.021
12	1 × COOH	2.78 ± 0.105
13	—	4.61 ± 0.113
14	—	5.48 ± 0.456
15	—	4.69 ± 0.689
16	—	5.51 ± 0.599
17	—	12.01 ± 1.695
18	—	30.37 ± 1.339
19	—	14.94 ± 0.958
20	—	6.369 ± 1.255
21[b]	13 × OH	572.47 ± 19.17

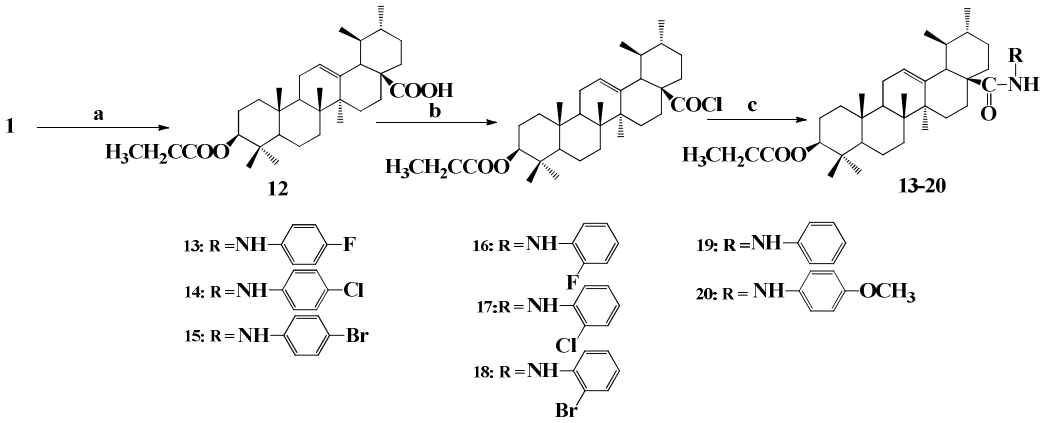
[a] The result summarized are the mean value of n=4 for IC_{50} values.
[b] Acarbose, positive control.



Scheme 1: Reagents and conditions: (a) BnCl,K2CO3,DMF,rt; (b) succinic anhydride or glutaric anhydride,DMAP,pyridine; (c) THF, Pd/C, 10%.



Scheme 2: Reagents and conditions: (a) ethyl bromoacetate, K_2CO_3,DMF, rt; (b) NaOH, THF/MeOH; (c) anhydride/Pyr/DMAP, r.t.



Scheme 3: Reagents and conditions: (a) anhydride/Pyr/DMAP, r.t. (b) $(COCl)_2$, CH_2Cl_2, r.t.; (c) CH_2Cl_2, Et_3N, RNH_2, r.t.