Accepted Manuscript

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PII: S1001-8417(18)30395-4
DOI: https://doi.org/10.1016/j.cclet.2018.10.009
Reference: CCLET 4673

To appear in: *Chinese Chemical Letters*

Received date: 1-6-2018
Revised date: 25-9-2018
Accepted date: 10-10-2018

Please cite this article as: Jiao P, Wang S, Liang S, Li M, Gao Q, Ji D, Chen Y, Li H, Ran F, Zhang Y, Zhang L, Zhou D, Xiao S, Facile preparation and characterization of novel oleanane-type triterpene functionalized β-cyclodextrin conjugates, *Chinese Chemical Letters* (2018), https://doi.org/10.1016/j.cclet.2018.10.009

Communication

Facile preparation and characterization of novel oleanane-type triterpene functionalized *β*-cyclodextrin conjugates

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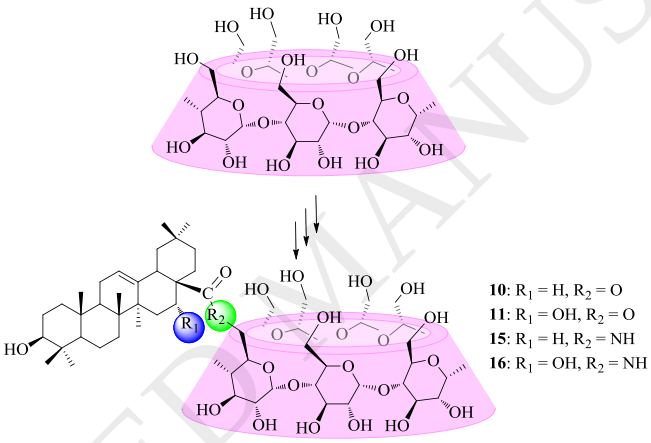
Graphical abstract



Four water-soluble *β*-CD-pentacyclic triterpene conjugates were synthesized *via* ester and amide linkages. All the conjugates showed lower hydrophobicity (Alog*P*) than their parent compounds while no significant cytotoxicity was found to HL-60, A549, Hela and Bel-7402 cells at concentrations up to 10 *μ*mol/L. Further anti-HCV entry activity and mechanism studies are under way in our laboratory.

ARTICLE INFO

Article history:
Received 1 June 2018
Received in revised form 25 September 2018
Accepted 30 September 2018
Available online

Keywords:
β-Cyclodextrin
DIBAL-H
Oleanolic acid
Echinocystic acid
Synthesis

ABSTRACT

Oleanolic acid (OA) and echinocystic acid (EA), two naturally occurring pentacyclic oleanane triterpenes, are gaining increasing attention due to their promising pharmacological activities. Conjugation with amphiphilic *α*(*β*)-cyclodextrin (CD) *via* "click chemistry" can improve their solubility and anti-HCV entry potency. In the present work, four water-soluble *β*-CD-pentacyclic triterpene conjugates were designed and synthesized, in which OA and EA was coupled to one of the primary hydroxyl groups of *β*-CD *via* ester and amide bonds. The structures of the conjugates were unambiguously determined by ^{1}H NMR, ^{13}C NMR and HRMS or MALDI-TOF-MS. All the conjugates showed lower hydrophobicity (Alog*P*) than their parent compounds and no significant cytotoxicity was found to HL-60, A549, Hela and Bel-7402 cells at concentrations up to 10 *μ*mol/L. Further anti-HCV entry activity and mechanism studies are under way in our laboratory.

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Pentacyclic triterpenes are secondary plant metabolites, widespread in fruit peels, leaves, and stem bark [1], with a few species containing up to 30% of their dry weight [2]. Oleanolic acid (OA, Fig. 1), an oleanane-type pentacyclic triterpene, has been isolated from more than 1,600 plant species, including many dietary and medicinal plants [3]. Numerous studies indicate natural OA and its saponins exhibit a wide range of pharmacological activities, such as antiviral, antitumor, anti-inflammation, and anti-microbial activities [4]. Due to its inherent bioactivities, availability and low cost, OA has been used as a starting molecule for the synthesis of oleanane triterpenes. A series of novel synthetic oleanane triterpenes have been prepared by chemical modifications of OA at three sites, including C-3 hydroxyl, $\Delta^{12\text{-}13}$ double bond and C-28 carboxylic acid [5]. Several synthetic derivative of OA, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) and its C-28 methyl ester (CDDO-Me) and C-28 imidazole (CDDO-Im) derivatives, have been tested in clinical trials [6]. Recently, we found that echinocystic acid (EA), an analog of OA with an extra hydroxyl group at C-16, displays substantial inhibitory activity on hepatitis C virus (HCV) entry with one derivative **Q8** (Fig. 1) showing EC_{50} at nanomolar level [7]. Despite their promising bioactivity, pentacyclic triterpenes are bulky, non-polar, and poorly soluble in water, which are major drawbacks for various applications, in particular, the development of drugs. For example, the solubility of OA in water is only about 0.05 μg/mL [8]. Hence, the synthesis of more water-soluble triterpene derivatives is needed. Some water-soluble derivatives have been synthesized by introducing polar sugar moieties at C-3 and/or C-28 [9].

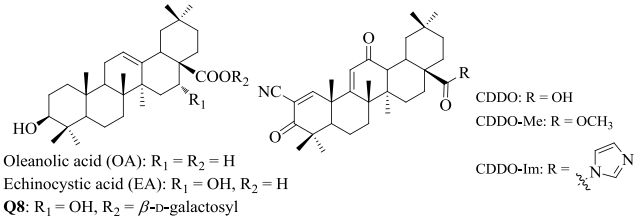
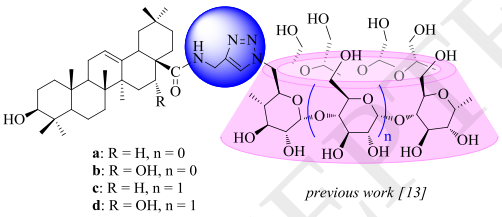


Fig. 1. The chemical structures of OA and its derivatives.

β-cyclodextrin (CD) is a macrocyclic oligomers of D-glucose with the secondary C-2 and C-3 hydroxyl groups on the secondary face and the primary C-6 hydroxyl group on the first face. This unique structure enables *β*-CD to possess high aqueous solubility. Due to the ability to form inclusion complex with a large range of hydrophobic drug molecules, the physicochemical properties of drugs are significantly modified after forming complexes with *β*-CD, such as the increased stability and water solubility, enhanced dissolution rate and bioavailability [10]. Several CD inclusion complexes with triterpenes have been studied [11]. Usually, the drugs are released quickly from the inclusion complex under physiological conditions. Alternatively, direct covalent linkage with CD has been suggested to improve the physicochemical properties of triterpines, which has been widely used for other water insoluble bioactive molecules, such as fluorouracil (5-FU) and folic acid [12].

In our previous studies, a series of water-soluble triazole-bridged *α*- and *β*-CD-pentacyclic triterpene conjugates have been synthesized *via* "click chemistry" (Fig. 2) [13]. As a continuation towards the elucidation of the relationship between the structure and anti-HCV entry activity of triterpene derivatives, we have synthesized conjugates **a-d** in which the triazolyl linker was replaced by ester or amide bond. Herein, we describe a simple method to prepare those *β*-CD derivatives and their lipophilicity (Alog*P*) along with their cytotoxicity to three human cancer cell lines.



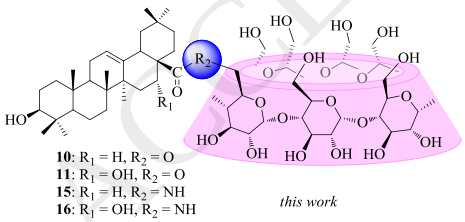
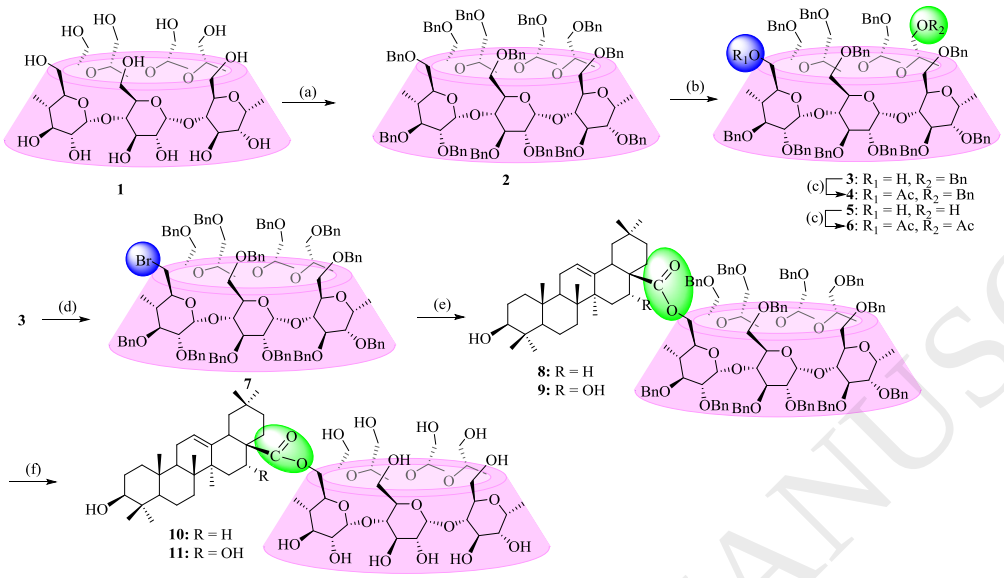


Fig. 2. Structure of novel 1:1 CD-pentacyclic triterpene conjugates. (Top: linked by triazol bond [13], bottom: linked by ester or amide bond).

As shown in Scheme 1, conjugation of *β*-CD with two oleanane-type triterpenes OA and EA *via* ester bond started from 6^{A}-hydroxy-per-*O*-benzyl-*β*-CD (**3**), a regioselective mono-debenzylated intermediate. **3** was prepared from the per-*O*-benzylated *β*-CD using diisobutylaluminium hydride (DIBAL-H) (30 equiv.) as a chemical "scalpel" as first introduced by P. Sinaÿ group [14]. In addition to the mono-debenzylated *β*-CD (**3**), the di-de-*O*-benzylated product **5** was also obtained in 20% yield after chromatography separation. The structures of **3** and **5** were further confirmed by their acetylated derivatives **4** and **6**, respectively. Initial attempts using classical methods to conjugate triterpenes OA and EA with **3** directly via ester bond yielded no desired product. Therefore, the compound **3** was converted to its bromide derivative **7** using the commercially available carbon tetrabromide (CBr_4) and triphenylphosphine (PPh_3) in

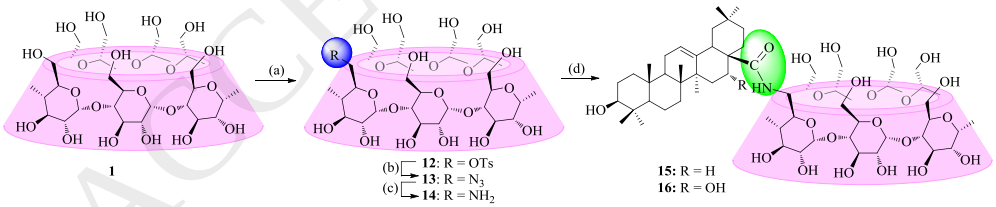
85% yield. It was characterized using ^{1}H and ^{13}C NMR, which gave the distinctive bromine methyl signals at 3.55 and 3.80 ppm (2 × H_6^A) and 34.59 ppm (C_6^A), respectively.

Conjugation of the key intermediate **7** with triterpenes EA and OA in the presence of K_2CO_3 provided compound **8** or **9** smoothly in 88%-92% yield, respectively. Fig. S1 in Supporting information presents the ^{1}H and ^{13}C NMR spectra of compound **9**. The triplet at 5.33 ppm (J = 3.0 Hz) refers to 1H, which, according to its ^{1}H-^{1}H and ^{1}H-^{13}C correlation spectra, should be assigned to H_{12}. The high-field doublets of doublet at 3.16 ppm (J = 4.4 and 11.1 Hz) and 3.05 ppm (J = 3.5 and 14.5 Hz), each referring to 1H, are assigned to H_3 and H_{18}, respectively. The ESI-HRMS mass spectrum of compound **9** recorded in the positive mode shows clearly an $[M/2+NH_4]^+$ ion at *m/z* 1712.8676 (calcd. isotopic mass for $^{12}C_{106}{}^{1}H_{122}{}^{14}N_1{}^{16}O_{19}$: 1712.8606), indicating that it was indeed connected *via* an ester bond. Similar patterns were also observed in ^{1}H NMR and ^{13}C NMR spectra of compound **8** of this series. Finally, de-*O*-benzylation of **8** or **9** was accomplished by hydrogenolysis to yield **10** or **11** in high yields (78%-91%).



Scheme 1. Synthesis of *β*-CD-oleanane-type triterpene conjugates **10** and **11** *via* ester bond. Reagents and conditions: (a) BnBr, NaH, DMF, 86%; (b) DIBAL-H (30 equiv.), toluene, 50 °C, 2 h 42.1% for compound **3** and 20.0% for compound **5**; (c) Ac_2O, pyridine, DMAP, 81%-85%; (d) CBr_4, PPh_3, CH_2Cl_2, 85%; (e) EA or OA, K_2CO_3, DMF, 62-82%; (f) Pd/C, H_2, CH_3OH, 78-91%.

A parallel experiment was carried out to conjugate pentacyclic triterpene with *β*-CD moiety through an amide bond rather than an ester bond. Such alteration might enhance the stability of pentacyclic triterpene-*β*-CD conjugates. As shown in Scheme 2, selective monotosylation of *β*-CD (**1**) according to the procedure described by Vizitiu *et al.* [15] with minor modifications afforded compound **12**, which was used without further purification for the nucleophilic substitution with NaN_3 in DMF to provide the mono-azide substituted derivative **13** in 8% yield over two steps. Reduction of the azide group of **13** with triphenylphospine followed by hydrolysis gave the key intermediate 6^A-amino-6^A-deoxy-*β*-CD **14**. Finally, conjugation of **14** with triterpenes OA and EA in the presence of *N*-ethyl-*N*'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC•HCl) afforded compounds **15** and **16** in moderate yields (43%-47%). Structural elucidation of the new derivatives was also done using 1D-NMR (^{1}H and ^{13}C), 2D-NMR and MALDI-TOF-MS techniques (Supporting information).



Scheme 2. Synthesis of *β*-CD-pentacyclic triterpene conjugates **15** and **16** *via* amide bond. Reagents and conditions: (a) H_2O, NaOH, TsCl, 9.4%; (b) NaN_3, DMF, 80 °C, 91%; (c) Ph_3P, DMF, NH_3 (aq), 63%; (d) EDC, HOBt, DMF, 43-47%.

The 1-octanol/water partition coefficient (log*P*) is a well-known measure of molecular lipophilicity. In a recent study, a negative correlation was found between the log*P* value of terpenes and their bioaccessibility ($r = -0.77$, $P < 0.001$) [16]. Therefore, decrease in the lipophilicity of pentacyclic triterpenes may increase the bioaccessibility. In this study, the calculated ALog*P* values based on Ghose and Crippen's method were determined using Pipeline Pilot software, Vers. 7.5 (Accelrys Corporation, San Diego, USA) [17]. We found that the targeting conjugates **10**/**11** and **15**/**16** showed increased hydrophilicity comparing with their parent compounds due to the introduction of *β*-CD (Table S1 in Supporting information). Compared with their analogs **b** and **d** [13a], the ALog*P* of conjugates **10** and **11** increased by 0.66 log units (triazol linker *vs.* ester linker) while the ALog*P* of conjugates **15** and **16** remained almost the same (trizolyl

linker *vs* amide linker). In addition, due to the 16-hydroxyl group of EA, the ALog*P* of conjugates **11** and **16** decreased by 1.1 log units (**10** *vs.* **11** and **15** *vs.* **16**), which means that their solubility in water are increased by ~ 12.5–fold.

Oleanane-type triterpenes are widely found in the plant kingdom as free acids or triterpene saponins linked with one or more sugar chains. Some natural pentacyclic triterpenes are shown weak cytotoxicity [18]. In this study, the cytotoxicity of the four conjugates (**10**, **11**, **15** and **16**) against human promyelocytic leukemia (HL-60), human cervical cancer (Hela), human lung cancer (A549), and human liver cancer (Bel-7402) cell lines was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at concentrations of 0.1-10 μmol/L [19]. Except conjugates **10**, **11** and **15** showed weak cytotoxicity to Hela cell at concentration of 1 μmol/L and 10 μmol/L (~ 23%-26%), no significant cytotoxicity was observed at concentrations up to 10 μmol/L for other three cancer cell (Table S2 in Supporting information). These results indicated that the synthesized *β*-CD-pentacyclic triterpene derivatives had no significant toxic effect to cells *in vitro*.

In summary, starting from natural *β*-CD, four water-soluble oleanane-type triterpene-*β*-CD conjugates **10/11** and **15/16** were synthesized *via* ester and amide linkage by a facile method. The new products were unambiguously characterized by ^{1}H NMR, ^{13}C NMR and HRMS or MALDI-TOF-MS. Compared with their parent compounds, they showed higher hydrophobicity (Alog*P*) with no significant cytotoxicity *in vitro*. This study supports that these conjugates are potential candidates for further evaluation as anti-HCV entry agents.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Nos. 81573269, 21572015, 21877007, 91753202 and 21702007) and the open funding of the State Key Laboratory of Phytochemistry and Plant Resources in West China. The authors gratefully acknowledge Dr. Hongwei Jin at State Key Laboratory of Natural and Biomimetic Drugs, Peking University for carrying out the ALog*P* determinations and Dr. Lijun Zhong at the Centre of Medical and Health Analysis, Peking University for MALDI-TOF experiments.

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