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Antidiabetic Ellagitannins from Pomegranate Flowers: Inhibition of α-Glucosidase and Lipogenic Gene Expression

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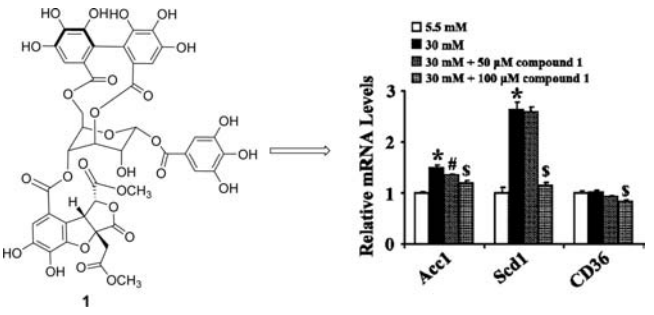
ABSTRACT



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Two new ellagitannins containing a rare 3-oxo-1,3,3a,8b-tetrahydrofuro[3,4-*b*]benzofuran moiety, namely punicatannins A (1) and B (2), were isolated from pomegranate (*Punica granatum*) flowers. Their structures with absolute configuration were determined by detailed analysis of spectroscopic data, electronic circular dichroism (ECD) calculation, and chemical hydrolysis. A plausible biogenetic route involving a key enzymatic 1,4-Michael addition is proposed. Punicatannin A showed potent inhibition of α-glucosidase and lipogenic gene expression.

The prevalence of diabetes is increasing rapidly worldwide. The International Diabetes Federation predicts that over 435 million people worldwide will be struggling with this disease by 2030 if no effective prevention and control programs are implemented. This global pandemic is driven by type 2 diabetes which contributes to the vast majority of all cases of diabetes.[1] While several synthetic drugs including sulfonylureas, biguanides, thiazolidinediones, and α-glucosidase inhibitors are available to treat type 2 diabetes, many have limited efficacy with significant associated side effects. This has led to the search for more effective compounds with less side effects, particularly natural products and dietary agents, for the management of type 2 diabetes.

Punica granatum L. (Punicaceae), commonly known as pomegranate, is commercially cultivated for its edible fruit throughout the drier parts of Southeast Asia, the Mediterranean region, and the United States. The flowers of this plant were used as a remedy for diabetes in the Unani and Ayurvedic medicinal systems.[2] Moreover, a pomegranate flower extract was reported to markedly lower blood

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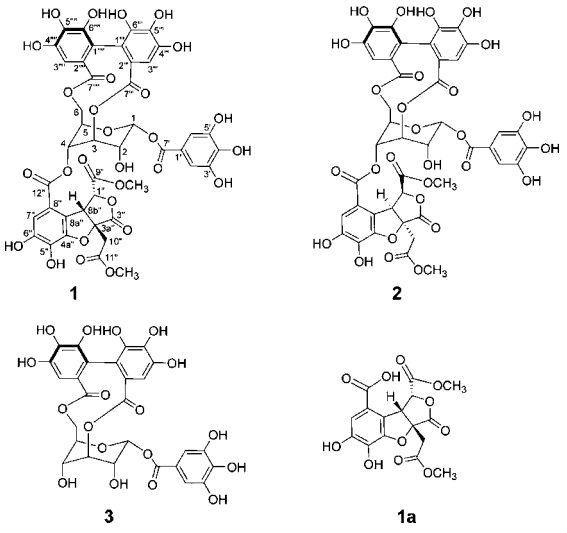
glucose levels in the Zucker diabetic fatty rat model.[3] However, there is limited knowledge on pomegranate flower constituents[4] and the bioactive antidiabetic compounds present therein remain unknown.

In continuation of our characterization of new antidiabetic compounds from natural sources,[5] this study sought to identify the bioactive compounds present in pomegranate flowers. Preliminary screening of a pomegranate flower extract showed promising α-glucosidase inhibitory activity (IC_{50} = 3.2 μg/mL). Subsequent bioassay-guided isolation afforded two unprecedented ellagitannins, named punicatannins A (**1**) and B (**2**), both containing a rare 3-oxo-1,3,3a,8b-tetrahydrofuro[3,4-*b*]benzofuran moiety attached to a ($^1C_4$)-glucopyranose core, along with a biosynthetically related compound, isocorilagin (**3**).[6] Herein we present the details of the isolation and structure elucidation of **1** and **2**, the evaluation of their antidiabetic activities, and a plausible biosynthetic pathway for these two compounds.



Compound **1** was isolated as a tan amorphous powder. The standard color reaction with ferric chloride (dark blue) and sodium nitrite−acetic acid (reddish brown) suggested that compound **1** was most likely an ellagitannin. Its molecular formula was established as $C_{43}H_{34}O_{28}$ with an index of hydrogen deficiency of 27 based on the HR-ESI-MS exhibiting an $[M-H]^-$ ion at *m*/*z* 997.1151 (calcd for $C_{43}H_{33}O_{28}$, 997.1158). The ^{1}H and ^{13}C NMR spectra [Supporting Information (SI) Table S2] of **1** showed signals due to a highly acylated hexopyranosyl moiety along with aromatic and carboxylic carbon resonances, strongly indicative of compound **1** being a hydrolyzable tannin. The presence of galloyl and hexahydroxydiphenoyl (HHDP) groups was readily determined by the ^{1}H NMR signals at δ_H = 7.08 (2H, s), δ_H = 6.72 (s), 6.83 (s) and characteristic ^{13}C NMR signals in the aromatic region. The small coupling constants of the protons of the glucopyranosyl unit indicated its $^1C_4$ conformation with the anomeric acyl group in an α-equatorial orientation consistent with isocorilagin (**3**). Analysis of HMBC data (Figure 1) permitted assignment of the galloyl and HHDP groups to C-1 and C-3, 6, respectively, by the correlations from H-1 to C-7′, from H-3 to C-7‴, and from H_2-6 to C-7⁗. The latter correlations further supported the $^1C_4$ conformation of the glucopyranosyl moiety carrying an HHDP unit at its C-3 and C-6 oxygen functions.[7]

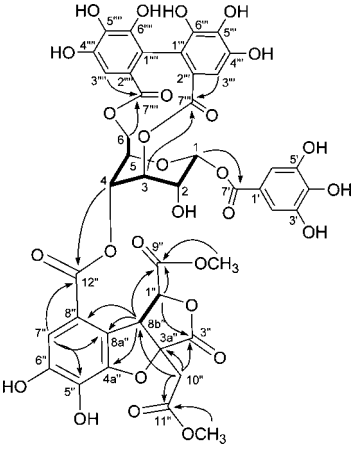


Figure 1. ^{1}H−^{1}H COSY (bold line) and key HMBC correlations (H→C) of **1**.

The remaining part of the molecule was established by analysis of 1D and 2D NMR data. Apart from the above functionalities, carbon resonances for six aromatic carbons (δ_C 112.7, 118.6, 119.1, 136.4, 147.8, 148.6), four ester carbonyls (δ_C 165.4, 169.9, 171.1, 175.0), two methines (δ_C 51.5, 80.9), an oxgenated quaternary (δ_C 89.6), one methylene (δ_C 39.3), and two *O*-methyls (δ_C 52.8, 53.0) were observed in the ^{13}C NMR spectrum. The chemical shifts of the aromatic carbon signals implied the presence of a pyrogallol ring. The HSQC spectrum permitted assignment of the protons to their bonding carbons, and the ^{1}H−^{1}H COSY and HMBC spectra (Figure 1) were applied to construct the structure of the remaining part of the molecule. The ^{1}H−^{1}H COSY correlation between H-8b″ and H-1″ revealed the linkage of C-8b″ and C-1″. In the HMBC spectrum, the mutual ^{1}H−^{13}C correlation between an aromatic singlet (δ_H = 7.12, H-7″) and the C-12″ ester carbonyl carbon (δ_C 165.4), C-5″, and C-8a″, indicated that the C-12″ ester carbonyl was linked at C-8″ of a pyrogallol-type ring. The HMBC correlations from H-8b″ to C-4a″, C-8″, and C-8a″, suggested that the CH-8b″ methine group was attached at C-8a″ of the pyrogallol-type ring. The HMBC correlations from H_2-10″ to C-3″ (δ_C 175.0), C-3a″ (δ_C 89.6), and C-8b″ (δ_C 51.5) indicated the contiguous linkage of C-3″, C-3a″, and C-8b″ and also

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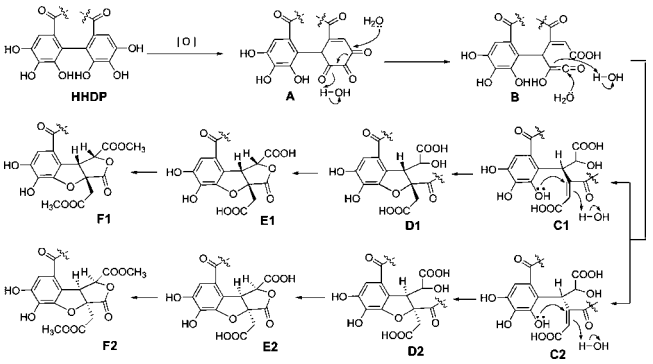
allowed the attachment of CH_2-10″ to C-3a″. A methoxycarbonyl group was attached to both C-10″ and C-1″ based on the HMBC correlations from H_2-10″ and the *O*-methyl proton signals (δ_H = 3.79) to C-11″ and from H-8b″, H-1″, and the *O*-methyl proton signals (δ_H = 3.31) to C-9″. The linkage of C-1″ and C-3″ via oxygen was determined by the HMBC correlation between H-1″ and C-3″. The index of hydrogen deficiency of 26 in compound **1** was accounted for by the isocorilagin moiety (17), four carbonyl groups (4), a pyrogallol ring (4), and a furan ring (1). The remaining index of hydrogen deficiency required the presence of an additional ring. Analysis of the ^{13}C NMR data suggested the presence of an ether bridge between oxygenated quaternary carbon C-3a″ and aromatic carbon C-4a″ to furnish a ring. Thus, the molecular structure of the remaining part was determined as the rare 5,6-dihydroxy-3a-(2-methoxy-2-oxoethyl)-1-(methoxycarbonyl)-3-oxo-1,3,3a,8b-tetrahydrofuro[3,4-*b*]benzofuran-8-carboxylic acid moiety.[8] Finally, the HMBC correlation between H-4 and C-12″ facilitated the placement of this structural unit at C-4 of the glucopyranosyl core via an ester bridge.

The relative configuration of the 5,6-dihydroxy-3a-(2-methoxy-2-oxoethyl)-1-(methoxycarbonyl)-3-oxo-1,3,3a,8b-tetrahydrofuro[3,4-*b*]benzofuran-8-carboxylic acid moiety was determined by the ROESY spectrum and the H-1″−H-8b″ coupling constant (9.6 Hz) reminiscent of the *cis*-configuration of these protons.[9] The ROESY correlation between H-8b″ and H-10″ indicated that H-8b″ and the C-3a″ 2-methoxy-2-oxoethyl group were cofacial.

A combination of electronic circular dichroism (ECD) analyses, chemical hydrolysis, and calculated ECD spectra were used to elucidate the absolute configuration of compound **1**. First, the HHDP moiety was assigned an *R*-configuration by the negative Cotton effect at 234 nm. Notably, a Cotton effect at 235 nm is a diagnostic criterion in determining the absolute configuration of HHDP units in ellagitannins, which is unaffected by the presence or absence of galloyl groups and the conformation of the glucose core.[10] Acid hydrolysis of **1** afforded D-glucose, which was identified by direct comparison with an authentic sample (SI S1). However, the challenging issue to determine the absolute configuration of the acyl group linked at C-4 in compound **1** still remained. Hydrolysis of **1** in hot water yields **1a**, which was confirmed by the ^1H NMR and mass spectra (SI S20 and S21). As the absolute configuration of **1a** could not be resolved directly by the analysis of its ECD curve, comparison between experimental and calculated ECD spectra using the time-dependent DFT method was successfully applied to resolve this issue.[11] The mandatory assigned (1″*R*, 3a″*R*, 8b″*R*)-configuration was selected for the conformational search of **1a** by using the OPLS2005 force field in the Schrodinger 9.2 MacroModel software package. After the detailed calculated ECD analysis (SI S4.1), the (1″*R*, 3a″*R*, 8b″*R*) absolute configuration of partial structure **1a** was unequivocally confirmed.

Compound **2** was isolated from the same fraction as **1** and had the same molecular formula ($C_{43}H_{34}O_{28}$) and similar UV, IR, ^1H, and ^{13}C NMR spectra as compound **1**. A combined analysis of ^1H, ^{13}C NMR (SI Table S2), $^1H-^1H$ COSY, HSQC, and HMBC data of **2** indicated that it possessed the same molecular structure as **1**. The glucopyranosyl core also had the same conformation and D-configuration. The experimental ECD spectrum exhibited a negative Cotton effect at 233 nm indicative of an HHDP moiety with an (*R*)-configured biphenyl bond. The anomeric galloyl group was assigned an α-equatorial orientation based on the ca. 1.0 Hz coupling constant of the anomeric proton. The 5,6-dihydroxy-3a-(2-methoxy-2-oxoethyl)-1-(methoxycarbonyl)-3-oxo-1,3,3a,8b-tetrahydrofuro[3,4-*b*]benzofuran-8-carboxylic acid moiety also showed the same relative configuration as **1**. Thus, **1** and **2** only differ as far as the absolute configuration of this rare structural moiety is concerned. Compound **2** is, thus, a (C-1″, C-3a″, C-8b″) quasi-enantiomer of compound **1** and accordingly named punicatannin B.

Scheme 1. Postulated Biosynthesis of the 4-Acyl Group of Punicatannins A (**1**) and B (**2**)



A possible biosynthetic pathway to the 5,6-dihydroxy-3a-(2-methoxy-2-oxoethyl)-1-(methoxycarbonyl)-3-oxo-1,3,3a,8b-tetrahydrofuro[3,4-*b*]benzofuran-8-carboxylic acid moiety of compounds **1** and **2** is postulated in Scheme 1. Its biosynthetic precursor is proposed to be an HHDP group, similar to that in, e.g., compound **1**, which is converted into **A** by oxidation and keto−enol tautomerism. Intermediate **A** would be cleaved into ketene **B** by hydrolysis.[12] This may be hydrolytically transformed into the enantiomeric intermediates **C1** and **C2** that may undergo a 1,4-Michael addition reaction to yield intermediates **D1** and **D2**. These may be converted into **E1** and **E2** by

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Table 1. α-Glucosidase Inhibitory Activities of Compounds **1**–**3**

no.	IC_{50} (μM)[a]
1	7.15 ± 0.27
2	12.39 ± 0.05
3	104.89 ± 7.73
acarbose[b]	208.60 ± 3.39

[a] IC_{50} values are shown as mean ± SD from three independent experiments. [b] Positive control.

trans-esterification. Methyl esterification of **E1** and **E2** would lead to the structural moieties **F1** and **F2** of compounds **1** and **2**.

Given the ethnomedicinal uses of pomegranate flowers for treating diabetes, we evaluated the isolated compounds for biological activities. First, compounds **1**–**3** and the clinical drug, acarbose, were tested for α-glucosidase inhibitory activities. This enzyme is a carbohydrate hydrolase that breaks down starch and α-linked di-, oligo-, and polysaccharides to glucose leading to elevated blood glucose levels. Thus, inhibition of the enzyme decreases the rate of digestion of carbohydrates and less glucose is absorbed. As shown in Table 1, compounds **1**–**3** showed potent and concentration-dependent α-glucosidase inhibitory activities (SI Figure S3). Remarkably, punicatannin A (**1**) was ca. 30 times more potent than acarbose (IC_{50} = 7.15 vs 208.60 μM, respectively).

Finally, based on compound availability, we selected compound **1** to evaluate its ability to inhibit lipogenic gene expression. It is known that obesity associated with insulin resistance and type 2 diabetes increases the risk of hepatic lipid accumulation. Lipid accumulation is also associated with increased *de novo* lipogenesis, and high glucose concentrations have been shown to induce lipid accumulation in HepG2 cells.[13] This is known to occur through the transcription factor, Srebp1c, which along with the lipogenic enzymes Acc-1, Fas, and Scd1 regulate lipogenesis. Furthermore, loss of Scd1 protects against obesity induced by decreased *de novo* lipogenesis,[14] and forced CD36 expression increases fatty acid uptake in HepG2 cells.[15] Here we showed that compound **1** prevents Acc1, Scd1, and CD36 induction in HepG2 cells exposed to high glucose (Figure 2). This suggests that compound **1** may repress the induction of lipogenic gene expression by high glucose exposure and could aid in the prevention of high glucose induced *de novo* lipogenesis and subsequently inhibit lipid accumulation.

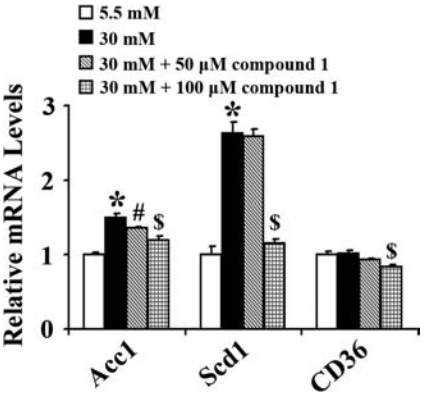

Figure 2. Compound **1** prevents elevation of Acc1, Scd1, and CD36 gene expression in HepG2 cells exposed to high glucose. (*) $P < 0.05$, group of high glucose compared to group of low glucose treatments in absence of compound **1**. (#) $P < 0.05$, group of high glucose compared to group of low glucose treatments in presence of compound **1** (50 μM). ($) $P < 0.05$, group of high glucose compared to group of low glucose treatments in presence of compound **1** (100 μM).

In summary, this work describes the discovery of two new ellagitannins containing the rare 3-oxo-1,3,3a,8b-tetrahydrofuro[3,4-*b*]benzofuran moiety. The abilities of these compounds to inhibit α-glucosidase enzyme activity and lipogenic gene induction could account for the reported antidiabetic properties of pomegranate flowers. Furthermore, the structure identified in these natural compounds could serve as a scaffold for the synthesis of structural analogs with enhanced antidiabetic activities.

Supporting Information Available. General experimental procedures, plant materials, extraction and isolation of compounds, hydrolysis of compound **1** and sugar analysis, α-glucosidase and lipogenic gene expression inhibition assays, calculated ECD analysis, tabulated NMR data and full NMR, MS spectra for compounds **1** and **2**, and ^{1}H NMR and MS spectra for compound **1a**. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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