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Original article

Potent vasorelaxant analogs from chemical modification and biotransformation of isosteviol

Orawan Wonganan [a], Chainarong Tocharus [b], Chonticha Puedsing [b], Sureeporn Homvisasevongsa [c], Oratai Sukcharoen [d], Apichart Suksamrarn [a,*]

[a] Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok 10240, Thailand
[b] Department of Anatomy, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand
[c] Division of Physical Science, Faculty of Science and Technology, Huachiew Chalermprakiet University, Samutprakarn 10540, Thailand
[d] Department of Biotechnology, Faculty of Science, Ramkhamhaeng University, Bangkok 10240, Thailand

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ABSTRACT

Isosteviol (**1**) has been reported to exhibit moderate vasorelaxant activity. In order to enhance the bioactivity of this compound, chemical modification of **1** to the dihydro analog, *ent*-16β-hydroxybeyeran-19-oic acid (**2**), was undertaken. Compound **2** was then converted to the corresponding acetate derivative, *ent*-16β-acetoxybeyeran-19-oic acid (**3**). Biotransformation of compounds **1**–**3** by the fungus *Cunninghamella echinulata* NRRL 1386 was investigated and the metabolites **4**–**9** were obtained. The substrates and their metabolites were subjected to in vitro rat aorta relaxant activity evaluation. The metabolite **4**, *ent*-7α-hydroxy-16-ketobeyeran-19-oic acid, exhibited the most highly potent activity, with EC_{50} of 3.46 nM, whereas the parent compound **1** showed relatively low activity (EC_{50} 57.41 nM). A 17-fold increase in vasorelaxant activity of the analog **4** relative to compound **1** is of particular significant. Compound **4** exerted vasorelaxant activity at particularly low concentration and the vasorelaxant profile reached maximum at relatively low concentration, especially when compared with acetylcholine, the positive control.

1. Introduction

Hypertension is one of the most common cardiovascular diseases that can cause coronary disease, myocardial infarction, stroke and sudden death and is the major contributor to cardiac failure and renal insufficiency. There are several classes of anti-hypertensive drugs, diuretics, β-blockers, calcium channel blockers, angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers and vasodilators [1]. These different classes of drugs have both advantages and disadvantages and some of them have adverse side effects [2,3]. It would therefore be beneficial for patients with hypertension if novel antihypertensive agents with lesser side effects are available.

Isosteviol (**1**, Scheme 1), a tetracyclic diterpenoid from the beyerane series, was obtained from acid hydrolysis of stevioside [4], a diterpenoid glycoside from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni. This non-caloric sweetening agent is 300 times sweeter than sucrose [5]. Several studies suggested that the non-sweetening agent **1** possesses a variety of biological activities including reducing blood pressure and cardioprotective effect [6–9], anti-hyperglycemic [10] and potential anti-tumor effects [11]. Previous reports of **1** and stevioside [12–16] on cardiovascular and related effects led us to investigate antihypertensive action of this type of compounds. This class of compounds is of special interest, since stevioside could be obtained from *S. rebaudiana* in large quantity [5,17] and compound **1** in turn was obtained in good yield from this glycoside by acid hydrolysis [4,9]. Moreover, the non-toxic or less toxic nature of this class of diterpenoids has prompted us to investigate antihypertensive activity of compound **1**. It has been reported that this non-sweetening compound exhibited moderate vasorelaxant activity [6], which might not be sufficiently potent for further drug development study. It was therefore of interest to see whether structural modification of **1** would give rise to analog(s) with considerably high vasorelaxant activity. The present work deals with chemical modification and microbial transformation of isosteviol (**1**) to analogs **2**–**9** (Scheme 1), some of which exhibited high vasorelaxant activity.

* Corresponding author. Tel.: +662 3190931; fax: +662 3191900.
E-mail addresses: s_apichart@ru.ac.th, asuksamrarn@yahoo.com (A. Suksamrarn).

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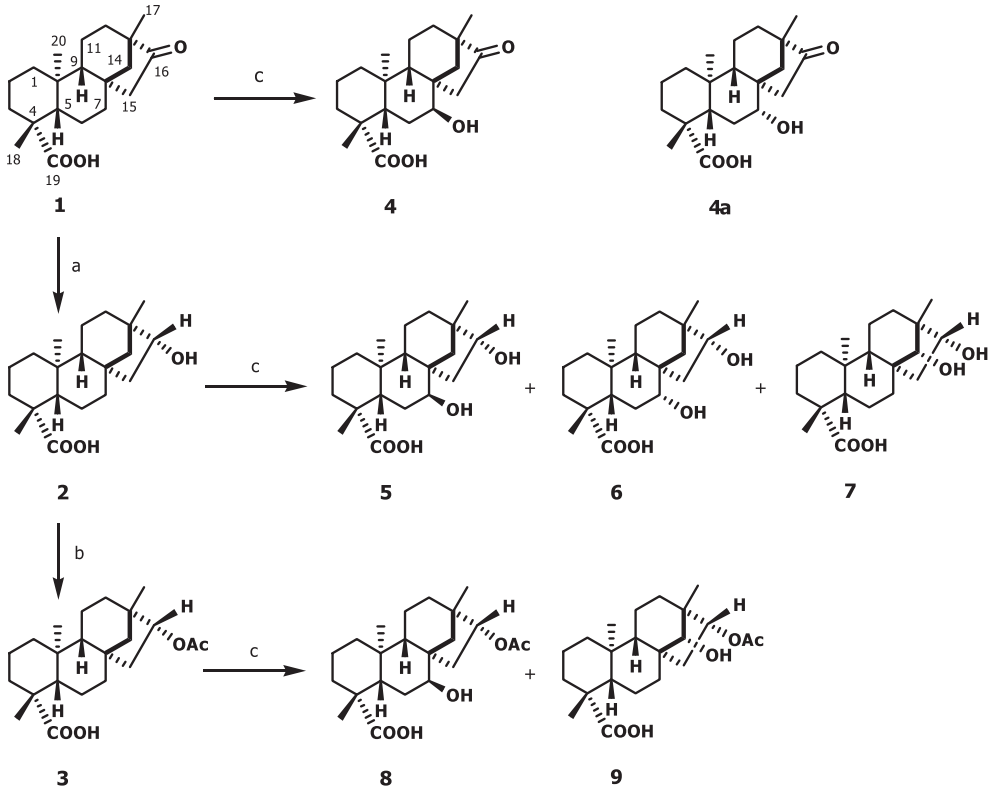

Scheme 1. Chemical modification of *ent*-16-ketobeyeran-19-oic acid (isosteviol, **1**) to the dihydro analog **2** and the corresponding acetate **3**, and microbial transformation of compound **1** to the hydroxylated analog **4**, compound **2** to the hydroxylated analogs **5**, **6** and **7**, and compound **3** to the hydroxylated analogs **8** and **9**. a: $NaBH_4$/THF; b: Ac_2O/pyridine; *c*: *Cunninghamella echinulata* NRRL 1386.

2. Chemistry

2.1. Chemical modification

Isosteviol (**1**) was chosen as the parent compound for structural modification to its analogs for vasorelaxant evaluation. We aimed to modify the keto function at the 16-position to the corresponding dihydro analogs. Acid hydrolysis of stevioside yielded **1** in 81%. Reduction of **1** with $NaBH_4$ gave the dihydro analog **2** in 97%. The reduction occurred exclusively from the β-face of the molecule. The presence of carbinol proton at δ 4.10 (br d, $J = 6.7$ Hz) confirmed the conversion of the keto group of **1** into the hydroxyl group in **2**. The spectroscopic (^{1}H NMR and mass spectra) data of **2** were consistent with the reported values [18,19]. Acetylation of compound **2** with acetic anhydride and pyridine gave the corresponding acetate **3** in 76%. The ^{1}H NMR spectrum of **3** showed a three-proton singlet signal at δ 2.02, thus indicating that acetylation has taken placed. A downfield shift of the carbinolic proton at C-16 also confirmed that acetylation has taken placed at the C-16 hydroxyl group.

2.2. Microbial transformation

The limited number of functional groups in isosteviol (**1**) has prevented us from further chemical modification. Microbial transformation is a powerful method for the regioselective and stereoselective introduction of hydroxyl group at un-activated position of beyerane diterpenoids including isosteviol [9,18–24]. Microbial transformation of isosteviol (**1**) with *Cunninghamella echinulata* NRRL 1386 produced the more polar metabolite **4** (see Scheme 1), which showed the $[2M-H]^-$ ion at *m/z* 667 consistent with the molecular formula $C_{20}H_{30}O_4$. Analysis of 1D and 2D NMR spectra revealed the presence of a hydroxyl group, which was located at C-7 by HMBC analysis. The ^{1}H and ^{13}C NMR spectra of **4** were consistent with the reported values of *ent*-7α-hydroxy-16-ketobeyeran-19-oic acid [22–24].

Incubation of **2** with the same fungus yielded three metabolites, **5**–**7**. Compound **5** was identified as *ent*-7α,16β-dihydroxybeyeran-19-oic acid by comparison of the NMR and MS data with literature [18,19]. The conversions of **1** and **2** by *C. echinulata* NRRL 1386 to the corresponding 7β-hydroxylated analogs **4** and **5**, respectively, have revealed the regio and stereoselectivity of the enzyme of this fungus.

Compound **6** displayed the $[M-H]^-$ ion at *m/z* 335.2208 in the HR-TOFMS (ESI$^-$), compatible with a molecular formula $C_{20}H_{32}O_4$. The IR spectrum suggested the presence of hydroxyl groups at 3624 and 3414 cm^{-1} and carboxyl group at 1701 cm^{-1}. The ^{13}C NMR and DEPT spectra revealed the presence of eight CH_2 and seven CH/CH_3 indicating the presence of a proton geminal to a new hydroxyl group at δ 75.7 in the ^{13}C NMR spectrum. The ^{1}H NMR spectrum of **6** revealed a new resonance at δ 3.70 and the HMBC spectra showed correlations with C-5 (δ 54.6), C-6 (δ 32.6), C-8 (δ 49.0), C-14 (δ 51.0) and C-15 (δ 34.3), thus confirming that hydroxylation has taken placed at C-7. In the ^{1}H NMR spectrum, a double doublet ($J = 11.5$ and 3.6 Hz) of H-7 was observed. The large coupling constant of H-7 indicated that it was in the axial position. The large *J* value resulted from axial–axial coupling between H-6ax and H-7. The hydroxyl group therefore adopted the equatorial orientation. This was in agreement with the reported product **4a** obtained from microbial transformation of **1** [21,24]. Furthermore, the orientation of the OH group at C-7 was also confirmed by the ROESY experiments. Thus, H-7 showed cross-peak with H-5 (δ 1.32), H-9 (δ 1.29) and H-14 (δ 1.08 and δ 2.46). On the basis of the spectroscopic data above led to the identification of **6** as *ent*-7β,16β-dihydroxybeyeran-19-oic acid.

The molecular formula of compound **7** was determined as $C_{20}H_{32}O_4$ from its HR-TOFMS (ESI$^+$) at *m/z* 359.2180 $[M+Na]^+$. The