α -Glucosidase inhibitory caged xanthenes from the resin of *Garcinia hanburyi*

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Abstract A yellow resin (gamboge) from *Garcinia hanburyi* has been widely used as folk medicine due to its antibacterial and antitumor activities. We isolated four α -glucosidase inhibitory compounds from the methanol extract of gamboge. The compounds (**1–4**) were identified as gambogic acid (**1**), moreollic acid (**2**), gambogic acid (**3**), and 10-methoxygambogenic acid (**4**), respectively through spectroscopic data including 2D-NMR and HREIMS. All compounds were examined in the enzyme inhibition assay against α -glucosidase to identify their inhibitory potencies and kinetic behavior. All compounds (**1–4**) showed enzyme inhibition against α -glucosidase, but the activity was significantly affected by the methoxy group on C-10 of ring A and pentenyl pyran moiety of ring D. For example, compound **1** (IC_{50} = 41.4 μ M) bearing pyran ring eight times effective than **4** (IC_{50} = 350.6 μ M) having geranyl group itself. Most active compound was found out to be gambogic acid (**1**) which was analyzed most abundant metabolite in gamboge by LC-ESI-MS/MS. In kinetic study, compounds **1** and **2** were proved as noncompetitive inhibitors.

Keywords Caged xanthenes · Gambogic acid · *Garcinia hanburyi* · α -Glucosidase · Noncompetitive inhibition

Introduction

α -Glucosidase (EC 3. 2. 1. 20) is a key enzyme that hydrolyzes starch and disaccharide to produce glucose. α -Glucosidase inhibitors have come to the target into the treatment of numerous diseases including type II diabetes, obesity, cancer, and HIV [1]. This enzyme inhibitors help to retard the cleavage of sugar and thereby control postprandial glucose level in diabetics and obesity [2]. On the other hand, the trimming of N-linked oligosaccharide within in $Glc_3Man_9GlcNAc_2$ glycoprotein is controlled by α -glucosidase I and II. The α -glucosidase in the sera involve in proliferation of neoplastic cells, which have structural changes of cell surface glycoconjugates. Thus, α -glucosidase inhibitor may lead to retard cancer growth. Finally, cellular signaling and recognition are principally orchestrated by glycoprotein [3]. α -Glucosidase inhibitors contribute to reducing the rate of viral proliferation in HIV infection [4,5]. The evaluation of α -glucosidase inhibitors with a broad biological spectrum from the natural sources may contribute to nutraceutical and medicinal field.

Garcinia hanburyi is small to a medium-sized tree and distributed in the Southern part of Thailand [6]. These species secrete a yellow gum resin called as gamboge by incision of stem or by breaking off the shoots. Gamboge has been widely used as a dye and folk medicine for potent purgative and infected wound [7]. Caged-polyprenylated xanthenes are leading metabolites and the source of the yellow coloration to gamboge. These caged xanthenes are also responsible for antibacterial, antitumor, antiviral and anti-inflammatory activities [8–10]. Most important biological studies have been focused on antitumor and anti-viral activity, which might have a deep correlation with α -glucosidase inhibition.

In this study, we isolated caged xanthenes and their structures were fully identified by spectroscopic data. The isolated xanthenes were examined α -glucosidase inhibition, of which inhibitory mechanism was estimated by double reciprocal equations of Lineweaver-Burk plots and Dixon plots. The natural abundance of the isolated compounds was analyzed by LC-ESI-MS/MS.

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Materials and Methods

Plant material and chemicals

The gamboge of *G. hanburyi* was provided from Haozhou Hongda Herbal Medicine Technology Ltd., China. Organic solvents for isolation processes were of first grade. The elution solvents for HPLC and MPLC were purchased as of analytical grade from Fisher (Fisher Scientific, Hampton, NH, USA). α -Glucosidase (EC 3. 2. 1. 20) originated from *Saccharomyces cerevisiae* source and was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The materials of column chromatography were silica gel (230–400 mesh, Merck, Kenilworth, NJ, USA) and C-18S (12 nm, S-20 μ m, YMC, Kyoto, Japan). The recycle HPLC and MPLC were carried out by using Triart C18 (S-5 μ m, 12 nm, and S-10 μ m, 12 nm, YMC).

Instruments

The Bruker AM 500 spectrometer (Bruker, Karlsruhe, Germany) was used for ^1H -NMR, ^{13}C NMR, and 2D NMR data. Mass spectrometry data were obtained by a JEOL JMS-700 (JEOL Ltd., Akishima, Japan). MPLC and recycle HPLC were conducted on Forte/R 100 (YMC Co., Ltd.) and LC-9130G NEXT (Jai Co., Ltd., Tokyo, Japan), respectively. The HPLC analysis was carried out by using the Agilent (Santa Clara, CA, USA) 1100 series system equipped with a diode array detector. Enzyme assay was screened by Spectra Max M3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Isolation and purification of compounds

The powder gamboge (30 g) of *G. hanburyi* was extracted using methanol (5 L) at room temperature to give dark extract (17.8 g). The methanol extract (10 g) was subjected to column chromatography on silica gel (10 $\times$ 40 cm, 230–400 mesh, 500 g) using hexane to ethyl acetate gradient (20:1 $\rightarrow$ 1:2) to give 10 fractions (A1–A10, each 200 mL). Fractions A5–6 (3.1 g) were fractionated via MPLC (250 $\times$ 20 mm, 12 nm, S-20 μ m, YMC) eluting with a gradual increase in MeOH (0–100%) in H₂O to afford 60 subfractions (B1–B60). The above MPLC process was repeated 10 times with 0.3 g each time. The subfractions B20–37 (1.2 g) enriched with compounds **1–4** were further chromatographed over recycle HPLC (250 $\times$ 20 mm, S-5 μ m, 12 nm, YMC). This recycle HPLC was repeated to afford compounds **1** (21 mg), **2** (14 mg), **3** (16 mg), and **4** (9 mg), respectively. All isolated compounds were identified on the basis of spectroscopic data and comparison with previous studies (Supplementary materials) [9,11,12].

Gambogic acid (**1**): yellow gum; HREIMS[M]⁺ 660.3295 (calcd for C₃₉H₄₈O₉ 630.3298), [α]_D = −108.0 (*c* = 0.1, CHCl₃); ^{13}C -NMR (CDCl₃) δ 208.7 (C12), 193.7 (C8), 173.0 (C30), 161.2 (C18), 156.3 (C16), 155.7 (C6), 140.4 (C27), 131.9 (C38), 131.2 (C33), 126.7 (C3), 125.0 (C37), 123.9 (C28), 122.6 (C32), 115.9 (C4), 108.8 (C17), 102.8 (C5), 101.8 (C7), 88.4 (C14), 86.2 (C13), 82.1 (C23), 80.9 (C2), 74.0 (C10), 55.8 (OCH₃), 47.9 (C9),

43.9 (C22), 43.5 (C11), 41.8 (C20), 29.7 (C25), 28.0 (C19), 27.7 (C26), 27.2 (C24), 25.7 (C39), 25.6 (C35), 22.9 (C36), 21.5 (C31), 20.5 (C29), 20.0 (C21), 18.1 (C34), 17.6 (C40).

Moreollic acid (**2**): yellow gum; HREIMS[M]⁺ 592.2672 (calcd for C₃₄H₄₀O₉ 592.2672), [α]_D = −98.8 (*c* = 0.1, CHCl₃); ^{13}C -NMR (CDCl₃) δ 208.6 (C30), 193.8 (C18), 171.5 (C16), 160.9 (C6), 156.4 (C27), 155.7 (C33), 138.3 (C28), 131.2 (C3), 127.3 (C32), 126.3 (C4), 122.5 (C17), 115.3 (C5), 109.1 (C7), 103.0 (C14), 101.9 (C13), 88.4 (C23), 86.3 (C2), 82.2 (C10), 78.4 (C2), 74.0 (C10), 55.8 (OCH₃), 47.9 (C9), 43.9 (C22), 43.5 (C11), 29.7 (C25), 28.6 (C24), 28.2 (C26), 28.0 (C19), 27.2 (C21), 25.6 (C35), 21.5 (C30), 20.6 (C31), 20.0 (C29), 18.1 (C34).

Gambogic acid (**3**): yellow gum; HREIMS[M]⁺ 628.3036 (calcd for C₃₈H₄₄O₈ 628.3036), [α]_D = −606.3 (*c* = 0.1, CHCl₃); ^{13}C -NMR (CDCl₃) δ 203.4 (C12), 178.9 (C8), 171.3 (C30), 161.5 (C18), 157.5 (C6), 157.4 (C16), 138.3 (C27), 135.3 (C10), 133.4 (C9), 131.8 (C38), 131.5 (C33), 127.6 (C28), 124.4 (C3), 123.9 (C37), 122.3 (C32), 115.9 (C4), 107.6 (C17), 102.7 (C5), 100.4 (C7), 90.9 (C14), 83.9 (C13), 83.8 (C23), 81.3 (C2), 49.0 (C22), 46.8 (C11), 42.0 (C20), 29.9 (C25), 29.3 (C24), 28.9 (C26), 27.7 (C19), 25.7 (C39), 25.6 (C35), 25.2 (C21), 22.7 (C36), 21.6 (C31), 20.7 (C29), 18.1 (C34), 17.6 (C40).

10-Methoxygambogenic acid (**4**): yellow gum; HREIMS[M]⁺ 662.3449 (calcd for C₃₉H₅₀O₉ 662.3455), [α]_D = −181.2 (*c* = 0.1, CHCl₃); ^{13}C -NMR (CDCl₃) δ 208.6 (C12), 193.8 (C8), 172.4 (C30), 163.3 (C18), 159.3 (C6), 153.9 (C16), 139.8 (C27), 137.6 (C19), 135.6 (C33), 131.6 (C38), 126.9 (C28), 124.0 (C37), 121.6 (C3), 121.5 (C32), 108.3 (C5), 106.4 (C17), 102.0 (C7), 88.3 (C14), 86.4 (C13), 82.2 (C23), 74.0 (C10), 55.8 (OCH₃), 47.8 (C9), 43.8 (C11), 43.3 (C22), 39.8 (C20), 29.7 (C25), 27.9 (C24), 27.2 (C26), 26.5 (C36), 25.8 (C39), 25.7 (C35), 21.9 (C21), 21.2 (C31), 20.6 (C4), 19.9 (C29), 18.0 (C34), 17.7 (C40), 16.2 (C19).

LC-ESI-MS/MS analysis

The column was a Triart C18 (S-5 μ m, 12 nm, YMC) and was operated at 25 °C. Chromatographic separation was performed by the linear gradient solvent system with mobile phase A (0.1% acetic acid) and mobile phase B (acetonitrile). The flow rate was 0.5 mL/min. The chromatogram was detected at 254 nm. The methanol extract of gamboge was ionized using electrospray ionization (ESI) source in positive and negative ion modes. Spray temperature was kept 500 °C. Nebulizer gas (N₂) and heater gas (N₂) were set at 40 and 50 psi, respectively. The capillary voltage was 4.5 kV/−4.5 kV. The curtain gas was kept at 25 psi. The ion spray voltage was wet to 5500 and −4500 V for ESI⁺ and ESI[−], respectively.

α -Glucosidase inhibitory assay

α -Glucosidase inhibitory activity was monitored via the conversion of *p*-nitrophenyl- α -D-glucospyranoside (*p*NPG) to *p*-nitrophenol by recording absorbance changes according to the previous studies [13]. First, test samples were dissolved in DMSO and

Fig. 1 Chemical structures of four isolated caged xanthenes (1-4) from the resin of *Garcinia hanburyi*

diluted to the corresponding concentration (0.125–2.0 mM). A typical 200 μ L assay volume contained 130 μ L of 50 mM potassium phosphate (pH 6.8), 40 μ L of *p*NPG as a substrate, 10 μ L of diluted samples, and 20 μ L of α -glucosidase solution (0.1 units/mL) on the 96 well plate each individual well. After 10 min of preincubation, the mixture was measured using the Spectra Max M3 Multi-Mode Microplate reader (Molecular Devices) at 405 nm. The inhibitory capacities of the isolated compounds were expressed as IC_{50} that is the half maximal inhibitory concentration.

% Inhibition = [(rate of control reaction – rate of sample reaction)/rate of control reaction] $\times$ 100.

Kinetic experiments

The enzyme kinetics were investigated in experiments using three range of substrate concentrations on the basis of K_m (125 μ M) and testing compounds at different concentrations on the basis of IC_{50} values. The kinetic parameters (K_m : Michaelis-Menten constant, V_{max} : maximal velocity) were analyzed using a nonlinear regression program, Sigma plot (SPCC Inc., Chicago, IL, USA). The inhibition constant (K_i) was determined according to Dixon plot [14].

Results and Discussion

Isolation and purification of compounds

The four α -glucosidase inhibitory compounds (1-4) were isolated from the gum resin of *G. hanburyi* called as gamboge by the repeated chromatographies over silica gel and octadecyl

functionalized silica gel. The compounds (1-4) were identified as caged xanthone derivatives: gambogic acid (1), moreollic acid (2), gambogic acid (3), and 10-methoxygambogic acid (4) by analysis of spectroscopic data (Supplementary material). Spectroscopic data of the isolated compounds (1-4) were in agreement with previous data.

Among the isolated compounds, we briefly described elucidation of compound 1 that is the most abundant metabolite in LC-ESI-MS/MS analysis. Compound 1 was isolated as a yellow powder with molecular formula $C_{39}H_{48}O_9$ established by the $[M]^+$ ion at 660.3295 (calcd 660.3298) in the HREIMS. The analysis of individual carbon atoms was conducted by 1H -NMR, ^{13}C -NMR, and the result of DEPT experiments. The compound 1 had the following functional groups: 5 methylenes (sp^3), 8 methines (sp^2), 1 methine (sp^3), 9 methyls and 16 quaternary carbons. The analysis of degrees of unsaturation indicated a hexacyclic skeleton, where two of the cyclic skeletons were responsible to make the caged structure. The unique methine proton H22 (δ_H 2.49, $J=7.70$, 3.30 Hz) in the center of the caged was proved by its COSY connectivities with H21b (δ_H 1.35). Two 1,1-dimethylallyl groups on C13 and C17 were confirmed by successive COSY connectivities of H26/H27/H29 and H31/H32/H34, respectively. Their locations were confirmed by HMBC correlation between H26 (δ_H 3.17) and C13 (δ_C 86.2), H31 (δ_H 3.25) and C17 (δ_C 108.8). The $[\alpha]_D$ value was determined to be -108.0 ($c=0.1$, $CHCl_3$), which is in agreement with previous data [12].

LC-ESI-MS/MS analysis

The caged xanthenes in the methanol extract of gamboge were

Fig. 2 (A) HPLC chromatograms measured at 254 nm of MeOH extracts from the resin of *Garcinia hanburyi*, (B-E) LC-ESI-MS/MS chromatograms of isolated caged xanthenes **1-4**, respectively

Fig. 3 (A) Effects of isolated caged xanthenes **1-4** on α -glucosidase for the hydrolysis of *p*-nitrophenyl- α -D-glycopyranoside. (B) the catalytic activity of α -glucosidase as a function of enzyme concentration at different concentrations of compound **1**. (C) Lineweaver-Burk plots and (D) Dixon plots for the inhibition of compound **1**. (E) Lineweaver-Burk plots and (F) Dixon plots for the inhibition of compound **2**

tentatively determined using LC-ESI-MS/MS. Complete chromatographic separation of caged xanthenes (**1-4**) was reached within 30 min (Fig. 2A). The nature of each HPLC peak was doubly verified by comparison with retention times the purified standard compounds and mass spectrometry. The positive ion mode of ESI used for compounds **1** and **3**, while compounds **2** and **4** were analyzed by the negative ion mode. The MS spectra of four compounds (**1-4**) are displayed in Fig. 2B-E. The peak 1 (t_R =11.2 min) had the molecular ion 591 $[M-H]^-$ and the major fragment at m/z 509 $[M-H]^- -82$ for compound **2** [15]. The peak 2 (t_R =16.4 min) had the molecular ion 661 $[M-H]^-$ and the major fragment ion at 461 $[M-$

$H]^- -200$) for compound **4** [16]. The molecular ion 629 $[M+H]^+$ and the major fragment at m/z 329 $[M+H]^+ -84-216$ were observed at peak 3 (t_R =21.5 min) for compound **3** [17]. The peak 4 (t_R =24.0 min) also showed the molecular ion 661 $[M+H]^+$ for compound **1** [17]. In particular, the most active compound **1** was the most abundant metabolite in the methanol extract of gamboge as shown in Fig. 2A.

α -Glucosidase inhibitory activity

The inhibitory potencies and capacities of the isolated caged xanthenes toward α -glucosidase were examined. The α -glucosidase

Table 1 α -Glucosidase inhibitory effect of isolated compounds 1–4

Compounds	IC ₅₀ (μ M) ^a	Kinetic mode (K_i , μ M) ^b
1	41.4 $\pm$ 2.2	Noncompetitive (44.8 $\pm$ 2.5)
2	180.6 $\pm$ 4.7	Noncompetitive (187.3 $\pm$ 3.9)
3	280.4 $\pm$ 3.2	NT ^c
4	350.6 $\pm$ 1.9	NT
Acarbose ^d	32.4 $\pm$ 0.7	NT

All compounds were examined in as set of experiments repeated triplicate; ^aIC₅₀ values of compounds represent the concentration of 50% enzyme activity loss. ^bValues of inhibition constant; ^cNT is not tested; ^dAcarbose was used as positive control

inhibitory potencies of the tested compounds are displayed in Table 1. A dose-dependent inhibition forward α -glucosidase was observed on all tested compounds (Fig. 3A). All caged xanthenes (**1–4**) inhibited α -glucosidase with IC₅₀ values of 41.4, 180.6, 280.4, and 350.6 μ M, respectively. The 2-methyl-2-pentenyl pyran moiety of ring D and methoxy group on C-10 of ring A played a critical role for α -glucosidase inhibition of caged xanthone. For example, compound **1** (IC₅₀=41.4 μ M) bearing pyran moiety of ring D was eight-fold effective than compound **4** (IC₅₀=350.6 μ M) having geranyl group itself. The 2-methyl-2-pentenyl group on the pyran ring was also important due to a four-fold better activity of compound **1** than compound **2** (IC₅₀=180.6 μ M). In comparison between compound **1** and **3** (IC₅₀=280.4 μ M), the main difference was a methoxy group on C-10.

In kinetic analysis, all compounds (**1–4**) showed the same relationship between enzyme activity and enzyme concentration. Figure 3B illustrated the inhibitory behavior of compound **1** against α -glucosidase, representatively. The different concentrations of compound **1** gave a family of straight lines on the plots of the initial velocity versus enzyme concentration. These straight lines passed through the origin. Increasing the inhibitor concentration resulted in lowering of the slope of line indicating that compound **1** was a reversible inhibitor. Finally, the kinetic behavior of α -glucosidase at a differing concentration of compounds (**1** and **2**) was studied. Both compounds **1** and **2** exhibited noncompetitive inhibition against α -glucosidase as shown in Lineweaver-Burk plots (Fig. 3C, E). All lines in the Lineweaver-Burk plots met at a nonzero point on the x-axis, which means no change of K_m value. The K_i values of **1** and **2** were determined to be 44.8 and 187.3 μ M, respectively, by Dixon plots (Table 1, Fig. 3D, and Fig. 3F).

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