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Methylenebisnicotiflorin: a rare methylene-bridged bisflavonoid glycoside from ripe Pu-er tea

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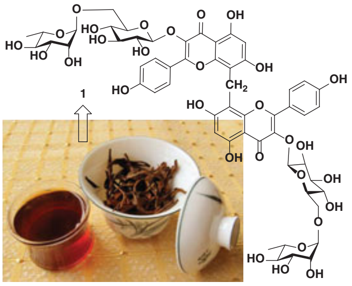

A new methylene-bridged dimeric flavonol glycoside, methylenebisnicotiflorin (**1**), was isolated from ripe Pu-er tea, along with 10 known flavonoids (**2**–**11**) and seven known phenolic compounds (**12**–**18**). The structure elucidation was based on spectroscopic analysis. Among them, 1,3-dihydroxyphenyl-2-*O*-sulfate (**13**), 2,3,4-trihydroxybenzoic acid (**16**) and 3,3′,4,4′-tetrahydroxybiphenyl (**18**) are reported from tea plants for the first time.

Keywords: ripe Pu-er tea; bisflavonoid glycoside; methylenebisnicotiflorin; methylene-bridged

1. Introduction

There is much epidemiological evidence that diets rich in phenolic secondary metabolites can reduce the incidence of non-communicable diseases such as cardiovascular diseases, diabetes, cancer and stroke (Crozier et al. 2009). Ripe Pu-er tea is rich in polyphenols, in particular, catechins, flavonoid glycosides and other simple phenols (Zhou et al. 2005; Xu et al. 2013; Tao et al. 2014). In China, especially in Yunnan province, ripe Pu-er tea is one of the most highly consumed beverages. Unlike black tea, which is the result of mechanical maceration of tea shoots triggers enzyme catalyzed oxidation (Subramanian et al. 1999), ripe Pu-er tea is based on mature tea leaves of *Camellia sinensis* var. *assamica* treated by microbial fermentation (Tao et al. 2014). The contents of tea polyphenols were highly changed during the post-fermentation process. For example, post-fermentation decreased the contents of catechin gallates and

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*Corresponding author. Email: zhangyj@mail.kib.ac.cn increased the content of gallic acid (Zhang et al. 2011). In our previous studies, a few catechin metabolites characteristic to the post-fermented Pu-er tea have been reported, such as puerins A and B (Zhou et al. 2005), puerins C – F (Tao et al. 2014) and carboxymethyl- and carboxyl-catechins (Tian et al. 2014). Moreover, catechin oxidation products (Jiang et al. 2011) and 8-C *N*-ethyl-2-pyrrolidinone substituted flavan-3-ols (Wang et al. 2014) were also reported from post-fermented tea. However, the condensed polyphenols, such as theaflavins and proanthocyanidins, which are the common constituents in black tea (Drynan et al. 2010), have not been found in ripe Pu-er tea. As one of our systematic studies on post-fermented tea, this paper reports the first isolation of a methylene-bridged dimeric polyphenol from the ripe Pu-er tea and its structure determination by spectroscopic methods.

2. Results and discussion

Repeated column chromatography (CC) over MCI-gel CHP20P, Sephadex LH-20, Toyopearl HW-40C and semi-preparative HPLC led to the isolation of a new methylene-bridged bisflavonoid glycoside, methylenebisnicotiflorin (**1**). In addition, 17 known compounds (**2**–**18**) were identified as 10 flavonoids, kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→3)-(4‴-*O*-acetyl)-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**2**), kaempferol-3-*O*-robinobioside (**3**), nicotiflorin (**4**), quercetin-3-*O*-robinobioside (**5**), rutin (**6**), kaempferol (**7**), quercetin (**8**), isovitexin (**9**), (2*R*)-isohemiphloin (**10**), and (2*S*)-isohemiphloin (**11**), and seven simple phenolics, 4-*O*-*p*-trans-coumaroyl quinic acid (**12**), 1,3-dihydroxyphenyl-2-*O*-sulfate (**13**), pyrogallol (**14**), protocatechuic acid (**15**), 2,3,4-trihydroxybenzoic acid (**16**), gallic acid (**17**) and 3,3′,4,4′-tetrahydroxybiphenyl (**18**), by comparison of their spectroscopic data with those reported in literatures (details shown in supplementary material). Compounds **13**, **16** and **18** are reported from tea plants for the first time.

Methylenebisnicotiflorin (**1**) was obtained as a yellow amorphous powder, and its molecular formula, $C_{55}H_{60}O_{30}$, was established from the negative HRESIMS at *m/z* 1199.3088 $[M-H]^-$ (calculated for $C_{55}H_{59}O_{30}$ 1199.3091) and ^{13}C NMR spectra. The IR spectrum of **1** displayed absorption bands at 3431 and 1647 cm^{-1}, ascribable to hydroxyl and α, β-unsaturated ketone groups, respectively. The absorption maxima at 204 and 275 nm in the UV spectrum indicated an extended conjugation. The ^1H and ^{13}C NMR spectra of **1** showed a set of signals [δ_H 5.36 (d, $J = 7.6$ Hz, H-1″), 3.70 (d, $J = 10.5$ Hz, H-6″a), 3.32 (m, H-6″b), 4.42 (s, H-1‴), 0.97 (d, $J = 6.0$ Hz, H-6‴); δ_C 101.6 (C-1″), 74.3 (C-2″), 76.4 (C-3″), 69.8 (C-4″), 75.8 (C-5″), 66.9 (C-6″), 100.9 (C-1‴), 70.4 (C-2‴), 70.7 (C-3‴), 71.9 (C-4‴), 68.4 (C-5‴), 17.9 (C-6‴)] attributable to one β-glucopyranosyl and one α-rhamnopyranosyl moieties. Acid hydrolysis of **1** afforded D-glucose and L-rhamnose, of which the absolute configurations were identified by GC analysis of their 1-(trimethylsilyl)-1*H*-imidazole derivatives (Hara et al. 1987). In addition, the left 15 carbon signals [δ 177.9 (C-4), 162.4 (C-7), 160.0 (C-4′), 159.0 (C-5), 156.8 (C-2), 154.1 (C-9), 133.0 (C-3), 131.0 (C-2′, 6′), 121.3 (C-1′), 115.2 (C-3′, 5′), 104.9 (C-8), 103.8 (C-10) and 98.5 (C-6)], as well as two two-proton doublet [δ_H 8.00 (d, $J = 8.7$ Hz, H-2′,6′), 6.84 (d, $J = 8.7$ Hz, H-3′,5′)] due to one 1,4-disubstituted benzene ring and one-proton aromatic singlet at δ 6.20 (s, H-6), were quite similar to those for kaempferol. The aforementioned NMR data closely resembled those of a flavonoid glycoside, nicotiflorin (kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside, **4**), (Zhou & Yang 2000). However, instead of the two A-ring aromatic methines (CH-6 and CH-8) in nicotiflorin, the NMR spectra of compound **1** displayed only one aromatic methine [δ_H 6.20 (s), δ_C 98.5 (CH)] in A-ring, and an additional quarternary aromatic carbon at δ_C 104.9 (C), as well as an additional benzylic methylene signal at δ_H 4.12 (s) and δ_C 16.5 (CH_2). The above data indicated that compound **1** should comprise symmetrically two nicotiflorin units connecting between two C-6 or two C-8 positions through a methylene bridge. The proposition coincided with the high molecular mass.

In the HMBC spectrum (Figure S1), correlations of OH-5 (δ 12.61) with the carbon at δ 159.0, 103.8 and 98.5, and the aromatic proton at δ 6.20 with the carbons at δ 162.4, 159.0, 104.9, and 103.8 could assign unambiguously these carbon signals as C-5 (δ 159.0), C-6 (δ 95.8), C-7 (δ 162.4), C-8 (δ 104.9), and C-10 (δ 103.8), respectively. Thus, an unresolved signal at δ_C 154.1 must be arisen from C-9. The CH_2 proton signal at δ_H 4.12 showing HMBC correlations with δ 162.4 (C-7), 104.9 (C-8) and 154.1 (C-9) revealed that the two nicotiflorin units in **1** were linked between their C-8 positions through this methylene bridge. Furthermore, HMBC correlations from H-1‴ (δ 4.42) to C-6″ (δ 66.9) revealed the 1→6 connectivity between rhamnosyl and glucosyl, while the chemical shift of C-2 [2.3 ppm upfield shift, compared with kaempferol (**7**)] suggested that the kaempferol aglycone was glycosylated at C-3. Therefore, the structure of compound **1** was assigned as two nicotiflorin units connecting between their C-8 positions through a methylene bridge and named methylenebisnicotiflorin (Figure 1).

In conclusion, the isolated phenolic compounds **1–18** could be divided into two groups, flavonoids (**1–11**) and simple phenols (**12–18**). Compound **1** is a novel methylene-bridged bisflavonoid glycoside, probably originated from formaldehyde during the microbial post-

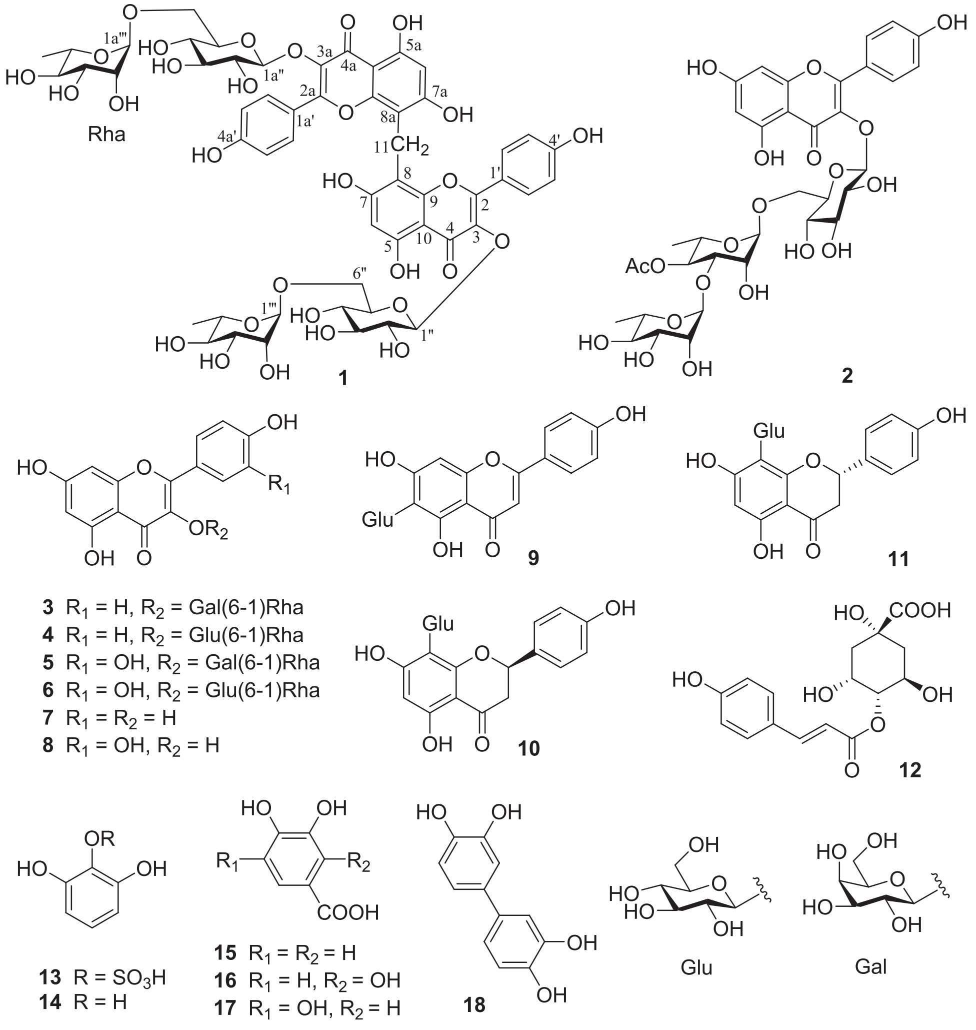

Figure 1. Structures of compounds **1** – **18** from ripe Pu-er tea.

fermentation (Hashimoto et al. 1989). Methylene-bridged bisflavonoids are rare in nature. To the best of our knowledge, only 21 analogues have been reported so far. For instance, oolonghomobisflavans A and B (Hashimoto et al. 1989), talienbisflavan A (Zhu et al. 2012) as well as bis (8-epicatechinyl)methane (Ma et al. 2014) (methylene-bridged dimeric flavan-3-ols) were reported from the commercial oolong tea, leaves of *Camellia taliensis*, and pericarps of *Litchi chinensis*, respectively. Furthermore, six methylene bisflavonoids were isolated from a western North America fern, *Pentagramma triangularis* (Roitman et al. 1993; Iinuma et al. 1994; Iinuma et al. 1997). Recently, tomoroside B, a methylene-bridged chalcone dimer, was reported from *Helichrysum zivojinii* (Aljančić et al. 2014). Nevertheless, methylenebisnicotiflorin (**1**) was the only symmetrical glycosylated methylene-bridged bisflavonoid reported from tea plants and the first dimeric polyphenol from the post-fermented tea. Compound **13** is a sulfate-conjugated phenolic compound, most probably produced by enzymatic conjugation of pyrogallol (**14**) with sulfate, which was previously reported from urine samples collected from volunteers after consumption of black tea (Daykin et al. 2005). The isolation of **13** from ripe Pu-er tea confirmed that a similar microbial conjugation of pyrogallol with sulfate probably happened during the post-fermentation process. Compounds **13**, **16** and **18** are reported from tea plants for the first time. Flavonoids (**2**–**11**) and simple phenolics (**12**, **14**, **15** and **17**) were common constituents reported from green tea and previous reports revealed that these compounds were important antioxidants present in tea.

3. Experimental

3.1. *General*

1D and 2D NMR spectra were recorded in CD_3OD or DMSO-d_6 with Bruker AM-400 or AVANCE III-600 NMR spectrometers (Bruker Co., Ettlingen, Germany) operating at 400 or 600 MHz for ^1H NMR and 100 or 150 MHz for ^{13}C NMR, respectively. ESIMS and HRESIMS were recorded on API QSTAR Pular-1 (Applied Biosystems, Foster City, CA, USA) and Agilent G6230 TOF mass spectrometers (Applied Agilent Technologies, Santa Clara City, CA, USA), respectively. UV spectroscopic data were measured on a Shimadzu-210A double-beam spectrophotometer (Shimadzu Corp., Kyoto, Japan). IR spectra of samples in KBr discs were recorded on a Bruker-Tensor-27 spectrometer (Bruker Co., Ettlingen, Germany) with KBr pellets. Column chromatography was done on Diaion HP20SS (Mitsubishi Chemical Co., Ltd., Tokyo, Japan), MCI-gel CHP-20P (75–150 μm, Mitsubishi Chemical Co., Ltd., Tokyo, Japan), Sephadex LH-20 (25–100 μm, Mitsubishi Chemical Co., Ltd., Tokyo, Japan), and Toyopearl HW-40C (50–100 μm, TOSOH, Japan). TLC was performed on precoated TLC plates (0.2–0.25 mm thickness, GF254 Silica gel, Qingdao Hailang Chemical Co., Ltd., Qingdao, China) with compounds visualised by spraying with anisaldehyde-sulfuric acid reagent and careful heating. Semi-preparative HPLC was performed on an Agilent 1260.

3.2. *Materials*

Commercial ripe Pu-er tea powder produced from the crude green tea prepared with leaves of *C. sinensis* var. *assamica* was purchased in Puer County, Yunnan Province, China. This material was identified by Prof. Ying-Jun Zhang, Kunming Institute of Botany (KIB), Chinese Academy of Sciences (CAS). A voucher specimen (KIB-Z-1202010) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, KIB, CAS.

3.3. *Extraction and isolation*

Commercial ripe Pu-er tea powder (2 kg) was dissolved in 100 L purified water and then centrifuged (TDL-5-A) at 5400 rpm for 10 min. The aqueous layer was diluted with another 100 L purified water and then ultra-filtered successively through Biomax 5 and 50 K

Polyethersulfone to give three fractions, with molecular weight of more than 50 K (440 g), between 50 and 5 K (540 g), and less than 5 K (840 g). The fraction of less than 5 K (840 g) was suspended in water and then successively partitioned with chloroform, ethyl acetate and *n*-BuOH, to yield $CHCl_3$ (130 g), EtOAc (60 g), *n*-BuOH (120 g) and H_2O (490 g) sub-fractions, respectively. The *n*-BuOH-soluble fraction (120 g) was applied to a Diaion HP20SS column chromatography (CC), eluting with H_2O containing increasing proportions of MeOH to give five fractions (Fr. 1–5). Fr. 1 (30.7 g) obtained by elution of purified water, was shown to mainly contain gallic acid (**17**) and polysaccharide by TLC analysis. Fr. 2 (12.0 g), obtained by elution of 20–40% MeOH, was subjected successively to CC over Toyopearl HW40C (0–40% MeOH), MCI-gel CHP20P (0–40% MeOH) and semi-preparative HPLC (flow rate: 3 mL/min, 7% CH_3CN in 1% trifluoroacetic acid) to yield compounds **12** (24 mg), **13** (42 mg), **14** (90 mg), **15** (2 mg), **16** (2 mg), **17** (200 mg) and **18** (3 mg). Fr. 4 (27.8 g), obtained by elution of 60–80% MeOH, was subjected successively to CC over Toyopearl HW40C (30–80% MeOH), MCI-gel CHP20P (30–80% MeOH), Sephadex LH-20 (MeOH) and semi-preparative HPLC (flow rate: 3 mL/min, 15–25% CH_3CN in 1% trifluoroacetic acid) to give **1** (4 mg), **2** (8 mg), **3** (24 mg), **4** (75 mg), **5** (20 mg), **6** (20 mg), **9** (28 mg), **10** (10 mg) and **11** (10 mg). Fr. 5 (6.3 g), obtained by elution of 80–100% MeOH, was subjected successively to CC over MCI-gel CHP20P (30–80% MeOH), and Sephadex LH-20 (MeOH) to get **7** (70 mg) and **8** (70 mg).

3.3.1. *Methylenebisnicotiflorin (1)*

Yellow amorphous powder; UV (MeOH), λ_{max} (logϵ): 275 (4.41), 204 (4.59); IR (KBr) ν_{max} 3431, 2926, 1647, 1509, 1438, 1364, 1178, 1066, 840, 579 cm^{-1}; ESIMS *m/z* 1199 $[M - H]^-$, HRESIMS *m/z* 1199.3088 $[M - H]^-$ (calcd for $C_{55}H_{59}O_{30}$, 1199.3091); ^1H NMR (600 MHz, DMSO-d_6): δ 12.61 (2H, s, OH-5,5a), 10.71 (2H, s, OH-7,7a), 10.17 (2H, s, OH-4′,4a′), 6.20 (2H, s, H-6,6a), 4.12 (2H, s, H-11), 8.00 (4H, d, $J = 8.7$ Hz, H-2′,6′,2a′,6a′), 6.84 (4H, d, $J = 8.7$ Hz, H-3′,5′,3a′,5a′), 5.36 (2H, d, $J = 7.6$ Hz, H-1″,1a″), 3.17 (2H, m, H-2″,2a″), 3.22 (2H, m, H-3″,3a″), 3.09 (2H, m, H-4″,4a″), 3.26 (2H, m, H-5″,5a″), 3.70 (2H, d, $J = 10.5$ Hz, H-6″a,6a″a), 3.32 (2H, m, H-6″b,6a″b), 4.42 (2H, brs, H-1‴,1a‴), 3.47 (2H, m, H-2‴,2a‴), 3.32 (2H, m, H-3‴,3a‴), 3.10 (2H, $J = 9.4$, 4.9 Hz, H-4‴,4a‴), 3.30 (2H, m, H-5‴,5a‴), 0.97 (6H, d, $J = 6.0$ Hz, H-6‴,6a‴); ^{13}C NMR (150 MHz, DMSO-d_6): δ 156.8 (C-2,2a), 133.0 (C-3,3a), 177.9 (C-4,4a), 159.0 (C-5,5a), 98.5 (C-6,6a), 162.4 (C-7,7a), 104.9 (C-8,8a), 154.1 (C-9,9a), 103.8 (C-10,10a), 16.5 (C-11), 121.3 (C-1′,1a′), 131.0 (C-2′,6′,2a′,6a′), 115.2 (C-3′,5′,3a′,5a′), 160.0 (C-4′,4a′), 101.6 (C-1″,1a″), 74.3 (C-2″,2a″), 76.4 (C-3″,3a″), 69.8 (C-4″,4a″), 75.8 (C-5″,5a″), 66.9 (C-6″,6a″), 100.9 (C-1‴,1a‴), 70.4 (C-2‴,2a‴), 70.7 (C-3‴,3a‴), 71.9 (C-4‴,4a‴), 68.4 (C-5‴,5a‴), 17.9 (C-6‴,6a‴).

3.4. *Acidic hydrolysis of compound 1*

Compound **1** (2.0 mg) was hydrolysed with 2 M HCl/1,4-dioxane (1:1, 4 mL) at 80°C for 4 h. The mixture was extracted with chloroform, and the water layer left was neutralised with Amberlite IRA-401, and the resin was removed by filtration. The filtrate was evaporated to dryness, and the resulting monosaccharides were dissolved in pyridine (2 mL). Then, L-cysteine methyl ester hydrochloride (1.5 mg) was added. After heating at 60°C for 1 h, trimethylsilylimidazole (1.5 mL) was added. The mixture was heated at 60°C for another 30 min. An aliquot (4 μL) of the supernatant was removed and directly subjected to GC analysis: column temperature, 180–280°C at 3°/min; carrier gas, N_2 (1 mL/min), injector and detector temperature, 250°C; split ratio, 1:50. The configurations of monosaccharides were determined by comparison of the retentions times (t_R values) of the corresponding derivatives with those of authentic samples. The GC (t_R values) of the D-glucose and L-rhamnose derivatives were 22.325 and 18.689 min, respectively.

Supplementary material

Supplementary material relating to this article is available online http://dx.doi.org/10.1080/14786419.2015.1065491: Identification of the known compounds **2–18**, key HMBC correlations of **1** (Figure S1), as well as 1D, 2D NMR and HRESIMS of compound **1**.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Note

1. The authors contributed equally to this paper.

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