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Puerins A and B, Two New 8-C Substituted Flavan-3-ols from Pu-er Tea

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Pu-er tea is a special treated fermented tea produced from crude green tea, which is prepared from the leaves of *Camellia sinensis* var. *assamica*. It is a traditional beverage having been used in China, particularly the southern areas, for a long time. Chemical investigation led to the identification of two new 8-C substituted flavan-3-ols, puerins A (**1**) and B (**2**), and two known cinchonain-type phenols, epicatechin-[7,8-*bc*]-4α-(4-hydroxyphenyl)-dihydro-2(3*H*)-pyranone (**3**) and cinchonain Ib (**4**), together with other seven known phenolic compounds, 2,2′,6,6′-tetrahydroxydiphenyl (**5**), (−)-epicatechin-8-*C*-β-D-glucopyranoside (**6**), (−)-epicatechin (EC) (**7**), (−)-epigallocatechin (EGC) (**8**), (±)-gallocatechin (GC) (**9**), gallic acid (**10**), and myricetin (**11**), in addition to caffeine (**12**). Their structures were determined by spectroscopic methods. The compounds **1**−**5**, which might be formed in the postfermentative process of Pu-er tea, were isolated from tea and theaceous plants for the first time.

KEYWORDS: Pu-er tea; *Camellia sinensis* var. *assamica*; phenolic compounds

INTRODUCTION

Tea is one of the most popular beverages consumed in the world, and its biological activities and health functions have been widely explored. It is produced from the leaves of tea plants, *Camellia sinensis* L. and *C. sinensis* var. *assamica* (Masters) Kitamura (Theaceae), which are evergreen shrubs or trees and usually pruned to 2−5 feet for cultivation. On the basis of the processing procedures, tea can generally be divided into green tea (nonfermented), oolong tea (semifermented), and black tea (fully fermented by oxidizing enzyme). Up to now, many chemical investigations have been carried out on tea, and a series of flavan-3-ols and hydrolyzable tannins in green tea (*1*, *2*), 8-*C*-ascorbyl-(−)-epigallocatechin 3-*O*-gallate and novel flavan-3-ols derivatives in oolong tea (*3*−*5*), and benzotropolone-type pigments in black tea (*6*, *7*), were reported.

Pu-er tea, a well-known tea from ancient times, was originally produced in the Yunnan province of China through a special postfermentative process, using crude green tea as raw materials, which were prepared from the leaves of *C. sinensis* var. *assamica*. It is increasingly liked by consumers not only because of the special flavor and taste but also the health-giving biological function. Pu-er tea was initially formed from long-distance transport of crude green tea on horseback. However, now, it is produced by a standardized postfermentative process with high temperatures and high humidity. The fresh leaves of *C. sinensis* var. *assamica*, the original plant material of Pu-er tea, were found to contain chalcan−flavan dimers and flavan-3-ol derivatives (*8*), and some flavonols and catechins were reported from the raw material (the crude green tea) of Pu-er tea by our group (*9*). However, the chemical constituents of Pu-er tea are thus far not known. In this investigation, we isolated a number of flavanoids and phenolic compounds from Pu-er tea and determined their structures by spectroscopic mehods.

MATERIALS AND METHODS

General. Optical rotations were measured on a P-1020 Polarimeter (JASCO, Tokyo, Japan). IR spectra were measured on an IR-450 spectrometer (Shimadzu, Kyoto, Japan) with KBr pellets. UV spectra were obtained on a 210A double-beam spectrophotometer (Shimadzu, Kyoto, Japan). ^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, HMQC, and HMBC spectra were recorded in acetone-d_6 with Bruker AM-400 and DRX-500 spectrometers operating at 500 and 400 MHz for ^{1}H and 125 and 100 MHz for ^{13}C, respectively. Coupling constants are expressed in Hertz, and chemical shifts are given on a δ (ppm) scale with tetramethylsilane as an internal standard. FABMS and HRFABMS were recorded on an AutoSpe 3000 spectrometer (VG, Manchester, U.K.) with glycerol as the matrix. Column chromatography was done on Diaion 101 resin (Shandong Lukang Pharmaceutical Co., Ltd.), Sephadex LH-20, 25−100 μm (Pharmacia Fine Chemical Co., Ltd.), MCI-gel CHP20P, 75−100 μm (Mitsubishi Chemical Co., Ltd.), and Toyopearl HW-40F (Tosoh Co., Ltd.). Thin-layer chromatography (TLC) was performed on precoated kieselgel 60 F254 plates, 0.2 mm thick (Merck), with benzene/ethyl formate/formic acid (3:6:1 or 2:7:1, v/v), and spots were detected by spraying with a 2% ethanolic $FeCl_3$ or anisaldehyde-H_2SO_4 reagent, followed by heating.

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Table 1. ^{13}C NMR Spectroscopic Data of Compounds **1** and **2** (δ Value, in Acetone-d_6)

C	1	2	C	1	2
2	79.4	80.2	13	31.9	32.6
3	66.5	67.2	14	23.8	24.4
4	29.2	29.5	15	29.2	29.5
5	155.3	157.6	16	35.7	36.4
6	95.8	96.1	17	12.5	12.6
7	156.7	157.6	1′	131.5	131.4
8	100.9	100.4	2′	114.7	106.9
9	156.3	156.0	3′	145.2	146.7
10	104.9	105.0	4′	145.2	135.0
11	53.1	54.0	5′	118.7	146.7
12	176.0	177.4	6′	115.7	106.9

Plant Materials. Pu-er tea, produced from the crude green tea prepared with leaves of *Camellia sinensis* var. *assamica*, was purchased at Linchang Tea Factory, Linchang County, Yunnan Province of China, and used for extraction and isolation of the chemical constituents.

Extraction and Isolation. Pu-er tea (1 kg) was extracted with 80% aqueous acetone at room temperature for 3 times (6, 3, and 3 L). After removal of the acetone under reduced pressure, the aqueous solution afforded precipitates, which were removed by filtration. The filtrate was extracted with $CHCl_3$ to give the crude compound **12** (8 g). The aqueous mother liquor was concentrated under reduced pressure to afford a residue (49 g), which was applied to a Diaion 101 resin column, eluting with H_2O and MeOH (each 1 L), successively. The MeOH eluate was concentrated to dryness and subjected to column chromatography over Sephadex LH-20, eluting with MeOH−H_2O (1:0−0:1) (each gradient at 300 mL) to afford five fractions. Further repeated column chromatography over MCI-gel CHP20P, Toyopearl HW-40F, and Sephadex LH-20 gave **3** (35 mg), **4** (40 mg), and **10** (60 mg) from fraction 1, **1** (40 mg), **2** (25 mg), and **7** (60 mg) from fraction 2, **6** (45 mg) and **11** (20 mg) from fraction 3, **8** (22 mg) and **9** (19 mg) from fraction 4, and **5** (80 mg) from fraction 5, respectively.

Puerin A (1). Pale brown amorphous powder, $[\alpha]^{21}_D$ +10.0 (*c* 0.005, MeOH). UV λ_{max} (MeOH): 207.5, 225.5, 282 nm. IR (KBr) ν_{max} (cm^{-1}): 3279, 1611, 1456, 1355, 1283, 1205, 1161, 1101, 1066, 975, 932. ^1H NMR (400 MHz, acetone-d_6) δ: 6.80 (1H, br s, H-2′), 6.76 (1H, d, J = 8.0 Hz, H-5′), 6.67 (1H, br d, J = 8.0 Hz, H-6′), 6.13 (1H, s, H-6), 5.36 (1H, br s, H-11), 4.80 (1H, br s, H-2), 4.20 (1H, m, H-3), 3.57, 2.52 (each 1H, m, H-13), 2.87 (1H, br d, J = 16.5 Hz, H-4b), 2.79 (2H, m, H-15), 2.72 (1H, br d, J = 16.5 Hz, H-4a), 2.38, 2.12 (each 1H, m, H-14), 2.21 (2H, m, H-16), 1.00 (3H, br s, H-17). ^{13}C NMR (100 MHz, acetone-d_6): see **Table 1**. FABMS (negative-mode): *m*/*z* 400 $[M]^-$. HRFABMS *m*/*z* 400.1514 $[M]^-$, calcd for $C_{22}H_{24}O_7$, 400.1522.

Puerin B (2). Pale brown amorphous powder, $[\alpha]^{21}_D$ +10.0 (*c* 0.005, MeOH). ^1H NMR (400 MHz, acetone-d_6) δ: 7.01 (2H, s, H-2′, 6′), 6.13 (1H, s, H-6), 5.38 (1H, br s, H-11), 4.76 (1H, br s, H-2), 4.22 (1H, m, H-3), 3.60, 2.52 (each 1H, m, H-13), 2.85 (1H, br d, J = 16.5 Hz, H-4b), 2.78 (2H, m, H-15), 2.72 (1H, br d, J = 16.5 Hz, H-4a), 2.37, 2.12 (each 1H, m, H-14), 2.22 (2H, m, H-16), 1.09 (3H, br s, H-17). ^{13}C NMR (100 MHz, acetone-d_6): see **Table 1**. FABMS (negative mode): *m*/*z* 415 $[M-H]^-$. HRFABMS: *m*/*z* 415.1553 $[M-H]^-$, calcd for $C_{22}H_{23}O_8$, 415.1393.

RESULTS AND DISCUSSION

The 80% aqueous acetone extract of commercial Pu-er tea was separated successively by partitioning with $CHCl_3$ and H_2O, and repeated Diaion 101 resin, Sephadex LH-20, MCI-gel CHP20P, and Toyopearl HW-40F column chromatography afforded two new phenolic compounds **1** and **2** (**Figure 1**). In addition, 10 known compounds were also obtained and identified as epicatechin-[7,8-*bc*]-4α-(4-hydroxyphenyl)-dihydro-2(3*H*)-pyranone (**3**) (*10*), cinchonain Ib (**4**) (*11*, *12*), 2,6,2′,6′-tetrahydroxybiphenyl (**5**) (*13*), (−)-epicatechin-8-*C*-*β*-D-glucopyranoside (**6**) (*14*), (−)-epicatechin (EC) (**7**) (*15*), (−)-epigallocatechin (EGC) (**8**) (*16*), (±)-gallocatechin (GC) (**9**) (*17*), gallic acid (**10**), myricetin (**11**) (*15*), and caffeine (**12**), respectively, on the basis of their physical and spectrascopic data and by comparison with reference values and authentic samples.

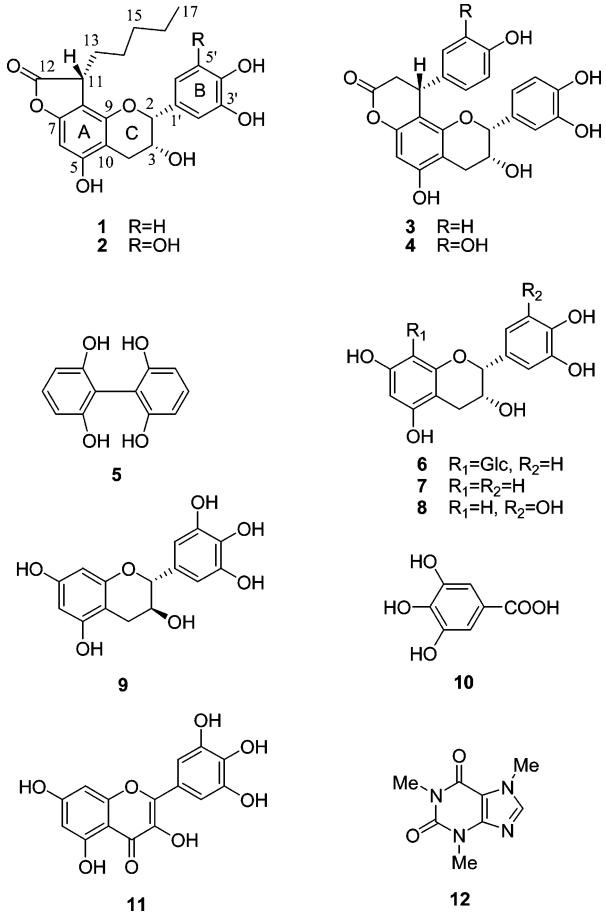

Figure 1. Structures of compounds isolated from Pu-er tea.

Compound **1** was obtained as a pale brown amorphous powder and showed UV absorptions at 207.5, 225.5, 282 nm. The negative HRFABMS exhibited a molecular ion peak at *m*/*z* 400.1514 $[M]^-$, corresponding to the molecular formula $C_{22}H_{24}O_7$. The occurrence of a flavan-3-ol skeleton in the molecule could be easily deduced from the ^1H NMR spectrum. The signals at δ 4.80 (s), 4.20 (m), and 2.70−2.85 (2H, m) ascribable to C_2, C_3, and C_4 protons on a flavan C ring, respectively, and ABX-type aromatic signals at δ 6.80 (1H, br s, H-2′), 6.76 (1H, br d, J = 8.0 Hz, H-5′), and 6.67 (1H, d, J = 8.0 Hz, H-6′) arising from the 3′,4′-dihydroxylated B-ring protons were closely related to those in (−)-epicatechin (**7**) (*15*). A singlet signal at high field in the aromatic region (δ 6.13) suggested that the A ring is pentasubstituted. The ^{13}C NMR and DEPT spectra (**Table 1**) revealed, in addition to 15 signals similar to those of epicatechin, compounds **3** (*10*) and **4** (*11*), the presence of a methine [δ_C 53.1 (d), δ_H 5.36] and *n*-pentyl [δ 23.8 (t), 29.2 (t), 31.9 (t), 35.7 (t), 12.5 (q)] groups, which were confirmed to be linked to each other by the ^1H-^1H COSY spectra. Furthermore, the

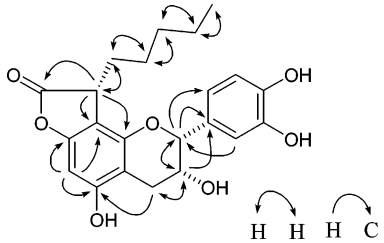

Figure 2. Important ^{1}H-^{1}H COSY and HMBC correlations of **1**.

appearance of a signal at δ 176.0 suggested the presence of a carboxyl function. This was supported by the IR spectrum showing a strong band at 1611 cm^{-1}, probably because of a β,γ-unsaturated five-membered lactone. In the HMBC spectrum of **1** (**Figure 2**), the methylene protons at δ 2.87 and 2.72 (H-4) were correlated with the carbon signal at δ 155.3, which was shown to correlate with the aromatic proton at δ 6.13, assigning the carbon signal at δ 155.3 as C-5 and the aromatic proton (δ 6.13) as C_6-H. In addition, HMBC correlation of the methine proton at δ 5.36 (H-11) with δ 156.3 (C-9) indicated the occurrence of a carbon−carbon linkage at the C_8 position in the epicatechin moiety. Other HMBC correlations including the methine at δ 5.36 (H-11) with the carboxyl group at δ 176.0 were also observed (**Figure 2**). On the basis of the above evidence, the five-membered lactone ring with an *n*-pentyl group attached was determined to be located at C-7 and C-8 positions of the epicatechin moiety and the structure of puerin A was established as shown in molecular **1**.

The molecular formula of compound **2** was determined to be $C_{22}H_{24}O_8$ based on the negative HRFABMS, which had one more oxygen atom than that of **1**. The ^{1}H and ^{13}C (**Table 1**) NMR spectroscopic features were consistent with those of **1**, suggesting a similar structure of **2** to that of **1**. The only difference was observed for their B ring in flavan-3-ol moiety. The typical ^{1}H and ^{13}C chemical shifts [δ_H 7.01 (2H, s), δ_C 131.4 (C-1′), 106.9 (C-2′, 6′), 146.7 (C-3′, 5′) and 135.0 (C-4′)] indicated the presence of a pyrogallol moiety as the B ring in **2**. Therefore, the structure of **2** was determined to be that of puerin B.

The configurations of C-11 methine in **1** and **2** were deduced by comparing their $[\alpha]_D$ with those of the known structural-related complex flavan-3-ols, which have the same substituent location in the flavan A ring and similar structures to **1** and **2**. Both epicatechin- (cinchonain Ia) and catechin-[7,8-*bc*]-4β-(3,4-dihydroxyphenyl)-dihydro-2(3*H*)-pyranone with a different C-3 configuration of the flavan-3-ol moiety but the same 4β configuration of the substituent in the flavan A ring showed the same negative $[\alpha]_D$, while epicatechin- (cinchonain Ib, **4**) and catechin-[7,8-*bc*]-4α-(3,4-dihydroxyphenyl)-dihydro-2(3*H*)-pyranone with the same 4α configuration of the substituent also gave the same positive $[\alpha]_D$ (*11*, *12*). This observation revealed that the positive or negative symbol of the $[\alpha]_D$ of these complex flavan 3-ols was determined by the substituent configuration (4α or 4β) in the flavan A ring. Accordingly, the configurations of the C-11 methine in **1** and **2** were deduced to be α because of their positive $[\alpha]_D$ values.

It is noticeable that the chemical constituents of Pu-er tea are distinctly different from those of the other kinds of tea, e.g., green tea, oolong tea, and black tea. Most of the characteristic flavan-3-ol derivatives in green tea, such as epigallocatechin 3-*O*-gallate (EGCG), EGC, and epicatechin 3-*O*-gallate (ECG), have disappeared, whereas the content of polyphenol polymers is remarkably enhanced in Pu-er tea. At the same time, several polyoxygenated compounds such as **1**−**5** are formed from the related catechin derivatives during the processing procedure of Pu-er tea. Compounds **1** and **2** are the first acylated flavan-3-ols with an aliphatic acid moiety linked to the flavan-3-ol A ring as a five-membered lactone through a C−C linkage. Both of the compounds **3** and **4** belonging to cinchonain-type flavan-3-ols are isolated from tea and tea plants for the first time. The former (**3**) is a phenylpropanoid−catechin isolated from the bark of *Ocotea porosa* (Lauraceae) (*10*), while the latter (**4**) was reported to be isolated from *Cinchona succirubra* (Rubiaceae) (*11*). Compound **5**, known as a synthetic chemical, is obtained from a natural source for the first time (*13*).

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