JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Carboxymethyl- and Carboxyl-Catechins from Ripe Pu-er Tea

Li-Wen Tian,[†,§] Mu-Ke Tao,[†,‡,§] Min Xu,[†] Jing Hu,[†] Hong-Tao Zhu,[†] Wen-Yong Xiong,[†] Dong Wang,[†] Chong-Ren Yang,[†] and Ying-Jun Zhang*[,†]

[†]State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, P. R. China

[‡]Chongqing Institute for Food and Drug Control, Chongqing 401121, P. R. China

Supporting Information

ABSTRACT: Ripe Pu-er tea, a special microbial postfermented tea originated from Yunnan Province, China, since ancient times, is made from green Pu-er tea prepared from the leaves of *Camellia sinensis* var. *assamica* (Theaceae). Chemical investigation on thearubigin (*n*-BuOH-soluble) fraction of the commercial ripe Pu-er tea, led to the identification of four new flavan-3-ol derivatives, 8-carboxymethyl-(+)-catechin (**1**), 8-carboxymethyl-(+)-catechin methyl ester (**2**), 6-carboxymethyl-(+)-catechin (**3**), and 6-carboxyl-(−)-gallocatechin (**4**), together with 18 known compounds, including other three flavan-3-ol derivatives (**5**−**7**), 10 flavonoid glycosides (**8**−**17**), two hydrolyzable tannins (**18** and **19**), two quinic acid derivatives (**20**−**21**), and a purine alkaloid (**22**). Flavonoid glycosides **8**−**11** are reported from tea plants for the first time. The thearubigin fraction of ripe Pu-er tea was qualitatively analyzed by HPLC, and gallic acid was found to be the major component. Compounds **4**, **6**−**17**, **21** and **22** were tested for their acute activities on insulin sensitivity in differentiated 3T3-L1 adipocytes, but none of them showed significant bioactivity at a concentration of 10 μM.

KEYWORDS: *ripe Pu-er tea, carboxymethyl-catechins, carboxylcatechins, insulin sensitivity activity*

INTRODUCTION

Ripe Pu-er tea is a well-known tea originated from Yunnan Province, China, since ancient times. It is classified as postfermented tea, based on the special processing procedures.[1] Briefly, the crude green tea, prepared from the leaves of *Camellia sinensis* var. *assamica* (Theaceae), is postfermented by microbes in a warm and humid environment under controlled condition. This is different from that of the black tea, which is fermented by the polyphenol oxidases existing in the tea leaves.[2] Owning to the microbial postfermentation, ripe Pu-er tea has a special flavor and taste on the basis of different chemical constituents from those of black tea and its original materials, a kind of crude green tea. The contents of catechins, the main constituents in fresh tea leaves and green tea, sharply decreased in ripe Pu-er tea. However, theaflavins, the characteristic constituents in black tea, were not found in ripe Pu-er tea.[3] A few catechin metabolites, such as catechin oxidation products,[4] cinchonain-type flavan-3-ols,[5] have been reported as the characteristics of postfermented tea in previous chemical investigations. These products were reported to be formed by nucleophilic addition or oxidation from catechins.[4,6] Our further chemical study on the *n*-BuOH-soluble fraction of the commercial ripe Pu-er tea led to the isolation of 22 compounds, including seven catechin derivatives (**1**−**7**), ten flavonoid glycosides (**8**−**17**), two hydrolyzable tannins (**18** and **19**), and two quinic acid derivatives (**20** and **21**), along with 7-methylxanthine (**22**). Most of the isolates were tested for their acute activities on insulin sensitivity in differentiated 3T3-L1 adipocytes. Herein, this paper describes the isolation, structure determination of the new compounds **1**−**4**, and insulin sensitivity activities of compounds **4**, **6**−**17**, and **21**−**22**.

MATERIALS AND METHODS

Generals. Column chromatography was performed on 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.). Semipreparative HPLC was performed using a Zorbax SB-C_{18} (Agilent, America) column (9.6 i.d. × 250 mm); the flow rate used was 5.0 mL/min, and the separation and detection were achieved using Waters 600 separation module and Waters 2487 detector. Thin-layer chromatography (TLC) was performed on precoated silica gel H plates, 0.2−0.25 mm thick (Qingdao Haiyang Chemical Co.), with benzene-ethyl formate-formic acid (2:7:1 or 3:6:1 v/v/v) or $CHCl_3$-MeOH-H_2O (7:3:0.5, v/v/v), and spots were detected by spraying with 2% ethanolic $FeCl_3$ or anisaldehyde-H_2SO_4 reagent followed by heating. ^{1}H, ^{13}C NMR, and 2D NMR spectra were recorded in acetone-d_6, methanol-d_4, or DMSO-d_6 with Bruker DRX-500 or Varian AV-600 spectrometers operating at 500 or 600 MHz for ^{1}H NMR and 125 or 150 MHz for ^{13}C NMR. Coupling constants were expressed in Hz and chemical shifts were given on a δ (ppm) scale. ESIMS and HRESIMS were recorded on an API QSTAR Pular-1 mass spectrometer. Optical rotations were obtained on a JASCO P-1020 polarimeter. UV spectroscopic data were measured on a Shimadzu-210A double-beam spectrophotometer. IR spectra of samples in KBr discs were recorded on a Bruker-Tensor-27 spectrometer with KBr pellets. CD spectra were obtained on a JASCO 810 spectrometer. Analytical HPLC was operated on a Waters 2695 separation module combined with the accessory of the Waters 2996 photodiode array detector, using a Zorbax SB-C_{18} (Agilent, America) column (4.6 i.d. × 250 mm). Water was purified in a Milli-Q (Millipore, America).

Received: July 31, 2014
Revised: November 18, 2014
Accepted: November 19, 2014
Published: November 19, 2014

dx.doi.org/10.1021/jf5036959 | *J. Agric. Food Chem.* 2014, 62, 12229−12234

Acetonitrile and methanol (chromatographic grade) were purchased from Merck (Darmstadt, FR, Germany).

Materials. Ripe Pu-er tea, produced from the crude green tea prepared with leaves of *C. sinensis* var. *assamica*, was provided by Menghai Tea Factory, Yunnan Province, China, in 2007. These materials were identified by Prof. Ying-Jun Zhang, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation. Ripe Pu-er tea (1.0 kg) was extracted with hot water (10 L, 100 °C for 30 min) for three times and filtered. The combined filtrate was concentrated to small volume (2 L) and then partitioned with $CHCl_3$, EtOAc, and *n*-BuOH, consecutively. The *n*-BuOH fraction (56.8 g) was applied to a MCI gel CHP20P column chromatography (CC), eluting with water containing increased proportions of MeOH (10% stepwise gradient, each 700 mL) to give six fractions 1−6. Fraction 2 (4.7 g) was chromatographed over MCI gel CHP20P (0−20% MeOH), Toyopearl HW-40F (0−40% MeOH), and Sephadex LH-20 (100% EtOH) columns to afford compound **20** (10.0 mg). Fraction 3 (6.0 g) was chromatographed over MCI gel CHP20P (0−20% MeOH) to yield five subfractions, Fr.3−1 to Fr.3−5. Fr.3−2 was repeatedly subjected to CC over Toyopearl HW-40F (0−20% MeOH) and MCI gel CHP20P (0−20% MeOH) to yield compound **21** (7.0 mg). Fr.3−4 was subjected to CC over Toyopearl HW-40F (0−20% MeOH) and MCI gel CHP20P (0−20% MeOH) to yield compound **18** (7.0 mg). Fr.3−5 was chromatographed over MCI gel CHP20P (30%−100% MeOH) and Toyopearl HW-40F (0−20% MeOH) to yield compound **19** (7.0 mg). Fraction 4 (14.3 g) was chromatographed over Sephadex LH-20 (0−50% MeOH) to yield six subfractions, Fr.4−1 to Fr.4−6.Fr.4−3 was chromatographed repeatedly over MCI gel CHP20P (10%−60% MeOH) and Toyopearl HW-40F (0−20% MeOH) to yield compounds **6** (26.0 mg), 7 (52.0 mg). and **8** (6.0 mg). Fr.4−4, 4−5 and 4−6 were repeatedly subjected to CC over Toyopearl HW-40F (0−30% MeOH), MCI gel CHP 20P (10%−30% MeOH), and semipreparative HPLC (26% MeOH) to yield compounds **1** (5.0 mg), **2** (3.0 mg), **3** (10.0 mg), **4** (60.0 mg), and **5** (9.0 mg). Fraction 5 (14.8 g) was chromatographed over Sephadex LH-20 (30−100% MeOH) to yield four subfractions, Fr.5−1 to Fr.5−4. Fr.5−1 was subjected to CC over Toyopearl HW-40F (40−70% MeOH), MCI gel CHP20P (30−70% MeOH) and silica gel ($CHCl_3$−MeOH−H_2O, 8:2:0.2−7.5:2.5:0.2) to yield compounds **9** (18.0 mg), **10** (9.0 mg), and **11** (10.0 mg). Fr.5−2 was repeatedly subjected to CC over Sephadex LH-20 (100% MeOH), MCI gel CHP20P (30−70% MeOH) and silica gel ($CHCl_3$−MeOH−H_2O, 8:2:0.2) to yield compounds **12** (24.0 mg), **13** (3.0 mg), and **22** (7.0 mg). Fr.5−4 was subjected to CC over MCI gel CHP20P (30−70% MeOH), and Toyopearl HW-40F (60−100% MeOH) to yield two mixtures of compounds **14** and **15** (24.0 mg), and **16** and **17** (22.0 mg).

*8-Carboxymethyl-(+)-Catechin (**1**).* A white amorphous powder; $[\alpha]_D^{16}$ +22.0° (*c* 0.18, MeOH); UV (MeOH), λ_{max} (logε): 282 (3.65), 204 (4.67) nm; IR (KBr) ν_{max} 3425, 2929, 2857, 1619, 1527, 1458, 1385, 1285, 1117 cm^{-1}; ^1H (600 MHz, acetone-d_6) and ^{13}C NMR (150 MHz, acetone-d_6) data, see Tables 1 and 2, respectively; ESIMS (negative ion mode) *m*/*z* 347 $[M-H]^-$, HRESIMS *m*/*z* 347.0768 $[M-H]^-$ (calcd for $C_{17}H_{15}O_8$, 347.0772).

*8-Carboxymethyl-(+)-Catechin Methyl Ester (**2**).* A white amorphous powder; $[\alpha]_D^{16}$ +21.6° (*c* 0.12, MeOH); UV (MeOH), λ_{max} (logε): 281 (3.67), 204 (4.76) nm; IR (KBr) ν_{max} 3442, 2927, 1621, 1526, 1457, 1383, 1284, 1202, 1115 cm^{-1}; ^1H (500 MHz, acetone-d_6) and ^{13}C NMR (125 MHz, acetone-d_6) data, see Tables 1 and 2, respectively; ESIMS (negative ion mode) *m*/*z* 361 $[M-H]^-$; HRESIMS *m*/*z* 361.0925 $[M-H]^-$ (calcd for $C_{18}H_{17}O_8$, 361.0928).

*6-Carboxymethyl-(+)-Catechin (**3**).* A white amorphous powder; $[\alpha]_D^{16}$ +7.6° (0.09, MeOH); UV (MeOH), λ_{max} (logε): 282 (3.68), 204 (4.71) nm; IR (KBr) ν_{max} 3440, 2926, 2854, 1618, 1528, 1454, 1385, 1286, 1198, 1118 cm^{-1}; ^1H (600 MHz, acetone-d_6) and ^{13}C NMR (150 MHz, acetone-d_6) data, see Tables 1 and 2, respectively; ESIMS (negative ion mode) *m*/*z* 347 $[M-H]^-$; HRESIMS *m*/*z* 347.0768 $[M-H]^-$ (calcd for $C_{17}H_{15}O_8$, 347.0772).

*6-Carboxyl-(−)-Gallocatechin (**4**).* A white amorphous powder; $[\alpha]_D^{16}$ −10.8° (*c* 0.19, MeOH); UV (MeOH), λ_{max} (logε): 260 (4.15), 217 (4.63), 195 (4.39) nm; IR (KBr) ν_{max} 3442, 2924, 2853, 1637, 1834, 1043 cm^{-1}; ^1H (500 MHz, acetone-d_6) and ^{13}C NMR (125 MHz, acetone-d_6) data, see Tables 1 and 2, respectively; ESIMS (negative ion mode) *m*/*z* 349 $[M-H]^-$; HRESIMS *m*/*z* 349.0552 $[M-H]^-$ (calcd for $C_{16}H_{13}O_9$, 349.0559).

Table 1. ^1H NMR Spectroscopic Data for 1−4 (in Acetone-d_6)

position	1	2	3	4
2	4.61 (d, 7.5)	4.56 (d, 7.5)	4.49 (d, 7.9)	4.42 (d, 8.0)
3	3.92 (ddd, 8.0, 7.5, 5.4)	3.92 (ddd, 8.2, 7.5, 5.3)	3.97 (ddd, 8.3, 7.9, 5.5)	3.93 (dd, 8.7, 4.9)
4	2.82 (dd, 16.0, 5.4)	2.78 (dd, 16.1, 5.3)	2.84 (dd, 15.8, 5.5)	2.67 (dd, 15.6, 4.9)
	2.52 (dd, 16.0, 8.0)	2.47 (dd, 16.1, 8.2)	2.50 (dd, 15.8, 8.3)	2.27 (dd, 15.6, 8.7)
6	6.10 (s)	6.05 (s)		
8			5.94 (s)	5.68 (s)
2′	6.87 (d, 1.9)	6.80 (s)	6.82 (d, 2.0)	6.42 (s)
5′	6.75 (d, 8.2)	6.74 (d, 8.2)	6.74 (d, 8.2)	
6′	6.71 (dd, 8.2, 1.9)	6.65 (d, 8.2)	6.42 (dd, 8.2, 2.0)	6.42 (s)
$-CH_2-$	3.51 (d, 16.7)	3.47 (d, 16.7)	3.49 (s)	
	3.43 (d, 16.7)	3.37 (d, 16.7)		
$-CH_3$		3.48 (s)		

Table 2. ^{13}C NMR Spectroscopic Data for 1−4 (in Acetone-d_6)

position	1	2	3	4
2	82.4	81.9	81.9	81.6
3	68.3	67.8	67.8	67.0
4	28.5	28.0	28.6	26.8
5	155.5	155.1	154.5	160.0
6	95.9	95.5	103.3	98.1
7	155.4	154.9	155.2	160.1
8	101.3	100.6	95.7	94.0
9	154.5	154.1	154.8	157.9
10	100.3	100.0	101.1	99.4
1′	132.1	131.6	131.5	130.4
2′	115.0	114.9	115.3	107.7
3′	145.6	145.2	145.4	145.7
4′	145.6	145.2	145.3	133.0
5′	115.7	115.7	115.9	145.7
6′	119.6	119.3	119.8	107.7
$-CH_2-$	29.0	28.8	29.8	
−COOR	174.8	174.3	176.3	177.3
$-CH_3$		51.9		

HPLC Analysis for Identification of the Constituents. The optimal mobile phase for analysis of fractions of *n*-BuOH fraction of Pu-er tea was a binary gradient elution system consisting of solvent A (Aceonitrile) and solvent B (Water containing 0.34% phosphoric acid). The gradient program used started from 4 to 40% of solvent A in solvent B within 40 min at a flow rate of 0.8 mL/min. The injection volume was 10 μL. The UV detection wavelength was monitored at 210 nm. The peaks were confirmed by the UV absorptions and retention times of authentic samples, which were 6.9 (**22**), 7.5 (gallic acid), 15.0 (**7**), 21.2 (**9**), 21.9 (**10**), 23.0 (**14**), 23.8 (**11**), 24.4 (**12**), 25.5 (**16**), and 26.9 (**13**) min, respectively (Figure 1).

Insulin Sensitivity Assay. Insulin sensitivity assays were performed using the method as reported previously.[7−9]

RESULTS AND DISCUSSION

Repeated column chromatography over MCI gel CHP20P, Sephadex LH-20, Toyopearl HW-40F and semi-preparative

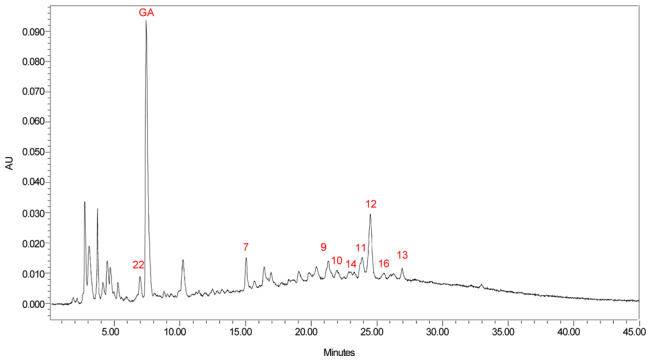

Figure 1. HPLC profile of *n*-BuOH-soluble fraction of ripe Pu-er tea (GA: gallic acid).

HPLC led to the isolation of four new flavan-3-ol derivatives **1**−**4** from the thearubigin (*n*-BuOH-soluble) fraction of the commercial ripe Pu-er tea. Moreover, 18 known compounds (**5**−**22**) were identified as three catechin derivatives, 8-carboxyl-(+)-catechin (**5**),[10] (+)-catechin-8-*C*-*β*-D-glucopyranoside (**6**),[11] and (−)-epicatechin-8-*C*-*β*-D-glucopyranoside (**7**),[11] 10 flavonoid glycosides, vicenin-2 (**8**),[12] isoschaftoside (**9**),[13] 2″-*O*-*β*-D-glucopyranosylvitexin (**10**),[14] quercetin 4′-*O*-*α*-L-rhamnopyranosyl-3-*O*-*α*-L-rhamnopyranosyl-(1 → 6)-*β*-D-glucopyranoside (**11**),[15] rutin (**12**),[16] nicotifiorin (**13**),[17] myricetin 3-*O*-*β*-D-glucopyranoside (**14**),[18] myricetin 3-*O*-*β*-D-galactopyranoside (**15**),[19] quercetin 3-*O*-*β*-D-glucopyranoside (**16**),[20] and quercetin 3-*O*-*β*-D-galactopyranoside (**17**),[21] two hydrolyzable tannins, 2-*O*-galloyl-D-glucose (**18**)[22] and 1-*O*-galloyl-*β*-D-glucopyranose (**19**),[23] two quinic acid derivatives, 3-*O*-galloylquinic acid (**20**)[24] and 3-*O*-caffeoylquinic acid (**21**),[25] and one purine alkaloid, 7-methylxanthine (**22**),[26] respectively, by comparing the spectra and physical data with those reported in the literature.

8-Carboxymethyl-(+)-catechin (**1**) was obtained as a white amorphous powder. Its molecular formula $C_{17}H_{16}O_8$ was established on the basis of the HRESIMS at *m*/*z* 347.0768 $[M-H]^-$ (calcd for $C_{17}H_{15}O_8$, 347.0772). The ^1H NMR spectrum of **1** showed the presence of three ABX coupled aromatic protons [*δ* 6.87 (d, *J* = 1.9 Hz, H-2′), 6.75 (d, *J* = 8.2 Hz H-5′), and 6.71 (dd, *J* = 8.2, 1.9 Hz, H-6′)], one aromatic singlet proton [*δ* 6.10 (*s*, H-6)], four catechin characteristic aliphatic protons [*δ* 4.61 (d, *J* = 7.5 Hz, H-2), 3.92 (ddd, *J* = 8.0, 7.5, 5.4 Hz, H-3), 2.82 (dd, *J* = 16.0, 5.4 Hz, H-4a), 2.52 (dd, *J* = 16.0, 8.0 Hz, H-4b)], and two mutually coupled aliphatic protons [*δ* 3.51, 3.43 (each 1H, *d*, *J* = 16.7, Hz)]. In ^{13}C NMR spectrum, 17 carbon signals were observed, and were further classified as two methylenes (δ_C 28.5, 29.0), four sp^2 methines (δ_C 95.9, 115.0, 115.7, 119.6), two sp^3 methines (δ_C 68.3, 82.4), one carboxylic carbon (δ_C 174.8) and other eight sp^2 quaternary carbons by HSQC and DEPT experiments. Comparison the ^1H and ^{13}C NMR data of **1** (Tables 1 and 2) with those of catechin,[27] indicated the presence of an additional carboxymethyl group in **1**, which should be substituted at C-6 or C-8 of the A-ring. The position of substitution was further identified by 2D NMR experiments. In the HMBC spectrum (Figure 2), the correlations from H-2 (δ_H 4.61) to C-9 (δ_C 154.5), and from H-4 (δ_H 2.82, 2.52) to C-5 (δ_C 155.5) and C-9, clearly assigned the signals of C-5 and C-9. The correlations of the additional methylene protons (δ_H 3.51, 3.43) with C-7 (δ_C 155.4), C-8 (δ_C 101.3), C-9 and the carboxylic carbon (δ_C 174.8) confirmed that the location of carboxymethyl unit was at

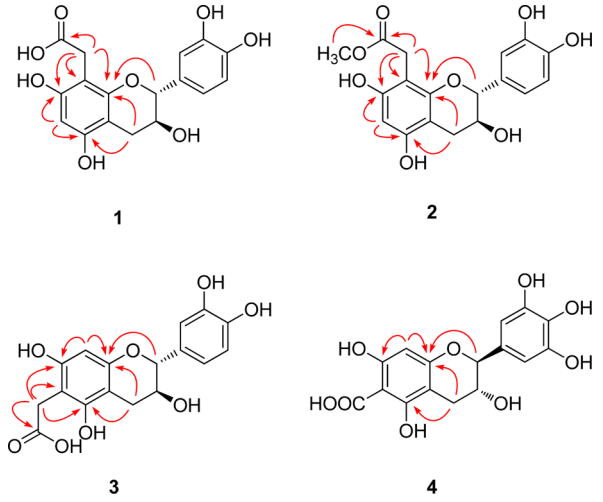

Figure 2. Key HMBC correlations (H → C) of **1**−**4**.

C-8. Other 2D NMR experiments further confirmed the planar structure of **1**, as shown in Figure 3. The coupling constant (7.5 Hz) between H-2 and H-3 indicated a 2,3-*trans* configuration. Further comparison the $[\alpha]_D$ of **1** ($[\alpha]_D^{16}$+ 22.0° (*c* 0.18, MeOH)) with (+)-catechin ($[\alpha]_D^{24}$ +15.5°(*c* 1.0, acetone))[27] and (−)-catechin ($[\alpha]_D$ −17.5° (*c* 0.16, MeOH))[28] revealed the 2*R*,3*S* absolute configurations in **1**. This can be confirmed by the CD spectrum, in which compound **1** gave a high-amplitude positive Cotton effect (CE) near 240 nm (Supporting Information Figure S17), corresponding to the ^1L_a electronic transition of the flavan-3-ols, consistent with 3*S*-configuration.[29] Based on the above evidence, the structure of **1** was determined to be 8-carboxymethyl-(+)-catechin.

8-Carboxymethyl-(+)-catechin methyl ester (**2**) was obtained as a white amorphous powder, possessing a molecular formula of $C_{18}H_{18}O_8$, as deduced from the HRESIMS measurement at *m*/*z* 361.0925 $[M-H]^-$ (calcd for $C_{18}H_{17}O_8$, 361.0928). The ^1H and ^{13}C NMR spectra of **2** (Tables 1 and 2) were closely resembled to those of **1**, except for an additional methoxy group (δ_H 3.48, δ_C 51.9) in **2**. In the HMBC spectrum of **2**, correlations of H-2 (δ_H 4.56, d, *J* = 7.5 Hz) with C-9 (δ_C 154.1), and the methylene protons (δ_H 3.47, 3.37) with C-7 (δ_C 154.9), C-8 (δ_C 100.6), C-9, and the carboxylic carbon (δ_C 174.3) confirmed that the carboxylmethyl group was attached to C-8. Moreover, the additional methoxy protons (δ_H 3.48) showed HMBC correlation with the carboxylic carbon (δ_C 174.3), revealing its linkage to the carboxyl group. Finally, the large coupling constant (7.5 Hz) between H-2 and H-3 in **2**, and the similar $[\alpha]_D$ value ($[\alpha]_D^{16}$ +21.6° (*c* 0.12, MeOH)) with **1**, suggested that **2** possesses the same 2*R*,3*S* configurations to **1**. In the CD spectrum, **2** showed a positive CE near 240 nm supported that **2** has a 2*R*,3*S*-configuration. Accordingly, compound **2** was determined as 8-carboxymethyl-(+)-catechin methyl ester.

6-Carboxymethyl-(+)-catechin (**3**), obtained as a white amorphous powder, has a same molecular formula of $C_{17}H_{16}O_8$ to **1**, as established by the HRESIMS (*m*/*z* 347.0768 $[M-H]^-$). The ^1H and ^{13}C NMR spectra of compound **3** were also closely resembled to those of **1**. In the HMBC spectrum of **3**, H-2 (δ_H 4.49) showed correlation with C-9 (δ_C 154.8), while H-4 (δ_H 2.84, 2.50) displayed correlations with C-5 (δ_C 154.5) and C-9 (δ_C 154.8). These led to the assignments of C-5 (δ_C 154.5), C-9 (δ_C 154.8), and C-7

dx.doi.org/10.1021/jf5036959 | J. Agric. Food Chem. 2014, 62, 12229−12234

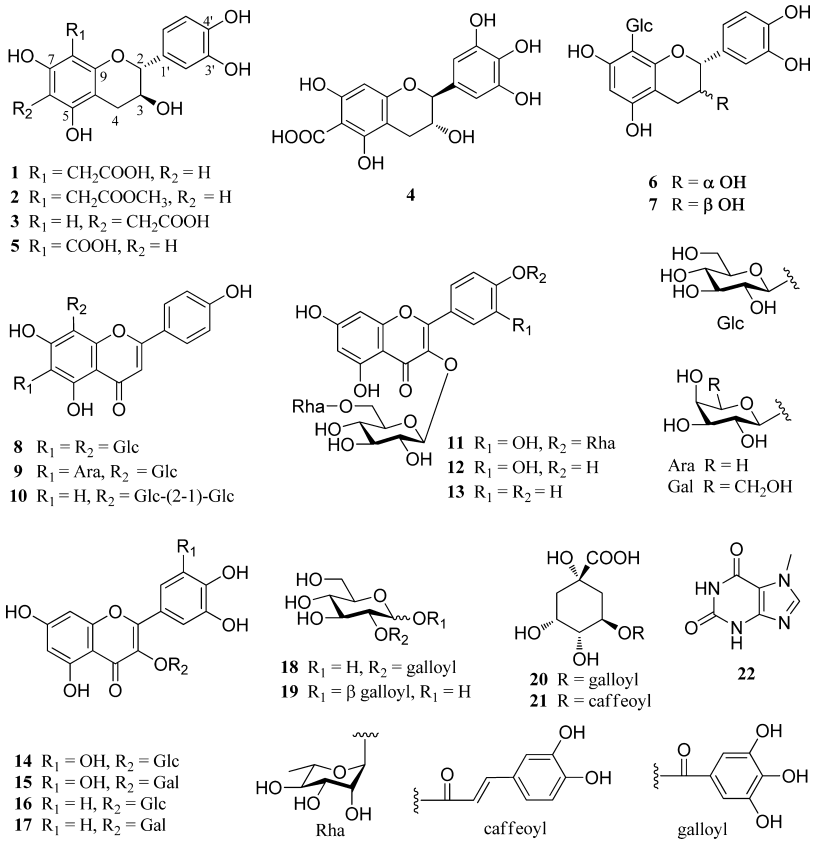

Figure 3. Compounds isolated from ripe Pu-er tea.

(δ_C 155.2) clearly. Moreover, the HMBC correlations of the carboxymethyl (δ_H 3.49) with C-5 (δ_C 154.5), C-7 (δ_C 155.2), and the carboxylic carbon (δ_C 176.3) revealed that the carboxymethyl group in **3** was attached to C-6 (Figure 2). Further analysis of HSQC and HMBC spectra of **3** confirmed its structure as shown in Figure 3. The similar $[\alpha]_D$ of **3** ($[\alpha]_D^{16}$ +7.6° (*c* 0.09, MeOH)) with **1**, together with the positive CE near 240 nm confirmed that **3** has the same 2*R*,3*S* configurations to **1**. Therefore, compound **3** was determined to be 6-carboxymethyl-(+)-catechin, the isomer of compound **1**.

6-Carboxyl-(−)-gallocatechin (**4**), obtained as a white amorphous powder, gave a quasi-molecular ion peak at *m*/*z* 349.0552 $[M-H]^-$ on the HRESIMS, corresponding to a molecular formula of $C_{16}H_{14}O_9$ for **4**. The molecular weight of **4** is 44 mass units larger than that of gallocatechin, corresponding to a carboxyl group. The ^1H and ^{13}C NMR data of **4** were very similar to those of gallocatechin,[27] except for the disappearance of one aromatic proton and the appearance of an additional carboxyl carbon (δ_C 177.3) signal. The above data suggested that one of the aromatic protons in gallocatechin was substituted by a carboxyl group in **4**. The position of substitution was identified by HMBC experiment. The HMBC correlations of H-2 (δ_H 4.42, d, *J* = 8.0 Hz) with the carbon at δ_C 157.9, and H-4 [δ_H 2.67 (dd, *J* = 15.6, 4.9 Hz), 2.27 (dd, *J* = 15.6, 8.7 Hz)] with the carbons at δ_C 160.0 and 157.9 could assign unambiguously these carbon signals as C-5 (δ_C 160.0) and C-9 (δ_C 157.9). Thus, an unresolved signal at δ_C 160.1 must be arisen from C-7. The HMBC correlations of aromatic proton at δ_H 5.68 with the C-7(δ_C 160.1) and C-9 (δ_C 157.9) indicated the position of aromatic proton to be H-8. Therefore, the carboxyl group was located on the C-6 position. A $J_{2,3}$ coupling constant of 8.0 Hz confirmed a 2,3-*trans* configuration. Further comparison the $[\alpha]_D$ of **4** ($[\alpha]_D^{16}$ −10.8° (*c* 0.19, MeOH)) with those of (−)-gallocatechin ($[\alpha]_D^{24}$ −11.2° (*c* 1.0, acetone))[27] and (+)-gallocatechin ($[\alpha]_D^{20}$ +150° (*c* 0.1, MeOH))[30] suggested that compound **4** has the same absolute configuration with (−)-gallocatechin, namely, 2*S*,3*R*-configuration. Moreover, **4** gave a high-amplitude negative CE near 240 nm, consistent with 3*R*-configuration. On the basis of the above evidence, the structure of **4** was deduced as 6-carboxyl-(−)-gallocatechin.

Thearubigin, having relative molecular weights between 700−4000 Da, is the main pigments in black tea considered generally as the *n*-BuOH soluble fraction. This fraction is presumed to be the polymers of catechins and/or theaflavins.[31] Inspired by concept of thearubigin in black tea, the *n*-BuOH soluble fraction of ripe Pu-er tea, the relative polar fraction after sequential solvent partitions, was qualitatively analyzed by HPLC analysis. As shown in Figure 1, compounds **7**, **9**−**14**, **16**, **22** and gallic acid (GA) were identified from the *n*-BuOH soluble fraction of ripe Pu-er tea, by comparison the retention time and UV profile with those of authentic samples under the same HPLC condition. The isolates constitute only a small portion of the *n*-BuOH-soluble ripe Pu-er tea pigments, albeit the visible peaks under wavelength of 210 nm in HPLC trace. The large unknown polymer pigments need to be further exploited.

Tea polyphenols from ripe Pu-er tea are reported to be active in antiobesity and antidiabetes,[32] of which, catechins are showing antiobesity and antidiabetes activities.[33] Most of the isolates, **4**, **6**−**17**, and **21**−**22** were tested for their acute activities on insulin sensitivity in differentiated 3T3-L1 adipocytes, but no significant activities were observed in these

dx.doi.org/10.1021/jf5036959 | J. Agric. Food Chem. 2014, 62, 12229−12234

compounds at a concentration of 10 μM. The above results suggested that the antidiabetes activity of catechins may not undergo the increasing insulin sensitivity.

In conclusion, the isolated phenolic compounds **1**−**21** could be divided into three groups, flavan-3-ol derivatives (**1**−**7**), flavonoid glycosides (**8**−**17**), and simple hydrolyzable tannins (**18**−**21**). Of them, **1**−**3** have additional carboxymethyl group in comparison with catechin. Compounds **4** and **5** are presumed to be formed through enzymatic Kolbe-Schmitt reaction from catechin.[34] Flavonoid glycosides **8**−**11** are reported from tea plants for the first time. Compound **11** have only been reported from *Zizyphus lotus* previously.[15] Simple hydrolyzable tannins (**18** and **19**) and quinic acid derivatives (**20** and **21**) were common constituents reported from green tea. The 7-methylxanthine (**22**) is the precursor of caffeine.[26] The *n*-BuOH-soluble fraction of ripe Pu-er tea was qualitatively analyzed by HPLC, from which nine compounds, **7**, **9**−**14**, **16**, **22** and gallic acid were identified by comparison the retention time and UV profile with those of authentic samples under the same HPLC condition, and gallic acid was found to be the major component. However, the compounds identified in HPLC trace only account for a small portion of *n*-BuOH-soluble fraction of ripe Pu-er tea pigment. The structure and function of the largely unknown polymer pigments are yet to be unveiled.

■ ASSOCIATED CONTENT

Supporting Information

The ^{1}H, ^{13}C, HSQC, HMBC, and CD spectra of compounds **1**−**4** in acetone-d_6 are available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +86-871-65223235. Fax: +86-871-65223235. E-mail: zhangyj@mail.kib.ac.cn.

Author Contributions

§L.-W.T. and M.-K.T. contributed equally to this paper.

Funding

This work was partly supported by the NSFC 21172224 and the 973 Program of Science and Technology of P. R. China (2011CB915503).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors are grateful to the staffs of the analytical group at State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, for measuring the spectroscopic data.

■ REFERENCES

(1) Yang, C. R.; Chen, K. K.; Zhang, Y. J. Classification of tea and definition of Pu-er tea. *Chaye Kexue Jishu* **2006**, *2*, 37−38 in Chinese.

(2) Drynan, J. W.; Clifford, M. N.; Obuchowicz, J.; Kuhnert, N. The chemistry of low molecular weight black tea polyphenols. *Nat. Prod. Rep.* **2010**, *27*, 417−462.

(3) Zhang, L.; Li, N.; Ma, Z. Z.; Tu, P. F. Comparison of the chemical constituents of aged pu-erh tea, ripened pu-erh tea, and other teas using HPLC-DAD-ESI-MSn. *J. Agric. Food Chem.* **2011**, *59*, 8754−8760.

(4) Jiang, H. Y.; Shii, T.; Matsuo, Y.; Tanaka, T.; Jiang, Z. H.; Kouno, I. A new catechin oxidation product and polymeric polyphenols of post-fermented tea. *Food Chem.* **2011**, *129*, 830−836.

(5) Zhou, Z. H.; Zhang, Y. J.; Xu, M.; Yang, C. R. Puerins A and B, two new 8-C substituted flavan-3-ols from Pu-er tea. *J. Agric. Food Chem.* **2005**, *53*, 8614−8617.

(6) Chen, H. F.; Takashi, T.; Gen Ichiro, N.; Toshihiro, F.; Kunihide, M. Phenylpropanoid-substituted catechins from *Castanopsis hystrix* and structure revision of cinchonains. *Phytochemistry* **1993**, *33*, 183−187.

(7) Prasad, C. N. V.; Anjana, T.; Banerji, A.; Gopalakrishnapillai, A. Gallic acid induces GLUT4 translation and glucose uptake activity in 3T3-L1 cells. *FEBS Lett.* **2010**, *584*, 531−536.

(8) Tan, M. J.; Ye, J. M.; Turner, N.; Hohnen-Behrens, C.; Ke, C. Q.; Tang, C. P.; Chen, T.; Weiss, H. C.; Gesing, E. R.; Rowland, A.; James, D. E.; Ye, Y. Antidiabetic activities of triterpenoids isolated from bitter melon associated with activation of the AMPK pathway. *Chem. Biol.* **2008**, *15*, 263−73.

(9) Xiong, W. Y.; Jordens, I.; Gonzalez, E.; McGraw, T. E. GLUT4 issorted to vesicles whose accumulation beneath and insertion into the plasma membrane are differentially regulated by insulin and selectively affected by insulin resistance. *Mol. Biol. Cell* **2010**, *21*, 1375−1386.

(10) Ballenegger, M. E.; Weith, A. J.; Rimbault, C. G.; Albert, A. I.; Courbat, P.; Tyson, R. G.; Thompson, D. G.; Palmer, D. R. Pharmceutical preparations containing (+)-cyanidan-3-ol derivatives, the use thereof, novel substituted (+)-cyanidan-3-ol derivatives, and processes for producing them. *Eur. Pat. Appl.* 96007A2. Dec. 7, 1983.

(11) Stark, T.; Hofmann, T. Application of a molecular sensory science approach to alkalized cocoa (*Theobroma cacao*): Structure determination and sensory activity of nonenzymatically *C*-glycosylated flavan-3-ols. *J. Agric. Food Chem.* **2006**, *54*, 9510−9521.

(12) Lu, Y. R.; Yeap Foo, L. Flavonoid and phenolic glycosides from *Salvia officinalis*. *Phytochemistry* **2000**, *55*, 263−267.

(13) Hilsenbeck, R. A.; Wright, S. J.; Mabry, T. J. *C*-Glycosylflavones from *Yeatesia viridiflora*. *J. Nat. Prod.* **1984**, *47*, 312−315.

(14) Segelman, A. B.; Segelman, F. P.; Star, A. E.; Wagner, H.; Seligmann, O. Structure of two *C*-diglycosylflavones from *Cannabis sativa*. *Phytochemistry* **1978**, *17*, 824−826.

(15) Maciuk, A.; Ghedira, K.; Thepenier, P.; Lavaud, C.; Zeches-Hanrot, M. A new flavonol glycoside from leaves of *Zizyphus lotus*. *Pharmize* **2003**, *58*, 158−159.

(16) Akbay, P.; Basaran, A. A.; Undeger, U.; Basaran, N. In vitro immunomodulatory activity of flavonoid glycosides from *Urtica dioica* L. *Phytother. Res.* **2003**, *17*, 34−37.

(17) Lee, H. B.; Kim, E. K.; Park, S. J.; Bang, S. G.; Kim, T. G.; Chung, D. W. Isolation and characterization of nicotiflorin obtained by enzymatic hydrolysis of two precursors in tea seed extract. *J. Agric. Food Chem.* **2010**, *58*, 4808−4813.

(18) Scharbert, S.; Holzmann, N.; Hofmann, T. Identification of the astringent taste compounds in black tea infusions by combining instrumental analysis and human bioresponse. *J. Agric. Food Chem.* **2004**, *52*, 3498−3508.

(19) Ye, G.; Huang, C. G. Flavonoids of *Limonium aureum*. *Chem. Nat. Compd.* **2006**, *42*, 232−234.

(20) Beck, M. A.; Haberlein, H. Flavonol glycosides from *Eschscholtzia californica*. *Phytochemistry* **1999**, *50*, 329−32.

(21) Yasukawa, K.; Takido, M. A flavonol glycoside from *Lysimachia Mauritiana*. *Phytochemistry* **1987**, *26*, 1224−1226.

(22) Haslam, E.; Uddin, M. The synthesis of 2- and 3-galloyl derivatives of arbutin. *Tetrahedron* **1968**, *24*, 4015−4020.

(23) Saijo, R.; Nonaka, G.-i.; Nishioka, I. Gallic acid esters of bergenin and norbergenin from *Mallotus japonicus*. *Phytochemistry* **1990**, *29*, 267−270.

(24) Zhou, Z. H.; Yang, C. R. Chemical constituents of crude green tea, the material of Pu-er tea in Yunnan. *Acta Bot. Yunnan.* **2000**, *22*, 343−350 in Chinese.

(25) Morishita, H.; Iwahashi, H.; Osaka, N.; Kido, R. Chromatographic separation and identification of naturally occurring chlorogenic acids by ^{1}H nuclear magnetic resonance spectroscopy and mass spectrometry. *J. Chromatogr. A* **1984**, *315*, 253−260.

(26) Ashihara, H.; Sano, H.; Crozier, A. Caffeine and related purine alkaloids: Biosynthesis, catabolism, function, and genetic engineering. *Phytochemistry* **2008**, *69*, 841−856.
(27) Seto, R.; Nakamura, H.; Nanjo, F.; Hara, Y. Preparation of epimers of tea catechins by heat treatment. *Biosci. Biotechnol. Biochem.* **1997**, *61*, 1434−1439.
(28) Cefarelli, G.; D'Abrosca, B.; Fiorentino, A.; Izzo, A.; Mastellone, C.; Pacifico, S.; Piscopo, V. Free-radical-scavenging and antioxidant activities of secondary metabolites from reddened cv. Annurca apple fruits. *J. Agric. Food Chem.* **2006**, *54*, 803−809.
(29) Slade, D.; Ferreira, D.; Marais, J. P. Circular dichroism, a powerful tool for the assessment of absolute configuration of flavonoids. *Phytochemistry* **2005**, *66*, 2177−215.
(30) Tian, Y. J.; Luo, Y. G.; Li, B. G.; Zhang, G. L. Chemical study on *Dendrobenthamia capitata* (Wall.) Hutch. *Nat. Prod. Res. Dev.* **2002**, *14*, 18−20.
(31) Haslam, E. Thoughts on thearubigins. *Phytochemistry* **2003**, *64*, 61−73.
(32) Zhang, D. Y.; Liu, Z. H.; Shi, Z. P.; Liu, Y. L.; Fu, D. H. Study on blood glucose-depressing and blood lipid-depressing function of Pu-er tea by high through screening method. *Chaye Kexue* **2005**, *26*, 49−53 in Chinese.
(33) Murase, T.; Misawa, K.; Haramizu, S.; Hase, T. Catechin-induced activation of the LKB1/AMP-activated protein kinase pathway. *Biochem. Pharmacol.* **2009**, *78*, 78−84.
(34) Kirimura, K.; Gunji, H.; Wakayama, R.; Hattori, T.; Ishii, Y. Enzymatic Kolbe−Schmitt reaction to form salicylic acid from phenol: Enzymatic characterization and gene identification of a novel enzyme, Trichosporon moniliiforme salicylic acid decarboxylase. *Biochem. Biophys. Res. Commun.* **2010**, *394*, 279−84.