JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Fuzhuanins A and B: The B-ring Fission Lactones of Flavan-3-ols from Fuzhuan Brick-Tea

Zhen-Mei Luo,[†,∥,§] Hai-Xia Du,[†,§] Li-Xiang Li,[†] Mao-Qiang An,[‡] Zheng-Zhu Zhang,[†] Xiao-Chun Wan,[†] Guan-Hu Bao,[†] Liang Zhang,[†] and Tie-Jun Ling*[,†]

[†]Key Laboratory of Tea Biochemistry and Biotechnology of Ministry of Education and Ministry of Agriculture, Anhui Agricultural University, 130 West Changjiang Road, Hefei 230036, P. R. China

[∥]Xiaogan Academy of Agricultural Sciences, 1 Yuanlin Road, Xiaogan 432000, Hubei, P. R. China

[‡]Yiyang Fu Cha Industry Development Co. Ltd., 690 North Datao Road, Yiyang 413000, P. R. China

Supporting Information

ABSTRACT: Fuzhuan brick-tea is a special dark tea prepared from the leaves of *Camellia sinensis* var. *sinensis*. Its production involves a fungal fermentation stage, which forms the unique flavors and functions by a series of biochemical reactions. Our phytochemical research of the material led to the isolation of two new B-ring fission lactones of flavan-3-ols, fuzhuanins A (**1**) and B (**2**). In addition, three other flavan-3-ol derivatives (**3**−**5**), three flavone *C*-glycosides (**6**−**8**), eight flavonoid *O*-glycosides (**10**−**17**), five simple phenolics (**19**−**23**), two norisoprenoid glycosides (**24**, **25**), two sesquiterpenoids (**26**, **27**), and theobromine (**28**), as well as two flavonoid anions (**9** and **18**), were also identified. The structures of these compounds were determined by spectroscopic methods. Compounds **4**, **19**, **20**, **22**−**24**, **26**, and **27** were reported for the first time in *Camellia* spp. and tea. Furthermore, HPLC analysis method was performed to compare the chemical constituents of the before/after fungal fermentation Fuzhuan brick-teas. Compound **1** was indicated as one of the major characteristic constituents generated in the fungal fermentation process. The IC_{50} value of the antiproliferative activity of **2** on HeLa cells was assayed as 4.48 μM. None of the isolated compounds showed any inhibition activity against the enteric pathogenic microbes at 800 μg/mL by the hole plate diffusion method.

KEYWORDS: *Fuzhuan brick-tea, dark tea, Camellia sinensis var. sinensis, fuzhuanin A, fuzhuanin B, B-ring fission lactones, HPLC analysis, antiproliferative activity, HeLa cells, enteric pathogenic microbes, antibacterial activity*

INTRODUCTION

As far as healthy benefits are concerned, tea is one of the best beverages in the world.[1−3] According to the manufacturing process, Chinese commercial teas are categorized as green tea, yellow tea, white tea, oolong tea, black tea, and dark tea.[1] The first occurrence of dark tea was documented in early 16th century in Ming Dynasty of China. In the following hundreds of years, several types of dark tea, such as Pu-erh tea, Qingzhuan brick-tea, Liubao tea, and Fuzhuan brick-tea, were developed. The manufacture of dark tea is unique for the microbial fermentation process, which is considered to be the key factor to form the special flavors and functions of dark tea.[1,4] Recently, dark teas are being paid more attention for the special functions of diabetic-healing, anti-HIV, antioxidation, and so on.[5−7] Some unique metabolites, such as cinchonain Ib and puerins A and B, have also been discovered in Pu-erh tea and other dark teas.[5,8]

As one of the major types of dark tea, Fuzhuan brick-tea (Figure 1) is known for remarkable antihyperlipidemia, antiobesity, antihyperglycemia, and antidysentery functions.[5,9−12] The manufacture of Fuzhuan brick-tea was described in details as panning, rapid pile fermentation, rolling, drying, softening with steam, piling, tea brick pressing, fungal fermentation, and drying.[4,13,14] Among these, fungal fermentation is the most important stage for the qualities of Fuzhuan brick-tea.[14,15] In this stage, the pretreated tea bricks are placed

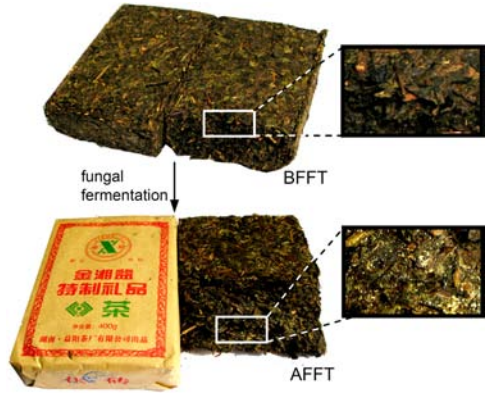

Figure 1. Pictures of BFFT and AFFT.

in a fungal fermentation workshop for two or three weeks. During this time, many fungi, such as *Eurotium* spp., *Debaryomyces* spp., and *Aspergillus* spp., grow dynamically within the tea leaves under controlled temperature and moisture conditions.[13] When the fungal fermentation process

Received: April 18, 2013
Revised: June 18, 2013
Accepted: June 18, 2013
Published: June 18, 2013

is terminated, the tea is predominated by "golden flora", i.e., *Eurotium* spp. (Figure 1).[13−15] Therefore, the bioactive compounds in Fuzhuan brick-tea are indicated as the metabolites of the complex micro-organisms.

As a part of our continuing phytochemical research on Fuzhuan brick-tea,[16,17] two new flavan-3-ol derivatives and other 26 constituents were isolated and identified. The isolated compounds were tested by hole plate diffusion method to evaluate the antibacterial activity. On the other hand, MTT method was applied to assay the antiproliferative activity of the new compounds (**1** and **2**) and other two flavan-3-ol derivatives (**3** and **4**). In addition, the principle constituents in the after fungal fermented Fuzhuan brick-tea (AFFT) were compared with those of the before fungal fermentation Fuzhuan brick-tea (BFFT, Figure 1) by HPLC analysis.

MATERIALS AND METHODS

General. UV spectra were obtained with a U-3010 spectrophotometer (Tokyo, Japan). Optical rotations were measured on a P-1020 polarimeter (Jasco, Tokyo, Japan). IR spectra were measured on a Thermo Nicolet 8700 FT-IR spectrophotometer. ^{1}H and ^{13}C NMR, ^{1}H−^{1}H COSY, HSQC, HMBC, and ROESY spectra were recorded with Bruker AM-400 spectrometers operating at 400 MHz for ^{1}H, and 100 MHz for ^{13}C. Coupling constants were expressed in Hz, and chemical shifts were given on a δ (ppm) scale with TMS as an internal standard. The HRESI-MS and HRAPCI-MS data were obtained with a Thermo LTQ Orbitrap XL LC/MS system by direct inlet. (Methanol was the solvent used for direct infusion. ESI conditions follow: sheath gas flow rate 20 arb; auxiliary gas flow rate 5 arb; capillary temperature 275 °C; isopray voltage 4.0 kV. APCI conditions follow: sheath gas flow rate 25 arb; auxiliary gas flow rate 10 arb; vaporizer temperature 250 °C; capillary temperature 275 °C; source current 5 μA.) The HPLC analysis was performed on a Waters 2695 separation module combined with a Waters 2489 UV detector. Separations were carried out using XP ODS-A C18 column (250 mm × 4.6 mm i.d., 5 μm, H&E Co. Ltd., P. R. China). Column temperature was set at 30 °C. The eluant was composed of mobile phase A (water containing 0.17% acetic acid) and mobile phase B (acetonitrile). The optimized gradient of mobile phase B was programmed as follows: 0−4 min, 6%; 4−16 min, from 6% to 14%; 16−22 min, from 14% to 15%; 22−32 min, from 15% to 18%; 32−37 min, from 18% to 29%; 37−45 min, from 29% to 45%; 45−50 min, 45%; 50−51 min, from 45% to 6%; then keep 6% for more 10 min. Elution was performed at a solvent flow rate of 1.0 mL/min. The injection volume was 10 μL. The UV detection wavelength was monitored at 280 nm. The preparative HPLC was performed on a Varian Prostar 325 HPLC instrument (Wakefield, RI) equipped with a Pursuit XRs 10 C_{18} 250 mm × 21.2 mm i.d. column, with 10% aqueous MeOH as the eluant. The flow rate was 15 mL/min. Detection was carried out at 254 nm. Silica gel 60 (200−300 mesh, Qingdao Marine Chemical CO. Ltd., Qingdao, P. R. China), Sephadex LH-20 (25−100 μm, Pharmacia Fine Chemical Co. Ltd., Sweden), YMC GEL ODS-A (50 μm, YMC Co. Ltd., Japan), polyamide (100−200 mesh, Luqiaosijia Biochemical Co. Ltd., Zhejiang, China), Toyopearl HW-40F (Tosoh Co. Ltd., Tokyo, Japan), and MCI-gel CHP20P (20−100 μm, Mitsubishi Chemical Co. Ltd., Japan) were used for column chromatography (CC). Polyamide CC was carried out with the eluant of 40% aqueous MeOH or ethyl acetate/methanol/H_2O (100:17:13, v/v). Aqueous MeOH from 0 to 100% (v/v) in increments of 10% was used as eluant for all Sephadex LH-20, Toyopearl HW-40F, and MCI-gel CC. Silica gel (GF254, Liangchen Chemical Co. Ltd., Huoshan, P. R. China) TLC experiments were performed with chloroform/methanol/H_2O (7:3:0.5, v/v), or ethyl acetate/methanol/H_2O (100:17:13, v/v), or benzene/ethyl formate/formoic acid (3:6:1 or 2:7:1, v/v), and spots were detected by UV illumination and detected by spraying with 0.2% ethanolic $FeCl_3$ or 10% sulfuric acid reagent followed by heating. Polyamide TLC (Taizhou Luqiao Sijia Biochemical Ltd., Taizhou, P. R. China) experiments were performed with chloroform/methanol/H_2O (7:3:0.5, v/v), or ethyl acetate/methanol/H_2O (100:17:13, v/v), with detection by spraying with 2% ethanolic $FeCl_3$.

Chemicals and Reagents. Noroxin was purchased from Shanghai Yan-an Pharmaceutical Co. Ltd., Shanghai, P. R. China. Berberine hydrochloride was purchased from Yabaoguangtai Pharmaceutical Co. Ltd., Pengzhou, Sichuan Province, P. R. China. The standards (purities > 98%) of gallic acid (GA), caffeine (CAF), (+)-catechin (C), (−)-epicatechin (EC), (+)-gallocatechin (GC), (−)-epigallocatechin (EGC), (+)-gallocatechin gallate (GCG), (−)-epigallocatechin gallate (EGCG), and (−)-epicatechin gallate (ECG) were purchased from Shanghai Winherb Medical Science Co. Ltd., Shanghai, P. R. China. Authentic samples **1**−**4** used for HPLC analysis were isolated in the present study. HPLC grade acetonitrile was purchased from Tedia (Philadelphia, PA). Beef extract and peptone were purchased from Beijing Aobo-xing Biotechnology Co. Ltd., Beijing, P. R. China. Agar powder was purchased form Hangzhou Microbial Reagent Co. Ltd., Hangzhou, Zhejiang Province, P. R. China. Sodium chloride and sodium hydroxide were purchased from Xuzhou Chemical Co. Ltd., Xuzhou, Jiangsu Province, P. R. China. 5-Fluorouracil, MTT, and DMSO in the antiproliferative assay were purchased from Sigma Chemical Co. Ltd.

Materials. Fuzhuan brick-tea for phytochemical research (produced in December 2006) was purchased from the major Fuzhuan brick-tea production factory, Yiyang Fu Cha Industry Development Co. Ltd., Yiyang, Hunan Province, P. R. China. The tea samples for HPLC analysis (BFFT and AFFT, respectively, produced in March and April 2012 as the same production batch, Figure 1) were supplied by the same company. The samples of AFFT and BFFT were stored in −20 °C before being prepared for HPLC analysis.

Extraction and Isolation. Fuzhuan brick-tea (3.6 kg) was processed as described before to obtain the *n*-BuOH fraction (500 g), which was fractionated by a Sephadex LH-20 CC to yield three subfractions (S1−S3).[16,17] After the isolation of 3*R*,9*R*-oxido-5-megastigmene,[17] the remaining parts of S1 (20 g) were integrated and fractionated by silica gel CC using CH_2Cl_2−MeOH−H_2O (18: 3: 0.5) as the eluant, resulting in 10 subfractions (S1-a to S1-j). Subfraction S1-d (30 mg) was separated by preparative HPLC to obtain **24** (3.0 mg) and **25** (8.0 mg). Subfraction S1-j (1.5 g) was subjected to a Sephadex LH-20 CC, yielding two subfractions (S1-ja, S1-jb). Subfraction S1-jb (46.7 mg) was subjected to an ODS CC eluted with methanol−H_2O (1:9) to yield **6** (5.5 mg), **7** (5.8 mg), and **18** (20.0 mg). Our previous work reported the isolation of isovitexin and astragalin from S2.[17] The remaining parts of S2 (30 g) were also combined and subjected to a MCI-gel CC to afford 11 subfractions (S2-a to S2-k). Subfraction S2-c (0.56 g) was subjected to repeated Sephadex LH-20 and Toyopearl HW-40F CC to yield **1** (84.8 mg), **21** (12.8 mg), **26** (4.4 mg), and **27** (3.0 mg). Subfraction S2-e (1.02 g) was subjected to Sephadex LH-20, Polyamide, and Toyopearl HW-40F CC to yield **5** (19.9 mg), **19** (12.0 mg), **22** (4.0 mg), and **28** (10.9 mg). Subfraction S2-g (3.04 g) was subjected to repeated Sephadex LH-20, ODS, Toyopearl HW-40F, and Polyamide CC to yield **2** (8.0 mg), **8** (112.3 mg), **9** (11.0 mg), **15** (23.4 mg), and **17** (45.2 mg). Further repeated CC over silica gel, Sephadex LH-20, Polyamide-gel, ODS, and Toyopearl HW-40F, as well as preparative HPLC, gave **3** (13.7 mg), **4** (6.6 mg), **10** (58.8 mg), **11** (25.1 mg), **12** (81.0 mg), **13** (86 mg), **14** (41.8 mg), **16** (13.0 mg), **20** (16.3 mg), and **23** (41.8 mg) from subfraction S2-i (1.52 g).

*Fuzhuanin A (**1**).* Colorless amorphous powder, $[\alpha]_D^{25}$ − 52 (*c* 0.0015, 75% EtOH). UV λ_{max} (MeOH) nm (log ε): 210.00 (4.66), 230.00 (3.98), 302.50 (3.85). IR (KBr) ν_{max} (cm^{-1}): 3265, 2853, 2650, 1702, 1645, 1629, 1564, 1514, 1471, 1381, 1326, 1289, 1241, 1205, 1182, 1154, 1107, 1048, 1018, 984, 892, 856, 830, 817, 776, 736, 719, 631, 515, 491, 458. ESI-MS: *m*/*z* 319 $[M - H]^-$, 639 $[2 M - H]^-$ in negative mode. HRESI-MS: *m*/*z* 321.059 45 ($[M + H]^+$, calcd for $C_{15}H_{13}O_8^+$, 321.060 49) in positive mode. For ^{1}H and ^{13}C NMR data, see Table 1.

*Fuzhuanin B (**2**).* Colorless gum, $[\alpha]_D^{25}$ + 97 (*c* 0.0096, MeOH). UV λ_{max} (MeOH) nm (log ε): 214.50 (3.89), 271.50 (2.62). IR (KBr) ν_{max} (cm^{-1}): 3420, 2970, 2925, 1710, 1629, 1609, 1520, 1470, 1381, 1314, 1265, 1208, 1182, 1149, 1108, 1063, 1042, 1019, 947, 823, 637, 586.

dx.doi.org/10.1021/jf401724w | J. Agric. Food Chem. 2013, 61, 6982−6990

Table 1. NMR Spectroscopic Data of 1 (in DMSO-d_6)

position	δ_C	δ_H (J in Hz)	HMBC (^{1}H to ^{13}C)
2	76.29	4.817 br s	C-3, 4, 8a, 1′, 2′, 6′
3	63.57	4.198 m	C-1′, 4a
4	27.90	2.724 dd (16.4, 4.4)	C-2, 3, 4a, 5, 8a
		2.527 dd (16.4, 2.8)	
4a	98.28		
5	156.51		
6	95.78	5.960 d (2.0)	C-4a, 5, 7, 8
7	156.69		
8	94.12	5.805 d (2.0)	C-4a, 6, 7, 8a
8a	154.50		
1′	157.47		
2′	111.04	6.247 t (1.2, 1.2)	C-2, 1′, 3′, 6′
3′	162.47		
4′	161.34		
5′	158.13		
6′	104.84	6.935 d (1.2)	C-2, 1′, 2′, 4′, 5′
3-O**H**		4.958 d (4.4)	C-2, 3, 4
5-O**H**		9.446 s	C-4a, 5, 6
7-O**H**		9.202 s	C-6, 7, 8

HRAPCI-MS: m/z 267.085 33 ([M + H]$^+$, calcd for $C_{13}H_{15}O_6^+$, 267.086 31) in positive mode. For ^{1}H and ^{13}C NMR data, see Table 2.

Table 2. NMR Spectroscopic Data of 2 (in DMSO-d_6)

position	δ_C	δ_H (J in Hz)	HMBC (^{1}H to ^{13}C)
2	73.55	3.807 br s	C-3, 4, 11, 12, 13
3	69.20	5.124 m	C-2, 4, 4a, 12
4	24.35	2.780 dd (17.6, 2.0)	C-2, 3, 4a, 5, 8a
		2.686 dd (17.6, 4.4)	C-2, 3, 4a, 5, 8a
4a	96.62		
5	156.43		
6	95.78	5.932 d (2.4)	C-4a, 5, 7, 8
7	156.63		
8	94.05	5.697 d (2.4)	C-4a, 6, 7, 8a
8a	154.04		
10	169.94		
11	39.85	2.410 d (18.0)	C-2, 10, 12, 13
		2.548 d (18.0)	C-2, 10, 12, 13
12	68.53		
13	25.31	1.333 s	C-2, 11, 12
5-O**H**		9.358 s	C-4a, 5
7-O**H**		9.032 s	C-6, 7, 8
12-O**H**		5.584 s	C-2, 11, 12, 13

Antibacterial Assays. Antibacterial activities were evaluated by the hole plate diffusion method as described before.[16] The test microorganisms were enteropathogenic *Escherichia coli* (EPEC), *Escherichia coli*, *Staphyloccocus aureus*, *Shigella dysenteriae*, and *Salmonella typhi*. Compounds **1**–**28**, noroxin (positive control-1), and berberine hydrochloride (positive control-2) were individually dissolved and diluted with DMSO to obtain serial concentrations of 800, 400, and 200, 100, 50, and 25 μg/mL. Seven 6 mm wide holes were bored with a sterilized steel borer into the nutrient agar media (beef extract 3 g, peptone 10 g, agar 17 g, NaCl 5 g, H_2O 1000 mL, pH 7.2) in each Petri dish (12 cm i.d.) inoculated with the test microorganism. The solution of the compound (60 μL) at a specific concentration was added into each of the holes. DMSO was used as the negative control. The plates were then incubated at 37 °C for 24 h. The inhibition zones around the holes were measured, and the minimal inhibitory concentration (MIC), which was defined as the lowest concentration able to inhibit any visible bacterial growth, was recorded. The assays were performed three times in order to guarantee reproducibility of results.

Cell Culture. HeLa cells, a generous gift from Dr. Zhijie Chang (Tsinghua University School of Medicine, Beijing, P. R. China), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, as well as 1% penicillin/streptomycin antibiotics. All the cells were kept at 37 °C in a 5% CO_2 containing atmosphere. Media and serum were purchased from Gibco (California).

Antiproliferative Assays. Antiproliferative assays of **1**–**4** were carried out by MTT colorimetric method, according to a reported method with slight modification.[18] Cells were plated in 96-well plates at a density of 8×10^4 cells/well and cultured for 24 h. 5-Fluorouracil (positive control) and **1**–**4** were, respectively, dissolved in DMSO to 10 mM, followed by 1000-fold dilution with DMEM to achieve concentration of 10 μM (containing 0.1% DMSO). For compound **2**, five serial 10-fold dilutions (10–0.001 μM) were prepared in DMEM. The sample solutions (200 μL each) were added in cells. The HeLa cells were reincubated for 24 h. Then, the medium was discarded, 100 μL of tetrazolium dye (MTT) solution (0.5 mg/mL) was added to each well, and the solution was incubated for an additional 4 h. Then, the medium was discarded again, and 100 μL aliquots of MTT dissolvent (10% SDS, 5% isobutanol in 0.01 M HCl) were added to each well. The plates were placed at 37 °C overnight to dissolve crystals, and read on a microplate reader at 560 nm using a Tecan Safire 2 microplate reader (Tecan, Switzerland). MTT solution with DMEM containing 0.1% DMSO (no compound), and with DMEM (no DMSO or compound), were, respectively, acted as negative and blank controls in microplate reading. Averages from three replicate wells were used for each sample and control, and each experiment was repeated three times. The averaged replicates for each compound at each concentration level were plotted against concentration. The IC_{50} values of fluorouracil and **2** were calculated by *FORECAST* function in Excel on the basis of the data acquired in the experiment.

Preparation of Tea Extracts for HPLC Analysis. The whole bricks of BFFT and AFFT were milled into powder and dehydrated, respectively. Each of the tea samples (2.500 g) was saturated in 70% aqueous methanol (100 mL) for 12 h at room temperature, during which an ultrasonic bath was carried out twice for 15 min. The extract was filtered through a 0.22 μm membrane filter for HPLC analysis. The experiments were performed in triplicate, and the results of quantification analysis were expressed as mean values with standard deviations.

Method Validation for the Quantification of the Main Constituents. The linear calibration curves contained five different concentrations of each reference compound by a series of appropriate dilution with methanol. All calibration curves were constructed by plotting the peak areas of the standard substances versus the corresponding concentration of the injected standard solutions to yield the following regression equations and ranges for quantificative analysis: $y = 22\,226\,755.3536x - 1440.149$ ($r^2 = 0.9996$, 0.000 908–0.010 89 mg/mL, GA); $y = 1467\,768.6252x - 937.9076$ ($r^2 = 0.9990$, 0.001 842–0.0221 mg/mL, GC); $y = 10\,623\,560.4301x + 14\,413.8333$ ($r^2 = 0.9998$, 0.006 25–0.1 mg/mL, **1**); $y = 1589\,947.1491x - 3289.9146$ ($r^2 = 0.9993$, 0.027 667–0.332 mg/mL, EGC); $y = 6042\,305.8486x - 239.5995$ ($r^2 = 0.9993$, 0.001 842–0.0215 mg/mL, C); $y = 27\,089\,834.5843x - 48\,601.9941$ ($r^2 = 0.9995$, 0.0347–0.347 mg/mL, CAF); $y = 6638\,070.6131x - 376.2343$ ($r^2 = 0.9994$, 0.012 722–0.152 667 mg/mL, EC); $y = 11\,076\,497.0512x - 174\,682.4199$ ($r^2 = 0.9954$, 0.054 33–0.5433 mg/mL, EGCG); $y = 6355\,730\,041.9395x - 133\,272.4409$ ($r^2 = 0.9988$, 0.0053–0.56 mg/mL, GCG); $y = 16\,296\,225.5893x - 254\,634.4686$ ($r^2 = 0.9947$, 0.0508–0.508 mg/mL, ECG).

Statistical Analysis. The data in Figure 5C,D and 7 were presented as means ±SD. The values were evaluated by the Student's t test using Excel software (Microsoft Software Inc.). Differences were considered as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

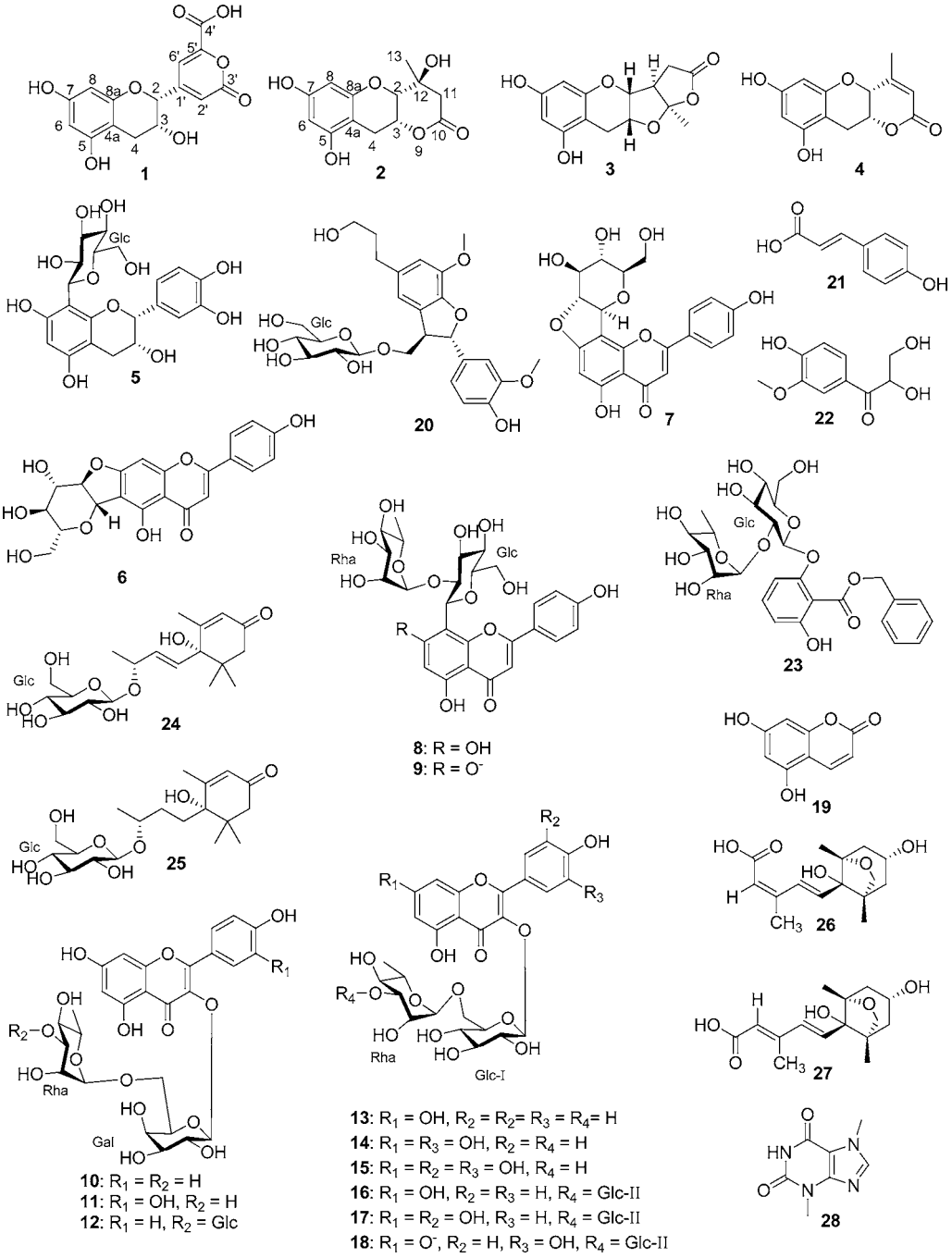

Figure 2. Structures of **1**−**28**.

RESULTS AND DISCUSSION

Isolation and Characterization. The 70% aqueous acetone extract of commercial Fuzhuan brick-tea was separated successively by partitioning with petroleum ether, $CHCl_3$, and *n*-BuOH. The *n*-BuOH fraction was separated by repeated silica gel, Sephadex LH-20, ODS, polyamide, MCI-gel CHP20P, and Toyopearl HW-40F CC. In addition to the 11 compounds reported in our previous paper,[17] two new flavan-3-ol derivatives, fuzhuanins A (**1**) and B (**2**), together with 24 known compounds and two flavonoid anions were yielded from this fraction. The known constituents were identified as three other flavan-3-ol derivatives, planchol A (**3**),[19] xanthocerin (**4**),[20] and (−)-epicatechin 8-*C*-*β*-D-glucopyranoside (**5**);[21] three flavone *C*-glycosides, chafurosides A (**6**) and B (**7**),[22] vitexin-2″-*α*-L-rhamnopyranoside (**8**);[23,24] eight flavonoid *O*-glycosides, biorobin (**10**),[25] quercetin-3-*O*-robinobioside (**11**),[26] kaempferol-3-*O*-[*β*-D-glucopyranosyl-(1→3)-*O*-*α*-L-rhamnopyranosyl-(1→6)-*O*-*β*-D-galactopyranoside] (**12**),[23] nicotiflorin (**13**),[27] rutin (**14**),[28] myricetin-3-*O*-rutinoside (**15**),[23,29] kaempferol-3-*O*-[*β*-D-glucopyranosyl-(1→3)-*O*-*α*-L-rhamnopyranosyl-(1→6)-*O*-*β*-D-glucopyranoside] (**16**),[23,30] quercetin-3-*O*-[*β*-D-glucopyranosyl-(1→3)-*O*-*α*-L-rhamnopyranosyl-(1→6)-*O*-*β*-D-glucopyranoside] (**17**);[31] five simple phenolic compounds, 5,7-dihydroxycoumarin (**19**),[32] (7*R*,8*S*)-dihydrodehydrodiconiferyl alcohol 9-*O*-*β*-D-glucopyranoside (**20**),[33] *p*-coumaric acid (**21**),[34] 2,3-dihydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one (**22**),[35] benzyl 2-neohesperidosyloxy-6-hydroxybenzoate (**23**);[36] two norisoprenoid glyco-

dx.doi.org/10.1021/jf401724w | J. Agric. Food Chem. 2013, 61, 6982−6990

sides, roseoside (**24**)[37] and icariside B_5 (**25**);[38] two sesquiterpenoids, dihydrophaseic acid (**26**)[39] and its isomer, namely 5-(3,8-dihydroxy-1,5-dimethyl-6-oxabicyclo[3.2.1]oct-8-yl)-3-methyl-2(*E*),4(*E*)-pentadienoic acid (**27**);[40] as well as theobromine (**28**).[41] The two flavonoid anions were identified as vitexin-2″-*α*-L-rhamnopyranoside-7-oxygen anion (**9**) (Supporting Information) and quercetin-3-*O*-[*β*-D-glucopyranosyl-(1→3)-*O*-*α*-L-rhamnopyranosyl-(1→6)-*O*-*β*-D-glucopyranoside]-7-oxygen anion (**18**) (Supporting Information), respectively (Figure 2).

Compound **1** was isolated as a colorless amorphous powder. Its HRESI-MS spectrum showed the $[M + H]^+$ ion at *m*/*z* 321.05945 (calcd 321.06049) corresponding to the molecular formula of $C_{15}H_{12}O_8$, with 10 degrees of unsaturation. The ^1H NMR spectrum of **1** showed typical proton signals for rings A and C of EC/EGC, at *δ* 4.817 (br s, H-2), 4.198 (m, H-3), 2.724 (dd, *J* = 16.4, 4.4 Hz, H-4a), 2.527 (dd, *J* = 16.4, 2.8 Hz, H-4b), 5.960 (d, *J* = 2.0 Hz, H-6), and 5.805 (d, *J* = 2.0 Hz, H-8), respectively.[42] The proton signals for ring B of EC/EGC were not observed in the spectrum. Instead, it showed two signals at *δ* 6.247 (1H, t, *J* = 1.2, 1.2 Hz) and 6.935 (1H, d, *J* = 1.2 Hz). Correspondingly, the ^{13}C NMR spectrum of **1** also revealed identical features of rings A and C for EC/EGC. Beside these, the spectrum showed additional signals of two tertiary carbons at *δ* 111.04 and 104.84, as well as four quaternary carbons at *δ* 157.47, 158.13, 161.34, and 162.47, respectively. This information suggested **1** to be a derivative of EC/EGC with modified B ring (MB). Considering the six carbons, three protons, and four oxygens (5 degrees of unsaturation) of MB, as well as the above NMR data, the moiety was supposed to be a ring with two carbonyl groups, which were supported by the IR absorption at 1702 cm^{-1}. In the HMBC spectrum of **1**, the proton signal at *δ* 4.817 (H-2) showed cross peaks with the ^{13}C signals at *δ* 157.47 (C-1′), 111.04 (C-2′), and 104.84 (C-6′), respectively (Figure 3). The ^{13}C signal at *δ* 111.04 (C-2′) showed correlation with the proton signal at *δ* 6.247 (H-2′) in the HSQC spectrum. The latter showed long-range correlations with the carbon signals at *δ* 157.47 (C-1′), 104.84 (C-6′), and 162.47 (C-3′), respectively (Figure 3). The HSQC spectrum also showed another correlation between the ^{13}C signal of *δ* 104.84 (C-6′) and the ^1H signal of *δ* 6.935 (H-6′), which was observed to have cross peaks with the carbon signals of *δ* 157.47 (C-1′), 111.04 (C-2′), 161.34 (C-4′), and 158.13 (C-5′), respectively, in the HMBC spectrum (Figure 3). Two possible structures of MB (MB-I, MB-II, Figure 3) were then deduced by combined analysis of the above information. These substructures were supported by the $^1H-^1H$ COSY correlations of H-2/H-2′/H-6′ (Supporting Information), which were caused by long-range couplings of allylic group system. MB-I, which is a seven-membered cyclic anhydride, was reported previously as an oxidation product of EC,[43] with characteristic NMR data of *δ* 114.2 and 108.2 for C-2′ and -6′, respectively (Figure 3). However, the HSQC and HMBC spectra of **1** revealed the NMR data of *δ* 111.04 and 104.84 for the two carbons, respectively. On the other hand, the anhydride carbonyl band to lower frequencies of 1724 cm^{-1}, which was caused by the ortho hydroxyl group,[43] was not observed in the IR spectrum of **1**. Therefore, the B ring of **1** was determined as MB-II (2-oxo-2*H*-pyran-6-carboxylic acid). The signal shape of the C-ring protons H-2 and H-3 in the ^1H NMR spectrum and the optical rotation of the compound indicated an unchanged stereochemistry in positions 2 and 3 compared to EC/EGC (Supporting Information).[43,44] On the base of the above evidence, the structure of **1** was determined and was shown in Figure 2. The compound was trivially named as fuzhuanin A.

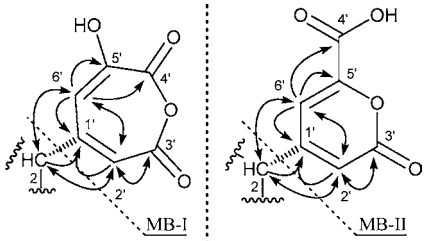

Figure 3. HMBC (^1H to ^{13}C) correlations and ^{13}C NMR data of MB-I and MB-II (#: ref 44).

Compound **2** was isolated as a colorless gum. Its molecular formula was confirmed as $C_{13}H_{14}O_6$ by the HRAPCI-MS data of 267.08533 (calcd for $C_{13}H_{15}O_6^+$, 267. 08631). Compound **2** was also suggested as a derivative of flavan-3-ols, by analysis of the characteristic proton signals at *δ* 5.932, 5.697 (both d, *J* = 2.4 Hz, H-6 and H-8), and *δ* 5.124 (m, H-3 in ester-form catechins), as well as *δ* 2.780 (dd, *J* = 17.6, 2.0 Hz, H-4a) and 2.686 (dd, *J* = 17.6, 4.4 Hz, H-4b) in the ^1H NMR spectrum (Supporting Information).[42] But it is interesting that the signal of H-2, which was identified by $^1H-^1H$ COSY correlation with *δ* 5.124 (H-3), was upfield shifted to *δ* 3.807. Furthermore, the signals of B-ring of normal catechins/epicatechins were not observed in the NMR spectra. Instead, the ^1H NMR spectrum showed signals for a methyl (*δ* 1.333, s), and a pair of germinal coupling protons (*δ* 2.410, 2.548, both d, *J* = 18.0 Hz). Also, the ^{13}C NMR showed signals of one CH_2 (*δ* 39.85) and one CH_3 (*δ* 25.31), as well as an oxygen-bearing quaternary carbon (*δ* 68.53) and an ester carbonyl (169.94). These information indicated **2** as a water addition derivative of **4**, which is another B-ring fission lactone derivative of flavan-3-ols.[20] The skeleton was confirmed by the HMBC analysis (Figure 4). In this spectrum, the signal of *δ* 3.807 (H-2) showed correlations with *δ* 39.85 (C-11), 68.53 (C-12), and 25.31 (C-13), respectively. The proton signal of *δ* 1.333 (H_3-13) showed long-range correlations with the carbon signals of *δ* 73.55 (C-2), 39.85 (C-11), and 68.53 (C-12), respectively. Also, both of the proton signals of *δ* 2.410, 2.548 (H_2-11) showed cross peaks with the carbon signals of *δ* 73.55 (C-2), 169.94 (C-10), 68.53 (C-12), and 25.31 (C-13), respectively, in the HMBC spectrum. The information of 1D and 2D NMR spectra was carried out to determine the stereochemistry of the compound. The signal shape of H-2 (broad singlet) and H-3 (m), and the observed cross-peak between the two protons in the ROESY spectrum, suggested a *cis* relation for H-2 and H-3 (Figure 4 and Supporting Information). The orientation of OH-12 was determined to be the same as those of H-2 and H-3, by the clear ROESY correlation between H-2 (*δ* 3.807), H-3 (*δ* 5.124), and O**H**-12 (*δ* 5.584) (Figure 4). Therefore, the stereochemistry of compound **2** was determined as

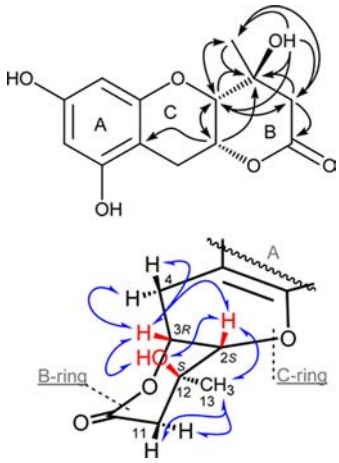

Figure 4. Key HMBC (black →, ^{1}H to ^{13}C) and NOESY (blue ↔) correlations of **2**.

rel.2*S*,3*R*,12*S*. The structure of **2** was identified as presented in Figure 2. The compound was named as fuzhuanin B.

Antiproliferative Assay on the Tumor Cells. The antiproliferative activities of **1**−**4** were evaluated by MTT methods. Before the assay for IC_{50} data, compounds **1**−**4** were preliminary surveyed for the potential antiproliferative activity on HeLa cells at 10 μM (Figure 5C). Fuzhuanin A (**1**) and xanthocerin (**4**) did not show any inhibition activity. Planchol A (**3**) showed moderate inhibition activity against the HeLa cells. Fuzhuanin B (**2**) showed significant antiproliferative activity as the cell viability was less than 75% of that of the negative control (Figure 5B,C). The inhibition activities of **2** (concentrations 10−0.001 μM) were then evaluated (Figure 5D). The IC_{50} value of the antiproliferative activity of **2** on HeLa cells was calculated as 4.48 μM (Supporting Information). As the result of positive control, the IC_{50} value of 5-fluorouracil was 1.68 μM.

Antibacterial Assay on Enteric Pathogenic Microbes. The antibacterial assay was performed by hole plate diffusion method. None of the isolated compounds showed any inhibition activity at the concentration of 800 μg/mL.

HPLC Analysis for Identification of the Main Constituents. The peaks were identified by the retention times of authentic samples, which were 6.28 (GA), 11.26 (GC), 14.51 (**1**), 16.63 (EGC), 18.47 (C), 18.81 (CAF), 22.97 (EC), 23.65 (EGCG), 26.09 (GCG), and 35.23 (ECG) min, respectively.[45] In addition, the peaks of fuzhuanin A (**1**) were confirmed by HPLC-DAD-MS method, in which the UV spectrum (200−400 nm) and MS of the chromatography peaks were compared with those of **1** (Supporting Information, S-Figure 2).

HPLC Quantitative Analysis. The main catechins, i.e., C, EC, GC, EGC, GCG, EGCG, and ECG, together with GA and CAF in BFFT and AFFT were characterized by the above method. The contents of these constituents were compared between BFFT and AFFT. As shown in Figures 6 and 7, the contents of GA, GC, EGC, GCG, EGCG, and ECG decreased markedly after fungal fermentation procedure. While the content of EC increased. The contents of C and CAF were not significantly different between BFFT and AFFT. It is interesting that the chromatographic peak of compound **1** was characterized in the HPLC of AFFT, but not in that of BFFT

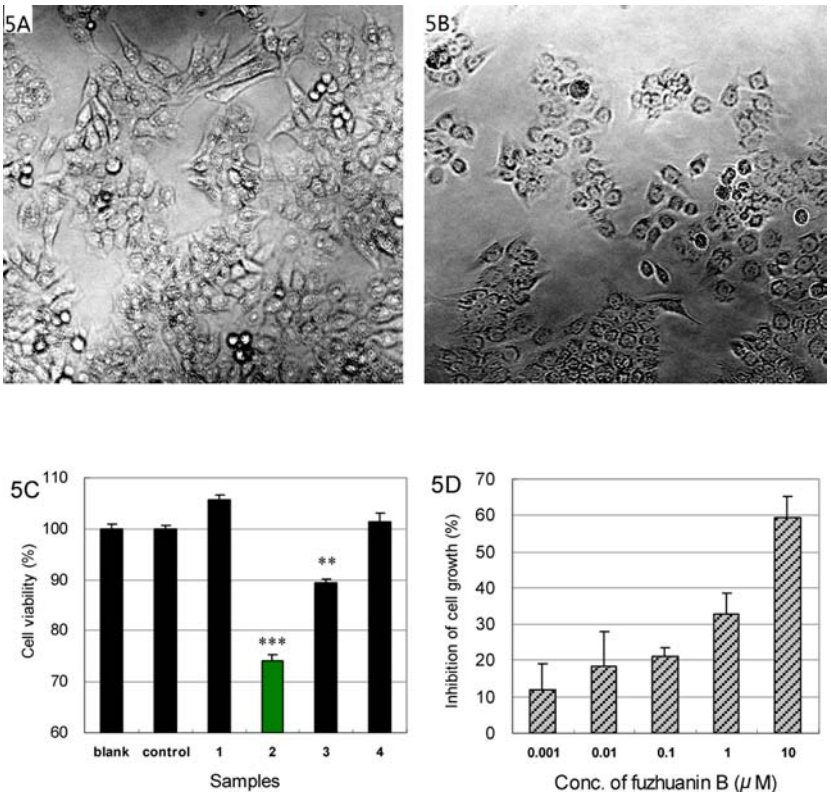

Figure 5. Results of antiproliferative assay of **1**−**4** on HeLa cells. Parts A and B showed effects of fuzhuanin B (**2**) on proliferation of HeLa cells (examined by light microscopy at 200× magnification) (5A, treatment with negative control for 24 h; 5B, treatment with **2** at 10 μM for 24 h). Part C showed the viability of HeLa cells after treatment of **1**−**4** for 24 h at 10 μM (**, $p < 0.01$ vs control; ***, $p < 0.001$ vs control). Part D showed the inhibition on HeLa cell growth with five serial concentrations of **2**.

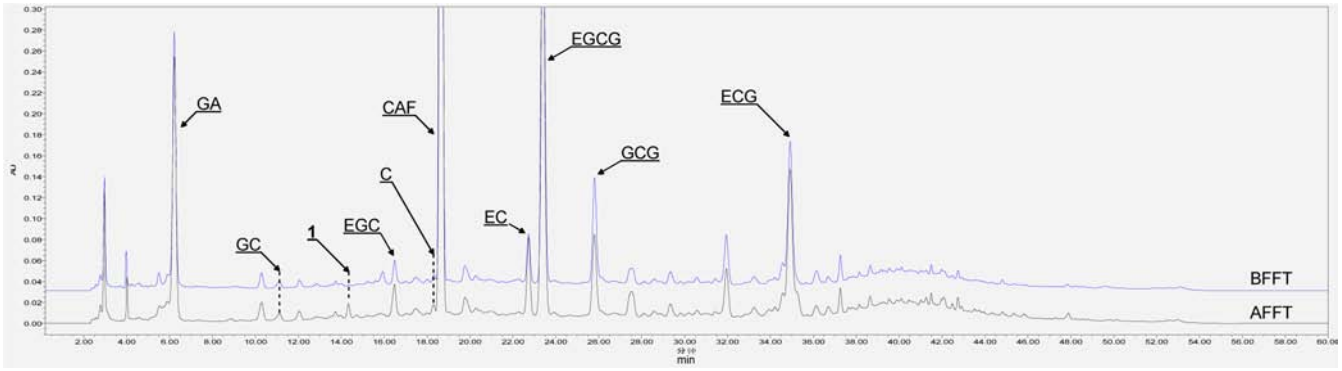

Figure 6. HPLC of BFFT and AFFT.

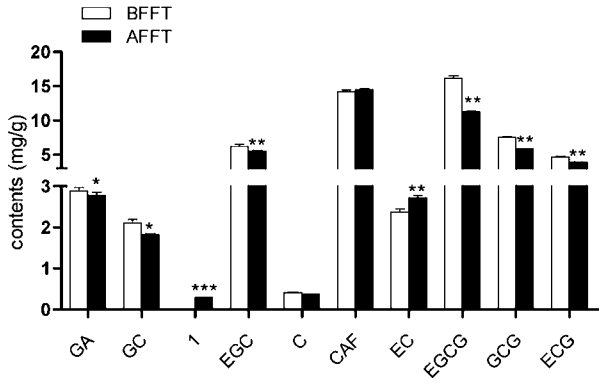

Figure 7. Quantification analysis of the main compounds in BFFT and AFFT. Differences were considered between BFFT and AFFT (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

(Figure 6 and Supporting Information), indicating **1** as one of the major characteristic constituents generated in fungal fermentation process of Fuzhuan brick-tea. The content of **1** in AFFT was 0.285 ± 0.0097 mg/g.

The proposed formation mechanisms of **1**−**4** were shown in Figure 8. The biochemical changes are rather complex in the production of Fuzhuan brick-tea, especially in the fungal fermentation process. In this stage, a complicated dynamic microbial ecosystem is established by many micro-organisms, including some dominant fungi such as *Eurotium* spp., *Debaryomyces* spp., and *Aspergillus* spp.[13] The microenvironment undoubtedly leads to a rather complicated phytochemical feature in fermented Fuzhuan brick-tea. On the other hand, some other similar flavan-3-ol derivatives were reported as the metabolites of EC or EGC by fermentation of teas or flavan-3-ols with fungi.[43−49] Therefore, compounds **2**−**4** were also

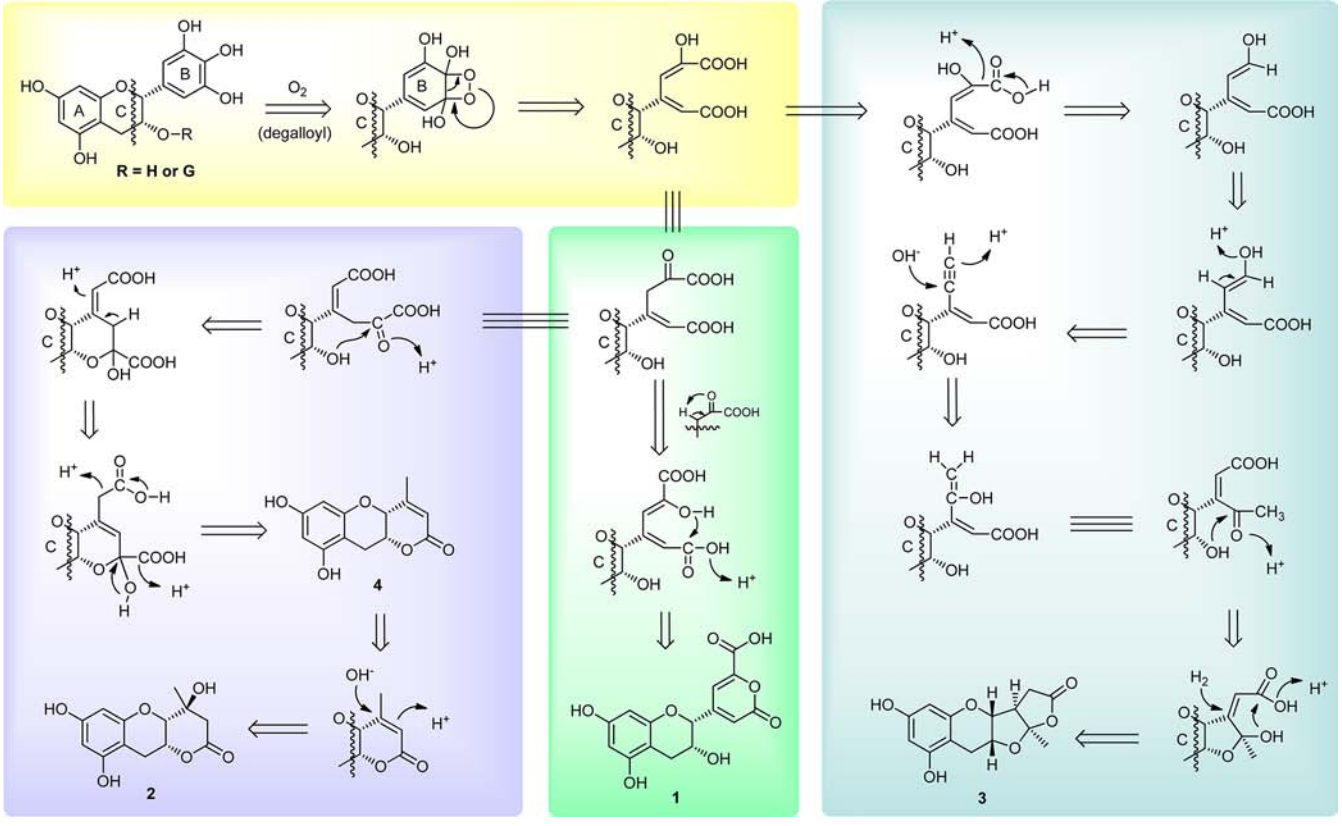

Figure 8. Proposed mechanisms for the formation of compounds **1**−**4**.

dx.doi.org/10.1021/jf401724w | J. Agric. Food Chem. 2013, 61, 6982−6990

supposed to be formed in the production process of Fuzhuan brick-tea, though these compounds were not able to be detected clearly in the HPLC analysis of BFFT or AFFT yet (Supporting Information).

In the present study, two new B-ring fission lactones of flavan-3-ols [fuzhuanins A (**1**) and B (**2**)] were isolated from fermented Fuzhuan brick-tea, together with other 24 known compounds, as well as two flavonoid anions. Fuzhuanin A was characterized as one of the major constituents generated in fungal fermentation process of Fuzhuan brick-tea. Fuzhuanin B showed significant antiproliferative activity against HeLa cell line with IC_{50} value of 4.48 μM. Despite the weak antibacterial activity of the isolated compounds, more biofunctional research should be performed to fuzhuanin A (**1**) and xanthocerin (**4**) for their potential proliferation on human cell (Figure 5C), such as stem cells. The antihyperlipidemia, antiobesity, antihyperglycemia, and AD (Alzheimer's disease)-healing activities of the compounds are also on the schedule of the research (ongoing experiments). In addition, several other unique chromatographic peaks were observed in the HPLC of AFFT compared to that of BFFT (Supporting Information, S-Figure 1), which indicated more interesting phytochemical work on this material in future.

■ ASSOCIATED CONTENT

Supporting Information

Spectra of **1**, **2**, **9**, **18**, the HPLC-UV and HPLC-PDAD-ESIMS of BFFT and AFFT, as well as the file about the results of IC_{50} of **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +86-551-65786401. Fax: +86-551-65786765. E-mail: lingtj@ahau.edu.cn.

Author Contributions

§These authors contributed equally to the paper.

Funding

This work was supported by Modern Agro-Industry Technology Research System, Ministry of Agriculture and Ministry of Finance, People's Republic of China, National Natural Science Foundation of China (Grant 31101335), and Natural Science Foundation of Anhui Province (Grant 10040606Q19), as well as Key Research Foundation of Science and Technology, Ministry of Education, P. R. China (Grant 209058).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Xin-Yi Liu, executive manager of Yiyang Fu Cha Industry Development Co. Ltd., for supply of the tea samples; Yu-Song Wang, University of Science and Technology of China, Chinese Academy of Sciences, for NMR measurements; and Prof. Hua-Rong Tan, Biotechnology Center of Anhui Agricultural University, for HPLC-DAD-MS measurements; as well as Jing Zhang, Key Laboratory of Tea Biochemistry and Biotechnology of Ministry of Education and Ministry of Agriculture, Anhui Agricultural University, for advice on HPLC analytical conditions.

■ ABBREVIATIONS USED

AFFT, the after fungal fermentation Fuzhuan brick-tea; BFFT, the before fungal fermentation Fuzhuan brick-tea; HPLC, high performance liquid chromatography; SI, Supporting Information; UV, ultraviolet; IR, infrared; NMR, nuclear magnetic resonance; COSY, correlated spectroscopy; HSQC, heteronuclear singular quantum coherence; HMBC, heteronuclear multiple bond coherence; NOESY, nuclear Overhauser effect spectroscopy; HR, high resolution; ESI-MS, electro-spray ionization mass spectrometry; APCI, atmospheric pressure chemical ionization

■ REFERENCES

(1) Wan, X. C. *Tea Biochemistry*, 3rd ed.; China Agriculture Press: Beijing, 2003; pp 268−275 (in Chinese).

(2) Yang, C. S.; Wang, X.; Lu, G.; Picinich, S. C. Cancer prevention by tea: Animal studies, molecular mechanisms and human relevance. *Nat. Rev. Cancer* **2009**, *9*, 429−439.

(3) Wang, Y.; Ho, C. T. Polyphenolic chemistry of tea and coffee: A century of progress. *J. Agric. Food Chem.* **2009**, *57*, 8109−8114.

(4) Jin, X. Y.; Chen, J. B.; Ji, K. W. *Tea Process Engineering*, 1st ed.; China Agriculture Press: Beijing, 2003; pp 45−125 (in Chinese).

(5) Zhang, L.; Zhang, Z. Z.; Zhou, Y. B.; Ling, T. J.; Wan, X. C. Chinese dark teas: Postfermentation, chemistry and biological activities. *Food Res. Int.* **2013**, in press. DOI: 10.1016/j.foodres.2013.01.016 (http://www.sciencedirect.com/science/article/pii/S0963996913000355).

(6) Du, W. H.; Peng, S. M.; Liu, Z. H.; Shi, L.; Tan, L. F.; Zou, X. Q. Hypoglycemic effect of the water extract of Pu-erh tea. *J. Agric. Food Chem.* **2012**, *60*, 10126−10132.

(7) Yamashita, Y.; Wang, L. H.; Zhang, T. S.; Nakamura, T.; Ashida, H. Fermented tea improves glucose intolerance in mice by enhancing translocation of glucose transporter 4 in skeletal muscle. *J. Agric. Food Chem.* **2012**, *60*, 11366−11371.

(8) 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.

(9) Yu, Z. Y.; Huang, J. A.; Yang, M. Z.; Zhang, Y.; Ou-Yang, M.; Fu, D. H. Research of the anti-diarrhea function of Fuzhuan tea. *Tea Sci.* **2009**, *29*, 465−469 (in Chinese).

(10) Wu, Y. Y.; Ding, L.; Xia, H. L.; Tu, Y. Y. Analysis of the major chemical compositions in Fuzhuan brick-tea and its effect on activities of pancreatic enzymes *in vitro*. *Afr. J. Biotechnol.* **2010**, *9*, 6748−6754.

(11) Fu, D. H.; Ryan, E. P.; Huang, J. A.; Liu, Z. H.; Weir, T. L.; Snook, R. L.; Ryan, T. P. Fermented *Camellia sinensis*, Fu Zhuan tea, regulates hyperlipidemia and transcription factors involved in lipid catabolism. *Food Res. Int.* **2011**, *44*, 2999−3005.

(12) Li, Q.; Liu, Z. H.; Huang, J. A.; Luo, G. A.; Liang, Q. L.; Wang, D.; Ye, X. Y.; Wu, C. B.; Wang, L. L.; Hu, J. H. Anti-obesity and hypolidemic effects of Fuzhuan brick tea water extract in high-fat diet-induced obese rats. *J. Sci. Food Agric.* **2013**, *93*, 1310−1316.

(13) Xu, A. Q.; Wang, Y. L.; Wen, J. Y.; Liu, P.; Liu, Z. Y.; Li, Z. J. Fungal community associated with fermentation and storage of Fuzhuan brick-tea. *Int. J. Food Microbiol.* **2011**, *146*, 14−22.

(14) Mo, H. Z.; Zhu, Y.; Chen, Z. M. Microbial fermented tea—A potential source of natural food preservatives. *Trends Food Sci. Technol.* **2008**, *19*, 124−130.

(15) Xu, X. Q.; Mo, H. Z.; Yan, M. C.; Zhu, Y. Analysis of characteristic aroma of fungal fermented Fuzhuan brick-tea by gas chromatography/mass spectrophotometry. *J. Sci. Food Agric.* **2007**, *87*, 1502−1504.

(16) Ling, T. J.; Wan, X. C.; Ling, W. W.; Zhang, Z. Z.; Xia, T.; Li, D. X.; Hou, R. Y. New triterpenoids and other constituents from a special microbial-fermented tea—Fuzhuan brick-tea. *J. Agric. Food Chem.* **2010**, *58*, 4945−4950.

(17) Luo, Z. M.; Ling, T. J.; Li, L. X.; Zhang, Z. Z.; Zhu, H. T.; Zhang, Y. J.; Wan, X. C. A new norisoprenoid and other compounds from Fuzhuan brick tea. *Molecules* **2012**, *17*, 3539−3546.
(18) Yang, S. G.; Zhang, X.; Sun, X. S.; Ling, T. J.; Feng, Y.; Du, X. Y.; Zhao, M.; Yang, Y.; Xue, D.; Wang, L.; Liu, R. T. Diverse ecdysterones show different effects on amyloid-β_{42} aggregation but all uniformly inhibit amyloid-β_{42}-induced cytotoxicity. *J. Alzheimers Dis.* **2010**, *22*, 107−117.
(19) Chang, J.; Case, R. Cytotoxic phenolic constituents from the roots of *Actinidia chinensis*. *Planta Med.* **2005**, *71*, 955−959.
(20) Zhang, W. X.; Bao, W. F. Studies on the chemical constituents of *Xanthoceras sorbifolia* Bunge. *Acta Pharmacol. Sin.* **2000**, *35*, 124−127 (in Chinese).
(21) Morimoto, S.; Nonaka, G. I.; Nishioka, I. Tannins and related compounds. XXXVIII.[1)] Isolation and characterization of flavan-3-ol glucosides and procyanidin oligomers from cassia bark (*Cinnamomum cassia* Blume). *Chem. Pharm. Bull.* **1986**, *34*, 633−642.
(22) Ishida, H.; Wakimoto, T.; Kitao, Y.; Tanaka, S.; Miyase, T.; Nukaya, H. Quantitation of chafurosides A and B in tea leaves and isolation of prechafurosides A and B from oolong tea leaves. *J. Agric. Food Chem.* **2009**, *57*, 6779−6786.
(23) Scharbert, S.; Holzmann, H.; 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.
(24) Sun, J. Y.; Yang, S. B.; Xie, H. X.; Li, G. H.; Qiu, H. X. Studies on chemical constituents from fruit of *Crataegus pinnatifida*. *Chin. Tradit. Herb. Drugs* **2002**, *33*, 483−486.
(25) Farkas, L.; Vermes, B.; Nógrádi, M. The final structures of robinin and biorobin and their total synthesis. *Phytochemistry* **1976**, *15*, 215−218.
(26) Brasseur, T.; Angenot, L. Flavonol glycosides from leaves of *Strychnos variabilis*. *Phytochemistry* **1986**, *25*, 563−564.
(27) Tomczyk, M.; Gudej, J.; Sochacki, M. Flavonoids from *Ficaria verna* Huds. *Z. Naturforsch., C: Biochem., Biophys., Biol., Virol.* **2002**, *57*, 440−444.
(28) Zhou, Z, H; Yang, C. R. Chemical constituents from crude green tea, the material of Pu-er tea in Yunnan. *Acta Bot. Yunnanica* **2000**, *22*, 343−350 (in Chinese).
(29) Kazuma, K.; Noda, N.; Suzuki, M. Malonylated flavonol glycosides from the petals of *Clitoria ternatea*. *Phytochemistry* **2003**, *62*, 229−237.
(30) Tanikawa, N.; Yoshida, K.; Kondo, T.; Mizuno, T.; Iwashina, T.; Nakayama, M. Urakunoside, a new tetraglycosyl kaempferol from petals of the Wabisuke camellia cv. Tarokaja. *Biosci. Biotechnol. Biochem.* **2011**, *75*, 2046−2048.
(31) Finger, A.; Engelhardt, U. H.; Wray, V. Flavonol glycosides in tea-kaempferol and quercetin rhamnodiglucosides. *J. Sci. Food Agric.* **1991**, *55*, 313−321.
(32) Yu, D. Q.; Yang, J. S. *Handbook of Analytical Chemistry*, 2nd ed.; Chemical Industry Press: Beijing, 1999; pp 447, 844−845 (in Chinese).
(33) Su, D. M.; Tang, W. Z.; Hu, Y. C.; Liu, Y. B.; Yu, S. S.; Ma, S. G.; Qu, J.; Yu, D. Q. Lignan glycosides from *Neoalsomitra integrifoliola*. *J. Nat. Prod.* **2008**, *71*, 784−788.
(34) Pouchert, C. J.; Behnke, J. *The Aldrich Library of ^{13}C and ^{1}H FT NMR Spectra*; Aldrich Chemical Company Inc.: Wisconsin; 1992, Vol. 2, pp 1051C; 1506A.
(35) Baderschneider, B.; Winterhalter, P. Isolation and characterization of novel benzoates, cinnamates, flavonoids, and lignans from Riesling wine and screening for antioxidant activity. *J. Agric. Food Chem.* **2001**, *49*, 2788−2798.
(36) Zhou, Y. J.; Li, B.; Liang, J. N.; Xu, S. X.; Sun, Q. S.; Guo, H.; Wu, L.; Tan, G. S. Five phenolic constituents from the leaves of *Quercus dentata*. *Chin. J. Org. Chem.* **2005**, *25*, 109−112.
(37) Otsuka, H.; Yao, M.; Kamada, K.; Takeda, Y. Alangionosides G−M: Glycosides of megastigmane derivatives from the leaves of *Alangium premnifolium*. *Chem. Pharm. Bull.* **1995**, *43*, 754−759.
(38) Miyase, T.; Ueno, A.; Takizawa, N.; Kobayashi, H.; Oguchi, H. Studies on the glycosides of *Epimedium grandiflorum* MORR. var. *thunbergianum* (MIQ.) NAKAI. III. *Chem. Pharm. Bull.* **1988**, *36*, 2475−2484.
(39) Ye, G.; Ma, C. H.; Huang, X. Y.; Li, Z. X.; Huang, C. G. Components of *Sophora alopecuroides* seeds. *Chem. Nat. Compd.* **2009**, *45*, 545−546.
(40) Martin, G. C.; Dennis, F. G., Jr.; MacMillan, J.; Gaskin, P. Hormones in pear seeds, 1: Levels of gibberellins, abscisic acid, phaseic acid, dihydrophaseic acid, and two metabolites of dihydrophaseic acid in immature seeds of *Pyrus communis* L. *J. Am. Soc. Hortic. Sci.* **1977**, *102*, 16−19.
(41) Zhao, N.; Gao, H. Y.; Sun, B. H.; Wu, L. J. Chemical constituents from the leaves of *Camellia sinensis*. *J. Shenyang Pharm. Univ.* **2007**, *24*, 211−214.
(42) Davis, A. L.; Cai, Y.; Davis, A. P.; Lewis, J. R. ^{1}H and ^{13}C-NMR assignments of some green tea polyphenols. *Magn. Reson. Chem.* **1996**, *34*, 887−890.
(43) Valcic, S.; Muders, A.; Jacobsen, N. E.; Liebler, D. C.; Timmermann, B. N. Antioxidant chemistry of green tea catechins. Identification of products of the reaction of (−)-epigallocatechin gallate with peroxyl radicals. *Chem. Res. Toxicol.* **1999**, *12*, 382−386.
(44) Valcic, S.; Burr, J. A.; Timmermann, B. N.; Liebler, D. C. Antioxidant chemistry of green tea catechins. New oxidation products of (−)-epigallocatechin gallate and (−)-epigallocatechin from their reactions with peroxyl radicals. *Chem. Res. Toxicol.* **2000**, *13*, 801−810.
(45) Xu, W. P.; Song, Q, S.; Li, D. X.; Wan, X. C. Discrimination of the production season of Chinese green tea by chemical analysis in combination with supervised pattern recognition. *J. Agric. Food Chem.* **2012**, *60*, 7064−7070.
(46) Wulandari, R. A.; Amano, M.; Yanagita, T.; Tanaka, T.; Kouno, I.; Kawamura, D.; Ishimaru, K. New phenolic compounds from *Camellia sinensis* L. leaves fermented with *Aspergillus* sp. *J. Nat. Med.* **2011**, *65*, 594−597.
(47) Kanegae, A.; Sakamoto, A.; Nakayama, H.; Nakazono, Y.; Yakashiro, I.; Matsuo, Y.; Tanaka, T.; Ishimaru, K. New phenolic compounds from *Camellia sinensis* L. fermented leaves. *J. Nat. Med.* **2012**, DOI: 10.1007/s11418-012-0704-5.
(48) Das, S.; Lamm, A. S.; Rosazza, J. P. N. Biotransformation of (+)-catechin to novel B-ring fission lactones. *Org. Process Res. Dev.* **2011**, *15*, 231−235.
(49) 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.