Feature-Based Molecular Networking Analysis of the Metabolites Produced by *in vitro* Solid-State Fermentation Reveals Pathways for the Bioconversion of Epigallocatechin Gallate

Hao-Fen Xie[†a], Ya-Shuai Kong[†a], Ru-Ze Li[†a], Hong Zhang[a], Lu-Lu Liu[a], Ting-Ting An[a], Rui Liu[a], Zi Yang[a], Jia-Ping Ke[a], Peng Zhang[a], Ming Zhao[d], Guan-Hu Bao[a], Zhong-Wen Xie[a], Da-Xiang Li[a], Xiao-Chun Wan[a], Qian-Ying Dai[a], Liang Zhang[a], Mao-Qiang An[e], Louis Felix Nothias[b], Ricardo R. Da Silva[b], Alexey V. Melnik[b], Michael J. Meehan[b], Pieter C Dorrestein[b], Yan-Hua Long[*c], Tie-Jun Ling[*a]

[a] State Key Laboratory of Tea Plant Biology and Utilization, Anhui Agricultural University, 130 Changjiang West Road, Hefei, Anhui, P. R. China

[b] Collaborative Mass Spectrometry Innovation Center, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, San Diego, CA, USA

[c] School of Life Sciences, Anhui Agricultural University, 130 Changjiang West Road, Hefei, Anhui, P. R. China

[d] College of Longrun Pu-erh Tea, Yunnan Agricultural University, Kunming, Yunnan, P. R. China

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

[†] These authors contributed equally to the work.

* Corresponding authors
(telephone: +86-551-65786401; fax: +86-551-65786765; E-mail address: lingtj@ahau.edu.cn)
(telephone: +86-551-65786129; fax: +86-551-65786129; E-mail address: longyanhua@ahau.edu.cn)

ABSTRACT

Flavan-3-ol B-ring fission derivatives (FBRFDs) are secondary metabolites that contribute to the unique properties of fermented dark teas. However, the FBRFD precursors and biochemistry are unclear. Fungal strains cultured from Fuzhuan brick tea (FBT) were incubated in an *in vitro* solid-state fermentation system containing *β*-cyclodextrin-embedded epigallocatechin gallate (EGCG), a potential precursor of FBRFDs. The produced metabolites were analyzed through a combination of targeted chromatographic isolation, non-targeted spectroscopic identification, and Feature-based Molecular Networking (FBMN) in the Global Natural Products Social Molecular Networking (GNPS) platform. Dihydromyricetin was identified for the first time, indicating that fungi possess a flavan-3-ol C-ring oxidation pathway. EGCG was verified as the precursor of dihydromyricetin and FBRFDs such as teadenol A and fuzhuanin A. The conversion was driven by the fungi rather than a hygrothermal effect. Isolates from Pezizomycotina showed much stronger abilities to convert EGCG to the B-/C-ring oxidation products than those from Saccharomycotina or Basidiomycota.

KEYWORDS: flavan-3-ol B-ring fission derivatives, FBRFDs, Fuzhuan brick tea, dark teas, solid-state fermentation system, fungi, *β*-cyclodextrin, *β*-CD, epigallocatechin gallate, EGCG, Global Natural Products Social Molecular Networking, GNPS, Feature-based Molecular Networking, FBMN, dihydromyricetin, oxidation, bioconversion, C-ring, teadenol A, fuzhuanin A, hygrothermal effect, Pezizomycotina

INTRODUCTION

Tea is one of the most popular beverages worldwide. Tea is classified into six categories, according to how the tea leaves are processed: green tea, white tea, yellow tea, oolong tea, black tea, and dark tea.[1] The production of dark tea in China has a long history, with diverse varieties including Fuzhuan brick tea (FBT), Pu-erh tea, Heizhuan brick tea, Huazhuan brick tea, Qingzhuan brick tea, Kangzhuan brick tea and Liubao tea.[1] Most dark teas are produced from the leaves of *Camellia sinensis* var. *sinensis*, except for Pu-erh tea, which uses *Camellia sinensis* var. *assamica*.[2,3] In recent years, dark teas have been studied for their health benefits, such as anti-diarrheal, anti-hyperlipidemic and anti-hyperglycemic activities.[4,5]

Unique to the dark teas, the manufacturing process includes a microbial fermentation stage, during which tea leaves (at 20 to 60% initial moisture) are piled together for a certain period of time, ranging from a half a day to a few months.[3] The piles for fermentation are huge as high as 3m, creating an environment where the heat produced by the microbes can increase the temperature, to as high as 80 °C, during fermentation.[6] In this post-fermentation stage, a series of complex chemical changes occur, which subsequently contribute to the unique quality characteristics and health benefits of dark teas.[4,5]

Tea contains numerous flavonoids and is especially high in flavan-3-ol compounds (flavanols). Epigallocatechin gallate (EGCG) is recognized as one of the most important bioactive compounds in tea leaves.[7,8] In dark teas, the EGCG content decreases considerably after microbial fermentation, while the levels of some bioactive derivatives of flavonoids, such as FBRFDs, rise.[9-12] FBRFDs are suspected to be an important bioactive compound in dark teas, and are likely derived through microbial fermentation from EGCG due to both structural similarities and the other known precursors in tea leaves.[5,10,13] However, the metabolism leading to FBRFDs remains unknown, so further investigation into the structures, bioactivities, and biosynthesis of FBRFDs in dark teas is critical.[4]

Recently, a number of FBRFDs, such as teasperin as well as teadenols A and B, have been detected in tea leaves after fermentation by some fungal strains.[14-16] However, there are still many unknowns, including the precursor(s) to FBRFDs, how the FBRFDs relate to other derivatives that also contain the C6-C3-C6 skeleton (such as quercetin, myricetin and their glycosides), and the relationship between the taxonomy of the microbes and the diverse structures of the derivatives. In fact, it even remains unknown whether the FBRFDs arise through the action of microbial enzymes or solely through the hygrothermal effect caused by microbial fermentation.[14-16]

To answer the above questions, a chemically defined, solid-state culture medium containing only EGCG, theanine, and caffeine, as well as some essential nutrients, would be ideal for investigation the products of fermentation by microbes isolated from dark teas. Both representative and predominant metabolites of tea leaves could be added in order to draw a much clearer relationship between the precursors and their microbial conversion products. In comparison to a liquid culture, a solid medium provides sufficient atmosphere for aerobic fungi. A solid medium also allows dissipation of the biologically generated heat, due to the smaller volume compared to the piles of tea leaves (Figure S1 in SI-1). However, EGCG is unstable in an open environment and in neutral-to-basic (pH >6) conditions.[17] Moreover, EGCG prevents agar from solidifying. These practical hurdles are among the reasons why there are few recent advances in this field.[13] In addition, the relative contents of the bioconverted products are generally at low levels and occur with a lot of structural diversity, further barring investigation by traditional strategies.

Oligosaccharides like *β*-cyclodextrin (*β*-CD) can be used shield target compounds from the environment by encapsulating unstable compounds within hydrophobic cavities. *β*-CD has been shown to protect the unstable ECGC in aqueous solvent.[18] We hypothesize that using *β*-CD-encapsulated ECGC will allow agar to solidify and create a solid medium. In addition, the EGCG in *β*-CD should be accessible to fungi due to the hydrolysis reactions of fungal cellulases.

In non-targeted metabolomics using chromatography and tandem mass spectrometry (MS), LC-MS data can be

better analyzed using Feature-based Molecular Networking (FBMN) in the Global Natural Product Social Molecular Networking (GNPS) environment.[19-21] To use the FBMN, the MS intensities and MS/MS spectral information of features are extracted from the raw data by a metabolomic software package, i.e., Optimus, MZmine2, MS-DIAL or XCMS, before being uploaded to GNPS for molecular networking.[19] The result of the molecular networking is visualized using Cytoscape, where each of the detected molecular features is represented by a node.[22] In the network, the nodes are connected by edges when the represented features have both a number of matched fragment ions and similar MS/MS profiles over a threshold value.[19-21] FBMN provides a convenient way to fully investigate the identifiers of each metabolite, including chromatography retention time and peak abundance, molecular numbers, molecular formulas, and MS/MS spectral similarity. FBMN compares the data for each identified feature to other features within the same sample, facilitating relational connections between metabolites. Therefore, FBMN has recently been used in an increasing number of research reports as a method for the comprehensive investigation of natural products.[23-26]

In this study, we first established a solid-state fermentation system that stabilizes EGCG by embedding it in β-CD. The fungi isolated from FBT were incubated in the system and screened for derivatives of EGCG within the LC-MS data using FBMN. Subsequently, one of the microbially converted flavan-3-ol derivatives was isolated and identified by targeted chromatography and spectroscopy. The bioconverted metabolites of EGCG were analyzed across samples fermented by 15 different fungal isolates from cultures on PDA supplemented with essential tea metabolites (caffeine, theanine and ECGC) using the FBMN technology. Finally, we searched for the possible pathways of these microbial conversions. These pathways will inform future research into the bioactive compounds present in dark teas.

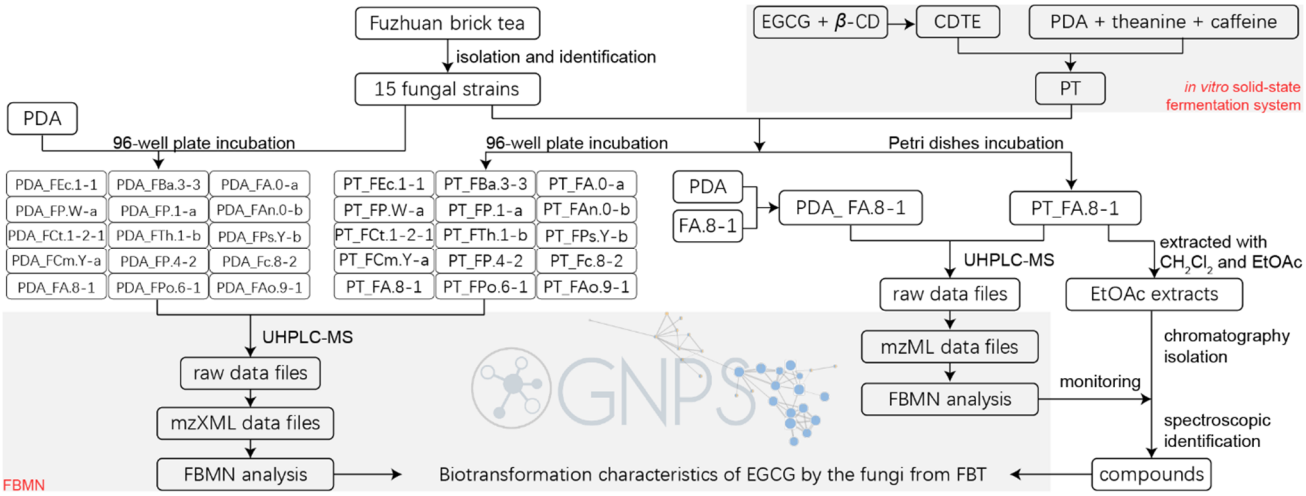

Figure 1. The workflow of the project.

MATERIALS AND METHODS

Instrumentation and Reagents

1D (^{1}H and ^{13}C NMR) and 2D [^{1}H-^{1}H correlated spectroscopy (COSY), heteronuclear multiple-bond coherence (HMBC)] NMR spectra were recorded in DMSO-d_6 on an Agilent DD2 600 MHz NMR spectrometer (Agilent, Inc., Santa Clara, CA, USA). Coupling constants were expressed in Hz. Chemical shifts were given on a δ (ppm) scale with TMS as the internal standard.

Semi-preparative HPLC was performed on a Waters e2695 combined with a Waters 2998 photodiode array detector (PDAD) (Waters, Milford, Massachusetts, USA). The semi-preparative chromatography column was an X Bridge Prep C18 (10 × 250 mm i.d., 5 μm) (Waters, Wexford, Ireland). The instrument parameters were as follows: injection volume, 30 μL; flow rate, 2.0 mL/min; column temperature, 30 °C. The eluent was composed of mobile phase A (water) and mobile phase B (acetonitrile). An isocratic elution was set at 12% phase B for the full chromatography time of 45 min. The UV detection wavelengths were 210 and 280 nm during the HPLC separation.

DNA quality was accessed by electrophoresis (Beijing Junyi Dongfang Electrophoresis Co. Ltd., Beijing, P. R. China). Column chromatography (CC) was performed using 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), or Toyopearl HW-40F (Tosoh Co. Ltd., Tokyo, Japan). Thin layer chromatography (TLC) using silica gel (GF254, Liangchen Chemical Co. Ltd., Huoshan, P. R. China) was performed with ethyl acetate/methanol/H_2O (100:17:13, v/v) or benzene/ethyl formate/formic acid (3:6:1, v/v). The TLC plates were detected by UV illumination, or by spraying with 0.2% ethanolic $FeCl_3$ for visualization of polyphenol dots, or detected by spraying with 10% sulfuric acid followed by heating. Polyamide TLC (Taizhou Luqiao Sijia Biochemical Ltd., Taizhou, P. R. China) was performed with ethyl acetate/methanol/H_2O (100:17:13, v/v), with detection by spraying with 2% ethanolic $FeCl_3$.

Pure, distilled water was purchased from Duksan Pure

Chemicals Co., Ltd. (Ulsan, Korea). Methanol, acetonitrile and formic acid were purchased from Thermo Fisher (Thermo Scientific, Waltham, MA, USA, for the LC-MS grade) or from Duksan Pure Chemicals Co., Ltd. (Ulsan, Korea, for the HPLC grade). Methanol, anhydrous ethanol, petroleum ether (PE), ethyl acetate (EtOAc) and dichloromethane (CH_2Cl_2) were purchased from Kelong Chemical Reagent Co., Ltd. (Chengdu, P. R. China). Deuterated dimethyl sulfoxide (DMSO-d_6, 99.9%) and deuteroxide (D_2O, 99.9%) were purchased from Cambridge Isotope Laboratories, Inc. (MA, USA). β-CD was kindly supplied by Prof. Qian-Ying Dai, Anhui Agricultural University. Millipore disposable syringe filters (0.22 μm) were bought from Merck Millipore Co., Ltd. (Cork, Ireland). PDA was purchased from Hangzhou Microbial Reagent Co., Ltd. (Hangzhou, P. R. China). EGCG, theanine and caffeine were bought from Sigma Chemical Co., Ltd. (USA). Plates (96-well) were bought from Corning Co., Ltd. (USA). The standards for UHPLC-MS analysis, including fuzhuanin A, teadenol A, planchol A, xanthocerin, teasperin, dihydromyricetin, EGCG, EGC and EC, were isolated and identified in our previous or current work.[10,11,27]

Materials

FBT and raw dark tea leaves were produced in July 2015 by a major Fuzhuan brick tea production factory, Yiyang Fu Cha Industry Development Co. Ltd. (Yiyang, Hunan Province, P. R. China). The tea materials were stored at -20 °C before treatment. In addition, 10 g of raw dark tea leaves were prepared before and after autoclaving (120 °C for 20 min) for validation of conversion of EGCG by the hygrothermal effect in tea leaves.

Strain Isolation and DNA Extraction of the Fungi

In addition to the finished FBT, several tea samples were collected during the manufacturing process of FBT at the 1st and the 7th day of microbial fermentation. Five grams of each tea sample was shaken (180 rpm) in 50 mL water for 30 min at 30 °C. The turbid liquid was serially diluted to 10^1, 10^2, 10^3, 10^4 and 10^5. A 100 μL suspension of each dilution was inoculated onto PDA in Petri dishes and incubated at 30 °C for 3—5 days, until the fungal colonies were clearly observed. Strains were isolated by the streak plate method. As a result, 15 fungal strains were isolated from the tea materials. The isolates were subcultured on PDA slants and stored at 4 °C for further analysis.

The genomic DNA of each strain was extracted using the benzyl chloride method, with some modifications.[28] Briefly, approximately 0.1 g fresh mycelia from each fungal strain, 0.5 mL of extraction buffer [100 mM Tris HCl (pH 9.0), 40 mM EDTA], 0.1 mL of 10% SDS, and 0.3 mL benzyl chloride were mixed in a tube and incubated at 50 °C for 1 h with missing by vortex every 10 min. Then 0.3 mL of 3 M NaAc (pH 5.2) was added and incubated at 4 °C for 15 min. After centrifugation at 98,000 g for 8 minutes, the supernatants were transferred to a tube containing equal volumes of chloroform, isopentanol and phenol (1:1:1, v/v), and shook for 5 minutes. After centrifugation at 98,000 g for 8 minutes, the supernatant was moved to another tube and was mixed with equal volumes chloroform and isopentanol, followed by centrifugation at 98,000 g for another 15 minutes. After that, the supernatant was transferred to another tube and mixed with one-tenth volume of both 3 M NaAc and isopropanol to precipitate the DNA after standing at -20 °C for 30 min. The DNA pellets were washed with 75% ethanol, centrifuged twice for 5 minutes at 98,000 g to discard the solvent, and dissolved in ddH_2O. The quality of each DNA sample was checked in an 0.8% agarose gel.

Polymerase chain reaction (PCR) was performed to amplify part of the ITS rRNA genes using primer ITS4 (5'-TCC TCC GCT TAT TGATATGC-3') and primer ITS5 (5'-GGA AGT AAAA GTC GTA ACA AGG-3'). Each 20 μL PCR reaction mixture contained 10 μL *Taq* PCR StarMix (2×), 1 μL of each Primer (2 μM), 10 ng DNA, and 7 μL ddH_2O. All samples were replicated three times. The PCR was performed using a GeneAmp PCR System 9700 (PE Applied Biosystems, USA) with the following standard procedure: initial denaturation at 98 °C for 1 min, followed by 30 cycles of 98 °C for 10 s, annealing at 50 °C for 30 s, 72 °C for 30 s, and final extension at 72 °C for 5 min. Then, the PCR products were analyzed by electrophoresis on a 2% (v/v) agarose gel (100 V, 40 min). The gel was stained with ethidium bromide, and the bands were photographed on an ultraviolet light transilluminator.

The sequencing of PCR products was performed by the Shanghai Sangon Biotech Co., Ltd. (Shanghai, P. R. China). The resulting sequences were compared with available reference sequences using Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi). Abbreviated names for the identified fungal isolates are documented in SI-2.

Preparation of specific growth media (PT)

Most fungi from dark teas are aerobic and grow on the surface of the piled tea leaves.[5,9] A solid-state medium would provide a growth environment that is more similar to that on tea leaves, compared to growth in submerged cultures. Since agar is not able to solidify when mixed with EGCG directly, EGCG was embedded in an oligosaccharide, which can potentially be degraded by a glycosidase of the fungi. The oligosaccharide β-CD (8.00 g) was dissolved in 100 mL water to obtain a saturated solution at 60 °C. EGCG (3.23 g) was dissolved in 5 mL anhydrous ethanol and slowly added to the β-CD solution. The mixture was stirred for 4 h at 60 °C, followed by standing at 4 °C for 12 h. Then the solution was freeze-dried to yield the product, β-CD embedded tea EGCG (CDTE). The CDTE structure was validated by NMR analysis (Figure S2 in SI-1).[18]

The embedded EGCG, theanine and caffeine were added to PDA to form a solid medium rich in components present in tea leaves, named PT (**P**DA mixed with the dominant compounds in **T**ea). PDA powder (4.3 g) was mixed with 125 mg theanine and 250 mg caffeine in 100 mL water before sterilization at 120 °C for 20 min (medium A). To make PT medium, 2.25 g CDTE was dissolved in 20 mL water, filtered through a 0.22-μm membrane, and added into medium A before it solidified (Figure 1). The prepared media was transferred into 96-well plates (500 μL/well) or poured in Petri dishes for fungal culturing.

Fungal Culture

For analysis and isolation of flavan-3-ol derivatives, spores of the *Aspergillus* isolate FA.8-1 (SI-2) were inoculated on the surface of PT in 1.8-L Petri dishes (about 45 mL PT for each dish).

The 15 isolated fungal strains were inoculated on 500 μL PT in 96-well plates (Figures 1, S1C and S1D) in triplicate. Some wells of PT were set as controls with no inoculation of any fungi (PT_control) (Figure S1 in SI-1). A series of controls were prepared with the spores of the 15 fungal strains on PDA in 96-well plates (Figures 1, S1A and S1B) to analyze the metabolites of the fungi without tea-specific components. Both the inoculated and uninoculated samples were incubated at 28 °C for 28 days before further treatment. The corresponding sample names can be found in Figure 1.

Isolation of Metabolites from PT_FA.8-1 in Petri Dishes

The PT from Petri dishes (a total of 1.8 L of media) were cut into small pieces (0.6 × 0.6 cm) after culturing and extracted with 1.5 L of methanol by ultrasonic extraction at room temperature for a total of 24 h (six times at 4 h each time). After vacuum removal of the methanol, the resulting aqueous solution was extracted successively with CH_2Cl_2 and EtOAc. The layers were separately concentrated to yield the CH_2Cl_2 fraction (fraction A, 0.3516 g) and the EtOAc fraction (fraction B, 0.9055 g). The EtOAc fraction was applied to a Sephadex LH-20 CC and eluted with MeOH, yielding nine subfractions (B1 to B9). Fraction B8 (398.8 mg) was analyzed by LC-MS and followed by a further purification through a silica gel column with an elution using PE-EtOAc (3:5 to 0:1) to yield five fractions (B8-a to B8-e). Fraction B8-b (54 mg) was then subjected to chromatography using a Sephadex LH-20 column and water-methanol (1:0 to 0:1) as the eluent, providing fractions B8-ba to B8-bf. Finally, fraction B8-ba was repeatedly subjected to semi-preparative HPLC, resulting in the isolation of compound **1** (20 mg; LC retention time 485 s; MS precursor ion *m/z* 321.061), which was monitored by LC-MS measurements step by step (figures not supplied). The NMR spectra and spectral data of **1** are listed in Figures S6-S9 and Table S2 in SI-1.

Extraction of Metabolites from PT/PDA Cultures of the 15 Fungal Strains in 96-well Plates

After incubation, the inoculated wells were occupied by plenty of mycelia (Figure S1 in SI-1). To each well (inoculated or control), 500 μL methanol was added, stirred, subjected to 60 min of supersonic extraction, and centrifuged at 1,500 g for 10 min. The supernatant was removed out of each well, respectively, and stored at -20 °C before the LC-MS measurement.

LC-ESI-Orbitrap-MS and LC-ESI-QTOF-MS Measurements

The samples PT_FA.8-1 and PDA_FA.8-1 from Petri dishes (Figure 1) were analyzed using an Acquity UPLC® HSS T3 column (100 × 2.1 mm, 1.8 μm) on an ultra-high performance liquid chromatography (UHPLC) system (Ultimate 3000, Dionex, Sunnyvale, CA, USA). Chromatographic separation was achieved using 0.075% formic acid in water (A) and acetonitrile with 0.075% formic acid (B) at a flow rate of 0.20 mL/min with the following gradient: 0 min, 2% B; 2 min, 14% B; 3.5 min, 15% B; 8 min, 16% B; 10 min, 25% B; 12 min, 30% B; 15.5 min, 100% B, 19.5 min, 100% B; 20 min, 2% B; 21 min, 100% B; 22 min, 2% B; and 25 min, 2% B. Column temperature was maintained at 40 °C. The MS data in positive mode were acquired on a Thermo Q-Exactive Focus (Thermo Fisher Scientific, MA, USA) mass spectrometer. The parameters were full-scan mode with a resolution of 70000 and a scan range of 100−1500 (*m/z*). The fragmentation spectra (MS/MS) were acquired using collision energies of 11, 20, and 60 eV as a stepped dissociation mode.

The extracts of the PDA/PT media incubated with the 15 different fungal strains in the 96-well plates (Figures 1 and S1) were analyzed using a UHPLC of 1290 Infinity system (Agilent) connected to a MicrOTOF-QII mass spectrometer (Bruker Daltonics) with an ESI source. UHPLC conditions were as follows: 1.7 μm C18 (150 × 2.1 mm) UHPLC column (Waters, MA, USA), column temperature, 40 °C; flow rate, 0.2 mL/min; mobile phase A, 99.9% water/0.1% formic acid (v/v); and mobile phase B, acetonitrile/0.1% formic acid (v/v). The linear gradient used for the chromatographic separation was the same as that of the LC-ESI-Orbitrap-MS measurement for the extracts of PDA/PT_FA.8-1. Mass spectra were acquired in positive ion mode over a range of *m/z* 80-1500. MS/MS fragmentation of the five most intense selected ions per spectrum was performed by stepped dissociation energy, ranging from 7-65 eV, to get diverse fragmentation patterns. Considering the possibility of cross contamination in the batch LC-MS detections, a process blank was set by washing the chromatography column every 6 runs.

LC-MS Data Processing

The raw data acquired from the LC-ESI-Orbitrap-MS were converted into .mzML (data files A) by MSConvert, while those acquired from UHPLC-QTOF-MS were converted into .mzXML file format (data files B) by Compass DataAnalysis Software (Version 4.2 Build 395, Bruker Daltonics) before being subjected to Optimus (version 1.5.1).[29] For both data files A and B, the MS intensities and the digitalized mass spectra of the detected features were extracted by the software, and exported to an MS^2 feature table and an .mgf file, respectively. The feature table contains the ID numbers, *m/z* values of the precursor ions, retention times from column chromatography, and the MS intensities of the detected features.[21]

The .mgf files for data files A and B were separately uploaded to the GNPS platform for molecular networking. After running the spectral clustering algorithm, two GraphML data sets were downloaded from the job status pages (for A; https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=7ea5e0a322ed44a58c0fa3f703e6aa96; and for B; https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=229c1dbbe66a4a258275dc6381b55e1b), and were imported into Cytoscape (version 3.7.2) along with the MS^2 feature tables for network visualization (Figures 3 and 6).[21,22] The heatmap and the hierarchical clustering for the FBRFDs and dihydromyricetin in the 15 different PT fungal cultures were performed by the R package ComplexHeatmap (Figure 6C).

RESULTS AND DISCUSSION

Identification of the Isolated Fungal Strains from FBT

The fungal strains isolated from the samples taken during the commercial processing of FBT were identified by DNA sequencing of the ITS gene and BLAST comparison in the NCBI database, followed by generation of a phylogenetic trees (Figure 2; SI-2). The isolated fungi belonged to two phyla. Most of the strains were Ascomycota, while FTh.1-b and FBa.3-3 were Basidiomycota. The Ascomycota isolates were distributed among two subphyla. Twelve strains were from subphylum Pezizomycotina, while the other one, FCm.9-1, was from Saccharomycotina. Since the isolation was based on colonies formed on culture media, the result indicated higher abundance of Pezizomycotina (80%) within the FBT fungal community.

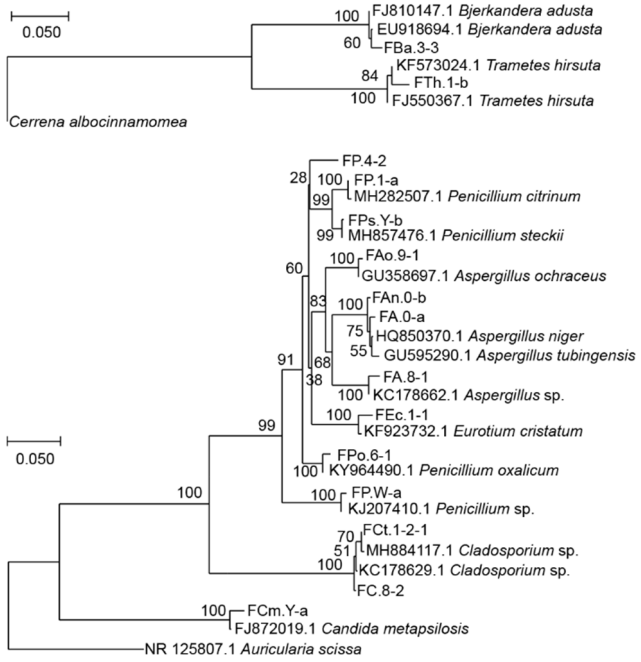

Figure 2. Two phylogenetic trees composed of the15 fungal strains isolated from FBT and some selected fungi from NCBI. The phylogenetic trees show the clustering results of the 15 fungal strains and their homologous sequences. *Auricularia scissa* and *Cerrena albocinnamomea* were used as outgroups for root plotting, which validated the identification of the fungal strains.

The identified isolates were *Aspergillus* spp., *Eurotium cristatum*, *Penicillium* spp., *Cladosporium* spp., and *Candida metapsilosis*. The identified fungi were similar to those previously reported as the dominant fungal community members in FBT, but no species from *Cyberlindnera*, *Wallemia*, *Trichosporon* or *Eucasphaeria* were obtained in this study.[9] It is possible that not all reported genera from FBT were obtained because of the inability to isolate natural microbiota due to cultural conditions and some uncultivable species.[30] An other reason might be the differences in the fungal species between product batches.

FBMN Analysis of the Metabolites in PT_FA.8-1

As *Aspergillus* is one the most abundant fungal genera in FBT,[9] isolate FA.8-1 (*Aspergillus* sp.) was selected and cultured in large quantities in order to investigate the bioconversion of EGCG by the dominant fungi of FBT using an approach that combined *in vitro* fermentation, LC-MS analysis, and feature annotation by FBMN. A molecular network of the metabolites from the *in vitro* culture of isolate FA.8-1 on media containing tea components (PT_FA.8-1) was built using FBMN (Figure 3). The molecular network demonstrated that EGCG was no longer present in the medium after 4 weeks of incubation, as evidenced by the absence of the node ($[M+H]^+$ 459.092) in the network. Correspondingly, some derivatives of EGCG were generated, represented as nodes connected by edges in a specific cluster (the blue nodes in Figure 3*a*). In molecular networking, nodes connecting by edges have both similar MS/MS spectral profiles, which are expressed as cosine scores, and a number of matched fragment ions between them. In other words, the features represented as nodes in this cluster are similar in their chemical structures.

By means of standard compounds matching (annotation level 1),[31] we discovered that EGC (feature ID: 1883), teadenol A (feature ID: 2959) and fuzhuanin A (feature ID: 1830) were indeed flavanol derivatives in PT_FA.8-1 (Figure 3), derived from EGCG by the fungus. In addition, a feature with ID 3298 was annotated as an isomer of fuzhuanin A by GNPS library matching.

The molecular networking of the FA.8-1 culture on PT medium (PT_FA.8-1) showed diverse structures for the derivatives of EGCG. The overview of the network demonstrated that the strain FA.8-1 has at least two mechanisms to bioconvert EGCG to FBRFDs: hydrolysis of the gallate of EGCG and oxidation of the B-ring of EGC, which can lead to several different products (Figure 3*c*). The predominant FBRFDs were teadenol A and fuzhuanin A.

In addition to the above features, there was a node (feature ID: 2829) connected to both teadenol A (ID: 2959) and fuzhuanin A (ID: 1830) in this cluster (in red number, Figure 3*a*). The feature represented by this node was indicated as another oxidized product of EGC, following the consensus clues of its neighboring nodes. This feature has the same precursor ion, *m/z* 321.0606, as fuzhuanin A (Figure 4), which indicated its molecular formula as $C_{15}H_{12}O_8$. Although the MS/MS of this feature was similar to that of fuzhuanin A, there were also some differences. A detailed investigation of their mass spectra indicated that one of the most characteristic MS/MS fragment ions of the feature was *m/z* 165.02, rather than *m/z* 183.03 of fuzhuanin A. The latter ion was thought to be the product of a retro-Diels-Alder reaction (Figure S3 in SI-1). Therefore, this information implied that the feature with ID 2829 was an oxidation product of EGC that differed from fuzhuanin A in the substructures of both ring B and ring C.

As far as we know, no compounds with such structural characters have been reported from any fungal species as metabolites of flavan-3-ols. This result indicated that there is a previously unknown metabolic pathway in at least *Aspergillus* spp.. Therefore, this feature was targeted by its LC retention time (485 s) and MS precursor ion (*m/z* 321.061) for the following isolation and detailed structural identification.

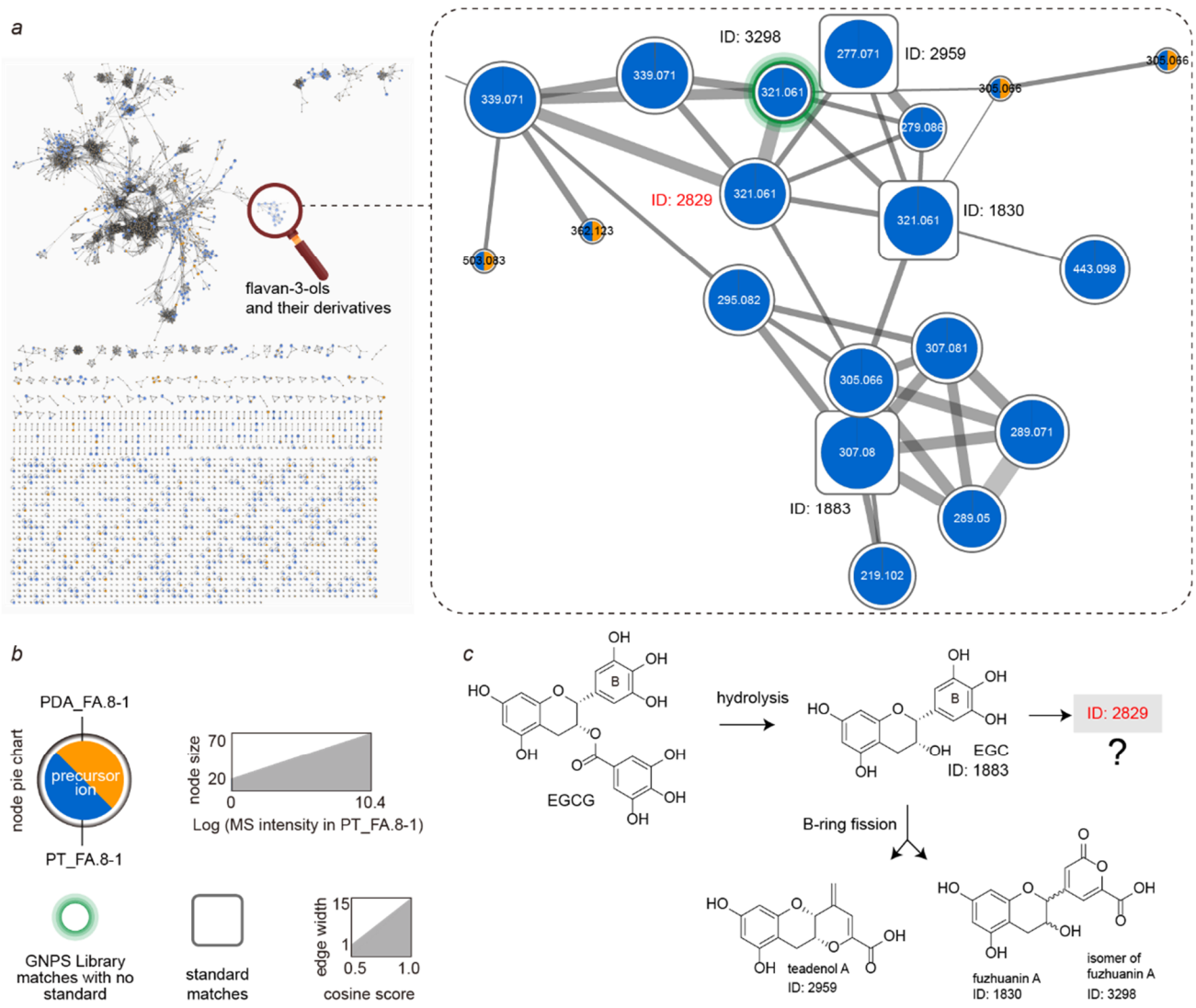

Figure 3. FBMN with partial annotation of the flavan-3-ols and their derivatives in PDA/PT_FA.8-1. *a*: The molecular network of all the detected features from both PT_FA.8-1 and PDA_FA.8-1. The nodes annotated as flavan-3-ols and their derivatives are enlarged, with IDs assigned for some key compounds (EGC with ID 1883; teadenol A with ID 2959; fuzhuanin A with ID 1830; isomer of fuzhuanin A with ID 3298). Blue color in pie chart indicates that the metabolite was present in cultures on PDA media containing the tea components ECGC, caffeine and theanine, while orange indicates that the metabolite was present on regular PDA. At least 15 derivatives of flavan-3-ol were present only in the PT cultures, including that standards EGC, teadenol A and fuzhuanin A (marked with a grey box), indicating that these are metabolites of FA.8-1 from EGCG. The thickness of the edges between the nodes (metabolites) relates to the cosine score of the MS/MS similarity, ranging from 0.5 to 1.0 *b*: Figure legends. *c*: Partial annotation along with the proposed metabolic pathways of four key flavan-3-ol derivatives.

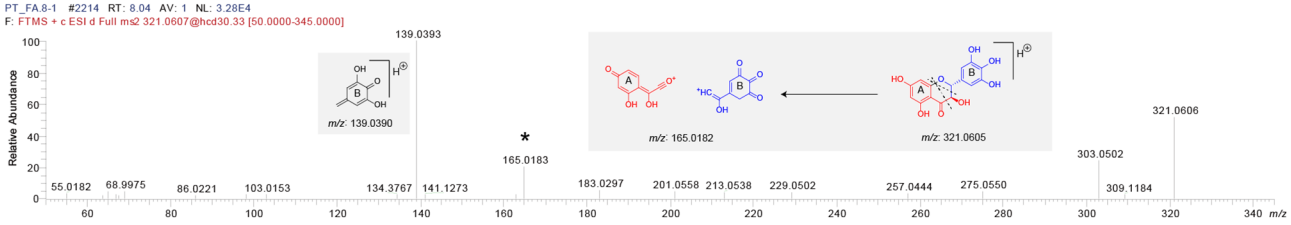

Figure 4. The MS/MS spectrum and partial annotation of the feature dihydromyricetin (ID: 2829).

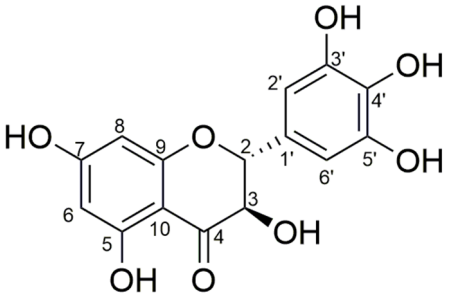

Figure 5. Structure of compound **1**.

Spectroscopic Identification of Compound 1

By means of targeted natural product isolation, compound **1**, an off-white powder, was purified from the extracts of PT_FA.8-1 (Figure 1). The compound had an identical $[M + H]^+$ signal, of *m/z* 321.0606 ($C_{15}H_{13}O_8^+$, calcd. 321.0605) (Figure 4), and LC retention time (485 s) as the feature with ID 2829 in the molecular network (Figure 3). The network analysis annotated compound **1** to be a derivative of flavan-3-ol, and indeed it showed signals of H-6 and H-8 in ring A (δ 5.88 and 5.93, both br s, 1H) as well as those in the triple-hydroxy substituted ring B [δ 6.40 (s), 2H] (Table S2 in SI-1). However, the typical proton signals of H-2, H-3, and H_2-4 for flavan-3-ols, which are generally distributed over the range of δ 2.5 to δ 5.0 ppm, were not observed in the spectrum.[32] On the other hand, there were two coupled doublets at δ 4.89 and 4.40 (J = 10.8 Hz) (Figure S6 in SI-1). By a combined analysis of a carbonyl carbon signal (δ 197.1) in the ^{13}C NMR spectrum (Table S2), we proposed a ring C of dihydroflavonol for the compound. A comparison of full NMR spectral data of **1** with dihydromyricetin in the literature confirmed our prediction (Figure 5).[33] In addition, we annotated the MS/MS spectrum of **1** using an *in silico* structure prediction platform based on MS fragments algorithms,[34] where the characterized fragment ion of *m/z* 165.08 was indicated as a result of fissions at ring C (Figure 4). The structural identification results proved the substructure prediction based on MS/MS analysis.

Interestingly, this is the first report on the ability of a fungal species to convert the C-ring structure of flavan-3-ols. A similar function was documented by the enzymes from two bacterial strains, i.e., *Pseudomonas* sp. and *Burkholderia oxyphila*, which converted CH_2-4 of epicatechin (or catechin) to carbonyl group.[35,36] The FBMN analysis validated the abilities of strain FA.8-1 to convert both ring B and ring C of flavan-3-ols in a defined media when in pure culture (Figure 3).

The current result inspired us to do a broader screening for other natural products of the fungi from dark tea. Therefore, we incubated all 15 fungal isolates on PT and on PDA, followed by another UHPLC-MS/MS-based FBMN analysis to generate another set of feature IDs (Figures 1 and 6).

FBMN of 15 Fungal Metabolites Derived from EGCG

UHPLC-MS is ultrasensitive in detecting nonvolatile metabolites and is a universal technique used for acquiring qualitative and quantitative data in current metabolomics research.[19,20] Annotation level 1, which identifies the structure of a feature by matching LC retention times in addition to full MS/MS data, is one of the most reliable annotation strategies in metabolomics.[31] The FBRFD standards used in this study, fuzhuanin A, teadenol A, xanthocerin, teasperin and planchol A, as well as the flavan-3-ol C-ring oxidation product, dihydromyricetin, were all isolated and identified by spectroscopy in our previous works or in this study.[10,11,27] In order to validate the bioconversion products of the fungal strains, the controls included the PT_control (no microbe inoculated) and incubation of the isolates on regular PDA media without the tea components (Figures 1, 6 and S1).

The FBMN using the LC-MS data from the PT_control samples showed no precursor signals of the known EGCG oxidation products, i.e., dihydromyricetin or any FBRFD, (Figure S10 in SI-1) and that the structure of EGCG was successfully protected by β-CD. In the autoclaved raw dark tea leaves, the corresponding features were not captured by FBMN (Figure S5 in SI-1), indicating that the hygrothermal effect does not drive the formation of any FBRFD or flavan-3-ol C-ring oxidation products in dark tea. In other words, the oxidation derivatives detected from the cultured PT samples were products converted by the fungi.

The overall FBMN analysis validated fuzhuanin A (ID: 250), teadenol A (ID: 298), xanthocerin (ID: 1754) and dihydromyricetin (ID: 2786) as fungal bioconversion products of EGCG (Figures 6A and S10). We considered that there must be some specific conversion steps in the fungi (route one and route three, Figure 7). We also noticed that planchol A (ID: 1720) and teasperin (ID: 1755) were not observed from any of the tested strains, although the two features were detected in FBT (Figure 6C). Perhaps these corresponding bioconversions are catalyzed by other microbes present in FBT that were not isolated from the material.[30]

Correlation between Fungal Taxonomy and Characteristics of the Bioconversions

A more detailed investigation allowed us to understand the relationship between fungal taxonomy and the isolate's abilities to convert flavan-3-ols. Among these fungi, isolates FTh.1-b and FBa.3-3 (both from the phylum Basidiomycota) and FCm.Y-a (the only one from subphylum Saccharomycotina) showed the weakest ability to bioconvert EGCG to the investigated derivatives (cluster-2, Figure 6C). The MS feature intensities of EGCG when FBa.3-3 and FCm.Y-a were cultured on PT media were almost the same as those in the control (Figure 6C), indicating that a high level of ECGC remained in these samples. For isolate FTh.1-b, although the EGCG level was significantly decreased, there was no corresponding metabolites with B-ring or C-ring conversions observed, implying that this isolate may have a completely different metabolic pathway compared to the other strains (Figure 6C). On the other hand, all isolates from subphylum Pezizomycotina showed much stronger abilities to convert EGCG to FBRFDs, i.e., fuzhuanin A (ID: 250) and teadenol A (ID: 298) (cluster-1, Figure 6C). This indicated that the isolates from Pezizomycotina shared a distinctive characteristic compared to the isolates from the other subphyla.

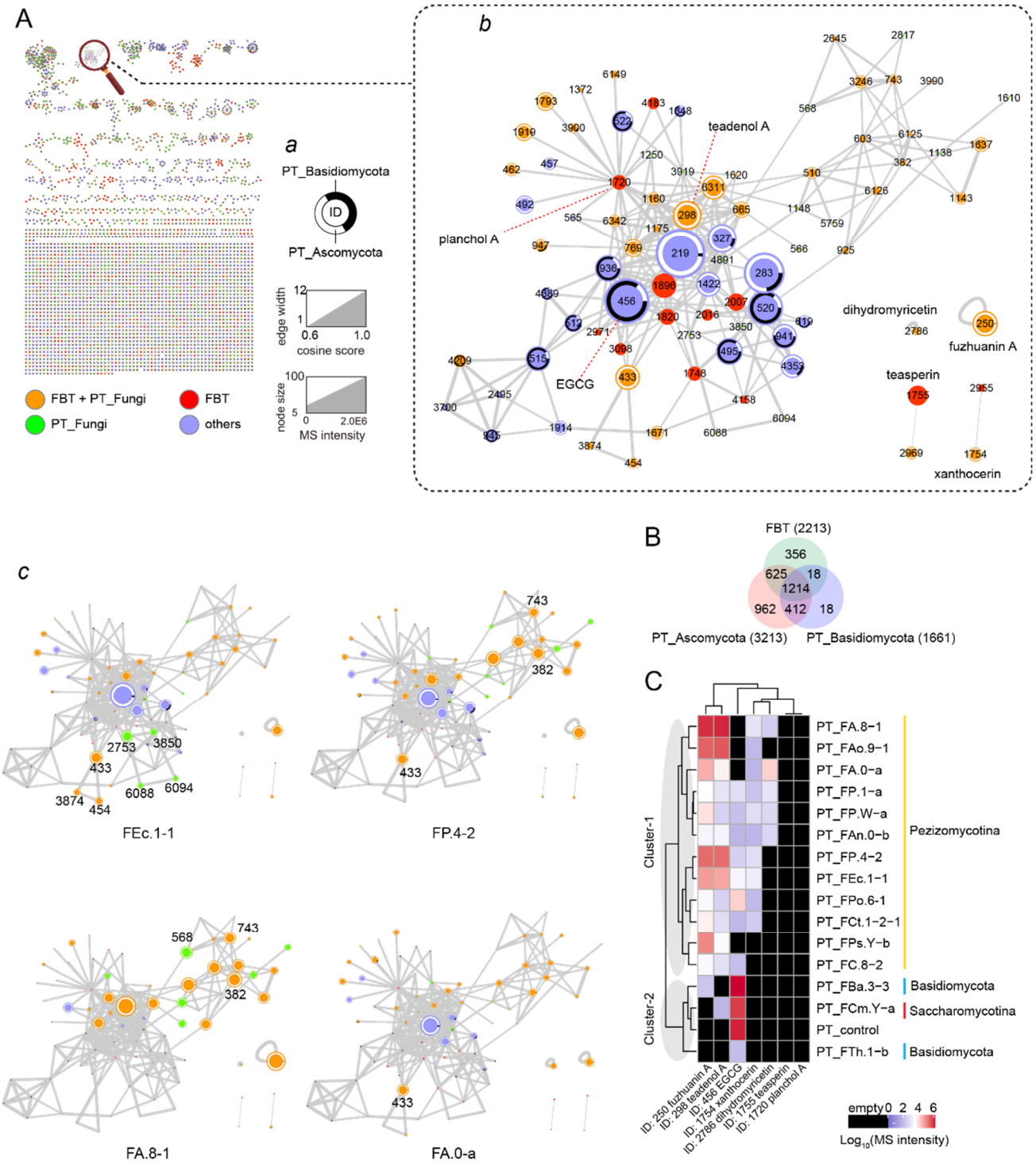

Figure 6. The FBMN and related Venn and heatmap analyses of the products of the 15 fungal strains when incubated on PDA or PDA containing theanine, caffeine and *β*-CD embedded tea EGCG (PT media). A. The FBMN of the PT incubated with the 15 fungal strains. In A, A*b*, and A*c*, features were labeled by node color that were present in only the FBT (red), in the PT culture (green), in both FBT and the PT culture (orange), and in others such as PDA culture or PT_control (blue) *a*: Figure legends (ID: feature ID). *b*: The node clusters of flavan-3-ols and their derivatives. The sizes of the nodes were plotted according to MS intensities of the features in the FBT sample. The black or white ring in the nodes of ID 250 (fuzhuanin A), ID 298 (teadenol A), ID 1754 (xanthocerin) and ID 2786 (dihydromyricetin) indicated them as the products of Ascomycota (white) rather than Basidiomycota (black). *c*: The node clusters of flavan-3-ols and their derivatives plotted according to MS intensities of the features in cultures of four isolates, PT_FEc.1-1 (FEc.1-1), PT_FP.4-2 (FP.4-2), PT_FA.8-1 (FA.8-1) and PT_FA.0-a (FA.0-a), respectively. B. A Venn diagram composed of the number of features in the three groups, i.e., FBT, PT_Ascomycota and PT_Basidiomycota. C. The heatmap representing MS intensities of the representative FBRFDs and dihydromyricetin in the samples. An empty value was presented in black. The phyla or subphyla of fungi were indicated by colored lines.

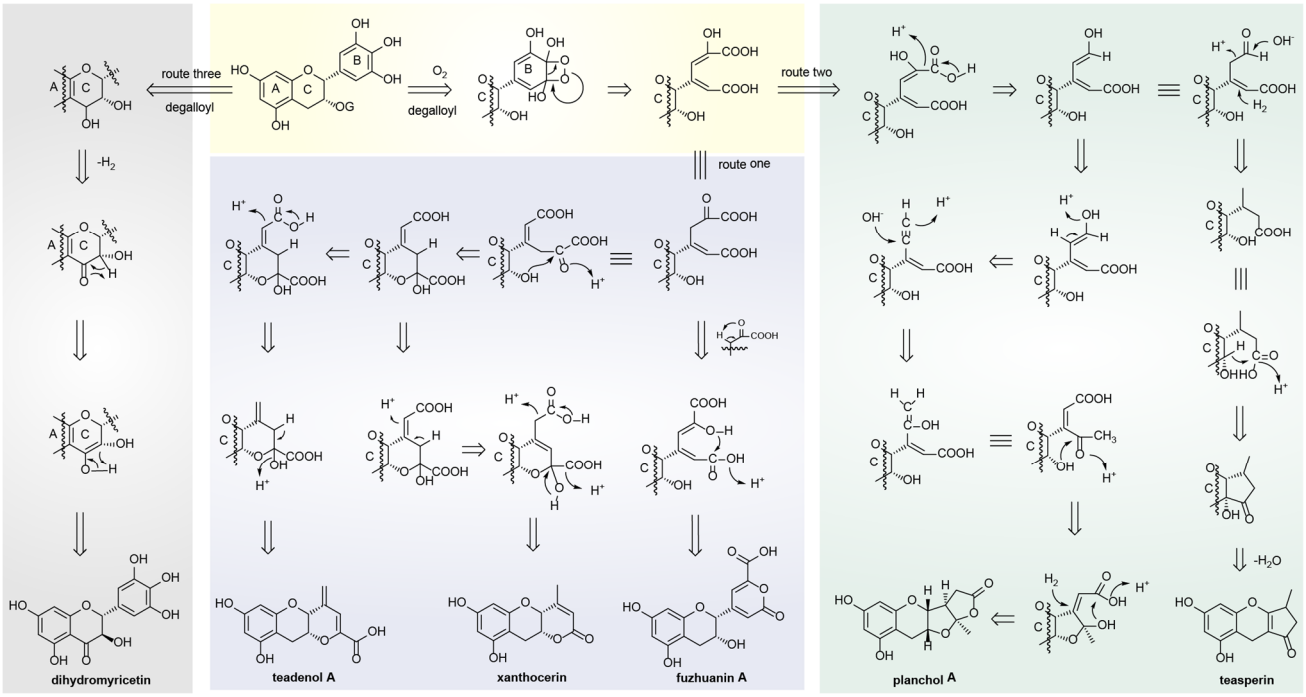

Figure 7. Proposed formation mechanisms of the FBRFDs and dihydromyricetin. Routes one and two are the proposed steps to fuzhuanin A, teadenol A, xanthocerin, planchol A and teasperin with additions to the reference.[10] Route three is the proposed formation route to dihydromyricetin.

The appearance of dihydromyricetin (ID: 2786) was restricted to some specific isolates of *Aspergillus* spp. and *Penicillium* spp., and was notably absent in those of *Eurotium* sp. and *Cladosporium* sp. (Figure 6C and SI-2). In view of this result, we might assume some species in the former two genera have evolved a specific metabolic pathway, which is proposed in the possible mechanisms for the formation of C-ring derivatives from EGCG (route three in Figure 7).

The solid-state fermentation system established in this work validated that the formation of flavan-3-ol derivatives in dark tea is significantly related to the fungal species present in the tea at the phylum/subphylum level.

FBMN-assisted Functional Analysis of the Potential Flavan-3-ol Enzymes in the Fungi

According to the latest proteomic study, oxidation on the B-ring of flavan-3-ols in fungi most likely initiates with a catechol dioxygenase.[37] However, there are still numerous complex steps required to go from the first fission of the rings of ECGC to the varied products (Figure 7).

A model of the three main pathways of the enzymes in the fungi responsible for production of the FRBDs and dihydromyricetin. Inspired by the occurrence of compounds derived from the B- or the C-ring of flavan-3-ol, we presumed some structures of hybrid oxidation products, i.e., the oxidation products on both B- and C-rings (Figure S4 in SI-1). However, no MS signal of these products was captured in the FBMN of the PT samples after careful *in silico* assisted annotations, i.e., CFM-ID and SIRIUS.[34,38] The result indicated that the corresponding enzymes in the fungi needs the original flavanol C ring as a substructural substrate to initialize B-ring oxidation, and vice versa (Figure 8).

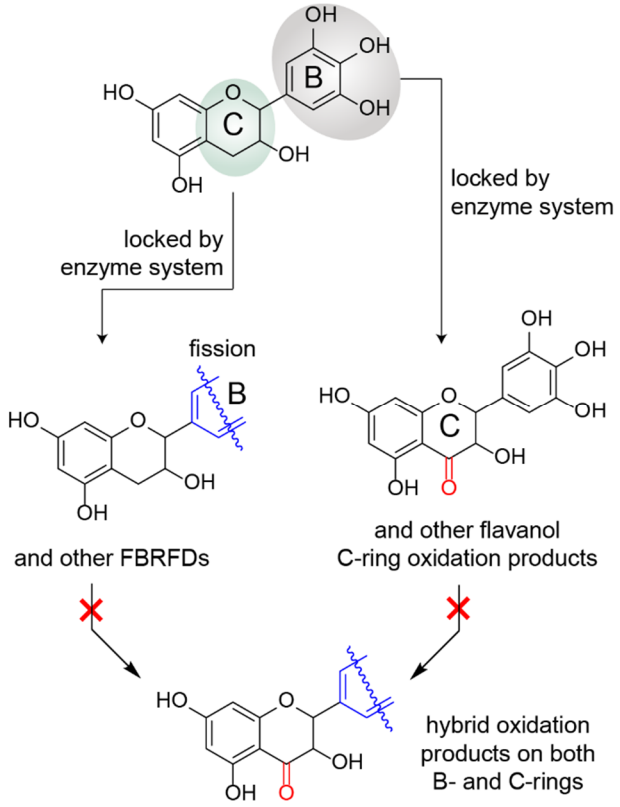

Figure 8. The characteristic oxidation of the B-ring or C-ring of EGCG by fungi isolated from fermented dark teas. Once either ring B or ring C is oxidized by an enzyme, the other ring is protected. The MS data showed that once ring B or ring C is oxidized, there is no further oxidation on the other ring, as no product were found with both rings oxidized.

Based on a brief summary on the references of the known flavan-3-ol derivatives so far, namely 6C/8C-DHHA-EGCG, 6C/8C-*N*-ethyl-2-pyrrolidinone-substituted flavan-3-ols, theaflavins, methylated EGCG, FBRFDs, and dihydroflavonols,[8,39,40] we propose that non-enzymatic reactions on EGCG/EGC lead to the modifications on ring A, while enzymatic reactions lead to those on rings B/C. This idea regarding enzymatic reaction positions on flavan-3-ols should be validated, as it may explain the formation of the diverse derivatives.

Other Results Based on the FBMN Analysis

The data indicated that some fungal strains should be further studied, as the MS intensities of some bioconverted products were relatively higher in their cultures (Figure 6A). These strains could be used to isolate the enzymes responsible for bioconversion of FBRFDs, and it is also possible that these fungal strains could produce other novel bioactive metabolites. Among the screened fungal isolates, four strains, FP.4-2 (*Penicillium* sp.), FEc.1-1 (*Eurotium cristatum*), FA.8-1 (*Aspergillus* sp.) and FA.0-a (*Aspergillus* sp.), showed a wider variety of products with high MS intensities. The corresponding products included, but were not limited to, the following features: ID 433 (*m/z* 613.157 from FEc.1-1, FP.4-2 and FA.0-a), ID 382 (*m/z* 339.072 from FP.4-2 and FA.8-1), ID 743 (*m/z* 295.081 from FP.4-2 and FA.8-1), ID 568 (*m/z* 553.135 from FA.8-1), as well as some features present only in the FEc.1-1 cultures, such as ID 3874 (*m/z* 469.135), ID 3850 (*m/z* 571.125), ID 2753 (*m/z* 557.11), ID 6094 (*m/z* 875.185), ID 6088 (*m/z* 861.167) and ID 454 (*m/z* 919.23), etc. (Figure 6A*c*). As far as we know, no flavan-3-ol derivatives with such precursor ions have previously been reported. Their structures, potential bioactivities and even flavors are a valuable line of future study.

As expected, fungi from phylum Ascomycota contributed more features to FBT than those from phylum Basidiomycota (1839 versus 1232, Figure 6B), which correlated with the number of isolates from Ascomycota (13 strains) and from Basidiomycota (2 strains). However, we were surprised that nearly 84% (1857 over 2213) of the metabolites found in the FBT samples were also found in the individual strain cultures on PT (Figure 6B). This is a potential yet important reason why different dark teas have characteristic flavors and health functions, as the microbiota of each kind of dark tea is unique.

The existence of dihydroflavonols in dark tea has been documented.[11] Although dihydroflavonols seem to be associated with general tea flavonols, we might have to change our view on the origin of this class of natural products in dark tea, and even in other materials containing flavan-3-ols, since dihydroflavonols could be products derived by microbial bioconversion of flavan-3-ols.

In view of both the diverse bioactivities of flavonoids and the wide-ranging bioconversion abilities of microorganisms, there is an increasing number of studies aiming to understand the metabolic pathways of microbes and to discover novel bioactive natural products.[41] We were surprised by the ubiquitous derivatives converted by fungi. It could be hypothesized that the microbes were forced to evolve various abilities to degrade and/or convert the flavonoids produced by plants, including flavan-3-ols, to reduce the risk of being harmed by these compounds.[42]

Embedding EGCG in β-CD, or the inclusion complex of β-CD and EGCG, was invented to prevent the oxidation of EGCG.[18] As far as we know, this is the first time to introduce this complex to solid-state microbial culture. There were some excellent explorations into the bioconversion of polyphenols by isolated microbes, including from dark tea samples, during liquid, anaerobic fermentation.[35,43-45] However, neither the exact structures nor the diversity of the products matched those extracted directly from dark teas, which is the result of solid-state, aerobic fermentation.[4,5] This result inspired us to develop a solid-state culture for fungal strains isolated from dark teas.

The *in vitro* solid-state fermentation system established in the current study supplied a convenient and reliable way for investigation of microbial conversion of EGCG and other unstable compounds. This system is especially useful for aerobic microorganisms. In addition, the system provides a platform where the bioconverted metabolites could be investigated by several approaches, e.g., MS imaging.[29] This system also provides a way to analyze products that require the actions of multiple microbes. More importantly, our work integrated FBMN, an emerging LC-MS data analysis technique, with an *in vitro* fermentation system (Figure 1). Compared to the traditional strategy of natural product chemistry research, i.e., isolating and identifying compounds with the occasional aid of manual inspection of LC-MS data, the strategy in this study is more efficient in exploring global results for the investigated material.

Nevertheless, there are still several drawbacks in this study. For example, the biomass of the fungi in the 96-well plate cultures was not quantified, although we did observe relatively equal mycelial abundance for the tested strains. Differences in growth might influence the bioconversion ratios among the strains (Figure 6C). In addition, more microbial strains and more standard compounds from dark teas are needed. The above points are why we could only draw relationships between the structure of the metabolites and the phylum or subphylum level of fungal taxonomy, and not to the family or genus levels (Figure 6C). On the other hand, bacteria in dark teas have been observed to modify the structures of flavan-3-ols as well. The corresponding investigation is on the way and will be reported in the near future.

ABBREVIATIONS USED

BLAST: basic local alignment search tool; CC: column chromatography; CDTE: β-CD embedded tea EGCG; CH_2Cl_2: dichloromethane; COSY: correlated spectroscopy; DMSO-d_6: deuterated dimethyl sulfoxide; D_2O: deuteroxide; EC: epicatechin; EGC: epigallocatechin; EGCG: epigallocatechin gallate; EtOAc: ethyl acetate; FBMN: feature-based molecular networking; FBRFDs: flavan-3-ol B-ring fission derivatives; FBT: Fuzhuan brick tea; GNPS: global natural product social; HMBC: heteronuclear multiple bond coherence; NMR: nuclear magnetic resonance; PCR: polymerase chain reaction; PDA: potato dextrose agar; PDAD: photodiode array detector; PE:

petroleum ether; PT: PDA mixed with the dominant compounds in tea; TLC: thin layer chromatography; UHPLC-ESI-MS: ultra-high performance liquid chromatography electro-spray ionization mass spectrometry; UV: ultraviolet.

ACKNOWLEDGEMENTS

We thank Pieter C. Dorrestein, director of Collaborative Mass Spectrometry Innovation Center, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, for supplying opportunities for UHPLC-ESI-QTOF-MS measurements. We thank Xin-Yi Liu, executive manager of Yiyang Fu Cha Industry Development Co. Ltd., for supplying the tea samples, and Hui-Min Guo, Biotechnology Center of Anhui Agricultural University, for the UHPLC-ESI-Orbitrap-MS measurements. We thank Michael J. Meehan and Alexey V. Melnik, Collaborative Mass Spectrometry Innovation Center, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, for assisting with the UHPLC-ESI-QTOF-MS measurements.

FUNDING

This work was supported by the National Natural Science Foundation of China (grant No. 31471720, 31760225), the U.S. National Institutes of Health for the Center for Computational Mass Spectrometry grant (P41 GM103484), the reuse of metabolomics data (R03 CA211211), and the tools for rapid and accurate structure elucidation of natural products (R01 GM107550), the China Scholarship Council during the visit of Tie-Jun Ling (201408775014) to University of California San Diego, the Modern Agro-Industry Technology Research System, Ministry of Agriculture & Ministry of Finance, People's Republic of China, National Key Research & Development Plan (2017YFD0400800), the Open Fund of the State Key Laboratory of Tea Plant Biology and Utilization (SKLTOF201801109), and the Key Grant for Innovative Province from the Department of Sciences and Technology of Anhui Province to Prof. Zhong-Wen Xie (grant No. 11080001).

SUPPORTING INFORMATION AVAILABLE DESCRIPTION

Figures S1 to S9 and Table S2 are available in SI-1.doc.
The full names along with some information of the 15 fungal strains are available in SI-2.xlsx.

REFERENCES

1. Wan, X. C. *Tea Biochemistry*, 3rd ed.; China Agriculture Press: Beijing, 2003; pp 268−275 (in Chinese).
2. Lv, H. P.; Zhang, Y. J.; Lin, Z.; Liang, Y. R. Processing and chemical constituents of Pu-erh tea: a review. *Food Res. Int*. **2013**, *53*, 608−618.
3. Jin, X. Y.; Chen, J. B.; Ji, K. W. *Tea Process Engineering*, 1st ed.; China Agriculture Press: Beijing, 2003; pp 45−125 (in Chinese).
4. Zhang, L.; Ho, C. T.; Zhou, J.; Santos, J. S.; Armstrong, L.; Granato, D. Chemistry and biological activities of processed *Camellia sinensis* teas: a comprehensive review. *Compr. Rev. Food Sci. Food Saf.* **2019**, *18*, 1474−1495.
5. Zhu, M. Z.; Li, N.; Zhou, F.; Ouyang, J.; Lu, D. M.; Xu, W.; Li, J.; Lin, H. Y.; Zhang, Z.; Xiao, J. B.; Wang, K. B.; Huang, J. A.; Liu, Z. H.; Wu, J. L. Microbial bioconversion of the chemical components in dark tea. *Food Chem.* **2020**, *312*, 126043.
6. All-China federation of supply and marketing cooperatives. *Technology regulations for Fu tea processing* (GH/T 1246-2019); Standards Press of China: Beijing, 2019 (in Chinese).
7. Nagle, D. G.; Ferreira, D.; Zhou, Y. D. Epigallocatechin-3-gallate (EGCG): chemical and biomedical perspectives. *Phytochemistry* **2006**, *67*, 1849−1855.
8. Sang, S.; Lambert, J. D.; Ho, C. T.; Yang, C. S. The chemistry and biotransformation of tea constituents. *Pharmacol. Res.* **2011**, *64*, 87−99.
9. Li, Q.; Huang, J. N.; Li, Y. D.; Zhang, Y. Y.; Luo, Y.; Chen, Y.; Lin, H. Y.; Wang, K. B.; Liu, Z. H. Fungal community succession and major components change during manufacturing process of Fu brick tea. *Sci. Rep*. **2017**, *7*, 1−9.
10. Luo, Z. M.; Du, H. X.; Li, L. X.; An, M. Q.; Zhang, Z. Z.; Wan, X. C.; Bao, G. H.; Zhang, L.; Ling, T. J. Fuzhuanins A and B: the B-ring fission lactones of flavan-3-ols from Fuzhuan brick-tea. *J. Agric. Food Chem.* **2013**, *61*, 6982−6990.
11. Zhu, Y. F.; Chen, J. J.; Ji, X. M.; Hu, X.; Ling, T. J.; Zhang, Z. Z.; Bao, G. H.; Wan, X. C. Changes of major tea polyphenols and production of four new B-ring fission metabolites of catechins from post-fermented Jing-Wei Fu brick tea. *Food Chem.* **2015**, *170*, 110−117.
12. Yue, Y.; Chu, G. X.; Liu, X. S.; Tang, X.; Wang, W.; Liu, G. J.; Yang, T.; Ling, T. J.; Wang, X. G.; Zhang, Z. Z. TMDB: A literature-curated database for small molecular compounds found from tea. *BMC Plant Biol*. **2014**, *14*, 243.
13. Meng, X. H.; Li, N.; Zhu, H. T.; Wang, D.; Yang, C. R.; Zhang, Y. J. Plant resources, chemical constituents, and bioactivities of tea plants from the genus *Camellia* section *Thea*. *J. Agric. Food Chem.* **2019**, *67*, 5318−5349.
14. 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**, *67*, 652−656.
15. 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.
16. Su, X. Q.; Zhang, G. J.; Ma, Y.; Chen, M.; Chen, S. H.; Duan, S. M.; Wan, J. Q.; Hashimoto, F.; Lv, H. P.; Li, J. H.; Lin, Z.; Zhao, M. Isolation, identification, and biotransformation of teadenol A from solid-state

fermentation of Pu-erh tea and *in vitro* antioxidant activity. *Appl. Sci.* **2016**, *6*, 161.

17. Wang, R.; Zhou, W. B.; Jiang, X. H. Reaction kinetics of degradation and epimerization of epigallocatechin gallate (EGCG) in aqueous system over a wide temperature range. *J. Agric. Food Chem.* **2008**, *56*, 2694−2701.

18. Ishizu, T.; Hirata, C.; Yamamoto, H.; Harano, K. Structure and intramolecular flexibility of *β*-cyclodextrin complex with (-)-epigallocatechin gallate in aqueous solvent. *Magn. Reson. Chem.* **2006**, *44*, 776−783.

19. Nothias, L.F.; Petras, D.; Schmid, R.; Dührkop, K.; Rainer, J.; Sarvepalli, A.; Protsyuk, I.; Ernst, M.; Tsugawa, H.; Fleischauer, M.; Aicheler, F.; Aksenov, A.; Alka, O.; Allard, P. M.; Barsch, A.; Cachet, X.; Caraballo, M.; Silva, R. R. D.; Dang, T.; Garg, N.; Gauglitz, J. M.; Gurevich, A.; Isaac, G.; Jarmusch, A. K.; Kameník, Z.; Kang, K. B.; Kessler, N.; Koester, I.; Korf, A.; Gouellec, A. L.; Ludwig, M.; Christian, M. H.; McCall, L. I.; McSayles, J.; Meyer, S. W.; Mohimani, H.; Morsy, M.; Moyne, O.; Neumann, S.; Neuweger, H.; Nguyen, N. H.; Nothias-Esposito, M.; Paolini, J.; Phelan, V. V.; Pluskal, T.; Quinn, R. A.; Rogers, S.; Shrestha, B.; Tripathi, A.; van der Hooft, J. J. J.; Vargas, F.; Weldon, K. C.; Witting, M.; Yang, H.; Zhang, Z.; Zubeil, F.; Kohlbacher, O.; Böcker, S.; Alexandrov, T.; Bandeira, N.; Wang, M.; Dorrestein, P. C. Feature-based Molecular Networking in the GNPS analysis environment. bioRxiv 2019, doi:10.1101/812404 https://www.biorxiv.org/content/10.1101/812404v1.

20. Wang, M. X.; Carver, J. J.; Phelan, V. V.; Sanchez, L. M.; Garg, N.; Peng, Y.; Nguyen, D. D.; Watrous, J.; Kapono, C. A.; Luzzatto-Knaan, T. L.; Porto, C.; Bouslimani, A.; Melnik, A. V.; Meehan, M. J.; Liu, W. T.; Crüsemann, M.; Boudreau, P. D.; Esquenazi, E.; Sandoval-Calderón, M.; Kersten, R. D.; Pace, L. A.; Quinn, R. A.; Duncan, K. R.; Hsu, C. C.; Floros, D. J.; Gavilan, R. G.; Kleigrewe, K.; Northen, T.; Dutton, R. J.; Parrot, D.; Carlson, E. E.; Aigle, B.; Michelsen, C. F.; Jelsbak, L.; Sohlenkamp, C.; Pevzner, P.; Edlund, A.; McLean, J.; Piel, J.; Murphy, B. T.; Gerwick, L.; Liaw, C. C.; Yang, Y. L.; Humpf, H. U.; Maansson, M.; Keyzers, R. A.; Sims, A. C.; Johnson, A. R.; Sidebottom, A. M.; Sedio, B. E.; Klitgaard, A.; Larson, C. B.; Boya P, C. A.; Torres-Mendoza, D.; Gonzalez, D. J.; Silva, D. B.; Marques, L. M.; Demarque, D. P.; Pociute, E.; O'Neill, E. C.; Briand, E.; Helfrich, E. J. N.; Granatosky, E. A.; Glukhov, E.; Ryffel, F.; Houson, H.; Mohimani, H.; Kharbush, J. J.; Zeng, Y.; Vorholt, J. A.; Kurita, K. L.; Charusanti, P.; McPhail, K. L.; Nielsen, K. F.; Vuong, L.; Elfeki, M.; Traxler, M. F.; Engene, N.; Koyama, N.; Vining, O. B.; Baric, R.; Silva, R. R.; Mascuch, S. J.; Tomasi, S.; Jenkins, S.; Macherla, V.; Hoffman, T.; Agarwal, V.; Williams, P. G.; Dai, J. Q.; Neupane, R.; Gurr, J.; Rodríguez, A. M. C.; Lamsa, A.; Zhang, C.; Dorrestein, K.; Duggan, B. M.; Almaliti, J.; Allard, P. M.; Phapale, P.; Nothias, L. F.; Alexandrov, T.; Litaudon, M.; Wolfender, J. L.; Kyle, J. E.; Metz, T. O.; Peryea, T.; Nguyen, D. T.; VanLeer, D.; Shinn, P.; Jadhav, A.; Müller, R.; Waters, K. M.; Shi, W. Y.; Liu, X. T.; Zhang, L. X; Knight, R.; Jensen, P. R.; Palsson, B.; Pogliano, K.; Linington, P. G.; Gutiérrez, M.; Lopes, N. P.; Gerwick, W. H.; Moore, B. S.; Dorrestein, P. C.; Bandeira, N. Sharing and community curation of mass spectrometry data with global natural products social molecular networking. *Nat. Biotechnol.* **2016**, *34*, 828−837.

21. Wang, M. X.; Jarmusch, A. K.; Vargas, F.; Aksenov, A. A.; Gauglitz, J. M.; Weldon, K.; Petras, D.; Da Silva, R.; Quinn, R.; Melnik, A. V.; Van der Hooft, J. J. J.; Caraballo-Rodríguez, A. M.; Nothias, L. F.; Aceves, C. M.; Panitchpakdi, M.; Brown, E.; Di Ottavio, F.; Sikora, N.; Elijah, E. O.; Labarta-Bajo, L.; Gentry, E. C.; Shalapour, S.; Kyle, K. E.; Puckett, S. P.; Watrous, J. D.; Carpenter, C. S.; Bouslimani, A.; Ernst, M.; Swafford, A. D.; Zúñiga, E. I.; Balunas, M. J.; Klassen, J. L.; Loomba, R.; Knight, R.; Bandeira, N.; Dorrestein, P. C. Mass spectrometry searches using MASST. *Nat. Biotechnol.* **2020**, *38*, 23−26.

22. Shannon, P.; Markiel, A.; Ozier, O.; Baliga, N. S.; Wang, J. T.; Ramage, D.; Amin, N.; Schwikowski, B.; Ideker, T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*. **2003**, *13*, 2498−504.

23. Wu, H. Y.; Ke, J. P.; Wang, W.; Kong, Y. S.; Zhang, P.; Ling, T. J.; Bao, G. H. Discovery of neolignan glycosides with acetylcholinesterase inhibitory activity from huangjinya green tea guided by ultra performance liquid chromatography-tandem mass spectrometry data and global natural product social molecular networking. *J. Agric. Food Chem.* **2019**, *67*, 11986−11993.

24. Reher, R.; Kim, H. W.; Zhang, C.; Mao, H. H.; Wang, M.; Nothias, L. F.; Caraballo-Rodriguez, A. M.; Glukhov, E.; Teke, B.; Leao, T.; Alexander, K. L.; Duggan, B. M.; Van Everbroeck, E. L.; Dorrestein, P. C.; Cottrell, G. W.; Gerwick, W. H. A convolutional neural network-based approach for the rapid annotation of molecularly diverse natural products. *J. Am. Chem. Soc.* **2020**, *142*, 4114−4120.

25. Nothias, L. F.; Nothias-Esposito, M.; Da Silva, R.; Wang, M. X; Protsyuk, I.; Zhang, Z.; Sarvepalli, A.; Leyssen, P.; Touboul, D.; Costa, J.; Paolini, J.; Alexandrov, T.; Litaudon, M.; Dorrestein, P. C. Bioactivity-based molecular networking for the discovery of drug leads in natural product bioassay-guided fractionation. *J. Nat. Prod.* **2018**, *81*, 758−767.

26. Hou, X. M.; Li, Y. Y.; Shi, Y. W.; Fang, Y. W.; Chao, R.; Gu, Y. C.; Wang, C. Y.; Shao, C. L. Integrating molecular networking and ^{1}H NMR to target the isolation of chrysogeamides from a library of marine-derived *Penicillium* fungi. *J. Org. Chem.* **2019**, *84*, 1228−1237.

27. 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.

28. Zhu, H.; Qu, F.; Zhu, L. H. Isolation of genomic DNAs from plants, fungi and bacteria using benzyl chloride. *Nucleic Acids Res*. **1993**, *21*, 5279.

29. Protsyuk, I.; Melnik, A. V.; Nothias, L. F.; Rappez, L.; Phapale, P.; Aksenov, A. A.; Bouslimani, A.; Ryazanov, S.; Dorrestein, P. C.; Alexandrov, T. 3D molecular cartography using LC-MS facilitated by Optimus and 'ili software. *Nat. Protoc.* **2018**, *13,* 134−154.

30. Wolfe, B. E.; Dutton, R. J. Fermented foods as experimentally tractable microbial ecosystems. *Cell* **2015**, *161*, 49−55.

31. Sumner, L. W.; Amberg, A.; Barrett, D.; Beale, M. H.; Beger, R.; Daykin, C. A.; Fan, T. W. M.; Fiehn, O.; Goodacre, R.; Griffin, J. L.; Hankemeier, T.; Hardy, N.; Harnly, J.; Higashi, R.; Kopka, J.; Lane, A. N.; Lindon, J. C.; Marriott, P.; Nicholls, A. W.; Reily, M. D.; Thaden, J. J.; Viant, M. R. Proposed minimum reporting standards for chemical analysis. *Metabolomics* **2007**, *3*, 211−221.

32. Davis, A. L.; Cai, Y.; Davies, A. P.; Lewis, J. R. ^{1}H and ^{13}C NMR assignments of some green tea polyphenols. *Magn. Reson. Chem*. **1996**, *34*, 887−890.

33. Zhang, Y.; Que, S.; Yang, X.; Wang, B.; Qiao, L.; Zhao, Y. Isolation and identification of metabolites from dihydromyricetin. *Magn. Reson. Chem.* **2007**, *45*, 909−916.

34. Djoumbou-Feunang, Y.; Pon, A.; Karu, N.; Zheng, J. M; Li, C.; Arndt, D.; Gautam, M.; Allen, F.; Wishart, D. S. CFM-ID 3.0: Significantly improved ESI-MS/MS prediction and compound identification. *Metabolites* **2019**, *9*, 72.

35. Jeffrey, A. M.; Knight, M.; Evans, W. C. The bacterial degradation of flavonoids-hydroxylation of the A-ring of taxifolin by a soil *Pseudomonad*. *Biochem. J*. **1972**, *130*, 373−381.

36. Otsuka, Y.; Matsuda, M.; Sonoki, T.; Sato-Izawa, K.; Goodell, B.; Jelison, J.; Navarro, R. R.; Murata, H.; Nakamura, M. Enzymatic activity of cell-free extracts from *Burkholderia oxyphila* OX-01 bio-converts (+)-catechin and (−)-epicatechin to (+)-taxifolin. *Biosci. Biotechnol. Biochem.* **2016**, *80*, 2473−2479.

37. Zhao, M.; Su, X. Q.; Nian, B.; Chen, L. J.; Zhang, D. L.; Duan, S. M.; Wang, L. Y.; Shi, X. Y.; Jiang, B.; Jiang, W. W.; Lv, C. Y.; Wang, D. P.; Shi, Y.; Xiao, Y.; Wu, J. L.; Pan, Y. H.; Ma, Y. Integrated meta-omics approaches to understand the microbiome of spontaneous fermentation of traditional Chinese Pu-erh tea. *mSystems* **2019**, *4*, e00680-19.

38. Dührkop, K.; Fleischauer, M.; Ludwig, M.; Aksenov, A. A.; Melnik, A. V.; Meusel, M.; Dorrestein, P. C.; Rousu, J.; Böcker, S. SIRIUS 4: a rapid tool for turning tandem mass spectra into metabolite structure information. *Nat. Methods* **2019**, *16*, 299−302.

39. Meng, Q.; Li, S. M.; Huang, J. Q.; Wei, C. C.; Wan, X. C.; Sang, S. M.; Ho, Q. T. Importance of the nucleophilic property of tea polyphenols. *J. Agric. Food Chem.* **2019**, *67*, 5379−5383.

40. Huang, W. L.; Wang, S. Y.; Sang, S. M.; Wan, X. C.; Wang, Y.; Ho, C. T. Quantification of ascorbyl adducts of epigallocatechin gallate and gallocatechin gallate in bottled tea beverages. *Food Chem.* **2018**, *261*, 246−252.

41. Cao, H.; Chen, X. Q.; Jassbi, A. R.; Xiao, J. B. Microbial biotransformation of bioactive flavonoids. *Biotechnol. Adv.* **2015**, *33*, 214−223.

42. Patel, K. D.; Scarano, F. J.; Kondo, M.; Hurta, R. A. R.; Neto, C. C. Proanthocyanidin-rich extracts from cranberry fruit (*Vaccinium macrocarpon* Ait.) selectively inhibit the growth of human pathogenic fungi *Candida* spp. and *Cryptococcus neoformans*. *J. Agric. Food Chem.* **2011**, *59*, 12864−12873.

43. 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.

44. Van't Slot, G.; Humpf, H. U. Degradation and metabolism of catechin, epigallocatechin-3-gallate (EGCG), and related compounds by the intestinal microbiota in the pig cecum model. *J. Agric. Food Chem.* **2009**, *57*, 8041−8048.

45. Van Dorsten, F. A.; Peters, S.; Gross, G.; Gomez-Roldan, V.; Klinkenberg, M.; De Vos, R. C.; Vaughan, E. E.; Van Duynhoven, J. P.; Possemiers, S.; Van de Wiele, T.; Jacobs, D. M. Gut microbial metabolism of polyphenols from black tea and red wine/grape juice is source-specific and colon-region dependent. *J. Agric. Food Chem.* **2012**, *60*, 11331−11342.