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Discovery of tanshinone derivatives with anti-MRSA activity via targeted bio-transformation

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ABSTRACT

Two potent anti-MRSA tanshinone glycosides (**1** and **2**) were discovered by targeted microbial biotransformation, along with rapid identification via MS/MS networking. Serial reactions including dehydrogenation, demethylations, reduction, glycosylation and methylation have been observed after incubation of tanshinone IIA and fungus *Mucor rouxianus* AS 3.3447. In addition, tanshinosides B (**2**) showed potent activities against serial clinical isolates of oxacillin-resistant *Staphylococcus aureus* with MIC values of 0.78 μg/mL. This is the first study that shows a significant increase in the level and activities of tanshinone glycosides relative to the substrate tanshinone IIA.

1. Introduction

Natural products have various biological activities and are a rich source of compounds in drug discovery [1–3]. However, drug discovery from natural products has diminished in the past two decades, because of decreased financial assistance, technical barriers to screening natural products using a high-throughput assay against molecular targets, and lack of reliability and efficient dereplication of natural products. The renaissance of natural products depends on the development of new techniques for quick identification of novel natural products, development of efficient tools for genome mining, and the discovery of new horizons for drug leads [4–12].

Lead compounds from natural sources and their structure-based functional optimization have become increasingly valuable as tools for investigation of the fundamental aspects of biological systems [13]. Biotransformation is regarded as enzyme-catalyzed reaction using enzymes, microorganisms or cell suspension cultures. It has been shown to be a powerful addition to the expanding array of synthetic strategies for the generation and optimization of lead compounds in drug discovery and development [14–20]. The advantages of biotransformation are incremental regio- and stereo selectivity and its environment friendly nature. Compared to organic synthesis, it is an alternative method of structural modification for obtaining optically pure compounds and providing efficient routes to targeted products. Currently, the challenge for biotransformation is to figure out strains which contains active enzymes to modification of the substrates. As depicted in the review of biotransformation [18–20], the discovery of potential strains needed hard and time-consuming screen traditionally. Hence, it is necessary to improve the classical screening method for a rapid and targeted screening platform [21].

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Our laboratory is committed to the study of new antibiotics by a bioassay-guided isolation approach, especially those are effective against drug-resistant pathogens [22–24]. Activity screening using the constructed Natural Product Library (NPL) was performed in our laboratory and the results revealed that tanshinone IIA (Tan IIA, **5**), the predominant substance in Chinese herb *Salvia miltiorrhiza*, possessed anti-bacterial activity against *Staphylococcus aureus* and has a minimum inhibitory concentration (MIC) of 3.125 μg/mL, while it was not effective against methicillin-resistant *S. aureus* (MRSA). Recently, the emergence of drug-resistant organisms, such as MRSA and vancomycin-resistant *S. aureus* (VRSA), has made treatment difficult and increased the rate of mortality [25,26]. We aimed to improve the anti-MRSA and anti-SA activities of Tan IIA by a targeted biotransformation screen. In our work, a rapid and efficient microbial screening procedure was developed utilizing a 24-well plate, according to the method developed by Stahl et al. [27,28]. A total of 203 microbial strains have been screened for their biotransformation activities on Tan IIA, including 87 fungi (Table S5) and 116 endophytic microbes from Traditional Chinese Medicines (TCMs) plants (Table S6). All strains were selected from our in-house strain library based on two criteria: a) Strains which can modify structures of terpenoids through biotransformation [29–31]; b) endophytic microorganisms isolated from plants, which are more likely to recognize and process natural products of plant origin.

Based on HPLC-DAD-UV analysis and activity evaluation, *Mucor rouxianus* 3.3447 was selected as the most promising strain since it showed the best activity among the fractions. Then, an MS/MS network was generated on the MS2 data set from biotransformed extract of *M. rouxianus* 3.3447, which revealed a number of new products structurally related to Tan IIA. Scale-up fermentation was performed and a series of new glycoside analogs of Tan IIA, tanshinosides A–D (**1**–**4**) (Fig. 1), were identified based on bioassay-guided purification and different NMR techniques. Thus, an MS/MS network-assisted microtiter-targeted biotransformation platform was established in this study as shown in Fig. 2.

2. Material and methods

2.1. General experimental details

The optical rotation was measured using a Perkin-Elmer Model 343 polarimeter with a 5 cm cell. The UV–vis spectra were obtained on a Cary 50 spectrophotometer. IR spectrum was recorded on a Nicolet 5700 FT-IR Microscope spectrometer (FT-IR Microscope Transmission). The NMR spectra were obtained on a Bruker Avance DRX600 spectrometer. Chemical shifts were calibrated internally against residual solvent signals (DMSO-d_6: δ_C 39.5, δ_H 2.50). HRESIMS measurements were obtained on Agilent 1200 HPLC/6520 QTOF-MS. ODS-A (Octadecylsilyl-A, YMC, 150 μm, AA12SA5) were used for purification. HPLC was performed using Agilent 1200 Series HPLC controlled using ChemStation Rev.B.02.01. Energy minimization (MM2) of structures was performed using Chem3D 12.0. (Cambridge Soft). Tanshinone IIA was purchased from Xi'an Hao-Xuan Bio-Tech Co., Ltd.

2.2. Micro-titer biotransformation screening

All strains were cultured on agar plates and stored in the fridge at 4 °C. Then, 1.0 mL sterile saline water (0.8% [m/v]) was added to the agar dish for each strain. After washing the spores to remove the mycelia, the conidial suspension was prepared by transferring 0.5 mL saline water into the wells of a 24-well cell culture plate (3 mL total volume capacity), which contained 0.5 mL of 50% glycerol. This procedure was repeated for each strain, and the 24-well cell plates (master plates) were stored at −80 °C until use. Each conidial suspension (200 μL) from the master plates was inoculated into a corresponding well in a 24-well plate (10 mL total volume capacity) (assay plates) containing 2 mL of the media using a multichannel pipette. For actinomycetes, ISP2 was chosen as the culture medium whereas for fungi, potato dextrose broth (PDB) was selected as the culture medium. After 72 h of incubation at 28 °C and 140 rpm in a shaker, 0.15 mg of Tan IIA in 20 μL methanol and

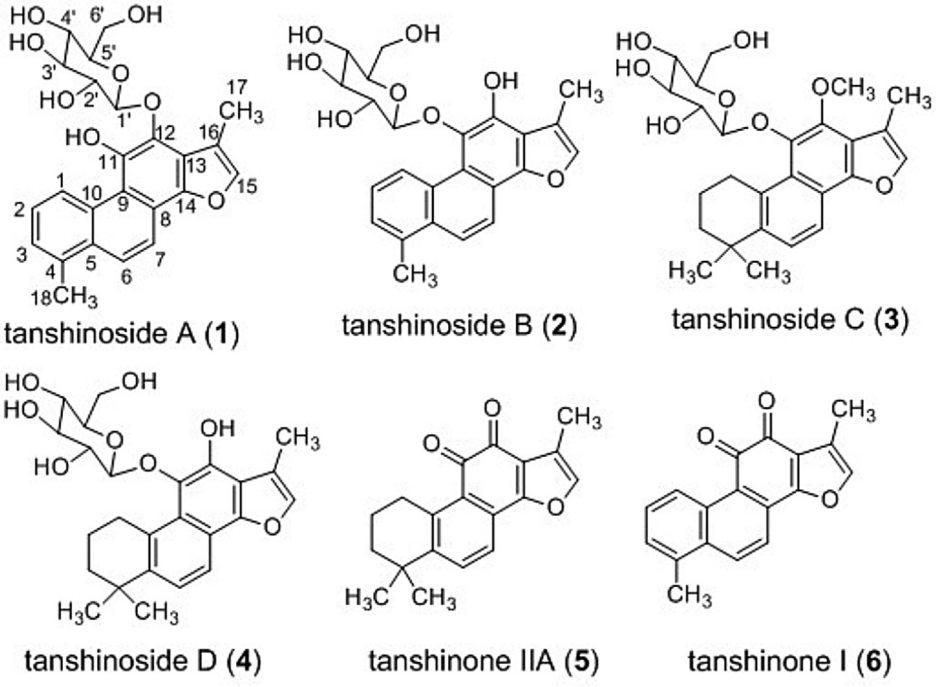

Fig. 1. Structure of isolated tanshinosides A–D (**1**–**4**), tanshinone IIA (**5**) and tanshinone I (**6**).

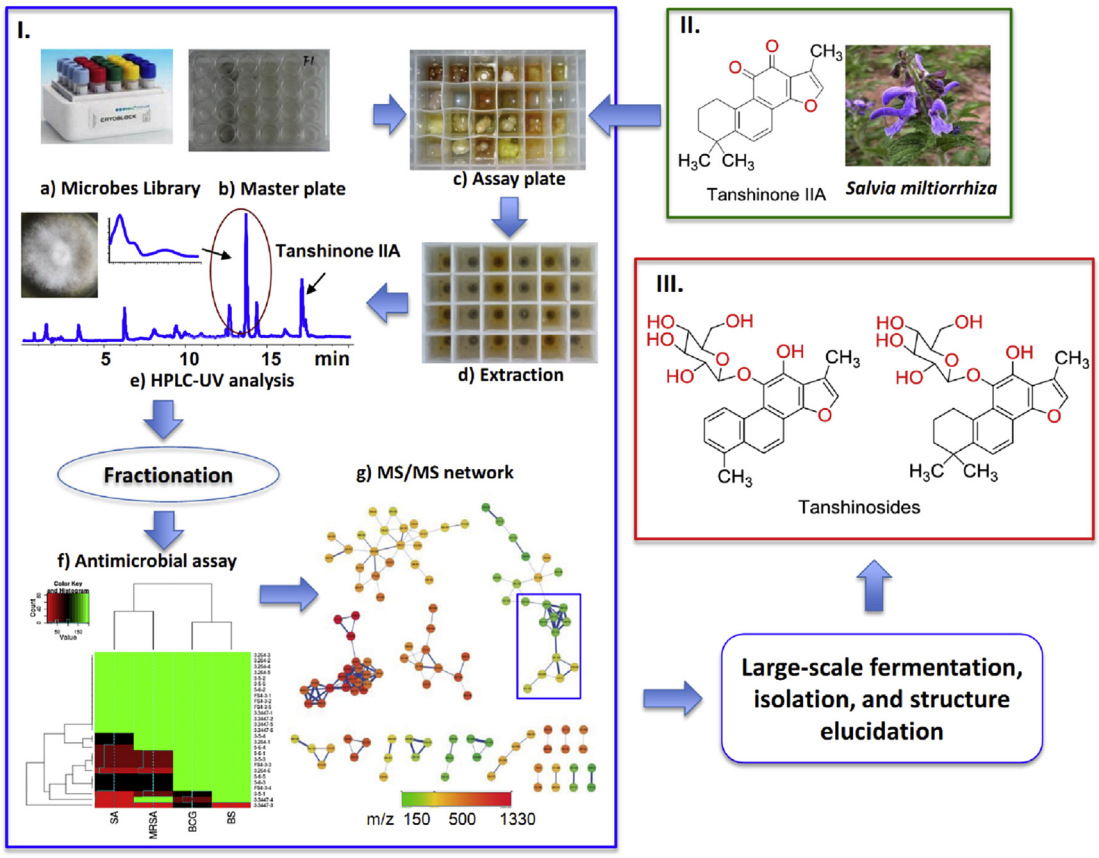

Fig. 2. Process of targeted micro-titer microbial biotransformation for anti-infective compounds. I. Rapid micro-titer microbial biotransformation screening: a) 203 microbes were selected for this study; b) Master plates with a spore solution were stored in a −80 °C freezer; c) Assay plates, containing 2 mL medium and 100 μL spores solution transferred from master plates; d) EtOAc extraction of the fermentation broth; e) HPLC-DAD-UV analysis of EtOAc extract. The samples containing potential new products were highlighted for further flash chromatographic fractionation; f) antibacterial evaluation of the fractions. A heat map was generated to indicate the most promising samples; g) The MS/MS data were collected and MS/MS networking was applied to identify the bioconverted products rapidly. II. Starting substrate: Tan IIA, isolated from TCM *S. miltiorrhiza*. III. Large-scale fermentation and isolation of biotransformed compounds and their structures were determined by means of spectroscopic techniques.

DMSO (1:1, v/v) was added to the wells of plates. The substrate controls used were sterile medium to which the substrate was added and incubated without microorganisms. The culture controls consisted of fermentation blanks in which the organisms were grown under identical conditions but without the substrate. All the samples were extracted with 2 mL EtOAc by ultrasonic processing for 10 min. The residue was prepared for the activity test and HPLC analysis.

2.3. NanoMate-LTQ-FT MS instrument parameters

Data collection was performed using a nanoMate ionization source coupled to a Thermo LTQ-FT-ICR Mass Spectrometer. All analyses were performed in positive ion mode in the mass range of *m/z* from 100 to 2000. Both the primary and nanospray capillaries were 150 μm o.d. × 50 μm i.d. The spray voltage was maintained between 2.0 and 3.0 kV depending on the solvent, with the solvents being acetonitrile-water-formic acid (6:4:1).

2.4. Molecular networking

The data was converted to the mzXML format, which is a text-based format used to represent mass spectrometry data describing the scan number, precursor *m/z*, and *m/z* and intensity of each ion observed in the MS/MS, using ReadW from Thermo or msconvert, part of the ProteoWizard package. Molecular networks were generated as previously described [9,10].

2.5. Cultivation, extraction, isolation, and purification

All microorganisms screened in our experiments were obtained from our in-house microorganism collections. Stock cultures of the fungi were stored on PDA slants (potato 20%, dextrose 2%, and agar 2%, natural pH) at 4 °C. Seed cultures were obtained by transferring fungi from stock cultures to PDB (potato 20% and dextrose 2%, natural pH). Flask scale biotransformation was carried out in 250 mL Erlenmeyer flasks containing 50 mL of medium. The flasks were placed on a rotary shaker operating at 220 rpm at 28 °C. After 3 days of incubation, the substrates (0.2 mL, 10 mg/mL, methanol and DMSO 1:1 [v/v]) were added into each flask, and these flasks were maintained under fermentation conditions for 5 days. The cultures were then pooled and filtered. The filtrates were extracted three times with equal volumes of EtOAc, and the extractions were evaporated *in vacuo* and analyzed by TLC and HPLC. The culture controls consisted of fermentation blanks in which fungi were grown without substrate but fed with the same amount of methanol and DMSO 1:1 [v/v]. Substrate controls were composed of sterile medium to which the substrate was added and incubated without fungi.

The preparative scale biotransformation of tanshinone IIA was carried out in 40 × 1000 mL flasks each containing 300 mL of PDB. Strain AS 3.3447 was incubated for 3 days before 15 mg of

tanshinone IIA (2 mL, methanol and DMSO 1:1 [v/v]) was fed to each flask. The incubation conditions and extraction process were the same as described above and afforded a crude extract (3.54 g). The extract was subjected to column chromatography on a silica gel (30 g). The column was eluted with cyclohexane-acetone (gradient, 100:1–1:1), which yielded fractions 1–6. Fraction 3 (cyclohexane-acetone 4:1, 500 mg) was chromatographed on ODS-MPLC (35 × 2.2 cm) column using the gradient elution from 10 to 80% acetonitrile/H_2O to provide 8 sub-fractions (A–H). Sub-fraction C (50 mg) was purified using reverse-phase HPLC (Zorbax XDB-C_8 250 × 9.4 mm, 5 mm column, 2 mL/min, isocratic elution with 45% ACN/H_2O) to yield tanshinoside A (**1**) (t_R = 25.4 min; 5.0 mg) and tanshinoside B (**2**) (t_R = 27.6 min; 3.0 mg). Then sub-fraction D (100 mg) was purified by reverse-phase HPLC (Zorbax SB-C_3 250 × 9.4 mm, 5 mm column, 2 mL/min, isocratic elution with 42% ACN/H_2O) to give tanshinoside C (**3**) (t_R = 28.0 min; 2.4 mg) and tanshinoside D (**4**) (t_R = 30.4 min; 8.0 mg).

2.6. Characterization of compounds

2.6.1. Tanshinoside A (**1**)

Red powder: $[\alpha]^{24}_D$ +48.6 (*c* 0.035, MeOH); UV (MeOH) λ_{max} (log ε) 273 (2.06), 196 (1.33) nm; NMR data, see Table 1 and Supporting Information Table S1.

2.6.2. Tanshinoside B (**2**)

Red powder: $[\alpha]^{24}_D$ + 30.0 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 273 (0.81), 194 (0.74) nm; NMR data, see Table 1 and Supporting Information Table S2.

2.6.3. Tanshinoside C (**3**)

Yellow powder: $[\alpha]^{24}_D$ + 101 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 260 (0.65) nm; NMR data, see Table 1 and Supporting Information Table S3.

2.6.4. Tanshinoside D (**4**)

Red powder: $[\alpha]^{24}_D$ + 115 (*c* 0.06, MeOH); λ_{max} (log ε) 260 (2.05), 253 (1.59) nm NMR data, see Table 1 and Supporting Information Table S4.

2.7. Bioassays

2.7.1. General antimicrobial assays

Antimicrobial assays were performed according to the Antimicrobial Susceptibility Testing Standards outlined by the Clinical and Laboratory Standards Institute (CLSI) using the bacteria *Staphylococcus aureus* (ATCC 6538), methicillin-resistant *Staphylococcus aureus* (MRSA), oxacillin-resistant *S. aureus* (8–24), oxacillin-resistant *S. aureus* (6281), *Pseudomonas aeruginosa* (PAO1), and *Bacillus subtilis* (ATCC 6633). For each organism, a loopful of glycerol stock was streaked on an LB-agar plate, which was incubated overnight at 37 °C. A single bacterial colony was selected and suspended in Mueller-Hinton Broth to approximately 1 × 10^5 CFU/mL. A two-fold serial dilution of each compound to be tested (4000–31.3 μg/mL in DMSO) was prepared, and an aliquot of each dilution (2 μL) was added to a 96-well flat-bottom microtiter plate (Greiner). Vancomycin and ciprofloxacin were used as positive controls and DMSO as the negative control. An aliquot (78 μL) of bacterial suspension was then added to each well (to give final compound concentrations of 100–0.78 μg/mL in 2.5% DMSO), and the plate was incubated at 37 °C aerobically for 16 h. Finally, the optical density of each well at 600 nm was measured with an EnVision 2103 Multi-label Plate Reader (Perkin-Elmer Life Sciences). The minimum inhibitory concentrations (MIC) were defined as the minimum concentration of each compound that inhibited visible bacterial growth. All the experiments were performed in triplicate [32].

2.7.1.1. Anti-BGC assay. The *Bacillus Calmette–Guérin* (BCG) antimicrobial assay was performed with a strain of *Mycobacterium*

Table 1
^{1}H (600 MHz) and ^{13}C NMR (150 MHz) NMR data (DMSO-d_6) of **1**–**4**.

Position	1		2		3		4	
	δ_H, mult (*J* in Hz)	δ_C	δ_H, mult (*J* in Hz)	δ_C	δ_H, mult (*J* in Hz)	δ_C	δ_H, mult (*J* in Hz)	δ_C
1	9.75, d (*8.6*)	126.2	9.75, m	127.6	2.73, m	27.5	2.98, 3.98, m	31.0
2	7.55, dd (*8.6*, *7.0*)	126.6	7.41, m	124.6	1.73, m	18.7	1.51, 1.85, m	20.0
3	7.48, d (*7.0*)	127.0	7.42, m	127.3	1.64, m	37.9	1.65, m	38.8
4	–	134.0	–	133.1	–	34.0	–	34.7
5	–	130.4	–	130.9	–	147.3	–	142.7
6	8.08, d (*9.2*)	123.3	7.90, d (*9.2*)	121.2	7.56, d (*8.2*)	127.8	7.40, d (*8.5*)	123.4
7	8.19, d (*9.2*)	118.3	8.11, d (*9.2*)	118.1	7.41, d (*8.2*)	127.7	7.83, d (*8.5*)	116.3
8	–	116.6	–	112.1	–	133.2	–	132.4
9	–	117.0	–	122.1	–	132.6	–	126.2
10	–	130.7	–	129.0	–	125.3	–	114.2
11	–	144.0	–	137.1	–	167.2	–	136.2
12	–	136.0	–	148.9	–	163.7	–	142.7
13	–	116.2	–	116.4	–	114.3	–	115.3
14	–	144.8	–	148.9	–	155.7	–	148.6
15	7.82, br.d (*1.5*)	142.4	7.84, s	141.7	7.50, s	140.7	7.74, s	141.1
16	–	120.0	–	116.4	–	120.8	–	116.7
17	2.47, s	9.49	2.43, s	9.53	2.09, s	9.67	2.38, s	9.50
18	2.74, s	20.1	2.68, s	20.2	1.29, s	31.5	1.30, s	31.5
19	–	–	–	–	1.29, s	31.5	1.28, s	32.5
1′	4.72, d (*8.0*)	106.6	4.73, d (*8.2*)	107.4	5.41, d (*7.6*)	94.7	4.41, d (*8.0*)	107.6
2′	3.48, m	74.1	3.62, m	74.3	3.02, m	72.1.	3.44, m	74.1
3′	3.32, m	76.1	3.31, m	76.1	3.21, m	77.7	3.23, m	76.1
4′	3.22, m	69.8	3.25, m	69.7	3.07, m	69.7	3.21, m	69.8
5′	3.22, m	77.5	3.03, m	77.1	3.21, m	76.5	3.05, m	77.1
6′	3.52, 3.71, m	61.0	3.31, 3.38, m	60.9	3.45, 3.69, m	61.0	3.44, 3.56, m	61.2
11–OH	10.03, br.s	–	–	–	–	–	–	–
12–OCH_3	–	–	–	–	3.65, s	51.2	–	–

bovis (BCG Pasteur 1173P2) containing a constitutive Green Fluorescent Protein (GFP) expression vector (pUV3583c-GFP) using direct readout of fluorescence as a measure of bacterial growth, as described previously. The BCG strain was pre-cultivated to mid-log phase (7 d) at 37 °C in Middlebrook 7H9 broth (40 mL; Difco) supplemented with 10% OADC enrichment (Becton Dickinson), 0.05% Tween-80, and 0.2% glycerol and then diluted to an OD_{600} of 0.025 with culture medium as bacterial suspension. A two-fold serial dilution of each compound to be tested (4000–31.3 μg/mL in DMSO), was prepared and an aliquot of each dilution (2 μL) was added to a 96-well flat-bottom microtiter plate (Greiner). Isoniazid was used as the positive control and DMSO as the negative control. An aliquot (78 μL) of the BCG-GFP bacterial suspension was then added to each well (to give final compound concentrations of 100–0.78 μg/mL in 2.5% DMSO), and the plate was incubated at 37 °C for 3 d. Mycobacterial growth was determined by measuring GFP-fluorescence using an EnVision 2103 Multi-label Plate Reader (Perkin-Elmer Life Sciences) with excitation at 485 nm and emission at 535 nm. All the experiments were performed in triplicate. MIC was defined as the minimum concentration that inhibited more than 90% of bacteria growth [33].

2.7.2. Time-kill experiments

Time-kill analysis was performed according to CLSI method M26-A.20. *S. aureus* (ATCC 6538) and methicillin-resistant *S. aureus* (MRSA, clinical strain obtained from Chaoyang hospital) was cultured overnight at 37 °C in MHB broth. The single colonies were picked and adjusted to approximately 10^5 CFU/mL with Mueller-Hinton broth as bacterial suspension. Either compound, adjusted to final concentrations of 0.5, 2, and 8 times the MIC, or DMSO, was then added. At 0, 2, 4, 6, 8, 10, and 24 h of incubation, 10 μL aliquots were taken from each group, serially diluted in MHB, plated onto LB plates, and incubated at 37 °C for 24 h for a viable count enumeration. The rates of killing were determined by measuring the reduction in numbers of viable bacteria ($\log_{10}$ CFU/mL) at 0, 1, 2, 4, 6, 8, 10, and 24 h with fixed concentrations of the compound. The experiments were performed in duplicate. If plates contained fewer than 10 cfu/mL, the number of colonies was considered to be below the limit of quantitation. Culture samples containing the compound were diluted at least 10-fold to minimize drug carryover to the LB plates. Bactericidal activity is defined as a $\geq$3-log reduction in the initial CFU count within 24 h. The compound was considered to be bacteriostatic at a concentration that reduced the original inoculum by 0–3 $\log_{10}$ CFU/mL within 24 h. Time-kill analyses on BCG were performed according to CLSI method M26-A.20. Experiments were performed in triplicate [34].

3. Results

A detailed HPLC analysis of the bioconverted crude extracts from Tan IIA by microbes and their corresponding crude extracts produced by microbes indicated that five strains, FS4-3, 3.3447, 3.246, 5–6, and 3–5 (Table S7), could produce new metabolites compared to the controls (Figures S1a–S1e), and the results were confirmed by flask scale (50 mL). As a result, there were four new small molecules produced by strain AS 3.3447 after incubation with the substrate Tan IIA. The UV spectra of these new substances were similar to those of Tan IIA, indicating that they were tanshinone analogs.

In order to increase the reliability of the bioassay on the biotransformed products, we tested the antimicrobial activities of the fractions from the crude extracts using five selected strains. This analysis removed the influence of the activity of the substrate Tan IIA and other natural products with antibacterial activities produced by the strain used for the biotransformation. A flash chromatography column was used to generate these fractions, and the activities were tested against four pathogens, i.e., *S. aureus*, MRSA, *B. subtilis*, and BCG. Four fractions, frs. 3447-3, 3447-4, 3.264-6, and 3-5-1, demonstrated significant *Staphylococcus* inhibiting activities. Meanwhile, fr. 3447-3 was also found to significantly inhibit *B. subtilis*. A heat map depicted in Figure S28, was generated and displayed different antibacterial activities among the 27 fractions originating from crude extracts of the highlighted strains. The shades of color represented MIC values, with red color indicating higher activity. Fraction 3.3447–3 was shown to be most active, followed by fr.3.3447-4.

The MS/MS networking was used to discover the molecular environments of microbes and dereplication of natural products [8–10]. Because structurally similar NPs share similar MS/MS fragmentation patterns, molecular families tend to cluster together within a network [9,10]. Successful generation of an MS/MS molecular network of biotransformed products by microbes could therefore be used to rapidly indicate and identify bioconverted products from substrates. The MS/MS data of the substrate, Tan IIA, acted as “seed” spectra, which provided the initial focal point in the network when processed with the MS/MS spectra from the biotransformed mixtures. Any nodes/clusters connected to that of Tan IIA on a generated molecular network can be considered as bioconverted products after careful confirmation by their MS2 patterns.

The MS/MS data of a crude extract of the fungus *M. rouxianus* 3.3447 treated with Tan IIA was obtained. An MS/MS molecular network was generated (Fig. 3a) and an FM3 layout was performed on Cytoscape. The tanshinones molecular family (boxed and enlarged) could be obviously mapped on the network and the ion signal at *m/z* 295.13 was determined to be a molecular ion of Tan IIA. The signals at *m/z* 441.15 and 459.20 were structurally similar to Tan IIA and can thus be considered as the potential biotransformed products. Analyzing the MS2 spectra of *m/z* 441 and 459 revealed that they both contained 162 Da shift peaks (Fig. 3b). These results indicated that glycosylation of Tan IIA occurred after treatment with the fungus *M. rouxianus* 3.3447.

Bioassay-directed fractionation and purification of a large-scale culture of *M. rouxianus* 3.3447 yielded four new glycosides of tanshinones, tanshinosides A (**1**), B (**2**), C (**3**), and D (**4**). All structures were assigned by detailed spectroscopic analysis as discussed below.

The molecular formula of tanshinoside A (**1**) was determined as $C_{24}H_{24}O_8$ from its HREIMS ($[M+H]^+$, *m/z* 441.1540, calcd. for $C_{24}H_{24}O_8H$, 441.1549). The ^{13}C NMR signals of the pyranose moiety at δ_C 106.6, 77.5, 76.1, 74.1, 69.8, and 61.0 together with the anomeric proton signal at δ_H 4.72 (d, J = 8.0 Hz) suggested the presence of a glucosyl group in the β-form [35]. Detailed analysis of the ^1H and ^{13}C NMR spectra of **1** revealed that the structure of the aglycone was very similar to that of tanshinone I (Tan I, **6**) (Figures S2–S3, Table 1), except that two carbonyl carbons on Tan I were replaced by two oxygen-bearing aromatic carbons (δ_C 144.0 and 136.0) on **1**. Tan I is a natural product previously reported from the TCM *Salvia miltiorrhiza* [36]. A careful comparison of the NMR data of **1** and Tan I revealed that **1** was a glycoside of the hydroquinone of Tan I. Substitution at C-12 of the glucosyl moiety of **1** could be determined by the HMBC correlations between the anomeric proton of glucose at δ_H 4.72 and δ_C 136.0 (C-12), and was further supported by the ROESY correlations between δ_H 9.75 (H-1) and the exchangeable hydrogen at δ_H 10.03 (brs) (11-OH) (Fig. 4, Figures S4–S8 and Table S1). Thus, the structure of **1** (Fig. 1) was established and all of the signals in the 2D NMR spectra were assigned unambiguously.

Tanshinoside B (**2**) was assigned the same molecular formula $C_{24}H_{24}O_8$ as **1** by HRESIMS ($[M+H]^+$, *m/z* 441.1540). The ^1H and ^{13}C

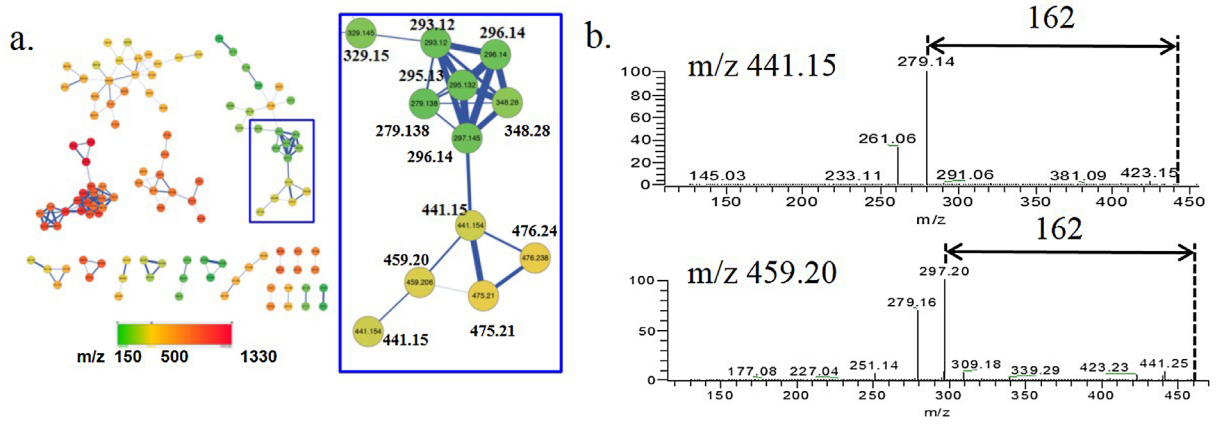

Fig. 3. Molecular network of tanshinone IIA biotransformation products of strain *M. rouxianus* AS 3.3447. (a) Tanshinones molecular family (boxed and enlarged) from the tanshinone IIA and fungus AS 3.3447 biotransformed products MS/MS clusters. (b) MS2 spectra of biotransformed products on tanshinone IIA (*m/z* 441.15 and 459.20) revealed glycosylation via biotransformation.

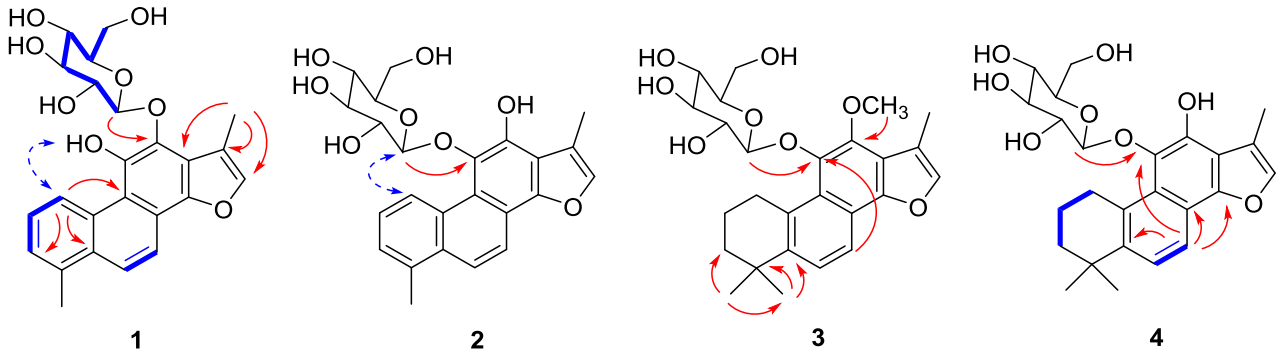

Fig. 4. Observed $^{1}H-^{1}H$ COSY, key HMBC, and ROESY correlations of **1**–**4**.

NMR data of **2** were very similar to those of **1** except the low-field shift of two oxygen-bearing aromatic carbons at δ_C 148.9 and 137.1 (Figures S9–S10, Table 1). The above evidences revealed that **2** might possess a different glycosylation position from **1**. The ROESY correlations of H-1 at δ_H 9.75 (m) and the anomeric proton at δ_H 4.73 (d, $J = 8.2$ Hz) (Fig. 4, Figures S11–S15 and Table S2) indicated that the glucosyl moiety was substituted at C-11 on **2**. The structure of tanshinoside B (**2**) was determined as shown in Fig. 1.

HRESIMS measurements of tanshinoside C (**3**) revealed an adduct ion ($[M+H]^+$ *m/z* 473.1919) consistent with the molecular formula of $C_{26}H_{32}O_8$. The ^{13}C spectrum displayed a set of NMR signals of the hexose moiety at δ_C 94.7, 77.7, 76.5, 72.1, 69.7, and 61.0. According to ^{1}H and ^{13}C NMR data of **3**, the structure of its aglycone was very similar to that of tanshinone IIA (Tan IIA, **5**) (Figures S16–S17, Table 1) [36], except that two carbonyl carbons on Tan IIA were replaced by two oxygen-bearing aromatic carbons (δ_C 167.2 and 163.7) on **3** and a carbon signal at δ_C 51.2 of $-OCH_3$. The HMBC correlations of an aromatic proton at δ_H 7.41 (d, $J = 8.2$ Hz) with C-11 at δ_C 167.2 and an anomeric proton at δ_H 5.41 (d, $J = 7.6$ Hz) and C-11 at δ_C 167.2 indicated that a glucosyl residue was linked to C-11 (Fig. 4, Figures S18–S21 and Table S3). The structure of **3** was identified (Fig. 1), and all the 2D NMR spectra signals were assigned unambiguously.

The molecular formula of tanshinoside D (**4**) was determined to be $C_{25}H_{30}O_8$ based on its HREIMS ($[M+H]^+$, *m/z* 459.2066,calcd. for 459.2019). Comparing the ^{1}H NMR data of **4** with that of **3** showed that the proton signal of the methoxyl group disappeared (Figures S22–S23, Table 1). It could be speculated that **4** was a glycosylation hydroquinone form of tanshinone IIA. The glycosylation position was determined by HMBC spectrum. The HMBC correlations of an aromatic proton at δ_H 7.83 (d, $J = 8.0$ Hz) with C-11 at δ_C 136.2 and an anomeric proton at δ_H 4.41 (d, $J = 8.0$ Hz) and C-11 at δ_C 136.2 indicated that a glucosyl residue was linked to C-11 (Fig. 4, Figures S24–S27 and Table S4). The structure of **4** was identified (Fig. 1), and all 2D NMR spectra signals were assigned unambiguously.

All of the isolated compounds were tested for their activities against *S. aureus*, MRSA, *B. subtilis*, BCG, and *P. aeruginosa* (Table 2). Tanshinosides A (**1**) and B (**2**) showed potent activities against both *S. aureus* and MRSA with MIC values ranging between 0.78 and 3.125 μg/mL. Tanshinoside C (**3**) was inactive with MIC values > 100 μg/mL, while tanshinoside D (**4**) showed weak activities against both *S. aureus* and MRSA with MIC values of 25 μg/mL. In order to further evaluate the pharmaceutical potential of **1** and **2**, their activities were measured against two clinical isolates of oxacillin-resistant *S. aureus* and **2** showed potent activity against oxacillin-resistant *S. aureus* (8–24) and (6281) with MIC values of 0.78 μg/mL. Comparison of the activities of **1** and **2** against the clinical isolates revealed that the glycosylation position of tanshinosides might be responsible for a different activity. However, Tan IIA did not show any activities against these resistant isolates.

In order to determine the bactericidal property of tanshinosides A (**1**) and B (**2**), dose-dependent experiments were carried out using *S. aureus* (ATCC 6538) and MRSA (clinical strain obtained from Chaoyang hospital) as model organisms (Fig. 5) [37]. The results indicated that **1** and **2** have similar potentials against killing

Table 2
Antimicrobial activities of tanshinosides A–D (**1**–**4**), Tan IIA and Tan I.

Organism (strain)	Minimum inhibitory concentration (μg/mL)						
	1	2	3	4	Tan IIA[a]	Tan I[b]	Control
Staphylococcus aureus (ATCC 6538)	0.78	1.56	>100	25	3.125	12.5	1[c]
Methicillin-resistant *S. aureus (MW2)*	1.56	3.125	>100	25	>100	50	1[c]
Oxacillin-resistant *S. aureus (8–24)*	3.125	0.78	>100	25	>100	50	1[c]
Oxacillin-resistant *S. aureus (6281)*	3.125	0.78	>100	25	>100	50	1[c]
Bacillus subtilis (ATCC 6633)	1.56	6.25	100	50	>100	>100	0.5[c]
Bacillus Calmette-Guérin (Pasteur 1173P2, BCG)	50	50	>100	50	3.125	25	0.05[d]
Pseudomonas aeruginosa (PAO1)	>100	>100	>100	>100	>100	>100	1[e]

[a] Tanshinone IIA.
[b] Tanshinone I.
[c] Vancomycin.
[d] Isoniazid.
[e] Ciprofloxacin.

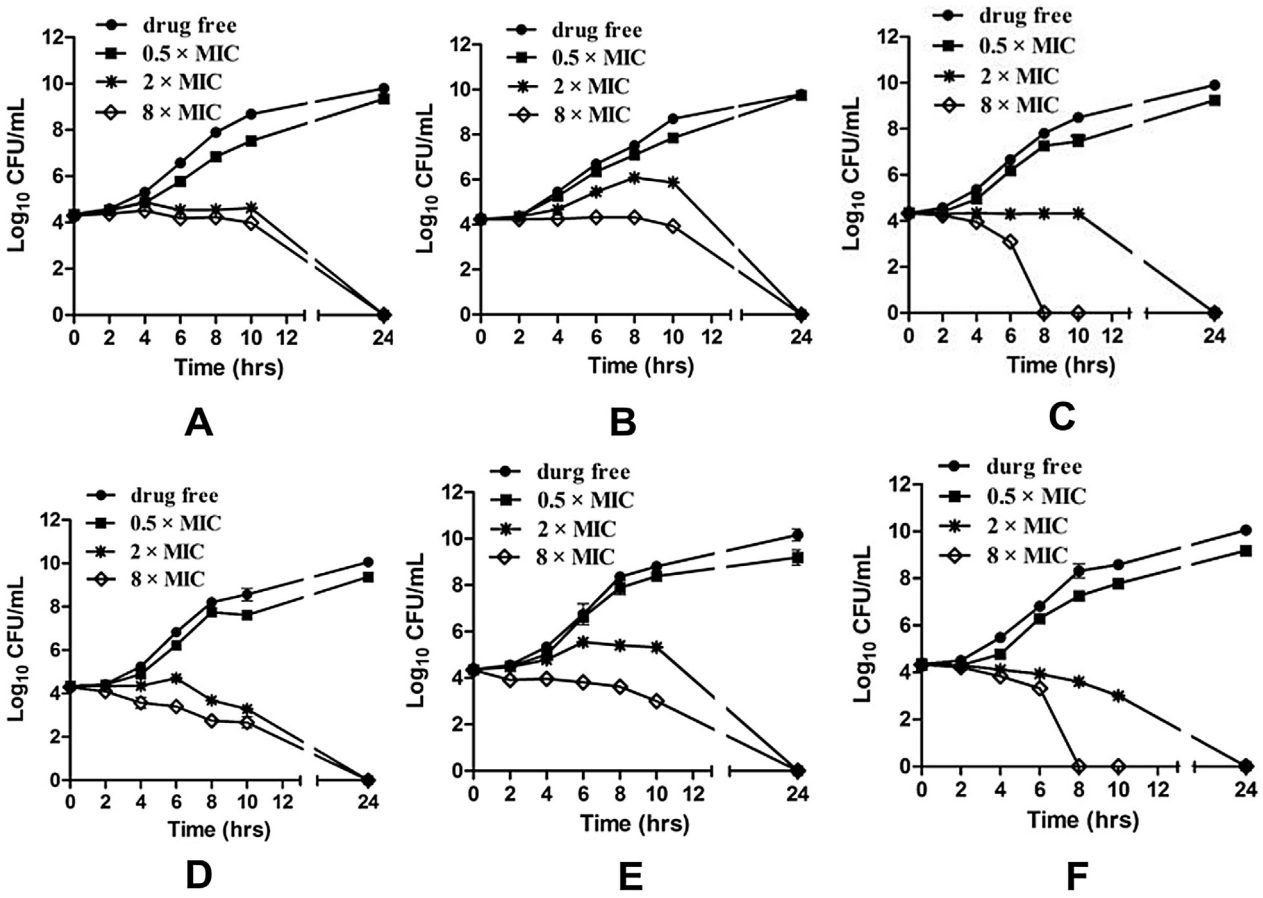

Fig. 5. The bactericidal activities of tanshinosides A (1) and B (2) against MRSA and *S. aureus* (SA). Compounds were added to cultures at time zero, and samples were processed as described in the Materials and Methods. A: in vitro time-kill curve of tanshinoside A (1) against MRSA; B: in vitro time-kill curve of tanshinoside B (2) against MRSA; C: in vitro time-kill curve of vancomycin against MRSA; D: in vitro time-kill curve of tanshinoside A (1) against SA; E: in vitro time-kill curve of tanshinoside B (2) against SA; F: in vitro time-kill curve of vancomycin against SA.

Staphylococcus bacteria as vancomycin in low concentrations, i.e. 2 × MIC. The present findings demonstrate that **1** and **2** possess a dose-dependent activity against MRSA and *S. aureus* on in vitro time-kill experiments. In addition, **1** showed a better activity against both MRSA and *S. aureus* than **2**.

4. Discussion and conclusion

The biotransformation of TCM-derived antibiotic Tan IIA by a fungus AS 3.3447 provided four new compounds, the glycosides of tanshinones, via a combination of dehydrogenation, demethylation, reduction, glycosylation, and methylation. Tanshinosides are serial structurally unique compounds that are not discovered from the secondary metabolites pathway of the tanshinone producing plant. This is the first report that describes tanshinone derivatives could be glycosylated by incubation with microbes. Compared to parent substrate, the activities of **1** and **2** against drug-resistant *S. aureus* clinical isolates were significant improved. This might

attribute to the glycosylation of the structure of Tan IIA. The glycosylation of natural products provided a wide range of unique substances and influence the pharmacological and pharmacokinetic properties of metabolites. Hence, glycosylation of natural products is used as a viable strategy to produce bioactive compounds with improved activity [38–40]. In addition, glycosides of Tan I (**1** and **2**) present more potent activities against most of the tested pathogens, compared to those of Tan IIA (**3** and **4**). This indicates that ring A might be the necessary functional group. Meanwhile, **1** and **2** showed different selectivity against these pathogens, which revealed that the substituted position of glucose moiety might correlate with their biological properties. As concluded, tanshinosides A (**1**) and B (**2**) might act as promising candidates for development as novel anti-MRSA therapeutic agents. The present approach provides a new cost-effective, sustainable and rapid strategy for drug discovery.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.synbio.2016.05.002.

References

[1] Li JWJ, Vederas C. Drug discovery and natural products: end of an era or an endless frontier. Science 2009;325:161–5.
[2] Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Nat Prod 2012;75:311–35.
[3] Cragg GM, Newman DJ. Natural products: a continuing source of novel drug leads. Biochim Biophys Acta 2013;1830:3670–95.
[4] Medema MH, Fischbach MA. Computational approaches to natural product discovery. Nat Chem Biol 2015;11:639–48.
[5] Milshteyn A, Schneider JS, Brady SF. Mining the metabiome: identifying novel natural products from microbial communities. Chem Biol 2014;21:1211–23.
[6] Kersten RD, Yang YL, Xu Y, Cimermancic P, Nam SJ, Fenical W, et al. A mass spectrometry-guided genome mining approach for natural product peptidogenomics. Nat Chem Biol 2011;7:794–803.
[7] Donia MS, Cimermancic P, Schulze CJ, Wieland Brown LC, Martin J, Mitreva M, et al. A systematic analysis of biosynthetic gene clusters in the human microbiome reveals a common family of antibiotics. Cell 2014;158:1402–14.
[8] Nguyen DC, Moree W, Lamsa A, Medema M, Zhao X, Gavilan R, et al. MS/MS networking guided analysis of molecule and gene cluster families. Proc Natl Acad Sci U. S. A 2013;110:E2611–20.
[9] Watrous J, Roach P, Alexandrov T, Heath BS, Yang JY, Kersten RD, et al. Telomere length in early life predicts lifespan. Proc Natl Acad Sci U. S. A 2012;109:1743–8.
[10] Yang JY, Sanchez LM, Rath CM, Liu X, Boudreau P, Bruns N, et al. Molecular networking as a dereplication strategy. J Nat Prod 2013;76:1686–99.
[11] Newman DJ, Cragg GM. Marine-sourced anti-cancer and cancer pain control agents in clinical and late preclinical development. Mar Drugs 2014;12:255–78.
[12] Cragg GM, Grothaus PG, Newman DJ. New horizons for old drugs and drug leads. J Nat Prod 2014;77:703–23.
[13] Nicolaou KC, Chen JS, Edmonds DJ, Estrada AA. Recent advances in the chemistry and biology of naturally occurring antibiotics. Angew Chem Int Ed Engl 2009;48:660–719.
[14] Pantaleone DP. Handbook of chiral chemicals. second ed. New York: Marcell Dekker Inc; 2006.
[15] Hanson JR. The microbiological transformation of diterpenoids. Nat Prod Rep 1992;9:139–51.
[16] Mahato SB, Majumdar I. Current trends in microbial steroid biotransformation. Phytochemistry 1993;34:883–98.
[17] Loughlin WA. Biotransformations in organic synthesis. Bioresour Technol 2000;74:49–62.
[18] Zhou LG, Wu JY. Development and application of medicinal plant tissue cultures for production of drugs and herbal medicinals in China. Nat Prod Rep 2006;23:789–810.
[19] Shah SA, Sultan S, Hassan NB, Muham-mad FK, Muhammad AB, Hussain FB, et al. Biotransformation of 17α-ethynyl substituted steroidal drugs with microbial and plant cell cultures: a review. Steroids 2013;78:1312–24.
[20] Venisetty R, Ciddi V. Application of microbial biotransformation for the new drug discovery using natural drugs as substrates. Curr Pharm Biotechnol 2003;4:153–67.
[21] Mufflera K, Leipolda D, Schellera M, Haasb C, Steingroewerb J, Bleyb T, et al. Biotransformation of triterpenes. Process Biochem 2011;46:1–15.
[22] Wang Q, Song F, Xiao X, Huang P, Li L, Monte A, et al. Abyssomicins from the South China Sea deep-sea sediment Verrucosispora sp.: natural thioether Michael addition adducts as antitubercular prodrugs. Angew Chem Int Ed Engl 2013;52:1231–4.
[23] Zhang L, Yan K, Zhang Y, Huang R, Bian J, Zheng C, et al. High-throughput synergy screening identifies microbial metabolites as combination agents for the treatment of fungal infections. Proc Natl Acad Sci U. S. A 2007;104:4606–11.
[24] Dai HQ, Wang J, Xin YH, Pei G, Tang SK, Ren B, et al. Verrucosispora sediminis sp. nov., a cyclodipeptide-producing actinomycete from deep-sea sediment. Int J Syst Evol Microbiol 2010;60:1807–12.
[25] Klein E, Smith DL, Laxminarayan R. Hospitalizations and deaths caused by methicillin-resistant Staphylococcus aureus, United States, 1999-2005. Emerg Infect Dis 2007;13:1840–6.
[26] Srinivasan A, Dick JD, Perl TM. Vancomycin resistance in staphylococci. Clin Microbiol Rev 2002;15:430–8.
[27] Fernandes P, Cabral J. Microlitre/millilitre shaken bioreactors in fermentative and biotransformation processes-a review. Biocatal Biotrans 2006;24:237–52.
[28] Stahl S, Greasham R, Chartrain MJ. Implementation of a rapid microbial screening procedure for biotransformation activities. Biosci Bioeng 2000;89:367–71.
[29] Zhang J, Guo H, Tian Y, Liu P, Li N, Zhou J, et al. Biotransformation of 20(*S*)-protopanaxatriol by Mucor spinosus and the cytotoxic structure activity relationships of the transformed products. Phytochemistry 2007;68:2523–30.
[30] Huang FX, Lin XH, He WN, Song W, Ye M, Yang WZ, et al. Two new oxidation products obtained from the biotransformation of asiatic acid by the fungus *Fusarium avenaceum* AS 3.4594. J Asian Nat Prod Res 2012;14:1039–45.
[31] Huang FX, Yang WZ, Ye F, Tian JY, Hu HB, Feng LM, et al. Microbial transformation of ursolic acid by *Syncephalastrum racemosum* (Cohn) Schroter AS 3.264. Phytochemistry 2012;82:56–60.
[32] Chen CX, Liu XT, Abdel-Mageed WM, Guo H, Hou WY, Jaspars M, et al. Nivetetracyclates A and B: novel compounds isolated from *Streptomyces niveus*. Org Lett 2013;15:5762–5.
[33] Wang JF, Dai HQ, Wei YL, Zhu HJ, Yan YM, Wang YH, et al. Antituberculosis agents and an inhibitor of the para-aminobenzoic acid biosynthetic pathway from Hydnocarpus anthelminthica seeds. Chem Biodivers 2010;7:2046–53.
[34] Barry AL, Craig WA, Nadler H, Reller LB, Sanders CC, Swenson JM. Methods for determining bactericidal activity of antimicrobial agents: approved guideline. Natl Comm Clin Lab Stand 1999;19:8–12.
[35] Markham KR, Ternai B, Stanley R, Geiger H, Mabry T. Carbon-13 NMR studies of flavonoids—III: naturally occurring flavonoid glycosides and their acylated derivatives. Tetrahedron 1978;34:1389–97.
[36] Cao CQ, Sun LR, Lou HR, Ji M. Chemical constituents of *Salvia miltiorrhiza* f. alba, Lishizhen Med. Mater Med Res 2009;3:63–4.
[37] Bogdanovich T, Esel D, Kelly LM, Bozdogan B, Credito K, Lin G, et al. Anti-staphylococcal activity of DX-619, a new des-F (6)-quinolone compared to those of other agents. Antimicrob Agents Chemother 2005;49:3325–33.
[38] Kapoor V, Kaur KA. Drug-glycosidation and drug development. Med Chem 2013;13:584–96.
[39] Thibodeaux CJ, Melançon CE, Liu HW. Unusual sugar biosynthesis and natural product glycodiversification. Nature 2007;446:1008–16.
[40] Singh S, Phillips Jr GN, Thorson JS. The structural biology of enzymes involved in natural product glycosylation. Nat Prod Rep 2012;29:1201–37.