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Microbial transformations of buagarofuran, an anti-anxietic agent

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ABSTRACT

19 structurally diverse metabolites (**2**–**20**) were isolated from the transformation of buagarofuran (**1**), an anxiety agent, by three filamentous fungal strains, *Cunninghamella echinulata* AS 3.3402, *Cunninghamella elegans* AS 3.3400, and *Absidia coerulea* AS 3.3538. Their structures and identified on the basis of extensive spectroscopic data (HRMS, NMR, IR, and CD) and chemical methods. All of the metabolites were oxidized products of **1**, and metabolite **20** was also one of mammalian metabolites. Metabolites **8**, **10**–**12**, and **20** significantly inhibited [^{3}H]-NE re-uptake, and **8** and **9** strongly inhibited [^{3}H]-5-HT re-uptake in the rat brain synaptosomes at 10 μM in vitro. The structural diversity of the microbial transformed metabolites of buagarofuran in the present report would give a favorable opportunity to probe its mammalian metabolism and better shed light on its pharmacokinetics and pharmacodynamics in vivo.

1. Introduction

Buagarofuran ((+)-(5*R*,7*R*,10*S*)-4-butyl-α-agarofuran, also named AF-5, **1**, Fig. 1), a derivative of α-agarofuran isolated from the Chinese traditional medicine Gharu-wood (*Aquillaria agallocha* Roxb.), is a novel drug candidate targeting anxiety disorder under phase II clinical trial in China. Buagarofuran was proved to possess significant anti-anxiety activity with a higher potency and a lower toxicity compared with diazepam and buspirone.[1,2] The anti-anxiety mechanism of buagarofuran was related to the modulation of central monoamine neurotransmitters, and it could greatly reduce the level of dopamine in homogenates of striatum, midbrain, and cortex to alleviate anxiety.[3] The metabolism study of buagarofuran revealed that various metabolites were detected when incubated with rat and human liver microsomal fractions. However, only two metabolites, 2-hydroxy- and 2-oxo-buagarofuran, were identified because of limited scale, ethnics, etc.[4] As reported, microbial transformations could be used for the preparation of mammalian drug metabolites.[5–7] Once identities of the microbial and mammalian drug metabolites have been established, the microbial metabolites can be prepared in sufficient quantity to be used in biological activity and toxicity tests, preparation of analytical standards, and so on. The advantages of using microorganisms for the production of metabolites are that the cultures are relatively easy to maintain and grow, and that scale-up to produce milligram or gram amounts is readily accomplished. In addition, a variety of metabolites could be obtained by microbial transformations, from which more active metabolites might be found.[8] In the above context, the microbial transformations of buagarofuran were systematically investigated in our group. Totally 31 strains were used for screening tests, three strains of which were selected for further preparative transformations to lead to the yields of 19 metabolites. Herein, we first report the microbial transformations of buagarofuran, the structural elucidation of the metabolites, and their inhibitory effects in vitro on 5-hydroxytryptamine (5-HT) and [^{3}H]-NE re-uptaking in rat brain synaptosomes.

2. Results and discussion

31 microbial strains (Table S1) belonging to prokaryotic organisms (including bacteria, actinomycetes) and eukaryotic organisms (including yeast, filamentous fungi), were used as biocatalysts for the biotransformations of buagarofuran (**1**). On the basis of TLC, HPLC, and LC–MS analyses, three strains of filamentous fungi designated to *Cunninghamella echinulata* AS 3.3402, *Cunninghamella elegans* AS 3.3400, and *Absidia coerulea* AS 3.3538, which showing good ability to convert buagarofuran, were selected for the further preparative biotransformations.

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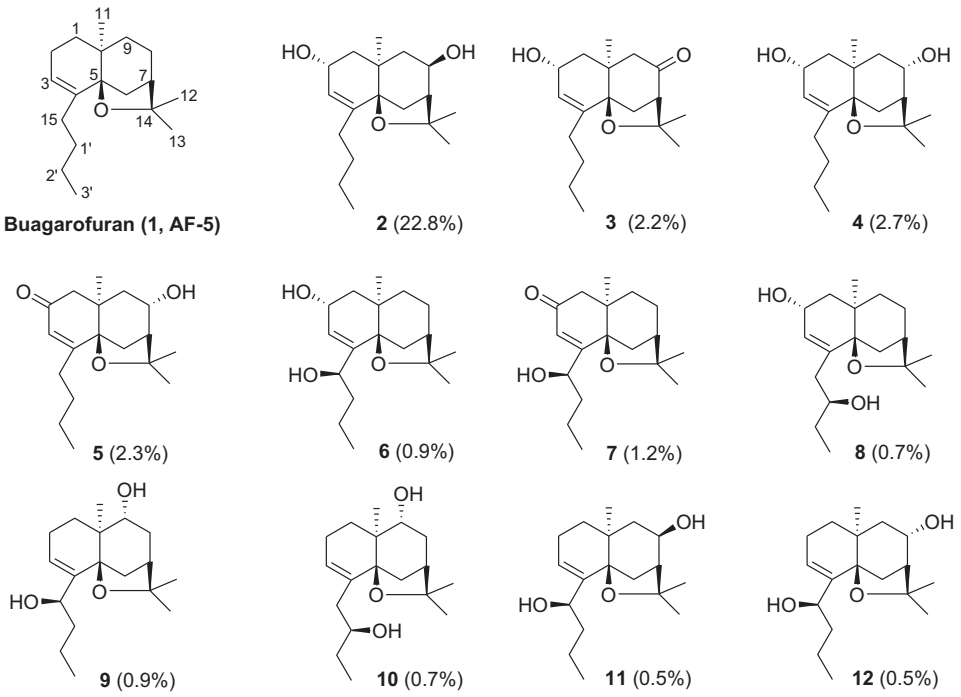

Fig. 1. The chemical structures of buagarofuran (**1**) and its metabolites (**2**–**12**) with *C. echinulata* AS 3.3402 (yields in parentheses).

2.1. Transformation of 1 by *C. echinulata* AS 3.3402

Following a standard two-stage fermentation protocol,[9] 11 new metabolites (**2**–**12**, Fig. 1) were obtained after incubation of **1** with cell cultures of *C. echinulata* for 4 days. These metabolites were purified by silica gel chromatography and semi-preparative HPLC. Their structures were identified on the basis of the extensive spectroscopic data (HRESIMS, IR, 1D NMR, 2D NMR, etc.) analysis and chemical methods. As shown, the major reactions were hydroxylations at various positions (C-2, C-8, C-15, and C-1′) and oxidations of alcohol to ketone (C-2 and C-8).

The molecular formula $C_{18}H_{30}O_3$ of **2** was determined by combined analyses of its HRESIMS (*m*/*z* $[M+H]^+$ 295.2257), ^{1}H NMR, and ^{13}C NMR spectroscopic data (Tables 1 and 2), suggesting the introduction of two additional OH groups as compared with **1**. It was further confirmed by the IR absorption at 3260 cm^{-1}. The ^{13}C NMR (Table 2) spectrum of **2** was similar to that of **1** except that the signals corresponding to C-2 (δ_C 23.1) and C-8 (δ_C 24.7) in **1** disappeared, while the oxygen-bearing methine signals C-2 (δ_C 64.8) and C-8 (δ_C 70.5) were observed. The loci of OH groups at C-2 and C-8 were further supported by the signals of H-2 and H-8 in **2** (Table 1), which significantly shifted downfield to δ_H 4.13 (t, *J*=4.8 Hz) and δ_H 4.06 (m) when compared with **1**. Furthermore, the HMBC correlations (Fig. 2) of H-2/C-1 and C-3, and H-8/C-9 and C-7 strongly supported the above deduction. The enhancement of integration value of H_3-11 was not observed when H-2 irradiated, and the

Table 1
^{1}H NMR spectroscopic data of **2**–**10**[a,b]

Position	**2**[a]	**3**[b]	**4**[b]	**5**[b]	**6**[a]	**7**[b]	**8**[a]	**9**[a]	**10**[a]
1	2.38 (dd, 13.2, 5.2) 1.59 (dd, 13.2, 6.8)	2.32 m 1.60 m	1.80 (dd, 15.0, 4.8) 1.54 (d, 15.0)	2.75 (d, 15.6) 1.96 (d, 15.6)	1.88 m, 1.36 m	2.80 (d, 15.6) 2.02 (d, 15.6)	1.88 (dd, 14.4, 6.0) 1.37 (d, 8.8)	1.48 s	1.44 m
2	4.13 (t, 4.8)	4.34 (t, 4.5)	4.22 (t, 4.8)		4.14 (t, 5.2)		4.11 (t, 5.1)	1.93 m	1.93 m
3	5.61 (d, 3.6)	5.78 (d, 3.3)	5.70 (d, 3.9)	5.90 s	5.91 (t, 4.4)	6.06 s	5.75 (d, 4.2)	5.96 (t, 3.6)	5.73 br s
6	1.97 (dd, 12.4, 5.6) 1.73 (d, 12.4)	2.66 m 2.17 m	2.38 (d, 11.1) 2.11 m	2.53 (dd, 2.4, 13.8) 2.26 m	2.44 m 1.84 m	2.66 (dd, 12.6, 4.8) 1.98 (d, 12.6)	2.20 (dd, 12.0, 3.6) 1.08 (d, 12.4)	1.85 (d, 12.9) 1.97 m	2.21 m 1.79 (d, 11.7)
7	2.04 m	2.66 m	2.20 m	2.26 m	1.94 m	1.84 m	1.97 m	1.97 m	1.97 m
8	4.06 m		4.15 br s	4.2 br s	1.73 m	1.75 m	1.72 m	1.56 m	1.55 m
9	2.07 m	2.32 m	2.00 m, 1.45 m	2.01 (d, 7.8), 1.61 (d, 7.8)	1.57 m 1.24 m	1.57 m 1.41 m	1.58 m, 1.27 (dd, 9.6, 6.0)	3.80 (dd, 11.4, 6.0)	3.80 (dd, 11.6, 6.0)
11	1.03 s	1.12 s	1.26 s	1.21 s	1.03 s	1.05 s	1.04 s	0.81 s	0.82 s
12	1.19 s	1.30 s	1.61 s	1.37 s	1.30 s	1.43 s	1.30 s	1.30 s	1.30 s
13	1.43 s	1.33 s	1.27 s	1.33 s	1.18 s	1.30 s	1.18 s	1.18 s	1.18 s
15	2.02 m	2.17 m	1.50 m	2.26 m	4.07 (dd, 8.0, 4.8)	4.35 (dd, 6.9, 5.1)	2.27 m 2.01 (d, 9.2)	4.02 (dd, 5.4, 5.1)	2.16 m, 1.94 m
1′	1.38–1.32 m	1.52 m	1.45 m	1.52 m	1.57 m	2.09 m	3.52 m	1.60 m	3.42 m
2′	1.38–1.32 m	1.45 m	1.37 m	1.44 m	1.48 m, 1.30 m	1.48 m, 1.30 m	1.48 m	1.30 m	1.47 m
3′	0.91 (t, 6.8)	0.96 (t, 6.6)	0.95 (t, 7.2)	0.96 (t, 6.6)	0.91 (t, 7.6)	0.97 (t, 7.5)	0.95 (t, 7.6)	0.91 (t, 7.2)	0.94 (t, 7.5)

[a] Data were measured in CD_3OD and recorded at 400 for MHz; Coupling constants (*J* in Hz) are given in parentheses. Assignments were based on gCOSY, HSQC, and HMBC experiments.
[b] Recorded at 300 MHz in $CDCl_3$.

Table 2
^{13}C NMR spectroscopic data of **2**–**10**[a,b]

Position	**2**[a]	**3**[b]	**4**[b]	**5**[b]	**6**[a]	**7**[b]	**8**[a]	**9**[a]	**10**[a]
1	42.0, CH_2	40.3, CH_2	42.1, CH_2	49.3, CH_2	42.4, CH_2	49.7, CH_2	42.5, CH_2	30.0, CH_2	31.6, CH_2
2	64.8, CH	64.2, CH	64.3, CH	199.2, C	64.4, CH	200.1, C	64.6, CH	23.0, CH_2	23.2, CH_2
3	128.3, CH	127.0, CH	126.2, CH	127.3, CH	128.4, CH	126.7, CH	131.1, CH	128.9, CH	131.5, CH
4	139.6, C	138.2, C	139.4, C	159.4, C	141.9, C	158.9, C	137.1, C	139.5, C	133.8, C
5	85.9, C	84.3, C	85.0, C	85.0, C	86.6, C	84.6, C	86.8, C	87.3, C	87.6, C
6	33.3, CH_2	32.2, CH_2	27.3, CH_2	27.3, CH_2	34.6, CH_2	33.9, CH_2	34.3, CH_2	33.5, CH_2	33.3, CH_2
7	51.8, CH	61.2, CH	51.3, CH	51.3, CH	45.3, CH	44.0, CH	45.5, CH	44.1, CH	44.2, CH
8	70.5, CH	210.0, C	68.4, CH	67.8, CH	24.7, CH_2	24.0, CH_2	24.7, CH_2	34.0, CH_2	34.0, CH_2
9	43.5, CH_2	52.0, CH_2	41.1, CH_2	41.9, CH_2	35.7, CH_2	33.9, CH_2	35.7, CH_2	74.5, CH	74.4, CH
10	38.5, C	39.6, C	36.3, C	40.6, C	37.4, C	41.1, C	37.4, C	42.9, C	42.9, C
11	25.9, CH_3	26.3, CH_3	26.2, CH_3	24.9, CH_3	25.5, CH_3	23.8, CH_3	25.5, CH_3	16.9, CH_3	16.8, CH_3
12	24.6, CH_3	23.7, CH_3	22.8, CH_3	22.7, CH_3	30.7, CH_3	30.6, CH_3	23.0, CH_3	23.2, CH_3	23.1, CH_3
13	31.9, CH_3	29.3, CH_3	30.5, CH_3	30.4, CH_3	23.1, CH_3	22.9, CH_3	30.9, CH_3	30.7, CH_3	30.1, CH_3
14	83.2, C	80.7, C	80.0, C	81.1, C	83.2, C	83.0, C	83.2, C	82.9, C	83.0, C
15	32.3, CH_2	31.1, CH_2	30.9, CH_2	31.3, CH_2	69.3, CH	70.4, CH	40.4, CH_2	69.1, CH	40.0, CH_2
1′	31.9, CH_2	30.4, CH_2	27.3, CH_2	29.5, CH_2	39.7, CH_2	38.2, CH_2	72.8, CH	39.7, CH_2	73.5, CH
2′	23.7, CH_2	22.7, CH_2	22.8, CH_2	22.6, CH_2	20.8, CH_2	19.4, CH_2	31.4, CH_2	20.9, CH_2	30.1, CH_2
3′	14.4, CH_3	14.0, CH_3	14.0, CH_3	13.9, CH_3	14.3, CH_3	13.8, CH_3	10.6, CH_3	14.3, CH_3	10.6, CH_3

[a] Data were measured in CD_3OD and recorded at 100 MHz; Assignments were based on gCOSY, HSQC, and HMBC experiments.
[b] Data were measured in $CDCl_3$ and recorded at 100 MHz.

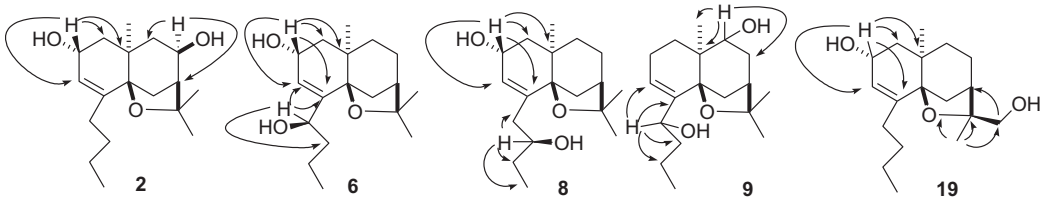

Fig. 2. Key HMBC correlations (H→C) of compounds **2**, **6**, **8**, **9**, and **19**.

irradiation of H-8 resulted in enhancement of H_3-11 in the NOE difference spectral experiments, suggesting the α-orientation of 2-OH and β-orientation of 8-OH. In addition, the assignment for positions 12 and 13 was determined according to NOE different spectrum experiments, mainly according to the NOE of H-7α/H_3-12. Thus, the structure of **2** was determined as 2α,8β-dihydroxy-buagarofuran. Metabolite **4** ($C_{18}H_{30}O_3$ established by HRESIMS) had NMR spectroscopic features similar to those of **2**, except that the ^1H NMR resonance of the H-8 (δ_H 4.06, m) in **2** instead of H-8 (δ_H 4.17, br s). In addition, the enhancement of H_3-11 was not observed when H-2 and H-8 were irradiated, which indicated that 2- and 8-OH groups were both α-oriented. Accordingly, **4** was determined as 2α,8α-dihydroxy-buagarofuran.

The molecular formula of **3** was established to be $C_{18}H_{28}O_3$ by combined analyses of its HRESIMS, ^1H NMR, and ^{13}C NMR spectroscopic data (Tables 1 and 2), with 2 amu less than that of **2**. The absence of H-8 signal (δ_H 4.06, m) and the presence of one carbonyl signal at δ_C 210.0 indicated that **3** was a C-8 ketonized product of **2**. It was further supported by the significant downshift of C-7 and C-9 at δ_C 61.2 and δ_C 52.0, respectively. The 2-OH group was determined to be α-configuration by NOE difference spectrum, in which the enhancement of H_3-11 was not observed when H-2 was irradiated. Thus, the structure of **3** was determined as 2α-hydroxy-8-oxo-buagarofuran.

The HRESIMS, ^1H and ^{13}C NMR spectroscopic data of **5** were in accordance with the molecular formula $C_{18}H_{28}O_3$, with 2 amu less than the corresponding value of **4**. The ^1H NMR spectrum of **5** showed the absence of oxygen-bearing methine signal of H-2 (δ_H 4.22, t, J=4.8 Hz) in **4**, whereas its ^{13}C NMR displayed the presence of one carbonyl signal at δ_C 199.2. Furthermore, the resonance for C-4 of **5** was deshielded significantly as compared with that of **4**. This implied that 2-OH in **4** was oxidized to a carbonyl moiety in **5**, which was confirmed by the IR absorption at 1667 cm^{-1} assigned to α,β-unsaturated carbonyl moiety. Therefore, **5** was 8α-hydroxy-2-oxo-buagarofuran.

The molecular formula $C_{18}H_{30}O_3$ of **6** was established by HRESI-positive mass spectrum at m/z $[M+Na]^+$ 317.2074, suggesting the substitution of two additional OH groups as compared with **1**. ^1H NMR (Table 1) showed that two methylene signals responsible for H-2 (δ_H 1.97, m) and H-15 (δ_H 1.36, m) in **1** disappeared, while a pair of oxygenated methine signals at H-2 (δ_H 4.14, t, J=5.2 Hz) and H-15 (δ_H 4.07, dd, J=8, 4.8 Hz) were observed. The HMBC correlations (Fig. 2) of H-2/C-1, C-3, C-4, and C-10, and H-15/C-3, C-4, C-5, C-1′, and C-2′ further supported this deduction. When H-2 was irradiated, the enhancement of H_3-11 was not observed, suggesting 2-OH was α-oriented. The absolute configuration of C-15 was determined using Mosher's method.[10–13] The (*S*)- and (*R*)-MTPA [α-methoxy-α-(trifluoromethyl) phenylacetyl] esters of **6** (**6a** and **6b**) were prepared using the corresponding (*R*)-(−)- and (*S*)-(+)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride (Fig. 3), respectively. The determination of the chemical shift differences $\Delta\delta(\delta_S-\delta_R)$ for the protons neighboring C-15 functionality led to the assignment of *S* or *R* configurations for C-15 of **6**. Although **6** has two hydroxy groups

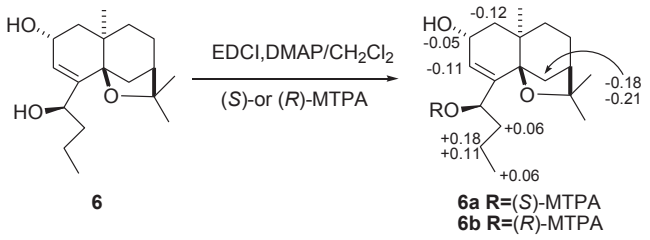

Fig. 3. $\Delta\delta(\delta_S-\delta_R)$ values obtained from the ^1H NMR spectra near C-15 of the MTPA esters **6a** and **6b**.

(2-OH and 15-OH), the steric hindrance of 2-OH from H_3-11 and H-3 resulted in selective esterification of 15-OH. The esterification of **6** with (*S*)-MTPA afforded a mixture of the (*S*)-MTPA monoester **6a** and the diester **6c** in a ratio of 3:1. While, in the esterification of **6** with (*R*)-MTPA, only monoester (2-ester) was observed and obtained. On the basis of these results, 15*R* configuration of **6** was determined. Thus, **6** was identified as (15*R*)-2α,15-dihydroxy-buagarofuran.

The HRESIMS, ^{1}H NMR, and ^{13}C NMR spectroscopic data (Tables 1 and 2) of **7** were in accordance with the molecular formula $C_{18}H_{28}O_3$, with 2 amu less than the corresponding value of **6**. The NMR spectroscopic data were similar to those of **6**, except the absence of oxygen-bearing methine signal corresponding to H-2 (δ_H 4.14, t, J=5.2 Hz) in **6**, and the presence of one carbonyl signal at δ_C 200.1. On the basis of the bulkiness rule for secondary alcohols, a negative Cotton effect at ~350 nm (the E band) in the $Rh_2(OCOCF_3)_4$-induced CD[14] spectrum (Fig. 4) supported the 15*R* configuration. Therefore, **7** was determined as (15*R*)-15-hydroxy-2-oxo-buagarofuran.

The molecular formula $C_{18}H_{30}O_3$ of metabolite **9** was established by HRESIMS at m/z 317.2085 $[M+Na]^+$. The ^{1}H NMR data of **9** (Table 1) were similar to those of **6** except that the signal corresponding to H-2 (δ_H 4.14, t, J=5.2 Hz) in **6** disappeared, while the oxygen-bearing methine signal at δ_H 3.80 (dd, J=6.0, 5.7 Hz, H-9) was observed. These indicated the introduction of OH group at C-9 position instead of C-2 position, which was further supported by that the signal of H_3-11 in **9** significantly shifted upfield to δ_H 0.81 (s) from δ_H 1.03 (s) in **6**. Additionally, the HMBC correlations (Fig. 2) of H-9/C-8, C-10, and C-11 solidly suggested the presence of 9-OH group. The enhancement of integration value of H_3-11 was not observed when irradiation of H-9 in the NOE difference spectral experiments, revealing that 9-OH was α-oriented. The absolute configuration of C-15 was determined using Mosher's method (Fig. 6).[11,12] The (*S*)- and (*R*)-MTPA esters of **9** (**9a** and **9b**) were prepared using the corresponding (*R*)-(−)-and (*S*)-(+)-MPTA, respectively. The determination of the chemical shift differences $\Delta\delta$ ($\delta_S-\delta_R$) for the protons neighboring C-15 functionality led to the assignment of *S* or *R* configurations for C-15 of **9**. On the basis of these results, 15*R* configuration was determined. Thus, **9** was determined as (15*R*)-9α,15-dihydroxy-buagarofuran.

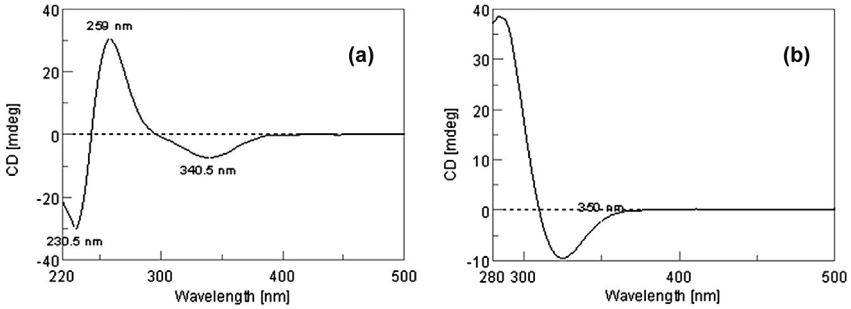

Fig. 4. Comparative CD spectra of **7**. a, CD spectrum of **7** recorded in MeOH; b, $Rh_2(OCOCF_3)_4$-induced CD spectrum of **7**.

Metabolite **8** showed a quasi-molecular ion peak at m/z 295.2241 $[M+H]^+$ in the HRESIMS spectrum, in agreement with the molecular formula $C_{18}H_{30}O_3$. The NMR spectroscopic (Tables 1 and 2) features of **8** were very similar to those of **6** except that the signal corresponding to H-15 (δ_H 4.07, dd, J=8.0, 4.8 Hz) in **6** disappeared, while another oxygen-bearing methine signal (δ_H 3.52, m) was observed. The HMBC correlations (Fig. 2) of the signals of δ_H 3.52/C-15, C-2′, and C-3′ indicated the presence of 1′-OH rather than 15-OH. Selective oxidation of the allylic 2-OH to carbonyl by active MnO_2[15] was undertaken to yield the derivative **8a** (Fig. 5). On the basis of the bulkiness rule for secondary alcohols, a positive Cotton effect at ~350 nm (the E band) in the $Rh_2(OCOCF_3)_4$-induced CD spectrum of **8a** (Fig. 5) supported the 1′*S* configuration. Thus, the structure of **8** was deduced to be (1′*S*)-2α,1′-dihydroxy-buagarofuran.

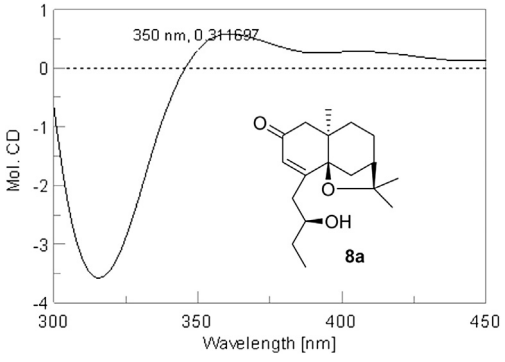

Fig. 5. $Rh_2(OCOCF_3)_4$-induced CD spectrum of **8a**.

The HRESIMS, ^{1}H and ^{13}C NMR spectroscopic data of **10** were in accordance with the molecular formula $C_{18}H_{30}O_3$. The NMR spectroscopic data (Tables 1 and 2) were analogous to those of **8** and **9**. Specifically, the spin−spin splitting pattern and coupling constants of H-9 (δ_H 3.80, dd, J=11.6, 6.0 Hz) were similar to those of **9**, while those of H-1′ in **10** was identical with those in **8**. Therefore, **10** was deduced as (1′*S*)-9α,1′-dihydroxy-buagarofuran.

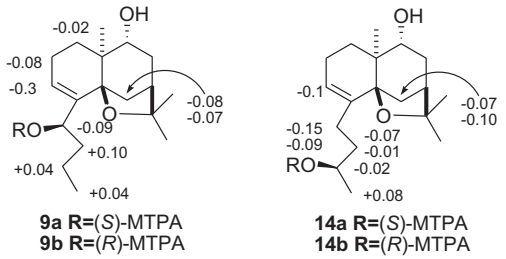

Fig. 6. $\Delta\delta(\delta_S-\delta_R)$ values obtained from the ^{1}H NMR spectra of the MTPA esters of **9** and **14**.

(+)-HRESI mass spectrum of **11** exhibited the quasi-molecular ion peaks at m/z $[M+H]^+$ 295.2246, suggesting the molecular formula of $C_{18}H_{30}O_3$ and the introduction of two OH groups as compared with **1**. The ^{1}H NMR (Table 3) data of **11** were similar to those of **6** except that the signal corresponding to H-2 (δ_H 4.14, t, J=5.2 Hz) in **6** disappeared, while another oxygen-bearing methine signal at δ_H 4.09 (m) was observed. The introduction of OH group at C-8 position was supported by comparing the signal of H-8 in **11** with

Table 3
^{1}H NMR spectroscopic data of **11**–**19**[a,b]

Position	**11**[a]	**12**[b]	**13**[b]	**14**[a]	**15**[b]	**16**[a]	**17**[b]	**18**[b]	**19**[a]
1	1.08 m	1.07 m	1.30 m	1.53 m	2.03 m, 1.42 m	1.47 m, 1.89 m	2.85 (d, 15.6)	2.75 (d, 15.6)	1.89 (dd, 14.4, 6.4)
							2.07 (d, 15.6)	1.96 (d, 15.6)	1.35 m
2	2.00 m	2.05 m	2.03 m	1.93 m	4.22 (t, 4.2)	4.04 m			4.09 (t, 5.2)
3	5.88 br s	5.92 (t, 3.3)	5.64 br s	5.58 br s	5.73 (d, 3.3)	5.62 br s	5.91 s	5.90 s	5.62 (d, 4.0)
6	2.49	2.17 m	2.26 (d, 12.0)	2.20 (d, 12.3)	2.33 m	2.21 (d, 12.6)	2.52 (dd, 12.6, 5.1)	2.66 m	2.29 (dd,12.0, 3.3)
	(dd, 13.2, 4.8)	1.85 m	1.75 (d, 12.0)	1.72 (d, 12.3)	1.76 (d, 15.0)	1.77 (d, 12.6)	1.82 (d, 12.6)	2.17 m	1.79 (d, 12.0)
	1.76 (d, 13.2)								
7	2.06 m	2.03 m	2.03 m	1.94 m	1.98 m	1.97 m	2.03 m	2.66 m	2.10 m
8	4.09 m	4.15 br s	1.56 m	1.53 m, 1.94 m	1.69 m	1.58 m	4.18 m		1.73 m
9	2.00 m	1.85	3.96 (dd, 11.1, 6.3)	3.76 (dd, 11.0, 5.7)	1.69 m, 1.32 m	3.82 (dd, 11.4, 6.6)	2.27 m	2.32 m	1.59 m, 1.26 m
		(dd, 15.3, 5.1)							
		1.66 m							
11	0.91 s	1.11 s	0.85 s	0.75 s	1.07 s	0.81 s	1.04 s	1.12 s	1.02 s
12	1.20 s	1.32 s	1.36 s	1.26 s	1.35 m	1.30 s	1.59 s	1.30 s	1.22 s
13	1.44 s	1.30 s	1.24 s	1.16 s	1.24 s	1.18 s	1.33 s	1.33 s	3.78 (d, 10.8)
									3.54 (d, 10.8)
15	4.04 m	4.21 m	2.11 m	2.10 m, 1.94 m	2.14 m, 2.23 m	1.38 m	1.75 m	2.17 m	2.02 m, 1.34 m
1′	2.06 m	2.08 m	1.52 m	1.53 m, 1.45 m	1.65 m	1.91 m	1.38–1.51 m	1.52 m	2.02 m, 1.34 m
2′	1.57 m	1.66 m	1.48 m	3.65 m	3.83 br s	1.30 m	1.32–1.51 m	1.45 m	1.34 m
3′	0.91 (t, 7.5)	0.96 (t, 6.9)	0.94 (t, 7.5)	1.09(d, 6.6)	1.22 (d, 6.0)	0.91 (t, 6.6)	0.96(t, 6.9)	0.96 (t, 6.6)	0.92 (t, 6.8)

[a] Data were measured in CD_3OD and recorded at 400 MHz for compounds **19**, others for 300 MHz; Coupling constants (*J* in Hz) are given in parentheses. Assignments were based on gCOSY, HSQC, and HMBC experiments.
[b] Data were measured in $CDCl_3$ and recorded at 300 MHz.

the methine proton signal of H-8 in **2**. 8-OH was determined to be β-oriented by NOE difference spectrum experiments, in which H_3-11 enhancement was observed as H-8 irradiated. Therefore, **11** was deduced to be (15*R*)-8β,15-dihydroxy-buagarofuran. Metabolite **12** had the same molecular formula of $C_{18}H_{30}O_3$ as **11**, and the spectroscopic data of **12** were very similar to those of **11**. Comparison of the spin–spin splitting pattern and coupling constants of H-8 in **12** and **11** indicated that α-orientation of H-8 in **12**. Thus, **12** was assigned as (15*R*)-8α,15-dihydroxy-buagarofuran.

2.2. Transformation of 1 by *A. coerulea* AS 3.3538

Three metabolites were obtained from the conversion of **1** by *A. coerulea* AS 3.3538, their structures were identified to be (15*R*)-9α,15-dihydroxy-buagarofuran (**9**), 9α-hydroxy-buagarofuran (**13**), and (2′*R*)-9α,2′-dihydroxy-buagarofuran (**14**) (Fig. 7). The predominant reaction was 9α-hydroxylation by this strain.

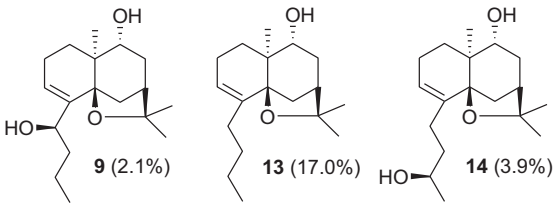

Fig. 7. The chemical structures of metabolites (**9**, **13**, **14**) of buagarofuran (**1**) with *A. coerulea* AS 3.3538 (yields in parentheses).

The molecular formula of $C_{18}H_{30}O_2$ of **13** was determined by HRESIMS with the quasi-molecular ion peak at *m*/*z* $[M+H]^+$ 279.2150, suggesting the substitution of an additional OH group as compared with **1**. The ^{1}H NMR (Table 3) data of **13** were similar to those of **1** except that the signal corresponding to H-9 (δ_H 1.17, m) in **1** disappeared, while an oxygen-bearing methine signal (δ_H 3.96, dd, *J*=11.0, 6.3 Hz) was observed. These indicated the introduction of OH group at C-9 position, which was further supported by the significantly shifting upfield of signal of H_3-11 to δ_H 0.85 (s) in comparison with **1**. The enhancement of H_3-11 was not observed when H-9 irradiated in the NOE difference spectra, revealing that 9-OH was α-oriented. Thus, the structure of **13** was determined as 9α-hydroxy-buagarofuran.

The MS and NMR spectroscopic data of **14** demonstrated that it was the 2′-OH derivative of **13**. This was verified by 2D NMR data analysis of **14**, which supported the 1D NMR data (Tables 3 and 4) assignments. It was further confirmed by the doublet of H_3-3′. The absolute configuration of C-2′ was determined by Mosher's method as before, and the results (Fig. 6) suggested that *R* configuration of C-2′. Therefore, **14** was determined to be (2′*R*)-9α,2′-dihydroxy-buagarofuran.

2.3. Transformation of 1 by *C. elegans* AS 3.3400

After incubation of **1** with cell cultures of *C. elegans* for 4 days, seven metabolites (**2**, **15**–**20**, Fig. 8) were obtained by a combination of open silica gel chromatography and semi-preparative HPLC purification. Their structures were elucidated based on the extensive spectroscopic data analyses. The occurred reactions included hydroxylation (C-8, C-9, etc.) and ketonization (C-2 and C-8 alcohols). Though the two strains of *C. echinulata* AS 3.3402 and *C. elegans* AS 3.3400 belong to the same genus, the generated metabolites of **1** were substantially different both in reaction position and in yield. It suggests the different enzymes (probably P450s) existence in the two different strains.

The NMR spectroscopic data (Tables 3 and 4) of **15** were similar to those of **14**, except the signal H-9 was disappeared, while H-2 at δ_H 4.22 (t, *J*=4.2 Hz) was observed, implying the presence of an OH group at C-2 rather than at C-9. The α-orientation of 2-OH was established according to the NOE difference spectrum, in which the enhancement of H_3-11 was not observed when irradiated the H-2. Thus, **15** was deduced as (2′*R*)-2α,2′-dihydroxy-buagarofuran.

The HRESIMS combined with the NMR data (Tables 3 and 4) indicated that metabolite **16** had the molecular formula $C_{18}H_{30}O_3$. The NMR data of **16** were very similar to those of **13** except that one additional oxy-bearing methine was observed, and the position was assigned to C-2 by HMBC correlations of H-2/C-1, and C-3. The enhancement of H_3-11 when H-2 irradiated suggested the β-orientation of 2-OH. Therefore, **16** was identified as 2β,9α-dihydroxy-buagarofuran.

The molecular formula $C_{18}H_{28}O_3$ of **17** was established by HRESIMS, IR, and NMR spectroscopic data (Tables 3 and 4). The

Table 4
^{13}C NMR spectroscopic data of **11**–**19**[a,b]

Position	**11**[a]	**12**[b]	**13**[b]	**14**[a]	**15**[b]	**16**[a]	**17**[b]	**18**[b]	**19**[a]
1	33.3, CH_2	32.2, CH_2	30.1, CH_2	30.2, CH_2	41.4, CH_2	39.6, CH_2	49.1, CH_2	48.4, CH_2	42.4, CH_2
2	23.3, CH_2	22.1, CH_2	22.1, CH_2	23.1, CH_2	64.4, CH	65.9, CH	199.2, C	197.8, C	64.7, CH
3	128.7, CH	128.3, CH	126.3, CH	128.1, CH	126.7, CH	131.5, CH	127.5, CH	127.7, CH	128.1, CH
4	139.3, C	138.6, C	135.9, C	136.9, C	139.2, C	138.6, C	159.0, C	157.7, C	140.0, C
5	86.1, C	85.3, C	85.5, C	87.7, C	84.8, C	87.1, C	84.0, C	84.3, C	87.0, C
6	33.2, CH_2	27.1, CH_2	33.7, CH_2	33.1, CH_2	33.2, CH_2	32.8, CH_2	32.7, CH_2	32.2, CH_2	34.0, CH_2
7	51.5, CH	51.3, CH	42.9, CH	44.3, CH	44.1, CH	44.0, CH	50.6, CH	61.1, CH	43.6, CH
8	69.4, CH	68.3, CH	32.1, CH_2	34.1, CH_2	23.9, CH_2	33.4, CH_2	70.0, CH	207.9, C	24.2, CH_2
9	43.5, CH_2	42.3, CH_2	74.1, CH	74.5, CH	34.2, CH_2	73.8, CH	42.5, CH_2	51.1, CH_2	35.9, CH_2
10	39.3, C	36.9, C	41.6, C	42.8, C	38.1, C	44.2, C	41.6, C	43.0, C	37.3, C
11	22.8, CH_3	22.9, CH_3	16.1, CH_3	16.8, CH_3	25.2, CH_3	17.6, CH_3	24.1, CH_3	26.2, CH_3	25.7, CH_3
12	24.8, CH_3	23.2, CH_3	22.9, CH_3	23.1, CH_3	22.7, CH_3	23.0, CH_3	24.2, CH_3	23.7, CH_3	25.7, CH_3
13	32.2, CH_3	30.8, CH_3	30.8, CH_3	30.4, CH_3	30.4, CH_3	30.3, CH_3	31.5, CH_3	29.2, CH_3	65.8, CH_2
14	82.8, C	80.0, C	81.1, C	83.0, C	81.6, C	82.8, C	82.7, C	81.8, C	85.3, C
15	70.8, CH	71.8, CH	31.3, CH_2	28.7, CH_2	27.1, CH_2	31.5, CH_2	31.6, CH_2	30.9, CH_2	32.1, CH_2
1′	39.5, CH_2	38.6, CH_2	29.2, CH_2	39.9, CH_2	37.8, CH_2	32.1, CH_2	29.5, CH_2	29.5, CH_2	32.1, CH_2
2′	20.8, CH_2	19.9, CH_2	22.8, CH_2	68.4, CH	67.5, CH	23.7, CH_2	22.5, CH_2	22.6, CH_2	23.8, CH_2
3′	14.3, CH_3	14.0, CH_3	14.1, CH_3	23.7, CH_3	24.0, CH_3	14.3, CH_3	13.9, CH_3	13.9, CH_3	14.4, CH_3

[a] Data were measured in CD_3OD and recorded at 100 MHz for compounds **11**–**14**, **17**, and **18**, and 150 MHz for **16**; Assignments were based on gCOSY, HSQC, and HMBC experiments.
[b] Data were measured in $CDCl_3$ and recorded at 100 MHz for compound **15**, **19** and 125 MHz for others.

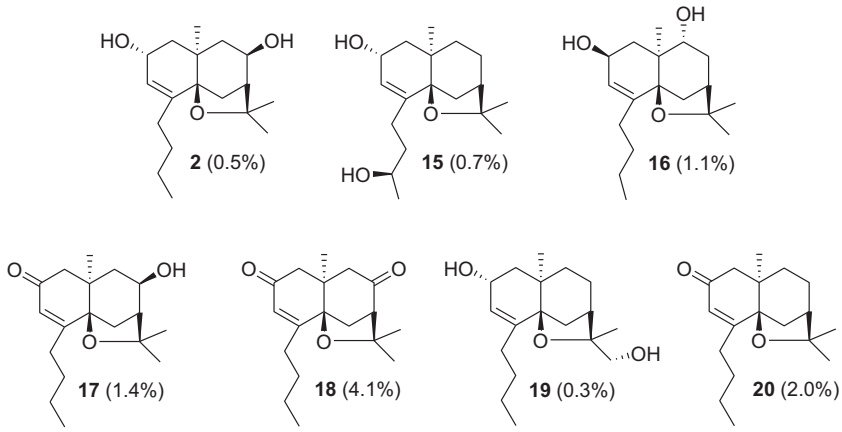

Fig. 8. The chemical structures of metabolites (**2**, **15**–**20**) of buagarofuran with *C. elegans* AS 3.3402 (yields in parentheses).

NMR data were similar to those of **5**. The different spin–spin patterns of H-8 in **17** and **5** indicated the different orientation of the introduced 8-OH group. It was confirmed by the enhancement of H_3-11 when H-8 irradiated. Accordingly, **17** was identified as 8β-hydroxy-2-oxo-buagarofuran.

The HRESIMS combined with the NMR data (Tables 3 and 4) indicated that **18** had the molecular formula $C_{18}H_{26}O_3$. The NMR data of **18** resembled those of **17**, except the presence of one carbonyl moiety instead of one OH group. The resonances for C-7 and C-9 were deshielded significantly to δ_C 61.1 and δ_C 51.1, respectively, as compared with that of **17**, further suggesting the carbonyl moiety at C-8. Thus, **18** was determined to be 2,8-dioxo-buagarofuran.

HRESI-positive mass spectrum of **19** exhibited two quasi-molecular ion peaks at $[M+H]^+$ 295.2243 and $[M+Na]^+$ 317.2091, consistent with the molecular formula of $C_{18}H_{30}O_3$. It suggested the substitution of two additional OH groups in comparison with **1**. The ^{1}H NMR spectrum (Table 3) of **19** was similar to that of **2** except that the signal corresponding to H_3-13 (δ_H 1.43, s) in **2** disappeared, while the oxy-bearing methylene signals at δ_H 3.78 (d, J=10.8 Hz) and δ_H 3.54 (d, J=10.8 Hz) were observed. These indicated the introduction of OH group at C-13 position, which was further supported by that the signal of C-13 in **19** significantly shifted downfield to δ_C 65.8 (t) compared with δ_C 31.9 (q) in **2**. Additionally, the HMBC correlations (Fig. 2) of H_2-13/C-7, C-12, and C-14 strongly confirmed the deduction. Accordingly, **19** was elucidated as 2α,13-dihydroxy-buagarofuran.

The HRESIMS combined with the NMR data (see Section 4.4.3) indicated that **20** had the molecular formula $C_{18}H_{28}O_2$, requiring 5 degrees of unsaturation. The NMR data of **20** resembled those of **17** except the disappearance of the resonance for 8-OH signal. Therefore, **20** was determined to be 2-oxo-buagarofuran, which was one of the metabolites of **1** incubated with rat and human liver microsomes in vitro.

2.4. Pharmacological evaluation of 5-hydroxytryptamine (5-HT) and [^{3}H]-NE re-uptaking in rat brain synaptosomes inhibitory activity

Serotonin and norepinephrine re-uptake inhibitors may be potential anti-anxiety. The in vitro anti-anxiety activity of the above 19 (**2**–**20**) metabolites along with buagarofuran (**1**) were preliminarily evaluated using rat brain synaptosomes by inhibiting both [^{3}H]-5-HT and [^{3}H]-NE re-uptake[16,17] using duloxetine as a positive control (Table 5). The results showed that metabolites **8**, **10**–**12**, and **20** exhibited remarkable in vitro anti-anxiety activity at 10 μM, inhibiting [^{3}H]-NE re-uptake in the rat brain

Table 5
The inhibitory effects on 5-hydroxytryptamine (5-HT)-re-uptaking in rat brain synaptosomes and re-uptake of [^{3}H]-NE experiments

Compounds	Inhibitory rates on 5-HT re-uptaking (%)	Inhibitory rates on [^{3}H]-NE re-uptaking (%)
Duloxetine	89.5	81.4
1	37.0	56.3
2	33.7	42.2
3	38.6	31.4
4	39.1	21.3
5	38.9	43.4
6	37.9	31.7
7	40.3	44.2
8	57.0	64.9*
9	50.0	8.2
10	45.1	60.5*
11	40.5	65.3*
12	35.9	66.9*
13	44.9	19.2
14	34.2	35.1
15	40.2	33.2
16	35.9	37.1
17	33.4	39.2
18	41.6	32.5
19	36.8	21.8
20	44.1	71.2

*$P<0.05$.

synaptosomes by 64.9%, 60.5%, 65.3%, 66.9%, and 71.2%, respectively. In addition, compounds **8** and **9** at 10 μM showed strongly in vitro inhibiting [^{3}H]-5-HT re-uptake in the rat brain synaptosomes by 57% and 50%, respectively. These metabolites, especially for **20**, one mammalian metabolite in vitro, displayed much more potent inhibitory activity on [^{3}H]-NE and (5-HT) re-uptaking than **1**. It suggested that metabolism of buagarofuran might generated pharmacologically active metabolites (bioactivation). These oxidized metabolites might be generated by P450 enzymes, one kind of common phase I metabolism enzymes in human and/or mammalian in vivo.

3. Conclusion

In summary, we report the successful microbial transformations of an anti-anxiety agent, buagarofuran, by three filamentous fungi, which led to the yield of 19 metabolites. The occurred reactions included mono-hydroxylation, di-hydroxylation, and oxidation of alcohol to ketone. The absolute configurations of the metabolites were determined by NOE difference spectral experiments, CD spectral data, and chemical methods. In particular, the $Rh_2(O\text{-}COCF_3)_4$-induced CD spectroscopic data together with the Mosher's methods, and selective oxidation of the double bond allylic hydroxyl group to carbonyl moiety were undertaken to determine the absolute configurations of several products. The structural diversity of the microbial transformed metabolites of buagarofuran in the present report would give a favorable opportunity to probe the mammalian metabolism of buagarofuran and better shed light on its pharmacokinetics and pharmacodynamics. Moreover, the more active metabolites generated both microbes and mammalian liver microsomes suggest that the anti-anxiety effects of buagarofuran in vivo might be attributed to the combination of the parent molecule and its active metabolite(s).

4. Experimental section

4.1. General experimental procedures

Optical rotations were measured on a Perkin–Elmer Model-343 digital polarimeter. The UV and CD spectra were recorded on a JASCO J-815 spectropolarimeter. The IR spectra were measured using a Nicolet 5700 FT-IR microscope spectrometer (FT-IR Microscope Transmission). NMR spectra were obtained on Mercury-300, Mercury-plus-400, and Bruker ARX-500 spectrometers. Chemical shifts (δ) are given in parts per million, and coupling constants (J) are given in Hertz (Hz). ESIMS data and HRESIMS spectra were carried out using an Agilent Technologies 6520 Accurate Mass Q-TOF LC/MS spectrometer. Column chromatography was performed with silica gel (200–300 mesh, Qingdao Marine Chemical Inc. Qingdao, PR China). Semi-preparative HPLC was carried out on a Shimadzu LC-6AD instrument equipped with a Shimadzu RID-10A detector and a YMC-Pack ODS-A column (250 mm×10 mm i.d., 5 μm) or a Grace Allsphere silica column (250 mm×10 mm, i.d., 5 μm). Analytical TLC was performed on pre-coated silica gel GF_{254} plates (Qingdao Marine Chemical Inc.), and visualized under UV light or by spraying with 10% H_2SO_4 in EtOH followed by heating at 120 °C. Unless otherwise stated, all chemicals were of analytic grade and obtained from commercially available sources and used without further purification.

4.2. Substrate, organisms, and cultivation conditions

Buagarofuran (**1**, 98% by HPLC) was gifted from Professor Dali Yin of Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, and characterized by comparison of the NMR data with the references.[1] The sample was dissolved in *N*,*N*-dimethylformamide (DMF) at the concentration of 50 mg/mL as stock solution before use. The strains including prokaryotic organisms and eukaryotic organisms listed in Table S1 were purchased from Chinese General Microorganism Culture Collection, Chinese Academy of Sciences, and kept on slats. The medium was used after autoclaving at 121 °C for 25 min. The seed cultures were prepared in 250 mL flasks with 70 mL liquid medium by incubating 2 days. A volume of 5 mL of the seed cultures was inoculated into each 1000 mL flask with 300 mL medium, and shaken at 120 r/min and 25 °C in the dark for the use of preparative biotransformation.

4.3. Screening biotransformations

For screening test, the microbial strains were incubated as described above. 2-day-old cell cultures were employed for biotransformation. A volume of 140 μL of substrate solution with final concentration of 100 mg/L was fed to one flask. The incubation was allowed to proceed for 4 days, after which the cultures were filtered and the filtrate was extracted with ethyl acetate (3×100 mL). The extract was dried with anhydrous Na_2SO_4 and concentrated under vacuum at 45 °C to give residue, and analyzed by TLC and HPLC.

4.4. Preparative biotransformations, extraction, isolation, and identification of metabolites

The 2-day-old seed cultures of the fungal strains were added to the 1000 mL Erlenmeyer flasks containing 300 mL of liquid medium. After 2 days of incubation, **1** (500 mg in 10 mL DMF for each strain) was evenly distributed into 5 L of cultures, and incubated at 25 °C in a rotary shaker at 120 r/min for 4 days. After which the cell cultures were filtrated. The filtrate was extracted with ethyl acetate (3×5 L), dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to afford the crude extract.

*4.4.1. Metabolites from **1** by C. echinulata AS 3.3402.* The crude extract (1.8 g) was subjected to silica gel column (200 g, 200–300 mesh) chromatography eluting with a gradient of petroleum ether (60–90 °C) in ethyl acetate to afford 11 fractions. The

fraction 10 (180 mg) was submitted to semi-preparative HPLC and eluted with *n*-hexane in ethyl acetate (5:1, v/v, 4.0 mL/min) to yield **2** (128 mg, 22.8%) and **8** (4 mg, 0.7%). Fraction 8 (170 mg) was fractionated by Sephadex LH-20 (MeOH) then purified by normal phase semi-preparative HPLC (*n*-hexane/ethyl acetate=5:1, v/v, 4.0 mL/min) to give **4** (15 mg, 2.7%) and **6** (5 mg, 0.9%). Fraction 7 (310 mg) was applied to reverse phase semi-preparative HPLC (30% ACN in H_2O, v/v) to afford **5** (13.0 mg, 2.3%), **9** (5.0 mg, 0.9%), **11** (3 mg, 0.5%), and **12** (3 mg, 0.5%). Fraction 6 was underwent chromatography through a Sephadex LH-20 column eluted with 30% $CHCl_3$ in MeOH (v/v) and further purified by reverse phase semi-preparative HPLC (35% ACN in H_2O, v/v, 3.0 mL/min) to yield **3** (9.0 mg, 2.2%), and **10** (4 mg, 0.7%). Fraction 5 (95 mg) was purified by reverse phase semi-preparative HPLC (35% ACN in H_2O, v/v, 3.0 mL/min) to give **7** (7 mg, 1.2%).

*4.4.1.1. 2α,8β-Dihydroxy-buagarofuran (**2**).* White powder, $[\alpha]_D^{20}$ +62.4 (*c* 0.50, MeOH); IR ν_{max} 3260, 2966, 2938, 2879, 1663, 1460, 1381, 1360, 1326, 1056, 1010, 882 cm^{-1}; ^1H (Table 1) and ^{13}C NMR data (Table 2); ESIMS *m/z* 295 $[M+H]^+$; HRESIMS (positive) *m/z* 295.2257 $[M+H]^+$ (calcd for $C_{18}H_{31}O_3$, 295.2268).

*4.4.1.2. 2α-Hydroxy-8-oxo-buagarofuran (**3**).* Pale oil, $[\alpha]_D^{20}$ −1.33 (*c* 0.30, MeOH); IR ν_{max} 3439, 2981, 2980, 2873, 1714, 1677, 1462, 1387, 1254, 1133, 1032, 903, 872 cm^{-1}; ^1H (Table 1) and ^{13}C NMR data (Table 2); ESIMS *m/z* 293 $[M+H]^+$; HRESIMS (positive) *m/z* 293.2096 $[M+H]^+$ (calcd for $C_{18}H_{29}O_3$, 293.2111).

*4.4.1.3. 2α,8α-Dihydroxy-buagarofuran (**4**).* White powder, $[\alpha]_D^{20}$ +13.3 (*c* 0.06, MeOH); IR ν_{max} 3376, 2996, 2968, 2925, 2862, 1714, 1678, 1660, 1459, 1365, 1295, 1136, 1085, 992, 871, 850 cm^{-1}; ^1H (Table 1) and ^{13}C NMR data (Table 2); ESIMS *m/z* 295 $[M+H]^+$, 611 $[2M+Na]^+$; HRESIMS (positive) *m/z* 295.2242 $[M+H]^+$ (calcd for $C_{18}H_{31}O_3$, 295.2268).

*4.4.1.4. 8α-Hydroxy-2-oxo-buagarofuran (**5**).* Pale oil, $[\alpha]_D^{20}$ −16.0 (*c* 0.16, MeOH); IR ν_{max} 3443, 2960, 2938, 2873, 1667, 1624, 1464, 1388, 1351, 1248, 1136, 1077, 994, 871 cm^{-1}; ^1H (Table 1) and ^{13}C NMR data (Table 2); ESIMS *m/z* 293 $[M+H]^+$; HRESIMS (positive) *m/z* 293.2105 $[M+H]^+$ (calcd for $C_{18}H_{29}O_3$, 293.2111).

*4.4.1.5. (15R)-2α,15-Dihydroxy-buagarofuran (**6**).* White powder, $[\alpha]_D^{20}$ +43.2 (*c* 0.07, MeOH); IR ν_{max} 3265, 2957, 2927, 2869, 1726, 1676, 1459, 1383, 1299, 1126, 1071, 1013, 958, 880 cm^{-1}; ^1H (Table 1) and ^{13}C NMR data (Table 2); ESIMS *m/z* 295 $[M+H]^+$, 611 $[2M+Na]^+$; HRESIMS (positive) *m/z* 317.2074 $[M+Na]^+$ (calcd for $C_{18}H_{30}NaO_3$, 317.2087).

*4.4.1.6. Preparation of (S)-(**6a**) and (R)-MTPA (**6b**) esters.* To a solution of **6** (5.0 mg, 0.017 mmol) in anhydrous dichloromethane (0.5 mL) were added (*S*)-MTPA chloride (15 mg, 0.064 mmol), EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] 10 mg, and DMAP [α-methoxy-α-(trifluoromethyl) phenylacetyl chloride] 10 mg, then the mixture was allowed to stir for 48 h at room temperature. The reaction was quenched by addition of 1.0 mL of H_2O, and the mixture was subsequently extracted with ethyl acetate (3×1.0 mL). The organic layers were combined, dried over anhydrous $NaSO_4$, and evaporated. The residue was subjected to column chromatography over silica gel chromatography using *n*-hexane/ethyl acetate (3:1) to yield the (*S*)-MTPA ester (**6a**, 1.1 mg). The same procedure was used to prepare the (*R*)-MTPA ester (**6b**, 0.8 mg). ^1H NMR (CD_3OD, 600 MHz, **6a**): δ_H 1.87 (m, H-1), 4.10 (t, *J*=5.4 Hz, H-2), 5.97 (d, *J*=4.2 Hz, H-3), 2.20 (dd, J_1=12.0 Hz, J_2=4.2 Hz, H-6a), 1.60 (d, *J*=12.0 Hz, H-6b), 1.65 (m, H-1′), 1.52 (m, H-2′a), 1.36 (m, H-2′b), 0.90 (t, *J*=7.8 Hz, H_3-3′). ^1H NMR (CD_3OD, 600 MHz, **6b**): δ_H 1.98 (m, H-1), 4.15 (t, *J*=5.4 Hz, H-2), 6.08 (d, *J*=4.2 Hz, H-3), 2.38 (dd, J_1=12.0 Hz, J_2=4.2 Hz, H-6a), 1.81 (d, *J*=12.0 Hz, H-6b), 1.59 (m, H-1′), 1.37 (m, H-2′a), 1.25 (m, H-2′b), 0.84(t, *J*=7.8 Hz, H_3-3′).

*4.4.1.7. (15R)-15-Hydroxy-2-oxo-buagarofuran (**7**).* Pale oil, $[\alpha]_D^{20}$ −11.8 (*c* 0.07, MeOH); IR ν_{max} 3424, 2964, 2934, 2873, 1670, 1459, 1382, 1229, 1148, 1131, 1071, 1009, 960, 885 cm^{-1}; ^1H (Table 1) and ^{13}C NMR data (Table 2); ESIMS *m/z* 293 $[M+H]^+$; HRESIMS (positive) *m/z* 293.2090 $[M+H]^+$ (calcd for $C_{18}H_{29}O_3$, 293.2111).

*4.4.1.8. (1′S)-2α,1′-Dihydroxy-buagarofuran (**8**).* White powder, $[\alpha]_D^{20}$ −12.7 (*c* 0.06, MeOH); IR ν_{max} 3370, 3253, 2949, 2926, 2858, 1651, 1459, 1291, 1007, 881 cm^{-1}; ^1H (Table 1) and ^{13}C NMR data (Table 2); ESIMS *m/z* 295 $[M+H]^+$; HRESIMS (positive) *m/z* 295.2241 $[M+H]^+$ (calcd for $C_{18}H_{31}O_3$, 295.2268).

*4.4.1.9. Preparation of **8a**.* A solution of 2.0 mg of **8** in 0.5 mL of ether and 5.0 mg of MnO_2 was stirred at room temperature for 4 h, after which the reaction was quenched with the addition of 10 mL of ethyl acetate. After purified by semi-preparative HPLC, 1.5 mg of **8a** was obtained. ^1H NMR (600 MHz, CD_3OCD) δ_H 2.05 (m, H-1), 5.91 (s, H-3), 2.66 (d, 15.6, H-6a), 2.20 (dd, 15.6, 9.6, H-6b), 1.92 (m, H-7), 1.75 (2H, m, H-8), 1.54 (m, H-9a), 1.27 (m, H-9b), 1.02 (3H, s, H-11), 1.37 (3H, s, H-12), 1.23 (3H, s, H-13), 2.42 (dd, 15.6, 3.6, H-15a), 2.32 (dd, 15.6, 5.4, H-15b), 3.56 (m, H-1′), 1.53 (2H, m, H-2′), 0.96 (3H, t, 7.8, H-3′).

*4.4.1.10. (15R)-9α,15-Dihydroxy-buagarofuran (**9**).* Pale oil, $[\alpha]_D^{20}$ +28.4 (*c* 0.07, MeOH); IR ν_{max} 3384, 2999, 2933, 2867, 1456, 1382, 1148, 1065, 1019, 969, 883, 855 cm^{-1}; ^1H (Table 1) and ^{13}C NMR data (Table 2); ESIMS *m/z* 295 $[M+H]^+$, 317 $[M+Na]^+$; HRESIMS (positive) *m/z* 317.2085 $[M+Na]^+$ (calcd for $C_{18}H_{30}NaO_3$, 317.2087).

*4.4.1.11. Preparation of (S)-(**9a**) and (R)-MTPA (**9b**) esters.* A sample of **9** (5.0 mg, 0.017 mmol), (*S*)-MTPA (15 mg, 0.064 mmol), and anhydrous dichloromethane (0.5 mL), EDC 10 mg, and DMAP 10 mg were allowed to react in a 5 mL tube at ambient temperature for 24 h. The mixture was evaporated to dryness and purified by normal phase semi-preparative HPLC (*n*-hexane/ethyl acetate=2:1, v/v) to afford the **9a** (1.0 mg): colorless oil; ^1H NMR (CD_3OD, 600 MHz) δ_H 1.68 (m, H-1), 1.90 (m, H-2), 5.77 (d, 3.6, H-3), 2.25 (dd, 9.6, 3.0, H-6a), 1.68 (d, 10.2, H-6b), 3.74 (dd, 10.8, 6.0, H_2-9), 1.70 (m, H-1′), 1.28 (m, H_2-2), 0.89 (t, 7.2, H_3-3′). Likewise, the reaction mixture was processed as described above for **9a** to afford **9b** (0.7 mg). Colorless oil; ^1H NMR (CD_3OD, 600 MHz) δ_H 1.70 (m, H-1), 1.98 (m, H-2), 6.07 (d, 3.6, H-3), 2.32 (dd, 10.2, 3.0, H-6a), 1.68 (d, 6.6, H-6b), 3.77 (dd, 10.8, 6.6, H_2-9), 1.60 (m, H-1′), 1.24 (m, H_2-2), 0.85 (t, *J*=7.8 Hz, H_3-3′).

*4.4.1.12. (1′S)-9α,1′-Dihydroxy-buagarofuran (**10**).* White powder, $[\alpha]_D^{20}$ −3.3 (*c* 0.03, MeOH); IR ν_{max} 3400, 2966, 2928, 2877, 1724, 1667, 1458, 1141, 1062, 1028, 983, 871 cm^{-1}; ^1H (Table 1) and ^{13}C NMR data (Table 2); ESIMS *m/z* 295 $[M+H]^+$; HRESIMS (positive) *m/z* 295.2246 $[M+H]^+$ (calcd for $C_{18}H_{31}O_3$, 295.2268).

*4.4.1.13. (15R)-8β,15-Dihydroxy-4-buagarofuran (**11**).* Pale oil, $[\alpha]_D^{20}$ +35.2 (*c* 0.13, MeOH); IR ν_{max} 3383, 2961, 2929, 2872, 1657, 1461, 1379, 1245, 1137, 1053, 1012, 959, 876 cm^{-1}; ^1H (Table 3) and ^{13}C NMR data (Table 4); ESIMS *m/z* 295 $[M+H]^+$, 317 $[M+Na]^+$; HRESIMS (positive) *m/z* 317.2104 $[M+Na]^+$ (calcd for $C_{18}H_{30}NaO_3$, 317.2087).

*4.4.1.14. (15R)-8α,15-Dihydroxy-4-buagarofuran (**12**).* White powder, $[\alpha]_D^{20}$ −4.8 (*c* 0.12, MeOH); IR ν_{max} 3409, 2960, 2931, 2873, 1708, 1669, 1462, 1384, 1136, 1023, 879 cm^{-1}; ^1H (Table 3) and ^{13}C

NMR data (Table 4); ESIMS *m/z* 295 $[M+H]^+$; HRESIMS (positive) *m/z* 317.2072 $[M+Na]^+$ (calcd for $C_{18}H_{30}NaO_3$, 317.2087).

*4.4.2. Metabolites from **1** by A. coerulea AS 3.3538.* The giving crude extract (0.9 g) was submitted to silica gel column (200 g, 200–300 mesh) chromatography eluting with a gradient of petroleum ether (60–90 °C) in acetone to afford 8 fractions. Compound **13** (90 mg, 17.0%) was crystallized from Fr. 4 (154 mg). Fr. 5 (132 mg) was purified by reverse phase semi-preparative HPLC (35% ACN in H_2O, v/v, 3 mL/min) to give **9** (12 mg, 2.1%). Fr. 8 (75 mg) was subjected to reverse phase semi-preparative HPLC to yield **14** (22 mg, 3.9%).

*4.4.2.1. 9α-Hydroxy-buagarofuran (**13**).* White powder, $[\alpha]_D^{20}$ +2.1 (*c* 0.15, MeOH); IR ν_{max} 3405, 2958, 2933, 2871, 1710, 1666, 1459, 1384, 1137, 1064, 1023, 973, 880 cm^{-1}; ^1H (Table 3) and ^{13}C NMR data (Table 4); ESIMS *m/z* 277 $[M+H]^+$; HRESIMS (positive) *m/z* 277.2150 $[M+H]^+$ (calcd for $C_{18}H_{29}O_2$, 277.2162).

*4.4.2.2. (2′R)-9α,2′-Dihydroxy-buagarofuran (**14**).* Pale oil, $[\alpha]_D^{20}$ +3.4 (*c* 0.26, MeOH); IR ν_{max} 3443, 2985, 2964, 2925, 2872, 1459, 1367, 1310, 1233, 1137, 1059, 967, 871, 852 cm^{-1}; ^1H (Table 3) and ^{13}C NMR data (Table 4); ESIMS *m/z* 295 $[M+H]^+$; HRESIMS (positive) *m/z* 295.2251 $[M+H]^+$ (calcd for $C_{18}H_{31}O_3$, 295.2268).

*4.4.2.3. Preparation of (S)-(**14a**) and (R)-MTPA (**14b**) esters.* The reaction mixture of **14** (5.0 mg, 0.017 mmol), (*S*)-MTPA (15 mg, 0.064 mmol), and anhydrous dichloromethane (0.5 mL), EDC 10 mg, and DMAP 10 mg were processed as described above for to afford **14a** and **14b**. Compound **14a**: colorless oil; ^1H NMR (CD_3OD, 600 MHz) δ_H 1.59 (m, H-1), 1.85 (m, H-2), 5.48 (s, H-3), 2.04 (d, 12.6, H-6a), 1.67 (d, 12.0, H-6b), 1.97 (m, H_2-7), 3.74 (dd, 11.4, 6.0, H_2-9), 1.85 (m, H-15a), 1.91 (m, H-15b), 1.59 (m, H-1′a), 1.52 (m, H-1′b), 1.35 (d, 6.0, H_3-3′). Compound **14b**: colorless oil; ^1H NMR (CD_3OD, 600 MHz) δ_H 1.58 (m, H-1), 1.90 (m, H-2), 5.58 (s, H-3), 2.14 (d, 14.4, H-6a), 1.74 (d, 12.6, H-6b), 2.00 (m, H_2-7), 3.76 (dd, 11.4, J_2=6.0 Hz, H_2-9), 2.0 (m, H_2-15), 1.65 (m, H-1′a), 1.53 (m, H-1′b), 1.27 (d, 6.0, H_3-3′).

*4.4.3. Metabolites from **1** by C. elegans AS 3.3400.* The residue (1.7 g) was subjected onto a silica gel chromatography column by eluting with petroleum/ether (60–90 °C)/acetone to provide 10 fractions (Fr. 1–Fr. 10). Fr. 7 (430 mg) was separated by Sephadex LH-20 column chromatography eluting with chloroform/MeOH (40:60, v/v) and further purified by semi-preparative reverse phase HPLC (37% ACN in H_2O, v/v, flow rate 3 mL/min) to afford **15** (4 mg, 0.7%), and **16** (6 mg, 1.1%). The Fr. 5 (430 mg) was separated by reverse phase HPLC column chromatography (37% ACN in H_2O, v/v, flow rate 3 mL/min) to **2** (3 mg, 0.5%) and **18** (23 mg, 4.1%). Fraction 4 (170 mg) was applied to semi-preparative reverse phase HPLC (30% ACN in H_2O, v/v, flow rate 3 mL/min) to afford **17** (8 mg, 1.4%), **19** (2 mg, 0.3%), and **20** (11 mg, 2.0%).

*4.4.3.1. (2′R)-2α,2′-Dihydroxy-buagarofuran (**15**).* White powder, $[\alpha]_D^{20}$ −6.0 (*c* 0.1, MeOH); IR ν_{max} 3398, 3069, 2927, 2861, 1721, 1620, 1546, 1448, 1395, 1227, 1063, 1016, 948, 819 cm^{-1}; ^1H (Table 3) and ^{13}C NMR data (Table 4); ESIMS *m/z* 317 $[M+Na]^+$; HRESIMS (positive) *m/z* 317.2084 $[M+Na]^+$ (calcd for $C_{18}H_{30}NaO_3$, 317.2087).

*4.4.3.2. 2β,9α-Dihydroxy-buagarofuran (**16**).* White powder, $[\alpha]_D^{20}$ −9.7 (*c* 0.07, MeOH); IR ν_{max} 3375, 2930, 2867, 1725, 1664, 1460, 1383, 1124, 1063, 1026, 978, 948, 880, 856 cm^{-1}; ^1H (Table 3) and ^{13}C NMR data (Table 4); ESIMS *m/z* 317 $[M+Na]^+$; HRESIMS (positive) *m/z* 317.2084 $[M+Na]^+$ (calcd for $C_{18}H_{30}NaO_3$, 317.2087).

*4.4.3.3. 8β-Hydroxy-2-oxo-buagarofuran (**17**).* Pale oil, $[\alpha]_D^{20}$ +16.5 (*c* 0.12, MeOH); IR ν_{max} 3330, 2961, 2935, 2874, 1715, 1676, 1462, 1387, 1254, 1132, 1032, 903, 873, 829 cm^{-1}; ^1H (Table 3) and ^{13}C NMR data (Table 4); ESIMS *m/z* 293 $[M+H]^+$; HRESIMS (positive) *m/z* 293.2111 $[M+H]^+$ (calcd for $C_{18}H_{29}O_3$, 293.2111).

*4.4.3.4. 2,8-Dioxo-buagarofuran (**18**).* Pale oil, $[\alpha]_D^{20}$ −41.3 (*c* 0.21, MeOH); IR ν_{max} 3444, 2957, 2932, 2872, 1710, 1670, 1462, 1384, 1254, 1134, 1012, 879 cm^{-1}; ^1H (Table 3) and ^{13}C NMR data (Table 4); ESIMS *m/z* 291 $[M+H]^+$; HRESIMS (positive) *m/z* 291.1948 $[M+H]^+$ (calcd for $C_{18}H_{27}O_3$, 291.1955).

*4.4.3.5. 2α,13-Dihydroxy-buagarofuran (**19**).* White powder, $[\alpha]_D^{20}$ +18 (*c* 0.1, MeOH); IR ν_{max} 3319, 2953, 2927, 2869, 1663, 1461, 1381, 1034, 1005, 948, 880 cm^{-1}; ^1H (Table 3) and ^{13}C NMR data (Table 4); ESIMS *m/z* 317 $[M+Na]^+$; HRESIMS (positive) *m/z* 295.2243 $[M+H]^+$ (calcd for $C_{18}H_{31}O_3$, 295.2268); *m/z* 317.2091 $[M+Na]^+$ (calcd for $C_{18}H_{30}NaO_3$, 317.2087).

*4.4.3.6. 2-Oxo-buagarofuran (**20**).* Colorless oil, $[\alpha]_D^{20}$ +3 (*c* 0.05, MeOH); IR ν_{max} 3298, 2959, 2931, 2869, 1675, 1624, 1460, 1383, 1258, 1145, 1013, 960, 889, 867, 819 cm^{-1}; ^1H NMR (300 MHz, CD_3OCD) δ_H 2.75 (d, 15.0, H-1a), 1.97 (d, 15.0, H-1b), 5.89 (s, H-3), 2.38 (dd, 12.5, 3.0, H-6a), 1.85 (d, 12.5, H-6b), 2.07 (m, H-7), 1.78 (2H, m, H-8), 1.48 (2H, m, H-9), 1.03 (3H, s, H-11), 1.42 (3H, s, H-12), 1.29 (3H, s, H-13), 2.21 (2H, m, H-15), 1.40 (4H, m, H-1′ and H-2′), 0.93 (3H, t, 7.5, H-3′); ^{13}C NMR (100 MHz, CD_3OCD) δ_C 49.6 (C-1), 199.4 (C-2), 127.3 (C-3), 159.8 (C-4), 84.8 (C-5), 33.4 (C-6), 44.1 (C-7), 24.1 (C-8), 29.6 (C-9), 40.6 (C-10), 23.7 (C-11), 22.6 (C-12), 30.3 (C-13), 82.6 (C-14), 34.0 (C-15), 31.1 (C-1′), 22.6 (C-2′), 13.9 (C-3′); ESIMS *m/z* 299 $[M+Na]^+$; *m/z* 276 $[M+H]^+$.

4.5. Inhibition of (5-HT) and [^{3}H]-NE-serotonin re-uptake bioassay

One male SD rat was decapitated and the brain was rapidly removed.[16,17] The whole brain minus cerebellum was weighed and homogenized in 19 volumes of ice-cold 0.32 mol/L sucrose solution by a Potter–Elvejhem homogenizer. The homogenate was centrifuged at 1000×*g* at 4 °C for 10 min. The supernatant was decanted and used for re-uptake experiments. 100 μL suspension was mixed with 50 μL drug/sample solution or krebs buffer. The total binding tubes and sample tested tubes were preincubated at 37 °C for 20 min, then added 400 μL 40.1 nmol/L [^{3}H]-5-HT ([^{3}H]-NE) solution in krebs buffer and incubated at 37 °C for 20 min. The non-specific binding tubes were preincubated in 0 °C ice water for 20 min, then added 400 μL 40.1 nmol/L [^{3}H]-5-HT ([^{3}H]-NE) solution in krebs buffer and incubated in 0 °C ice water for 20 min. All tubes were filtrated by a cell harvester immediately after incubation. The fiberglass filter membrane was washed five times by ice-cold saline, then put into scintillation vials, and counted in 4 mL of liquid scintillation cocktail. The percent inhibition of sample on [^{3}H]-5-HT ([^{3}H]-NE) re-uptake=(cpm of total binding tubes−cpm of sample tubes)/(cpm of total binding tubes−cpm of nonspecific binding tubes)×100%. The percent inhibition at each sample concentration was the mean of three replicates. IC_{50} values were calculated by log-probit analysis.

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Supplementary data

Selected ^{1}H NMR, ^{13}C NMR, DEPT, gHSQC, HMBC, NOE, and IR spectra of compounds **2**–**19**, and the list of microorganism strains for screening transformation of buagarofuran. Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tet.2014.04.011.

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