Epigenetic modification in histone deacetylase deletion strain of *Calcarisporium arbuscula* leads to diverse diterpenoids

Jian Bai\(^a\), Rong Mu\(^a\), Man Dou\(^a\), Daojiang Yan\(^a\), Bingyu Liu\(^a\), Qian Wei\(^a\), Jun Wan\(^a\), Yi Tang\(^{a,b}\), Youcai Hu\(^a,\*\)

\(^a\)State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China

\(^b\)Department of Chemical and Biomolecular Engineering, Department of Chemistry and Biochemistry, University of California, Los Angeles, CA 90095, USA

Received 27 August 2017; received in revised form 21 September 2017; accepted 15 October 2017

**Abstract** Epigenetic modifications have been proved to be a powerful way to activate silent gene clusters and lead to diverse secondary metabolites in fungi. Previously, inactivation of a histone H3 deacetylase in *Calcarisporium arbuscula* had led to pleiotropic activation and overexpression of more than 75% of the biosynthetic genes and isolation of ten compounds. Further investigation of the crude extract of *C. arbuscula*\(^Δ\)hdaA strain resulted in the isolation of twelve new diterpenoids including three cassanes (1–3), one cleistanthane (4), six pimaranes (5–10), and two isopimaranes (11 and 12) along with two known cleistanthane analogues. Their structures were elucidated by extensive NMR spectroscopic data analysis. Compounds 2 and 4 showed potent inhibitory effects on the expression of MMP1 and MMP2 (matrix metalloproteinases family) in human breast cancer (MCF-7) cells.

© 2018 Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

---

**KEY WORDS**

*Calcarisporium arbuscula*; Calcarisporic acids; Pimarane; Diterpenoid; Matrix metalloproteinase inhibitor; Epigenetic genome mining

---

\*Corresponding author.

E-mail address: huyoucai@imm.ac.cn (Youcai Hu).

Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.
1. Introduction

_Calcarisporium arbuscula_, an endophytic fungus lives in the flesh of healthy fruit-bodies of mushrooms, mainly produces the adenosine triphosphate synthase inhibitors aurovertins B and D, which structurally contain a 2,6-dioxabicyclo[3.2.1]-octane ring. In our prior genome mining investigation, genome sequencing of _C. arbuscula_ revealed a large number of potential secondary metabolite gene clusters (68, of which 9 contain terpene synthases), which are significantly more than the two predominant metabolites aurovertins B and D, suggesting that most gene clusters are silent or lowly expressed in axenic cultures. Epigenetic modifications have been proved to be a powerful way to activate silent gene clusters in fungi. Deletion of _hdaA_, which encodes a histone deacetylase (HDAC), has led to the globally pleiotropic activation and overexpression of more than 75% of the biosynthetic genes and the isolation of diverse ten compounds of which four possess new structures, including three cassanes and a novel meroterpenoid derived from the esterification between an anthraquinone and a cyclohexene ring with a vinyl and a methyl groups located at C-13. So far, diterpenoids have exhibited diverse structural features and a wide range of interesting biological activities, such as cytotoxic, antimicrobial, anti-inflammatory, and antiviral properties, attracting more and more attention.

Careful analysis of the crude extract from the _C. arbuscula_ Δ_hdaA_ strain by UPLC–MS showed the presence of numerous minor ingredients that could not be identified due to sample limitations. To further explore the potential of the mutant strain Δ_hdaA_ and ongoing search for diverse new bioactive natural products, we fermented in a larger scale using potato dextrose agar (PDA) culture. Subsequently, investigation of the PDA fermentation extract resulted in the isolation of twelve new diterpenoids including three cassanes (1–3), one cleistanthene (4), six pimaranes (5–10), and two isopimaranes (11 and 12), together with two known cleistanthanes analogues (13 and 14, Fig. 1). Herein, details of the isolation, structure characterization, and bioactivities of 1–14 are reported.

2. Results and discussion

Compound 1 was isolated as a colorless amorphous solid with [α]_D_20^20 +30.0 (c 0.10, MeOH). The IR spectrum of 1 showed absorption bands for hydroxyl (3490 cm⁻¹), carbonyl (1724 cm⁻¹), and carbon–carbon double bonds moieties (1649 cm⁻¹). Its molecular formula was determined as C_20H_27O_5 by negative HR-ESI-MS at m/z 347.1864 [M−H]⁻ (Calcd. for C_20H_27O_5, 347.1864) combined with NMR spectroscopic data (Tables 1 and 2). The ¹H NMR spectrum of 1 showed signals attributable to a vinyl group [δ_H 6.25 (dd, J = 17.7 and 10.9 Hz, H-16), 5.09 (d, J = 17.7 Hz, H-17a), and 4.92 (d, J = 10.9 Hz, H-17b)], an olefinic proton [δ_H 5.66 (br s, H-12)], an oxygen-bearing methine [δ_H 4.34 (m, H-3)], a secondary methyl [δ_H 1.08 (d, J = 7.0 Hz, H-15)] and two tertiary methyl [δ_H 1.50 (s, H-18) and 0.89 (s, H-20)]. The ¹³C NMR and HSQC data revealed the presence of 20 carbons, including three methyls, four methylenes, four methines (including one O-methine), three sp³ quaternary carbons with one oxygenated, four olefinic carbons accounting for an olefin and a vinyl group, a carboxylic carbon (δ_C 175.1), and a ketone carbon (δ_C 211.7). These data accounted for all the NMR resonances of 1 and four of the seven unsaturation degrees, indicating 1 as a tricyclic compound.

Analysis of the ¹H–¹H COSY NMR data (Fig. 2) of 1 identified the C-1–C-3 moiety, the C-16–C-17 vinyl group, and the C-7/8/9/10/11 carbon. In the HMBC spectrum (Fig. 2) of 1, correlations from the olefinic proton H-16 to C-12, C-13, and C-14 indicated that C-13 was connected to C-12, C-14, and C-16, respectively, establishing a cyclohexene ring with a vinyl and a methyl groups located at C-13 and C-14, respectively. HMBC correlations from the methyl H_3-20 to C-1, C-5, C-9, and C-10 led to the connections of C-1, C-5, C-9, and C-10 to C-14, C-8, C-9, and C-10, respectively. The HMBC correlations of H_2-18/C-3, C-4, C-5, and C-19 and H_2-7/C-5 and C-6 located two hydroxy groups at C-3 and C-5, respectively, a carboxyl group at C-19, and a ketone group at C-6, establishing the tricyclic cassane type diterpene skeleton. Based on these data, the planar structure of 1 was established as shown in Fig. 1 which is closely resemble to sonomolide B and hawaiinolide F.

![Figure 1](image_url) Structures of compounds 1–14.
The relative configuration of 1 was deduced by analysis of NOESY spectrum. NOESY correlations (Fig. 3) of H-1-20 with H-8 and H-2 with H-1 indicated that these protons were in the β-orientation, whereas those of H-15 with H-9 suggested the α-axial orientation of the methyl at C-14. NOESY cross peaks of H-9 with H3-18, indicating the α-orientation for OH-5 and Me-18. Thus, the relative configuration of 1, which was very similar as those of hawaiinolides F and G, was established.

The absolute configuration of 1 was determined by analysis of its CD spectrum. The negative Cotton effect observed at 320 nm (Calcd. for C_{20}H_{29}O_5, 349.2020). The 1H NMR and 13C NMR spectroscopic data (Tables 1 and 2) showed the presence of two tertiary methyls, three quaternary carbons with one oxygenated, four olefinic carbons accounting for an exocyclic olefin, and one carboxylic carbon ([M−H]− (Calcd. for C_{20}H_{29}O_5, 333.2071)).

The 1H NMR and 13C NMR spectroscopic data (Tables 1 and 2) of 3 were similar to those of 2. The major difference was that signals for a methylene at δ_C 27.8 (C-6), δ_H 4.0 (J = 13.8, 2.4 Hz), and δ_H 3.9 (J = 10.2, 4.9 Hz), were replace resonances for an oxygenated methine at δ_C 69.8 (C-6) and δ_H 4.0 (br s, H-17). HMBC correlations from H2-6 to C-5, C-7, C-8, and C-10. HMBC correlations from H-1 (δ_C 3.57) to C-3, C-5, C-10, and C-20, suggested that C-1 was substituted by a hydroxy group. In addition, the relative configuration of 3 was deduced from NOESY correlations and by comparison to that of 2. NOESY correlations of H-20 with H-1 and H-8 indicated the β-orientation of OH-1, whereas those of OH-5 with H-18, OH-1, and H-9, and of H-15 with H-9 revealed that they were in the α-orientation. Thus, 3 was elucidated as 1α, 5α-dihydroxy-15α-cassane-12, 16-dien-19-oic acid and designated as calcarisporic acid C.

Compound 4, colorless needles with [α]_{D}^{20} +10.2 (c 0.17, MeOH), was determined to have the molecular formula of C_{20}H_{30}O_{5} by negative HR-ESI-MS at m/z 349.2030 [M−H]− (Calcd. for C_{20}H_{30}O_{5}, 349.2020) and NMR spectroscopic data. Comparing the 1D NMR spectra (Tables 1 and 2) of 4 with those of 1, signals due to an oxygenated methine were observed at δ_H 4.04 (br s, H-6) and δ_C 69.8 in the NMR spectra of 2, with the disappearance of the ketone carbon at δ_C 211.7 (C-6) in 1. The HMBC correlations from the oxygenated methine proton at δ_H 4.04 (br s, H-6) to C-4, C-5, C-7, C-8, and C-10, indicated the location of the hydroxyl group at C-6. According to NOESY correlations, the relative configuration of 2 was same as that of 1, except for the presence of correlation of H-6 with H-18, which suggested the β-configuration of OH-6. Therefore, 2 was established as 3α, 5α, 6β-trihydroxy-15α-cassane-12, 16-dien-19-oic acid and named calcarisporic acid B.

The molecular formula of compound 3 was determined as C_{20}H_{30}O_{5} on the basis of negative HR-ESI-MS at m/z 333.2083 [M−H]− (Calcd. for C_{20}H_{29}O_{5}, 333.2071).
group connected to C-13 and C-14, respectively, establishing a
cyclohexane of C ring moiety with an exocyclic ole
fin at C-13/C-17 and a vinyl group attached to C-13 fused to the B ring unit at C-8/C-9. Thus, the planar structure of 4 was established (Fig. 1), a tricyclic cleistanthane diterpene, which closely resembled that of zythiostromic acid A isolated from Zythiostroma sp. with [α]20
D −31.7 (c 0.35, MeOH). This suggested that the two compounds are diastereomers. Comparison of the chemical shifts and coupling constants in the 1H NMR spectra of 4 and zythiostromic acid A indicated difference only in the signals attribute to H-14 and H-15. This suggested that 4 was the C-14 epimer of zythiostromic acid A, which were confirmed by NOESY correlations (Fig. 3) of H-14 with H-9, and of H-8 with H-15 and H2-20, while other NOESY correlations of 4 were the same as those of zythiostromic acid A. Therefore, 4 was established as 3α, 5α, 6β-trihydroxy-15β-
cleistanthane-13(17), 15-dien-19-oic acid and named calcarisporic acid D.

Compound 5 was obtained as colorless needles. Its molecular formula was C20H30O5, as determined by negative HR-ESI-MS at m/z 349.2036 [M – H]. Analysis of its 1H and 13C NMR data (Tables 1 and 3) revealed the presence of three tertiary methyls, six methylenes, two oxygenated methines, four sp3 quaternary carbons with one oxygenated, four olefinic carbons accounting for a tetra-substituted olefin and a vinyl group, and a carboxylic carbon (δC 178.7). Comparison of the NMR spectroscopic data of 5 with those of 2 suggested that 5 possessed the same A ring moiety as 2. Interpretation of the 1H–1H COSY NMR data (Fig. 2) of 5 identified the C-6–C-7 moiety, the C-11–C-12 unit, and the C-15–C-16 vinyl group. HMBC correlations (Fig. 2) from H-6 to C-4, C-5, and C-10 suggested that

| No. | 1              | 2              | 3              | 4              |
|-----|----------------|----------------|----------------|----------------|
| 1   | 1.94, overlap  | 1.70, td (13.8, 3.5) | 3.57, d (3.0) | 1.66, overlap  |
| 2   | 1.59, m       | 1.14, d (12.5)  | 2.10, overlap  | 1.98, overlap  |
| 3   | 2.31, m       | 1.98, overlap   | 1.56, d (11.6) | 1.64, overlap  |
| 4   | 2.03, overlap  | 1.5, m         | 1.72, td (13.7, 4.0) | 1.5, m      |
| 5   | 4.34, br d (5.5) | 4.22, br s     | 1.72, td (13.8, 4.0) | 1.5, m      |
| 6   | 4.04, br s    | 2.03, td (13.8, 2.4) | 5.21, br s    | 3.92, br s    |
| 7   | 3.14, t (12.9) | 2.11, m       | 1.87, overlap  | 1.62, overlap  |
| 8   | 1.94, overlap  | 1.77, m       | 1.52, overlap  | 1.41, m       |
| 9   | 2.71, m       | 2.09, m       | 2.56, m       | 1.72, overlap  |
| 10  | 2.22, m       | 2.01, overlap  | 2.12, overlap  | 1.70, overlap  |
| 11  | 2.02, overlap  | 1.95, overlap  | 1.84, overlap  | 1.00, qd (13.0, 3.6) |
| 12  | 5.66, br s    | 5.63, t (3.5)  | 5.63, t (3.8)  | 2.35, d (12.9) |
| 13  | 2.47, m       | 2.32, m       | 2.38, m       | 2.25, t (9.8)  |
| 14  | 1.50, d (7.0) | 0.87, d (6.8) | 0.92, d (6.9) | 5.62, ddd (17.2, 9.8, 9.8) |
| 15  | 6.25, dd (17.7, 10.9) | 6.20, dd (17.6, 10.9) | 6.20, dd (17.6, 10.9) | 5.13, dd (10.2, 2.0) |
| 16  | 5.09, d (17.7) | 5.08, d (17.6) | 5.07, d (17.6) | 4.64, d (1.1)  |
| 17  | 4.92, d (10.9) | 4.90, d (10.9) | 4.89, d (10.9) | 4.48, br s     |
| 18  | 1.50, s       | 1.36, s       | 1.12, s       | 1.32, s       |
| 19  | 0.89, s       | 0.96, s       | 0.70, s       | 0.91, s       |
| 1-OH |            |               | 5.50, d (4.0) |               |
| 3-OH |            | 6.23, br s    | 6.23, d (2.5) |               |
| 5-OH |            | 6.10, br s    | 5.15, s       | 6.20, br s    |
| 6-OH |            |               | 7.21, br s    |               |
| 19-OH |           | 12.11, s      | 14.32, br s   |               |

*aNMR data (δ) were measured at 500 MHz in CD3OD for 1 and at 600 MHz in DMSO-d6 for 2–4.

Figure 2 1H–1H COSY and key HMBC correlations of compounds 1, 4, 5 and 10.
Compound 7 was assigned the molecular formula C_{20}H_{29}O_{4} by negative HR-ESI-MS at m/z 333.2077 [M–H]⁻ (Calcd. for C_{20}H_{29}O_{4}, 333.2071). One less oxygen than that of 5. Comparison of the NMR data of 7 with those of 5 indicated that an α-methine [δ_H 4.10, δ_C 73.7] at C-3 in 5 was replaced by a methylene [δ_H 1.83 and 1.74, δ_C 32.8] in 7. This suggested that 7 was the 3-dehydroxy analog of 5, which was confirmed by HMBC correlations from H-2 to C-1, C-2, C-4, and C-5. NOESY correlations of H-11β (δ_H 1.94) with H-20 and H-15, of H-14 with H-17 and H-12α (δ_H 1.43), and of H-18 with H-3 (δ_H 1.83), H-3β (δ_H 1.74), and H-6α (δ_H 1.93) indicated that the relative configuration of 7 was similar as that of 5. Thus, 7 was established as 5α,14β-dihydroxy-pimarane-8(9), 15-dien-19-oic acid and named calcarisporic acid G.

Compound 8 was determined to have the molecular formula C_{20}H_{30}O_{4} by negative HR-ESI-MS at m/z 347.2239 [M–H]⁻ (Calcd. for C_{20}H_{30}O_{4}, 347.2228). The NMR data (Tables 1 and 3) of 8 were closely similar to those of 7. The major differences were that an additional methoxy group was presented in 8, and the resonance for C-14 was downfield-shifted by 11.4 ppm in comparison with 7. This indicated that 8 was the 14-methoxy derivative of 7, which was verified by HMBC correlations from H-14 to the additional methoxy group (δ_C 62.1), C-8, C-9, C-13, and C-15. The relative configuration of 8 was identical with that of 7 according to NOESY spectrum. Consequently, 8 was elucidated as 5α-hydroxy-14β-methoxy-pimarane-8(9), 15-dien-19-oic acid and designated as calcarisporic acid H.

The molecular formula of compound 9 was deduced to be C_{20}H_{30}O_{4} by negative HR-ESI-MS at m/z 333.2077 [M–H]⁻ (Calcd. for C_{20}H_{30}O_{4}, 333.2071). The ¹H and ¹³C NMR spectra (Tables 1 and 4) of 9 showed close resemblance to those of 5, with the exception of the signals assigned to C-5, where the oxygenated quaternary carbon (δ_C 80.5) in 5 was replaced by a methine (δ_H 1.79, δ_C 46.5) in 9. This suggested that 9 was the 5-dehydroxy analog of 5, which was confirmed by HMBC correlations from H-5 to δ_C 179 (δ_H 1.79) to C-1, C-4, C-6, C-10, C-18, C-19, and C-20. NOESY correlations of H-15 with H-5 (δ_H 1.79) and H-3, of H-11β (δ_H 1.92) with H-20 and H-15, and of H-14 with H-17 and H-12α (δ_H 1.43) indicated that the relative configuration of 9 was in accordance with that of 5. Therefore, 9 was elucidated as 3α,14β-dihydroxy-pimarane-8(9), 15-dien-19-oic acid and designated as calcarisporic acid I.

Compound 10 was assigned the molecular formula C_{20}H_{32}O_{4} by negative HR-ESI-MS at m/z 333.2069 [M–H]⁻ (Calcd. for C_{20}H_{32}O_{4}, 333.2071). The ¹H and ¹³C NMR data (Tables 1 and 4) showed the presence of three tertiary methyls, seven methylenes, five sp³ quaternary carbons with two oxygenated, four olefinic carbons accounting for a trisubstituted olefin and a vinyl group, and a carbonylic carbon (δ_C 180.2). Comparison of the NMR spectroscopic data of 10 with those of 7 suggested that 10 possessed the same A ring unit as 7. Analysis of the ¹H–¹H COSY spectrum (Fig. 2) of 10 revealed the C-6–C-7 moiety, the C-11–C-12 unit, and the C-16–C-17 vinyl group. HMBC
Table 3  

| No. | 5      | 6      | 7      | 8      |
|-----|--------|--------|--------|--------|
| 1   | 1.97, overlap | 1.96, overlap | 1.78, dd (12.9, 4.1) | 1.77, overlap |
| 2   | 2.31, m | 2.30, m | 1.97, overlap | 1.96, overlap |
| 3   | 4.10, br s | 4.07, br s | 1.83, d (12.7) | 1.83, d (12.8) |
| 6   | 2.48, m | 2.47, m | 2.94, m | 2.50, m |
| 7   | 2.55, m | 2.51, m | 2.94, m | 2.51, m |
| 11  | 2.02, overlap | 1.99, overlap | 1.94, overlap | 1.92, overlap |
| 12  | 1.67, m | 1.59, m | 1.66, m | 1.58, m |
| 14  | 3.47, br s | 3.24, br s | 3.48, br s | 3.24, br s |
| 15  | 5.76, dd (17.7, 11.0) | 5.80, dd (17.7, 11.0) | 5.77, dd (17.7, 11.0) | 5.80, dd (17.7, 11.0) |
| 16  | 4.96, bd (17.7) | 4.99, dd (17.7, 1.4) | 4.96, dd (17.7, 1.4) | 4.99, dd (17.7, 1.0) |
| 17  | 1.01, s | 1.05, s | 1.01, s | 1.05, s |
| 18  | 1.46, s | 1.45, s | 1.23, s | 1.24, s |
| 20  | 1.01, s | 1.01, s | 1.00, s | 1.00, s |
| OMe| 3.51, s | 3.51, s | 3.52, s | 3.52, s |

*NMR data (δ) were measured at 500 MHz in CD3OD for 5, 6, and 8, and at 600 MHz in CD3OD for 7.

A detailed comparison of the NMR spectroscopic data (Tables 1 and 4) of 12 with those of 7 revealed that they possessed the identical planar structure, while weak differences were attributed to C-12, C-14, C-15, and C-17, suggesting that the two compounds were diastereomers. This was further verified by NOESY correlations of H-11/β (δH 2.02) with H2-20 and H17-1, and of H-14 (δH 3.30) with H17-1. Thus, 12 was elucidated as 5α,14α-di-OH-pimarane-8(14), 15-dien-19-oic acid and designated as calcarisporic acid L.

The known compounds were identified as hawaiinolide G13 (13) and 14-epi-zythiostromic acid B14 (14), respectively, by comparing their NMR spectroscopic data with those reported in literatures.

It was worth mentioning that the pimarane and isopimarane type diterpenes (5–12) were isolated from the genus Calcarisporium for the first time. This discovery further supported the proposed pathway for the biosynthesis of these diterpenes (Scheme 1), where they could be synthesized from the same diterpene biosynthetic gene cluster, and the cassane and cleistanthane type diterpenoids both derived from the (iso) pimaranes, in which migration of either the C-13 methyl or vinyl group to C-14 led to the cassane or cleistanthane type diterpenoids5,24,25. Subsequently, three types of diterpenoids skeleton were heavily oxidized to form the characteristic diterpenoids above, encouraging us to search more diverse structures in this mutant strain.

Cytotoxic activities of compounds 1–14 were evaluated against two human cancer cell lines (MCF-7 and HepG-2) in the MTT assay, with taxol as a positive control (IC50 5.0 ± 0.4 nmol/L, respectively). None of them exhibited significant activities in the concentration range of 10−5−10−7 mol/L.

The matrix metalloproteinases (MMPs) family can degrade various protein components in the extracellular matrix and plays a key role in tumor invasion and metastasis26,27. Therefore, we tested the inhibitory effects of the isolated compounds on the expression of
MMPI and MMP2 in human breast cancer (MCF-7) cells by Western blot assay. Due to the sample limitation, compounds 2, 4, 6, and 7 were evaluated for this inhibitory activity firstly. Experimental results (Fig. 5) indicated that the four tested compounds had no significant inhibitory effects on the viability of MCF-7 cells, but the expression level of MMPI and MMP2 could be affected obviously. Western blot showed that both 2 and 4 could reduce MMP2 expression significantly at 200 μmol/L for 24 h with significant difference from that in DMSO control group. Meanwhile, 4 could also reduce MMPI expression significantly. This indicated that the activities of 2 and 4 may be associated with expression inhibition of MMP proteinases family, and although 2 and 4 exhibited no cytotoxicities against tested tumor cell lines, they could function in suppressing tumor invasion and metastasis and possesses potential antitumor value. Comparing the structure of 4 with that of 2, the major difference was that a vinyl group at C-14 in 4 and a methyl group at C-14 in 2 were observed. Both 4 and 2 possessed an unsaturated unit connected to C-13. These suggested that the vinyl group attached to C-14 contributed as a functional group and the substituted group at C-14 may play an important role in the inhibitory effect on the expression level of MMPI and MMP2.

In addition, compounds 1–14 were assessed for their anti-inflammatory inhibitory activities against the lipopolysaccharide (LPS)-induced NO production in murine microglial BV2 cells as well as their antioxidant activities against rat liver microsomal lipid peroxidation induced by Fe⁴⁺-cystine in vitro. All compounds were inactive for anti-inflammatory and antioxidant activities at the concentrations of 10⁻⁵ and 10⁻⁶ mol/L, respectively.

3. Conclusions

Twelve new diterpenoids, calcarisporic acids A–L, including three cassanes (1–3), one cleistanthane (4), six pimaranes (5–10), and two isopimaranes (11 and 12) along with two know cleistanthanes analogues (13 and 14), were isolated from the mutant strain ΔhdaA of Calcarisporium arbuscula, in which an encoding histone deacetylase gene (and hdaA) was deleted. Compounds 5–12 are the first examples of pimarane and isopimarane type diterpenes isolated from the genus Calcarisporium. In addition, the inhibitory effects of four compounds (2, 4, 6 and 7) on the expression of MMPI and MMP2 in MCF-7 cells were evaluated. Compounds 2 and 4 significantly inhibited the expression level of MMPI and MMP2, and are promising as a potential anti-metastatic agent for the treatment of human breast cancer. Our results further present the potential of genome mining-guided discovery of bioactive natural products in fungi.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured on an Autopol IV automatic polarimeter (Rudolph Research Co.). UV spectra were measured on a JASCO V-650 spectrophotometer. CD spectra were measured on a JASCO J-815 spectrometer. IR spectra were recorded on a Nicolet 5700 FT-IR spectrometer using a FT-IR microscope transmission method. NMR spectra were recorded at 600 or 500 MHz for 1H NMR and 150 or 125 MHz for 13C NMR, respectively, on a Bruker AVIIIHD 500 (Bruker Corp., Karlsruhe, Germany) or a VNS-600 or an Inova 500 instrument (Varian Associates Inc., Palo Alto, CA, USA) in CD3OD or DMSO-d6 with solvent peaks used as references at 25 °C. ESI-MS and analytical HPLC were performed on a Waters ACQUITY H-Class UPLC–MS with QDA mass detector (ACQUITY UPLC® BEH, 1.7 μm, 50 mm × 2.1 mm, C18 column) using positive and negative mode electrospray ionization. HR-ESI-MS data were taken on an Agilent 6520 Accurate-Mass Q-TOF LC/MS spectrometer (Agilent Technologies, Ltd., Santa Clara, CA, USA).

| No. | 9 | 10 | 11 | 12 |
|-----|---|----|----|----|
| 1   | 1.56, overlap | 1.61, dd (13.6, 3.7) | 1.54, overlap | 1.76, overlap |
|     | 1.52, overlap | 1.31, br d (13.6) |               | 1.45, overlap |
| 2   | 2.17, m | 1.99, dd (13.5, 3.8) | 2.19, m | 1.98, overlap |
|     | 1.63, m | 1.48, overlap | 1.63, dd (14.4, 3.0) | 1.45, overlap |
| 3   | 4.00, t (2.50) | 1.88, d (14.0) | 4.01, br s | 1.85, d (13.2) |
|     |               | 1.52, overlap |               | 1.69, m |
| 5   | 1.79, d (12.2) |               | 1.79, dd (9.3, 3.9) |               |
| 6   | 1.90, overlap | 2.54, td (13.9, 6.0) | 2.02, overlap | 2.45, overlap |
|     | 1.84, td (12.1, 5.3) | 1.92, dd (14.7, 5.2) | 1.90, overlap | 1.95, overlap |
| 7   | 2.34, m | 2.32, tdd (13.5, 5.8, 2.2) | 2.47, br d (13.5) | 2.43, overlap |
|     | 1.95, dd (18.0, 4.5) | 2.22, dd (15.5, 4.9) | 1.86, overlapped | 2.08, overlap |
| 11  | 2.07, m | 1.71, m | 2.03, overlap | 2.02, overlap |
|     | 1.92, overlap | 1.50, overlap | 1.91, overlap | 1.93, overlap |
| 12  | 1.57, overlap | 1.59, overlap | 1.73, m | 1.74, m |
| 14  | 3.63, br s | 5.37, br s | 3.22, br s | 3.30, br s |
| 15  | 5.86, dd (17.7, 10.9) | 5.70, dd (17.4, 10.5) | 6.00, dd (18.0, 10.5) | 6.01, dd (17.4, 10.4) |
| 16  | 4.98, dd (17.7, 1.1) | 4.96, dd (10.5, 1.7) | 5.00, dd (18.0, 1.0) | 5.01, dd (17.4, 1.5) |
|     | 4.94, dd (10.9, 1.1) | 4.91, dd (17.4, 1.7) | 4.99, dd (10.5, 1.0) | 5.00, dd (10.4, 1.5) |
| 17  | 0.98, s | 1.03, s | 0.89, s | 0.92, s |
| 18  | 1.27, s | 1.28, s | 1.28, s | 1.23, s |
| 20  | 0.92, s | 0.95, s | 0.96, s | 1.06, s |

a) NMR data (δ) were measured at 500 MHz in CD3OD for 9–12.
Semi-preparative HPLC was performed on a LabAlliance Series III pump equipped with a LabAlliance model 201 UV detector, using YMC-Pack ODS-A column (250 mm × 10 mm, 5 μm). Column chromatography (CC) was carried out on silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden), or reversed phase C18 silica gel (50 μm, YMC, Kyoto, Japan). TLC was carried out on glass precoated silica gel GF254 plates (Qingdao Marine Chemical Co., Ltd., China). Spots were visualized under UV light or by spraying with 10% H2SO4 in 95% EtOH followed by heating.

4.2. Fungal material

Fungus Calcarisporium arbuscula NRRL 3705 (ATCC® 46034™) is the wild type strain. The ΔhdaA mutants were constructed by replacement of hdaA by hph (Hygromycin resistance gene). The mutant strains were grown on 20 L of solid PDA media (BD) divided into 200 large 150 × 15 mm2 Petri dishes at room temperature for 40 days.

4.3. Extraction and isolation

The fermented material was extracted repeatedly with EtOAc (3 × 5.0 L), and the organic solvent was evaporated to dryness under vacuum to afford the crude extract (14.2 g), which was partitioned between MeOH and hexane. The MeOH fraction (13.5 g) was evaporated to dryness under vacuum, and separated by reversed phase C18 silica gel vacuum liquid chromatography with a gradient of MeOH (20%–100%) in H2O to give eleven fractions (F1–F11).

Scheme 1 Proposed biosynthetic pathways for compounds 1–14.

Figure 5 Effects of compounds 2, 4, 6, and 7 on the expressions of MMP1 and MMP2 in MCF-7 cells. (A) Cells were treated with different test compounds at 200 μmol/L or DMSO as control for 24 h. The expressions of MMP1 and MMP2 were determined by Western blot. Representative immunoblots are shown and GAPDH is the loading control. (B) Relative expression level of MMP1 is calibrated by GAPDH. (C) Relative expression level of MMP2 is calibrated by GAPDH. Each value represents the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01 versus DMSO control.
4.3.1. Calcarisporic acid A (1)
Colorless amorphous solid; \([\alpha]_D^{20} +30.0 (c 0.10, \text{MeOH})\); UV (MeOH) \(\lambda_{\text{max}}^{\text{logg}}\) (4.68 nm) CD (c 1.4 × 10\(^{-3}\) mol/L, MeOH) \(\lambda_{\text{max}} (\Delta\epsilon)\) 240 (−0.29), 288 (+0.13), 320 (−0.12) nm; IR \(v_{\text{max}}\) 3490, 3086, 2930, 1809, 1724, 1649, 1603, 1457, 1391, 1223, 1099, 994, 897, 852, 760 cm\(^{-1}\); \(^1\)H NMR (CD\(_3\)OD, 500 MHz) data see Table 1; ESI-MS \(m/z\) 347 [M−H]\(^+\); HR-ESI-MS: \(m/z\) 347.1864 [M−H]\(^+\) (Calcld. for C\(_{21}\)H\(_{29}\)O\(_4\), 347.1864).

4.3.2. Calcarisporic acid B (2)
Colorless granules (MeOH); \([\alpha]_D^{20} +51.7 (c 0.19, \text{MeOH})\); UV (MeOH) \(\lambda_{\text{max}}^{\text{logg}}\) (231 (4.96) nm; IR \(v_{\text{max}}\) 3307, 3198, 2938, 2612, 1694, 1652, 1602, 1516, 1468, 1436, 1276, 1081, 1004, 955, 893, 769, 732, 592 cm\(^{-1}\); \(^1\)H NMR (DMSO-d\(_6\), 600 MHz) data see Table 2, \(^{13}\)C NMR (DMSO-d\(_6\)), 150 MHz) data see Table 1; ESI-MS \(m/z\) 349 [M−H]\(^+\); HR-ESI-MS: \(m/z\) 349.2030 [M−H]\(^+\) (Calcld. for C\(_{20}\)H\(_{28}\)O\(_4\), 349.2030).

4.3.3. Calcarisporic acid C (3)
Colorless amorphous solid; \([\alpha]_D^{20} +83.3 (c 0.19, \text{MeOH})\); UV (MeOH) \(\lambda_{\text{max}}^{\text{logg}}\) (231 (4.81) nm; IR \(v_{\text{max}}\) 3309, 3183, 2974, 2930, 1688, 1652, 1606, 1462, 1271, 1117, 962, 949, 929, 893, 798, 711, 647, 535 cm\(^{-1}\); \(^1\)H NMR (DMSO-d\(_6\), 600 MHz) data see Table 2, \(^{13}\)C NMR (DMSO-d\(_6\)), 150 MHz) data see Table 1; ESI-MS \(m/z\) 333 [M−H]\(^+\); HR-ESI-MS: \(m/z\) 333.2083 [M−H]\(^+\) (Calcld. for C\(_{20}\)H\(_{28}\)O\(_4\), 333.2071).

4.3.4. Calcarisporic acid D (4)
Colorless needles (MeOH); \([\alpha]_D^{20} +10.2 (c 0.17, \text{MeOH})\); UV (MeOH) \(\lambda_{\text{max}}^{\text{logg}}\) (204 (4.50) nm; IR \(v_{\text{max}}\) 3456, 3367, 3073, 2965, 2320, 1694, 1645, 1509, 1465, 1421, 1297, 1166, 1003, 965, 918, 771, 725, 608, 568 cm\(^{-1}\); \(^1\)H NMR (DMSO-d\(_6\), 600 MHz) data see Table 2, \(^{13}\)C NMR (DMSO-d\(_6\)), 150 MHz) data see Table 1; ESI-MS \(m/z\) 349 [M−H]\(^+\); HR-ESI-MS: \(m/z\) 349.2031 [M−H]\(^+\) (Calcld. for C\(_{20}\)H\(_{28}\)O\(_4\), 349.2030).

4.3.5. Calcarisporic acid E (5)
Colorless amorphous solid; \([\alpha]_D^{20} =-12.0 (c 0.20, \text{MeOH})\); UV (MeOH) \(\lambda_{\text{max}}^{\text{logg}}\) (206 (4.37) nm; IR \(v_{\text{max}}\) 3339, 3080, 2935, 1701, 1638, 1460, 1380, 1267, 1009, 948, 912, 855, 706, 672 cm\(^{-1}\); \(^1\)H NMR (CD\(_3\)OD, 500 MHz) data see Table 3, \(^{13}\)C NMR (CD\(_3\)OD, 150 MHz) data see Table 1; ESI-MS \(m/z\) 349 [M−H]\(^+\); HR-ESI-MS: \(m/z\) 349.2036 [M−H]\(^+\) (Calcld. for C\(_{20}\)H\(_{28}\)O\(_4\), 349.2030).

4.3.6. Calcarisporic acid F (6)
Colorless amorphous solid; \([\alpha]_D^{20} =-21.0 (c 0.13, \text{MeOH})\); UV (MeOH) \(\lambda_{\text{max}}^{\text{logg}}\) (204 (4.58) nm; IR \(v_{\text{max}}\) 3495, 3274, 3079, 2934, 1701, 1635, 1461, 1383, 1238, 1089, 1005, 939, 907, 864, 690, 665 cm\(^{-1}\); \(^1\)H NMR (CD\(_3\)OD, 500 MHz) data see Table 3, \(^{13}\)C NMR (CD\(_3\)OD, 125 MHz) data see Table 1; ESI-MS \(m/z\) 363 [M−H]\(^+\); HR-ESI-MS: \(m/z\) 363.2178 [M−H]\(^+\) (Calcld. for C\(_{21}\)H\(_{31}\)O\(_4\), 363.2177).

4.3.7. Calcarisporic acid G (7)
Colorless needles (MeOH); \([\alpha]_D^{20} +16.0 (c 0.20, \text{MeOH})\); UV (MeOH) \(\lambda_{\text{max}}^{\text{logg}}\) (204 (4.47) nm; IR \(v_{\text{max}}\) 3544, 3353, 2934, 1686, 1621, 1467, 1433, 1377, 1280, 1255, 1065, 941, 914, 800, 675 cm\(^{-1}\); \(^1\)H NMR (CD\(_3\)OD, 600 MHz) data see Table 3, \(^{13}\)C NMR (CD\(_3\)OD, 150 MHz) data see Table 1; ESI-MS \(m/z\) 347 [M−H]\(^+\); HR-ESI-MS: \(m/z\) 347.2239 [M−H]\(^+\) (Calcld. for C\(_{21}\)H\(_{31}\)O\(_4\), 347.2228).

4.3.8. Calcarisporic acid H (8)
Colorless amorphous solid; \([\alpha]_D^{20} +12.0 (c 0.15, \text{MeOH})\); UV (MeOH) \(\lambda_{\text{max}}^{\text{logg}}\) (205 (4.51) nm; IR \(v_{\text{max}}\) 3570, 3147, 2937, 1724, 1638, 1606, 1458, 1383, 1274, 1200, 1073, 973, 937, 854, 796, 675 cm\(^{-1}\); \(^1\)H NMR (CD\(_3\)OD, 500 MHz) data see Table 3, \(^{13}\)C NMR (CD\(_3\)OD, 125 MHz) data see Table 1; ESI-MS \(m/z\) 347 [M−H]\(^+\); HR-ESI-MS: \(m/z\) 347.2239 [M−H]\(^+\) (Calcld. for C\(_{21}\)H\(_{31}\)O\(_4\), 347.2228).
4.3.9. Calcarisporic acid I (9)
Colorless amorphous solid; $[\alpha]_D^{20} +56.9$ (c 0.19, MeOH); UV (MeOH) $\varepsilon_{\max}$ (log ε) 206 (4.50) nm; IR $\varepsilon_{\max}$ 3427, 2970, 1687, 1639, 1537, 1425, 1377, 1254, 1146, 1090, 999, 917, 882, 802, 698 cm$^{-1}$; $^1$H NMR (CD$_3$OD, 125 MHz) data see Table 4; $^{13}$C NMR (CD$_3$OD, 500 MHz) data see Table 4; HR-ESI-MS: m/z 333 [M–H]$^-$; HR-ESI-MS: m/z 333.2077 [M–H]$^-$ (Calcd. for C$_{20}$H$_{29}$O$_4$, 333.2071).

4.3.10. Calcarisporic acid J (10)
Colorless amorphous solid; $[\alpha]_D^{20} +103.9$ (c 0.22, MeOH); UV (MeOH) $\varepsilon_{\max}$ (log ε) 205 (4.41) nm; IR $\varepsilon_{\max}$ 3382, 3080, 2905, 2871, 1693, 1455, 1413, 1378, 1357, 1199, 1090, 999, 917, 882, 802, 698, 621 cm$^{-1}$; $^1$H NMR (CD$_3$OD, 125 MHz) data see Table 4; $^{13}$C NMR (CD$_3$OD, 125 MHz) data see Table 1; ESI-MS m/z 333 [M–H]$^-$; HR-ESI-MS: m/z 333.2069 [M–H]$^-$ (Calcd. for C$_{20}$H$_{29}$O$_4$, 333.2071).

4.3.11. Calcarisporic acid K (11)
Colorless amorphous solid; $[\alpha]_D^{20} +85.9$ (c 0.16, MeOH); UV (MeOH) $\varepsilon_{\max}$ (log ε) 204 (4.52) nm; IR $\varepsilon_{\max}$ 3498, 2930, 2865, 1707, 1639, 1458, 1391, 1308, 1278, 1002, 981, 917, 798, 685 cm$^{-1}$; $^1$H NMR (CD$_3$OD, 500 MHz) data see Table 4; $^{13}$C NMR (CD$_3$OD, 125 MHz) data see Table 1; ESI-MS m/z 333 [M–H]$^-$; HR-ESI-MS: m/z 333.2077 [M–H]$^-$ (Calcd. for C$_{20}$H$_{29}$O$_4$, 333.2071).

4.3.12. Calcarisporic acid L (12)
Colorless amorphous solid; $[\alpha]_D^{20} +126.9$ (c 0.12, MeOH); UV (MeOH) $\varepsilon_{\max}$ (log ε) 204 (4.59) nm; IR $\varepsilon_{\max}$ 3498, 2930, 2865, 1707, 1639, 1458, 1391, 1308, 1278, 1002, 981, 917, 798, 685 cm$^{-1}$; $^1$H NMR (CD$_3$OD, 500 MHz) data see Table 4; $^{13}$C NMR (CD$_3$OD, 125 MHz) data see Table 1; ESI-MS m/z 333 [M–H]$^-$; HR-ESI-MS: m/z 333.2084 [M–H]$^-$ (Calcd. for C$_{20}$H$_{29}$O$_4$, 333.2071).

4.4. Cytotoxicity assay
Compounds 1–14 were tested for cytotoxic activities against MCF-7 (human breast cancer) and HepG-2 (human liver cancer) cell lines by the MTT method, as described in the literature.

4.5. Inhibitory effects on the expression of MMP1 and MMP2 in MCF-7 cells
Compounds 2, 4, 6, and 7 were tested for the inhibitory effects on the expression of MMP1 and MMP2 in human breast carcinoma cell line MCF-7 by Western blot. MCF-7 cells were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science, and was cultured in DMEM medium (Gibco, Invitrogen Corporation) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL benzyll penicillin G, and 100 mg/L streptomycin under a humidified atmosphere of 5% CO$_2$ at 37 °C for 24 h. MCF-7 cells were treated with different test compounds at 200 μmol/L or DMSO as control for 24 h. Then, cells were collected and lysed. The lysates were clarified by 12,000 × g centrifugation at 4 °C for 30 min. Total proteins were quantified with a microplate spectrophotometer using the bicinchoninic acid assay (BCA assay). Then equal amount of proteins were separated by SDS-polyacrylamide gel (SDS-PAGE) and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The blots were incubated with primary antibodies overnight at 4 °C, followed by second antibodies at the room temperature for 0.5 h. Immuno-reactive protein bands were detected by the ECL detection system (Amersham Bioscience, Piscataway, NJ, USA).

4.6. Anti-inflammatory activity assay
Compounds 1–14 were assessed for their anti-inflammatory inhibitory activities against the lipopolysaccharide (LPS) induced NO production in murine microglial BV2 cell line using the Griess method, as described in the literature. Curcumin was used as the positive control (inhibitory rate 66.0 ± 2.5% at 10$^{-3}$ mol/L).

4.7. Antioxidant assay
Compounds 1–14 were assessed for their antioxidant activities against rat liver microsomal lipid peroxidation induced by Fe$^{3+}$-cystine in vitro, as described in the literature. Curcumin was used as the positive control (inhibitory rate 95.0 ± 3.5% at 10$^{-3}$ mol/L).

Acknowledgments
This work was supported financially by National Natural Science Foundation of China (Nos. 21502233 and 81522043), CAMS Initiative for Innovative Medicine (CAMS-I2M-I-010), the PUMC Youth Fund (33320140175), and the State Key Laboratory Foundation for Excellent Young Scientists to Youcai Hu (GTZB201401). We are grateful to the Department of Instrumental Analysis, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College for the spectroscopic measurements and Prof. Xiaoguang Chen and Prof. Dan Zhang for the cytotoxicity, anti-inflammatory, and antioxidant assays.

Appendix A. Supplementary material
Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.apsb.2017.12.012.

References
1. Watson P. Calcarisporium arbuscula living as an endophyte in apparently healthy sporophores of Russula and Lactarius. Trans Br Mycol Soc 1955;38:409–14.
2. Osselton MD, Baum H, Beechey RB. Isolation, purification and characterization of aurovertin B. Biochem Soc Trans 1974;2:200–2.
3. van Raaij MJ, Abrahams JP, Leslie AG, Walker JE. The structure of bovine F1-ATPase complexed with the antibiotic inhibitor aurovertin. J Chem Soc Chem Commun 1974;409:–7.
4. Mulheim LJ, Beechey RB, Leworthy DP, Osselton MD. Aurovertin B, a metabolite of Calcarisporium arbuscula. J Chem Soc Chem Commun 1974;21:874–6.
5. Mao XM, Xu W, Li D, Yin WB, Chooi YH, Li YQ, et al. Epigenetic genome mining of an endophytic fungus leads to the pleiotropic characterization of aurovertin. Angew Chem Int Ed 2015;54:7592–6.
6. Cichewicz RH. Epigene manipulation as a pathway to new natural products. Angew Chem Int Ed 2015;54:7592–6.
8. Wu G, Zhou H, Zhang P, Wang X, Li W, Zhang W, et al. Polyketide production of pestaloficiols and macrodilidi ficiolides revealed by manipulations of epigenetic regulators in an endophytic fungus. *Org Lett* 2016;18:1832–5.

9. Fan A, Mi W, Liu Z, Zeng G, Zhang P, Hu Y, et al. Deletion of a histone acetyltransferase leads to the pleiotropic activation of natural products in *Metastizium robertsii*. *Org Lett* 2017;19:1686–9.

10. Zheng Y, Ma K, Lyu H, Huang Y, Liu H, Liu L, et al. Genetic manipulation of the COP9 signalosome subunit PICciE leads to the discovery of pestaloficiols in *Pestalotiopsis fici*. *Org Lett* 2017;19:4700–3.

11. Li MH, Li QQ, Zhang CH, Zhang N, Cui ZH, Huang LQ, et al. An isopimarane diterpenoid from the stems of *Euonymus oblongifolius*. *Phytochemistry* 2017;135:144–50.

12. Liu N, Wang S, Lou HX. A new pimarane-type diterpenoid from moss *Pseudoleskeella papillosa* (Lindb.) Kindb. *Acta Pharm Sin B* 2013;4:273–80.

13. Chen S, Zhang Y, Niu S, Liu X, Che Y. Cytotoxic cleistanthane and cassane diterpenoids from the entomogenous fungus *Ceratotheca ceae* in China. *Acta Pharm Sin B* 2013;4:256–9.

14. Chen S, Zhang Y, Niu S, Liu X, Che Y. Cytotoxic cleistanthane and cassane diterpenoids from the entomogenous fungus *Pseudoleskeella papillosa* (Lindb.) Kindb. *Acta Pharm Sin B* 2012;3:256–9.

15. Li F, Ma J, Li CJ, Yang JZ, Zhang D, Chen XG, et al. Bioactive sesquiterpenes and macrodiolide cations and macrodiolide cations and macrodiolide cations and macrodiolide cations from the roots of *Paraconiothyris gerrardii*. *Fitoterapia* 2014;99:236–42.

16. Shiono Yoshihito, Ogata Kota, Koseki Takuya, Murayama Tetsuya, Funakoshi T. A cleistanthane diterpene from a marine-derived *Daphnane diterpenoids* from the roots of *Illicium dunnianum*. *Phytochemistry* 2010;71:711–7.

17. Park M, Han J, Lee CS, Soo BH, Lim KM, Ha H. Carnosic acid, a phenolic diterpene from rosemary, prevents UV-induced expression of matrix metalloproteinases in human skin fibroblasts and keratinocytes. *Exp Dermatol* 2013;22:336–41.

18. Ma S-G, Tang W-Z, Liu Y-X, Hu Y-C, Yu S-S, Zhang Y, et al. Prenylated C6-C3 compounds with molecular diversity from the roots of *Illicium oligandrum*. *Phytochemistry* 2011;72:115–25.

19. Zhang L, Luo RH, Wang F, Jiang MY, Dong ZJ, Yang LM, et al. Highly functionalized daphnane diterpenoids from *Trigonostemon thyroides*. *Org Lett* 2010;12:1525–8.

20. Zhang L, Luo RH, Wang F, Dong ZJ, Yang LM, Zheng YT, et al. Daphne diterpenoids isolated from *Trigonostemon thyroides* as HIV-1 antivirals. *Phytochemistry* 2010;71:1879–83.

21. Kirk DN. The chiroptical properties of carbonyl compounds. *Tetrahedron* 1986;42:777–818.

22. Bai J, Chen H, Fang ZF, Yu SS, Wang WJ, Liu Y, et al. Sesquiterpenes from the roots of *Illicium dunnianum*. *Phytochemistry* 2012;80:137–47.

23. Ayer WA, Khan AQ. Zythiostromic acids, diterpenoids from an antifungal *Zythiostroma* species associated with aspen. *Phytochemistry* 1996;42:1647–52.

24. Peters RJ. Two rings in them all: the labdane-related diterpenoids. *Nat Prod Rep* 2010;27:1521–30.

25. Gao Y, Houzatko KB, Peters RJ. Terpenoid synthase structures: a so far incomplete view of complex catalysis. *Nat Prod Rep* 2012;29:1153–75.

26. Ayer WA, Khan AQ. Zythiostromic acids, diterpenoids from an antifungal *Zythiostroma* species associated with aspen. *Phytochemistry* 1996;42:1647–52.

27. Peters RJ. Two rings in them all: the labdane-related diterpenoids. *Nat Prod Rep* 2010;27:1521–30.

28. Shiono Yoshihito, Ogata Kota, Koseki Takuya, Murayama Tetsuya, Funakoshi T. A cleistanthane diterpene from a marine-derived *Daphnane diterpenoids* from the roots of *Illicium dunnianum*. *Phytochemistry* 2010;71:711–7.

29. Park M, Han J, Lee CS, Soo BH, Lim KM, Ha H. Carnosic acid, a phenolic diterpene from rosemary, prevents UV-induced expression of matrix metalloproteinases in human skin fibroblasts and keratinocytes. *Exp Dermatol* 2013;22:336–41.

30. Ma S-G, Tang W-Z, Liu Y-X, Hu Y-C, Yu S-S, Zhang Y, et al. Prenylated C6-C3 compounds with molecular diversity from the roots of *Illicium oligandrum*. *Phytochemistry* 2011;72:115–25.

31. Qu J, Fang L, Ren X-D, Liu Y, Yu S-S, Li L, et al. Bisindole alkaloids from the roots of *Illicium dunnianum*. *Phytochemistry* 2011;72:115–25.

32. Zhang L, Luo RH, Wang F, Jiang MY, Dong ZJ, Yang LM, et al. Highly functionalized daphnane diterpenoids from *Trigonostemon thyroides*. *Org Lett* 2010;12:1525–8.

33. Zhang L, Luo RH, Wang F, Dong ZJ, Yang LM, Zheng YT, et al. Daphne diterpenoids isolated from *Trigonostemon thyroides* as HIV-1 antivirals. *Phytochemistry* 2010;71:1879–83.

34. Kirk DN. The chiroptical properties of carbonyl compounds. *Tetrahedron* 1986;42:777–818.

35. Bai J, Chen H, Fang ZF, Yu SS, Wang WJ, Liu Y, et al. Sesquiterpenes from the roots of *Illicium dunnianum*. *Phytochemistry* 2012;80:137–47.

36. Ayer WA, Khan AQ. Zythiostromic acids, diterpenoids from an antifungal *Zythiostroma* species associated with aspen. *Phytochemistry* 1996;42:1647–52.

37. Peters RJ. Two rings in them all: the labdane-related diterpenoids. *Nat Prod Rep* 2010;27:1521–30.

38. Gao Y, Houzatko KB, Peters RJ. Terpenoid synthase structures: a so far incomplete view of complex catalysis. *Nat Prod Rep* 2012;29:1153–75.

39. Khagisov FZ, Podobed OV, Gracheva TS, Salziev KD, Grachev SV, Berezov TT. Role of matrix metalloproteinases and their inhibitors in tumor invasion and metastasis. *Biochemistry* 2003;68:711–7.

40. Park M, Han J, Lee CS, Soo BH, Lim KM, Ha H. Carnosic acid, a phenolic diterpene from rosemary, prevents UV-induced expression of matrix metalloproteinases in human skin fibroblasts and keratinocytes. *Exp Dermatol* 2013;22:336–41.

41. Ma S-G, Tang W-Z, Liu Y-X, Hu Y-C, Yu S-S, Zhang Y, et al. Prenylated C6-C3 compounds with molecular diversity from the roots of *Illicium oligandrum*. *Phytochemistry* 2011;72:115–25.

42. Qu J, Fang L, Ren X-D, Liu Y, Yu S-S, Li L, et al. Bisindole alkaloids from the roots of *Illicium dunnianum*. *Phytochemistry* 2011;72:115–25.

43. Zhang L, Luo RH, Wang F, Jiang MY, Dong ZJ, Yang LM, et al. Highly functionalized daphnane diterpenoids from *Trigonostemon thyroides*. *Org Lett* 2010;12:1525–8.

44. Zhang L, Luo RH, Wang F, Dong ZJ, Yang LM, Zheng YT, et al. Daphne diterpenoids isolated from *Trigonostemon thyroides* as HIV-1 antivirals. *Phytochemistry* 2010;71:1879–83.