Beshanzoides A–D, unprecedented cycloheptanone-containing polyketides from *Penicillium commune* P-4-1, an endophytic fungus of the endangered conifer *Abies beshanzuensis*

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A number of previously undescribed (1–7) and structurally related known (8–17) isobenzofuran-type polyketides were obtained from the fermentation of *Penicillium commune* P-4-1, an endophytic fungus isolated from the fresh trunk bark of the critically endangered conifer *Abies beshanzuensis*. Beshanzoides A–D (1–4, resp.) feature a cycloheptanone-containing isobenzofuran ring system hitherto unknown, which might be biosynthesized via two steps of aldol reactions starting from a common co-occurring isobenzofuran-type polyketide as the precursor. The new structures were elucidated by spectroscopic methods, electronic circular dichroism data, and single crystal X-ray diffraction analyses. Beshanzoid E (5) showed antimicrobial activity (MIC: 16 μg mL⁻¹) against *Staphylococcus aureus*, whereas (±)-strobide A (10) inhibited (MIC: 16 μg mL⁻¹) *Candida albicans*. Cyclopaldic acid (12) and 3-O-methyl-cyclopaldic acid (13) exhibited inhibitory effects against acetyl-CoA carboxylase 1 (ACC1) with IC₅₀ values of 0.96 and 11.77 μM, respectively. Compound 12 also inhibited (IC₅₀: 7.56 μM) ATP-citrate lyase (ACL).

**Introduction**

The loss of plant diversity significantly exacerbates the complications in the discovery of new natural products (NPs)-derived drugs owing to rare and endangered plants (REPs) being better botanical sources. An important action for the conservation of REPs is to provide valuable resources for researchers for new chemical entities (NCEs). Pinaceae is one of the top privileged drug-prolific plant families. However, a considerable part of Pinaceae plants became ‘vulnerable (VN), ‘endangered (EN), ‘critically endangered (CR), or even ‘extinct (EX)’. There were 39 species listed in the China Plant Red Data Book published in 1992, and the situation is increasingly getting worse. To make the best of these valuable resources, we keep paying special attention to REPs in Pinaceae with the hope of stimulating active protection and utilization of these fragile plant species in a sustainable way, especially by investigating the renewable needles and twigs from those large trees distributed and managed in the wild or cultivated in botanic gardens.

*Abies beshanzuensis* M. H. Wu, among the above mentioned 39 species endemic to China, has been regarded as one of the 12 CR-grade plant species in the world by IUCN (International Union for Conservation of Nature and Natural Resources) since 1987. Only three living *A. beshanzuensis* trees nowadays grow in the wild at the summit of Baishanzu Mountain located at Zhejiang Province. In a preceding work, several rare *Abies* sesquiterpenoids with interesting structures and bio-activities were obtained from the mass-limited (400 g only) shed barks, and the findings immediately attracted much attention among synthetic chemists and pharmacologists.

Microbes are always important sources for drug discovery. Microorganisms living in special environments have been found to yield a large number of bioactive NPs with novel skeletons. The significances of chemistry and biology of plant endophytic fungi have been well documented. REPs species generally possess extended lifespans and survive in unique habitats, which hints at the existence of special endophytic...
fungi that could yield unique bioactive secondary metabolites.\textsuperscript{11,12} The plant sample of \textit{A. beshanzuensis} is too precious but extremely limited to be collected on a large scale for further phytochemical investigation, we thereafter turned to its endophytic fungi.\textsuperscript{11} This conifer (growing up in a special environmental setting) is still very attractive and promising, blow people away. It encouraged us to carry out a further research of its endophytic fungus.

In this study, we investigate the secondary metabolites of \textit{Penicillium commune} P-4-1, an endophytic fungus isolated from the fresh trunk bark of the title plant. As a result, seven previously undescribed (17–20) isobenzofuran-type polyketides (Fig. 1 and 2) were isolated and characterized. Reported herein are the isolation and characterization of three 5,6-dihydroxy-4-methylphthalides (8, 9, 10), 6-ethylphthalide (11), 9-hydroxy-4-methylphthalide (12), 3-hydroxy-4-methylphthalide (13), and 17-ethylphthalide (14), respectively. The 13C-NMR spectrum of 1 displayed two secondary methyl groups (δ\textsubscript{C} 1.45 [3H, d, \(J = 6.5\) Hz], 1.15 [3H, d, \(J = 6.1\) Hz]), one tertiary methyl group (δ\textsubscript{C} 3.95 [3H, s]), and one methoxy group (δ\textsubscript{C} 3.95 [3H, s]), and two oxymethine resonances (δ\textsubscript{C} 5.61 [1H, d, \(J = 10.6\) Hz], 4.26 [1H, qd, \(J = 6.5, 2.7\) Hz]). In addition, proton signals assigned to a methylene group (δ\textsubscript{H} 1.45 [3H, d, \(J = 6.5\) Hz], 2.60 [1H, br dd, \(J = 16.6, 5.0\) Hz]), 2.60 [1H, br dd, \(J = 16.6, 12.8\) Hz]) and two methine protons (δ\textsubscript{H} 3.61 [1H, m], 2.47 [1H, dd, \(J = 10.6, 2.7\) Hz]) were also readily distinguished. The 13C-NMR spectrum of 1 with the aid of HSQC NMR experiment showed 30 carbon resonances (Table 1) classified as four methyls (δ\textsubscript{C} 21.3, 23.1, 16.3, 9.0), one methylene (δ\textsubscript{C} 35.5), four methines (δ\textsubscript{C} 60.2, 157.8, 148.9, 119.3, 116.1, 109.7), an ester carbonyl (δ\textsubscript{C} 167.8), and a ketonic carbonyl (δ\textsubscript{C} 213.5). The aforementioned 1D NMR data indicated that 1 is partly similar to the co-occurring (±)-strobide A (10),\textsuperscript{13} an isobenzofuranone derivative. This was further confirmed by the HMBC cross-peaks from H-3 to C-1/C-3a, from H-17 to C-5/C-6/C-7, and from OCH\textsubscript{3} to C-7. In addition, the \(1^\text{H}-1^\text{H}\) COSY correlations of H-2/H-3/H-15 and H-3/H-11/H-12/H-13 as well as the HMBC correlations from H-2 to C-3a/C-4/C-5/C-10, from H-15 to C-8/C-9/C-10, from H-11 to C-3/C-3a/C-10/C-12, and from H-3 to C-10 suggested the isobenzofuranone ring should be integrated into a bicyclo[5.4.0]undecane backbone (Fig. 3). Therefore, the planar structure of 1 was defined as the first representative of a 5/6/7 tricyclic isobenzofuranonetype skeleton.

The relative configuration of 1 was determined by analysis of the proton–proton coupling constants (Table 1) and NOESY interactions (Fig. 3). The magnitude of \(J_{\text{H}\overset{\text{H}}{\rightarrow}11}\) (10.6 Hz) indicated that H-3 and H-11 adopted trans-axial orientations. The clear NOESY correlation between H-3 /H-9 (Fig. 3) revealed their co-facial relationship and both to be β-oriented. Finally, a single-crystal X-ray diffraction experiment with Ga K\textsubscript{\alpha} radiation for 1 unambiguously defined its absolute configuration

**Fig. 1** Chemical structures of compounds 1–4.

**Table 1** Chemical structures of compounds 5–17.
Based on its HRESIMS (m/z 321.1326 [M + H]+; calcd for C17H21O6, 321.1333) and 13C-NMR data (Table 1), the molecular formula C17H20O6 of beshanzoide B (2) was found to be the same as compound 1. The 1H- and 13C-NMR data of compound 2 highly resembled those of 1 (Table 1), indicating that both 2 and 1 share the same planar structure (Fig. 1). Similarly, the large coupling constant (J = 10.6 Hz) between of H-3 and H-11 indicated both protons adopted trans-axial orientations. The NOESY correlation of H-3/H-9 indicated that 2 and 1 even have the same relative configurations at C-3, C-9, and C-11; however, the ECD spectra of 2 and 1 displayed totally reversed Cotton effects (CEs) around 225 and 290 nm (Fig. 5), exciting by the chromophores of isobenzofuranone ring and cycloheptanone. The absolute configurations at C-3, C-9, and C-11 in 2 were hence elucidated as 3R,9R,11R,12S, respectively. Both 1 and 2 are not enantiomers, otherwise they should have the same set of NMR data. This means that the absolute configuration at C-12 in 2 should be consistent with 1. In fact, the chemical shifts of H-12 (δH 4.44 for 2; 4.26 for 1) and C-12 (δC 67.7 for 2; 70.5 for 1) were obviously differentiated with each other (Table 1), which was attributed to the contrary neighboring stereogenic center (C-11: R in 2, S in 1). Thus, compounds 2 and 1 are diastereoisomers, and the absolute configuration of 2 is determined as 3R,9R,11R,12S.

Table 1 1H and 13C NMR (δ in ppm, J in Hz) of 1–4

| No. | δH  | δC  | δH  | δC  | δH  | δC  | δH  | δC  |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1   | —   | 167.8 | —   | 167.8 | —   | 167.8 | —   | 167.8 |
| 3   | 6.31 d (10.6) | 75.5 | 6.29 d (10.6) | 76.8 | 6.41 d (10.7) | 75.9 |
| 3a  | —   | 148.9 | —   | 148.6 | —   | 115.8 | —   | 115.4 |
| 4   | —   | 116.1 | —   | 116.1 | —   | 160.1 | —   | 160.2 |
| 5   | —   | 160.2 | —   | 119.3 | —   | 119.2 | —   | 119.3 |
| 6   | —   | 119.3 | —   | 157.8 | —   | 157.7 | —   | 157.8 |
| 7   | —   | 191.7 | —   | 109.7 | —   | 109.7 | —   | 109.6 |
| 2   | 2.60 br dd (16.6, 12.8) | 35.5 | 2.58 br dd (17.0, 12.8) | 35.8 | 2.71 br dd (17.1, 12.0) | 35.0 |
| 3   | 3.03 br dd (16.6, 5.0) | 42.7 | 3.00 br dd (17.0, 5.2) | 42.6 | 3.09 br dd (17.1, 4.7) | 42.4 |
| 8   | 3.61 m | 42.6 | 3.64 m | 42.6 | 3.57 m | 42.6 |
| 9   | —   | 213.5 | —   | 213.0 | —   | 205.8 |
| 10  | —   | 213.5 | —   | 213.0 | —   | 205.8 |
| 11  | 2.47 dd (10.6, 2.7) | 66.7 | 2.30 dd (10.6, 1.9) | 66.7 | 4.02 d (10.7) | 73.4 |
| 12  | 4.26 qd (6.5, 2.7) | 70.5 | 4.44 qd (6.4, 1.9) | 67.7 | 4.24 s (10.7) | 30.7 |
| 13  | 1.45 d (6.5) | 23.1 | 1.27 d (6.4) | 22.2 | 2.34 s | 9.1 |
| 14  | 2.18 s | 9.0 | 2.17 s | 9.0 | 2.19 s | 9.1 |
| 15  | 1.15 d (6.1) | 16.3 | 1.13 d (6.3) | 16.0 | 1.14 d (6.3) | 16.6 |
| 7-OMe | 3.95 s | 62.3 | 3.95 s | 62.3 | 3.96 s | 62.3 |

a Assignments were made by a combination of 1D and 2D NMR experiments. b Measured in acetone-d6 (600 MHz). c Measured in acetone-d6 (150 MHz).

[absolute structure parameter: 0.01(15)] to be (3S,9S,11S,12S) (Fig. 4).

Based on its HRESIMS (m/z 321.1326 [M + H]+; calcd for C17H21O6, 321.1333) and 13C-NMR data (Table 1), the molecular formula C17H20O6 of beshanzoide B (2) was found to be the same as compound 1. The 1H- and 13C-NMR data of compound 2 highly resembled those of 1 (Table 1), indicating that both 2 and 1 share the same planar structure (Fig. 1). Similarly, the large coupling constant (J = 10.6 Hz) between of H-3 and H-11 indicated both protons adopted trans-axial orientations. The NOESY correlation of H-3/H-9 indicated that 2 and 1 even have the same relative configurations at C-3, C-9, and C-11; however, the ECD spectra of 2 and 1 displayed totally reversed Cotton effects (CEs) around 225 and 290 nm (Fig. 5), exciting by the chromophores of isobenzofuranone ring and cycloheptanone.15 The absolute configurations at C-3, C-9, and C-11 in 2 were hence elucidated as 3R,9R,11R,12S. Both 1 and 2 are not enantiomers, otherwise they should have the same set of NMR data. This means that the absolute configuration at C-12 in 2 should be consistent with 1. In fact, the chemical shifts of H-12 (δH 4.44 for 2; 4.26 for 1) and C-12 (δC 67.7 for 2; 70.5 for 1) were obviously differentiated with each other (Table 1), which was attributed to the contrary neighboring stereogenic center (C-11: R in 2, S in 1). Thus, compounds 2 and 1 are diastereoisomers, and the absolute configuration of 2 is determined as 3R,9R,11R,12S.

Fig. 3 Observed key 2D NMR correlations of 1.

Fig. 4 The ORTEP drawing of 1.

Fig. 5 Experimental ECD spectra of compounds 1–4.
Beshanzoide C (3) and D (4) were firstly isolated as a mixture of enantiomers possessing a molecular formula of C_{12}H_{12}O_{6} determined by an [M + H]^{+} ion at m/z 319.1184 in the HRESIMS and 13C NMR data (Table 1), indicating 3 (4) has one more IHD than compounds 1 and 2. Consistent with this, the 1H- and 13C-NMR spectroscopic data of 3 (4) revealed the presence of a ketocarbonyl group rather than the oxymethine at C-12 in 1 and 2. This was corroborated by the HMBC correlations from H-3/H-11/H-13 to C-12. Like 1 and 2, the coupling constant of J_{H3/H11} (10.7 Hz) and the NOE correlation of H-3/H-9 in compounds 3 and 4 indicated that they share the same relative configurations at C-3, C-9, and C-11. Interestingly, as H-11 connects with two ketonic carbonyl groups (C-10 and C-12), the hydrogen atom becomes reactive and was partly replaced by the deuterium atom in the deuterated solvents, which weakened the intensity of the signal of H-11 in the 1H-NMR spectrum to some extent (the relative integral reads 0.28). As a result, H-3 (δ_{H} 6.41) appeared to be a doublet ridden by a singlet. This phenomenon of deuterium involving a keto–enol tautomerism has been also found for ivenonoid A, a limonoid previously isolated from Khaya ivorensis.\[\text{[33]}\]

The enantiomeric mixture 3 (4) were subsequently proven by the flat ECD curve and the near-zero specific rotation value. The following racemic resolution by chiral HPLC separation afforded the enantiomers with equal amount. Finally, the absolute configurations of purified 3 and 4 were assigned as 3S, 9S, 11S and 3R, 9R, 11R, respectively, by comparing their ECD spectra with those of 1 and 2 (Fig. 5). As expected, these were further supported by their opposite optical rotation values \[\{[\alpha]_{25}^{25} +25.0 (c 0.02, MeOH)\} \text{ for 3; } -25.0 (c 0.02, MeOH) \text{ for 4}.\]

With a protonated ion peak at m/z 253.0703 [M + H]^{+} in the HRESIMS, beshanzoide E (5) was found to have the molecular formula of C_{10}H_{10}O_{5}. Comparative analysis of its NMR spectroscopic data (Table 2) with those of to the co-occurring 7-carboxy-4-hydroxy-6-methoxy-5-methylphthalide (8), an iso-benzofuranone derivative just recently isolated from marine-derived fungus Aspergillus sp. IMCASMF180035,\[\text{[44]}\] revealed that the structure of 5 is almost the same as that of 8 except for an additional methoxy group \[\delta_{H} 3.91 (3H, s), \delta_{C} 53.1\] appearing in 5. The connection of this methoxy group with C-9 was confirmed by the HMBC correlation from δ_{H} 3.91 to δ_{C} 167.9 (C-9). Therefore, compound 5 was defined as a methylated derivative of 8, which might be an artifact formed during the isolation.

The molecular formula C_{10}H_{10}O_{5} of beshanzoide F (6) was determined by its HRESIMS (m/z 233.0418 [M + Na]^{+}, calcd for C_{10}H_{10}O_{5}Na, 233.0420) and 13C NMR data (Table 2). The 1H-NMR spectrum (Table 2) showed signals for two equivalent oxymethylene protons (δ_{H} 5.18, 2H, s), one methoxyl group (δ_{H} 3.88, 3H, s) and one methyl group (δ_{H} 2.15, 3H, s). The 1C-NMR spectrum displayed one ester carbonyl (δ_{C} 171.7), six quaternary aromatic (δ_{C} 152.8, 152.7, 153.8, 133.2, 120.6 and 108.6), one methylene (δ_{C} 68.5), one methoxyl (δ_{C} 62.5), and one methyl group (δ_{C} 9.0). The above data indicated that compound 6 shares the same carbon skeleton with 5, while four substitutent groups on aromatic ring in 6 are two hydroxyls, one methyl, and one methoxy. The key HMBC correlations between H-3 (δ_{H} 5.18) and C-4 (δ_{C} 133.2) and from the methyl group to C-5 (δ_{C} 152.7), C-6 (δ_{C} 120.6) and C-7 (δ_{C} 152.8) suggested that the methyl group is attached to C-6 in 6. However, the location of the methoxy group remains unknown. Another set of 1H-NMR data were thus, acquired in DMSO-d_{6} with two hydroxyl proton signals appearing at δ_{H} 9.39 and δ_{H} 9.56, which were not highly chelated by intramolecular hydrogen bond. The NOE correlations of 4-OH (δ_{H} 9.39)/H-3 (δ_{H} 5.13), 5-OH (δ_{H} 9.56)/H-8 (δ_{H} 2.06), and 7-OH (δ_{H} 3.78)/H-8 (δ_{H} 2.06) revealed that the methoxy was connected to C-7, and the two hydroxyls were connected to C-4 and C-5, respectively.

| Table 2 | 1H and 13C NMR (δ in ppm, J in Hz) of 5, 6, 8, and 9 |
|---|---|---|---|---|---|---|---|
| No. | δ_{H} | δ_{C} | δ_{H} | δ_{C} | δ_{H} | δ_{C} | δ_{H} | δ_{C} |
| 1 | 171.2 | | 171.7 | 171.2 | | 171.2 | 169.1 |
| 3 | 5.26 s | 69.3 | 5.18 s | 5.15 s | 68.5 | 5.26 s | 69.2 | 6.52 s |
| 3a | 122.5 | | 135.8 | 121.8 | 148.0 |
| 4 | 153.2 | | 152.7 | 121.8 |
| 5 | 126.5 | | 126.6 |
| 6 | 158.8 | | 158.4 |
| 7 | 118.2 | | 119.5 |
| 7a | 130.4 | | 130.3 |
| 8 | 2.25 s | 9.9 | 2.15 s | 2.06 s | 9.0 | 2.25 s | 9.9 | 4.71 s |
| 9 | 167.9 | | 169.3 | 2.16 s |
| 6-OH | 3.80 s | 62.7 | | 3.84 s | 62.6 |
| 7-OH | 3.88 s | 3.78 s | 62.5 | | 3.97 s |
| 8-OH | 3.91 s | 53.1 | | 3.43 s | 58.7 |
| 9-OH | 9.39 s | 5.96 s |

* Assignments were made by a combination of 1D and 2D NMR experiments. * Measured in CD_{3}OD (400 MHz). * Measured in CD_{3}OD (150 MHz).

* Measured in DMSO-d_{6} (600 MHz).
Beshanzoide G (7) was isolated as a pair of HPLC inseparable mixture with one set of NMR data. The molecular formula C_{22}H_{23}O_{11} of 7 was deduced from its HRESIMS (m/z 485.1044 [M + Na]^+), calcld for C_{22}H_{23}O_{11}Na, 485.1054). The 1H-NMR spectrum provided one oxymethine (6.55, 1H, s), two equivalent oxymethylene protons (δ_H 4.83, 2H, s), one methoxyl group (δ_H 3.96, 3H, s) and one methyl group (δ_H 2.13, 3H, s). The 13C NMR spectrum showed one ester carbonyl (δ_C 169.0), six quaternary aromatic (δ_C 163.3, 158.9, 148.2, 121.7, 116.3, and 110.1), one methine (δ_C 97.6), one methylene (δ_C 65.6), one methoxy (δ_C 62.5), and one methyl group (δ_C 9.0). Based on the above data, compound 7 was proposed to have a completely symmetric framework. Compared with the reported compound (±)-strobride A (10), the signals are similar except for the appearance of two equivalent oxymethylene protons linked with the benzene ring in 7 instead of such a methyl group in 10. Therefore, compound 7 might be structured as a dimer by two (±)-strobride A fractions linked by an ether bond. The HMBC correlations from H-8 (δ_H 4.83) to C-3a (δ_C 148.2), C-4 (δ_C 116.3) and C-5 (δ_C 163.3), from H-9 (δ_H 2.13) to C-5 (δ_C 163.3), C-6 (δ_C 121.7), and C-7 (δ_C 158.9), as well as from 7-OCH3 (δ_H 3.96) to C-7 (δ_C 158.9) confirmed the substituents in 7. Additional HMBC correlations from H-8 (δ_H 4.83) to C-8 (δ_C 65.6) ensure the ether linkage from C-8 to C-8'. The optical rotation value is zero, which suggested an equal proportion of C3-3'-enantiomers. The phenomenon of related naturally occurring racemic phthalide mixtures have been well documented for (±)-strobride A and 3-O-methyl cyclopolic acid. A detailed explanation was given in 1992 by K. K. Ogilvie et al.

The molecular formula C_{12}H_{14}O_{6} of compound 9 (ref. 26) was determined by its HRESIMS. The 1H-NMR and 13C-NMR spectrum data (Table 2) indicated that the methylene in 9 is connected with a methoxy rather than forming a dimer, which was confirmed by the HMBC correlation from 8-OCH3 (δ_H 3.43) to C-8 (δ_C 67.7). This structure can be found in SciFinder database (CAS # 1083201-20-0), but there is no reference available.

Beshanzoides A-D (1–4) are the first representatives of polyketides possessing a cycloheptanone-containing isobenzofuran ring system. A plausible biogenetic pathway for them is proposed as shown in Scheme 1. Briefly, the co-occurring cyclopaldic acid (12) could be regarded as the biogenetic precursor of 1–4. Similar to the proposed bio-synthesis pathway of stealthin F, a butanone was connected to 12 through an aldol reaction. After dehydration, an ACP (acyl carrier protein) joined to extend the chain and form a structure of 10, 12-dione, which is regarded as a common intermediate in the formation of fatty acid and polyketide. Following intramolecular aldol reaction would generate compounds 3 and 4 possessing a seven membered ring. 3 and 4 could be reduced by a ketoreductase to yield compounds 1 and 2, respectively.

Naturally occurring compounds from fungi (mainly polyketides and alkaloids) have been often reported to have antibiotic and anti-tumor bioactivities. Therefore, all the isolates were evaluated for their anti-bacteria, anti-fungi and cytotoxicity activities. In the anti-bacteria screening, growth inhibition against Escherichia coli, Salmonella typhimurium, Shigella flexneri (Gram-negative), and Staphylococcus aureus (Gram-positive) were tested (Table S1†), while amoxicillin and chloromycetin were used as positive controls. Only compound 5 was found to inhibit S. aureus with an MIC value of 16 µg mL\(^{-1}\). Meanwhile, Candida albicans SC5314 was taken as the research strain in the anti-fungus screening (Table S1† fluconazole was used as positive control). Compound 10 exhibited an MIC value of 16 µg mL\(^{-1}\) against C. albicans. The cytotoxicity of each isolate against the human colon cancer RKO cell lines was evaluated using the CCK8 method, but none of them displayed cytotoxicity against the RKO cell line at 10 µM.

In addition, as part of an on-going project towards the discovery of novel NPs-derived agents for treatments of hyperlipidemia and metabolic disorders associated with the abnormal glucose and lipid metabolism (e.g., T2DM), all the isolates were also tested for their inhibitory activity against acetyl-coenzyme A carboxylase alpha transcript variant 1 (ACC1) and adenosine triphosphate (ATP)-citrate lyase (ACL), two potential drug targets for both fatty acid and cholesterol biosynthesis. As a result, compounds 12 and 13 displayed inhibitory activity against ACC1 with IC\(_{50}\) values of 0.96 ± 0.11 and 11.77 ± 1.72 µM, respectively. ND-630 (CAS # 1434635-54-7) was used as the positive control (IC\(_{50}\): 1.56 ± 0.07 nM). Compound 12 also showed significant inhibition activity against ACL with an IC\(_{50}\) value of 7.56 ± 1.38 µM. BMS-303141 (CAS # 943962-47-8) was used as the positive control (IC\(_{50}\): 0.20 ± 0.01 µM).

Conclusions

All things considered, most studies on plant-derived secondary metabolites and/or their bioactivities are primarily focusing on the macroscopic organisms (e.g., leaves, twigs, and barks) of these plants. As a result, microbial communities, especially of conifers remain largely untouched and are underexplored.

Scheme 1 A plausible biosynthetic pathway for compounds 1–4.

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resources for chemical and biological diversities. In addition, the populations of some medically valuable conifers are risking extinction due to the subsequent deforestation along with environmental changes. In an effort to protect these precious plants by gleaning valuable secondary metabolites from their endophytes, four unprecedented cycloheptanone-containing polyketides (beshanzoides A-D, 1–4) and a few structurally related new derivatives (5–7) were isolated and characterized from *Penicillium commune* P-4-1, an endophytic fungus isolated from the critically endangered plant *Abies beshanzuensis*. The discovery of structurally and biologically interesting polyketides extended the diversities of the secondary metabolites from endophytic fungi. It is the first time that isobenzofuranone derivatives were found to show remarkable inhibitory activity against ACC1 and ACL. We believe more glamorous discoveries will be unraveled from the endophytic fungi of this critically endangered and promising conifer.

The fungus of this critically endangered and promising conifer.

**Experimental**

**General experimental procedure**

Optical rotations were determined using a Rudolf Autopol IV polarimeter. UV absorptions were observed on a Hitachi U-2900E while IR spectra were recorded on an Avatar 360 FTIR spectrometers. ECD spectra were recorded on a JASCO-810 spectropolarimeter. All NMR experiments were measured with Bruker Avance III 400 MHz or 600 MHz spectrometers. Chemical shifts are shown in δ (ppm) and the residual solvent peaks of CD$_3$OD, acetone-$d_6$, or DMSO-$d_6$ were used as references. X-ray data were collected on a Bruker D8 Venture diffractometer (Ga Kα). HRESIMS were measured on an AB SCIEX Triple TOF 6500 spectrometer. Semi-preparative HPLC was performed on a Waters e2695 system with both 2998 prominence diode array 5600 spectrometer. Semi-preparative HPLC was performed on a Waters Sun PDA detector and a Daicel Chiralpak IC column (5.0 μm, 250 × 10 mm) was equipped for purifications. The chiral HPLC separations were operated on a Shimadzu LC-20AT system with an SPD-M20A PDA detector and a Daicel Chiralpak IC column (5 μm, 250 × 4.6 mm). Column chromatography (CC) was carried out with silica gel (100–200 and 200–300 mesh, Qingdao Marine Chemical Co. Ltd., China), MCI gel CHP20P (75–150 μm, Mitsubishi Chemical Industries, Japan), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden). Thin layer chromatography (TLC) was performed on pre-coated plates (HSGF254, Yantai Jiangyou Silica Gel Development Co., Ltd., Yantai, China). TLC spots were visualized under both 254 and 365 nm UV light and by heating after sprayed with 10% (v/v) H$_2$SO$_4$/EtOH reagent.

**Fungal material**

The fresh trunk bark of *A. beshanzuensis* was collected from the Baishanzu Mountain located at Lishui, Zhejiang Province, in April 2014. The plant sample was identified by Dr Ke-Jun Cheng (Lishui Institute of Agricultural Science, China). The bark was airlifted to the laboratory in ice packs within 24 h and stored at 4 °C until processed. The endophytic fungus P-4-1 was isolated from the bark and identified as *Penicillium commune* by comparing its ITS sequence (GenBank Accession No. MW721174) with corresponding sequences in public database (NCBI).

**Fermentation, extraction, and isolation**

The *Penicillium commune* P-4-1 was cultivated at 28 °C on autoclaved solid rice culture medium in 86 Erlenmeyer flasks (500 mL), each containing 60 g of rice and 80 mL of ddH$_2$O. After 30 days’ incubation, mediums were dried and soaked in EtOAc (60 min × 3 times) to afford a dark brown EtOAc extract (91.6 g). The EtOAc extract was subjected to a silica gel column (100–200 mesh) chromatography with a gradient-elution system, PE–EtOAc (20 : 1 → 10 : 1 → 5 : 1 → 2 : 1 → 1 : 1, neat EtOAc, v/v) and EtOAc–MeOH (1 : 1 → 0 : 1, v/v) to obtain seven fractions (Fr. 1 to Fr. 7), according to TLC analysis. Fr. 2 (528 mg) was separated over silica gel (PE-acetone, 5 : 1 → 2 : 1 → 1 : 1 → neat acetone) to give five subfractions (Fr. 2-A to Fr. 2-E). From Fr. 2-C (110 mg), compound 11 (13.6 mg, $t_R$ = 8.7 min) was fractionated by chromatograph column over Sephadex LH-20 (MeOH) followed by semi-preparative HPLC (MeCN–H$_2$O 32 : 68). Fr. 2-D (21 mg) was purified by semi-preparative HPLC (MeCN–H$_2$O 33 : 67), and compound 15 (3.5 mg, $t_R$ = 11.5 min) was isolated. Fr. 3 (3.1 g) was subjected to a silica gel column (PE-acetone, 10 : 1 → 5 : 1 → 2 : 1 → 1 : 1) to give Fr. 3-A to Fr. 3-D. Fr. 3-C (78 mg) was further fractionated by and semi-preparative HPLC (MeCN–H$_2$O 32 : 68) and compound 16 (2.5 mg, $t_R$ = 12.0 min) was yielded. Fr. 4 (8.2 g) was fractionated into eight sub-fractions (Fr. 4-A to Fr. 4-H) by an MCI gel column with a gradient of MeOH–H$_2$O (30% → 50% → 70% → 90% → 100% MeOH). By employing semi-preparative HPLC purification system (MeCN–H$_2$O, 22 : 78), compounds 10 (1.2 mg, $t_R$ = 19.2 min) and 6 (1.5 mg, $t_R$ = 18.2 min) were obtained from Fr. 4-C (13 mg), Fr. 4-F (340 mg) was further chromatographed by silica gel to afford seven subfractions (Fr. 4-F-1 to Fr. 4-F-7). Compounds 12 (3.0 mg, $t_R$ = 19.6 min) and 14 (0.5 mg, $t_R$ = 12.9 min) were purified from Fr. 4-F-5 (26 mg) while compounds 1 (5.0 mg, $t_R$ = 18.0 min) and 2 (3.0 mg, $t_R$ = 22.0 min) purified from Fr. 4-F-7 (33 mg) by semi-preparative HPLC (MeCN–H$_2$O 36 : 64 /MeCN–H$_2$O 32 : 68). In a similar way, compounds 13 (6.2 mg, $t_R$ = 10.0 min; MeCN–H$_2$O, 55 : 45) and 3 (4) (2.1 mg, $t_R$ = 16.2 min; MeCN–H$_2$O, 43 : 57) were isolated from Fr. 4-G (100 mg). Fr. 5 (2.1 g) was separated by silica gel column (PE-acetone, 10 : 1 → 5 : 1 → 2 : 1 → 1 : 1), followed by semi-preparative HPLC (MeCN–H$_2$O, 51 : 49) to get compound 17 (1.0 mg, $t_R$ = 5.0 min). Separation of Fr. 6 (5.2 g) on an MCI column with a stepwise gradient elution of MeOH–H$_2$O (50 : 50 → 70 : 30 → 90 : 10 → 100 : 0) collected seven sub-fractions (Fr. 6-A to Fr. 6-G). Compound 8 (30 mg) was recrystallized from Fr. 6-A (200 mg). Fr. 6-E (130 mg) was further fractioned by silica gel with PE-acetone system (4 : 1 → 3 : 1 → 2 : 1 → 1 : 1) into twelve parts (Fr. 6-E-1 to Fr. 6-E-12). Semi-preparative HPLC afforded compounds 9 (1.9 mg, $t_R$ = 10.5 min), 5 (1.7 mg, $t_R$ =
10.5 min), and 7 (3.1 mg, t<sub>R</sub> = 13.2 min) from Fr. 6-E-4 (11 mg; MeCN–H<sub>2</sub>O, 29: 71), Fr. 6-E-5 (24 mg; MeCN–H<sub>2</sub>O, 33: 67), and Fr. 6-E-10 (7 mg; MeCN–H<sub>2</sub>O, 32: 68), respectively. The mixture of enantiomers 3 and 4 (1.2 mg) was purified by chiral HPLC (hexane-isopropanol, 78: 22). Compounds 3 (0.5 mg, t<sub>R</sub> = 22.6 min) and 4 (0.5 mg, t<sub>R</sub> = 24.9 min) were obtained.

### Compound characterization

Beshanzoide A (1) white powder, mp 206.4–208.9 °C, [α]<sub>D</sub><sup>20</sup> +80.9 (c 0.06, MeOH); UV (MeOH) λ<sub>max</sub> (log ε) 216 (4.3), 262 (3.9) nm; ECD (c 1.00 × 10<sup>−4</sup> M, MeOH) ε<sub>max</sub> (Δε) 225 (−5.6), 290 (+5.1) nm; IR (film) ν<sub>max</sub> (cm<sup>−1</sup>) 3469 (br), 1719, 1702, 1589, 1275, 1116 and 1024 cm<sup>−1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, see Table 1; ESIMS m/z 321 [M + H]<sup>+</sup>, 343 [M + Na]<sup>+</sup>; HRESIMS m/z 321.1329 [M + H]<sup>+</sup> (calc for C<sub>17</sub>H<sub>20</sub>O<sub>6</sub>, 321.1333, Δ = −0.1 ppm).

Beshanzoide B (2) white powder, [α]<sub>D</sub><sup>20</sup> −89.0 (c 0.08, MeOH); UV (MeOH) λ<sub>max</sub> (log ε) 216 (4.1), 262 (3.7) nm; ECD (c 1.30 × 10<sup>−4</sup> M, MeOH) λ<sub>max</sub> (Δε) 222 (−1.6), 237 (−1.9), 307 (+7.1) nm; <sup>1</sup>H and <sup>13</sup>C NMR data, see Table 1; ESIMS m/z 319 [M + H]<sup>+</sup>, 343 [M + Na]<sup>+</sup>; HRESIMS m/z 319.1184 [M + H]<sup>+</sup> (calc for C<sub>17</sub>H<sub>20</sub>O<sub>6</sub>, 319.1176, Δ = 2.4 ppm).

Beshanzoide C (3) white powder, [α]<sub>D</sub><sup>20</sup> +25.0 (c 0.02, MeOH); UV (MeOH) λ<sub>max</sub> (log ε) 216 (3.9), 262 (3.5) nm; ECD (c 1.30 × 10<sup>−4</sup> M, MeOH) λ<sub>max</sub> (Δε) 221 (−2.2), 237 (−1.6), 305 (−7.2) nm; <sup>1</sup>H and <sup>13</sup>C NMR data, see Table 1; ESIMS and HRESIMS see beshanzoide C (3).

Beshanzoide E (4) white powder, [α]<sub>D</sub><sup>20</sup> −30.0 (c 0.02, MeOH); UV (MeOH) see beshanzoide C (3); ECD (c 1.30 × 10<sup>−4</sup> M, MeOH) λ<sub>max</sub> (Δε) 221 (−2.2), 237 (−1.6), 305 (−7.2) nm; <sup>1</sup>H and <sup>13</sup>C NMR data, see Table 1; ESIMS and HRESIMS see beshanzoide C (3).

### Antimicrobial assays

The antibiotic properties of compounds 1–17 against Escherichia coli, Salmonella typhimurium, Shigella flexneri (Gram-negative), Staphylococcus aureus (Gram-positive) and Candida albicans (fungi) were evaluated by microdilution method. Amoxicillin and chloromycetin were the positive controls for antibacterial assays, and fluconazole was the antifungal positive control. Test bacteria or fungi were counted and seeded at the appropriate concentrations in sterile 96-well microplates. Isolated metabolites and positive controls were individually tested at 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 μg mL<sup>−1</sup>. Assays were incubated at 35 °C for 16 (bacteria) or 24 (fungi) hours. MIC (minimum inhibitory concentration) was the lowest concentration of the tested compounds that could completely inhibit the growth of bacteria (fungi). The MIC values were determined by a combination of naked eyes observations and optical density (OD) measurements.

### ACC1 inhibitory activity assay

ADP-Glo™ Kinase Assay (Promega, Madison, WI) was used to assess the activity of ACC1. The kinase assay was carried out in a 384-well plate (ProxiPlate™-TM-384 Plus, PerkinElmer) with 2.0 μL of ACC1 and 1.0 μL of test compounds with different concentrations and pre-incubate for 0.5 hours at room temperature. Then the 2.0 μL of ATP was added to each well and kept for 60 min at room temperature. After the enzymatic reaction, 2.5 μL of ADP-Glo reagent was added to each well to terminate the kinase reaction and deplete the unconsumed ATP within 60 min at room temperature. In the end, 5.0 μL of kinase detection reagent (reagent 2) was added to each well and incubated for 1 h to simultaneously convert ADP to ATP. The luminescence signal was measured by a PerkinElmer En-Vision reader (PerkinElmer, MA, USA). The known inhibitor ND-630 (ref. 30) was used as the positive control.
ACL inhibitory activity assay

The same as the ACC1 inhibitory activity assay, the assay was performed using ADP-Glo™ Kinase Assay (Promega, Madison, WI). The ACL inhibitory activity was quantified by the amount of ADP generated of the enzymatic reaction. The kinase assay was carried out in a 384-well plate (ProxiPlateTM-384 Plus, PerkinElmer) in a volume of 5 μL reaction mixture, containing 2.0 μL of ACL, 2.0 μL of ATP, and 1.0 μL of test compounds with different concentrations. Reactions in each well was kept going for 30 min under 37 °C. After that, 2.5 μL of ADP-Glo™ reagent was added to terminate the kinase reaction and deplete the unconsumed ATP within 60 min at room temperature. Finally, 5.0 μL of kinase detection reagent (reagent 2) was added to each well and incubated for 1 h to simultaneously convert ADP to ATP. The luminescent signal was measured using an En-Vision multilabel plate reader (PerkinElmer, MA, USA) and BMS-303141 (ref. 31) was taken as the positive control.

Conflicts of interest

There are no conflicts to declare.

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