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Thiocytochalasins A–D, four sulfur-containing cytochalasans from an endophytic fungus *Phoma multirostrata* XJ-2-1

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ABSTRACT

Four unprecedented sulfur-containing cytochalasans, thiocytochalasins A–D (**1**–**4**), were isolated from an endophytic fungus *Phoma multirostrata* XJ-2-1. Thiocytochalasins A (**1**) and B (**2**) feature a novel 5/6/14/5 tetracyclic scaffold, which are the first examples of cytochalasan containing a thiophene moiety. Thiocytochalasins C (**3**) and D (**4**) are epimeric cytochalasan homodimers formed *via* a thioether bridge. Their structures with absolute configurations were established by detailed analysis of the HRESIMS, NMR, and X-ray crystallography. The plausible biogenetic pathway of **1**–**4** was postulated. Compounds **3** and **4** exhibited significant cytotoxicity against CT26 cells with IC_{50} values of 0.85 and 0.76 μmol/L, respectively.

Sulfur-containing natural products are a large class of multifunctional molecules existing in both terrestrial and marine organisms. To date, more than 1000 sulfur-containing natural products have been reported, many of which exhibit potent biological activities and pharmacological properties [1]. For centuries, sulfur has continued to maintain its status as the dominating heteroatom integrated into a set of 362 sulfur-containing FDA approved drugs (besides oxygen or nitrogen) [2]. Sulfonamides, thioethers, sulfones, and penicillin are the most common scaffolds in sulfur containing drugs, which are well studied both on synthesis and application during the past decades [2–4].

Cytochalasans are a group of fungal-derived polyketide-nonribosomal peptide synthetase (PKS-NRPS) natural products characterized by a perhydro-isoindolone core fused with a macrocyclic ring, exhibiting high structural diversity and a broad-spectrum of bioactivities. They are majorly produced by the genera *Aspergillus*, *Chaetomium*, *Xylaria*, *Helminthosporium*, *Penicillium*, *Zygosporium*, *Metarhizum*, *Rosellinia*, *Ascochyta*, *Hypoxylon*, *Spicaria*, and *Phoma* [5–8]. In recent decades, more than 500 cytochalasans have been reported [5,9–15], which are generally classified into six groups, namely cytochalasins, chaetoglobosins, pyrichalasins, aspochalasins, alachalasins, and trichalasin [5,6]. Cytochalasins, a group of cytochalasans with a phenylalanine unit incorporated into the polyketide backbone, is the largest group of cytochalasans [5].

Phoma species are a rich source of bioactive secondary metabolites including cytochalasins [16], and the first cytochalasan was isolated from a culture broth of a *Phoma* sp. in 1966 [17]. *Phoma multirostrata* XJ-2-1, an endophytic fungus, was isolated from the medicinal plant *Parasenecio albus* collected from Xinning County, Hunan Province of China. In the previous study, we reported ergocytochalasin A, a merocytochalasan consisting of one cytochalasin moiety and one ergosterol moiety with a 5/6/14/6/5/6/6/6 fused octacyclic ring system, and nine new cytochalasins multirostratins B–J, from *P. multirostrata* XJ-2-1 [9,10]. To search for more novel metabolites with unique structures and significant bioactivities, the fungus *P. multirostrata* XJ-2-1 was further investigated, which led to the identification of four unprecedented sulfur-containing cytochalasans, thiocytochalasins A–D (**1**–**4**) (Fig. 1). To the best of our knowledge, only five sulfur-containing cytochalasans have been reported before [14,18], of which bisaspochalasins B and C are cytochalasan dimers connected by thioether bond [14]. Thiocytochalasins A (**1**) and B (**2**) feature an unprecedented 5/6/14/5 tetracyclic scaffold, which are the first examples of cytochalasan fused with a thiophene moiety. Thiocytochalasins C (**3**) and D (**4**) are epimeric cytochalasan homodimers formed *via* a thioether bridge. Herein, the details of the isolation, structure elucidation, plausible biogenetic pathway, as well as biological activities of thiocytochalasins A–D (**1**–**4**) are reported.

Thiocytochalasin A (**1**), colorless needles, possessed a molecular formula of $C_{31}H_{35}NO_5S$ as determined by the HRESIMS ($[M+Na]^+$, *m/z* 556.2127, calcd. for $C_{31}H_{35}NO_5SNa$, 556.2134), requiring 15 double bond equivalents (DBEs), which was consistent with the ^{13}C

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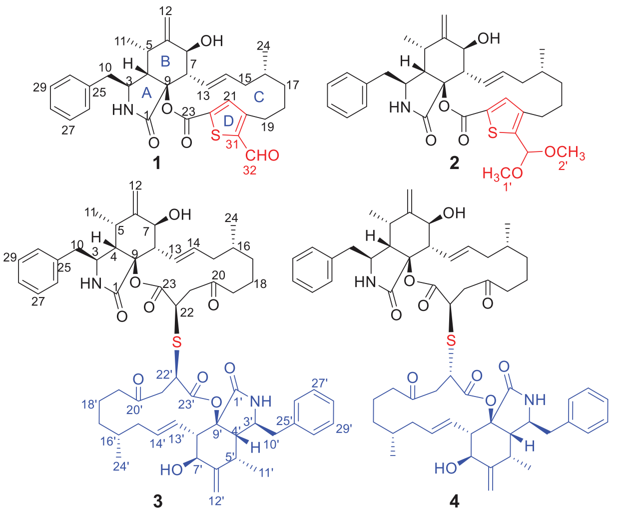


Fig. 1. Chemical structures of compounds **1**–**4**.

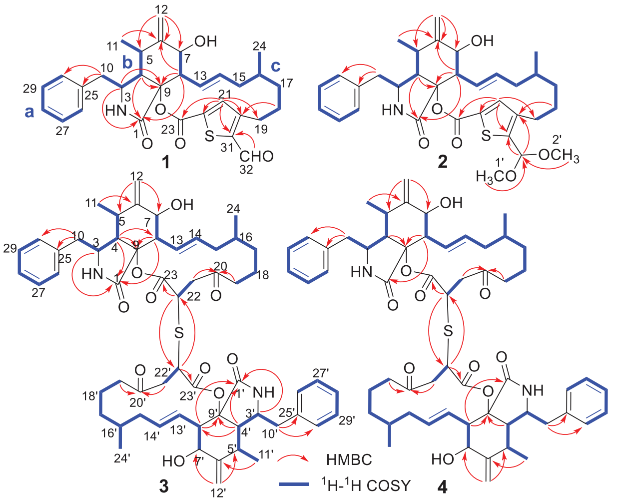


Fig. 2. Key HMBC and $^1H-^1H$ COSY correlations of **1**–**4**.

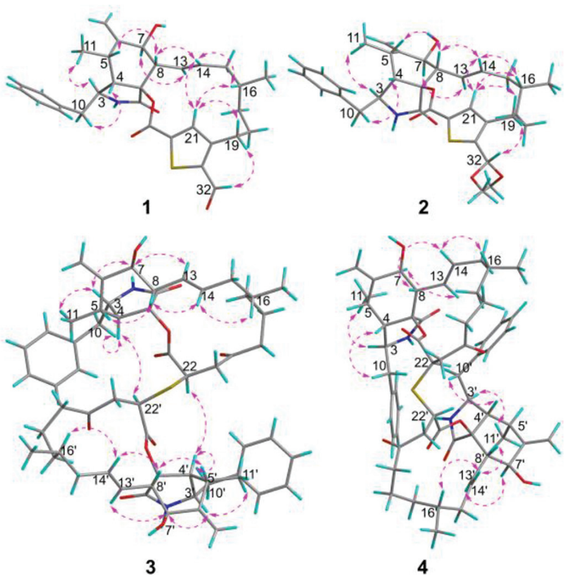


Fig. 3. Key NOESY correlations of **1**–**4**.

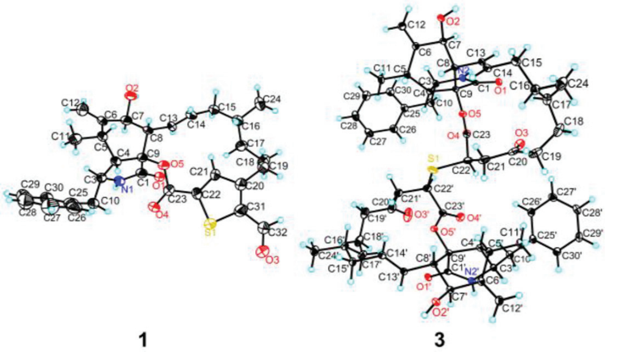


Fig. 4. X-ray ORTEP drawing of compounds **1** and **3**. Displacement ellipsoids are drawn at the 50% probability level.

NMR data. Its IR spectrum showed absorptions for hydroxy (3419 cm^{-1}), ester carbonyl (1717 cm^{-1}), conjugated aldehyde and/or amide (1668 cm^{-1}), and phenyl (1604 and 1494 cm^{-1}) groups. The ^1H NMR data (Table S1 in Supporting information) of **1** showed two doublet methyls at δ_H 1.01 (d, 3H, $J = 6.7$ Hz) and 1.15 (d, 3H, $J = 6.6$ Hz); five olefinic protons at δ_H 5.23 (br s, 1H), 5.47 (br s, 1H), 5.77 (ddd, 1H, $J = 15.2$, 10.6, 3.4 Hz), 5.90 (ddd, 1H, $J = 15.2$, 9.6, 2.2 Hz), and 7.93 (s, 1H); one monosubstituted phenyl group with five coupled aromatic protons at δ_H 7.24 (d, 2H, $J = 7.6$ Hz), 7.25 (m, 1H), and 7.33 (t, 2H, $J = 7.6$ Hz); one aldehyde proton at δ_H 10.05 (s, 1H). The ^{13}C NMR data (Table S1), with the aid of DEPT and HSQC experiments, exhibited 31 carbon resonances, comprising three carbonyls (δ_C 160.8, 170.1, and 182.7), six aromatic carbons, eight olefinic carbons, one sp^3 quaternary carbons, six sp^3 methines, five sp^3 methylenes, and two methyls. These functionalities accounted for 11 out of the 15 DBEs, indicating four additional rings in the structure of **1**. The aforementioned NMR data, along with the previous reports of the chemical investigation on *P. multirostrata* EA-12 [9,10], suggested that **1** was likely a cytochalasin.

The planar structure of **1** was established by interpretation of its 2D NMR spectra (Fig. 2). Analysis of the ^1H-^1H COSY spectrum of **1** revealed the presence of three spin coupling fragments **a**–**c** which are shown with blue bold bonds (Fig. 2). Then, those fragments with the nonprotonated carbons and heteroatoms were assembled by examination of the HMBC spectrum to furnish the planar structure of **1**. The HMBC cross-peaks of H_2-10/C-25 and C-30 indicated the monosubstituted phenyl group attached to C-10. The HMBC correlations of H-3/C-1, C-9, H-4/C-1, C-9, H_3-11/C-6, H_2-12/C-5, C-6, C-7, H-8/C-1, C-6, C-9 delineated the perhydro-isoindolone core (rings A and B). The construction of ring C was established by the HMBC networks of H_2-18/C-20, H-21/C-19, C-22, C-23. The HMBC cross-peaks of H-21/C-20, C-22, C-31, H-32/C-31, allowed the establishment of the unique thiophene ring D, which bridged to the ring C at C-20 and C-22, and simultaneously suggested that the aldehyde group is attached to C-31. Thus, the gross structure of **1** was elucidated as shown in Fig. 1.

The relative configuration of compound **1** was fixed by the analysis of the ^1H-^1H coupling constants (Table S1) and the NOESY correlations (Fig. 3). The *E*-geometry of Δ^{13} double bond was deduced from the large coupling constant between H-13 and H-14 ($J_{13,14} = 15.2$ Hz). The NOESY correlations of H-3 with H_3-11 revealed that H-3 and H_3-11 were positioned on the same side of the molecule and randomly assigned as α-oriented, while H-4, H-5, H-8 and H-16 were assigned to be β-oriented by the NOESY cross-peaks of H-4/H_2-10; H-8/H-5 and H14; H-14/H-16. The α orientation of H-7 was determined by the NOESY correlation between H-7 and H-13. To confirm the structure assigned for compound **1** and determine its absolute configuration, quality crystals were successfully obtained from recrystallization in CH_2Cl_2/MeOH/H_2O

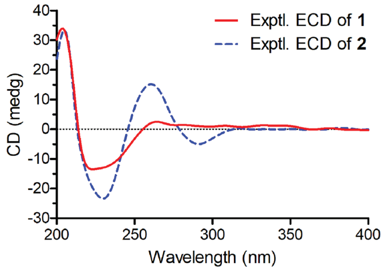


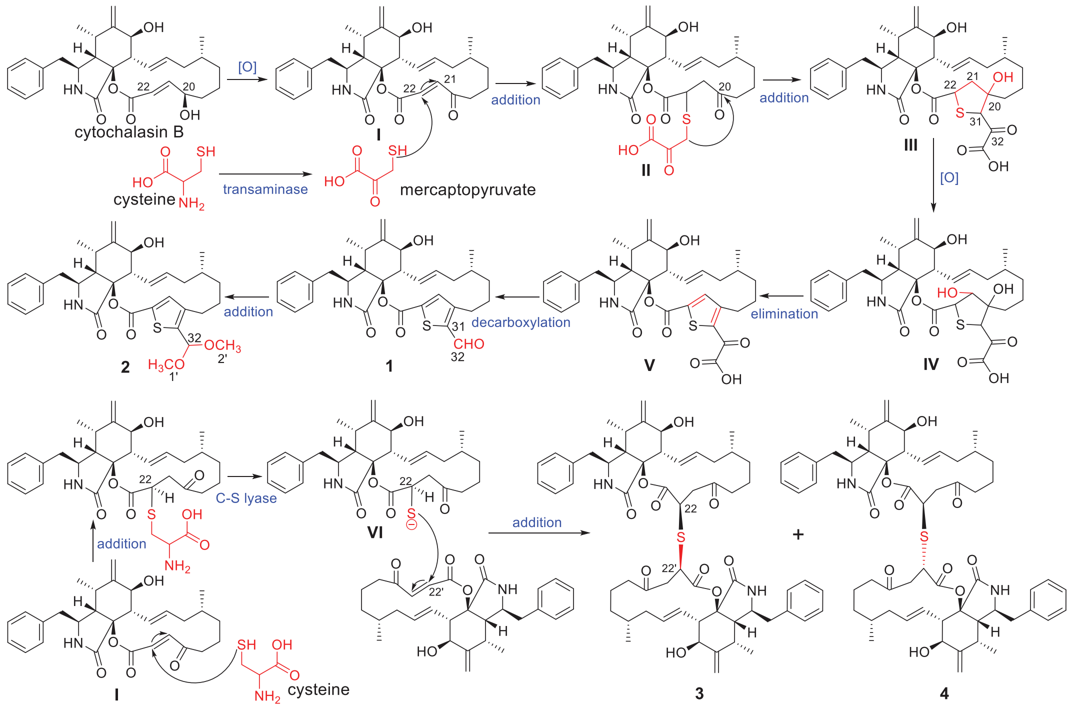
Fig. 5. Experimental ECD spectra of **1** and **2**.

(10:10:1) at room temperature, and then subjected to single-crystal X-ray diffraction (XRD) analysis using Ga Kα radiation (Fig. 4). The X-ray crystallography result not only secured the above structural assignment for **1** but also established its absolute configuration (3*S*, 4*S*, 5*S*, 7*S*, 8*S*, 9*S*, 16*R*) by the excellent Flack parameter [0. 12 (2)].

Thiocytochalasin B (**2**) was obtained as white powder. Its molecular formula was deduced to be $C_{33}H_{41}NO_6S$ from (+)-HRESIMS ion peak at *m/z* 602.2542 $[M+Na]^+$ (calcd. for $C_{33}H_{41}NO_6SNa$, 602.2552) and the ^{13}C NMR data (Table S1), indicating 14 DBEs. The IR spectrum revealed the presence of hydroxy (3383 cm^{-1}), carbonyl (1711 cm^{-1}), and terminal double bond (1640 and 972 cm^{-1}). Detailed comparison of the NMR data between **1** and **2** (Table S1) indicated they had identical carbon skeleton. The main difference was that the C-32 aldehyde group in **1** was replaced by a dimethoxy methine group in **2**, which was confirmed by the HMBC correlations (Fig. 2) from H_3-1′ (δ_H 3.36) and H_3-2′ (δ_H 3.32) to C-32 (δ_C 98.7). The NOESY correlations of **2** disclosed that this compound had the same relative configuration as that of compound **1** (Fig. 3). Finally, the absolute configuration of **2** was identified by comparison of its ECD spectrum with that of **1** (Fig. 5).

Thiocytochalasin C (**3**) was isolated as colorless needles with the molecular formula of $C_{58}H_{72}N_2O_{10}S$ from its HRESIMS ion peak at *m/z* 1011.4811 $[M+Na]^+$ (calcd. for $C_{58}H_{72}N_2O_{10}SNa$, 1011.4805), requiring 24 DBEs. The ^{13}C NMR (Table S2 in Supporting information) displayed only 29 resonances for **3**, suggesting that compound **3** was a symmetrical cytochalasin dimer linked by a central bridged sulfur atom. Its IR absorption bands at 3359 and 1717 cm^{-1} suggested the presence of hydroxy and carbonyl groups, respectively. Further analysis of the 1D NMR data (Table S2) revealed that the cytochalasin monomer in **3** closely resembled the known compound cytochalasin B_6 [19], with the only difference being the replacement of a methylene (δ_C 29.0, C-22) in cytochalasin B_6 by a methine (δ_C 42.1, C-22) in **3**. This was confirmed by the ^1H-^1H COSY correlation of H_2-21/H-22, and the HMBC cross-peaks from H_2-19 and H_2-21 to C-20, and from H-22 to C-23 (Fig. 2). Considering the symmetry of the molecule, this change led to the deduction that the two monomers were connected by virtue of a sulfur bridge between C-22 and C-22′, which was supported by the HMBC correlation of H-22/C-22′ (Fig. 2). The relative configuration of **3** was determined by analysis of the NOESY spectrum (Fig. 3). The NOESY correlations of H-3/H-7 and H_3-11 indicated the assignment of the α-configuration for these protons. The β-configuration for H-4, H-8 and H_3-16 was fixed according to the NOESY correlations of H-4/H-10; H-8/H-5, H-14; and H-14/H-16. However, the relative configuration of C-22 could not be resolved because of the absence of specific evidence. Fortunately, a suitable crystal was obtained from a solution of CH_2Cl_2/MeOH (1:1). The single-crystal X-ray diffraction analysis using Cu Kα radiation (Fig. 4) with Flack parameter [0.009 (10)] corroborated the structural assignment of **3** based on NMR and identified its absolute configuration as 3(3′)*S*, 4(4′)*S*, 5(5′)*S*, 7(7′)*S*, 9(9′)*S*, 16(16′)*R*, 22(22′)*R*.

Thiocytochalasin D (**4**), white powder, had the same molecular formula of $C_{58}H_{72}N_2O_{10}S$ as **3**, as deduced from its HRESIMS ion peak at *m/z* 1011.4821 $[M+Na]^+$ (calcd. for $C_{58}H_{72}N_2O_{10}SNa$, 1011.4805) and the ^{13}C NMR data (Table S3 in Supporting information). Its IR absorption bands at 3421 and 1713 cm^{-1} suggested the presence of hydroxy and carbonyl groups, respectively. A detailed comparison of the NMR data of **4** and **3** (Tables S2 and S3) indicated that the two compounds are structurally related, except that the structural symmetry present in **3** was broken in **4**, as the ^{13}C



Scheme 1. Plausible biosynthetic pathway of compounds **1**–**4**.

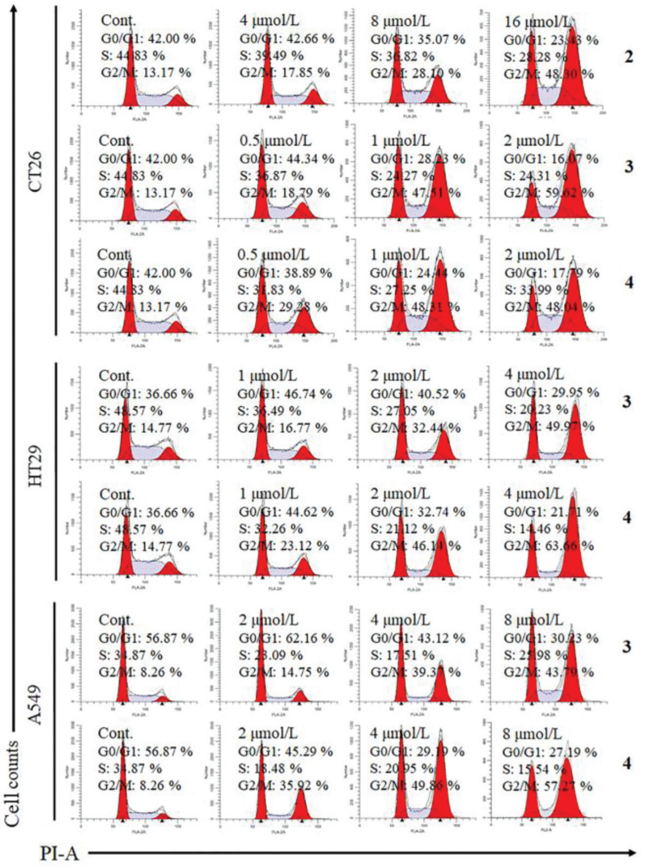


Fig. 6. Cell cycle analysis of CT26, HT29 and A549 cells after 24 h treatment with compounds **2**–**4** at different concentrations using PBS as a negative control (Cont.).

NMR of **4** showed 58 signals. Further analysis of the 2D NMR spectra (Fig. 2) constructed the planar structure of **4**, which was the same as that of **3**. The only difference between **4** and **3** was the opposite configuration at C-22′, which was determined by comparison of their chemical shifts and coupling constants as H-22′ at δ_H 3.91 (dd, 2H, $J = 11.4$, 3.1 Hz) for **3** while H-22′ at δ_H 4.14 (dd, 1H, $J = 7.8$, 3.5 Hz) for **4**. The relative configuration of other chiral centers in **4** was assigned as the same as that of **3** by their similar NMR data and NOESY correlations (Fig. 3). As cytochalasins shared the same biogenetic pathway, the absolute configuration of **4** was assigned as 3(3′)*S*, 4(4′)*S*, 5(5′)*S*, 7(7′)*S*, 9(9′)*S*, 16(16′)*R*, 22*R*, 22′*S*. The structure of **4** was hence established as shown in Fig. 1.

The plausible biosynthesis pathway of thiocytochalasins A–D (**1**–**4**) is proposed in Scheme 1. On the basis of the systematic report of enzymatic carbon-sulfur bond formation in natural product biosynthesis [20], cysteine is considered to be the sulfur source of thiocytochalasins A–D (**1**–**4**). And cytochalasin B, a main cytochalasin from this strain [9], is postulated to be the precursor. First, cytochalasin B was reduced to give a vital intermediate **I** *via* an oxidation reaction, which subsequently involved a nucleophilic addition to C-22 in the α,β-unsaturated 1,4-diketone moiety of intermediate **I** with mercaptopyruvate to produce the thio intermediate **II**. The intermediate **II** underwent a series of reactions including addition, oxidation, elimination, and decarboxylation to generate thiocytochalasin A (**1**) with a 2,3,5-trisubsituted thiophene moiety. Then, thiocytochalasin B (**2**) was produced by an addition of **1**. The cysteine could conduct a nucleophilic addition to intermediate **I**, leading to an intermediate **VI** which could generate a sulfur anion to couple with another monomer intermediate **I** to produce a pair of epimeric cytochalasan homodimers thiocytochalasins C (**3**) and D (**4**) decorated with a thioether bridge.

Compounds **1**–**4** were evaluated for their cytotoxicity against MCF-7 human breast adenocarcinoma, HepG2 human hepatocellular carcinoma, CT26 murine colon carcinoma, HT-29 human colon carcinoma, and A549 human lung carcinoma cell lines using MTT method. As a result, compounds **1** and **2** showed modest cytotoxicity against the tested cell lines with IC_{50} values of 4.17–23.41 μmol/L (Table S4 in Supporting information). Compounds **3** and **4** displayed strong cytotoxicity against four cell lines with IC_{50} values from 0.76 μmol/L to 7.52 μmol/L, and exhibited moderate cytotoxicity against HepG2 cells (Table S4). Subsequently, the effects of **2**–**4** on cell cycle progression of CT26, HT29 and A549 cells were investigated. The results showed that they could concentration-dependently induce G2/M cell cycle arrest in these cells, especially compounds **3** and **4** could significantly arrest cell cycle G2/M phase of CT26 cells at the concentration of 1 μmol/L (Fig. 6).

In conclusion, the discovery of thiocytochalasins A–D (**1**–**4**) from an endophytic fungus *P. multirostrata* XJ-2-1 has expanded the scaffolds of cytochalasan with sulfur-containing moiety. In particular, identification of the thiocytochalasins A (**1**) and B (**2**), first examples of cytochalasans fused with a thiophene moiety, is of great importance in the research field of cytochalasans. It is noteworthy that two cytochalasan homodimers with thioether bridge have been reported from *Aspergillus flavipes* before, and thiocytochalasins C (**3**) and D (**4**) exhibit their differences on the type of cytochalasan monomer and non-selective addition at C-22′ in the α,β-unsaturated 1,4-diketone moiety compared to the reported compounds bisaspochalasins B and C [14]. In addition, the significant cytotoxicity of compounds **3** and **4** against five cancer cell lines especially against CT26 cells with IC_{50} values of 0.85 and 0.76 μmol/L, respectively, is better than the previously reported cytochalasin monomer from this strain [9], which might provide an idea to boost the discovery of antitumor candidates for pharmaceutical chemists.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2022.03.063.

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