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# JBIR-137 and JBIR-138, new secondary metabolites from *Aspergillus* sp. fA75

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Natural products are considered to be good sources for the screening of lead compounds of clinical drugs. We performed many drug screenings employing a variety of assay systems with crude extracts of microbial cultures as a traditional natural product library. In some assay systems, effective application of our crude extract library was difficult without making some improvements. From this viewpoint, we started to construct a purified natural compounds library from cultures of microorganisms. To achieve this, we established the high-throughput detection system for microbial secondary metabolites using UPLC-UV-evaporative light-scattering (ELS)-MS system, and more than 1000 compounds, which we have already isolated, were analyzed by the common analytic method and in our database. The registered compounds in microbial cultures are automatically identified with our system, which allows us easily to pick up unregistered compounds. The unregistered compounds are isolated from the cultures and store in our library. Because *Aspergillus* species are known to produce more than 950 documented bioactive compounds such as mevinolin, aflatoxin and citrinin,[1] their secondary metabolites are an important source to obtain various bioactive compounds. Therefore, we attempted to obtain secondary metabolites from cultures of *Aspergillus*. During chemical screening based on our analytic system, we isolated a new janthitrem derivative named JBIR-137 (**1**) and a novel metabolite JBIR-138 (**2**), together with the known compounds, a tremorgenic agent janthitrem B[2] and 6-hydroxycyclopiamine B[3] from the culture of *Aspergillus* sp. fA75 (Figure 1). This paper describes the fermentation, isolation, structural elucidation, and briefly, biological activity of **1** and **2**.

*Aspergillus* sp. fA75 was isolated from a soil sample collected in the forest at Noda, Chiba Prefecture, Japan. The strain was cultivated in 50-ml test tubes, each containing 15 ml potato dextrose medium (24 g $l^{-1}$; BD Biosciences, San Jose, CA, USA). The test tubes were shaken reciprocally (320 r.p.m.) at 27 °C for 2 days. Aliquots (4 ml) of the culture were transferred to 500-ml Erlenmeyer flasks containing brown rice 15 g (Akitakomachi, Yamagata, Japan), bacto–yeast extract 30 mg (BD Biosciences), sodium tartarate 15 mg, $K_2HPO_4$ 15 mg and water 45 ml. The flasks were incubated statically at 27 °C for 14 days.

The culture (10 flasks) was extracted with 80% aq. $Me_2CO$ (200 ml per flask), and the extract was filtered. After concentration *in vacuo*, the aqueous residue was extracted with EtOAc (600 ml × 3). The EtOAc layer was dried over anhydrous $Na_2SO_4$ and evaporated *in vacuo*, yielding a dark brown gum (815 mg). The extract was fractionated using normal-phase medium-pressure liquid chromatography (Purif-Pack SI-30, Shoko Scientific Co., Yokohama, Japan) with a gradient system of *n*-hexane–EtOAc (0–25% EtOAc) followed by the stepwise solvent system of $CHCl_3$–MeOH (0, 2, 5, 10, 20, 30 and 100% MeOH) to obtain five fractions (5% fraction-1, 5% fraction-2, 5% fraction-3, 10 and 20–30%). The fractions were monitored by UPLC-UV-ELS-MS system. Compound **1** was isolated from the 5% MeOH fraction-1 (26.5 mg) by reversed-phase HPLC using a CAPCELL PAK C18 MG II column (5.0 μm, 20 i.d. × 150 mm; Shiseido, Tokyo, Japan) with 85% aq. MeOH containing 0.1% formic acid (flow rate 10 ml $min^{-1}$, Retention time (Rt) = 11.6 min). From the 5% MeOH fraction-2 (89.1 mg), janthitrem B[2] (5.0 mg) was isolated by HPLC preparation. The 5% MeOH fraction-3 (45.6 mg) was applied to gel filtration chromatography (Sephadex LH-20, GE Healthcare BioSciences AB, Uppsala, Sweden) eluting with $CHCl_3$–MeOH (1:1) to yield crude **2** (45.6 mg). The obtained material was further purified by the HPLC (40% aq. MeOH containing 0.1% formic acid, Rt = 17.3 min) to yield pure **2** (9.9 mg). The 6-hydroxycyclopiamine B[3] (3.7 mg) was purified from the 20–30% MeOH fraction (124.7 mg) using LH-20 column chromatography and HPLC.

JBIR-137 (**1**) was obtained as a colorless amorphous solid: $[\alpha]^{22}$-66 (MeOH; *c* 0.13); UV $\lambda_{max}$ nm (ε): 281 (30 300), 290 (32 200) and 374 (5800) in MeOH; IR ($\nu_{max}$): 3400 (hydroxy), 1618, 1455, 1371 (aromatic and pyrrole) $cm^{-1}$. Its molecular formula was determined

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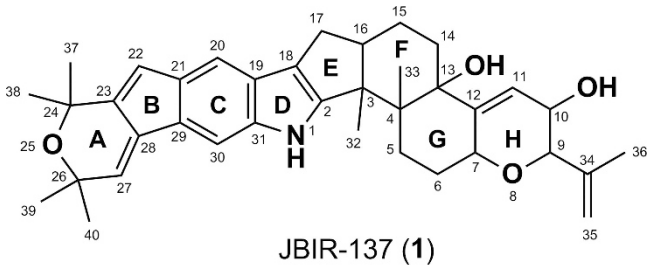


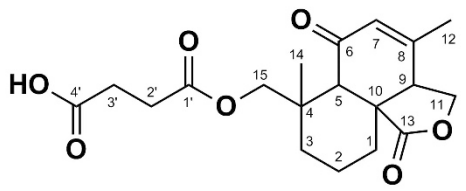


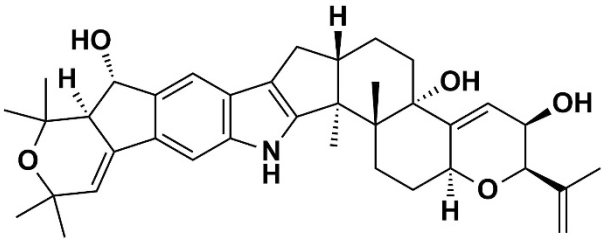


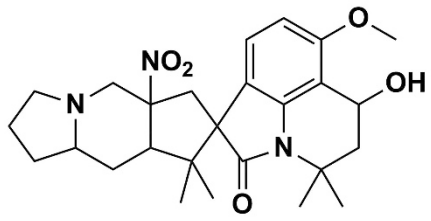


**Figure 1** Structures of **1**, **2**, janthitrem B and 6-hydroxycyclopiamine B.

to be $C_{37}H_{45}NO_4$, with 16 index of hydrogen deficiency by high-resolution ESI-MS (*m/z* 566.3273 $[M–H]^-$, calcd for $C_{37}H_{44}NO_4$: 566.3270). The $^1$H-, $^{13}$C- and heteronuclear single-quantum coherence NMR data (Table 1) showed 37 carbon signals including 7 methyls, 6 methylenes (one olefinic), 9 methines (3 oxygen-bearing, 2 aromatic and 3 olefinic) and 15 quaternary carbons (5 $sp^3$ and 10 $sp^2$). The planar structure of **1** was clarified on the basis of double-quantum-filtered COSY and constant time-HMBC (CT-HMBC)[4] experiments (Figure 2a), as described below.

In the CT-HMBC spectrum, $^1$H–$^{13}$C long-range correlations from a singlet methyl proton $H_3$-36 ($\delta_H$ 1.78) to the oxymethine carbon C-9 ($\delta_C$ 80.3), olefinic quaternary carbon C-34 ($\delta_C$ 143.0) and exo-methylene carbon C-35 ($\delta_C$ 111.5) proved the presence of an isopropenyl group. The sequence from H-9 ($\delta_H$ 3.86) to the olefinic methine H-11 ($\delta_H$ 5.71, $\delta_C$ 118.3) through the oxymethine proton H-10 ($\delta_H$ 3.96, $\delta_C$ 64.0) was observed in the COSY spectrum. In addition to the $^1$H spin system, HMBC correlations from H-9 to the oxymethine carbon C-7 ($\delta_C$ 74.8) and C-11, from H-10 to the olefinic quaternary carbon C-12 ($\delta_C$ 149.4), and from H-11 to C-7 and C-12 revealed a six-membered ether ring (ring H). In consideration of all the above correlations, the structure of ring H was determined to be a 3,6-dihydro-2*H*-pyran bearing an oxygen and an isopropenyl group at C-10 and C-9, respectively. The $^1$H–$^1$H spin systems from methylene protons $H_2$-5 ($\delta_H$ 2.61, $\delta_H$ 1.65) to the oxymethine proton H-7 ($\delta_H$ 4.62) through methylene protons $H_2$-6 ($\delta_H$ 2.18, $\delta_H$ 1.93), together with the HMBC correlations from H-11 to the oxygenated quaternary carbon C-13 ($\delta_H$ 77.6), from $H_2$-6 and $H_2$-5 to the quaternary carbon C-4 ($\delta_C$ 43.5), and from a singlet methyl proton $H_3$-33 ($\delta_H$ 1.03) to C-4, C-5 ($\delta_C$ 27.9) and C-13, indicated that ring G is a cyclohexane bearing an oxygen and a methyl group at C-13 and C-4, respectively. A $^1$H–$^1$H spin-coupling system from methylene protons $H_2$-14 ($\delta_H$ 1.95, $\delta_H$ 1.66) to methylene protons $H_2$-17 ($\delta_H$ 2.64, $\delta_H$ 2.36) through methylene protons $H_2$-15 ($\delta_H$ 2.05, $\delta_H$ 1.66) and a methine proton H-16 ($\delta_H$ 2.80) was observed. $^1$H–$^{13}$C long-range couplings from a singlet methyl proton $H_3$-32 ($\delta_H$ 1.30) to the aromatic quaternary carbon C-2 ($\delta_C$ 154.5), quaternary carbons C-3 ($\delta_C$ 51.8), C-4 and the methine carbon C-16 ($\delta_C$ 50.7), from the methylene protons $H_2$-14 to the oxymethine carbon C-13, and from the methylene protons $H_2$-17 to the aromatic quaternary carbons C-2 and C-18 ($\delta_C$ 117.6), indicated the ring moieties E and F.

Strong *m*-couplings from the aromatic proton H-20 ($\delta_H$ 7.11) to aromatic carbons C-29 ($\delta_C$ 128.6) and C-31 ($\delta_C$ 139.2), and from the aromatic proton H-30 ($\delta_H$ 7.48) to aromatic carbons C-19 ($\delta_C$ 126.0) and C-21 ($\delta_C$ 136.9), allowed the assignment of a benzene-ring substructure (ring C). The $^1$H–$^{13}$C long-range couplings from the aromatic proton H-20 to C-18 and C-19 indicated that the benzene-ring moiety was substituted at C-18.

The remaining substructures were established as follows. Singlet methyl protons $H_3$-37/$H_3$-38 ($\delta_H$ 1.51) were long-range coupled to each other and to the oxygenated quaternary carbon C-24 ($\delta_C$ 74.6) and aromatic quaternary carbon C-23 ($\delta_C$ 141.6). Another set of singlet methyl protons $H_3$-39/$H_3$-40 ($\delta_H$ 1.44) were long-range coupled to each other and to the oxygenated quaternary carbon C-26 ($\delta_C$ 74.0) and the aromatic methine carbon C-27 ($\delta_C$ 128.2). The aromatic methine proton H-27 ($\delta_H$ 6.47) was long-range coupled to the aromatic carbons C-23, C-28 ($\delta_C$ 135.2) and C-29, revealing the sequence from C-24 to C-26 through C-23, C-28 and C-27, and showed the substituted position of C-28 to be at C-29. In addition to these correlations, $^1$H–$^{13}$C long-range couplings from the aromatic proton H-22 ($\delta_H$ 6.36) to aromatic carbons C-21, C-23, C-28 and C-29 indicated the formation of a 5-membered ring structure (ring B). The HMBC correlations from the aromatic protons H-20 and H-30 to the aromatic carbons C-22 and C-28, respectively, also supported these connections. Finally, the molecular formula of **1** and the $^{13}$C chemical shifts at C-24 and C-26 indicated a 2,2,6,6-tetramethyl-3,6-dihydro-2*H*-pyran and an indole-like moieties (rings C and D). Thus, the planar structure of **1** was revealed as shown in Figure 1. The structure of **1** is closely related to the janthitrems that were reported as tremorgenic mycotoxins.[5]

The partial relative configuration was determined from the NOESY spectrum and the corresponding coupling constants. A small coupling constant between H-9 and H-10 ($<1$ Hz) and strong NOE between H-9 and H-10 determined the relative configuration of the ring H as shown in Figure 2b. In the same manner, the NOESY correlations among H-9, H-10, H-7, H-5a, H-32 and H-15a indicated that these protons are located on the same side in the molecule. On the other hand, the NOEs among H-33, H-6b, H-14b and H-16 showed that these protons are on the opposite side from $CH_3$-32. Consequently, the relative configuration of ring E-H was established as shown in Figure 2b.

**Table 1** $^{13}C$ and $^{1}H$ NMR spectroscopic data for JBIR-137 (1) and JBIR-138 (2)

| *1* | | | *2* | | |
|---|---|---|---|---|---|
| *Position* | $\delta_C$ | $\delta_H$, *mult (J in Hz)* | *Position* | $\delta_C$ | $\delta_H$, *mult (J in Hz)* |
| 2 | 154.5 | | 1 | 33.8 | a, 2.34, br d (13.2) |
| 3 | 51.8 | | | | b, 1.73, ddd (4.2, 13.8, 13.8) |
| 4 | 43.5 | | 2 | 18.9 | a, 1.60, br d (10.8) |
| 5 | 27.9 | a, 2.61, br dd (5.0, 13.5) | | | b, 1.55, m |
| | | b, 1.65, ovl[f] | 3 | 36.1 | a, 1.96, br d (13.2) |
| 6 | 29.0 | a, 2.18, m | | | b, 1.05, ddd (1.8, 13.2, 13.2) |
| | | b, 1.93, m | 4 | 37.2 | |
| 7 | 74.8 | 4.62, dd (1.5, 9.0) | 5 | 55.5 | 2.82, s |
| 9 | 80.3 | 3.86, br s | 6 | 197.4 | |
| 10 | 64.0 | 3.96, br d (6.0) | 7 | 131.1 | 5.91, s |
| 11 | 118.3 | 5.71, dd (1.5, 6.0) | 8 | 156.7 | |
| 12 | 149.4 | | 9 | 54.1 | 3.14, d (6.0) |
| 13 | 77.6 | | 10 | 49.0 | |
| 14 | 34.7 | a, 1.95, m | 11 | 68.0 | a, 4.63, dd (6.0, 10.2) |
| | | b, 1.66, ovl[f] | | | b, 4.38, d (10.2) |
| 15 | 22.1 | a, 2.05, m | 12 | 20.7 | 1.99, s |
| | | b, 1.66, ovl[f] | 13 | 178.9 | |
| 16 | 50.7 | 2.80, m | 14 | 27.4 | 1.23, s |
| 17 | 28.0 | a, 2.64, dd (8.0, 10.0) | 15 | 66.6 | a, 5.15, d (11.4) |
| | | b, 2.36, dd (10.0, 13.0) | | | b, 4.66, d (11.4) |
| 18 | 117.6 | | 1′ | 174.3 | |
| 19 | 126.0 | | 2′ | 30.0[g] | 2.60[h], br s |
| 20 | 110.6 | 7.11, s | 3′ | 30.4[g] | 2.62[h], br s |
| 21 | 136.9 | | 4′ | 176.2 | |
| 22 | 122.8 | 6.36, d (2.0) | | | |
| 23 | 141.6 | | | | |
| 24 | 74.6 | | | | |
| 26 | 74.0 | | | | |
| 27 | 128.2 | 6.47, d (2.0) | | | |
| 28 | 135.2 | | | | |
| 29 | 128.6 | | | | |
| 30 | 105.4 | 7.48, s | | | |
| 31 | 139.2 | | | | |
| 32 | 16.6 | 1.30, s | | | |
| 33 | 20.13[a] | 1.03, s | | | |
| 34 | 143.0 | | | | |
| 35 | 111.5 | a, 5.14, br s | | | |
| | | b, 4.96, br s | | | |
| 36 | 20.12[a] | 1.78, s | | | |
| 37 | 31.8[b] | 1.51[d], s | | | |
| 38 | 31.8[b] | 1.51[d], s | | | |
| 39 | 31.2[c] | 1.44[e], s | | | |
| 40 | 31.2[c] | 1.44[e], s | | | |

NMR spectra were taken on a Varian NMR System 500 NB CL in MeOH-$d_4$ with the residual solvent peak as an internal standard ($\delta_C$ 49.0, $\delta_H$ 3.31 p.p.m.).
[a−e, g,h]Interchangeable.
[f]Overlapped with other signals.

JBIR-138 (**2**) was obtained as a colorless amorphous solid: $[\alpha]^{22}_D$ −31 (MeOH; *c* 0.5); UV $\lambda_{max}$ nm (ε): 230 (13 900) in MeOH. The molecular formula of **2** was established as $C_{19}H_{24}O_7$ (index of hydrogen deficiency = 8) by high-resolution ESI-MS (*m/z* 363.1447 $[M–H]^-$, calcd for $C_{19}H_{23}O_7$ 363.1444). The IR absorption ($\nu_{max}$ 3446, 1766, 1724 and 1674 $cm^{-1}$) indicated the presence of hydroxy, γ-butyrolactone, carboxylic acid and α,β-unsaturated ketone functional groups. The assignments of the $^{1}H$ and $^{13}C$ NMR spectroscopic data were tabulated in Table 1.

The COSY spectrum showed a sequence from methylene protons $H_2$-1 ($\delta_H$ 2.34, $\delta_H$ 1.73) to methylene protons $H_2$-3 ($\delta_H$ 1.96, $\delta_H$ 1.05) through methylene protons $H_2$-2 ($\delta_H$ 1.60, $\delta_H$ 1.55), and a $^{1}H$ spin coupling between a methine proton H-9 ($\delta_H$ 3.14) and a methylene proton H-11a ($\delta_H$ 4.63). The CT-HMBC spectrum showed $^{1}H$–$^{13}C$ long-range correlations (Figure 2c) as follows: from a singlet methyl proton $H_3$-14 ($\delta_H$ 1.23) to an $sp^3$ quaternary carbon C-4 ($\delta_C$ 37.2), a methylene carbon C-3 ($\delta_C$ 36.1), a methine carbon C-5 ($\delta_C$ 55.5) and an oxymethylene carbon C-15 ($\delta_C$ 66.6); from $H_2$-2 to C-4 and a quaternary carbon C-10 ($\delta_C$ 49.0); and from $H_2$-1 to C-10 and C-5. These correlations indicated the presence of a cyclohexane ring bearing a methyl group and an oxymethylene group at the C-4 position. The strong HMBC correlations from the allylic methyl

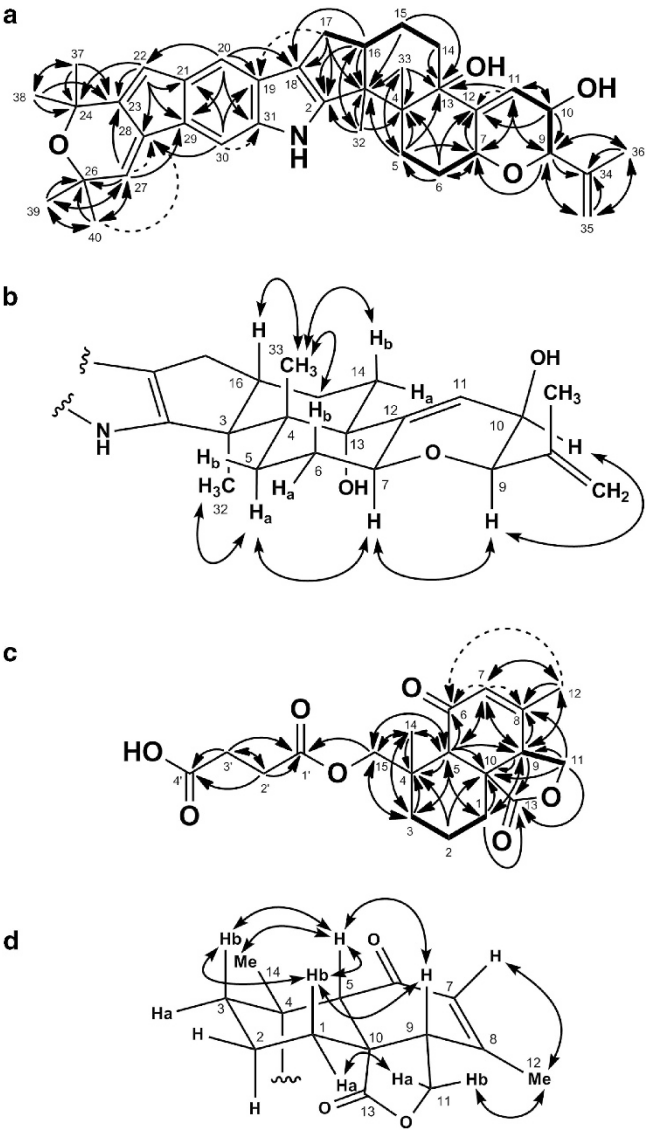


**Figure 2** (**a**) Key COSY and HMBC correlations of **1**. COSY: bold line; HMBC ($^1$H to $^{13}$C): solid arrow ($J_{CH}=8$ Hz) and dashed arrow ($J_{CH}=3$ Hz). (**b**) Partial relative configuration of **1**. (NOESY correlation: arrow) (**c**) Key COSY and HMBC correlations of **2**. COSY: bold line; HMBC: solid arrow (strong) and dashed arrow (weak). (**d**) Key NOESY correlations of **2**.

proton $H_3$-12 ($\delta_H$ 1.99) to the olefinic methine carbon C-7 ($\delta_C$ 131.1), the deshielded olefinic quaternary carbon C-8 ($\delta_C$ 156.7), and the methine carbon C-9 ($\delta_C$ 54.1), and weak correlations from $H_3$-12 and the olefinic methine proton H-7 ($\delta_H$ 5.91) to the $\alpha,\beta$-unsaturated ketone carbonyl carbon C-6 ($\delta_C$ 197.4), showed a sequence from C-6 to C-9. The direct connectivity between C-5 and C-6 was revealed by HMBC correlations from the singlet methine proton H-5 ($\delta_H$ 2.82) to C-6 and C-7, which showed an octalone moiety. The $^1$H–$^1$H spin coupling between H-9 and H-11a together with the common $^1$H–$^{13}$C long-range couplings from these protons to C-8, C-10 and the carbonyl carbon C-13 ($\delta_C$ 178.9) indicated a $\gamma$-lactone moiety, the presence of which was supported by the IR absorption at 1766 cm$^{-1}$ *vide ante*. Additionally, the long-range correlation from H-1 to C-13 demonstrated the direct connectivity between C-13 and C-10, establishing the condensation of the octalone and $\gamma$-butyrolactone ring moieties. Thus, a sesquiterpene structure was determined (Figure 2c). A succinic acid moiety was elucidated from the HMBC correlations from methylene protons $H_2$-2′ ($\delta_H$ 2.60) and $H_2$-3′ ($\delta_H$ 2.62) to carbonyl carbons C-1′ ($\delta_C$ 174.3) and C-4′ ($\delta_C$ 176.2). An ester linkage between C-15 and C-1′ was revealed by the HMBC correlation from the oxymethylene protons $H_2$-15 ($\delta_H$ 5.15, $\delta_H$ 4.66) to C-1′. Thus, the gross structure of **2** was elucidated (Figure 1).

The relative configuration of the sesquiterpene moiety of **2** was determined by the NOESY spectrum. NOESY correlations among H-1b, H-3b, H-5, H-9 and H-14 suggested that these protons are on the same face of the molecule. The correlations between H-1a and H-11a implied the relative configuration of the $\gamma$-butyrolactone ring shown in Figure 2d. To the best of our knowledge, the backbone of **2** has not yet been reported as a secondary metabolite produced by microorganisms.

The cytotoxic activities of **1**, **2**, janthitrem B and 6-hydroxycyclopiamine B against human ovarian adenocarcinoma SKOV-3 cells were examined by using the WST-8 ((2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) colorimetric assay (Cell Counting Kit; Dojindo, Kumamoto, Japan). After administered the compounds for 72 h, **1** and janthitrem B exhibited weak cytotoxic activities against SKOV-3 cells with the $IC_{50}$ of 12.5 and 37.6 μM, respectively. To the contrary, **2** and the 6-hydroxycyclopiamine B did not show cytotoxicity ($IC_{50}>100$ μM). Although janthitrem B and its derivatives were known as tremorgenic agents, their cytotoxic effects have not been reported. Further studies on biological activities of **1** are under way.

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