The Journal of Antibiotics (2010) 63, 251–253
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NOTE

Allantopyrone A, a new α-pyrone metabolite with potent cytotoxicity from an endophytic fungus, *Allantophomopsis lycopodina* KS-97

Yoshihito Shiono[1], Miwa Yokoi[1], Takuya Koseki[1], Tetsuya Murayama[1], Nobuhiro Aburai[2] and Ken-ichi Kimura[2]

The Journal of Antibiotics (2010) **63,** 251–253; doi:10.1038/ja.2010.18; published online 12 March 2010

Keywords: *Allantophomopsis lycopodina*; Allantopyrone A; apoptosis; endophytic fungus; islandic acids; α-pyrone

An endophyte is a fungal or bacterial microorganism that can exist within the living tissue of most plant species without causing pathogenic effects. An endophyte produces a great number and diversity of classes of biologically derived compounds within the host plant, which have been implicated in the protection of its host against pathogens and herbivores.[1] Endophytic microorganisms are an excellent source of structurally diverse molecules that have potential therapeutic value.[2] Our previous investigation of endophytic fungi isolated from plants in Japan resulted in the isolation of some bioactive and structurally unique metabolites.[3–5] In our continuing efforts to discover biologically active compounds among the secondary metabolites of endophytes, we have investigated the fungal strain *Allantophomopsis lycopodina* KS-97, isolated from a tree branch. In this paper, we describe the isolation, structural elucidation, cytotoxicity and the preliminary apoptosis-inducing activity of a new compound, allantopyrone A (**1**), and of a known compound, islandic acid-II methyl ester (**2**) (Figure 1a).

KS-97 was isolated from a dead beech branch collected in April 2005 on the periphery of the Gassan stock farm in Yamagata, Japan. The producing strain KS-97 was cultivated on sterilized unpolished rice (20 g per Petri dish ×50; total of 1000 g) at 25 °C for 4 weeks. Purification of the fungal metabolites was guided by UV absorption and the characteristic coloration by TLC. An acetone extract from the fermented and unpolished rice was concentrated and then partitioned between ethyl acetate (EtOAc) and water. The purification of the EtOAc layer was guided by the intense characteristically blue coloration with a vanillin–sulfuric acid solution on the TLC plates. The EtOAc layer was chromatographed on a silica gel column using a gradient of *n*-hexane-EtOAc (0–100) to give fractions 1–11 (Fr. 1-1 to 1-11).

Fr. 1–7 (*n*-hexane-EtOAc, 40:60, 0.8 g) was subjected to silica gel column chromatography by eluting with $CHCl_3$ and an increasing ratio of EtOAc (0–100). Eleven fractions (Fr. 2-1 to 2-11) were obtained. Fr. 2–6 ($CHCl_3$-EtOAc, 50:50, 0.05 g) was subjected to octa decyl silyl (Fuji Silysia, Chemical, Kasugai, Japan) column chromatography using a mixture of MeOH–H_2O (80:20) to afford compound **1** (15 mg). Fr. 2–5 ($CHCl_3$-EtOAc, 40:60, 0.04 g) was purified by octa decyl silyl column chromatography using a mixture of MeOH–H_2O (80:20) to afford compound **2** (1.5 mg).

The structure of islandic acid-II methyl ester (**2**) (Figure 1a) was established on the basis of FAB–MS, ^{1}H-, ^{13}C-NMR, ^{1}H–^{1}H COSY, ^{13}C–^{1}H COSY and HMBC data. The ^{1}H-NMR and IR data were identical to those previously reported in literature.[6]

Allantopyrone A (**1**) (Figure 1a) was obtained as a yellow powder. The molecular formula of **1** was deduced as $C_{17}H_{20}O_7$ from HR–FAB–MS, requiring eight degrees of unsaturations. The UV spectrum of **1** exhibited absorption maxima at 230, 260 and 330 nm, which suggests that it contains a conjugated double-bond system in its structure. The IR spectral data showed absorption bands at 3478 and 1714 cm^{-1}, indicating the presence of hydroxyl and ester groups, respectively. The ^{13}C-NMR (Table 1) and DEPT spectra in $CDCl_3$ of **1** indicated the presence of two methyls, three sp^3 methylenes, six sp^2 methines and six sp^2 quaternary carbons including two carbonyl carbons. Based on the analysis of the ^{1}H- and ^{13}C-NMR data (Table 1) combined with the ^{13}C–^{1}H COSY spectrum, the presence of two *trans*- and one *cis*- olefins (δ_H 5.53 (1H, d, J=11.4 Hz), 6.11 (1H, m), 6.56 (1H, t, J=11.4 Hz), 6.70 (1H, d, J=15.4 Hz), 6.87 (1H, dt, J=15.4, 4.1 Hz), 7.37 (1H, dd, J=14.9, 11.4 Hz), δ_C 114.1, 116.6, 128.1, 140.0, 141.2, 146.2), two singlet and one multiplet methylenes attached to an oxygen atom (δ_H 4.38 (2H, m), 4.51 (2H, s), 5.08 (2H, s), δ_C 54.0, 56.4, 61.9), one methoxyl group (δ_H 4.12 (3H, s), δ_C 62.6) and one olefinic doublet methyl (δ_H 1.87 (3H, d, J=6.7 Hz), δ_C 18.6) was detected. The results of the ^{1}H–^{1}H COSY experiment with **1** revealed

[1]Department of Bioresource Engineering, Faculty of Agriculture, Yamagata University, Yamagata, Japan and [2]Laboratory of Chemical Biology, Faculty of Agriculture, Iwate University, Iwate, Japan
Correspondence: Dr Y Shiono, Department of Bioresource Engineering, Faculty of Agriculture, Yamagata University, 1-23 Wakabamachi, Tsuruoka, Yamagata 997-8555, Japan.
E-mail: yshiono@tds1.tr.yamagata-u.ac.jp
Received 3 October 2009; revised 14 January 2010; accepted 10 February 2010; published online 12 March 2010

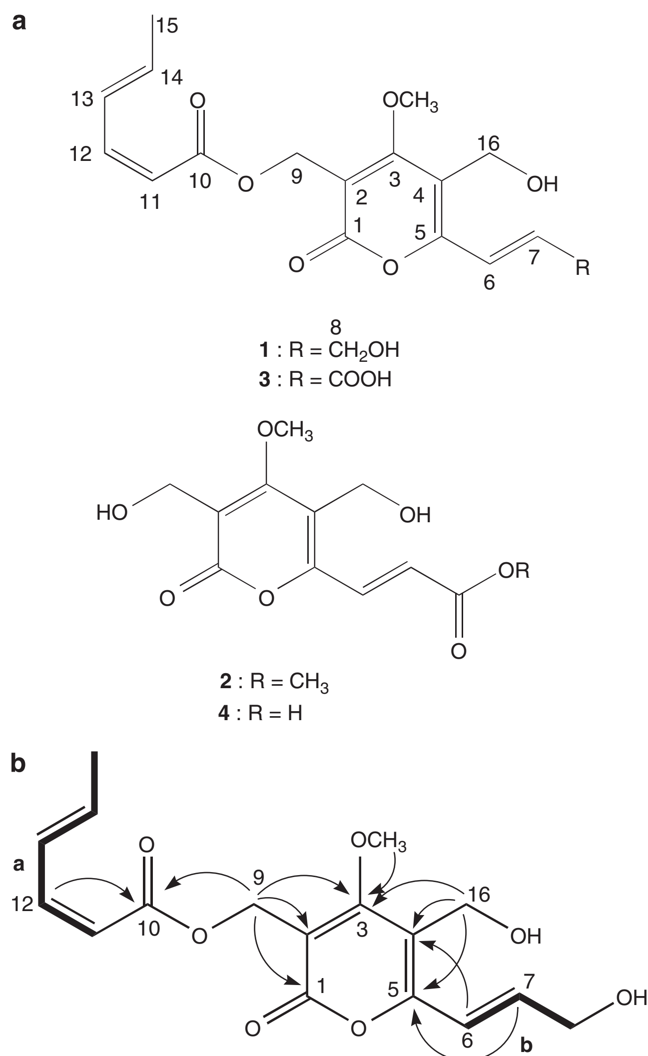

Figure 1 Structures of compounds **1**, **2**, **3** and **4** (**a**), and ^{1}H–^{1}H COSY (bold lines) and HMBC (arrows) correlations observed for **1** (**b**).

two partial structures (**a** and **b**), which are indicated by bold lines in Figure 1b. In the HMBC spectrum of **1** (Figure 1b), HMBCs from the singlet methylene (H-9) to C-1, C-2 and C-3, from the other singlet methylene (H-16) to C-3, C-4 and C-5, from the methoxyl group to C-3, and from the allylic protons (H-6 and H-7) to C-5 indicated that the signals were accommodated on a 3,5,6-trisubstituted 4-methoxy-2-pyrone ring in which the partial structure **b** was incorporated. Furthermore, the assignment of the 2-pyrone ring (α-pyrone) was also confirmed by the diagnostic ^{13}C resonances at δ_C 164.0, 106.1, 171.2, 113.1 and 157.4, and by the typical lactone IR band at 1666 cm^{-1}.[7,8] In addition, HMBC from H-12 to C-10 suggested that there is a methyl (2*Z*,4*E*)-hexadienoyl moiety (**a**) in this molecule. Furthermore, the substitutional downfield shift for H-9 and the HMBC between H-9 and C-10 revealed the location of the hexadienoyl to be at C-9. Therefore, the gross structure of **1** was determined to be that shown in Figure 1a, which, to our knowledge, is the first report of this fungal metabolite.

A similar compound, islandic acid-I (**3**) (Figure 1a), was previously isolated from *Penicillium islandicum* Sopp. as a mycotoxin.[9] Subsequently, further chemical study of the fungus led to the isolation of islandic acid-II (**4**) (Figure 1a). Islandic acid-I (**3**) showed a 100% inhibition of cell growth against Yoshida sarcoma cells at a concentration of 100 μg/ml (286 μM). In addition, the methyl ester of **3** showed a 100% growth inhibition at 1.0 μg/ml (2.7 μM), which was 100-fold higher than the activity of unmodified **3**. In our study, the cytotoxicities of **1** and **2** were evaluated in HL60 cells by using the MTT assay and showed cytotoxicity against HL60 cells at IC_{50} values of 0.32 and 6.55 μM, respectively.[10] Further, in order to determine the possible involvement of **1** and **2** in apoptosis induction, we evaluated the generation of the typical DNA ladder pattern, which represents internucleosomal fragmentation and is observed when cells undergo apoptosis.[10] HL60 cells treated with either **1** or **2** for 6 h manifested the characteristic DNA fragmentation pattern by apoptosis dose dependently (Figure 2). Although both compounds have an α,β-unsaturated carbonyl group in the molecule, the cytotoxicity and apoptosis-inducing activity of **1** were 20 times stronger than those of **2**, suggesting that the reactivity of **1** against a molecular target in HL60 cells is stronger than that of **2**.

Table 1 ^{1}H- and ^{13}C-NMR data for **1**

No.	δ_C	δ_H	*HMBC*
1	164.0 s		
2	106.1 s		
3	171.2 s		
4	113.1 s		
5	157.4 s		
6	140.0 d	6.70 (1H, d, 15.4)	4, 5, 8
7	116.6 d	6.87 (1H, dt, 15.4, 4.1)	5
8	61.9 t	4.38 (2H, m)	6
9	56.4 t	5.08 (2H, s)	1, 2, 3, 10
10	166.1 s		
11	114.1 d	5.53 (1H, d, 11.4)	12, 13
12	146.2 d	6.56 (1H, t, 11.4)	10, 14
13	128.1 d	7.37 (1H, dd, 14.9, 11.4)	11, 15
14	141.2 d	6.11 (1H, m)	12, 15
15	18.6 q	1.87 (3H, d, 6.7)	13, 14
16	54.0 t	4.51 (2H, s)	3, 4, 5
OMe	62.6 q	4.12 (3H, s)	3

^{13}C- (100 MHz) and ^{1}H- (400 MHz) NMR spectra were taken on JEOL NMR system EX-400 (JEOL Ltd, Tokyo, Japan) in $CDCl_3$; the solvent peak was used as internal standard (δ_C 77.0, δ_H 7.26); and values in parentheses are coupling constants in Hz.

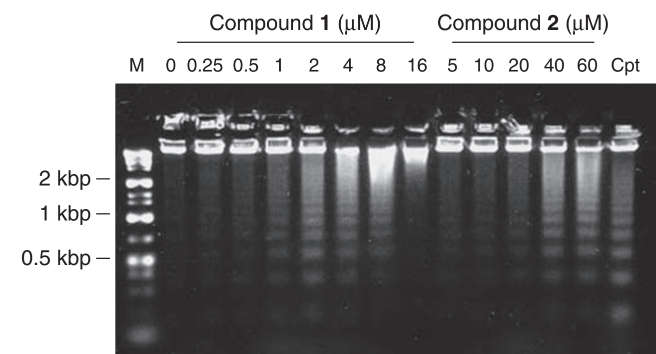

Figure 2 DNA fragmentations induced by compounds **1** or **2** in HL60 cells. Cells (5×10^5 cells ml^{-1}) were treated with indicated concentrations of **1** or **2** for 6 h. DNA was analyzed by 2% agarose gel electrophoresis and DNA ladder was visualized by staining the gel with ethidium bromide. The left side of the lane is a DNA size marker, and the right side shows positive control cells treated with 200 nM camptothecin (Cpt).

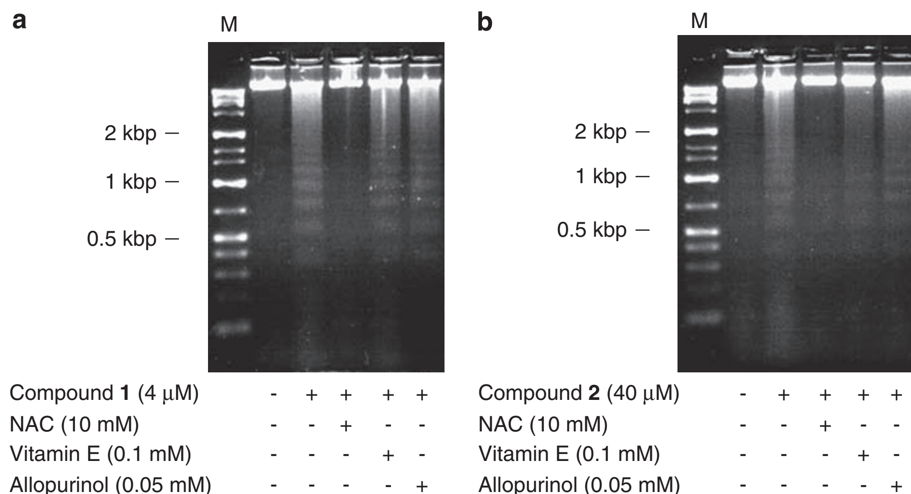

Figure 3 Effect of various anti-oxidant agents on the DNA fragmentation induced by compounds **1** (**a**) or **2** (**b**) in HL60 cells. The method is the same as described in Figure 2, except for the addition of various anti-oxidant agents before 1 h of the reaction with **1** (4 μM) or **2** (40 μM).

As *N*-acetyl-L-cysteine (NAC) is known to bind to the α,β-unsaturated carbonyl group by Michael-type addition, and also possesses anti-oxidant activity, we tested the effect of NAC on **1**- or **2**-induced apoptosis in HL60 cells. NAC showed inhibitory activity, but **1**-induced apoptosis was not inhibited by other anti-oxidant agents, vitamin E or allopurinol.[11,12] When the cells were treated with **2**, apoptosis was suppressed not only by NAC but also by vitamin E partially (Figure 3b). After 1 h of mixing with NAC methyl ester and with **1** in pH 7.5 aqueous buffered solution, a new peak (retention time: 2.7 min) was observed following the peak decrease of **1** (retention time: 14.7 min) in HPLC analysis (Capcell pak C_{18} column 150×4.6 mm inner diameter (Shiseido, Tokyo, Japan); flow rate: 1.0 ml min^{-1}; mobile phase: MeOH–H_2O (45:55, v/v) for **1** or MeOH–H_2O (30:70, v/v) for **2**). However, the peak of **2** on HPLC (retention time: 12.0 min) was unchanged under the same method as **1**. Although the mechanism for inducing apoptosis in **1** is not fully understood, these results show a different mechanism of apoptosis for each.

1: Yellow amorphous powder; UV (MeOH) λ_{max} (ε)=230 nm (31 000), 260 nm (19 000), 330 nm (12 000), IR (KBr) $\nu_{max}\chi\mu^1$=3475, 2878, 2845, 1714, 1666, 1637, 1165, 952, FAB–MS: *m/z* 337 $(M+H)^+$, HR–FAB–MS *m/z* 337.1287 (calcd for $C_{17}H_{20}O_7$+H, 337.1287), for ^{1}H- and ^{13}C-NMR, see Table 1.

ACKNOWLEDGEMENTS

We thank Ms Teiko Yamada of the Faculty of Agriculture at Tohoku University for the HR–FAB–MS measurements.

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