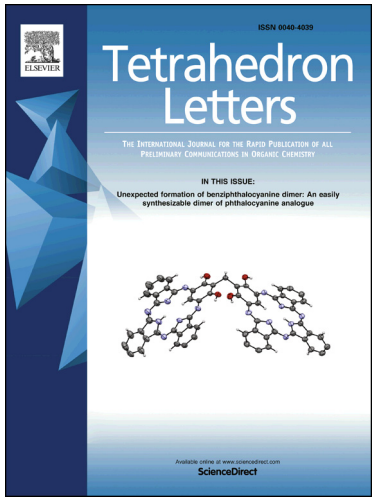

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Nectrianolins A, B, and C, new metabolites produced by endophytic fungus *Nectria pseudotrichia* 120-1NP

Nanang Rudianto Ariefta[a, b, *], Priska Kristiana[b, c], Handojo Hadi Nurjanto[d], Hiroyuki Momma[e], Eunsang Kwon[e], Tatsuya Ashitani[b], Keitaro Tawaraya[b], Tetsuya Murayama[b], Takuya Koseki[b], Hiroshi Furuno[f], Narandulam Usukhbayar[a], Ken-ichi Kimura[a], Yoshihito Shiono[a, b, *]

[a]The United Graduate School of Agricultural Sciences, Iwate University, Morioka, Iwate 020-8550, Japan

[b]Department of Food, Life, and Environmental Science, Faculty of Agriculture, Yamagata University, Tsuruoka, Yamagata 997-8555, Japan

[c]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada, Bulaksumur, Yogyakarta 55281, Indonesia

[d]Department of Silviculture, Faculty of Forestry, Universitas Gadjah Mada, Bulaksumur, Yogyakarta 55281, Indonesia

[e]Research and Analytical Center for Giant Molecules, Graduate School of Science, Tohoku University, Sendai, Miyagi 980-8576, Japan

[f]Institute for Materials Chemistry and Engineering (IMCE), Kyushu University, Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

[*] Corresponding authors. Tel. /Fax: +81-235-28-2873. E-mail: nanang.ariefta@gmail.com (N. R. Ariefta); e-mail: yshiono@tds1.tr.yamagata-u.ac.jp (Y. Shiono)

Abstract

Two sesquiterpene-epoxycyclohexenone conjugates, nectrianolins A (**1**) and B (**2**), together with a sesquiterpene, nectrianolin C (**3**), were isolated from the brown rice culture of *Nectria pseudotrichia* 120-1NP, an endophytic fungus isolated from *Gliricidia sepium*. Their structures were determined on the basis of 1D-/2D-NMR spectroscopy and HRESIMS data analyses in combination with chemical means. Nectrianolins A-C (**1**-**3**) exhibited cytotoxic activity against both HL-60 and HeLa cells.

Keywords

Endophyte; *Nectria pseudotrichia*; Cytotoxicity; Nectrianolins.

Introduction

Endophytes are ubiquitous microorganisms that are found in all plant species.[1] These microorganisms maintain a balance symbiotic relationship with their hosts, *i.e.*, this association is apparently asymptomatic and avirulent.[2] Secondary metabolites produced by endophytes are rich sources of diverse and pharmacologically active metabolites.[2–4] Environmental factors (*e.g.*, light, temperature, pH, and nutrients) play an important role in production of secondary metabolites by endophytes. These factors are associated with pathogenicity and fungal tolerance to stress including drought or heat stress.[5] The Indonesian tropical forest has a large diversity of plants due to its complex geological history, large number of islands, and tropical climate. Thus, the Indonesian tropical forest is a rich potential source for the isolation of endophytic fungi and their associated metabolites.[6] Several strains of endophytic fungi were isolated by examining different trees in the Wanagama forest of Indonesia. One strain 120-1NP was isolated from the inner tissue of *Gliricidia sepium* healthy stem, which was subsequently identified as *Nectria pseudotrichia*. The *Nectria* genus is a

saprophyte and considered as a phytopathogen that induced both stem-end rot of avocadoes[7] and canker on trees.[8] However, this genus is also a rich source of interesting secondary metabolites with diverse structures and biological activities.[8–10] Purification of the extract derived from fungal culture of *N. pseudotrichia* 120-1NP led to the isolation of the following three novel compounds: two sesquiterpene-epoxycyclohexenones **1** and **2**, along with the sesquiterpene congener **3**. In this study, we also presented the comprehensive structural elucidation and characterization of these compounds.

Results and Discussion

Compound **1**[11] was obtained as a colorless crystal. Its molecular formula was determined to be $C_{22}H_{32}O_5$ through a combination of HRESIMS (*m/z* 399.2142 $[M+Na]^+$) and NMR data. The molecular formula of **1** revealed seven degrees of unsaturation. The IR absorptions at 3332 and 1677 cm^{-1} suggested the presence of hydroxyl and α,β-unsaturated carbonyl groups. The ^{13}C NMR results (Table 1) along with the HMQC data of **1** revealed the presence of 22 carbon resonances and confirmed the presence of four methyls, five methylenes, seven methines, one ketone carbonyl, and five quaternary carbons. The ^1H NMR spectroscopic data (Table 1) showed proton resonances, which include a doublet methyl at δ_H 0.68 (3H, d, J = 6.9 Hz, H-14), two singlet methyls at δ_H 0.70 (3H, s, H-15) and 1.50 (3H, s, H-13), an aliphatic methine at δ_H 1.53 (1H, m, H-5), two methylenes at δ_H 1.29 (2H, m, H-4), 1.85 (1H, m, H-3a), and 1.89 (1H, m, H-3b), and an olefinic methine at δ_H 5.36 (1H, s, H-2). These proton resonances are consistent with the presence of a substituted cyclohexene ring. The double bond position was assigned by HMBC correlations from the olefinic methyl (H-13) to two sp^2 carbons at δ_C 139.4 (C-1) and 124.5 (C-2) and to the quaternary carbon at δ_C 40.4 (C-6). Additional HMBC correlations from H-2, H-4, H-5, H-14, and H-15 to C-6 confirmed that the structure of **1** contained a 1,5,6-trimethyl-cyclohexene moiety (Fig. 1). The presence of a

3-methyl-2-penten-1-ol moiety was clarified using HMBC correlations from Me-12 to C-8, C-9 and C-10, and from H-11 to C-9 and C-10.

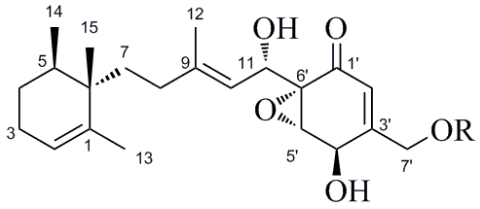
Table 1
^{1}H (600 MHz) and ^{13}C NMR (150 MHz) spectroscopic data of **1**-**3** in pyridine-d_5

Pos.	Nectrianolin A (**1**)		Nectrianolin B (**2**)		Nectrianolin C (**3**)	
	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
1	139.4, C		139.5, C		137.5, C	
2	124.5, CH	5.36, s	124.5, CH	5.37, s	125.7, CH	5.69, m
3	25.7, CH_2	a: 1.85, m b: 1.89, m	25.7, CH_2	a: 1.85, m b: 1.89, m	25.8, CH_2	a: 1.82, m b: 1.88, m
4	27.1, CH_2	1.29, m	27.1, CH_2	1.29, m	27.2, CH_2	1.31, m
5	33.3, CH	1.53, m	33.3, CH	1.53, m	33.4, CH	1.62, m
6	40.4, C		40.5, C		40.5, C	
7	34.8, CH_2	a: 1.36, td (5.5, 13.1) b: 1.42, td (3.4, 13.7)	34.8, CH_2	a: 1.37, m b: 1.42, m	35.1, CH_2	a: 1.41, m b: 1.46, m
8	34.6, CH_2	a: 1.56, m b: 1.81, m	34.7, CH_2	a: 1.58, m b: 1.82, m	34.3, CH_2	a: 1.88, m b: 1.91, m
9	141.2, C		141.2, C		139.6, C	
10	124.1, CH	5.68, d (8.9)	123.9, CH	5.66, d (9.6)	124.5 CH	5.39, br s
11	63.4, CH [a]	5.63, d (8.9)	63.5, CH	5.61, d (8.9)	58.9, CH_2	4.39, d (6.9)
12	17.2, CH_3	1.92, s	17.3, CH_3	1.93, s	16.6, CH_3	1.67, s
13	19.2, CH_3	1.50, s	19.2, CH_3	1.51, s	19.3, CH_3	1.52, s
14	15.8, CH_3	0.68, d (6.9)	15.9, CH_3	0.68, d (6.2)	15.9, CH_3	0.73, d (6.1)
15	20.9, CH_3	0.70, s	20.9, CH_3	0.71, s	21.1, CH_3	0.76, s
1’	194.6, C		194.1, C			
2’	121.1, CH	6.78, s	122.6, CH	6.49, s		
3’	162.3, C		154.9, C			
4’	63.4, CH [a]	5.11, s	64.3, CH	5.13, s		
5’	60.4, CH	4.58, s	60.4, CH	5.09, s		
6’	61.7, C		61.6, C			
7’	63.4, CH_2	a: 4.64, d (17.2) b: 5.02, d (17.2)	63.4, CH_2	a: 5.12, d (16.8) b: 5.38, d (16.8)		
1”			171.5, C			
2”			44.7, CH_2	a: 2.61, dd (4.8, 15.1) b: 2.71, dd (8.2, 15.1)		
3”			63.3, CH	4.55, m		
4”			24.0, CH_3	1.33, d (6.2)		

[a] Overlapped signals.

The HMBC correlation from H-7 to C-6 indicated that the pentenol was located at C-6. Further analysis of the HMBC of **1** revealed correlations from H-2’ to C-6’, H-4’ to C-2’ along with C-5’ and H-7’ to C-3’ and C-2’. These data suggested the presence of an oxygenated cyclohexenone skeleton as well as hydroxymethyl and hydroxyl moieties. The presence of an epoxy group at C-5’ and C-6’ was deduced from the chemical shift of the ^{13}C

NMR signals at these positions. The presence of this epoxy group was also supported by the ^{1}H NMR data acquired in CD_3OD since H-5' appeared at the epoxy region (δ_H 3.73 d, J = 1.2 Hz), compared to H-4', which appeared at the hydroxyl region (δ_H 4.47 s). The HMBC correlations from H-11 to C-5' and C-6' indicated a link between C-11 and C-6' of the cyclohexenone moiety (Fig. 2).



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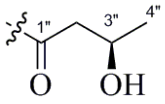
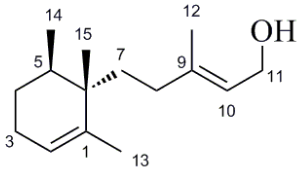
2 R= 



3

Fig. 1. Structures of **1**-**3**.

The relative configuration of the cyclohexene ring in **1** was determined on the basis of ^{13}C NMR shifts compared to the reported compounds and 1D NOE (Fig. S8). The ^{13}C NMR shifts of C-5, C-6, C-14, and C-15 were similar with those of stelliosphaerol A isolated from *Stelliosphaera formicum*[12] [δ_C 34.6 (C-5), 41.6 (C-6), 16.3 (C-14), and 21.5(C-15)]. These carbons have a *cis* orientation that is slightly different from the *trans* orientation in subersin[13] [δ_C 37.8 (C-5), 39.5 (C-6), 16.0 (C-14), and 26.6 (C-15)] and oculatolide[14] [δ_C 37.7 (C-5), 39.5 (C-6), 15.9 (C-14), and δ_C 26.4 (C-15)]. The NOEs data showed correlations from Me-14 to H_2-7, and Me-15 to H-4, but not from Me-15 to H-5. These data showed that the Me-15 has a pseudo-axial orientation along with the Me-14 and C-7 have a pseudo-equatorial

orientation. Further, NOE correlation between H-12 and H-11 indicated an *E*-form double bond at C-9 and C-10. The *trans* orientation for both the epoxide and C-4'-OH groups were assigned on the basis of the analysis of its ^{3}J coupling constant of 0 Hz for H-4' and H-5'.

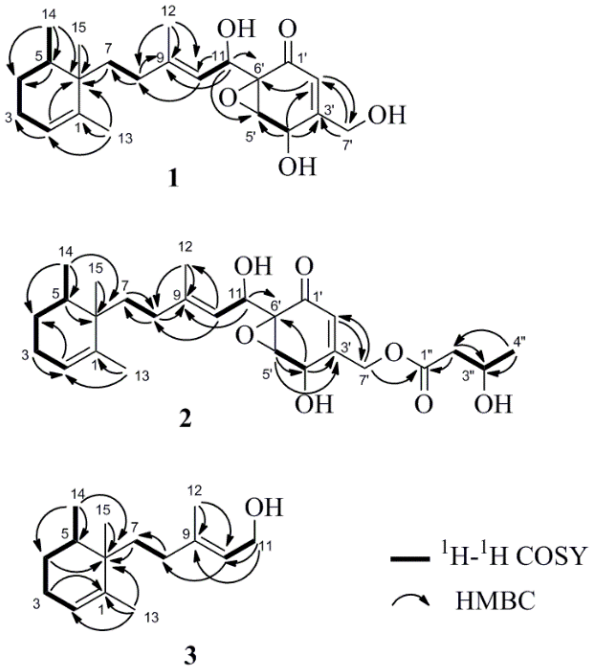


Fig. 2. ^{1}H-^{1}H COSY and key HMBC correlations of **1**-**3**.

The absolute configuration of epoxycyclohexenone was deduced by circular dichroism (CD) analysis. The CD spectrum of **1** generated negative Cotton effects at 240 nm (-4.2) and 340 nm (-2.2). The CD data was compared with that of 13-hydroxylmacrophorin A (4'*R*, 5'*R*, 6'*R*) isolated from *Microdiplodia* sp. TT-12[15] [CD (MeOH) Δε: 240 (-4.6), 334 (+2.86)]. This comparison suggested that the C-4'-OH in **1** was β-oriented, identical to that observed in 13-hydroxylmacrophorin A (4'*R*). In contrast, the epoxide at C-5' and C-6' in **1** was α-oriented (5'*S*, 6'*S*), which is opposite of that observed in 13-hydroxylmacroporin A. This was supported by a similar relationship between the CD spectra of neomacrophorin III isolated from *Trichoderma* sp. 1212-03[16] [ECD (MeOH) Δε: 243 (-6.1), 339 (-3.2)] and myrothecol B isolated from *Myrothecium* fungus[17] [CD (MeOH) Δε: 249 (-5.5), 334 (-3.8)], where the C-4'-OH and epoxide at C-5' and C-6' were *trans* oriented (4'*R*, 5'*S*, 6'*S*). Furthermore, single

crystal was obtained from a methanol solution of **1** and was suitable for X-ray crystallography. Final refinement of the diffraction data resulted in a small Flack parameter -0.1(3), allowing the assignment of the absolute configuration of **1** (Fig. 3).[18]

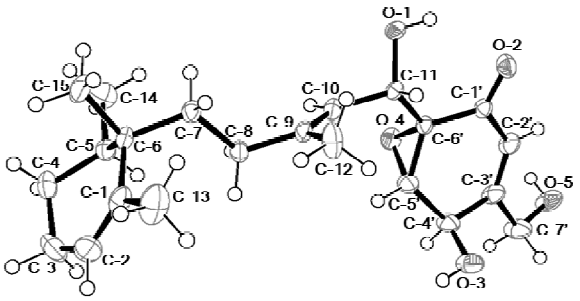


Fig. 3. ORTEP drawing of **1**, showing 50% probability ellipsoids.

Compound **2**[19] was isolated utilizing a less polar eluent system than **1**, which indicated that **2** is a less polar compound compared to **1**. The HRESIMS data of **2** showed a sodium adduct ion at *m/z* 485.2509 $[M+Na]^+$, suggesting the molecular formula $C_{26}H_{38}O_7$ with eight degrees of unsaturation. The molecular formula of **2** was 86 units larger than the molecular formula of **1**. The ^1H and ^{13}C NMR spectroscopic data (Table 1) of **2** closely resembled those of **1** except for several additional resonances at δ_C 171.5 (C-1"), 44.7 (C-2"), 63.3 (C-3"), 24.0 (C-4"), δ_H 2.61 (dd, *J* = 4.8, 15.1 Hz, H-2a"), 2.71 (dd, *J* = 8.2, 15.1 Hz, H-2b"), 4.55 (m, H-3"), and 1.33 (d, *J* = 6.2 Hz, H-4"), which indicated characteristic signals for a 3-hydroxybutanoate moieties. In the HMBC analysis of **2**, correlations from H-7' to C-1" indicated that the butanoate moiety was connected to C-7' (Fig. 2). Compound **2** exhibited identical ^{13}C NMR chemical shifts (Table 1) and CD data [CD (MeOH) Δε: 248 (-4.2), 340 (-1.9)] to those of **1**. These data indicated that **1** and **2** have the same configuration of the cyclohexene (C-5, C-6 and C-11) and epoxycyclohexenone (C-4', C-5' and C-6') moieties.

The absolute configuration at C-3" of **2** was determined by alkaline methanolysis. The resulting product was compared to the authentic standards by chiral GC-MS. Injection of the pentane extract of hydroxylate into GC-MS with a CycloSil-B capillary column was

monitored by the 3-hydroxybutanoate ion ($[M-CH_3]^+$ *m/z* = 103) generating a signal at 12.937 min. This signal was verified with a mixture of standard methyl (*S*/*R*)-3-hydroxybutanoate that appeared at 12.800 and 12.938 min, respectively, under the same conditions. The configuration of C-3" was then determined to be the (*R*)-form. The structure of **2** was finally determined as shown in Fig. 1.

The molecular formula of **3**[20] was assigned as $C_{15}H_{26}O$ by HRESIMS analysis (*m/z* 245.1876 $[M+Na]^+$) in combination with NMR data, which indicated only three degrees of unsaturation. In the NMR data of **3**, characteristic upfield signals at δ_C 19.3 (C-13), 15.9 (H-14), 21.1 (C-15), δ_H 1.52 (H-13), 0.73 (H-14), and 0.76 (H-15) revealed the presence of a 1,5,6-trimethyl-cyclohexene moiety. Furthermore, signals at δ_C 35.1 (C-7), 34.3 (C-8), 139.6 (C-9), 124.5 (C-10), 58.9 (C-11), δ_H 1.41 (H-7a), 1.46 (H-7b), 1.88 (H-8a), 1.91 (H-8b), 5.39 (H-10), and 4.39 (H-11) revealed the presence of a 2-methylbutene moiety. Intensive HMBC analysis confirmed that the planar structure of **3** has the same sesquiterpene part of **1**. The critical difference between **1** and **3** was the absence of the epoxycyclohexenone group and the appearance of a new oxygenated methylene at C-11 in compound **3**. The absence of the epoxycyclohexenone group in **3** was also supported by the finding that **3** lacked the IR absorbance at ν_{max} 1677 cm^{-1} that was present in **1** and **2**. The relative configuration of **3** was determined to be identical to **1** on the basis similar ^{13}C NMR chemical shifts shown in Table 1. The structure of **3** was elucidated and is shown in Fig. 1.

We propose that the biosynthetic pathway of **3** derived from a farnesyl pyrophosphate, which is different from the biosynthetic pathway of **1** and **2** that are derived from a farnesyl pyrophosphate and a gentisyl alcohol. Compounds **1** and **2** have a rearranged monocyclofarnesyl skeleton (which is uncommon to sesquiterpene-epoxycyclohexane conjugates) instead of a bicyclofarnesyl skeleton which is present in macrophorins[21], neomacrophorins[16], myrothecols[17], and craterellins.[22]

Compounds **1**, **2**, and **3** were evaluated for their in vitro cytotoxicity against HL-60 and HeLa cell lines by the MTT method using a published protocol.[23] Compounds **1**, **2**, and **3** exhibited cytotoxic activity against the HL-60 cell lines with IC_{50} values of 1.7, 1.5 and 10.1 μM, respectively. Additionally, compound **1**, **2**, and **3** exhibited cytotoxicity against the HeLa cell lines with IC_{50} values of 34.7, 16.6 and 52.1 μM, respectively.

The present study clarified the structures of **1** and **2** as sesquiterpene-epoxycyclohexenone conjugates. The structure of **3** was also elucidated, and was found to be derived from the farnesyl pyrophosphate. Ascochlorin[24], cylindrols[25], LL-Z1272s[26], moverastins[27] ilicicolins C, and F[28] have been reported to be prenylated phenolic derivatives. All of these compounds consist of the farnesyl-derived cyclohexanone and the gentisyl-derived methyl benzaldehyde moieties. It is of particular interest that **1** and **2** contain the epoxycyclohexenone moiety instead of the benzaldehyde group. Further study of the mechanisms of action by **1**, **2** and **3** is underway.

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Conflict of interest

The authors of present manuscript have declared that no competing of interests.

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11. *Nectrianolin A* (**1**): colorless crystal; $[\alpha]_D^{25}$ -30.7 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 220 (3.96) nm; CD (3.7 x 10^{-5} M, MeOH) λ_{max} (Δε) 248 (-4.2), 333 (-2.2); IR (KBr) ν_{max} 3332, 2935, 1677, 1249, 1045, 732 cm^{-1}; ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5) data, see Table 1; HRESIMS (positive-ion mode) *m/z* 399.2142 ($[M+Na]^+$ calcd. for $C_{22}H_{32}NaO_5$, 399.2147).

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18. Crystal data for *nectrianolin A* (**1**): $C_{22}H_{32}O_5$, M = 376.49; orthorhombic system, space group $P2_12_12_1$, a = 8.4487(7) Å, b = 9.8313(9) Å, c = 24.200(2) Å, V = 2010.1(3) Å^3, Z = 4, d = 1.241 g/cm^3, $F(000)$ = 812. A crystal of dimensions 0.400 × 0.300 × 0.100 mm^3 was used for measurement. All measurements were made on a Rigaku XtaLAB mini diffractometer using graphite monochromated Mo-Kα radiation, with linear absorption coefficient μ(Mo-Kα) = 0.864 cm^{-1}. The data were collected at a temperature of -123 ± 1°C to a maximum 2θ value of 55.0°. Of the 21057 reflections were collected, where 4601 were unique (R_{int} = 0.0322) and were collected for the analysis. All calculations were performed using the CrystalStructure crystallographic software package except for refinement, which was performed using SHELXL Version 2013/4. The final reliability factors are R_1 = 0.0565, wR_2 = 0.1756, the goodness of fit on F^2 was equal to 1.09, and Flack parameter was -0.1(3). Crystallographic data for the structure reported in this paper have been deposited at the Cambridge Crystallographic Data Centre under reference number CCDC 1556508. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1 EZ, UK, and (fax. +44-(0)-1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

19. *Nectrianolin B* (**2**): yellow oil; $[\alpha]_D^{25}$ -43.2 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 219 (4.97) nm; CD (3.7 x 10^{-5} M, MeOH) λ_{max} (Δε) 248 (-4.2), 340 (-1.9); IR (KBr)

ν_{max} 3444, 2962, 1677, 1407, 1110, 740 cm^{-1}; ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5) data, see Table 1; HRESIMS (positive-ion mode) *m/z* 485.2509 ($[M+Na]^+$ calcd. for $C_{26}H_{38}NaO_7$, 485.2515).

20. *Nectrianolin C* (**3**): yellow oil; $[\alpha]_D^{25}$ +23.3 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 222 (2.87) nm; IR (KBr) ν_{max} 3421, 2923, 1635, 1380, 1091, 798 cm^{-1}; ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5) data, see Table 1; HRESIMS (positive-ion mode) *m/z* 245.1876 ($[M+Na]^+$ calcd. for $C_{15}H_{26}NaO$, 245.1875).
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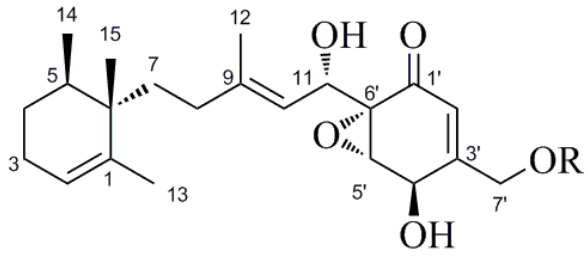
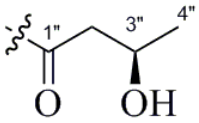
Table 1
^{1}H (600 MHz) and ^{13}C NMR (150 MHz) spectroscopic data of **1**-**3** in pyridine-d_5

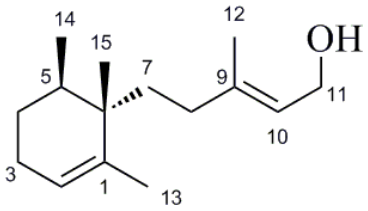
Pos.	Nectrianolin A (**1**)		Nectrianolin B (**2**)		Nectrianolin C (**3**)	
	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
1	139.4, C		139.5, C		137.5, C	
2	124.5, CH	5.36, s	124.5, CH	5.37, s	125.7, CH	5.69, m
3	25.7, CH_2	a: 1.85, m b: 1.89, m	25.7, CH_2	a: 1.85, m b: 1.89, m	25.8, CH_2	a: 1.82, m b: 1.88, m
4	27.1, CH_2	1.29, m	27.1, CH_2	1.29, m	27.2, CH_2	1.31, m
5	33.3, CH	1.53, m	33.3, CH	1.53, m	33.4, CH	1.62, m
6	40.4, C		40.5, C		40.5, C	
7	34.8, CH_2	a: 1.36, td (5.5, 13.1) b: 1.42, td (3.4, 13.7)	34.8, CH_2	a: 1.37, m b: 1.42, m	35.1, CH_2	a: 1.41, m b: 1.46, m
8	34.6, CH_2	a: 1.56, m b: 1.81, m	34.7, CH_2	a: 1.58, m b: 1.82, m	34.3, CH_2	a: 1.88, m b: 1.91, m
9	141.2, C		141.2, C		139.6, C	
10	124.1, CH	5.68, d (8.9)	123.9, CH	5.66, d (9.6)	124.5 CH	5.39, br s
11	63.4, CH [a]	5.63, d (8.9)	63.5, CH	5.61, d (8.9)	58.9, CH_2	4.39, d (6.9)
12	17.2, CH_3	1.92, s	17.3, CH_3	1.93, s	16.6, CH_3	1.67, s
13	19.2, CH_3	1.50, s	19.2, CH_3	1.51, s	19.3, CH_3	1.52, s
14	15.8, CH_3	0.68, d (6.9)	15.9, CH_3	0.68, d (6.2)	15.9, CH_3	0.73, d (6.1)
15	20.9, CH_3	0.70, s	20.9, CH_3	0.71, s	21.1, CH_3	0.76, s
1’	194.6, C		194.1, C			
2’	121.1, CH	6.78, s	122.6, CH	6.49, s		
3’	162.3, C		154.9, C			
4’	63.4, CH [a]	5.11, s	64.3, CH	5.13, s		
5’	60.4, CH	4.58, s	60.4, CH	5.09, s		
6’	61.7, C		61.6, C			
7’	63.4, CH_2	a: 4.64, d (17.2) b: 5.02, d (17.2)	63.4, CH_2	a: 5.12, d (16.8) b: 5.38, d (16.8)		
1”			171.5, C			
2”			44.7, CH_2	a: 2.61, dd (4.8, 15.1) b: 2.71, dd (8.2, 15.1)		
3”			63.3, CH	4.55, m		
4”			24.0, CH_3	1.33, d (6.2)		

[a] Overlapped signals.



1 R= H

2 R= 



3

Fig. 1. Structures of **1**-**3**.

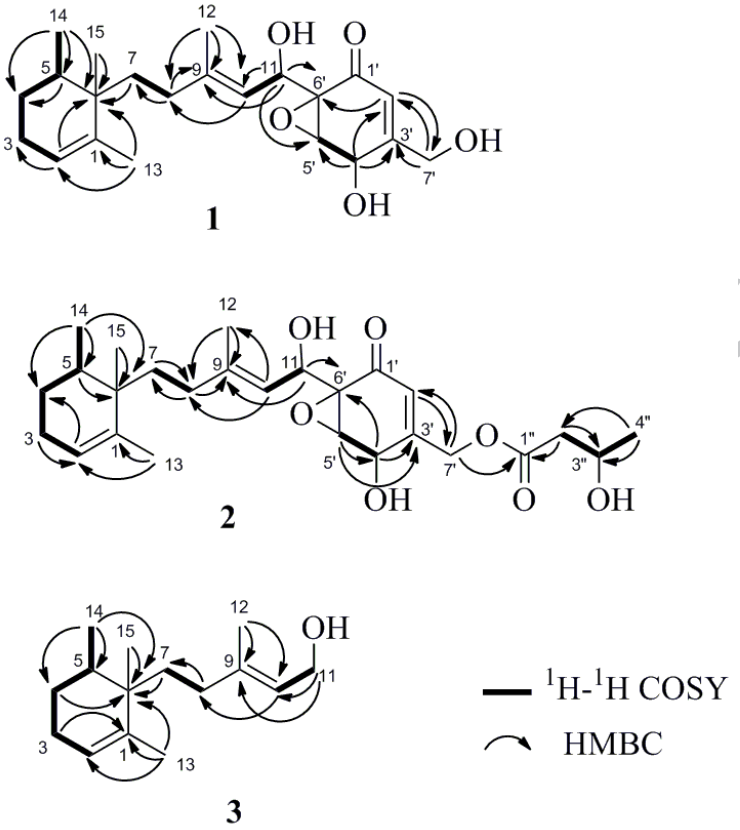


Fig. 2. ^{1}H-^{1}H COSY and key HMBC correlations of **1**-**3**.

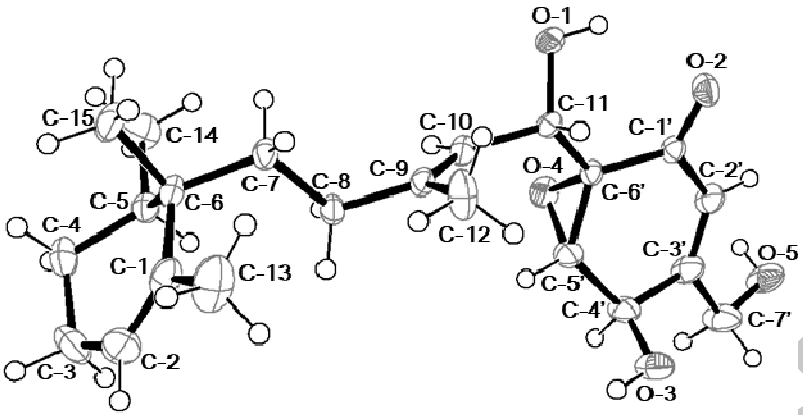
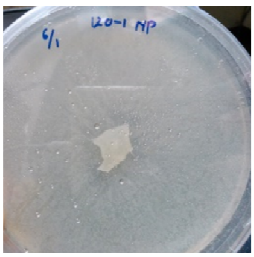
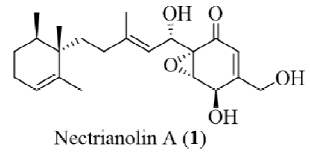


Fig. 3. ORTEP drawing of **1**, showing 50% probability ellipsoids.

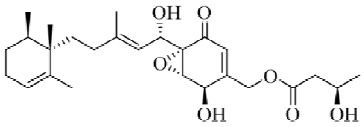
Graphical Abstract



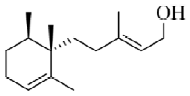
Nectria pseudotrichia 120-1NP



Nectrianolin A (**1**)



Nectrianolin B (**2**)



Nectrianolin C (**3**)

Highlights

- Chemical study of metabolites produced by fungal endophyte *Nectria pseudotrichia* 120-1NP.
- Isolation of two sesquiterpene-epoxycyclohexenone conjugates and its one sesquiterpene congener from fungal endophyte.
- Their structures were elucidated on the basis of spectroscopy analyses.