# Production of Sesquiterpene-Type Phytoalexins by Hairy Roots of *Hyoscyamus albus* Co-treated with Cupper Sulfate and Methyl Jasmonate

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**The production of sesquiterpene-type phytoalexins with a vetispyradiene skeleton by *Hyoscyamus albus* hairy roots induced by methyl jasmonate (MeJA) was reported in a previous paper. The production pattern on co-treatment with cupper sulfate and MeJA ($CuSO_4$–MeJA) showed a TLC profile differing from that on treatment with MeJA. Thus, we studied the production of phytoalexins on hairy root culture involving co-treatment with $CuSO_4$–MeJA. In the experiment, many sesquiterpene-type phytoalexins with a vetispyradiene skeleton were isolated, most of which were different from the products reported in the previous paper. Here, we isolated four new phytoalexins (1—4) along with known compounds 5—10 from the culture medium of *H. albus* hairy roots co-treated with MeJA–$CuSO_4$. The structures of the new compounds (1—4) were determined as: (3*R*,4*S*,5*R*,7*S*,9*R*)-3-acetoxy-9-(2-methylpropionyloxy)solavetivone (1), (3*R*,4*S*,5*R*,7*S*,9*R*)-3-hydroxy-9-(3-methylbutanoyloxy)solavetivone (2), (3*R*,4*S*,5*R*,7*S*,9*R*)-3-acetoxy-9-(3-methyl-butanoyloxy)solavetivone (3), and (3*R*,4*S*,5*R*,7*S*,9*R*)-3-acetoxy-9-(3-methyl-2-butenoyloxy)-solavetivone (4) based on MS and NMR including 2D-NMR data. These findings indicated that the production of phytoalexins in *H. albus* hairy roots yielded different products based on treatment with different chemicals ($CuSO_4$, MeJA, and MeJA–$CuSO_4$).**

**Key words** *Hyoscyamus albus*; hairy root; solavetivone; phytoalexin; sesquiterpene; co-treatment with cupper sulfate–methyl jasmonate

The gram-negative bacteria *Agrobacterium rhizogenes* is a plant pathogen. On infecting plants it induces adventitious roots, known as "hairy roots." Hairy roots have an excellent growth capacity in simple culture systems without requiring the addition of any exogenous plant hormones.[1,2] Furthermore, hairy roots are known to produce secondary metabolites at a high yield compared to those of undifferentiated plant cell suspensions and their mother plants. So, hairy roots were expected to provide a culture system for the production of pharmacologically important natural products and the biotransformation of organic compounds into useful biologically active compounds. Thus, many medicinal plants have been transformed to hairy roots, and there have been many related reports on the efficient production of useful natural products[3—7] and biotransformation of organic substances into useful compounds.[8—10] Furthermore, hairy roots were expected to become a simple and easy experimental system for the biosynthesis of secondary metabolites such as phytoalexins, and plant gene engineering.[11,12] Elicitation of hairy roots on culture with heavy metal ions such as cupper ion, organic chemicals such as jasmonic acid and biological substances such as yeast extract was studied, and found to enhance defense processes in plants and the production of secondary metabolites such as phytoalexins.[13,14] Especially, there were many reports concerning induction by plant hormones, jasmonic acid, and methyl jasmonate (MeJA). In many cases, MeJA enhanced the production of pharmacologically important secondary metabolites such as defense compounds, phytoalexins,[15—17] and defence systems in plants.[18]

We reported the production of a coumarin-type phytoalexin, umbelliferone, in *Pharbitsis nil* hairy roots.[19] The *P. nil* hairy roots also showed substrate specific and strong reactivity on the glucosylation of low-molecular-weight phenolic compounds, and strong reactivity on the reduction of a formyl group on the aromatic ring to a hydroxymethyl group.[20,21] Solanaceae, such as *Solanum*, *Hyoscyamus*, and *Atropa* sp., were used as medicinal plants, yielding alkaloids such as nicotine and tropan alkaloids as the main constituents, but they produced sesquiterpene-type phytoalexins, such as lubimine[22] and solavetivone,[23] after treatment with biotic and abiotic elicitors. We reported the production of sesquiterpene-type phytoallexins in *Hyoscyamus albus* (Solanaceae) hairy roots treated with MeJA,[24] and the efficient production of tropane alkaloids in *H. niger* hairy roots.[25] We also reported the identification of a strongly expressed gene in *H. niger* hairy roots, related to and enhancing lateral root formation in *H. niger* hairy roots.[26] We previously found that products from *H. albus* hairy roots treated with $CuSO_4$, MeJA, and co-treatment of $CuSO_4$–MeJA showed different production patterns in terms of the TLC profile. So, we attempted to produce sesquiterpene-type phytoallexins in *H. albus* hairy roots cell culture by co-treatment with $CuSO_4$–MeJA, and carried out the isolation and structural elucidation of the resulting phytoalexins. This paper reports the isolation of four new sesquiterpenes, **1**—**4** and their structures.

## Results and Discussion

Hairy roots were induced in *H. albus* by infection with *A. rhizogenes* employing the leaf disc method. The hairy roots were co-treated with $CuSO_4$–MeJA (100 $\mu$g/ml each), and the culture medium was extracted with EtOAc at three weeks after treatment. From the EtOAc extract, four new compounds (**1**—**4**) were isolated along with known compounds

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Table 1. $^{1}$H-NMR Data of Compounds **1**—**4** (in $CDCl_3$)

| H-No. | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| H-1 | 5.93 (1H, s) | 5.90 (1H, s) | 5.93 (1H, s) | 5.93 (1H, s) |
| 3 | 5.77 (1H, d, $J$=11.5 Hz) | 4.30 (1H, d, $J$=12.0 Hz) | 5.80 (1H, d, $J$=12.0 Hz) | 5.84 (1H, d $J$=12.0 Hz) |
| 4 | 2.41 (1H, dq, J=11.5, 7.2 Hz) | 2.04 (1H, overlap) | 2.40 (1H, dq, $J$=12.0, 7.2 Hz) | 2.41 (1H, dq, $J$=12.0, 7.2 Hz) |
| 6 | 2.11 (1H, dd, $J$=14.6, 7.6 Hz) | 2.03 (1H, overlap) | 2.00 (1H, dd, $J$=14.4, 9.6 Hz) | 2.05 (1H, overlap) |
| | 2.06 (1H, dd, $J$ = 14.6, 8.6 Hz) | 1.96 (1H, overlap) | 2.11 (1H, dd, $J$=14.4, 7.2 Hz) | 2.03 (1H, overlap) |
| 7 | 2.93 (1H, br qui, $J$=6.8 Hz) | 2.96 (1H, br qui, $J$=7.2 Hz) | 2.94 (1H, br qui, $J$=7.2 Hz) | 2.94 (1H, br qui, $J$=6.6 Hz) |
| 8 | 2.28 (1H, ddd, $J$=13.7, 7.6, 6.1 Hz) | 2.20 (1H, ddd, $J$=13.2, 6.6, 5.5 Hz) | 2.26 (1H, overlap) | 2.10 (1H, overlap) |
| | 2.02 (1H, overlap) | 2.03 (1H, overlap) | 2.07 (1H, overlap) | 2.35 (1H, overlap) |
| 9 | 5.26 (1H, t, $J$=7.8 Hz) | 5.23 (1H, t, $J$=7.8 Hz) | 5.28 (1H, t, $J$=8.4 Hz) | 5.36 (1H, t, $J$=8.4 Hz) |
| 12 | 4.83 (1H, s) | 4.76 (1H, s) | 4.83 (1H, s) | 4.85 (1H, s) |
| | 4.80 (1H, s) | 4.73 (1H, s) | 4.80 (1H, s) | 4.80 (1H, s) |
| 13 | 1.77 (3H, s) | 1.70 (3H, s) | 1.77 (3H, s) | 1.78 (3H, s) |
| 14 | 1.32 (3H, d, $J$=7.2 Hz) | 1.43 (3H, d, $J$=7.2 Hz) | 1.32 (3H, d, $J$=7.2 Hz) | 1.32 (3H, d, $J$=7.2 Hz) |
| 15 | 1.99 (3H, s) | 1.94 (3H, s) | 2.00 (3H, s) | 2.00 (3H, s) |
| 2′ | 2.62 (1H, m) | 2.13 (1H, dd, $J$=15.0, 7.2 Hz) | 2.27 (2H, overlap) | 5.76 (1H, s) |
| | | 2.10 (1H, dd, $J$=15.0, 7.2 Hz) | | |
| 3′ | 1.18 (3H, d, $J$=7.0 Hz) | 2.00 (1H, overlap) | 2.08 (1H, overlap) | |
| 4′ | 1.15 (3H, d, $J$=7.0 Hz) | 0.85 (3H, d, $J$=7.2 Hz) | 0.95 (3H, d, $J$=6.6 Hz) | 1.92 (3H, s) |
| 5′ | | 0.87 (3H, d, $J$=6.6 Hz) | 0.93 (3H, d, $J$=6.6 Hz) | 2.17 (3H, s) |
| 2″ | 2.18 (3H, s) | | 2.20 (3H, s) | 2.19 (3H, s) |

(**5**—**10**), as shown in Experiments. The structures of known compounds (**5**—**7**) were determined and identified based on MS and NMR data as follows; 3-hydroxysolavetivone (**5**),[24] 3,9-dihydroxysolavetivone (**6**),[24] and 3-hydroxy-9-(3-methyl-2-butenoyloxy)solavetivone (**7**).[24] Compound **8** was identified as 3-acetoxysolavetivone isolated as an exudate of *Solanum abutiloides*.[27] Compound **9** was identified as 11,12-dihydroxy-vetisoyra-1(10)-en-2-one isolated from potato tubers infected with pathogens.[28] Compound **10** was identified as 13-hydroxysolavetivone reported as a biotransformation product of solavetivone on cell suspension culture[29] (Fig. 1).

Compound **1** was obtained as a viscous, pale yellow oil. The molecular formula of **1** was determined as $C_{21}H_{30}O_5$ based on a pseudo-molecular ion peak at $m/z$ 385.1969 $[M+Na]^+$ in HR-electrospray ionization (ESI)-MS. The UV spectrum of **1** showed a maximum absorption at 245 nm. The $^{1}$H-NMR data of **1** showed the presence of three doublet methyl groups [$\delta_H$ 1.15 (3H, d, $J$=7.0 Hz) and 1.18 (3H, d, $J$=7.0 Hz), and 1.32 (3H, d, $J$=7.2 Hz)], two singlet vinyl methyl groups [$\delta_H$ 1.99 (3H, s) and 1.77 (3H, s)], an acetyl group [$\delta_H$ 2.18 (3H, s)], an exomethylene group [$\delta_H$ 4.83 (1H, s) and 4.80 (1H, s)], a singlet olefine proton [$\delta_H$ 5.93 (1H, s)], two methine protons on oxygen bearing carbons [$\delta_H$ 5.77 (1H, d, $J$=11.5 Hz) and 5.26 (1H, t, $J$=7.8 Hz)], and some methine and methylene protons, as shown in Table 1. The $^{13}$C-NMR data of **1** showed the presence of twenty-one carbon signals, such as four olefinic carbons ($\delta_C$ 165.2, 147.0, 126.8, 109.5), two oxygen bearing methine carbons ($\delta_C$ 79.8, 74.9), a carbonyl carbon ($\delta_C$ 193.4), two ester carbonyl carbons ($\delta_C$ 176.6, 170.4), an acetyl methyl carbon ($\delta_C$ 20.8), *i*-propyl ($\delta_C$ 34.1, 19.1, 18.7) of a *t*-butyloyl moiety, and so on as shown in Table 2. These data showed that **1** was a solavetivone derivative with acetyloxy and 2-methylpropionyloxy moieties. That is a diacyl derivative of 3,9-dihydroxysolavetivone (**6**). The proton signals at C-3 and C-9 of **1** shifted down-field ($\delta_H$5.77, 5.26) compared with those of 3,9-dihydroxysolavetivone (**6**) ($\delta_H$ 4.75, 4.41). The carbon signals at C-2, 4, 5 and 8 shifted to the upper-fields compared with those of (**6**) (see Table 2). These data indicated

Table 2. $^{13}$C-NMR Data of Sesquiterpenes Produced by *H. albus* Hairy Roots

| C-No. | 1 | 2 | 3 | 4 | 6 |
|---|---|---|---|---|---|
| C-1 | 126.8 | 125.2 | 126.8 | 126.8 | 125.0 |
| 2 | 193.4 | 199.5 | 193.5 | 193.7 | 200.5 |
| 3 | 74.9 | 73.3 | 74.7 | 78.8 | 73.6 |
| 4 | 44.2 | 47.9 | 44.3 | 44.3 | 48.3 |
| 5 | 54.2 | 54.4 | 54.2 | 54.3 | 55.8 |
| 6 | 40.6 | 40.7 | 40.4 | 40.8 | 40.7 |
| 7 | 42.5 | 42.5 | 42.5 | 42.6 | 42.6 |
| 8 | 37.1 | 37.2 | 37.0 | 37.4 | 40.4 |
| 9 | 79.8 | 79.8 | 79.6 | 78.7 | 80.7 |
| 10 | 165.2 | 167.1 | 165.1 | 165.2 | 168.6 |
| 11 | 147.0 | 147.1 | 147.1 | 147.2 | 147.9 |
| 12 | 109.5 | 109.4 | 109.5 | 109.4 | 108.8 |
| 13 | 21.3 | 21.3 | 21.3 | 21.3 | 21.4 |
| 14 | 13.7 | 13.6 | 13.6 | 14.8 | 13.7 |
| 15 | 21.5 | 21.5 | 21.4 | 21.4 | 21.8 |
| 1′ | 176.6 | 172.3 | 172.5 | 165.7 | |
| 2′ | 34.1 | 43.6 | 43.4 | 115.2 | |
| 3′ | 18.7 | 25.7 | 25.8 | 159.2 | |
| 4′ | 19.1 | 22.3 | 22.3 | 27.6 | |
| 5′ | | 22.3 | 22.2 | 20.8 | |
| 1″ | 170.4 | | 170.4 | 170.4 | |
| 2″ | 20.8 | | 20.8 | 20.9 | |

that **1** was acyloxylaed at the C-3 and C-9 positions. The plane structure of **1** was confirmed by heteronuclear multiple bond connectivity (HMBC) data. The HMBC data of **1** showed H–C long-range correlations as follows; H-3 ($\delta_H$ 5.77, d, $J$=11.5 Hz) with C-2, 4, 5, 14, and 1″ ($\delta_C$ 193.4, 44.2, 54.2, 13.7, 170.4, respectively); Me-14 ($\delta_H$ 1.32, d, $J$=7.2 Hz) with C-3, 4, and 5 ($\delta_C$ 74.9, 44.2, 54.2, respectively); Me-15 ($\delta_H$ 1.99, s) with C-1, 5, and 10 ($\delta_C$ 126.8, 54.2, 165.2, respectively); Me-13 ($\delta_H$ 1.77, s) with C-7, 11, and 12 ($\delta_C$ 42.5, 147.0, 109.5, respectively); H-12 ($\delta_H$ 4.83, s, 4.80, s) with C-7, 11, and 13 ($\delta_C$ 42.5, 147.0, 21.3, respectively); H-4 ($\delta_H$ 2.41, dq, $J$=11.5, 7.2 Hz) with C-2, 3, 5, 6, 9, and 14 ($\delta_C$ 193.4, 74.9, 54.2, 40.6, 79.8, 13.7, respectively); H-9 ($\delta_H$ 5.26, t, $J$=7.8 Hz) with C-4, 5, 7, 8, 10, and

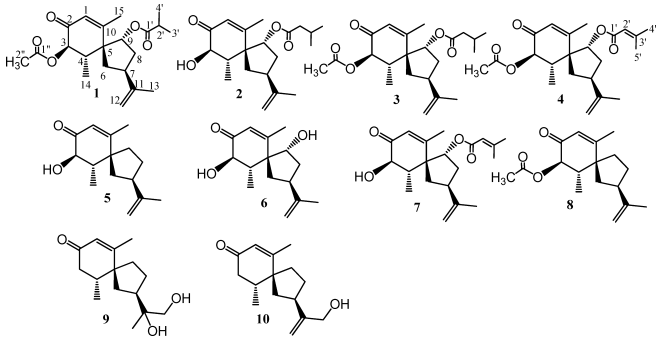

Fig. 1. Structures of Sesquiterpenes Isolated from *H. albus* Hairy Roots

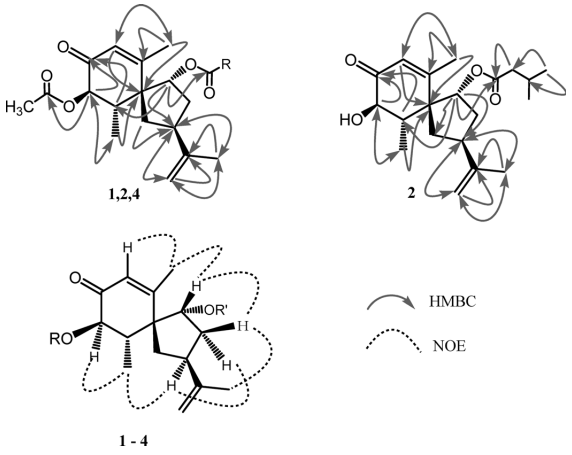


Fig. 2. Key HMBC and NOESY Correlations of Sesquiterpenes from *H. albus*

1′ ($\delta_C$ 44.2, 54.2, 42.5, 37.1, 165.2, 176.6, respectively); and so on as shown in Fig. 2. The HMBC data showed that the plane structure of **1** was 3-acetoxy-9-(2-methylpropionyloxy)vetispyra-1(10),11-dien-2-one. In nuclear Overhauser effect spectroscopy (NOESY) data, Me-14 showed correlations with H-3 and H-7; Me-15 with H-1 and H-9; and so on as shown in Fig. 2. From these data, the relative configuration was determined as shown in Fig. 1. This was supported by the coupling constant ($J$=12.6 Hz) between H-3 and H-4 (diaxial configuration). The absolute configuration of **1** should be same as the previously reported derivatives of (3*R*,4*S*,5*R*,7*S*,9*R*)-3,9-dihydroxysolavetivone based on the circular dichroism (CD) Cotton effects ($[\theta]_{315}$ −362, $[\theta]_{239}$ +10462). Thus, the structure of **1** was determined as (3*R*,4*S*,5*R*,7*S*,9*R*)-3-acetoxy-9-(2- methylpropionyloxy)solavetivone.

Compound **2** was obtained as a viscous, pale yellow oil. The molecular formula of **2** was determined as $C_{20}H_{30}O_4$ based on the molecular ion peak at $m/z$ 334.2154 $[M]^+$ in HR-electron impact ionization-mass spectra (EI-MS). The IR spectrum of **2** showed absorption bands at 3480 (OH), 2960 (CH) 1734 (ester carbonyl), 1676 (conjugated carbonyl), and 1254 (C–O) $cm^{-1}$. The $^1$H-NMR data of **2** showed the presence of three doublet methyl groups [$\delta_H$ 1.43 (3H, d, $J$=7.2 Hz), 0.87 (3H, d, $J$=6.6 Hz), and 0.85 (3H, d, $J$=7.2 Hz)], two olefine methyl groups [$\delta_H$ 1.94 (3H, s) and 1.70 (3H, s)], two oxygen bearing methine groups [$\delta_H$ 4.30 (1H, d, $J$=12.0 Hz) and 5.23 (1H, t, $J$=7.8 Hz)], an exomethylen group [$\delta_H$ 4.76 (1H, s) and 4.73 (1H, s)], and an olefine proton [$\delta_H$ 5.90 (1H, s)]. The $^{13}$C-NMR spectrum of **2** showed the presence of a conjugated carbonyl carbon ($\delta_C$ 199.5), an ester carbonyl carbon ($\delta_C$ 172.3), four olefinic carbons ($\delta_C$ 167.1, 147.1, 125.2, 109.4), and two oxygen bearing methine carbons ($\delta_C$ 79.8, 73.3). These facts indicated that **2** was a 3-methylbutanoyloxy ester of **6**. The proton signal at C-3 of **2** appeared in an upper-field ($\delta_H$ 4.30) compared with that of **1** ($\delta_H$ 5.77). This indicated that **1** had a free hydroxyl group at C-3. The position of the 3-methylbutanoyloxy group and plane structure of **2** were determined from the HMBC experiment of **2**. The H–C long-range correlations in the HMBC spectrum are shown in Fig. 2. Me-14 ($\delta_H$ 1.43, 3H, d, $J$=7.2 Hz) showed an H–C long-range correlation with C-3, 4 and 5 ($\delta_C$ 73.3, 47.9, 54.4, respectively); Me-15 ($\delta_H$ 1.94, 3H, s) with C-1, 5, and 10 ($\delta_C$ 125.2, 54.4, and 167.1, respectively); Me-13 ($\delta_H$ 1.70, 3H, s) with C-7, 11, and 12 ($\delta_C$ 42.5, 147.1, 109.4, respectively); H-3 ($\delta_H$ 4.30, 1H, d, $J$=12.0 Hz) with C-2, 4, 5, and 14 ($\delta_C$ 199.5, 47.9, 54.4, 13.6, respectively); H-9 ($\delta_H$ 5.23, 1H, t, $J$=7.8 Hz) with C-4, 6, 7, 8, 10, and 1′ ($\delta_C$ 47.9, 40.7, 42.5, 37.2, 167.1, 172.3, respectively); H-1 ($\delta_H$ 5.90, 1H, s) with C-3, 5 and 15 ($\delta_C$ 73.3, 54.4, 21.5, respectively); and so on as shown in Fig. 2. From these data, the plane structure of **2** was determined as a 9-*O*-3-methylbutanoyl derivative of **6**. In NOESY data, Me-14 showed nuclear Overhauser effect (NOE) correlations with H-3 and H-7; Me-15 with H-1 and H-9; and so on as shown in Fig. 2. Thus, the structure of 2 was determined as *rel*-(3*R*,4*S*,5*R*,7*S*,9*R*)-3-hydroxy-9-(3-methylbutanoyloxy)-solavetivone.

Compound **3** was obtained as a viscous, pale yellow oil. The molecular formula of **3** was determined as $C_{22}H_{32}O_5$ based on the pseudo-molecular ion peak at $m/z$ 377 $[M+H]^+$ in ESI-MS and 22 carbon signals in the $^{13}$C-NMR spectrum. The UV spectrum of **3** showed absorption at 245 nm. The $^1$H-NMR data of **3** showed the presence of an acetyl group [$\delta_H$ 2.20 (3H, s)], three doublet methyl groups [$\delta_H$ 1.32 (3H, d, $J$=7.2 Hz), 0.95 (3H, d, $J$=6.6 Hz), and 0.93 (3H, d, $J$=6.6 Hz)], two vinyl methyl groups [$\delta_H$ 2.00 (3H, s), 1.77 (3H, s)], two oxygen bearing methine groups [$\delta_H$ 5.80 (1H, d, $J$=12.0 Hz), 5.28 (1H, t, $J$=8.4 Hz)], an exomethylene group [$\delta_H$ 4.83 (1H, s), 4.80 (1H, s)], and an olefin proton [$\delta_H$ 5.93 (1H, s)]. The $^{13}$C-NMR spectrum of **3** showed the presence of two ester carbonyl carbons ($\delta_C$ 172.5, 170.4), a conjugated carbonyl carbon ($\delta_C$ 193.5), four olefin carbons ($\delta_C$ 165.1, 147.1, 126.8, 109.5), two oxygen bearing methine carbons ($\delta_C$ 74.7, 79.6), and so on as shown in Table 2. These facts indicated that **3** was an acetyl and 3-methylbutanoyl ester derivative of **6**. The positions of acyl moieties and the plane structure of **3** were determined from HMBC experiments, as shown in Fig. 2; Me-14 ($\delta_H$ 1.32, 3H, d, $J$=7.2 Hz) showed an H–C long range correlation with C-3, 4, and 5 ($\delta_C$ 74.7, 44.3, 54.2, respectively); Me-15 ($\delta_H$ 2.00, 3H, s) with C-1, 5 and 10 ($\delta_C$ 126.8, 54.2, 165.1, respectively); Me-13 ($\delta_H$ 1.77, 3H, s) with C-7, 11, and 12 ($\delta_C$ 42.5, 147.1, 109.5, respetively); M-4′ and 5′ ($\delta_H$ 0.95, 3H, d, $J$=6.6 Hz, 0.93, 3H, d, $J$=6.6 Hz) with C-2′ and 3′ ($\delta_C$ 43.4, 25.8); H-3 ($\delta_H$ 5.80, 1H, d, $J$=12.0 Hz) with C-2, 4, 5, 14, and 1″ ($\delta_C$ 193.5, 44.3, 54.2, 13.6, 170.4, respectively); H-9 ($\delta_H$ 5.28, 1H, t, $J$=8.4 Hz) with C-4, 5, 7, 10, and 1′ ($\delta_C$ 44.3, 54.2, 42.5, 165.1, 172.5, respectively); and so on as shown in Fig. 2. These facts indicated that the plane structure of **3** was 3-ace-

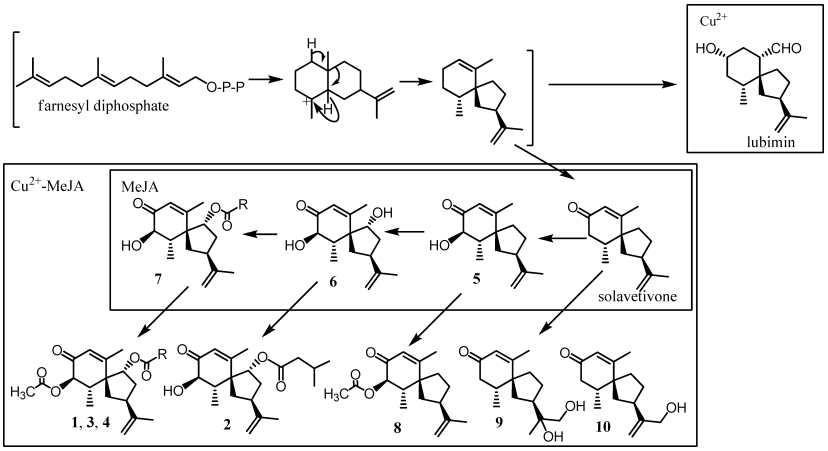


Fig. 3. Production of Sesquiterpenes by *H. albus* Hairy Roots Treated with $CuSO_4$, MeJA, and $CuSO_4$-MeJA, Respectively

toxy-9-(3-methylbutanoyloxy)solavetivone. The relative configuration of **3** was determined from the data generated by the NOESY experiment. Me-14 showed an NOE correlations with H-3 and H-7, Me-15 with H-1 and H-9, and so on as shown in Fig. 2. These data indicate that the stereochemistry of **3** showed a *rel*-(3*R*,4*S*,5*R*,7*S*,9*R*)-configuration. Thus, the structure of **3** was determined as *rel*-(3*R*,4*S*,5*R*,7*S*,9*R*)-3-acetoxy-9-(3-methylbutanoyloxy)solavetivone.

Compound **4** was obtained as a viscous, pale yellow oil. The molecular formula of **4** was determined as $C_{22}H_{30}O_5$ based on the pseudo-molecular ion peak at *m/z* 375 $[M+H]^+$ in ESI-MS and 22 carbon signals in the $^{13}$C-NMR spectrum of **4**. The UV spectrum of **4** showed absorption at 247 nm. The $^1$H-NMR data of **4** showed the presence of four singlet methyl groups on olefine carbons [$\delta_H$ 1.78 (3H, s), 1.92 (3H, s), 2.00 (3H, s) and 2.17 (3H, s)], an acetyl group [$\delta_H$ 2.19 (3H, s)], a doublet methyl group [$\delta_H$ 1.32 (3H, d, $J$=7.2 Hz)], an exomethylene group [$\delta_H$ 4.85 (1H, s) and 4.80 (1H, s)], two olefin protons [$\delta_H$ 5.93 (1H, s) and 5.76 (1H, s)], and two oxygen bearing methine groups [$\delta_H$ 5.84 (1H, d, $J$=12.0 Hz) and 5.36 (1H, t, $J$=8.4 Hz)]. The $^{13}$C-NMR data of **4** showed the presence of two ester carbonyl groups ($\delta_C$ 170.4, 165.7), a conjugated carbonyl group ($\delta_C$ 193.7), six olefin carbons ($\delta_C$ 165.2, 159.2, 147.2, 126.8, 115.2, 109.4), two oxygen bearing methane carbons ($\delta_C$ 78.8, 78.7), a quaternary carbon ($\delta_C$ 54.3), and several methine and methylene carbons, as shown in Table 2. These data indicated that **4** was an acetyl and 3-methyl-2-butenoyl derivative of **6**. Plane structure of **4** was determined from the HMBC correlations. The HMBC data of **4** showed H–C long-range correlations as follows; Me-14 ($\delta_H$ 1.32, 3H d, $J$=7.2 Hz) with C-3, 4, and 5 ($\delta_C$ 78.8, 44.3, 54.3, respectively); Me-15 ($\delta_H$ 2.00, 3H, s) with C-1, 5, and 10 ($\delta_C$ 126.8, 54.3, 165.2, respectively); Me-13 ($\delta_H$ 1.78, 3H, s) with C-7, 11, and 12 ($\delta_C$ 42.6, 147.2, 109.4, respectively); Me-4′ and 5′ ($\delta_H$ 1.92, 3H, s, 2.17, 3H, s) to C-2′ and 3′ ($\delta_C$ 115.2, 159.2, respectively); H-3 ($\delta_H$ 5.84, d, $J$=12.0 Hz) with C-2 ,4, 5, 14, and 1″ ($\delta_C$ 193.7, 44.3, 54.3, 14.8, 170.4, respectively); H-9 ($\delta_H$ 5.36, 1H, t, $J$=8.4 Hz) with C-4, 6, 7, 10, and 1′ ($\delta_C$ 44.3, 40.8, 42.6, 165.2, 165.7, respectively); H-1 ($\delta_H$ 5.93, 1H, s) to C-2, 5, and 15 ($\delta_C$ 193.7, 54.3, 21.4, respectively); and so on as shown in Fig. 2. These data indicated that the structure of **4** was 3-acetoxy-9-(3-methyl- 2-butenoyloxy)solavetivone. The stereochemistry of **4** was determined from NOESY experiment. Me-14 showed NOE correlations with H-3 and H-7; Me-15 with H-1 and H-9; Me-4′ with H-2′, and so on as shown in Fig. 2. Thus, the structure of **4** was determined to be *rel*-(3*R*,4*S*,5*R*,7*S*,9*R*)-3-acetoxy-9-(3-methyl- 2-butenoyloxy)solavetivone.

Most samples did not undergo CD data analysis due to their scarcity, and only **1** and **6** had their CD spectra measured. **1** and **6** showed the same tendency (negative Cotton effect at near 310 nm and positive Cotton effect at near 248 nm) with those of previously reported compounds with a 3,9-dioxysolavetivone structure.[24] Thus, the absolute configuration of these compounds should be same as those of previously reported compounds.[24] The absolute configurations of new compounds having a 3,9-dioxysolavetivone structure should be concluded to be (3*R*,4*S*,5*R*,7*S*,9*R*).

*H. albus* hairy roots induced by with $CuSO_4$ yield lubimine as a single product, which was reported as phytoalexin in Solanaceae plants.[22] The production of lubimine was identified as the only main spot on TLC (data not shown). On the other hand, the hairy roots elicited with MeJA yielded solavetivone as a major product along with several other minor products.[24] Solavetivone was also reported as a phytoalexin in Solanaceae plants.[23] The TLC profile of products induced by co-treatment with MeJA–$CuSO_4$ showed a difference with those on treatment with $CuSO_4$ and MeJA, respectively. Further, we isolated new products derived by co-treatment with $CuSO_4$–MeJA. These results indicated the following; elicitation with $CuSO_4$ yielded lubimine as the only phytoalexin, but elicitation with MeJA induced oxygenation and acyl processes in the hairy roots. Furthermore, co-treatment with MeJA–$CuSO_4$ induced acetyl processing to OH at C-3 and a characteristic oxidation process of the isopropenyl group (Fig. 3). Thus, it was interesting that elicitation with different chemicals induced different biosynthetic steps. In this study, 10 compounds were isolated, of which three compounds (**5**—**7**) were identical to those isolated in the previous study,[24] but seven compounds were isolated for the first time from the *H. albus* hairy root culture system.

## Experimental

**General Procedures** UV data were recorded on a Hitachi U2001 spec-

trophotometer. IR data were recorded on a JASCO FT/IR-6300 spectrometer with ATR. CD spectrum was recorded on a JASCO J-820 spectropolarimeter. $^{1}$H- and $^{13}$C-NMR data were recorded on a Bruker-DRX (600, 150 MHz, respectively) spectrometer using $CDCl_3$ as the solvent and tetramethylsilane (TMS) as an internal standard. HR-EI-MS data were recorded on a JEOL-HX110 mass spectrometer. ESI-MS and HR-ESI-MS data were recorded on a Waters Micromass Q-Tof micro mass spectrometer. Preparative and analytical HPLC were carried out on reverse phase columns (Mighty sil RP-18, C-8 and C-4, Kanto Chemical Co., Ltd.) using $CH_3CN$-$H_2O$ and MeOH-$H_2O$ solvent systems at 210 nm detection. Analytical TLC was carried out on pre-coated Kiesel gel 60$F_{254}$ (Merck).

**Hairy Root Induction and Cultivation** Hairy roots were induced from bacteria-free *H. albus* germinates inoculated with *Agrobacterium rhizogenes* (MAFF03-01724) by the leaf disc method, as reported previously.[24] The hairy roots were sub-cultured at four-week intervals in Murashige and Skoog (MS) culture medium supplemented with half inorganic nutrients and 3% sucrose (1/2 MS) at 25 °C.

**Production and Isolation of Phytoalexines** Hairy roots of *H. albus* were subcultured in 32 flasks of 500 ml containing 200 ml of MS medium for 3 weeks. To the culture were added 100 $\mu$M of $CuSO_4$ and 100 $\mu$M of MeJA with further culturing for 7 d. The medium was collected by filtration and extracted with ethyl acetate (EtOAc) twice. The EtOAc solution was evaporated *in vacuo*. The extract was dissolved in methanol (MeOH). The MeOH-soluble part (1.5 g) was subjected to HPLC purification using an octadecyl silica (ODS) column (Might sil RP-18, Kanto Chemical Co., Ltd.) with $CH_3CN$–$H_2O$ (55 : 45) to yield seven fractions, Fr-1 (80 mg), 2 (44 mg), 3 (20 mg), 4 (25 mg), 5 (50 mg), 6 (21 mg), and the recovered fraction (1.1 g). Fr. 1 was purified by HPLC using a C-8 column (Might sil C-8, Kanto Chemical Co., Ltd.) and MeOH–$H_2O$ (43 : 57) to give comp. **5** (2.1 mg), **6** (3.0 mg), and **8** (10.1 mg). Fr. 3 was purified by HPLC using the C-8 column and $CH_3CN$–$H_2O$ (43 : 57) to give comp. **7** (5.0 mg). Fr. 4 was purified by HPLC using the C-4 column (Might sil C-4, Kanto Chemical Co., Ltd.) and $CH_3CN$–$H_2O$ (38 : 62) to give comp. **2** (4.3 mg), **9** (5.4 mg), and **10** (6.2 mg). Fr. 5 was purified by HPLC using an ODS column and $CH_3CN$–$H_2O$ (35 : 65) to give **1** (6.5 mg). Fr. 6 was purified by HPLC using the C-4 column and $CH_3CN$–$H_2O$ (40 : 60) to give comp. **3** (7.0 mg) and **4** (3.3 mg).

**Compound 1** Viscous, pale yellow oil, HR-ESI-MS; *m*/*z* 385.1969 $[M+Na]^+$ (Calcd 385.1991 for $C_{21}H_{30}O_5Na$), UV; $\lambda_{max}$ nm ($\varepsilon$); 245 (6078) (MeOH), CD $[\theta]_{315}$ −362, $[\theta]_{239}$ +10462 ($5.5\times10^{-4}$ M, MeOH), $^{1}$H-NMR data are shown in Table 1 and $^{13}$C-NMR data in Table 2.

**Compound 2** Viscous, pale yellow oil, HR-EI-MS; *m*/*z* 334.2154 $[M]^+$ (Calcd 334.2154 for $C_{20}H_{30}O_4$), IR $\nu_{max}$ $cm^{-1}$; 3480, 2960, 1734, 1676, 1375, 1290, 1254, 1182 (KBr), $^{1}$H-NMR data are shown in Table 1 and $^{13}$C-NMR data in Table 2.

**Compound 3** Viscous, pale yellow oil, ESI-MS; *m*/*z* 377 $[M+H]^+$, UV; $\lambda_{max}$ nm; 245 (MeOH), $^{1}$H-NMR data are shown in Table 1 and $^{13}$C-NMR data in Table 2.

**Compound 4** Viscous, yellow oil, ESI-MS; *m*/*z* 375 $[M+H]^+$, UV; $\lambda_{max}$ nm; 247 (MeOH), $^{1}$H-NMR data are shown in Table 1 and $^{13}$C-NMR data in Table 2.

**Compound 6** Viscous, colorless oil, HR-ESI-MS; *m*/*z* 273.1472 $[M+Na]^+$ (Calcd 273.1467 for $C_{15}H_{22}O_3Na$), $[\theta]_{314}$ −1750, $[\theta]_{248}$ +13750 ($8\times10^{-4}$ M, MeOH), $^{13}$C-NMR data are shown in Table 2.

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