Phytoalexins from Hairy Roots of *Hyoscyamus albus* Treated with Methyl Jasmonate

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Received May 28, 1998

The treatment of hairy roots of *Hyoscyamus albus* with copper sulfate (Cu^{2+}) and methyl jasmonate (JAMe) produced several phytoalexins having the vetispyrane skeleton. Lubimin and solavetivone were isolated after treatment with Cu^{2+}. Seven sesquiterpenoid phytoalexins were isolated from the culture medium after treatment with JAMe, including lubimin, solavetivone, 3-hydroxysolavetivone and four new compounds (**1**−**4**). Structures of the new compounds were elucidated to be (3*R*,4*S*,5*R*,7*S*,9*R*)-3-hydroxy-9-tigloyloxysolavetivone (**1**), (3*R*,4*S*,5*R*,7*S*,9*R*)-3-hydroxy-9-(3-methylbutenoyloxy)-solavetivone (**2**), (3*R*,4*S*,5*R*,7*S*,9*R*)-3-hydroxy-9-isobutanoyloxysolavetivone (**3**); and (3*R*,4*S*,5*R*,7*S*,9*R*)-3,9-dihydroxysolavetivone (**4**). The induction pattern of phytoalexins in hairy roots treated with JAMe was different in those treated with Cu^{2+}, and co-treatment with JAMe and Cu^{2+} gave only solavetivone.

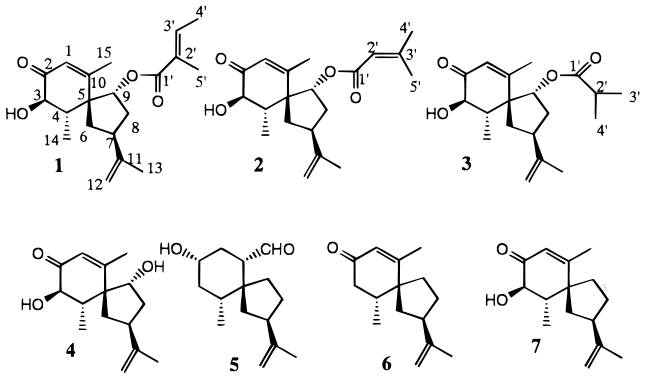
The transformed adventitious roots, so-called hairy roots, induced by the *Ri* plasmid of *Agrobacterium rhizogenes*, are expected to be a valuable culture system for production of secondary metabolites.[1−3] Thus, we induced hairy roots of *Hyoscyamus niger* L. (Solanaceae) and reported the production of hyoscyamine by these hairy roots.[4] Sesquiterpene phytoalexins, such as lubimin and rhishitin,[5,6] have been identified from Solanaceous plants, and we expected the production of similar phytoalexins in the hairy roots of *Hyoscyamus* plants.

Jasmonic acid (JA) and its methyl ester, methyl jasmonate (JAMe), are known to enhance the production of phytoalexins and other secondary metabolites such as taxoids,[7] alkaloids,[8] and shikonine.[9] They are also signal transducers[10−12] in the wound response of plants and are found concomitant with the induction of phytoalexins by expression of genes.[13]

We induced hairy roots of *H. albus* by co-culture methods with *A. rhizogenes* (MAFF 03−01724), and treated those with Cu^{2+} or JAMe to produce phytoalexins. This paper deals with the production of new phytoalexins by treatment of *H. albus* hairy roots with JAMe.

Results and Discussion

Hairy roots of *H. albus* were induced by infection with *A. rhizogenes* (MAFF 03−01724). JAMe was added to a three-week-old culture of the hairy roots in Murashige and Skoog (MS) medium and cultured for seven days. Seven compounds, **1**−**4**, lubimin (**5**), solavetivone (**6**), and 3-hydroxysolavetivone (**7**) were isolated using reversed phase HPLC (ODS) column. The known compounds, **5**−**7** were identified from their spectral data as solavetivone,[14] lubimin,[5] and 3-hydroxy-solavetivone,[15] respectively.



The EIMS of **1** gave a molecular ion at *m*/*z* 332, consistent with a molecular formula of $C_{20}H_{28}O_4$. The ^1H NMR spectrum of **1** indicated two doublet methyl [δ 1.53 (J = 7.0 Hz), 1.81 (J = 7.0 Hz)], three singlet methyl (δ 1.78, 1.83, 2.03), two carbinyl [δ 4.33 (1H, d, J = 12.0 Hz), 5.47 (1H, t, J = 8.0 Hz)], one exomethylene (δ 4.81, br s), and two olefin [δ 5.95 (s), 6.85 (br q, J = 7.0 Hz)] proton signals. These data were similar to those of **6** except for the presence of two carbinyl groups and a C_5 side chain in **1**. The UV spectrum of **1** was also consistent with a solavetivone skeleton. The ^{13}C NMR spectrum of **1** exhibited 20 carbon signals, consisting of a conjugated carbonyl (δ 199.7), a conjugated carboxyl (δ 167.1), six olefinic carbons (δ 109.3, 125.1, 128.3, 138.7, 147.1, 167.1), and two carbinyl carbons at δ 73.1 and 79.7. From these data, **1** was thought to be an acyl derivative of dihydroxysolavetivone. To confirm the solavetivone skeleton of **1**, the position of the oxygenated moieties and the structure of the acyl moiety, an HMBC experiment was carried out. The HMBC of **1** showed the expected correlations due to the spiro-structure, α,β-unsaturated ketone, 3-hydroxy group, 9-acyloxy group, isopentenyl moiety, and tigloyl groups as shown in Figure 1. The coupling constant of carbinyl proton at C-3 was 12 Hz, from which the conformation of hydroxyl and methyl groups at C-3 and C-4 of **1** was determined to be diequatorial. The relative configuration

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10.1021/np980214i CCC: $15.00
Published on Web 10/24/1998

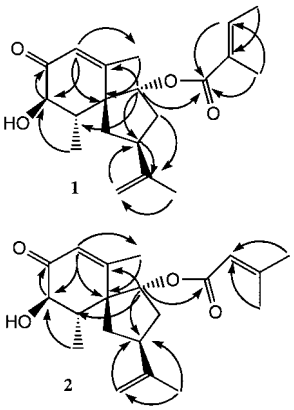

Figure 1. Significant correlations observed in the HMBC spectra of **1** and **2**.

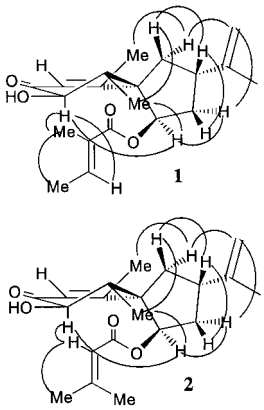

Figure 2. Stereostructures and difference NOEs of **1** and **2**.

of **1** was determined from coupling constants and the difference NOE experiment as shown in Figure 2. The presence of a tigloyl group was confirmed from the correlation between Me-4′ and Me-5′ in the NOE experiment. Thus, the structure of **1** was determined to be 3-hydroxy-9-tigloyloxy-solavetivone. The CD spectrum of **1** showed a negative Cotton effect ([θ] −3210 at 315 nm) and a positive Cotton effect ([θ] +62 930 at 235 nm). These Cotton effects were applied to the empirical rules[16,17] for n→π* and π→π* transitions of an α,β-unsaturated ketone. From these data, the absolute configuuration of **1** was determined to be *3R,4S,5R,7S,9R*. Thus, the structure of **1** is (*3R,4S,5R,7S,9R*)-3-hydroxy-9-tigloyloxysolavetivone.

The EIMS of **2** gave a molecular ion at *m/z* 332, consistent with a molecular formula of $C_{20}H_{28}O_4$. ^{1}H and ^{13}C NMR spectra of **2** indicated the same vetispyran skeleton as that of **1** (see ^{1}H and ^{13}C NMR data of **1** and **2** in Tables 1 and 2, respectively). The ^{1}H NMR spectrum of **2** showed the presence of a C_5-acyl group differing from that of **1**. The acyl group had two methyl groups [δ 1.90 (d, J = 1.0 Hz), 2.16 (d, J = 1.0 Hz)] with long-range coupling to an olefinic proton and an olefinic proton (br s) with a long-range coupling to two methyl groups. Those observations were confirmed by an H−H COSY experiment. Thus, the acyl group was deduced to be 3-methyl-2-butenoate. The plane structure of **2** was confirmed using HMBC as shown in Figure 1. The relative configuration of **2** was deduced to be same as in **1** from the coupling constants and the difference NOE experiment (Figure 2). Thus, the structure of **2** was determined to be 3-hydroxy-9-(3-methylbutenoyloxy)solavetivone. The CD spectrum of **2** showed Cotton effects similar to those found in **1**. From these data, the absolute configuration of **2** was determined to be the same as in **1**. Thus, the structure of **2** is (*3R,4S,5R,7S,9R*)-3-hydroxy-9-(3-methylbutenoyloxy)solavetivone.

The EIMS of **3** gave a molecular ion at *m/z* 302, consistent with a molecular formula of $C_{19}H_{28}O_4$. The ^{1}H and ^{13}C NMR spectra of the vetispyran moiety of **3** showed the same chemical shifts and the same signal pattern as those of **1** and **2**. The ^{1}H NMR spectrum also indicated the presence of an isopropyl group [δ 1.14 (3H, d, J = 7.2 Hz), 1.18 (3H, d, J = 6.8 Hz), 3.43 (1H, m)]. The ^{13}C NMR spectrum of **3** indicated an ester carbonyl group (δ 176.5), suggesting that the acyl group of **3** was an isobutanoyl moiety. Thus, the structure of **3** was deduced to be 3-hydroxy-9-isobutanoyloxysolavetivone. The CD spectrum of **3** showed Cotton effects similar to those of **1** and **2**. Thus, the structure of **3** was determined to be (*3R,4S,5R,7S,9R*)-3-hydroxy-9-isobutanoyloxysolavetivone.

The EIMS of **4** gave a molecular ion at *m/z* 250, consistent with a molecular formula of $C_{15}H_{22}O_3$. This molecular formula corresponded to the vetispyran portion of **1**. The ^{1}H and ^{13}C NMR spectra of **4** were identical with those of the deacyl part of **1** except for H-9 and the carbon signals around C-9. The signal of H-9 was shifted upfield [δ 4.41 (1H, t, J = 8.0 Hz)]. The signal of C-9 was shifted slightly downfield (δ 80.8), and those of C-5 and C-8 were also shifted downfield (δ 55.8 and 40.4, respectively). This gave further proof that the position of the acyl group on **1**, **2**, and **3** was at C-9. The structure of **4** was therefore deduced to be 3,9-dihydroxysolavetivone. The CD spectrum of **4** gave Cotton effects similar to those found in **1**. Thus, the structure of **4** was determined to be (*3R,4S,5R,7S,9R*)-3,9-dihydroxysolavetivone.

The *H. albus* hairy root culture treated with JAMe gave seven vetispyran-type sesquiterpenes (**1**−**7**). Of these **1**, **2**, and **5** were isolated in relatively higher yield. From the medium of the same culture treated with Cu^{2+}, **5** was isolated along with a small amount of **6**. Co-treatment with JAMe and Cu^{2+} gave **6** in high yield along with small amounts of **5**.

Compounds **1**−**3** and **6** showed antibacterial activity against the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* as shown in Table 3. Only compound **1** showed antifungal activity against *Botrytis cinerea*.

Experimental Section

General Experimental Procedures. NMR spectra, including NOE, HMBC, and HMQC experiments were obtained in $CDCl_3$ at 500 MHz (^{1}H) and 125 MHz (^{13}C) using JEOL α-500 spectrometer. Chemical shifts are reported relative to TMS; EIMS was taken on a JEOL JMS-AX 505W mass spectrometer. CD spectra were recorded on a JASCO J-20A spectropolarimeter. UV spectra were recorded on a Hitachi U3410 spectrophotometer. TLC was carried out on precoated Si gel 60F_{254} plates (Merck).

Hairy Root Induction. Seeds of *H. albus* were obtained from Tsukuba Medicinal Plant Research Station, National Institute of Health Sciences, and were surface-sterilized using 0.05% sodium hypochlorite for 15 min. After being rinsed several times with sterile distilled H_2O, the seeds were transferred to woody plant (WP) medium,[18] supplemented with 3% sucrose and 0.2% gellan gum adjusted to pH 5.7, and incubated at 25 °C and 16 h of light. The leaves and cotyledons of two-week-old seedlings were cut into approximately 1.0-cm pieces and exposed to a 1:1000 diluted solution of an overnight culture of *A. rhizogenes* (MAFF 03-01724) in YEB medium. After 30 min, the segments were transferred to the WP agar medium containing 0.5% cefotaxime to suppress bacterial growth. Developing roots from the segments were subcultured on the fresh WP medium 3 times at 10-day intervals. Vigor-

Table 1. ^{1}H NMR Data (δ) of Compounds **1**−**4** (500 MHz, $CDCl_3$)[a]

H	1	2	3	4
1	5.85 (s)	5.95 (s)	5.96 (s)	5.98 (s)
3	4.33 (d, J = 12.0)	4.40 (d, J = 12.0)	4.35 (d, J = 12.0)	4.45 (d, J = 12.0)
4	2.13 (m)	2.14 (m)	*b*	2.14 (m)
6	2.12 (dd, J = 14.6,7.3)	2.15 (m)	*b*	*b*
	2.05 (dd, J = 14.6,9.2)	2.01 (m)	*b*	*b*
7	2.97 (m)	2.97 (m)	2.97 (m)	2.98 (m)
8	2.25 (ddd, J = 13.9,8.1,8.0)	2.29 (m)	*b*	*b*
	2.14 (m)	2.12 (m)	*b*	*b*
9	5.47 (t, J = 8.0)	5.35 (t, J = 7.5)	5.29 (t, J = 8.0)	4.41 (t, J = 8.0)
12	4.81 (s)	4.81 (s)	4.80 (s)	4.74 (s)
13	1.78 (s)	1.78 (s)	1.78 (s)	1.77 (s)
14	1.53 (d, J = 7.0)	1.52 (d, J = 7.0)	1.50 (d, J = 7.2)	1.51 (d, J = 7.2)
15	2.03 (s)	2.02 (s)	2.01 (s)	1.97 (s)
2′		5.65 (br s)	*b*	
3′	6.55 (br q)		1.18 (d, J = 6.8)	
4′	1.81 (d, J = 7.0)	1.90 (d, J = 1.0)	1.14 (d, J = 7.0)	
5′	1.83 (s)	2.16 (d, J = 1.0)		

[a] Assignments of **1** and **2** were confirmed by HMBC, HMQC, and H−H COSY experiments. [b] Signals overlapped.

Table 2. ^{13}C NMR Data (δ) of Compounds **1**−**4** (125 MHz, $CDCl_3$)[a]

C	1	2	3	4
1	125.1	125.2	125.3	125.1
2	199.7	199.7	199.6	200.4
3	73.1	73.2	73.5	73.7
4	47.8	48.1	50.0	48.4
5	54.5	54.6	54.6	55.8
6	41.2	40.8	41.1	40.8
7	42.4	42.6	42.8	42.7
8	37.4	37.5	37.6	40.5
9	79.7	79.8	80.2	80.8
10	167.1	167.3	167.1	168.6
11	147.1	147.2	147.2	147.9
12	109.3	109.3	109.5	108.9
13	21.3	21.3	21.4	21.3
14	13.5	13.5	13.5	13.7
15	21.4	21.4	21.6	21.4
1′	167.5	165.6	176.5	
2′	128.3	115.3	34.3	
3′	138.7	159.1	19.3	
4′	14.6	27.5	18.9	
5′	12.4	20.3		

[a] Assignments of **1** and **2** were carried out by the aid of HMBC and HMQC experiments.

Table 3. Minimal Inhibitory Concentration of Compounds **1**−**4** and **6** against *S. aureus* and *B. subtilis*

compound	*S. aureus* (μg/mL)	*B. subtilis* (μg/mL)
1	100	250
2	100	250
3	250	250
4	500	500
6	100	100

ously growing hairy roots free from *A. rhizogenes* were selected from young root tips. The transferred hairy roots were tested for the presence of mikimopin, according to Tanaka et al.[19]

Antimicrobial Activity. The test was carried out using the agar plate dilution method. Samples were dissolved in soybean casein digest (SCD) on agar plate, in the case of bacteria, and potet dextrose (PD) on agar plate, in the case of fungi, in each concentration, and test bacteria (Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*) and fungi (*Fusarium oxysporium* f. sp. *lycopersici* and *Botrytis cinera*) were applied to the agar surface of SCD and PD, respectively, and incubated for 2 days at 30 °C. The results were observed from the formation of the colonies and spores, respectively.

Isolation of Phytoalexins. Hairy roots of *H. albus* were subcultured in 32 flasks of 500 mL including 200 mL of MS medium for 3 weeks. To the culture was added 100 μM of JAMe, and it was cultured for 7 days. The medium was collected by filtration and extracted with EtOAc. The EtOAc-soluble fraction (550 mg) was dissolved in MeOH and subjected to HPLC using a reversed-phase column (YMC, ODS) with CH_3CN-H_2O (65:35) as mobile phase and detection at 210 nm. Preparative separation of the EtOAc-soluble fraction gave **1** (3.1 mg), **2** (9.1 mg), **3** (2.8 mg), **4** (6.0 mg), **5** (12.4 mg), **6** (4.9 mg), and **7** (2.2 mg).

Production of Phytoalexins by Treatment with Cu^{2+}. Cu^{2+} was added to three-week-old hairy root culture of *H. albus* in 600 μM and cultured for 3 days. The cultured medium was extracted with EtOAc. TLC of the EtOAc soluble fraction was carried out on Si gel plates.

Production of Phytoalexins by Co-treatment with JAMe and Cu^{2+}. To three-week-old hairy roots of *H. albus* were added 100 μM of JAMe and 600 μM of Cu^{2+}, and this was cultured for 3 days. The cultured medium was extracted with EtOAc. TLC of the EtOAc-soluble fraction was carried out on Si gel plates.

***(3R,4S,5R,7S,9R)*-3-Hydroxy-9-tigloyloxysolavetivone (1):** EIMS *m/z* 332 $[M]^+$ $C_{20}H_{28}O_4$, *m/z* 314 $[M - H_2O]^+$ $C_{20}H_{26}O_3$, *m/z* 232 $[M - C_5H_8O_2]^+$ $C_{15}H_{20}O_2$; CD (MeOH) $[\theta]_{315}$ −2698, $[\theta]_{240}$ +25 992; UV (MeOH) λ_{max} 241, 221 nm; ^{1}H NMR, Table 1; ^{13}C NMR, Table 2.

***(3R,4S,5R,7S,9R)*-3-Hydroxy-9-(3-methylbutenoyloxy)-solavetivone (2):** EIMS *m/z* 332 $[M]^+$ $C_{20}H_{28}O_4$, *m/z* 314 $[M - H_2O]^+$ $C_{20}H_{26}O_2$, *m/z* 232 $[M - C_5H_8O_2]^+$ $C_{15}H_{20}O_2$; CD (MeOH) $[\theta]_{315}$ −3210, $[\theta]_{235}$ +62 934 (MeOH); UV (MeOH) λ_{max} 243, 224 nm. ^{1}H NMR, Table 1; ^{13}C NMR, Table 2.

***(3R,4S,5R,7S,9R)*-3-Hydroxy-9-isobutanoylsolavetivone (3):** EIMS *m/z* 320 $[M]^+$ $C_{19}H_{28}O_4$, *m/z* 302 $[M - H_2O]^+$ $C_{19}H_{26}O_3$; CD (MeOH) $[\theta]_{310}$ −2490, $[\theta]_{235}$ +38 400; UV (MeOH) λ_{max} 240 nm; ^{1}H NMR, Table 1; ^{13}C NMR, Table 2.

***(3R,4S,5R,7S,9R)*-3,9-Dihydroxysolavetivone (4):** EIMS *m/z* 250 $[M]^+$ $C_{15}H_{22}O_3$, *m/z* 232 $[M - H_2O]+$ $C_{15}H_{20}O_2$; CD (MeOH) $[\theta]_{310}$ −5290, $[\theta]_{242}$ +49 520; UV (MeOH) λ_{max} 248 nm; ^{1}H NMR, Table 1; ^{13}C NMR, Table 2.

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NP980214I