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ANTIMICROBIAL STRESS COMPOUNDS FROM *HYPOCHOERIS RADICATA*

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Key Word Index—*Hypochoeris radicata*; Asteraceae; stress compound; sesquiterpenoids; alkenals; fungitoxins.

Abstract—The exudates of *Hypochoeris radicata* leaves stressed with cupric chloride afforded two eudesmane- and guaiane-type sesquiterpenes, and two alkenals, (2*E*,4*E*)-6-hydroxyhexadienal and (2*E*,4*E*)-hexadienedial (mucondialdehyde). Their structures were determined by spectroscopic and synthetic methods.

INTRODUCTION

During a survey of antimicrobial stress metabolites in Asteraceae plants, we found that some fungitoxins were inducibly produced in the leaves of *Hypochoeris radicata* irradiated with ultraviolet rays. It was confirmed that these compounds were also released into cupric chloride solution in which the leaves were steeped. In the present study, we identified new fungitoxic compounds, aliphatic hydroxyalkenal, alkenedial, and eudesmane- and guaiane-type sesquiterpenes from the exudates of *H. radicata* leaves stressed by cupric chloride.

Earlier studies by Bohlmann and Bohlmann [1], and Ohmura *et al.* [2] revealed the presence of a pentacyclic triterpene, a phenylpropanoid, a phenylbutanoid glucoside, and six sesquiterpenes and 17 sesquiterpene glucosides (guaianolides, germacranolides and eudesmanes). Structually related guaiane- and eudesmane-type sesquiterpenes have also been identified from other *Hypochoeris* species [3–5].

Even though induced fungitoxins (for example phytoalexins) are not very common in Asteraceae plants [6], in addition to the present compounds we have already confirmed a fungitoxic guaianolide lettucenin A in the exudates of dandelion leaves stressed with cupric chloride [7, 8]. However, various sesquiterpene stress metabolites have been isolated from Solanaceae, for example, rishitin and its relatives from potato and tomato, and capsidiol from pepper [9]. Usually stress metabolites are induced by various stresses, drying, mechanical injury, irradiation and heavy metal ions as well as microbial invasion. It is not distinguished if the compounds identified by the present study were formed *de novo* by gene derepression or action of the constitutive enzyme(s) in the cells.

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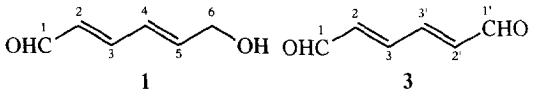
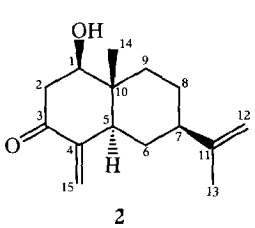
RESULTS AND DISCUSSION

The antimicrobial compounds inducibly produced by *Hypochoeris radicata* leaves and accumulated in a cupric chloride solution were extracted with ethyl acetate, and fractionated by usual procedures (see Experimental) to afford **1**–**4**.

During the fractionation and purification, **1** and **3** showed unstable and volatile properties. Compound **1** was successfully isolated in poor yield and exhibited ^{1}H NMR signals attributable to conjugated *trans*-coupling (J = 15.2 Hz) with olefinic protons resonating at δ6.17 (α), 7.14 (β), 6.58 (γ) and 6.37 (δ) showing a coupling sequence from an aldehydic proton (δ9.58). The H_δ showed a further coupling to an oxygenated methylene at δ4.36 (2H, *d*, J = 4.7 Hz). The EI-mass spectrum exhibited only a weak peak at m/z 112 expected to be the parent ion associated with a base peak at m/z 83.

Further experiments were impossible because of insufficient amounts of the isolated compounds. The estimated compound **1**, (2*E*,4*E*)-6-hydroxyhexadienal has been prepared from dimethyl (2*E*,4*E*)-hexadienedioate via (2*E*,4*E*)-hexadienedial (mucondialdehyde) by Goon *et al.*





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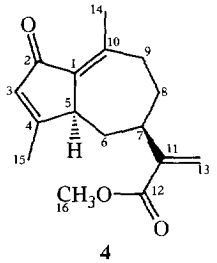

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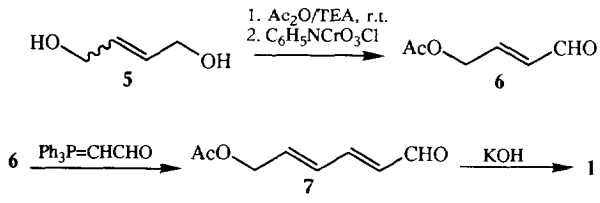

Scheme 1. Synthetic route for (2*E*,4*E*)-6-hydroxyhexadienal (**1**).

[10]. In the present study, **1** was synthesized from 2-butene-1,4-diol (*E*:*Z* = 7:3) according to Scheme 1. Chromatographic (TLC) and spectroscopic (^{1}H NMR) analyses revealed the identity of natural and synthetic **1**.

The identification of **1** suggested that a possible precursor of **1**, mucondialdehyde (**3**), might be found in the exudate. In our recent survey, various plants stressed by biotic and abiotic agents yielded **3** detectable as a potent fungitoxin [11]. Although the isolated compound corresponding to **3** on TLC plates was revealed to be denatured when concentrated to dryness in the air, the ^{1}H NMR signals and GC-mass spectrum were identical to those of mucondialdehyde isolated from *Chenopodium album* [11]. As with authentic mucondialdehyde, **3** exhibited a reddish orange colour or a dull blue colour after spraying TLC plates with 2,4-dinitrophenylhydrazine and *N*,*N*-dimethyl-*p*-phenylenediamine [12], respectively. It has been proposed that **3** appearing as a stress metabolite may be derived from linole(n)ic acid mediated by hydroperoxidation and β-scission in the general lipoxygenase pathway, even though neither of the precursors of **3**, nor the relevant enzymes have been confirmed yet [11].

Mucondialdehyde (**3**) has been identified as a metabolite of benzene catalysed by the microsomal enzymes in mouse liver [13]. An *in vitro* experiment using NADH, alcohol dehydrogenase and **3** as a substrate yielded (2*E*,4*E*)-hexadiene-1,6-diol via (2*E*,4*E*)-6-hydroxyhexadienal (**1**) [10].

Mucondialdehyde (**3**), possessing a palindromic α,β-unsaturated aldehyde structure, shows various kinds of physiological activities, for example, antimicrobial [14], mutagenic [15, 16], inhibitory effects on erythropoiesis and bone marrow function in female mice [17, 18], toxicity to erythrocytic and granulocytic colony growth [19], and reducing hepatic glutathione level and cytochrome P_{450} [20, 21].

The molecular weight of **2** was 234.1615 determined by HREI-mass spectrometry which indicated the molecular formula $C_{15}H_{22}O_2$ (requires M_r 234.1620). The ^{1}H NMR signals detected in pyridine-d_5, ^{1}H–^{1}H COSY, HMQC and HMBC revealed the structure of **2**. The hydroxylated methine proton (δ3.92) clearly H–H correlated to a methylene proton (δ3.13 and 2.82), which were assigned to be α-carbonylmethylene protons. The carbonyl group deshielded one of the exomethylene protons which resonated at δ5.12 and 6.07, and both C-2 and C-15 methylene protons were correlated to the carbonyl carbon (δ198.8) by HMBC spectroscopy. The eudesmane skeleton was confirmed by the detailed analyses of ^{1}H–^{1}H COSY, HMQC and HMBC spectra. The relative stereochemistry of **2** was established by the observation of NOEs between H-1 and H-5, H-5 and H-7, and H_β-2 and Me-14. This compound was induced by stress with cupric chloride, however, the structure is closely related to the known *Hypochoeris* glucoside (hypochoeroside K) [2]. The detailed NMR data for **2** are summarized in Table 1.

The molecular ion at *m/z* 260.1419 of **4** detected by HREI-mass spectrometry indicated the molecular formula $C_{16}H_{20}O_3$ (requires 260.1413). The ^{1}H NMR spectrum of **4** was similar to that of isohypoglabric acid except for the methyl signal at δ3.79 [5]. These results indicated that the structure of **4** was isohypoglabric acid methyl ester. The relative stereostructure of **4** was confirmed by the detection of NOE between H-5 and H-7. The detailed NMR data for **4** are summarized in Table 1.

The structure of **4** thus identified resembles known guaiane-type sesquiterpenes in *H. radicata* [2]. However, **4** is unique in *Hypochoeris* guaianoids as it possesses a non-oxygenated C-15, and an oxidized but unlactonized C_3 side chain. Compounds **1**–**3** were found only in the exudates of stressed leaves, whilst it was not clear if **4** was an induced metabolite.

EXPERIMENTAL

General. Mp: uncorr. Analytical and prep. thin-layer separations were carried out on Merck pre-coated silica gel plates (60 F_{254}; layer thickness 0.25 mm). TLC bioautography was employed to detect antifungal constituents by using the Merck plates and *Cladosporium herbarum* as the test fungus [22]. Details relating to the TLC solvent systems are given at the appropriate points. Instrumental analyses (UV, MS and NMR) were undertaken using the equipment and conditions previously described [23].

Plant material and induction of stress metabolites. Aerial parts of *Hypochoeris radicata* were collected on the campus of Hokkaido University in October and stressed with Cu^{2+} by soaking 200 g each of the leaves for 24 hr in 1 l of 3 mM cupric chloride soln in a plastic vat (40 × 70 × 20 cm^3). The exudates from 6 kg (fr. wt) of the leaves were extracted with EtOAc.

Isolation of antifungal compounds. The crude extracts equivalent to 0.50 g of fresh leaves treated with the $CuCl_2$ soln and H_2O were applied to TLC bioautography to detect the inducibly produced antifungal components. Some inhibitory zones on silica gel plates including **2** (R_f at 0.31) and **3** (R_f at 0.40) developed in hexane–EtOAc (2:3) were remarkable and characteristic of the $CuCl_2$ treated fr. The other two fungitoxins (**1** and **4**) masked with some constitutive materials in the first TLC bioautography became detectable in the further column frs at R_f 0.47 (**4**) and R_f 0.26 (**1**) on TLC plates developed in the same solvents.

The crude extract (*ca* 0.95 g) of the exudates released from 6 kg of *H. radicata* leaves into the cupric chloride soln was at first fractionated by silica gel (20 g) CC. Column frs (50 ml each) and the ratio of hexane–EtOAc as eluting solvent were as follows: Fr. 1–3, 7:3; Fr. 4–6,

Table 1. NMR spectral data of **2** and **4** (^{13}C: 125 MHz, ^{1}H: 500 MHz)

	2			4	
Position	C(C_6D_6)	H (pyridine-d_5)	HMBC	C($CDCl_3$)	H($CDCl_3$)
1	76.2	3.92 *ddd* (5.0, 6.0, 11.3)	H-14, H_α-9, H_α-2, H_β-2	136*	
2	46.4	H_α 3.13 *dd* (6.0, 17.5)		197*	
		H_β 2.82 *dd* (11.3, 17.5)			
3	198.8		H_α-2, H_β-2, H_a-15, H_b-15	133.6	6.05 *s*
4	148.6		H_b-15	172*	
5	44.6	1.65 *m*	H-14, H-1	49.2	3.15 *d* (11.8)
6	29.2	H_α 1.82 *ddd*	H_α-7, H_β-8	36.2	H_α 2.12 *d* (11.8)
		H_β 1.35 *m*			H_β 0.98 *q* (11.8)
7	45.6	H 1.95 *dddd* (3.3, 3.4, 12.6, 12.6)	H_β-8, H-13, H_a-12, H_b-12	43.5	2.86 *t* (11.8)
8	26.7	H_α 2.38 *ddd*-like (2.8, 3.3, 13.2)	H_α-9, H_β-6, H_β-9	31.1	H_α 1.91 *m*
		H_β 1.46 *dddd* (3.1, 12.6, 12.6, 13.2)	H-7		H_β 1.38 *m*
9	36.8	H_α 1.35 *m*	H_β-8, H-1	37.7	H_α 2.50 *t* (13.7)
		H_β 2.07 *ddd* (2.8, 3.1, 11.5)			H_β 2.22 *dd* (7.1, 13.7)
10	38.9		H-1, H_α-2, H_β-2, H_β-9, H-14	171*	
11	150.1			146*	
12	109.5	H_a 4.85 *br s*	H-13	168*	
		H_b 4.88 *br s*			
13	21.3	3H 1.77 *s*	H_a-12, H_b-12	123.7	H'_a 5.45 *s*
					H'_b 6.19 *s*
14	10.0	3H 0.99 *s*	H-1, H-5, H_α-9, H_β-9	20.5	3H 2.37 *s*
15	118.5	H_a 5.12 *dd* (1.5, 2.5)		16.6	3H 2.06 *s*
		H_b 6.07 *dd* (1.5, 2.3)			
16				52.0	3H 3.79 *s*
HO		6.68 *d* (5.0)			

*Quaternary carbons were detected by HMBC and the chemical shift value are approximate.

3:2; and Fr. 7–9, 1:1. Combined frs 2 and 3 (209 mg) containing **2**–**4** were rechromatographed over 20 g of silica gel. The charged column was continuously eluted with a mixture of hexane–EtOAc (7:3). The eluate from 20 to 60 ml afforded **4**, and **3** and **2** were found in the eluates from 70 to 120 ml, and from 150 to 180 ml, respectively.

The eluate containing **2** was concd and subjected to prep. TLC in hexane–EtOAc (3:2). The major component eluted from the TLC band was successively subjected to prep. TLC in $CHCl_3$–MeOH (50:1, R_f 0.17–0.29) to yield **2**, 24.4 mg. Needles mp 112–114° were obtained after further purification by HPLC: Inertsil ODS column (20.0 × 250 mm) eluted with MeCN–H_2O (7:3) at a flow rate of 5.0 ml min^{-1}, R_t 17–18 min monitored at UV 254 nm.

The frs containing **3** were further purified by prep. TLC in $CHCl_3$–MeOH (40:1, R_f 0.57) and subjected to ^{1}H NMR and GC-MS analyses. When monitored by a UV detector at 254 nm, **3** was found at *ca* R_t 4.2 min by HPLC: Inertsil ODS column (6.0 × 250 mm) eluted with MeOH–H_2O (7:3) at a flow rate of 0.8 ml min^{-1}.

Early column frs containing **4** were subjected to HPLC: Inertsil ODS column (20.0 × 250 mm) eluted with MeOH–H_2O (4:1) at a flow rate of 5.0 ml min^{-1}. The major peak detectable at R_t 26–28 min was collected to give 1.5 mg of pure **4**.

One of the first column frs, fr. 6, exhibited a 2,4-dinitrophenylhydrazine positive and fungitoxic component (**1**, R_f 0.26 developed in hexane–EtOAc, 2:3), a part of which (*ca* 1.0 mg) was isolated by HPLC: Inertsil ODS column (4.6 × 250 mm) eluted around R_t 4–5 min with MeOH–H_2O (7:3) at a flow rate of 0.8 ml min^{-1}.

Physicochemical properties of Hypochoeris *fungitoxins*

(2E,4E)-6-*Hydroxyhexadienal* (**1**). Isolated compound, ^{1}H NMR ($CDCl_3$, 500 MHz): δ4.36 (2H, *d*, J = 4.7 Hz, H-6), 6.17(1H, *dd*, J = 15.2 and 7.8 Hz, H-2), 6.37 (H, *dt*, J = 15.2 and 4.7 Hz, H-5), 6.58 (1H, *dd*, J = 15.2 and 10.8 Hz, H-4), 7.14 (1H, *dd*, J = 15.2 and 10.8 Hz, H-3), 9.58 (1H, *d*, J = 7.8 Hz, H-1).

Synthetic compound **1**. Pale yellow oil, 2,4-dinitrophenylhydrazine: (+), reddish orange. FIMS *m/z* (rel. int.): 112 $[M]^+$ (100). EIMS *m/z* (rel. int.): 112 $[M]^+$ (4.2), 94 $[M - H_2O]^+$ (7.0), 83 $[M - CHO]^+$ (100), 68 (60), 57 (8.9), 55 (45), 41 (23). The ^{1}H NMR spectrum was identical to that of natural product except for the detection of OH signal at *ca* 2.1.

Compound **2**. Needles, mp 112–114°, $[\alpha]_D^{22}$ −40.0° ($CHCl_3$; *c* 0.1). UV λ_{max}^{MeOH} nm (ε): 243 (12400), 291 (1600). FDMS: *m/z* (rel. int.): 234 $[M]^+$ (100). HREI-MS: *m/z* 234.1615 ($C_{15}H_{22}O_2$ requires 234.1620). EIMS *m/z* (rel. int.): 234 $[M]^+$ (74), 119 (51), 107 (56), 105 (64), 93 (64),

91 (72), 79 (62), 67 (52), 41 (100), 40 (67). NMR data in Table 1.

Mucondialdehyde (**3**). GCMS (GC: column, 5% PEG-20M, 3 mm i.d. × 1 m; temp. prog. 70–210° at 10° min^{-1}; He, 40 ml min^{-1}; R_t 11.9 min identical to that of authentic mucondialdehyde [24]) *m/z* (rel. int.): 110 $[M]^+$ (94), 82 (19), 81 (95), 54 (15), 53 (100), 51 (22), 50 (18), 39 (21). The ^1H NMR spectrum of **3** in C_6D_6 at 500 MHz was confirmed to be identical to that of mucondialdehyde isolated from *Chenopodium album* [11].

Compound **4**. Oil. FIMS *m/z* (rel. int.): 260 $[M]^+$ (100), HREI-MS *m/z* 260.1419 ($C_{16}H_{20}O_3$ requires 260.1413). EIMS *m/z* (rel. int.): 260 $[M]^+$ (70), 201 (29), 200 (45), 185 (27), 159 (28), 148 (100), 133 (44), 105 (47), 91 (44), 41 (40), 39 (26). UV λ_{max}^{MeOH} nm: 250. CD λ_{max}^{MeOH} nm (rel. int.): 302 (+ 0.2), 239 (+ 1.0). NMR data in Table 1.

Preparation of (2E,4E)-6-*hydroxyhexadienal* (**1**) (Scheme 1). To a mixture of 2-butene-1,4-diol (**5**, *E*:*Z* = 7:3, 5.14 g) and Ac_2O (5.70 g) in dioxane (25 ml) was added 5.77 g of triethylamine dropwise under stirring, and further stirred for 4 hr. After acidification, the products were extracted with EtOAc. The concd extracts were subjected to silica gel CC with hexane EtOAc, 3:1 as eluting solvent to yield 4-acetoxy-2-buten-1-ol and 1,4-diacetoxy-2-butene in 37 and 22% yields, respectively. 4-Acetoxy-2-butene-1-ol: EIMS *m/z* (rel. int.): 112 $[M - H_2O]^+$ (1.4), 70 $[M - CH_2CO]^+$ (31), 60 (15), 43 (100). ^1H NMR ($CDCl_3$, 270 MHz): δ 2.08 (3H, *s*, Ac), 4.18 (2H, *d*, *J* = 4.6 Hz, CH_2OH), 4.58 (2H, *d*, *J* = 5.6 Hz, CH_2OAc), 5.80–5.99 (2H, *m*, CH = CH).

The allyl alcohol (*E*:*Z* = 7:3, 1.0 g) was oxidized with pyridinium chlorochromate as usual and worked-up to yield (2*E*)-4-acetoxy-butenal (**6**, in 66% yield after CC) [25]. Compound **6**; EIMS *m/z* (rel. int.): 99 $[M - CHO]^+$ (1.5), 68 (18), 43 (100). ^1H NMR ($CDCl_3$, 270 MHz): δ 2.14 (3H, *s*, Ac), 4.86 (2H, *dd*, *J* = 4.3 and 2.0 Hz, $-CH_2OAc$), 6.29 (1H, *ddt*, *J* = 15.8, 7.8 and 2.0 Hz, $=C\underline{H}CHO$), 6.83 (1H, *dt*, *J* = 15.8 and 4.3 Hz, $=C\underline{H}-CH_2OAc$), 9.59 (1H, *d*, *J* = 7.8 Hz, CHO).

Commercially available formylmethytriphenylphosphonium chloride (1.0 g) was stirred in 10 ml of EtOH, to which was added 0.30 g triethylamine. After 1 hr stirring, the reaction mixt. was reduced to dryness. The resulting crude ylide (1.24 g) and (*E*)-4-acetoxy-2-butenal (**6**, 140 mg) were refluxed in 20 ml C_6H_6 for 17 hr under N_2 gas. The reaction mixture was concd *in vacuo* and applied to silica gel CC (hexane EtOAc, 2:1) to yield (2*E*,4*E*)-6-acetoxy-2,4-hexadienal 63 mg (**7**, in 37% yield), a part of which was stood for 2 hr in dioxane aq. 2.3 M KOH (5:1) at room temp. After being neutralized, the hydrolysate was extracted with Et_2O, and finally purified by prep. TLC in $CHCl_3$–MeOH, 9:1 (R_f *ca* 0.39) to yield pure **1**.

Compound **7**. FIMS *m/z* (rel. int.): 154 $[M]^+$ (100). EIMS *m/z* (rel. int.): 94 $[M - MeCO_2H]^+$ (17), 81 (40), 43 (100). ^1H NMR ($CDCl_3$, 270 MHz): δ 2.12 (3H, *s*, Ac), 4.72 (2H, *d*, *J* = 5.5 Hz, $AcOCH_2$), 6.19 (1H, *dd*, *J* = 15.4 and 7.9 Hz, $C\underline{H}CHO$), 6.27 (1H, *dt*, *J* = 15.3 and 5.5 Hz, $AcOCH_2C\underline{H}$), 6.53 (1H, *dd*, *J* = 15.3 and 10.7 Hz, $CH_2-CH=C\underline{H}$), 7.10 (1H, *dd*, *J* = 15.4 and 10.7 Hz, $C\underline{H}=CH-CHO$), 9.59 (1H, *d*, *J* = 7.9 Hz, CHO). Physicochemical properties of synthetic **1**, see those of the natural product.

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