

Sesquiterpenoids in Root Exudates of *Solanum aethiopicum*

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Five known sesquiterpenoids, solavetivone, lubimin, lubiminoic acid, aethione and lubiminol were isolated from the root exudates recovered from *Solanum aethiopicum* by a newly proposed method using charcoal. Quantitative analysis of the sesquiterpenoids in the root exudates of *S. aethiopicum* and *S. melongena* suggested that relatively large amounts of the sesquiterpenoids were exuded from the roots. Antifungal activity of the sesquiterpenoids against *Fusarium oxysporum* and *Verticillium dahliae* was also examined.

Introduction

Chemical studies on the constituents of the roots of solanaceous rootstocks have been conducted in order to reveal their resistance against soil-borne pathogens (Nagaoka *et al.*, 1987, 1993 and 1995; Yoshihara *et al.*, 1988a and 1988b). Wild relatives are often used as mating sources of resistant cultivars as well as rootstocks in solanaceous plants. *Solanum aethiopicum* L., one of the wild relatives, is resistant against soil-borne pathogens such as *Fusarium oxysporum* f. sp. *melongenae*, *Verticillium dahliae* and *Ralstonia solanacearum*, and is utilized as a rootstock and a source of resistance against *R. solanacearum* (Ano *et al.*, 1991). In a previous study (Nagase *et al.*, 2001), nine sesquiterpenoids (**1–9**) were isolated from the roots of *S. aethiopicum*. These vetispirane-type sesquiterpenoids are known as the phytoalexin of Solanaceae. Sesquiterpenoids in the root exudates of *S. aethiopicum* were investigated to estimate their effects on soil-borne plant pathogens existing in rhizospheres.

This report deals with the isolation of five sesquiterpenoids, solavetivone (**1**), lubimin (**5**), lubiminoic acid (**7**), aethione (**9**) and lubiminol (**10**) from the root exudates of *S. aethiopicum*, which were recovered by a newly proposed

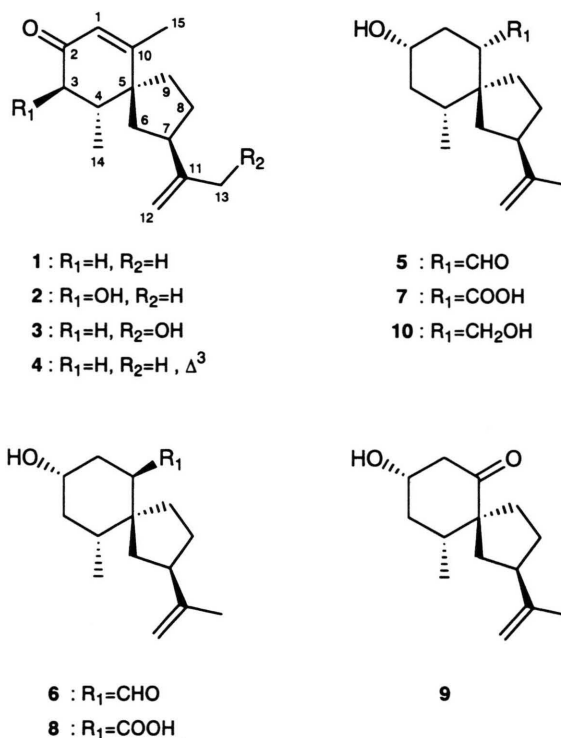


Fig. 1. Structures of sesquiterpenoids from roots and root exudates of *S. aethiopicum*.

method using charcoal. Comparative quantification of sesquiterpenoids in the roots and the root exudates of *S. aethiopicum* and *S. melongena* was carried out by GC-MS. Antifungal activities of the sesquiterpenoids against *F. oxysporum* f. sp. *melongenae* and *V. dahliae* were also examined.

Results and Discussion

Lipophilic exudates from the roots of *S. aethiopicum* were adsorbed to charcoal by cultivating the plants in pots filled with a charcoal-vermiculite mixture. The mixture detached from the roots after cultivation was eluted with ethanol and ethyl acetate to recover the lipophilic exudates containing sesquiterpenoids. The ethanol eluate and the ethyl acetate eluate were evaporated and extracted with the ethyl acetate. Chromatographic purification of ethyl acetate extracts gave five sesquiterpenoids (**1**, **5**, **7**, **9** and **10**). The ¹H and MS spectral data of these sesquiterpenoids were identical to those of solavetivone (**1**) (Coxon *et al.*, 1974), lubimin (**5**) (Katsui *et al.*, 1977; Ewing, 1990), lubiminoic acid (**7**), aethione (**9**) (Nagase *et al.*, 2001) and lubiminol (**10**) (Katsui *et al.*, 1977; Ewing, 1990). These sesquiterpenoids except **10** have been isolated from the roots of *S. aethiopicum* (Nagase *et al.*, 2001). This newly proposed procedure seems to be available for the recovery of lipophilic exudates from roots, and is much simpler and easier than the method of Tang and Young (1982). Application of this method to other plants and other-type compounds should be further investigated. In addition, the absolute configurations of **7** and **8**, which have been presumed in a previous study (Nagase *et al.*, 2001), were confirmed by comparing their specific rotations with

those of **7** and **8** converted from **5** and **6** with sodium chlorite, respectively.

Selected sesquiterpenoids (**1**, **2**, **5**, **9** and **10**) in the roots and the root exudates of *S. aethiopicum* grown in the field and the greenhouse were quantified by GC-MS to roughly estimate their contents in different growth conditions. The sesquiterpenoid contents of the roots and the root exudates of *S. melongena* (cultivar: Senryo), which is susceptible to *F. oxysporum* f. sp. *melongenae* and *V. dahliae*, were also analyzed to assess the differences between species. Both plants were cultivated in the field and in pots filled with a charcoal-vermiculite mixture, and the roots were collected after cultivation, and then the root exudates were recovered from the charcoal-vermiculite mixture. The results are summarized in Table I. The sesquiterpenoid contents of the root exudates were calculated as mg per kg of harvested roots to contrast with those of the roots. The sesquiterpenoid contents of the roots in the field were far larger than those in the greenhouse, suggesting that soil environments, especially soil microbial properties, affect the generation of sesquiterpenoids. The roots of *S. aethiopicum* and *S. melongena* grown in the field accumulated considerably greater amounts of solavetivone (**1**) and lubimin (**5**), respectively, while the contents of the roots in the greenhouse exhibited no differences between species. Yoshihara *et al.* (1988) described similar results in their comparative study on sesquiterpenes in the roots of resistant rootstock (*S. melongena* × *S. integrifolium*) and *S. melongena* (cultivar: Senryo). The sesquiterpenoid contents of the root exudates were quite larger than those of the roots. This indicates that the roots of *Solanum* plants exude a relatively large quantity of sesquiterpenoids, which

Table I. Sesquiterpenoid contents of the roots and the root exudates of *S. aethiopicum* and *S. melongena* grown in the field and the greenhouse.

Plant	Source	Sesquiterpenoid contents (mg/kg fresh roots)				
		1	2	5	9	10
<i>S. aethiopicum</i>	root (field)	331.5	35.0	63.3	n.d.	n.d.
	root (pot)	1.4	0.2	1.7	n.d.	n.d.
	exudate (pot)	6.6	n.d.	50.3	30.2	3.8
<i>S. melongena</i>	root (field)	76.7	n.d.	421.8	n.d.	n.d.
	root (pot)	n.d.	n.d.	1.1	n.d.	n.d.
	exudate (pot)	1.3	n.d.	20.7	2.6	0.3

n.d.; not detectable.

Table II. Sesquiterpenoid contents of the roots and the root exudates of *S. aethiopicum* and *S. melongena* in test tube experiments. Standard deviations are shown in parentheses.

Plant	Source	Roots (g/tube)	Sesquiterpenoid contents ($\mu\text{g}/\text{tube}$)				
			1	2	5	9	10
<i>S. aethiopicum</i>	root	2.1 (0.2)	n.d.	n.d.	1.2 (1.5)	n.d.	n.d.
	exudate	2.1 (0.2)	1.1 (0.9)	n.d.	21.5 (11.9)	1.1 (0.7)	1.7 (1.5)
<i>S. melongena</i>	root	1.9 (0.7)	n.d.	n.d.	1.5 (1.0)	n.d.	n.d.
	exudate	1.9 (0.7)	1.1 (1.0)	n.d.	47.6 (30.5)	5.9 (4.5)	5.3 (1.8)

n.d.; not detectable.

have possible effects on microorganisms including soil-borne pathogens in the rhizospheres. Lubimin (**5**) was the major component of the sesquiterpenoids in the root exudates of both plants, and seems to exist mostly in the root exudates of *S. aethiopicum* rather than *S. melongena*, although degradation and transformation of the sesquiterpenoids by soil microorganisms are not considered. Adsorption of the sesquiterpenoids to charcoal possibly reduces the bioavailability to degraders.

To recover the sesquiterpenoids from the root exudates on a small scale, cultivation of *S. aethiopicum* and *S. melongena* was examined in test tubes filled with a charcoal-vermiculite mixture for two weeks. The amounts of sesquiterpenoids recovered from the roots and the root exudates in the test-tube experiments were enough to analyze by GC-MS (Table II). The results were similar to the pot experiments as mentioned above, except that the sesquiterpenoid contents in the root exudates of *S. melongena* were greater than those of *S. aethiopicum*. These data provided no obvious relationship between resistance against soil-borne pathogens and sesquiterpenoids in the root exudates. Desjardins *et al.* (1995) suggested that the dry rot resistance of the potato tuber did not correlate with the concentrations of sesquiterpenes elicited by arachidonic acid. Quantitative evaluation of the sesquiterpenoids in the root exudates of *Solanum* plants should be further studied.

In spore germination tests, all the tested compounds showed antifungal activity against *F. oxysporum* and/or *V. dahliae* at 100 $\mu\text{g}/\text{ml}$ (Table III). The structure-activity relationship in the case of *F. oxysporum* was different from that in the case of *V. dahliae*. Compounds **1**, **3**, **5** and **6** possess stronger activity against *F. oxysporum* than **2**, **4** and **9**, but compounds **7** and **8** do not. The inhibitory

Table III. Effects of **1**–**9** on spore germination of *F. oxysporum* and *V. dahliae* at 100 $\mu\text{g}/\text{ml}$.

Compound	Spore germination (% control)	
	<i>F. oxysporum</i>	<i>V. dahliae</i>
1	30	14
2	60	8
3	30	106
4	60	18
5	5	42
6	30	54
7	100	44
8	120	34
9	85	32

effects of lubimin (**5**) and epilubimin (**6**) on spore germination of *F. oxysporum* would be attributed to the formyl group. All the compounds except **3** exhibited antifungal activity against *V. dahliae*, and compounds **1**, **2** and **4** gave especially strong activities. The isopropenyl group of the sesquiterpenoids may be essential to inhibit spore germination of *V. dahliae*. The antifungal activity of solavetivone (**1**) and anhydro- β -rotunol (**4**) against various fungi in spore germination tests has been reported by Harris and Dennis (1976).

Solavetivone (**1**) and lubimin (**5**) restrained radial growth of *F. oxysporum*, but stimulated the growth of *V. dahliae* at 50 and 100 $\mu\text{g}/\text{ml}$ as shown in Table IV. Apparently, these compounds produced several effects on soil-borne pathogens.

Experimental

General experimental procedures

NMR: Bruker AMX-500 instrument, 500 MHz (^1H NMR) and 125 MHz (^{13}C NMR) in CDCl_3 . MS: JEOL JMS-AX500 and JEOL JMS-SX102A

Table IV. Effects of **1** and **5** on growth of *F. oxysporum* and *V. dahliae*.

Compound	Concn. ($\mu\text{g/ml}$)	% growth	
		<i>F. oxysporum</i>	<i>V. dahliae</i>
1	50	68	108
	100	28	143
5	50	50	148
	100	23	184

mass spectrometer. IR: Perkin Elmer System 2000 FT-IR spectrometer. Polarimeter: JASCO DIP-350 digital polarimeter. Silica gel 60 F₂₅₄ TLC plates (thickness, 0.25 mm; Merck, Darmstadt, Germany) were used for analytical TLC. CC was carried out on silica gel 60 N (Kanto Chemicals, Tokyo, Japan) and silica gel 60 (Merck, Darmstadt, Germany).

Recovery of root exudates

The plants of *S. aethiopicum* L. were grown in small pots (0.5 l) filled with peatmoss and perlite (2:1 v/v) for two months in the greenhouse at 20°C on average. After the pot cultivation the plants were transplanted to 25 pots filled with 2 l of a mixture of charcoal and vermiculite (1:3 v/v), and cultivated for a month. Liquid fertilizer was used as a nutrient source. The mixtures of charcoal and vermiculite were carefully separated from the roots of *S. aethiopicum* in a plastic container filled with tap water after the cultivation. The recovered mixture of vermiculite and charcoal (50 l) was halved and put into two stainless steel columns (diameter, 39 cm; height, 39 cm). Each steel column has a sieve of 1 mm at 5 cm from the bottom, and a drain-pipe plugged with absorbent cotton on the lowest side. Each mixture of charcoal and vermiculite (25 l) was rinsed with 12.5 l of water, and eluted sequentially with 12.5 l of EtOH, another 12.5 l of EtOH and 12.5 l of EtOAc. Each eluate was evaporated to dryness *in vacuo*, and extracted with 100 ml of EtOAc three times after the addition of 100 ml of distilled water. The EtOAc extracts dried over Na₂SO₄ were concentrated to dryness, and gave the E-1 fraction from the first EtOH eluate, the E-2 fraction from the second EtOH eluate and the E-3 fraction from the EtOAc eluate. The E-3 fraction combined with the

E-2 fraction, called the ES fraction, was used for isolation of the sesquiterpenoids, since the TLC analysis of these fractions suggested that the sesquiterpenoids were mainly included in the E-3 fraction, and a small amount of sesquiterpenoids was in the E-2 fraction.

Isolation

The ES fraction (468 mg) from 25 plants was subjected to CC on silica gel and sequentially eluted with CHCl₃ and CHCl₃-MeOH (97:3 v/v) to yield 15 fractions (Fr. 1–15). Compounds **1** (5.2 mg), **5** (32.0 mg), **7** (0.6 mg), **9** (1.2 mg) and **10** (0.7 mg) were isolated from Fr. 6, Fr. 8, Fr. 14, Fr. 10 and Fr. 14 by further chromatography, respectively. A bioautographic technique according to Homans and Fuchs (1970) was used with *F. oxysporum* f. sp. *melongenae* for the detection of antifungal sesquiterpenoids. Anisaldehyde-H₂SO₄ was also used as a spray reagent on TLC chromatograms to detect sesquiterpenoids. The NMR spectral data are not shown.

Solavetivone (1): Colorless oil; $[\alpha]_D^{20}$ -95.3° (c 0.27, CHCl₃); EIMS *m/z* (rel. int.): 218.1640 (calcd. for C₁₅H₂₂O, 218.1670) [M]⁺ (100), 203 [M-Me]⁺ (33).

Lubimin (5): Colorless oil; $[\alpha]_D^{20}$ +32.8° (c 0.32, CHCl₃); EIMS *m/z* (rel. int.): 236.1748 (calcd. for C₁₅H₂₄O₂, 236.1776) [M]⁺ (91), 218 [M-H₂O]⁺ (34), 193 (100).

Lubiminoic acid (7): White solid; $[\alpha]_D^{20}$ +35.4° (c 0.06, CHCl₃); EIMS *m/z* (rel. int.): 252.1761 (calcd. for C₁₅H₂₄O₃, 252.1725) [M]⁺ (19), 234 [M-H₂O]⁺ (78), 107 (100).

Aethione (9): Colorless oil; EIMS *m/z* (rel. int.): 222.1632 (calcd. for C₁₄H₂₂O₂, 222.1621) [M]⁺ (100), 207 [M-Me]⁺ (17), 204 [M-H₂O]⁺ (16).

Lubiminol (10): White solid; $[\alpha]_D^{20}$ +24.3° (c 0.12, CHCl₃); FDMS *m/z* (rel. int.): 238 (100); EIMS *m/z* (rel. int.): 238.1943 (calcd. for C₁₅H₂₆O₂, 238.1932) [M]⁺ (15), 220 [M-H₂O]⁺ (30), 202 [M-2H₂O]⁺ (31), 148 (100).

Chemical conversions

Sesquiterpenes **5** and **6** isolated from the roots of *S. aethiopicum* in a previous study (Nagase *et al.*, 2001) were used for their oxidation to **7** and **8**.

Oxidation of 5 to 7: An aq. soln. of NaClO₂ (20 μmol) was added slowly to the soln. of **5** (2 mg, 8.5

μmol) in 0.4 ml of *t*-BtOH containing 2-methyl-2-butene (50 μmol) plus 0.1 ml of aq. NaH_2PO_4 (10 μmol), and reacted at room temp. for 1 hr. The reacted soln. was acidified and extracted with CH_2Cl_2 . Silica gel CC of the CH_2Cl_2 extracts yielded **7** (1.2 mg). Data for **7**: White solid; $[\alpha]_{\text{D}}^{20} +40.5^\circ$ (c 0.12, CHCl_3); EIMS m/z (rel. int.): 252.1727 (calcd. for $\text{C}_{15}\text{H}_{24}\text{O}_3$, 252.1725) $[\text{M}]^+$ (33), 234 $[\text{M}-\text{H}_2\text{O}]^+$ (82), 40 (100); The ^1H and ^{13}C NMR spectra of **7** agreed with those of **7** isolated in the previous study (Nagase *et al.*, 2001).

Oxidation of 6 to 8: Compound **6** (2.4 mg, 10 μmol) was oxidized with NaClO_2 in the same way as described in the oxidation of **5**, and purification of the reacted product yielded **8** (1.6 mg). Data for **8**: White solid; $[\alpha]_{\text{D}}^{20} -19.4^\circ$ (c 0.16, CHCl_3); EIMS m/z (rel. int.): 252.1720 (calcd. for $\text{C}_{15}\text{H}_{24}\text{O}_3$, 252.1725) $[\text{M}]^+$ (25), 234 $[\text{M}-\text{H}_2\text{O}]^+$ (75), 40 (100). The ^1H and ^{13}C NMR spectra of **8** agreed with those of **8** isolated in the previous study (Nagase *et al.*, 2001).

Pot cultivation

Plants of *S. aethiopicum* L. and *S. melongena* L. (cv. Senryo) were cultivated in pots filled with a mixture of charcoal and vermiculite, and the sesquiterpenoids were recovered from the mixture in the same way as described for the recovery of root exudates. Yields of the ES fractions from the root exudates of *S. aethiopicum* and *S. melongena* were 518 mg and 745 mg per 25 pots, respectively. Separated fresh roots of *S. aethiopicum* (0.56 kg) and *S. melongena* (0.8 kg) were extracted with 70% EtOH (3 l). Each extract was filtered and evaporated to 200 ml *in vacuo*. The aq. soln. was extracted with 200 ml of Et_2O three times, and the Et_2O extracts dried over Na_2SO_4 were concentrated to dryness *in vacuo*. Small amounts of the ES fractions from the exudates and the Et_2O extracts from the roots were dissolved in EtOAc at 0.2 mg/ml for GC-MS analysis.

Field cultivation

The plants of *S. aethiopicum* L. and *S. melongena* L. (cv. Senryo) grown in the greenhouse for two months were transplanted to the field in the Agricultural Experimental Farm of Hokkaido University. Fresh roots (1 kg) of the plants grown in the field for three months were collected and

extracted with 70% EtOH (5 l). The EtOH extracts were evaporated to 400 ml, and extracted with an equal volume of Et_2O three times. The Et_2O extracts dried over Na_2SO_4 were concentrated to dryness, and the residues were dissolved in adequate volumes of EtOAc and diluted to 0.1 mg/ml in EtOAc for GC-MS analysis.

Test tube experiment

S. aethiopicum L. and *S. melongena* L. (cv. Senryo) were grown in small pots filled with vermiculite (100 ml) at 25 °C in an incubator. After four weeks the plants were transplanted to test tubes for plant culture (2.5 cm i.d. $\times$ 10 cm) containing a charcoal-vermiculite mixture (30 ml) for 1 plant per tube, and grown at 25 °C for 2 weeks in the incubator. Liquid fertilizer was used as a nutrient source. Each charcoal-vermiculite mixture was carefully detached from the roots in a beaker filled with distilled water after the cultivation. The detached mixture was collected on a filter paper affixed to a funnel, rinsed with 30 ml of water, and eluted successively with 30 ml of EtOH and EtOAc. The EtOH and EtOAc eluate were combined, evaporated, and extracted with 10 ml of EtOAc three times. The EtOAc extracts dried over Na_2SO_4 were concentrated to dryness *in vacuo*, and dissolved in 2 or 5 ml of EtOAc for GC-MS analysis. Fresh roots removed from the charcoal-vermiculite mixture were extracted with 25 ml of 70% EtOH, and then the extracts were evaporated and extracted with 15 ml of EtOAc three times. The EtOAc extracts were dissolved in 2 ml of EtOAc after evaporation. All treatments were performed with three replicates.

GC-MS analysis

The samples were analyzed in full-scan mode with a Finnigan-MAT GCQ gas chromatograph-mass spectrometer equipped with a capillary column of DB-17 (0.25 mm i.d. $\times$ 30 m; film thickness, 0.25 μm ; J&W Scientific, Folsom, USA). The column oven was held at 120 °C for 1 min, heated to 210 °C at 10 °C/min and then held at 210 °C for 5 min. The column oven was then heated to 250 °C at 10 °C/min and held at 250 °C for 11 min. Flow rate of He gas as a carrier was 1 ml/min. Injector and ion source temperatures were 250 and 200 °C, respectively. Ionization was done by elec-

tron ionization (EI). Scanning range and rate were m/z 50–300 and one time per second, respectively. Under these conditions, the sesquiterpenoids had the following retention times: **1**, 11.54 min; **2**, 13.18 min; **5**, 14.14 min; **9**, 12.30 min; **10**, 16.22 min. Quantification of **1**, **2**, **5**, **9** and **10** was carried out by monitoring the selected ions at m/z 190, 176, 236, 222 and 220 in the full-scan data, respectively. The external standard solutions of **1**, **2**, **5**, **9** and **10** at 2, 4, 6, 8 and 10 $\mu\text{g/ml}$ were prepared using compounds isolated from the root exudates and roots of *S. aethiopicum*.

Bioassay on spore germination

Sesquiterpenoids (**1**–**9**) isolated from the roots of *S. aethiopicum* in a previous study (Nagase *et al.*, 2001) were used for spore germination tests. The spores of *F. oxysporum* f. sp. *melongenae* and *V. dahliae* were harvested by irrigating each individual culture grown in potato dextrose agar (PDA) slants with 10 ml of sterile distilled water. The spores collected by filtration with gauze and centrifugation were suspended in 1 ml of sterile distilled water for spore germination tests. Each sesquiterpenoid (**1**–**9**) was dissolved in 0.4% glucose/EtOH (9:1 v/v) at a concentration of 200 $\mu\text{g/ml}$. Equal volumes of a spore suspension and a sesquiterpenoid soln. were mixed, and 4 μl of the mixture was pipetted onto a microscope slide. Each slide was put into a Petri dish, and incubated at 25 °C for 20 h in the dark. Moistened filter papers were affixed to the inside of the lid and the

bottom of the Petri dish to maintain humidity. After incubation, the percentage of germinating spores was determined, and the percentage control of spore germination was calculated by comparing with germination in the absence of the test compounds. All treatments were duplicated.

Bioassay on radial growth

Two sesquiterpenes (**1** and **5**) were dissolved in EtOH at both 5 and 10 mg/ml, and applied to the bioassay. Each sesquiterpene soln. (20 μl) was mixed with 2 ml of PDA medium in a small test tube and then transferred to a Petri dish (5 cm in diameter). Mycelial blocks of *F. oxysporum* f. sp. *melongenae* and *V. dahliae* were excised from the growing margins of each individual PDA plate with a cork borer (5 mm in diameter), and placed in the center of plates containing the sesquiterpenes. The inoculated plates were incubated at 23°C in the dark for 4 days in the case of *F. oxysporum*, and for 14 days in the case of *V. dahliae*. After the incubation radial growth on the sesquiterpene-amended media was measured, and divided by the radial growth of the control to provide the percentages of growth. All the procedures were duplicated.

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