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ALTERSOLANOL-RELATED COMPOUNDS FROM THE CULTURE LIQUID OF *ALTERNARIA SOLANI*

NOBUYUKI OKAMURA,* KEIJI MIMURA, HIROYUKI HARAGUCHI,† KAZUSHI SHINGU,‡ KAZUMOTO MIYAHARA‡ and AKIRA YAGI

Faculty of Pharmacy and Pharmaceutical Sciences and †Engineering, Fukuyama University, Fukuyama, Hiroshima 729-02, Japan; ‡Faculty of Pharmaceutical Sciences, Setsunan University, 45-1, Hirakata, Osaka 573-01, Japan

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Key Word Index—*Alternaria solani*; Hyphomycetes; 5-methylsulphonylmethylenealtersolanol A; 5-methylaltersolanol A; antimicrobial activity.

Abstract—Two new crystalline pigments, 5-methylsulphonylmethylenealtersolanol A and 5-methylaltersolanol A, together with tetrahydroaltersolanol B, were isolated from the culture liquid of a strain of *Alternaria solani*, a pathogen of tomato *Lycopersicon esculentum*. Their structures have been established from spectroscopic studies, and the structures of 5-methylsulphonylmethylenealtersolanol A and 5-methylaltersolanol A were shown to be 6-methoxy-5-methylsulphonylmethylene-3β-methyl-1β,2α,3α,4β,8-pentahydroxy-1,2,3,4-tetrahydroanthraquinone and 6-methoxy-3β,5-dimethyl-1β,2α,3α,4β,8-pentahydroxy-1,2,3,4-tetrahydroanthraquinone, respectively.

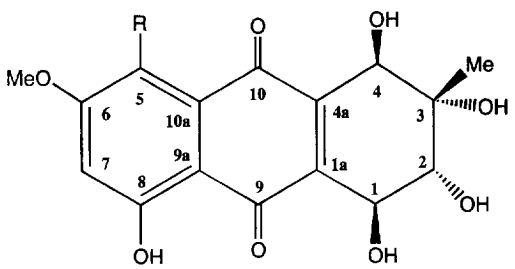
INTRODUCTION

Structures of twelve tetrahydroanthraquinone pigments in a strain of *Alternaria solani*, indicating an inhibitory activity against Gram-positive bacteria and *Pseudomonas aeruginosa*, have been reported in preceding papers [1, 2]. By use of HPLC analysis [3] several unknown peaks, together with those of known altersolanol-related compounds, were found in the culture liquid. Repeated MCI-gel CHP 20P and Sephadex LH-20 chromatographies of the culture liquid led to the isolation of **1**–**3**. This paper deals with the isolation and characterization of these compounds and their antimicrobial activities.

RESULTS AND DISCUSSION

Sixty percent aqueous MeOH fraction obtained by a stepwise gradient elution on a MCI-gel CHP 20P column was rechromatographed over a MCI-gel CHP 20P column eluted with 40% MeOH. As a preliminary separation method, this chromatography effectively excluded major known altersolanol-related compounds from minor **1**–**3** which were subsequently purified by further chromatography. On HPLC analysis with the photodiode-array detection, peaks of **1** and **2** showed the characteristic UV-vis spectrum of altersolanol A–H.

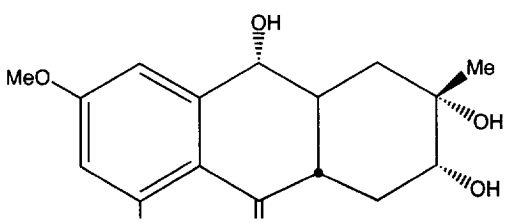
Compound **1** (5-methylsulphonylmethylenealtersolanol A) obtained as orange red needles, mp 218–222°, $[\alpha]_D^{26}$ − 292.5° shows absorption maxima at 203, 224, 273 and 431 nm in the UV-vis. spectrum. The negative FAB-MS spectrum exhibited fragment ion peaks at *m/z*: 428 M^-, 349 $[M - CH_3SO_2]^-$ and 79



R

1 CH_2-SO_2-Me

2 Me



$[CH_3SO_2]^-$, and HR-positive FAB-MS spectrum showed the $[M+H]^+$ ion peak suggesting the molecular formula $C_{18}H_{20}O_{10}S$ at *m/z*: 429.0856. This result was supported by elemental analysis. The ^1H and ^{13}C NMR signals were similar to those of altersolanol A, except for additional proton and carbon signals due to methyl (δ_H 2.92; δ_C 50.7) and methylene (δ_H 4.99, 5.19; δ_C 42.4) groups, the lack of C-5 proton signal and the down-field shift (6.8 ppm) of C-5 carbon signal as shown in Table 1 and 2. In addition, the coupling constants between the protons and the hydroxy protons at C_1, C_2 and C_4 in D_2O-exchanged ^1H NMR spectrum of **1** corresponded with those of altersolanol A (Table 1). Furthermore, the methylene signal correlated to C-6 and C-10a on HMBC spectrum, and thereby the methylene group must be connected to C-5 of altersolanol A shown in Fig. 1. Finally, the methylsulphonyl signal correlated to the methylene signal on NOESY spectrum, as shown in Fig. 1. Accordingly, **1** is 5-methylsulphonylmethylenealtersolanol A.

Compound **2** (5-methylaltersolanol A) obtained as red plates, mp 228–229°, $[\alpha]_D^{24}$ − 178.9°, shows absorption maxima at 202, 223, 273 and 443 nm in the UV-vis. spectrum, and the spectral data of ^1H and ^{13}C NMR closely related to those of altersolanol A [4], except for another proton and carbon signals due to methyl group (δ_H 2.41; δ_C 12.2) attached to the aromatic ring at C-5 or C-7 shown in Tables 1 and 2. The coupling constants between the protons and the hydroxy protons at C_1, C_2 and C_4 in D_2O-exchanged ^1H NMR spectrum of **2** corresponded with those of altersolanol A (Table 1). On positive FAB-MS, **2** gave fragments ion peaks at *m/z*: 351 $[M+1]^+$ and 335 $[M-CH_3]^+$, and the molecular formula, $C_{17}H_{18}O_8$, also gave a methylaltersolanol structure for **2**. The disappearance of the proton signal at C-5 on the ^1H

Table 2. ^{13}C NMR spectral data of altersolanols (DMSO-d_6)

	Altersolanol A	1	2
C-1	68.3	68.2	68.1
C-2	73.6	73.7	73.7
C-3	72.7	72.9	72.9
C-4	68.3	68.4	68.4
C-5	106.4	123.4	113.2
C-6	162.9	164.5	164.3
C-7	105.6	103.5	104.6
C-8	165.1	162.3	163.5
C-9	188.1	188.7	188.8
C-10	183.3	186.1	186.0
C-1a	144.2	143.2	143.5
C-4a	141.8	142.8	143.3
C-9a	109.2	109.1	110.0
C-10a	132.9	129.9	131.3
Me-3	22.2	22.3	22.3
Me-5		12.2	
MeO-6	56.1	56.6	57.1
CH_2-5			42.4
Me-S			50.7

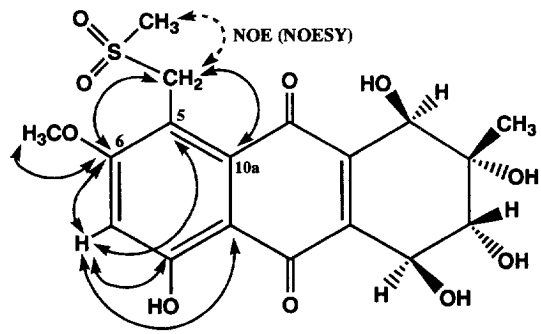


Fig. 1. HMBC and NOESY correlations of **1**.

Table 1. ^1H NMR spectral data of altersolanols (DMSO-d_6)

	Altersolanol A	1	2
H-1	4.48 *m*	4.49 *m*	4.49 *dd* (7.0, 5.9)
D*	*d* (6.8)	*d* (7.3)	*d* (7.3)
H-2	3.64 *m*	3.62 *m*	3.63 *t* (7.0)
D*	*d* (6.8)	*d* (7.3)	*d* (7.3)
H-4	4.32 *d* (5.9)	4.38 *d* (6.1)	4.36 *d* (6.5)
D*	*s*	*s*	*s*
H-5	7.02 *d* (2.4)		
H-7	6.83 *d* (2.4)	6.85 *s*	6.99 *s*
Me-3	1.24 *s*	1.25 *s*	1.24 *s*
Me-5		2.41 *s*	
OMe-6	3.91 *s*	3.93 *s*	3.96 *s*
OH-1	5.05 *d* (5.9)	4.96 *d* (5.6)	5.05 *d* (5.9)
OH-2	4.90 *d* (6.8)	4.82 *d* (6.9)	4.84 *d* (6.6)
OH-3	4.48 *s*	4.48 *s*	4.46 s
OH-4	5.70 *d* (5.9)	5.60 *d* (6.1)	5.64 *d* (6.5)
OH-8	12.15 *s*	12.91 *s*	12.90 *s*
Other			S-Me: 2.92 CH_2-5: 4.99 *d* (12.4)

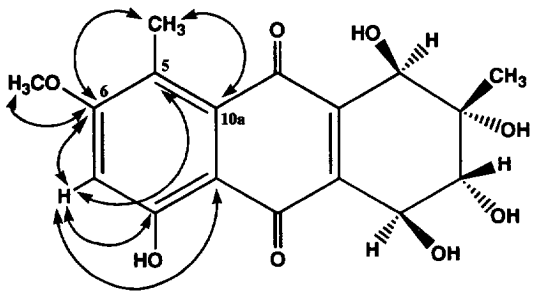


Fig. 2. HMBC correlations of **2**.

NMR spectrum and the marked down-field shift (17 ppm) of the carbon signal at C-5 on the ^{13}C NMR spectrum indicated that the methyl group is attached at C-5 of altersolanol A. This fact was clarified by the examination of the partial structure on the HMBC spectrum shown in Fig. 2. Accordingly, these data established the structure of **2** in which the C-5 proton in altersolanol A is displaced by a methyl group.

Compound **3** was isolated as cream-colored needles. **3** was determined to be tetrahydroaltersolanol B [5] as one of the metabolites of *A. solani* by comparing the ^1H and ^{13}C NMR spectra with those of the literature data.

In an earlier study of the culture liquid, altersolanol G attaching a methyl group at C_1 in altersolanol A was isolated [2], and **1** and **2** which have additional methyl and methylsulphonylmethylene groups at C_5 in altersolanol A, respectively, were identified in the present study. Incorporation of methyl and methylsulphonylmethylene groups into altersolanol A and relationship between **3** and altersolanol B are biosynthetically unclear.

The antimicrobial activity of **1**, **3** and altersolanol A was examined by the broth dilution method [6]. Altersolanols A and G inhibit the growth of Gram-positive bacteria, *Micrococcus luteus* IFO 3333 and *Staphylococcus aureus* NCTC 8530, and *Pseudomonas aeruginosa* IFO-3080, whereas **1** and **3** did not show any activity even at 50 μg ml^{-1}.

EXPERIMENTAL

The NMR spectra were recorded at 600 MHz (^1H) and 150 MHz (^{13}C) at a probe temperature of 35° using tetramethylsilane (TMS) as an internal reference. Positive and negative ion FAB-MS (using glycerol and triethanolamine as the matrix, respectively) were taken. Column chromatography was carried out on MCI-gel CHP 20P (75–150 μm, Mitsubishi Chemical Industries), Sephadex LH-20 (25–100 μm, Pharmacia Fine Chemicals) and Cosmosil 40C_{18}-PREP (Nacalai Tesque Inc.). HPLC analysis was performed on a Wako Wakosil-II 5C18 HG (5 μm, 4.6 mm i.d. × 150 mm, Wako Pure Chemical) with a Tosoh CCPD pump acetonitrile–H_2O (18:82); 3–20 min, linear change to acetonitrile–H_2O (90:10) [3].

Strain and cultivation. The strain of *A. solani* was isolated from a diseased tomato leaf in the green house of the University. The isolated fungus was cultured in the medium [1].

Antimicrobial assay. The minimal growth inhibitory concentrations (MIC) of altersolanol related compounds against a variety of screened microorganisms were measured by two-fold serial broth dilution method. Bacteria were cultured in 3% nutrient broth at 37°. After 2 days, the growth of bacteria was examined spectrometrically as the absorbance at 660 nm, and that of filamentous fungi with the naked eye. MIC was defined as the lowest concentration of the test compounds at which growth was below 0.03 in absorbance or not visible [6].

Isolation. The dark red culture medium (66 l) was subjected to MCI-gel CHP 20P column chromatography using a stepwise gradient elution with H_2O–MeOH as solvent. The 60% MeOH eluate (30 l) was applied to a MCI-gel CHP 20P column with 40% MeOH to furnish fractions I (1.07 g) and II (12.0 g). Fr. I was rechromatographed on a MCI-gel CHP 20P column with 20% MeOH and recrystallized with MeOH. The mother liquid was recrystallized from MeOH–H_2O to yield **1** (54.5 mg). Fr. II was further chromatographed on a MCI-gel CHP 20P column with 50% MeOH and a Sephadex LH-20 column with 50% MeOH followed by recrystallized from acetone to give **3** (39.8 mg). The mother liquid of **3** was separated on a Cosmosil 40C_{18}-PREP column with 25% MeOH and a Sephadex LH-20 with 30% MeOH and recrystallized from acetone–H_2O to yield **2** (4.7 mg).

5-Methylsulphonylmethylenealtersolanol A (**1**). Orange-red needles (MeOH–H_2O), mp 218–223°, $[\alpha]_D^{26}$ − 292.5° (EtOH, *c* 0.021). Negative FAB-MS *m/z*: 428 M^-, 349 $[M - CH_3SO_2]^-$, 79 $[CH_3SO_2]^-$. HR-positive FAB-MS *m/z*: Found 429.0856 $[M + H]^+$ ($C_{18}H_{21}O_{10}S$ requires 429.0857). *Anal.* Calcd for $C_{18}H_{20}O_{10}S$: C, 50.46; H, 4.71. Found: C, 50.50; H, 4.76. UV λ_{max}^{EtOH} nm (log ε): 203 (4.33), 224 (4.53), 273 (4.16), 431 (3.68). ^1H and ^{13}C NMR: Tables 1 and 2.

5-Methylaltersolanol A (**2**). Red plates (acetone–H_2O), mp 228–229°, $[a]_D^{24}$ − 178.9° (EtOH, *c* 0.038). Positive FAB-MS *m/z*: 351 $[M + 1]^+$, 335 $[M - CH_3]^+$. HR-EIMS *m/z*: Found 350.09983 $[M]^+$ ($C_{17}H_{18}O_8$ requires 350.10012). UV λ_{max}^{EtOH} nm (log ε): 202 (4.10), 223 (4.38), 273 (3.95), 443 (3.55). ^1H and ^{13}C NMR: Tables 1 and 2.

Tetrahydroaltersolanol B (**3**). Cream-colored needles (acetone), mp 240–244°, $[\alpha]_D^{24}$ − 41.2° (EtOH, *c* 0.068). Positive FAB-MS *m/z*: 309 $[M + 1]^+$, 277 $[M - CH_3O]^+$. UV λ_{max}^{EtOH} nm (log ε): 217 (4.22), 230 (4.04), 280 (4.21), 318 (3.82). ^1H NMR (DMSO-d_6): δ 1.17 (3H, *s*, CH_3), 1.21 (1H, *dd*, J = 13.4, 12.1 Hz, H-4$_a$), 1.47 (1H, *m*, J = 12.5, 12.1, 11.7 Hz, H-1$_a$),

4.0 Hz, H-1a), 3.78 (3-OH), 3.82 (3H, *s*, OMe), 4.29 (1H, *br*, *d*, H-10), 4.43 (2-OH), 5.62 (10-OH), 6.35 (1H, *d*, $J = 2.4$ Hz, H-7), 6.71 (1H, *dd*, $J = 2.4$, 1.2 Hz, H-5). ^{1}H NMR (DMSO-d_6 + D_2O): δ 1.17 (3H, *s*, CH_3), 1.22 (1H, *dd*, $J = 13.4$, 12.1 Hz, H-4_{ax}) 1.47 (1H, *q*, $J = 12.5$, 12.1 Hz, H-1_{ax}), 1.96 (1H, *m*, $J =$ 12.3, 12.1, 10.9, 3.6 Hz, H-4a), 2.13 (1H, *m*, $J = 12.5$, 4.4, 4.0 Hz, H-1_{eq}), 2.17 (1H, *dd*, $J = 13.4$, 3.6 Hz, H-4_{eq}), 2.46 (1H, *m*, $J = 12.3$, 12.1, 4.0 Hz, H-1a), 3.28 (1H, *dd*, $J = 11.7$, 4.4 Hz, H-2), 3.82 (3H, *s*, OMe), 4.29 (1H, *br*, *d*, H-10), 6.35 (1H, *d*, $J = 2.4$ Hz, H-7), 6.71 (1H, *dd*, $J = 2.4$, 1.2 Hz, H-5). ^{13}C NMR (DMSO-d_6): δ 26.9 (Me), 29.2 (C-1), 41.1 (C-4), 41.6 (C-4a), 46.9 (C-1a), 55.5 (OMe), 69.5 (C-3), 70.7 (C-10), 73.4 (C-2), 98.9 (C-7), 103.9 (C-5), 109.1 (C-9a), 151.6 (C-10a), 164.3 (C-8), 165.7 (C-6), 203.1 (C-9).

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