Dimers of Altersolanol A from *Alternaria solani*

George Lazarovits, R. William Steele, and Albert Stoessl

Agriculture Canada, London Research Centre, 1400 Western Road, London, Ontario, Canada, N6G 2V4

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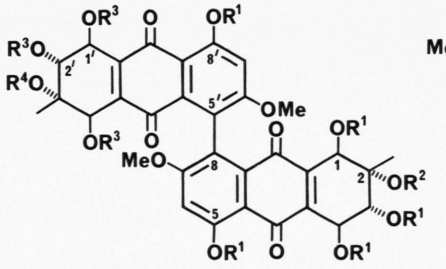
Altersolanol A Dimers, Fungal Metabolites, Potato Pathogen, Phytotoxins

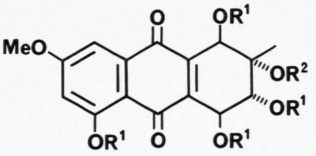
Weakly phytotoxic, water-soluble pigments from culture filtrates of the potato early blight organism *Alternaria solani* have been identified by ^{1}H NMR, UV, mass spectrometric, and acetylation studies as the pair of rotamers represented by structure **1a**.

Introduction

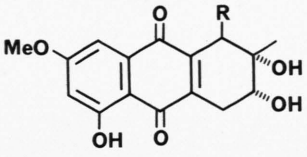
Alternaria solani, a pathogen of solanaceous plants and well-known as the fungus causing early blight of potato, has been a rich source of interesting secondary metabolites. These include (but are not limited to) the tetrahydroanthraquinones altersolanol A (**2a**), altersolanol B (**3**) and dactylariol (**4**), as well as several closely related anthraquinones (**5a–c**) [1–4]. All these compounds, together with two further anthraquinones (**5d,e**), have been reported also as metabolites of *A. porri* [5–7]. The relationship be-



	R^1	R^2	R^3	R^4
(1a)	H	H	H	H
(1b)	Ac	H	Ac	H
(1c)	Ac	Ac	Ac	H
(1d)	Ac	Ac	Ac	Ac

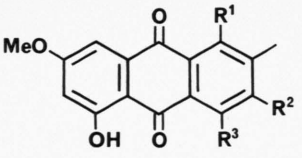


	R^1	R^2
(2a)	H	H
(2b)	Ac	H
(2c)	Ac	Ac

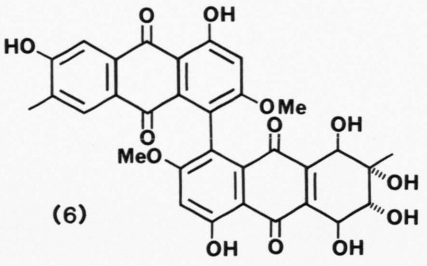


(3) R=H

(4) R=OH



	R^1	R^2	R^3
(5a)	H	OH	H
(5b)	H	H	H
(5c)	H	OH	H
(5d)	H	H	OH
(5e)	OH	H	OH



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Reprint requests to Dr. A. Stoessl.

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tween *A. solani* and *A. porri* is unclear. The three tetrahydroanthraquinones have been found to be phytotoxic by routine *in vitro* tests [8, 9] but a definitive phytopathological evaluation of their significance in host-parasite relations has yet to be undertaken.

In the course of studies on the effects of the melanization inhibitor tricyclazole on *A. solani* [10], we observed the accumulation, in aqueous culture filtrates, of red pigments that were not readily extracted into lipophilic solvents. Nevertheless, the two major components of the pigment mixture could be isolated in pure form and were identified as the two possible rotamers, I and II, of the altersolanol A dimer **1a**, on the evidence given below. It should be noted at the outset that Suemitsu and co-workers recently reported [11, 12] the isolation, from *A. porri,* of the alterporriols A and B as rotamers (atropisomers) sharing the common structure **6**. This is derived from the coupling of one molecule of altersolanol A (**2a**) with one molecule of macrosporin (**5a**) whereas, of course, the dimers **1a** are composed of two altersolanol A moieties.

Materials

Common solvents were redistilled; other chemicals were of reagent grade. Biochemicals were of the best quality available. Water employed in high performance liquid chromatography (HPLC) was purified using a Milli-Q Water system (Millipore Corp.) and all HPLC mobile phases were filtered through 0.2 μm Zetapor membranes (Cuno Inc., Meriden, Conn., U.S.A.).

Alternaria solani was strain 83 W, obtained from naturally occurring segregates of isolate ATTC 44204, and was maintained on Czapek-Dox agar modified by the addition of mineral salts and vitamins as described previously [10].

Liquid cultures were grown at 24 °C in the dark in 1 l Roux bottles on a Czapek-Dox medium (100 ml/bottle) modified as above but prepared without agar.

Methods and Results

General

Evaporations were done at the reduced pressure of the water aspirator. Unless otherwise noted, analytical thin layer chromatography (TLC) was on precoated sheets of silica gel (Macherey-Nagel Polygram Sil G/UV_{254}, 0.25 mm); preparative TLC on precoated plates (Baker 250 SiF, 0.25 mm) prewashed with solvent. The solvents employed were $CHCl_3$/MeOH/HOAc, 90:10:0.1 by vol. for native pigments, 95:5:0.1 for derived octa – and nonaacetates, and 98:2:0.1 for decaacetates. HPLC was performed with a Waters liquid chromatography system equipped with two model 510 pumps, a U6K sample injector, model 490 programmable multiwavelength detector and a model 840 data and control station. Preparative separations were made on a 10 mm × 25 cm Vydac Phenyl column (The Separations Group, Hesperia, CA., U.S.A.) and analytical separations on a 3.9 mm × 7.5 cm Nova-Pak Phenyl column (Waters Scientific Ltd.), with a 25 min linear gradient of 5 to 50% v/v CH_3CN in 10 mM aqueous trifluoracetic acid (TFA).

Melting points were determined on the Kofler Hot Stage and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 photoelectric polarimeter. UV Spectra were recorded in 96% EtOH with a Shimadzu UV-240 instrument. ^1H NMR spectra were obtained with a Varian XL 200 system, with methanol-d_4 (native pigments) or $CDCl_3$ (derived acetates) as solvents and tetramethylsilane (TMS) as internal reference. Mass spectra were measured with a Finnigan MAT 8230 system in the EI mode at 70 eV and a probe temperature of 300 °C.

Isolation of pigments

(a) Cultures grown on agar medium were extracted with 2 changes of EtOAc/MeOH (1:1). The extract was concentrated till mainly aqueous, acidified with HOAc and extracted with EtOAc. The aqueous phase was evaporated to dryness and the residue extracted with MeOH. The MeOH-soluble material from several runs was pooled and stored at 4 °C. This material was adsorbed on SiO_2 (7.5 g; Camag DFO) by evaporation from MeOH, and applied to the top of a column of 120 g SiO_2 in EtOAc/MeOH/HOAc (95:5:0.1). Elution with this solvent (10 ml fractions) gave dimer II, free of I, in fractions 30–36, and a mixture of I and II in fractions 37–95. Preparative TLC of the latter (on Macherey-Nagel Polygram Sil G; solvent as above) gave complete resolution of the dimers (*ca.* 5 mg each).

(b) In a subsequent run, the aqueous filtrate from 13-day old cultures on liquid Czapek-Dox medium (5 × 100 ml) was adjusted to pH 4.0 with HOAc and centrifuged at 30,000 *g* × 20 min. The supernatant

was concentrated to one-tenth vol., made 5% in MeOH, and centrifuged again, as above, before preparative HPLC. Separations (3 runs) were developed at 3 ml/min as follows: 40 min elution with 5% (v/v) CH_3CN in 10 mM aqueous TFA; 5 min linear gradient to 10% CH_3CN in 10 mM TFA; thereafter, isocratically. Altersolanol A eluted between 50–55 min and dimers I and II between 70–85 and 100–115 min, respectively. Individual peaks were collected and further purified by rechromatography as above.

Dimer I (7.5 mg; accidental losses) was a homogeneous (HPLC, TLC, ^{1}H NMR) but amorphous red powder, only very slowly but eventually completely soluble in H_2O or MeOH, insoluble in $CHCl_3$, EtOAc, C_6H_6; λ_{max} nm (ε) 458 (10,450), 274 (22,100), 224 (54,700), $[\alpha]_D^{24} - 1065°$ (c, 0.0034% in EtOH).

Dimer II (30 mg) was an orange glass, homogeneous by the same criteria as I, but very readily soluble in H_2O or MeOH, insoluble in $CHCl_3$, EtOAc, C_6H_6; λ_{max} nm (ε) 456 (9,650), 274 (22,500), 225 (56,800), $[\alpha]_D^{24} - 702°$ (c, 0.01% in EtOH).

Dimer I acetates

Dimer I (*ca.* 5 mg) was kept overnight at room temperature in pyridine (0.9 ml) and Ac_2O (0.6 ml). After evaporation to dryness, the product was separated by preparative TLC into I-nonaacetate (IAc_9; 1.6 mg) as faster-moving, minor product, and octaacetate (IAc_8; 5.8 mg). The latter crystallized from hot MeOH/$CHCl_3$; stout orange-red prisms, m.p. 312–17 °C dec., λ_{max} nm (ε) 402 (8,100), 269 (39,000), 220 (62,800); $[\alpha]_D^{24} - 220°$ (c, 0.25% in $CHCl_3$); *m/e* 1006 (M^+, 0.02%), 964 (M − 42, 0.8%), 922 (M − 2 × 42, 2.1%), 886 (M − 2 × 60, 0.04%), 844 (M − 2 × 120 − 42, 0.3%), 802 (M − 2 × 60 − 2 × 42, 0.6%), 760 (M − 2 × 60 − 3 × 42, 0.9%), 724 (M − 4 × 60 − 42, 0.8%), 682 (M − 4 × 60 − 2 × 42, 1.8%), 640 (M − 4 × 60 − 3 × 42, 2.8%), 598 (M − 4 × 60 − 4 × 42, 6.7%) and 61 (100%) *inter al.* Exact mass calculated for $C_{46}H_{44}O_{23}$ ($C_{48}H_{46}O_{24} - C_2H_2O$): 964.2273; found 964.2342 ± .0024.

The I-octaacetate (5.8 mg) in Ac_2O (0.2 ml) at 0 °C was treated with a small drop of 20% $HClO_4$. Ice was added after 1 h, the product was extracted into $CHCl_3$, washed with two small portions H_2O, combined with the qualitatively identical product (TLC, several systems) from the analogous reaction of IAc_9 (1.6 mg), and separated from a small amount (0.3 mg) of by-product by preparative TLC (in toluene/EtOAc/HOAc 5:4:1). The yellow I-decaacetate (IAc_{10}; 4.6 mg) obtained was recrystallized from hot MeOH/$CHCl_3$; plates, (3.8 mg), m.p. chiefly, 304–7 °C, $[\alpha]_D^{24} - 252°$ (c, 0.075% in $CHCl_3$), λ_{max} nm (ε) 402 (8,300), 269 (35,800), 220 (58,600).

Dimer II acetates

Acetylation of dimer II (15 mg) in pyridine (2.7 ml) as above, and chromatography of the product over SiO_2 (Camag DS5, 12 g, 1 ml fractions) gave II-nonaacetate ($IIAc_9$; 2.4 mg, glass, fractions 2–4) and octaacetate ($IIAc_8$; 16.5 mg, glass, fractions 5–10). The latter was obtained micro-crystalline by precipitation from $CHCl_3$ with CCl_4; λ_{max} nm (ε) 402 (7,700), 269 (38,500), 221 (56,700); $[\alpha]_D^{24} - 136°$ (c, 0.25% in $CHCl_3$); ms essentially as for IAc_8. Exact mass found, 964.2347. Further acetylation of $IIAc_8$ (8.9 mg, $HClO_4$ catalysis) and preparative TLC gave the decaacetate ($IIAc_{10}$; 7.4 mg) as an amorphous solid, λ_{max} nm (ε) 402 (6,400), 270 (30,400), 221 (50,000); $[\alpha]_D^{24} - 106°$ (c, .06% in $CHCl_3$). The same decaacetate was obtained also by $HClO_4$ – catalyzed acetylation of $IIAc_9$ (TLC).

Phytotoxicity of altersolanol A and its dimers

The phytotoxicity of **2a** and dimers I and II was assessed by injecting 25–50 µl of aqueous solutions into the intercellular spaces of tomato leaves. Altersolanol A caused necrosis at injected sited at concentrations as low as 2.5 µg/ml whereas dimers I and II showed toxicity only above 100 µg/ml. At concentrations below this, or with water, no visible reactions were observed at injected sites.

Discussion

On acetylation, in pyridine, the two pigments, I and II, gave two corresponding pairs of acetates, easily separable by preparative TLC and formulated as IAc_8 and IAc_9, and $IIAc_8$ and $IIAc_9$, respectively. On further acetylation, with perchloric acid catalysis, each pair of compounds gave only one major product, IAc_{10} and $IIAc_{10}$, respectively. The ^{1}H NMR parameters of these compounds are collected in Table I, together with the data for altersolanol A (**2a**) and its tetra- and pentaacetates **2b** and **2c**. It is immediately apparent that IAc_9 and $IIAc_9$ give rise to twice as many resonances, other than those from the acetyl groups, as any of the other compounds. This

Table I. ^{1}H NMR spectra[a].

		H-8	H-6	H-4	H-1	H-3	MeO	ArOAc	ROAc	C-2-Me
2a[b]		7.11 (d, 2.5)	6.70 (d, 2.5)	4.82 (d, 7.4)	4.71	3.84 (d, 7.4)	3.90	–	–	1.43
1a	(I)[b]	–	6.78	4.72 (d, 7.5)	4.27	3.79 (d, 7.5)	3.68	–	–	1.345
1a	(II)[b]	–	6.81	4.72 (d, 7.4)	4.29	3.775 (d, 7.4)	3.795	–	–	1.345
2b		7.43 (d, 2.6)	6.82 (d, 2.6)	6.27 (d, 7.6)	6.18	5.34 (d, 7.6)	3.78	2.36	2.14, 2.10, 2.04	1.28
1b	(IAc_8)	–	6.785	6.21 (d, 7.3)	5.825	5.27 (d, 7.3)	3.655	2.39	2.10, 2.05, 1.94	1.17
1b	($IIAc_8$)	–	6.835	6.20 (d, 7.5)	5.835	5.11 (d, 7.5)	3.705	2.40	2.08, 2.04, 1.985, 1.945	1.17
1c	(IAc_9)	–	6.80	6.24 (d, 7.3)	6.75	5.46 (d, 7.3)	3.67	2.40	2.115, 2.057, 1.95	1.47
		–	6.79	6.225 (d, 7.2)	5.79	5.28 (d, 7.2)	3.66	2.395	2.11, 2.054, 1.99, 1.95	1.18
1c	($IIAc_9$)	–	6.84	6.23 (d, 7.3)	6.74	5.31 (d, 7.3)	3.724	2.42	2.09, 2.05, 1.96, 1.95	1.48
		–	6.844	6.21 (d, 7.3)	5.84	5.12 (d, 7.3)	3.720	2.40	2.09, 2.05, 1.95	1.17
1d	(IAc_{10})	–	6.78	6.27 (d, 7.4)	6.765	5.455 (d, 7.4)	3.65	2.39	2.12, 2.06, 1.97, 1.95	1.47
1d	($IIAc_{10}$)	–	6.85	6.23 (d, 7.4)	6.75	5.30 (d, 7.4)	3.73	2.42	2.09, 2.08, 2.05, 1.96	1.49
2c		7.475 (d, 2.65)	6.83 (d, 2.65)	6.25) (d, 7.5)	7.125	5.53 (d, 7.5)	3.905	2.36	2.12, 2.09, 2.03, 1.96	1.555

[a] To TMS as internal reference, in $CDCl_3$ except where noted; multiplicites and observed coupling constants *J*, in Hz, in parentheses.
[b] In methanol-d_4.

leads directly to the conclusion that I and II must be symmetrical dimers, each affording a symmetrical octa- and decaacetate but also a nonaacetate in which only one of a pair of hydroxyls has remained unacetylated, thereby conferring magnetic non-equivalence to the two halves of the molecule. Comparison of the ^{1}H NMR data further reveals that the dimers and their acetates contain the same 1,2,3,4-tetrahydro-1,2,3,4-tetraoxynaphthoquinone moiety as altersolanol A (**2a**), as evidenced by the close similarity of chemical shifts and vicinal couplings of the pertinent protons. The conserved structural and conformational identity of this moiety is illustrated also by the marked downfield shifts of H-1 (and H-1′) and the C-2 methyls that accompany the acetylation of the free tertiary hydroxyl group present in **2b**, IAc_8, $IIAc_8$, and in one of the halves of IAc_9 and $IIAc_9$. Finally, the compounds also exhibit very similar resonances for the 5-OAc and 7-OMe groups. In contrast, the spectra of the dimers differ markedly from those of the monomers in that the AB pattern characteristic of H-6 and H-8, in **2** and derivatives, is replaced by a singlet resonance with the chemical shift typical of H-6, and the absence of any signal for H-8. The UV spectra of the dimers (λ_{max} *ca.* 458 nm) and their acetates (λ_{max} *ca.* 405 nm) show a bathochromic shift relative to altersolanol A (λ_{max} 422 nm) and its acetates (λ_{max} 388 nm) which is similar to that reported for skyrin (λ_{max} 462 nm) *vis-à-vis* its monomer emodin (λ_{max} 437 nm) [13]. Mass spectral data, measured for the octaacetates, are also consistent with their formulation as dimers of the constitution $C_{32}H_{22}O_8(COCH_3)_8$.

The sum of these data establish that the compounds are rotamers (atropisomers), derived from altersolanol A (**2a**) by dimerization through C-8 and C-8′ which are represented by the common projections **1a** for I and II, **1b** for IAc_8 and $IIAc_8$, **1c** for IAc_9 and $IIAc_9$, and **1d** for IAc_{10} and $IIAc_{10}$. Similar instances of isomerism due to restricted rotation are familiar from dimeric anthraquinones and anthrones [14], coumarins [15], and most pertinently, the recently described alterporriols A and B [11, 12]. The remarkable upfield shift observed for one of the

acetoxy groups in the ^{1}H NMR spectrum of alterporriol A heptaacetate, but not of the B derivative, was ascribed by Suemitsu [11] to differential shielding by the aromatic macrosporin moiety of the isomer, and allowed a tentative assignment of configurations. No clearly significant shielding differences are observable for I and II or any of the three pairs of derived acetates. An assignment of their configurations will therefore become possible only with the preparation of a pair of partly aromatized derivatives or, preferably, an X-ray determination.

In the case of dimeric anthraquinones, there is evidence to indicate that they are not derived by coupling of their constituent monomers but instead, are probably formed in parallel with the latter from shared precursors [14]. In consonance with this view, nearly all of these compounds appear to have been isolated in only one of the two possible antipodal forms. It is therefore of interest that dimers I and II were found by analytical HPLC to be present in equal amounts in all culture extracts that were examined. This suggests that they may be formed by the oxidative coupling of **2a**. The precise nature of this process, and of that leading to the alterporriols, needs further investigation.

The two dimers were found to be only very weakly phytotoxic when tested by injection into tomato leaves. However, in using this assay, we discovered that altersolanol A itself is much more toxic than previously believed. A fuller study of this matter is in progress.

Acknowledgements

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