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Note

Analysis of naturally occurring reduced anthraquinones obtained from *Alternaria porri* (Ellis) Ciferri by high-performance liquid chromatography

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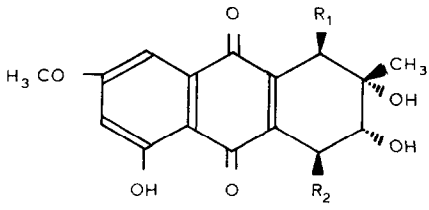
Altersolanol A, altersolanol B and dactylariol have been found as metabolic pigments produced by *Alternaria solani*[1,2], *Alternaria porri*[3–5] and *Dactylaria lutea*[6]. Of these pigments, altersolanol B has been reported by Sturdik and Drobnica[7] to show antiprotozoal activity. Recently, we found that these three pigments inhibit elongation of the root in seeds of lettuce and stone-leek, and also that altersolanol A shows antimicrobial activity against Gram-positive and -negative bacteria[8]. The occurrence of these reduced anthraquinones as metabolic pigments in some microorganisms is of interest from the viewpoint of biogenesis, pharmacology and chemotaxonomy. Their structures are shown in Fig. 1.

As anthraquinones are pharmaceutically important constituents of plants, many analytical methods such as paper, thin-layer, gas–liquid and column chromatography have been reported[9]. Little information is available, however, concerning the high-performance liquid chromatography (HPLC) of anthraquinones[9]. This paper describes an HPLC procedure for the separation and identification of altersolanol A, altersolanol B and dactylariol. In addition we studied the quantitative relationship among them during the fermentation period when *Alternaria porri* was cultured on Brian's T medium.



		R_1	R_2
Altersolanol	A	OH	OH
Altersolanol	B	H	H
Dactylariol		OH	H

Fig. 1. Structures of reduced anthraquinones.

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EXPERIMENTAL AND RESULTS

Reagents

Altersolanol A, altersolanol B and dactylariol were those isolated and identified as metabolic pigments of *Alternaria porri* (Ellis) Ciferri.

Instrumentation

HPLC was performed on a Hitachi 655 liquid chromatograph equipped with a UV detector and gradient apparatus, operating at 254 nm for all assays.

HPLC of reduced anthraquinones

Of several column packings and solvent systems investigated, a YMC A-314 column (Shimakyu Chemicals, octadecyl-silica type of reversed-phase column) with methanol–water (65:35) as the solvent system provided superior resolution of the reduced anthraquinones. A stainless-steel column (300 × 6 mm I.D.) used and a flow-rate of 1 ml/min. Samples of 10 μl were injected on to the column. As shown in Fig. 2, retention times were altersolanol A 7.09 min (k' = 0.30), dactylariol 8.45 min (k' = 0.55) and altersolanol B 15.50 min (k' = 1.82). These values were directly related to polarity. It is possible to separate all three compounds in less than 16 min.

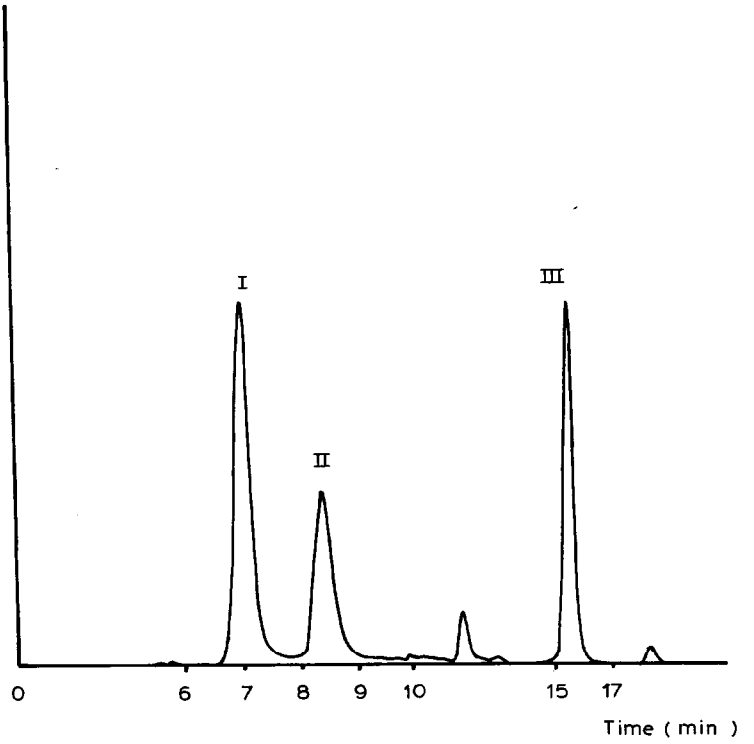

Fig. 2. HPLC of reduced anthraquinones. I, Altersolanol A; II, dactylariol; III, altersolanol B.

Application to the quantitative analysis of reduced anthraquinones produced by Alternaria porri

(a) Fungus. Alternaria porri (Ellis) Ciferri was isolated and donated by Prof. Emeritus M. Hiura and Prof. Y. Matsui, Rakuno College.

(b) Culture conditions and extraction of pigments. Brian's T medium was used as a culture medium. A number of 500-ml erlenmyer's flasks containing 200 ml of the medium were sterilized in an autoclave for 20 min at 2.3 atm and 120°C. The fungi, cultured on an agar slant for 7–10 days, was inoculated into the flasks, which were then kept at 28°C. After fermentation for 1 week, 10 ml of the culture liquid were taken and extracted first with *n*-hexane to remove lipids and then with ethyl acetate (2 × 10 ml). The ethyl acetate extracted coloring matter, and this extract was denoted B-1 (2.4 mg). Similarly, after fermentation for 2 and 3 weeks, further ethyl acetate extracts were taken and denoted B-2 (5.6 mg) and B-3 (6.6 mg), respectively.

(c) Quantitative analyses of altersolanol A, dactylariol and altersolanol B during the fermentation period. The linearity of the detector response was verified using a series of methanol solutions containing reduced anthraquinones. Fig. 3 shows that the detector response is linear at levels of up to about 10–70 ppm of the reduced anthraquinones. The detection limits, defined by the amount that produces a detector signal twice the height of the detector noise level, with an injection volume of 5 μl, were 0.4, 1 and 2 ppm for altersolanol A, dactylariol and altersolanol B, respectively.

Extracts B-1 (2.4 mg), B-2 (5.6 mg) and B-3 (6.6 mg) were dissolved in methanol (5 ml), then 5 μl of each were subjected to HPLC analysis under the conditions given above. The concentrations of altersolanol A, dactylariol and altersolanol B were calculated from the detector responses (peak areas) by using the following linear equations of the calibration graphs in Fig. 3, obtained using the method of least squares:

altersolanol A: $b = 12.0 \cdot 10^6 a + 9.7 \cdot 10^2$

dactylariol: $b = 13.5 \cdot 10^6 a - 142.9 \cdot 10^2$

altersolanol B: $b = 10.4 \cdot 10^6 a - 347.5 \cdot 10^2$

where a (g/l) is the concentration and b is the peak area (detector response).

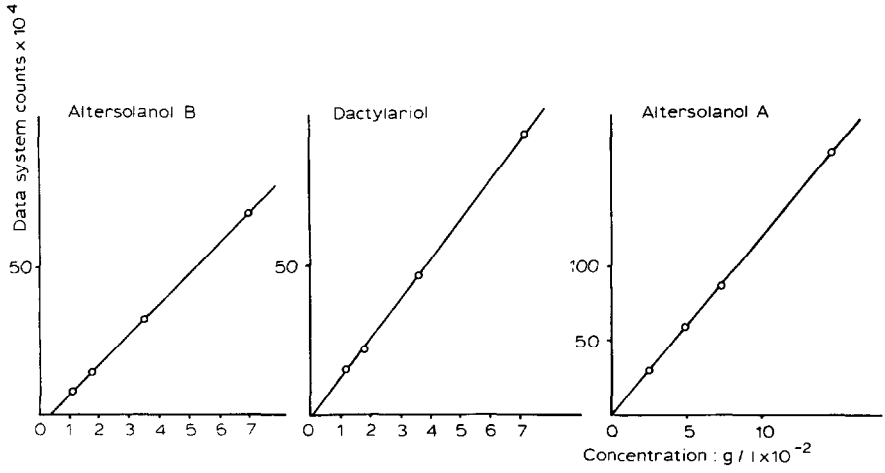

Fig. 3. Linearity of detector responses.

TABLE I

RELATIVE ERRORS CALCULATED FROM LINEAR EQUATION

Compound	*True concentration (g/l)*	*Concentration from linear equation (g/l)*	*Relative error* (%)*
Altersolanol A	0.147	0.1470	0
	0.074	0.0731	$5.74 \cdot 10^{-1}$
	0.049	0.0493	$6.46 \cdot 10^{-1}$
	0.025	0.0250	o
Dactylariol	0.0713	0.0713	0
	0.0360	0.0361	$3.22 \cdot 10^{-1}$
	0.0176	0.0175	$3.59 \cdot 10^{-1}$
	0.0123	0.0124	$3.58 \cdot 10^{-1}$
Altersolanol B	0.0698	0.0698	0
	0.0350	0.0349	$1.28 \cdot 10^{-1}$
	0.0176	0.0177	$4.47 \cdot 10^{-1}$
	0.0116	0.0155	$5.52 \cdot 10^{-1}$

* Relative error = (concentration from linear equation/true concentration) × 100.

In order to calculate how much the mean value of a set of results differs from the true value, the relative error was calculated (Table I). These results indicate that the linear equations are suitable for practical applications.

The results obtained from analyses of extracts B-1, B-2 and B-3 are given in Table II, and show that the contents of altersolanol A, dactylariol and altersolanol B in the culture liquid continued to increase for up to 14 days and the increase in the altersolanol B concentration was particularly large. However, after 21 days the altersolanol A content decreased abruptly, whereas that of altersolanol B continued to increase and that of dactylariol was found to be unchanged. Stoessl and Unwin[2], using *Alternaria solani* and studying the incorporation of [1,2-$^{13}C_2$]acetate, demonstrated that altersolanol A is metabolized to altersolanol B, macrosporin (7-methoxy-3,5-dihydroxy-2-methylanthraquinone) and dactylariol. Our results suggest that in the early period of fermentation altersolanol A is first formed, then it is

TABLE II

VARIATION OF ALTERSOLANOL A, DACTYLARIOL AND ALTERSOLANOL B IN THE CULTURE LIQUID AT DIFFERENT STAGES OF FERMENTATION

Compound	*7 days*			*14 days*			*21 days*		
	mg/l	*s**	*mol (· 10^{-6})*	*mg/l*	*s**	*mol (· 10^{-6})*	*mg/l*	*s**	*mol(·10^{-6})*
Altersolanol A	6.3	0.08	18.6	11.5	0.30	34.2	0.9	0.05	2.8
Dactylariol	2.0	0.17	6.3	3.9	0.22	12.2	3.8	0.10	11.8
Altersolanol B	7.6	0.17	24.7	27.8	0.03	90.1	32.6	0.25	105.0
Total	15.9		49.6	43.2		136.5	37.3		119.6

* s = standard deviation (mg/l).

converted into altersolanol B and dactylariol, supporting the validity of the pathways proposed by Stoessl and Unwin.

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