Journal of Chromatography, 503 (1990) 282–287
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM 22 141

Note

High-performance liquid chromatographic determination of alterporriol D and E in fermentation of *Alternaria porri* (Ellis) Ciferri

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(First received March 20th, 1989; revised manuscript received October 20th, 1989)

In the course of our investigation on pigments of *Alternaria porri* (Ellis) Ciferri, the causal fungus of black spot disease, we have isolated five modified bianthraquinone pigments from the culture liquid, named alterporriol A (Ap-A,**1**)[1], B (Ap-B,**1**)[2], C (Ap-C, **2**)[3], D (Ap-D, **3**)[4] and E (Ap-E, **3**)[4], and determined their chemical structures. Of these pigments, Ap-A, -B and -C consist of macrosporin (Mac, **4**)[5] and altersolanol A (As-A, **5**), both of which are metabolic pigments of *Alternaria solani*[6] and *Alternaria porri*[7] and Ap-A and -B are atropisomers of each other. Likewise, Ap-D and -E were found to be atropisomers of each other and their planar and spatial structures are shown in Fig. 1 (**3**). Recently, Lazarovits *et al.*[8] reported the dimers of As-A from the culture liquid of *Alternaria solani* and presented their planar structures. Comparing the structures, spectral data and other physico-chemical properties of Ap-D and -E with those of the dimers of As-A just mentioned, they were found to be the same.

Previously, we reported the high-performance liquid chromatographic (HPLC) determination of As-A, Mac and Ap-A, Ap-B and Ap-C in the fermentation of *Alternaria porri* in order to explore the biosyntheses of Ap-A, Ap-B and Ap-C[9]. The structures of Ap-D and Ap-E show that they consist of two moieties of As-A. So far as the biosyntheses of Ap-D and -E are concerned, two pathways can be considered, namely, whether As-A is first metabolized and then two moieties of As-A bond to Ap-D and -E, or Ap-D and -E are first metabolized and then their C–C linkages connecting the monomeric halves are cleaved into two halves of the molecule, two moieties of As-A. This paper deals with the HPLC determination of Ap-D and -E during the period of fermentation in order to explore their metabolic pathways.

0021-9673/90/$03.50

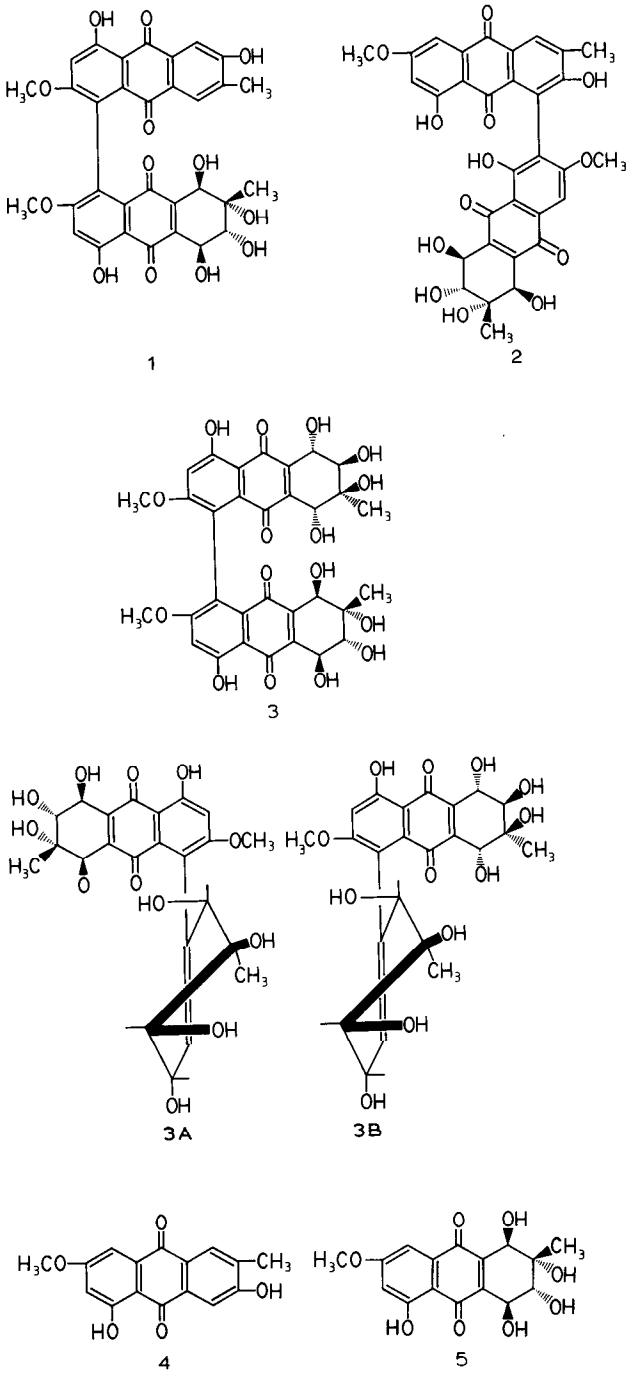

Fig. 1. Structure of pigments **1** = Alterporriol A and B; **2** = alterporriol C; **3** = alterporriol D and E **3A** = spatial structure of alterporriol D (*R*-form); **3B** = spatial structure of alterporriol E (*S*-form); **4** = macrosporin; **5** = altersolanol A.

EXPERIMENTAL

Material

As-A, Ap-D and -E were isolated as metabolic pigments of *Alternaria porri* (IFO 9762), which was isolated and donated by the Institute for Fermentation, Osaka, Japan (IFO).

High-performance liquid chromatography

HPLC was performed on a Shimadzu LC-6A liquid chromatograph equipped with a UV detector operating at 254 nm for all assays. The solvent system used was 0.05 *M* ammonium dihydrogenphosphate (adjusted to pH 2.5 with phosphoric acid–acetonitrile (41:9). The column used was a YMC A-312 (Yamamura Chemical Labs.), commercially packed with reversed-phase octadecylsilica (5 μm) (150 mm x 6.0 mm I.D.); the mobile phase flow-rate was 1.0 ml/min. Samples of 10 μl were injected onto the column.

Fermentation and extraction of pigments

A 2% (w/v) sucrose solution of onion decoction was used as a culture medium. A number of 500-ml erlenmyer flasks containing 200 ml of the medium were sterilized in a autoclave for 20 min at 2.3 bar and 120°C. The fungi, cultured on agar for 7–10 days, were inoculated into the flasks, which were then kept at 25°C. After fermentation for 2 days, 10 ml of the culture liquid were taken and extracted with *n*-hexane (4 × 10 ml) to remove lipids. The aqueous layer obtained was called S-2. By a similar procedure, the filtrates corresponding to fermentation periods of 5, 7, 14, 21 and 28 days were designated S-3, S-4, S-5, S-6 and S-7, respectively, plus S-1 for the blank.

RESULTS AND DISCUSSION

Determination of As-A, Ap-D and Ap-E during the fermentation period

The data obtained as mean values of nine experiments and those of one representative experiment are given in Tables I and II. The chromatograms of pigments

TABLE I

VARIATION OF PIGMENTS AT DIFFERENT STAGES OF FERMENTATION

Mean values of nine experimental results are shown. *A* = peak-area ratio to I.S.; *B* = weight ratio to I.S.; *C* = concentration (mg/ml), *S* = standard deviation (mg/ml).

Sample	*Altersolanol A*				*Alterporriol D*				*Alterporriol E*			
	A	*B*	*C*	*S*	*A*	*B*	*C*	*S*	*A*	*B*	*C*	*S*
S-1	–	–	–	–	–	–	–	–	–	–	–	–
S-2	0.783	0.610	0.061	0.013	0.274	0.013	0.001	0.0006	0.284	0.023	0.002	0.0015
S-3	5.469	4.354	0.435	0.14	0.917	0.132	0.013	0.0078	1.619	0.278	0.028	0.021
S-4	6.609	5.265	0.527	0.18	2.777	0.476	0.048	0.022	2.970	0.536	0.054	0.018
S-5	4.107	3.266	0.327	0.12	2.955	0.509	0.051	0.012	3.289	0.597	0.060	0.025
S-6	0.685	0.532	0.053	0.036	2.685	0.459	0.046	0.020	2.676	0.480	0.048	0.022
S-7	0.640	0.496	0.050	0.025	2.004	0.333	0.033	0.013	2.273	0.403	0.040	0.017

TABLE II

VARIATION OF PIGMENTS OBSERVED IN ONE REPRESENTATIVE EXPERIMENT AT DIFFERENT STAGES OF FERMENTATION

Symbols as in Table I.

Sample	*Altersolanol A*				*Alterporriol D*				*Alterporriol E*			
	A	*B*	*C*	*S*	*A*	*B*	*C*	*S*	*A*	*B*	*C*	*S*
S-1	–	–	–	–	–	–	–	–	–	–	–	–
S-2	1.25	0.983	0.098	$1.1 \cdot 10^{-3}$	0.074	0.010	0.001	$1.7 \cdot 10^{-5}$	0.076	0.012	0.001	$3.1 \cdot 10^{-5}$
S-3	5.68	4.52	0.452	$5.9 \cdot 10^{-3}$	1.01	0.149	0.015	$1.8 \cdot 10^{-4}$	0.877	0.136	0.014	$2.8 \cdot 10^{-4}$
S-4	7.32	5.83	0.583	$1.7 \cdot 10^{-2}$	3.26	0.565	0.057	$1.1 \cdot 10^{-3}$	2.43	0.433	0.043	$5.3 \cdot 10^{-4}$
S-5	2.49	1.97	0.197	$2.9 \cdot 10^{-3}$	3.41	0.593	0.059	$1.1 \cdot 10^{-3}$	2.64	0.473	0.047	$1.0 \cdot 10^{-3}$
S-6	0.874	0.683	0.068	$2.1 \cdot 10^{-3}$	2.90	0.499	0.050	$5.6 \cdot 10^{-4}$	2.37	0.421	0.042	$1.4 \cdot 10^{-3}$
S-7	0.911	0.712	0.071	$2.4 \cdot 10^{-3}$	2.13	0.356	0.036	$7.5 \cdot 10^{-4}$	1.72	0.297	0.030	$3.1 \cdot 10^{-4}$

and the internal standard (I.S.) are shown in Fig. 2, in which the retention times (t_R) were 6.0 min (Ap-D, capacity factor, $k' = 1.71$), 7.8 min (As-A, $k' = 2.23$) and 9.6 min (Ap-E, $k' = 2.74$). We used the internal standard method for quantitation and benzoic acid ($t_R = 14$ min, $k' = 4.00$) was used as the internal standard for As-A, Ap-D and Ap-E. For example, methanolic solutions of As-A (1 mg/ml) (0.4, 0.6, 0.8, 1.0 and 1.2 ml) were placed in sample vials and 1-ml portions of methanolic solutions of benzoic acid (1 mg/ml) were added. After the volumes had been adjusted to 10 ml with methanol, 10-μl portions of each were subjected to HPLC under the conditions mentioned above. By plotting the peak-area ratio against sample weight a calibration graph for As-A was obtained. The calibration graphs for Ap-D and Ap-E were

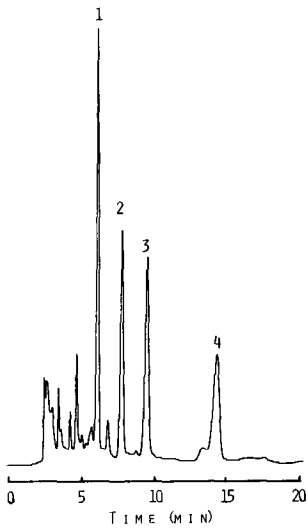

Fig. 2. Chromatograms of pigments and I.S. Peaks: 1 = alterporriol D (26.3%); 2 = altersolanol A (22.9%); 3 = alterporriol E (22.1%); 4 = benzoic acid (13.6%).

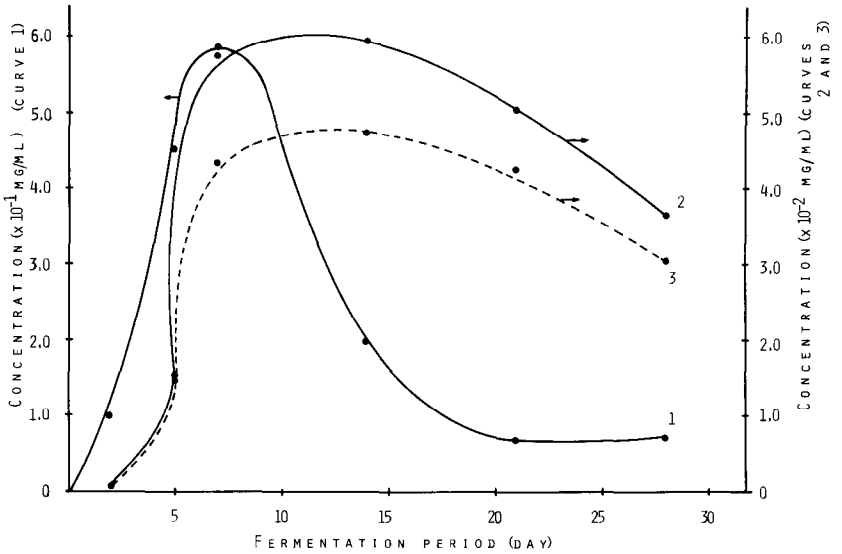

Fig. 3. Relationship between concentration of pigments and fermentation periods: 1 = altersolanol A; 2 = alterporriol D; 3 = alterporriol E.

obtained by using the same concentrations as that of As-A[9]. The limits of detection, based on a signal-to-noise ratio of 10 for As-A, Ap-D and Ap-E, were 0.1 μg/ml.

The concentrations of As-A, Ap-D and Ap-E were calculated from the detector responses (peak areas) by using the method of least squares:

$$\text{As-A: } y = (0.799x - 1.57 \cdot 10^{-2}) \cdot 0.1$$
$$\text{Ap-D: } y = (0.185x - 3.77 \cdot 10^{-2}) \cdot 0.1$$
$$\text{Ap-E: } y = (0.191x - 3.12 \cdot 10^{-2}) \cdot 0.1$$

where y is the concentration of the pigment (mg/ml) and x is the ratio of the peak area of the pigment to that of the internal standard (I.S.).

As a practical procedure, benzoic acid (1 mg) was dissolved in each of culture liquids S-1 to S-7 and then 10 μl of each were subjected to HPLC under the conditions given above.

The combined results obtained from nine fermentation experiments indicated that As-A was detected after fermentation for 2 days, Ap-D and Ap-E were not or only slightly detected at this time and the content of As-A was found to increase continuously for 7 days and then gradually to decrease, whereas those of Ap-D and Ap-E were found to increase continuously for up to 14 days and then gradually to decrease, as shown in Fig. 3. It is of interest that the amounts of Ap-D and Ap-E are similar throughout the fermentation period, as shown in Tables I and II.

We conclude that As-A is first formed in the early period of fermentation, and then two moieties of As-A are bonded to Ap-D and Ap-E when fungus is cultured on onion decoction medium.

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