ISOLATION AND CHARACTERIZATION OF NON-PROTEIN CHROMOPHORE AND ITS DEGRADATION PRODUCT FROM ANTIBIOTIC C-1027

Sir:

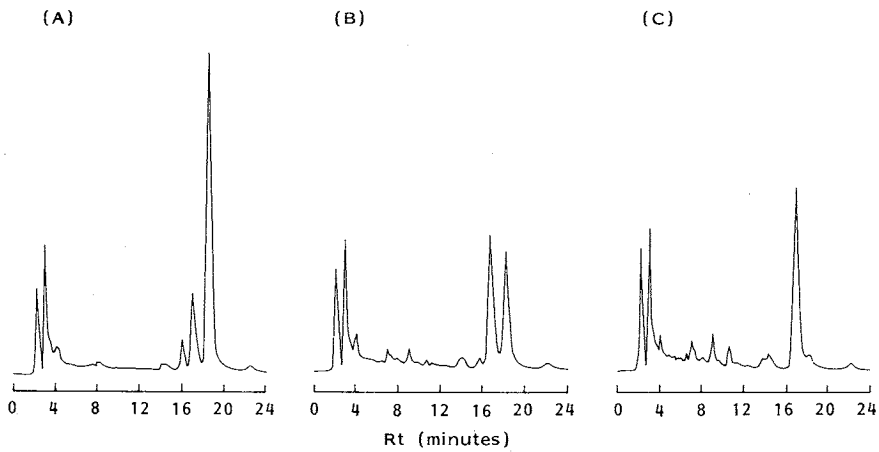
The new antitumor antibiotic C-1027, which also exhibits antibacterial activity, is an acidic protein containing a non-protein chromophore and was isolated from a culture filtrate of *Streptomyces globisporus* C-1027[1~3]. The protein moiety of C-1027 has been characterized as a single polypeptide chain cross-linked by two disulfide bonds with MW of 10,500 daltons[4]. Recently, we reported that the chromophore of C-1027 plays a predominant role in expression of the biological activity and may inhibit tumor cell growth by causing DNA cleavage with subsequent inhibition of DNA synthesis[5]. The C-1027 chromophore, which has an absorption shoulder between 340 and 360 nm, was found to be extractable from purified C-1027 with organic solvents such as methanol and ethyl acetate under the alkaline condition, but the chromophore so obtained was extremely labile.

In this communication, we present data on the isolation procedure, characterization, and biological activity of the C-1027 chromophore fraction, and show the structure of one of its degradation products.

The fermentation conditions used were the same as described previously[1]. For preparation of the chromophore fraction, the lyophilized fraction containing antibiotic C-1027 that had been precipitated from a culture filtrate with ammonium sulfate was dissolved in 0.2 M phosphate buffer (pH 8.0) and then extracted three times with an equal volume of ethyl acetate. After evaporation, the chromophore-containing residue was dissolved in methanol and kept at room temperature in a dark room. At this step, the time course of changes in the HPLC profile of the chromophore extract was determined and is shown in Fig. 1. The freshly prepared chromophore extract showed mainly three peaks on the HPLC, as shown in (A) of Fig. 1, and these were tentatively named components I, II, and III based on their respective order of elution (Fig. 2). UV spectra of the two components II and III were very similar and both had a broad absorption spectrum with its peak centered at 345 nm (Fig. 2). The component corresponding to peak III was present in the greatest abundance and possessed most of the *in vitro* biological activity. But there was diminished activity in this fraction with time of storage, which was attributable to decomposition of peak III material to products with earlier Rt's. Since component II appeared to be comparatively more stable than component III, whose $t_{1/2}$ was less than 1 hour, we attempted to isolate and purify

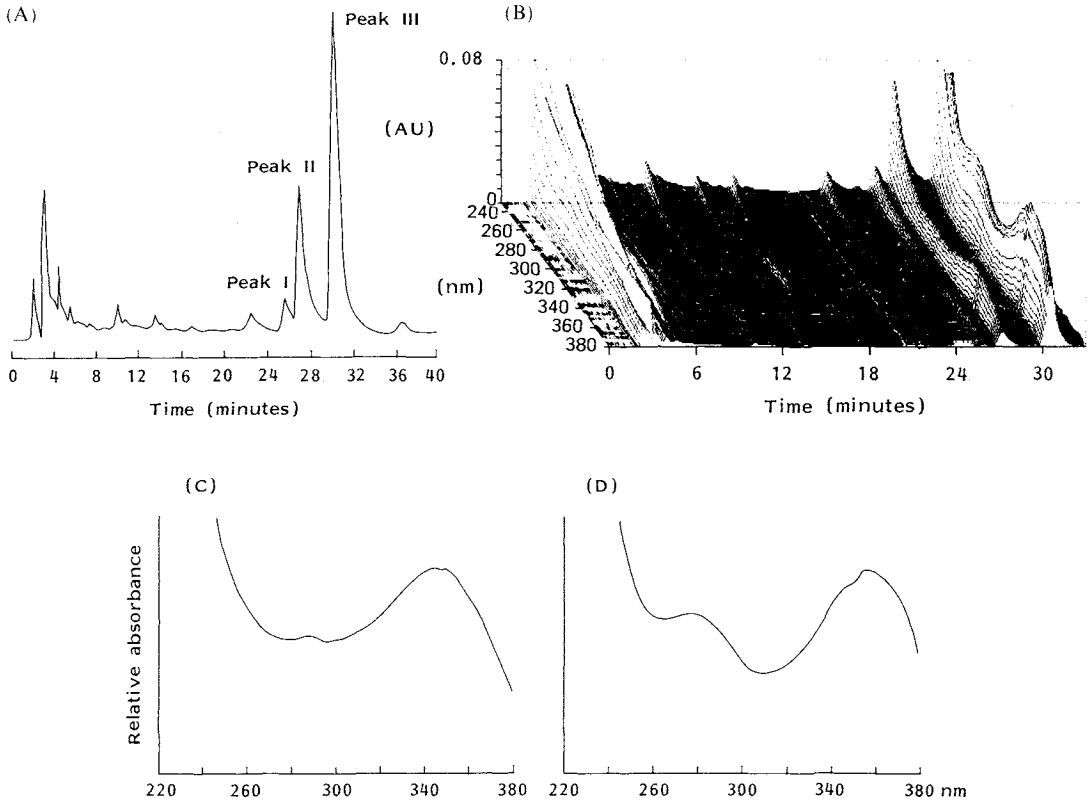
Fig. 1. Time course of changes in C-1027 chromophore components in MeOH solution at room temperature.

(A) No storage, (B) after 1 hour of storage, (C) after 4 hours of storage.



Analytical conditions of HPLC are as follows: Column, Develosil ODS 5 (250×4.6 mm, i.d.); solvent, acetonitrile-20 mM phosphate buffer (pH 7.0) (50:50); flow rate, 1.0 ml/minute; detector, UV (350 nm, 0.04 a.u.f.s.).

Fig. 2. Three-dimensional analysis of C-1027 chromophore components by photodiode array detector.
(C) UV spectrum of peak II, (D) UV spectrum of peak III.



Analytical conditions of HPLC are as follows: Column, Develosil ODS 5 (250 × 4.6 mm, i.d.); solvent, acetonitrile - 20 mM phosphate buffer (pH 7.0) (45 : 55); flow rate, 1.0 ml/minute; detector, Shimadzu SPD-M6A system. The chromophore complex showed mainly three peaks on HPLC and that were tentatively named components I, II, and III based on their respective order of elution.

component II.

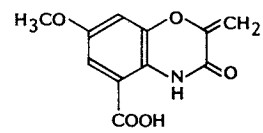
The chromophore fraction (27 mg) was prepared by ethyl acetate extraction from the crude protein (*ca.* 5 g) precipitated by adding ammonium sulfate to broth filtrate. When chromophore extract was kept in methanol (20 ml) at 5°C in a dark room overnight, most of it converted to component II, followed by further decomposition into more polar products. After concentration, the resultant residue was dissolved in a small volume of methanol and was chromatographed on an ODS column (50 × 1.5 cm, i.d., Yamazen Co.) with acetonitrile - 20 mM phosphate buffer (pH 7.0) (45 : 55) as eluant. The active eluted fractions, after evaporation, were pooled and extracted with ethyl acetate to give 1 mg of chromophore component II. It gave a single peak on analytical HPLC under the chromatographic conditions given in the legend of Fig. 1. The low yield of component II obtained may have been due to loss by degradation. Determination of the MW was done by use of the ionization technique of FAB-MS. The FAB mass spectrum of component II was characterized by *quasi*-molecular ion peak $(M+H)^+$ at *m/z* 846 together with the appearance of $(M+Na)^+$ at *m/z* 868, and the molecular formula was determined as $C_{43}H_{44}N_3O_{13}Cl$ by HR positive FAB-MS (Calcd: 846.2640, Found: *m/z* 846.2643). However, the structural elucidation of purified component II has been unsuccessful so far because of its small quantity.

During these purification processes, a natural degradation product of chromophore component II, possessing acidic property, was detected on HPLC. Further, we found that this degradation compound remained as a stable compound when extracted from the culture filtrate with ethyl acetate under the acidic

condition. Therefore, this degradation product (II-F_3) was isolated from the broth filtrate by the following steps: The broth filtrate (5 liters) obtained was adjusted to pH 3 and then extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was subjected to silica gel column (250 × 20 mm, i.d., Merck) chromatography, and the column was eluted with ethyl acetate - hexane (1 : 2). Further purification was achieved by ODS column chromatography (300 × 8 mm, i.d., Yamazen Co.) with acetonitrile - 20 mM phosphate buffer (pH 7.0) (15 : 85). The fractions containing degradation product II-F_3 were evaporated to remove the solvent and extracted with ethyl acetate to give 5 mg of degradation product II-F_3 as pale yellow powder. It showed a single peak on HPLC under the following analytical conditions: column, TSKgel ODS-120T (250 × 4.6 mm, i.d.); solvent, a linear gradient of acetonitrile in concentration from 20% to 80% in 20 mM phosphate buffer (pH 7.0); flow rate, 1.0 ml/minute; detector, UV (350 nm, 0.04 a.u.f.s.). The Rt of purified II-F_3 was 10.5-minute. Physico-chemical properties of II-F_3 were as follows: EI-MS *m/z* 235 ($M^{+\cdot}$); UV λ_{max}^{MeOH} nm (ε) 217 (29,000), 340 (8,700); IR (KBr disk) cm^{-1} 3435, 3220, 1665, 1635, 1610, 1510, 1380, 1205, 1050, 720; ^1H NMR (DMSO-d_6) δ 10.57 (br s), 7.14 (d, J=2.8 Hz), 7.00 (d, J=2.8 Hz), 5.47 (d, J=1.7 Hz), 5.13 (t, J=1.5 Hz).

The UV spectrum of II-F_3 thus obtained was similar to that of the methyl ester of an alkaline degradation product of the chromophore from the antitumor protein auromomycin, an antibiotic similar to C-1027[4], as reported by KUMADA *et al.*[6], recently. For the preparation of its methyl ester, II-F_3 (5 mg) was dissolved in 2 ml of water, to which was added 50 μl of 1 M silver nitrate. The resultant precipitate was suspended in 0.5 ml of acetonitrile and then 20 μl of 10% methyl iodide - acetonitrile was added. After having been kept for 1 hour at room temperature, the concentrate was separated by preparative TLC on Silica gel F_{254} (Merck) developed with hexane - ethyl acetate (2 : 1) to yield 2 mg of II-F_3 methyl ester. The physico-chemical properties of II-F_3 methyl ester agreed fully with those reported for the degradation product of the auromomycin chromophore in terms of UV, IR, EI-MS, and ^1H NMR spectra given by KUMADA *et al.* On the basis of these results, the chemical structure of degradation product II-F_3 was proposed to be 3,4-dihydro-7-methoxy-2-methylene-3-oxo-2*H*-1,4-benzoxazine-5-carboxylic acid, as shown in Fig. 3. The UV spectrum of degradation product

Fig. 3. Structure of degradation product II-F_3 derived from the C-1027 chromophore.



II-F_3 resembled that of component II. In addition, the ^1H NMR spectrum obtained from isolated chromophore component II showed the benzoxazine ring to be part of the structure, and indicated degradation product II-F_3 to be connected by an ester linkage to a highly-unsaturated unknown moiety as the remaining portion of the molecule. SHIBUYA *et al.*[7] synthesized benzoxazine derivatives related to II-F_3 to study their DNA-cleaving activity, and found that one of them caused single-strand breakage of covalently closed circular (ccc)-DNA in the presence of Cu^{++}. Although degradation product II-F_3, and also its benzoxazine derivatives, has no biological activity, it would be useful to know about the correlation of the II-F_3 structure with the DNA-cleaving activity of the native C-1027 chromophore.

NAOI *et al.*[8] reported only one kind of chromophore to be present in macromomycin and auromomycin. However, the whole structure of the auromomycin chromophore has not yet been elucidated. The HPLC profile of C-1027 chromophore complex seems to be different from that of macromomycin and of auromomycin. A more detailed study on the structure of chromophore component II is now in progress.

The antimicrobial activity of freshly prepared C-1027 chromophore fraction against a number of organisms is shown in Table 1, in comparison with those activities of intact antibiotic C-1027 and its antagonist C-1027-AG. The chromophore fraction possessed activity against not only Gram-positive bacteria but also a lower level of activity against most of the Gram-negative bacteria tested, while C-1027 displayed activity against Gram-positive bacteria and against some strains of Gram-negative bacteria. C-1027-AG, a protein with a primary structure identical to that of the protein moiety (apoprotein) of C-1027, had no activity against any bacteria tested, even at high concentrations. Furthermore, when KB carcinoma cells were exposed to the chromophore fraction for 3 days in tissue culture, the ED_{50} value obtained was less than 0.01 ng/ml, while that of C-1027 was 0.1 ng/ml, as reported previously[5]. However, the ED_{50} value of

Table 1. Antimicrobial spectra of C-1027, the freshly prepared chromophore extract (C-1027-chr) and C-1027-AG as apoprotein.

Test organism	MIC (μg/ml)		
	C-1027	C-1027-chr	C-1027-AG
Staphylococcus aureus FDA 209P	0.39	1.56	>100
S. aureus Terajima	0.39	0.78	>100
S. aureus Smith	0.20	0.78	>100
S. epidermidis IFO 3762	0.39	1.56	>100
S. citreus	0.39	1.56	>100
Streptococcus faecalis subsp. *liquefaciens*	0.20	0.78	>100
Micrococcus luteus ATCC 10240	0.39	1.56	>100
M. luteus ATCC 9341	0.20	1.56	>100
Bacillus subtilis ATCC 6633	0.39	1.56	>100
B. cereus IFO 3001	0.20	0.78	>100
Escherichia coli IFO 3972	>50	25	>100
E. coli K-12	>50	25	>100
E. coli NIHJ	>50	25	>100
E. coli B IFO 13168	0.78	3.13	>100
E. coli B IAM 1268	0.39	1.56	>100
E. coli ATCC 27166	0.20	1.56	>100
Proteus vulgaris IID OX 19	>50	50	>100
Klebsiella pneumoniae ATCC 29665	>50	50	>100
Salmonella typhimurium	>50	>50	>100
Serratia marcescens IFO 12648	>50	25	>100
Pseudomonas aeruginosa IFO 13275	>50	>50	>100

The MIC was determined by serial agar dilution method using nutrient agar at an inoculum of 10^6 cells/ml after a 21-hour incubation at 37°C.

Antibiotic C-1027 and its antagonist C-1027-AG were isolated by the same purification procedure as described in our previous report[4].

isolated chromophore component II was 0.6 μg/ml; and its degradation product II-F_3 was not active against KB cells ($ED_{50} \geqq 10\,\mu$g/ml) or against microbial cells (MIC $\geqq 100\,\mu$g/ml). Thus the isolated protein-free chromophore component was much more labile than the native C-1027, although the chromophore fraction possessed temporarily more potent activity. These results suggest that antibacterial activity, as well as cytotoxic activity, of C-1027 is due to its chromophore and that the protein moiety may be required for stabilizing the chromophore. Further, native C-1027 and the chromophore fraction exhibited a different antimicrobial spectrum. This difference may be related to differences in permeability of the target cells to these molecules.

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