

C-1027 Eneidyne Chromophore: Presence of Another Active Form and Its Chemical Structure

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(Received for publication December 24, 1998)

Antibiotic C-1027, a member of a family of potent antitumor chromoproteins, was isolated from the culture supernatant of *Streptomyces globisporus* C-1027¹⁾, and found to display antimicrobial activity as well as to have potent cytotoxic effects²⁾. C-1027 was shown to consist of two components, an acidic protein of 110 amino acid residues, previously sequenced^{3,4)}, and a non-protein chromophore extractable with organic solvents, which was very labile in the free form as is the case for chromophores of known chromoproteins such as neo-carzinostatin (NCS), auromomycin, actinoxanthin, kedarcidin, and maduropeptin^{5,6)}. By a detailed analyses of the ¹H and ¹³C NMR spectra with the aid of 2D-NMR technique of aromatized C-1027 chromophore III (Chr-III), the native C-1027 chromophore IV (Chr-IV) was shown to possess a 9-membered 1,5 diyn-3-ene core structure in a 16-membered macrocyclic ring together with benzoxazolate and aminosugar moieties as side chains^{7~9)} (Fig. 1). The biological activity of the chromophore has been attributed to its ability to produce DNA damage following rearrangement to form a diradical species which abstracts hydrogen atoms from deoxyribose even in the absence of reducing agent^{10~12)}, in contrast to other enediynes antibiotics that required a reducing agent. It also has been shown that the apoproteins of chromoprotein antibiotics, as seen with C-1027, show a high degree of amino acid sequence homology, stabilize the chromophores¹⁾ and additionally function as a targeting vehicle for the chromophore¹³⁾.

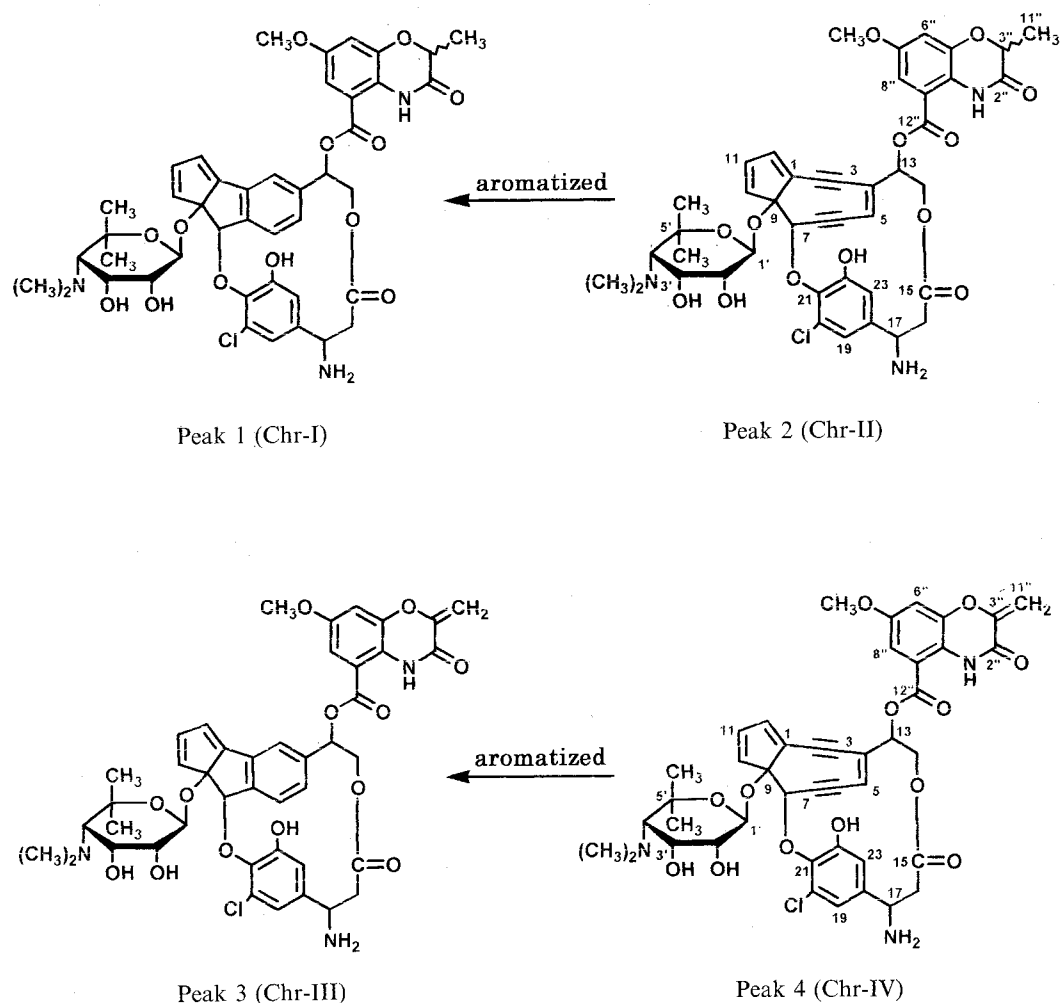
Recently, for further toxicological evaluation and formulation studies, we have attempted to improve the purification procedure to make it more suitable for large-scale operations. In the present work, we deal with

the isolation, structural elucidation, and biological activities of another active chromoprotein, tentatively termed NeoC-1027, that we found during large-scale production. In particular, we report the isolation and structure of two chromophore components, NeoC-1027 chromophore I (Chr-I) and -II, which could be separated from Chr-III and -IV by reversed-phase HPLC of chromophore preparations freshly extracted from the chromoprotein fraction with EtOAc in the dark.

Fermentation was carried out for 96 hours at 27°C under aeration of 0.3 v.v./minute and agitation at 25 rpm in a 2-kl tank fermentor containing 1-kl of the production medium composed of glycerol 2.0%, dextrin 2.0%, fishmeal (bonito, Wako Pure Chemical) 0.5%, proteose peptone 0.05% (Difco) (pH 7.0 before sterilization). The broth filtrate (96 liters) obtained after removing the mycelial cake was treated according to the purification procedures for C-1027, as shown in Fig. 2. The antibiotics were recovered by precipitation with ammonium sulfate at pH 4, and purified by the successive steps of salting out with a UF membrane (SEP-3013, Asahi Chemical Co.), and by hydrophobic and ion-exchange column chromatography with Butyl S and DEAE A-50 cellulofine (Seikagaku Inc.), respectively. At the final step, C-1027 was found to be homogeneous upon the high-pressure liquid chromatographic (HPLC) analysis on TSK gel Ether-5PW and TSK gel 2000GSW columns (Tosoh Co., Ltd.), as shown in previous reports^{1,3)}. However, HPLC analysis of the extract just after fractionating the crude chromoprotein-preparation suggested the presence of another biologically active chromophore separable from its protein moiety, as monitored by typical UV absorption at both 280 and 350 nm and antibacterial activity against *Micrococcus luteus* ATCC 9341. Therefore, separation of this new complex from the chromophore/protein complex of C-1027 could be achieved by careful reversed-phase HPLC analysis of each fraction eluted from a hydrophobic TSK gel phenyl-5PW column (20 × 5.5 cm, i.d.) with a linear gradient concentration of 1.5 M (NH₄)₂SO₄ in 0.1 M phosphate buffer and 0.1 M phosphate buffer (pH 6.0) for 300 minutes at a flow rate of 8 ml/minute. The respective bio-active fractions containing C-1027 and NeoC-1027 were separately pooled, desalted with a UF membrane, and then lyophilized as chromophore/protein complex to yield 890 mg and 240 mg, respectively. Both C-1027 and NeoC-1027 possessed apoprotein with

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Fig. 1. Structure of chromophore components Chr-I and -II of NeoC-1027 and Chr-III and -IV of C-1027.



The chromophore components corresponding to peaks 1, 2, 3, and 4 in Fig. 3-a were tentatively named as Chr-I, -II, -III, and -IV, respectively.

similar physico-chemical properties such as amino acid composition, molecular weight and iso-electric point, as described previously for C-1027³⁾.

As shown in Table 1, both compounds showed similar antibacterial activities with minimum inhibitory concentrations (MICs) of $\leq 0.05 \sim 0.2 \mu\text{g/ml}$ against Gram-positive bacteria and $0.39 \sim 0.78 \mu\text{g/ml}$ against some strains of Gram-negative bacteria such as *Escherichia coli* on Mueller-Hinton agar (Difco) after an 18-hour incubation at 37°C . NeoC-1027 Chr-I had antibacterial activity with MICs of 100, 50 and $100 \mu\text{g/ml}$, respectively, against *Enterococcus faecalis* IFO 12968, *E. faecium* ATCC 6056 and *Micrococcus luteus* ATCC 9341 among

Gram-positive and -negative bacteria tested. In addition, NeoC-1027 exhibited potent cytotoxic activity against KB cells even at concentrations less than 0.1 ng/ml , when the KB cells ($2 \times 10^3 \text{ cells/ml}$) were exposed to the antibiotic for 3 days at 37°C in Eagle's minimal essential medium supplemented with 10% fetal calf serum. However, Chr-I of NeoC-1027 did not show cytotoxicity even at the concentration of $10 \mu\text{g/ml}$. The *in vivo* antitumor effect of NeoC-1027 was examined by intravenous administration on day 1, 5, 9 to male CDF₁ mice (6 weeks old) subcutaneously implanted with Colon 26 adenocarcinoma fragments ($2 \times 2 \times 2 \text{ cm}^3$). NeoC-1027 showed a significant inhibition of tumor growth (74%)

Fig. 2. Procedure for purification of antibiotic C-1027 and NeoC-1027 in large-scale production.

Fermentation broth (96 liters)
centrifuge at 12,000 r.p.m for 30 min
Supernatant
Ammonium sulfate fractionation
60 % saturation, pH 4
centrifuge at 12,000 r.p.m for 30 min
Precipitate
Butyl S cellulofine column chromatography
a stepwise elution, 1.8 M ~ 1.4 M (NH ₄) ₂ SO ₄
in 0.01 M Na ₂ HPO ₄ -KH ₂ PO ₄ buffer, pH7.0
Desalting and concentration
DEAE A-500 cellulofine column chromatography
0.01 M Na ₂ HPO ₄ -KH ₂ PO ₄ buffer-elution
Desalting with a UF-membrane and concentration
DEAE A-500 cellulofine column chromatography
a stepwise elution, 0.05 M ~ 0.675 M NaCl solution
TSK gel phenyl-5PW column chromatography
a linear gradient concentration-elution, 1.5 M ~ 0 M (NH ₄) ₂ SO ₄
in 0.1 M Na ₂ HPO ₄ -KH ₂ PO ₄ buffer, pH6.0
Active fractions of C-1027 and NeoC-1027
Desalting with a UF-membrane and concentration
Lyophilization
Purified powder (C-1027 890 mg, NeoC-1027 240 mg)

Table 1. Antimicrobial spectrum of C-1027 and NeoC-1027.

Test organism	MIC (μg/ml)	
	C-1027	Neo C-1027
<i>Staphylococcus aureus</i> FDA 209P	≤0.05	0.1
<i>S. aureus</i> Terajima	≤0.05	0.1
<i>S. aureus</i> Smith	≤0.05	≤0.05
<i>S. epidermidis</i> IFO 3762	≤0.05	0.1
<i>Enterococcus faecium</i> ATCC 6056	≤0.05	≤0.05
<i>Micrococcus luteus</i> ATCC 9341	0.1	0.2
<i>Bacillus subtilis</i> ATCC 6633	≤0.05	≤0.05
<i>B. cereus</i> IFO 3001	0.2	0.2
<i>B. subtilis</i> H17 (rec ⁺)	≤0.05	0.1
<i>B. subtilis</i> M45 (rec ⁻)	≤0.05	≤0.05
<i>Escherichia coli</i> NIHJ	50	> 50
<i>E. coli</i> K-12 C600	> 50	> 50
<i>E. coli</i> B	0.39	0.78
<i>E. coli</i> ATCC 27166 (NIH)	0.39	0.78
<i>Klebsiella pneumoniae</i> ATCC 29665	> 50	> 50
<i>Proteus vulgaris</i> IID OX-19	> 50	> 50
<i>Salmonella typhimurium</i> G-46	> 50	> 50
<i>Serratia marcescens</i> IFO 12648	> 50	> 50
<i>Enterobacter cloacae</i> IFO 13535	> 50	> 50
<i>Pseudomonas aeruginosa</i> IFO 13275	> 50	> 50

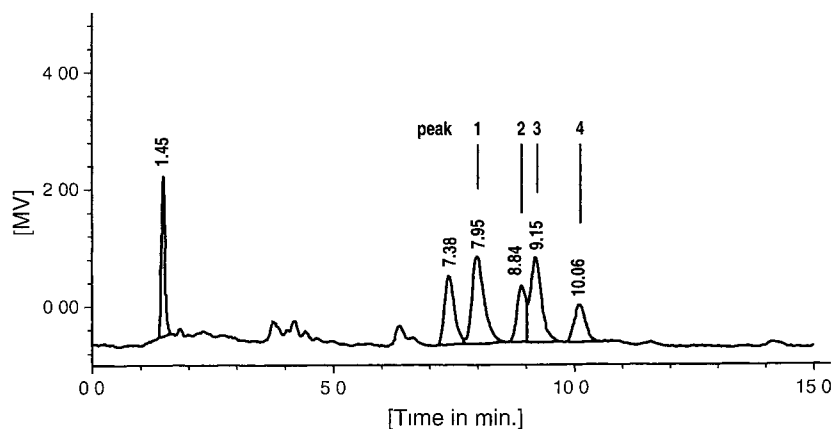
The minimum inhibitory concentration (MIC) was determined by the serial agar dilution method using Mueller-Hinton agar at an inoculum size of 10⁶ cells/ml and a 18-hour incubation at 37°C.

at a dose of 0.05 mg/kg/day on day 12 (mortality: 0/6).

A typical chromatogram from the reversed-phase HPLC of the chromophore extract from the chromoprotein fraction is shown in Fig 3-a. The four main peaks were tentatively named as Chr-I, -II, -III, and -IV on the basis of their respective order of elution. As shown in preceding communications⁷⁻⁹, we deduced the structure of C-1027 Chr-IV from the cycloaromatization mechanism leading to the more stable reaction product C-1027 Chr-III. Therefore, the time course of degradation of NeoC-1027 Chr-II in several organic solvents was examined at the room temperature by means of sequential HPLC analysis. As judged by the rate of appearance of degradation product in organic solvent, Chr-II was effectively converted into Chr-I in EtOH (Fig. 3-b), as

seen in the relationship between Chr-III and -IV of C-1027⁹). Accordingly, the present studies to elucidate the structure of the active component NeoC-1027 Chr-II were performed with the more stable reaction product, NeoC-1027 Chr-I, since Chr-II is highly unstable and undergoes cycloaromatization. The crude chromophore fraction prepared by an EtOAc extraction of protein-precipitate (20 g) at alkaline pH was added to EtOH and then subjected by preparative HPLC on Inertsil ODS-2 column (250 × 20 mm, i.d.) with CH₃CN/0.01 N-HCl (30:70) as a mobile phase with detection of UV-350 nm at flow rate of 8 ml/minute. After removal of CH₃CN, the eluted fraction was lyophilized to obtain the purified Chr-I as its HCl salt (1 mg). It gave a single peak under the analytical HPLC conditions given in the legend in

Fig. 3-a. Typical chromatogram from the reversed-phase HPLC of chromophore components.



The sample was prepared as an EtOAc extract, after the precipitate, obtained by treatment of the broth filtrate with ammonium sulfate, was dissolved in 0.1 M K_2HPO_4 solution.

HPLC conditions:

Column: Inertsil ODS-2 (150 × 4.6 mm, i.d.)

Temperature: 40°C

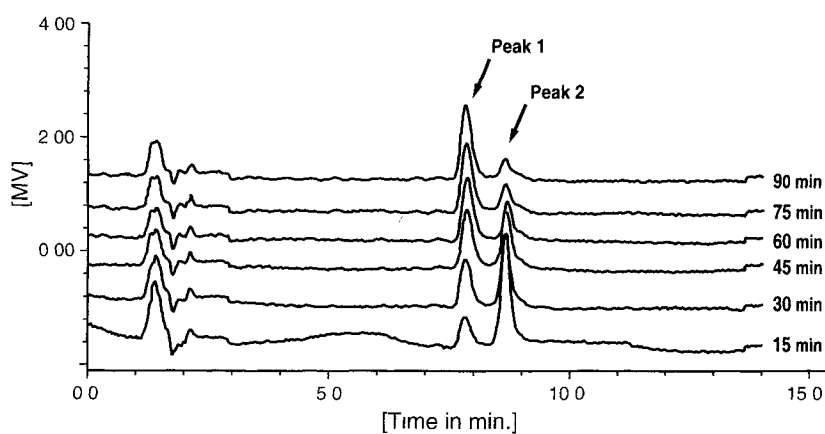
Mobile phase: 20 mM phosphate buffer (pH 6.86)/ CH_3CN (50 : 50)

Flow rate: 1.0 ml/minute

Detection: UV-350 nm

Apparatus: Tosoh SC-8010 system

Fig. 3-b. Time course of degradation of active peak 2.



The freshly prepared chromophore fraction Chr-II of NeoC-1027 was added to EtOH and stood at room temperature for the time indicated in the Figure.

HPLC conditions:

Column: Inertsil ODS-2 (250 × 20 mm, i.d.)

Temperature: room temperature

Mobile phase: 0.01 N-HCl/ CH_3CN (30 : 70)

Flow rate: 8.0 ml/minute

Detection: UV-350 nm

Apparatus: Tosoh SC-8010 system

Table 2. ^{13}C and ^1H -NMR chemical shifts (δ , ppm) of NeoC-1027 Chr-I in $\text{DMSO}-d_6$ and CD_3OD .

Position	NeoC-1027 Chr-I (in CD_3OD)				NeoC-1027 Chr-I ($\text{DMSO}-d_6$)		
	δ_{C} (ppm)	Multiplicity	HMBC ^a	δ_{H} (ppm)	Multiplicity	δ_{H} (ppm)	Multiplicity
1	152.85, 152.80	s	6.11, (6.69, 6.70), 6.76, (7.50)				
2	137.93	s	6.11, (6.69, 6.70), 7.44				
3	118.74	d	6.06, 6.95	7.56, 7.54	1H, brs	7.48	1H, s
4	141.36	s	4.26, 6.06, 7.44				
5	127.49	d	6.06, (7.54, 7.56)	6.95	1H, dd, 8, 1.5 Hz	6.87	1H, dd, 8, 1.5 Hz
6	129.18	d		7.44	1H, d, 8 Hz	7.24	1H, d, 8 Hz
7	148.43	s	6.95, (7.54, 7.56)				
8	85.98	d	7.44	6.11	1H, s	6.02	1H, brs
9	102.05	s	4.52, 6.11, 6.76				
10	136.99	d	(6.69, 6.70)	6.76	1H, d, 1 Hz	6.66	1H, d, 5 Hz
11	139.08	d	(6.69, 6.70), 6.76	6.76	1H, d, 1 Hz	6.68	1H, dd, 5.5, 2 Hz
12	128.33, 128.30	d	6.76	6.70, 6.69	1H, brs	6.61	1H, s
13	74.41	d	(1.50, 1.49)	6.06	1H, t, 7.5 Hz	5.97	1H, dd, 11, 5 Hz
14	66.97	t	6.06	4.26	2H, d, 7.5 Hz	4.23	1H, dd, 10, 5 Hz
						4.13	1H, dd, 11, 10 Hz
15	169.29	s	2.59, 4.26, 4.40				
16	42.51	t	4.40	3.05	1H, dd, 12, 3 Hz	3.02	1H, dd, 13, 2 Hz
				2.59	1H, t, 12 Hz	2.39	1H, t, 13 Hz
17	52.95	d	2.59, 6.15, 6.94	4.40	1H, dd, 12, 3 Hz	4.23	1H, overlapped
NH ₃ -17						8.63	3H, brs
18	134.50	s	2.59, 4.40				
19	116.50	d	4.40, 6.15	6.94	1H, d, 2 Hz	6.97	1H, s
20	131.48	s	6.15, 6.94				
21	141.91	s	6.15, 6.94				
22	153.15	s	6.15				
OH-22						8.19	1H, brs
23	115.36	d	4.40, 6.94	6.15	1H, d, 2 Hz	6.04	1H, d, 2 Hz
1'	93.84	d	3.11, 4.25	4.52	1H, d, 8 Hz	4.53	1H, brs, 7 Hz
2'	71.17	d	4.25	3.11	1H, dd, 8, 3.5 Hz	2.93	1H, brs
OH-2'							
3'	68.73	d		4.25	1H, overlapped	4.07	1H, brs
OH-3'							
4'	71.96	d	1.53, 2.98, 4.25	3.18	1H, d, 3 Hz	3.10	1H, brs
N(CH ₃) ₂ -4'	44.90	q	2.98, 3.18	2.98	6H, s	2.83	6H, brs
NH-4'						9.05	1H, brs
5'	76.61	s	1.53, 3.18, 4.25, 4.52				
CH ₃ -6' α	32.56	q	1.53, 3.18	1.53	6H, s	1.46	3H, s
CH ₃ -6' β	21.72	q	1.53, 3.18			1.44	3H, s
NH-1''						9.82	1H, s
2''	168.69	s	(1.50, 1.49), (4.68, 4.67)				
3''	74.12	d	4.26, 6.95, (7.56, 7.54)	4.68, 4.67	1H, q, 7 Hz	4.79	1H, q, 7 Hz
5''	146.19	s	(4.68, 4.67), 6.90				
6''	110.21	d	7.34	6.90	1H, d, 3 Hz	7.04	1H, d, 3 Hz
7''	156.75	s	3.84, 6.90, 7.34				
OCH ₃ -7''	56.52	q		3.84	3H, s	3.82	3H, s
8''	109.89	d	6.90	7.34	1H, d, 3 Hz	7.29	1H, d, 3 Hz
9''	115.07, 115.04	s	7.34				
10''	124.54	s	6.90, 7.34				
CH ₃ -11''	16.50, 16.48	q	(4.68, 4.67)	1.50, 1.49	3H, d, 7 Hz	1.42	3H, d, 7 Hz
12''	166.48	s	6.06, 7.34				

^{13}C and ^1H NMR spectra were recorded at 100 MHz and 400 MHz, respectively, at 30°C; Coupling constants are given in Hz.

^a Figures in parentheses indicate a pair of signals corresponding to the diastereomers of NeoC-1027 Chr-I.

Fig. 3-a.

The UV-absorption spectrum of NeoC-1027 Chr-I showed close similarity to that of Chr-III around 205, 229 (sh), 278, and 355 nm. FAB-MS spectra at positive mode; 870 (M+Na)⁺, *m/z* 848 (M+H)⁺ and at negative mode; *m/z* 846 (M-H)⁻ of Chr-I showed its nominal molecular weight to be 847. Furthermore, HRFAB-MS [found *m/z* 848.2741, calcd. *m/z* 848.2797 for C₄₃H₄₆N₃O₁₃Cl+H] suggested the molecular formula as C₄₃H₄₆N₃O₁₃Cl. By comparison with the molecular weight of C-1027 Chr-III (MW 845), Chr-I was deduced to be a hydrogenated compound of Chr-III. The ¹H and ¹³C NMR studies of Chr-I were performed in both DMSO-*d*₆ and MeOH-*d*₄ solutions at 30°C, and these results are summarized in Table 2. The ¹H NMR spectrum in DMSO-*d*₆ solution showed a doublet methyl signal and quartet methine signal at 1.42 ppm and 4.79 ppm, respectively. Moreover, exo-methylene signals corresponding to the oxazine side chain of Chr-III were not observed. This indicated that saturation of the exo-methylene group in oxazine side chain of Chr-III has occurred. Based on the ¹H NMR spectrum in DMSO-*d*₆ solution, Chr-I seemed to be a single component, but some signals split into two peaks, when MeOH-*d*₄ was used as solvent. Further, when the detailed NMR analyses including ¹³C NMR, HMQC, and HMBC experiments were performed in MeOH-*d*₄ solution, Chr-I was suspected to be a mixture consisting of two isomers. The signal splitting in the ¹H NMR and ¹³C NMR spectra was quite small except for the proton signals of the doublet methyl (1.50 and 1.49 ppm) and quartet methine (4.68 and 4.67 ppm). Consequently, these components were considered to be stereochemical isomers (diastereomers) at the methyl group in the oxazine side chain; and the respective structures of Chr-I and -II in NeoC-1027 were determined to be those shown in Fig. 1. Taking the relationship between NeoC-1027 Chr-I and Chr-II into consideration, Chr-II should be a corresponding enediyne mother compound, as seen in the relationship between Chr-III and -IV in C-1027.

We and SUGIURA *et al.*^{14,15)} reported that analysis of the fluorescence and circular dichroism spectra of C-1027, apoprotein, and chromophore revealed a specific 1:1 binding of chromophore to the apoprotein, and significant interaction of the oxazine group with apoprotein in the chromophore/apoprotein complex. Recent studies have showed that removal of the oxazine group in the aromatized form of C-1027 chromophore resulted in a 400-fold reduction in DNA binding affinity¹⁶⁾. Further, NMR experiments and distance-

restrained molecular dynamics computations for the complex of the DNA oligomer with the aromatized product Chr-III have indicated intercalative and minor-groove binding of the oxazine and amino sugar moieties, respectively¹⁷⁾. Thus, observations of DNA intercalation by the oxazine moiety of C-1027 suggest the conservation of a unique class of DNA-binding elements, as also occurs with the anthranilate of esperamicin A₁¹⁸⁾. The difference in the structures of Chr-II and Chr-IV might provide us a clue as to the participation of the tertiary structure of the apoprotein in the chromophore binding and the detailed elucidation for function of oxazine side chain in chromophore in an intercalative DNA binding mode in C-1027 antibiotic. In practice, separation of C-1027 and Neo-C-1027 may be achieved by taking advantage of the slight difference in the structure of their Chr-II and IV in the purification of these compounds on a hydrophobic column of TSK gel phenyl-5PW (Tosoh Co., Ltd.).

Acknowledgments

The authors are indebted to Dr. NORIO UNEMI, Director of our laboratory, and to Dr. WU JIAN-PO, Director of the Institute of Medicinal Biotechnology in The People's Republic of China, for their valuable comments and suggestions during the course of this study.

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