CONFORMATIONS OF PROTEIN MOIETIES AND CHROMOPHORE-PROTEIN INTERACTIONS IN THE ANTITUMOR ANTIBIOTICS, MACROMOMYCIN AND AUROMOMYCIN, CHARACTERIZED BY IR AND CD SPECTRAL ANALYSIS

NOBUHIKO MIWA

Department of Antibiotics, National Institute of Health
Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

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The free chromophores (chr) extracted from macromomycin (MCR) and auromomycin (AUR) showed different IR spectra in KBr tablets and different CD spectra in ethanol or methanol solution. Chr-MCR contained a greater amount of alkyl moieties than chr-AUR as shown by much stronger IR absorbance at 2960 cm^{-1}.

The IR spectra of MCR, AUR and their apo-proteins (apo) in D_2O showed that major parts of the proteins formed antiparallel β-pleated sheets. Apo-AUR exhibited a faster H-D exchange than AUR, indicating existence of more extensive "nonmotile parts" of the β-sheet formed through the chromophore-protein interaction in AUR. However, apo-MCR exhibited a slightly faster H-D exchange than MCR. This difference presumably was associated with the lower content of the chromophore (1.4 wt%) in MCR than that in AUR (8.2 wt%).

Chr-AUR was extracted from the intact antibiotic with methanol more efficiently than chr-MCR. Further, a marked bathochromic effect by the chromophore-protein interaction was observed in the CD and UV absorption for MCR, but not for AUR. These results indicated that chr-MCR was tightly bound to the protein moiety while most of chr-AUR was loosely bound.

MCR and AUR, antitumor antibiotics isolated from *Streptomyces macromomyceticus*,[1,2] are composed of proteins (11,700 daltons) of similar amino acid composition[2] and non-protein chromophores.[2~4] AUR is converted to MCR by Amberlite XAD-7 column chromatography releasing the chromophore.[2] The author and co-workers have previously shown that both MCR and AUR exhibit a differential cytotoxic effect to normal diploid, virus-transformed and carcinoma human lung cultured cells, and that the chromophore moieties are responsible for this differential effect.[4]

MCR and AUR are inferred to contain the same chromophore but to be different in their chromophore contents, as judged from similar elution profiles in high pressure liquid chromatography and similar antibacterial spectra of the two chromophores.[3] However, the author *et al.* have found that free chr-AUR is inactivated more rapidly than chr-MCR in the serum-containing medium,[4] suggesting that there may be some differences between the two chromophores. The chemical structures of the chromophores are still unknown. Conformations of the protein moieties and the chromophore-protein interaction modes of these antibiotics have not been reported.

In this paper, the author will show that chr-MCR and chr-AUR differ in the CD and IR spectra, and that the major parts of the protein moieties from antiparallel β-pleated sheet as reported for another protein antitumor antibiotic, neocarzinostatin.[5,6] It will be also indicated that the chromophore-protein interaction in MCR is more intensive than that in AUR.

Abbreviations: MCR, macromomycin; AUR, auromomycin; chr, chromophore; apo, apo-protein; rec, reconstituent; IR, infrared; CD, circular dichroism; UV, ultraviolet.

Materials and Methods

Antibiotics

MCR and AUR were generously provided by Kanegafuchi Chemical Industry Co., Ltd., Takasago, Hyogo, Japan.

Extraction of Chromophores from MCR and AUR

Chr-MCR and chr-AUR were extracted with ethanol and methanol precooled at −20°C, respectively, in the dark as previously described[4] and used immediately. The residual protein fractions were rinsed twice with the alcohols and used as apo-MCR and apo-AUR. Amounts of the chromophores and apo-proteins were expressed as equivalent weights of intact MCR and AUR before extraction.

Reconstitution of MCR and AUR

Rec-MCR and rec-AUR were obtained by mixing the chromophores with the apo-proteins at 20 and 10 mg/ml, respectively, as previously described.[4] Free chromophores were removed by PD-10 columns (Pharmacia Fine Chemicals) packed with Sephadex G-25M.

CD Spectra

The CD measurements were performed at 21°C under constant nitrogen flush with a Jasco spectropolarimeter J-40. The CD data were expressed in terms of mean residue ellipticity, $[\theta]$, in deg·cm^2·dmole^{-1}. The mean residue weight of 92.9 obtained from amino acid analysis[2] was used. The reference media such as ethanol, methanol and 0.05 M phosphate buffer (pH 7.5) μ=0.15; KCl (Buffer I) were successively measured after the measurements of chr-MCR, chr-AUR and the other compounds, respectively. The ellipticity was corrected by subtracting these reference values.

UV Absorption Spectra

The spectra were read on a Hitachi double beam-spectrophotometer 124. The molar absorption coefficients were calculated on the basis of a molecular weight of 12,500 for MCR and AUR.[2]

IR Spectra and H-D Exchange

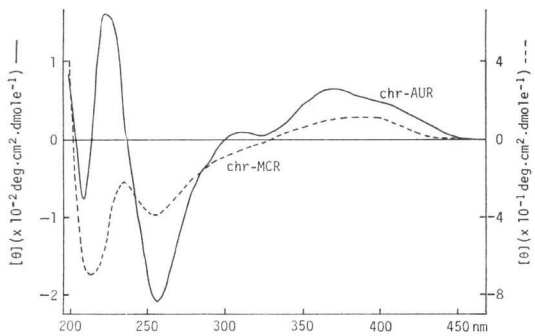
The IR measurements were carried out at 21°C with a Jasco double beam-infrared spectrophotometer IR-G with polystyrene film for calibration. The spectra of chr-MCR and chr-AUR were measured in KBr tablets after evaporation of the solvent at −40°C in the dark. The spectra of MCR, AUR and their apo-proteins were measured at pD 7.5 in D_2O (30 mg/ml) with KRS-5 cells of 0.05 mm path length. The H-D exchanges were conducted at 21°C in the dark.

Results

CD and UV Absorption Spectra of MCR, AUR, Their Chromophores and Apo-proteins

Chr-MCR and chr-AUR exhibited different CD spectra (Fig. 1). Apo-MCR and apo-AUR showed no CD band above 320 nm attributed to the chromophores (Figs. 2 and 3), indicating that the two apo-proteins contained no chromophores.

Fig. 1. CD spectra of the chromophores extracted from MCR and AUR.



MCR, AUR and their apo-proteins showed CD spectra in the far UV differing from the typical CD spectra of many other water-soluble globular proteins.[7] The CD spectra of apo-MCR and apo-AUR exhibited similar wavelengths to those of the β-pleated sheet: positive (231 nm) and negative (213 nm) extrema and two

Fig. 2. CD spectra of MCR, its apo-protein and reconstituent.

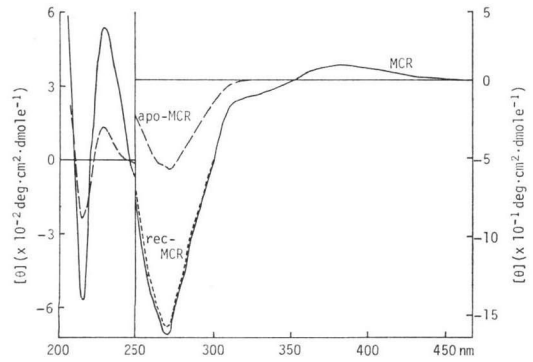


Fig. 3. CD spectra of AUR, its apo-protein and reconstituent.

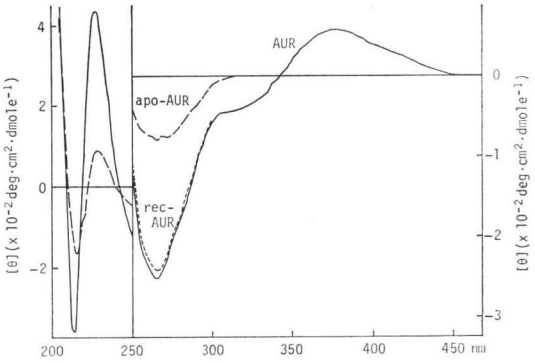
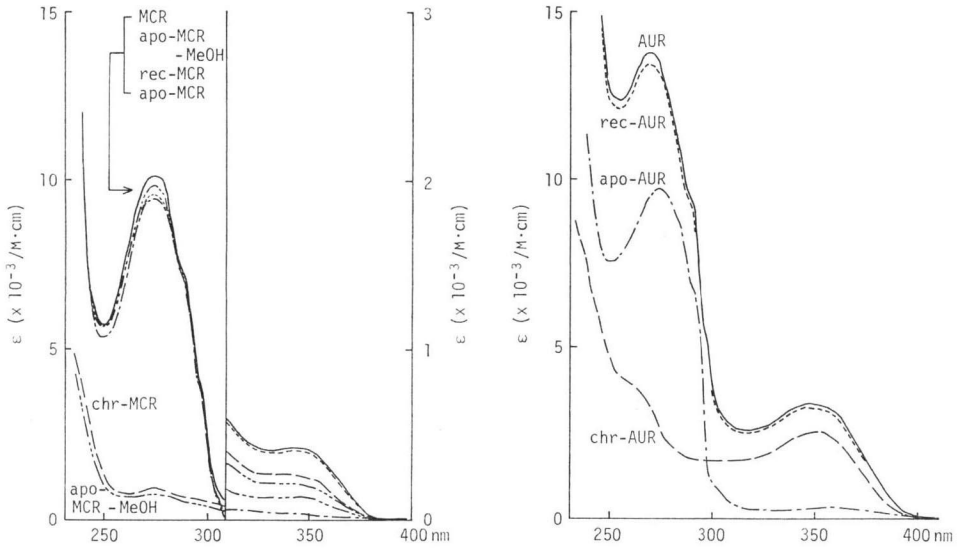


Fig. 4. Absorption spectra of MCR, AUR and their derivatives.

Chr-MCR-MeOH and apo-MCR-MeOH represent the chromophore and residual protein of MCR separated with methanol, respectively. Other derivatives were prepared as described in "Materials and Methods".



crossover points (225 and 204 nm),[7] though showing ellipticities much lower than those of the β-pleated sheet.

There was a slight difference in the absorption spectra between chr-MCR (λ_{max} 342 and 276 nm) and chr-AUR (λ_{max} 352 nm; shoulder, around 265 nm) (Fig. 4). The CD and absorption spectra of the chromophores did not change whether the solvent was ethanol or methanol.

Extraction Efficiency of the Chromophores from MCR and AUR

The maximal ellipticities at 256~272 nm and maximal absorption coefficients at 340~353 nm of MCR, AUR, their chromophores and apo-proteins separated with the alcohols, and their reconstituents were assessed from the CD and absorption spectra shown in Figs. 2~4 (Table 1). The maximal absorption coefficient (ε_{max}) of free chromophores extracted from MCR with methanol (ε=210; 340 nm) was 60% of that of the total chromophores (ε=350), while the ε_{max} of chromophores extracted with ethanol (ε=280; 342 nm) was 90% of that of the total chromophores (ε=310), indicating that chr-MCR was ex-

Table 1. Bathochromic effect in CD and UV absorption spectra by binding of the apo-proteins to the chromophores.

	$-[\theta]_{\lambda_{max}}$ (λ_{max}: nm)			Bathochromicity coefficient (%)
	Free chromophore	Protein	Sum	
Intact MCR	—	162 (270)	162	71
Rec-MCR	—	156 (269)	156	64
EtOH-treated MCR	39 (256)	56 (272)	95	—
Intact AUR	—	250 (265)	250	−13
Rec-AUR	—	241 (266)	241	−16
MeOH-treated AUR	207 (257)	79 (266)	287	—

	$\varepsilon_{\lambda_{max}}$ (λ_{max}: nm)			Bathochromicity coefficient (%)
	Free chromophore	Protein	Sum	
Intact MCR	—	430 (345)	430	39
Rec-MCR	—	410 (345)	410	32
MeOH-treated MCR	210 (340)	140 (345)	350	13
EtOH-treated MCR	280 (342)	30 (340)	310	—
Intact AUR	—	3440 (347)	3440	22
Rec-AUR	—	3250 (347)	3250	15
MeOH-treated AUR	2630 (352)	190 (353)	2820	—

The CD and absorption data are obtained from Figs. 1~3 and 4, respectively. The bathochromicity coefficient represents the ratio of increase in ellipticity or absorbance of a chromophore-protein complex to the sum of ellipticities or absorbances of both a free chromophore and a protein.

Table 2. Weight recovery in extraction of the chromophores from MCR and AUR with ethanol and methanol, respectively.

	Amount (mg)	Recovery (%)	Content (%)
MCR	76.41	100.0	100.0
Chr-MCR	1.07	1.4	1.4
Apo-MCR	74.65	97.7	98.6
AUR	36.99	100.0	100.0
Chr-AUR	2.98	8.1	8.2
Apo-AUR	33.74	91.2	91.8

tracted with ethanol more efficiently than with methanol. On the other hand, the ε_{max} of free chr-AUR extracted from AUR with methanol (ε=2,630; 352 nm) was 93% of that of the total chromophores (ε=2,820), indicating that chr-AUR was extracted with methanol more efficiently than chr-MCR. The contents of chr-MCR and chr-AUR were 1.4 and 8.2 wt%, respectively, when estimated from the weight recovery of extraction (Table 2).

Bathochromic Effects in CD and UV Absorption by Binding of the Apo-proteins to the Chromophores

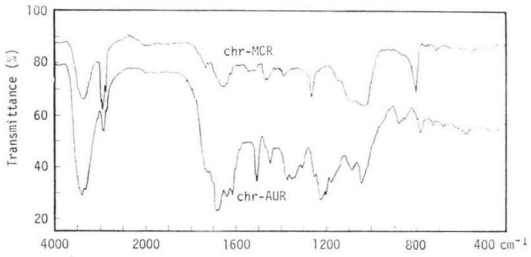
Both intact MCR and rec-MCR showed 71 and 64% greater mean residue ellipticities near 270 nm, respectively, than the sum of the mean residue ellipticities of free chr-MCR and apo-MCR separated by ethanol extraction (Table 1). The bathochromic effect was observed also for the absorption near 345 nm attributed to the chromophore moiety of MCR. Further, MCR treated with methanol exhibited a bathochromic effect of 13% in 345 nm absorbance, and this value was between the values of holo-MCR (39 and 32%) and ethanol-treated MCR (0%) (Table 1), suggesting that the methanol-treated MCR had a considerable amount of the chromophore yet bound to the protein moiety. In contrast, AUR and rec-

AUR did not show any bathochromic effects in the CD at 266 nm, and showed the smaller effects than that of holo-MCR in the absorption at 347 nm.

IR Spectra and H-D Exchange of MCR, AUR and Their Derivatives

Chr-MCR and chr-AUR showed different IR spectra (Fig. 5). Chr-MCR exhibited an absorption at 2960 cm^{-1} attributed to alkyl groups, which was greater than that of chr-AUR. The treatment of the chromophore with 1 N HCl at 0°C for 2 hours in the dark eliminated the peaks of 1661 cm^{-1} for chr-MCR and 1685, 1511 and 1226 cm^{-1} for chr-AUR, with loss of cytocidal activity toward carcinoma A549 cells (data not shown). This HCl treatment, however, did not affect the absorption band centered at 3430 cm^{-1}, suggesting that this absorption was assigned to O–H stretching vibration of hydroxyl groups.

Fig. 5. IR spectra of the chromophores of MCR and AUR in KBr tablets.



MCR, AUR and their apo-proteins showed similar IR spectra in amide I and II regions in D_2O solution (Fig. 6). Such spectral charac-

Fig. 6. IR spectra of MCR, AUR and their apo-proteins in amide I and II regions at various times after D_2O addition.

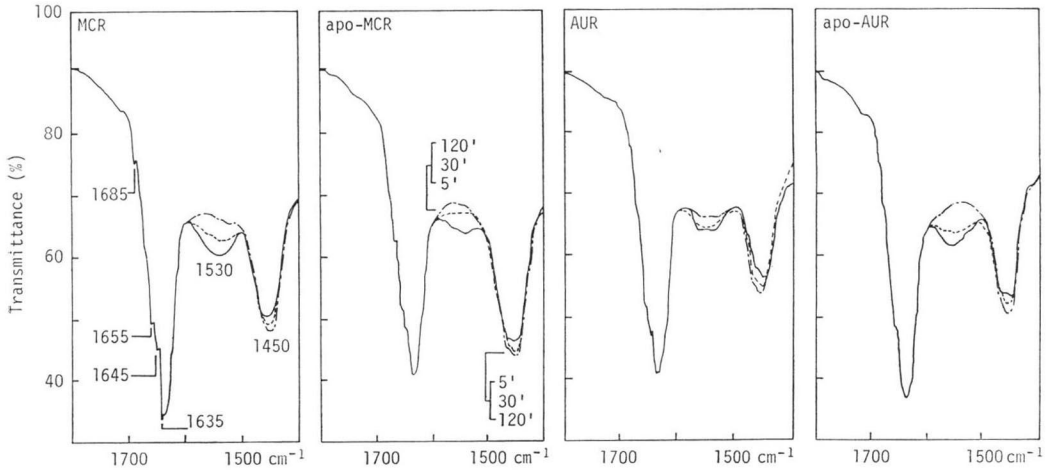


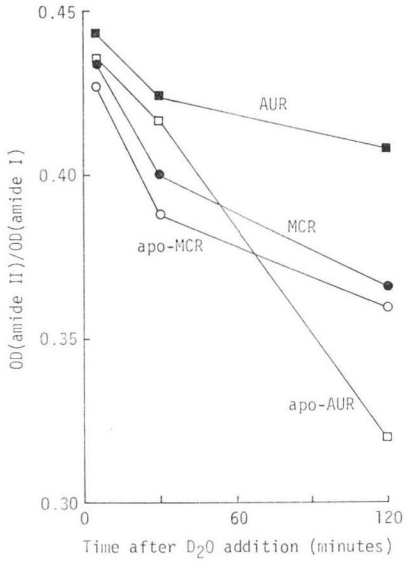
Table 3. Characteristics of IR spectra of MCR and AUR and their apo-proteins measured in D_2O at pD 7.5.

	Designation	Amide I frequency (cm^{-1})		Amide II frequency (cm^{-1})		Assigned conformation
		Obsd.	Calcd.	Obsd.	Calcd.	
MCR, Apo-MCR	$\nu(0, \pi)$	1685 w	1690 w	1530 s	1530 s	Antiparallel β-structure
	$\nu(\pi, 0)$	1635 s	1630 s	—	—	
AUR	—	1655 w	1655 s	—	1535 s	Random coil
Apo-AUR	$\nu(0)$	1645 w	1650 s	—	1515 w	α-Helix
	$\nu(5\pi/9)$	—	1650 m	—	1545 s	

s, m and w represent strong, medium and weak absorptions, respectively. The observed frequencies obtained from Fig. 6 were compared with the calculated frequencies[8] of reference conformations.

Fig. 7. Time course of H-D exchange in MCR, AUR and their apo-proteins.

OD values in amide I and II regions were obtained from Fig. 6.



teristics as a strong absorption for amide I near 1635 cm^{-1}, a weak one at 1685 cm^{-1} and an intense amide II frequency around 1530 cm^{-1} were similar to those of antiparallel β-pleated sheet proteins (Table 3).[8]

After addition of D_2O to lyophilized MCR, AUR and their apo-proteins, amide II absorption decreased, while amide I absorption did not change and amide II absorption increased (Fig. 6). The decrease in the ratio of amide II absorbance to amide I absorbance, an index of the proportion of H-D exchange, was more marked in apo-AUR than in AUR, whereas it was only slightly greater in apo-MCR than in MCR (Figs. 6 and 7).

Discussion

Chr-MCR and chr-AUR exhibit different CD and IR spectra (Figs. 1 and 5). However, the two chromophores show similar analytical elution profiles in high pressure liquid chromatography and similar antibacterial spectra.[3] Therefore, their chemical structures appear to be different but resemble each other. This difference may be related to the previous result that the cytocidal activity of free chr-AUR is lost in a serum-containing medium more rapidly than that of chr-MCR.[4]

The CD spectra of apo-MCR and apo-AUR have the wavelengths of positive (228 and 229 nm, respectively) and negative (215 nm, in common) extrema (Figs. 2 and 3) similar to those of β-pleated sheet.[7] However, the spectra show anomalously low mean residue ellipticities as compared to those of many other water-soluble proteins,[7] and therefore the protein conformations of MCR and AUR are unexplained from the CD spectra. MCR and AUR are unusually resistant to pronase digestion,[4,11] which seems to be due to an extraordinarily packed polypeptide folding that may be associated with these atypical CD spectra in the far UV.

The IR spectra of MCR and AUR in D_2O (Fig. 6) show that the major parts of the protein form antiparallel β-pleated sheets (Table 3), as shown for intact neocarzinostatin.[5,6] This is compatible with the amino acid composition[2] of MCR or AUR: Glycine, which destabilizes α-helix,[9] is the most abundant amino acid (20 residues/127 residues). Further, the protein has many threonine (18/127) and valine residues (16 or 17/127) which preferentially compose β-sheet.[9]

Apo-MCR and apo-AUR form the β-sheet in the same manner as MCR and AUR as shown by the IR spectra (Fig. 6). However, H-D exchange velocity in NH-CO of apo-AUR is greater than that in holo-AUR (Fig. 7). These results show that holo-AUR and apo-AUR are similar in the IR spectra reflecting a static configuration of the β-sheet, but different in the H-D exchange reflecting a dynamic configuration freedom denoted by "motility".[10] The "nonmotile part" of the β-sheet in holo-AUR appears to be more extensive than that in apo-AUR. On the other hand, apo-MCR exhibits an only slightly faster H-D exchange than that of MCR (Fig. 7). This difference between AUR and MCR presumably is associated with the difference in the chromophore contents of MCR and AUR (Table 2). A high chromophore content in AUR is likely to increase the nonmotile parts of the β-sheet more markedly through the chromophore-protein interaction.

The interaction between chr-MCR and apo-MCR is too intense to facilitate the full extraction of chr-MCR from holo-MCR with methanol (Table 1). In contrast, chr-AUR appears to interact with apo-AUR so weakly as to be efficiently released from the protein moiety by methanol extraction (Table

1).[4,12,18] This stronger interaction in MCR than that in AUR is suggested also by the fact that bathochromic effects in the CD and UV absorption are marked for MCR but not for AUR (Figs. 2~4 and Table 1). This bathochromic effect probably ensues from rigid immobilization of the chromophore by its ambient protein which functions as an "auxochrome".

The weaker chromophore-protein interaction in AUR explains the fact that chr-AUR is efficiently released by Amberlite XAD-7 chromatography[2] and protein denaturation with urea.[11] Moreover, the more intensive interaction in MCR accounts for the findings that addition of reductants such as $NaBH_4$ and dithiothreitol is indispensable for scission of isolated DNA by holo-MCR, but not by holo-AUR[14] or chr-MCR.[15] The reductant can cleave the S-S bridges of the protein moiety so as to induce polypeptide unfolding, which promotes chromophore release from the protein, resulting in transformation into the active species. Two S-S bridges are contained in neocarzinostatin,[17] which is homologous with MCR and AUR in the amino acid sequence at least for the greater part of *N*-terminal 31 residues.[16]

Chr-MCR contains a hydrophobic part as shown by a much stronger IR absorption at 2960 cm^{-1} (Fig. 5) attributed to alkyl moiety than that of chr-AUR. The van der Waals' force between this hydrophobic part of chr-MCR and a hydrophobic region in apo-MCR is likely to contribute to the more intensive chromophore-protein binding. Hydrophobic bonds between hydrophobic parts[18,19] of the chromophore and hydrophobic regions[17] of the protein are regarded as an important factor of the interaction in neocarzinostatin.[20]

Acknowledgments

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References

1) CHIMURA, H.; M. ISHIZUKA, M. MAEDA, S. HORI, K. KIMURA, J. IWANAGA, T. TAKEUCHI & H. UMEZAWA: A new antibiotic, macromomycin, exhibiting antitumor activity and antibacterial activity. J. Antibiotics 21: 41~49, 1968
2) YAMASHITA, T.; N. NAOI, T. HIDAKA, K. WATANABE, Y. KUMADA, T. TAKEUCHI & H. UMEZAWA: Studies on auromomycin. J. Antibiotics 32: 330~339, 1979
3) NAOI, N.; T. MIWA, T. OKAZAKI, T. WATANABE, T. TAKEUCHI & H. UMEZAWA: Studies on the reconstitution of macromomycin and auromomycin from the chromophore and protein moiety. J. Antibiotics 35: 806~813, 1982
4) MIWA, N.; S. MIZUNO & S. OKAMOTO: in preparation.
5) MAEDA, H.; H. SHIRAISHI, S. ONODERA & N. ISHIDA: Conformation of antibiotic protein, neocarzinostatin, studied by plane polarized infrared spectroscopy, circular dichroism and optical rotatory dispersion. Int. J. Peptide Protein Res. 5: 19~26, 1973
6) SAMY, T. S. A.; M. ATREYI, H. MAEDA & J. MEIENHOFER: Selective tryptophan oxidation in the antitumor protein neocarzinostatin and effects on conformation and biological activity. Biochemistry 13: 1007~1014, 1974
7) CHANG, C. T.; C.-S. C. WU & J. T. YANG: Circular dichroic analysis of protein conformation: inclusion of the β-turns. Anal. Biochem. 91: 13~31, 1978
8) MIYAZAWA, T. & E. R. BLOUT: The infrared spectra of polypeptides in various conformation: amide I and II bands. J. Am. Chem. Soc. 83: 712~719, 1961
9) CHOU, P. Y. & G. D. FASMAN: Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. 47: 45~48, 1978
10) HVIDT, A.: Discussion of the pH dependence of the H-D exchange of proteins. Compt. Rend. Trav. Lab. Carlsberg 34: 299~317, 1964
11) SUZUKI, H.; S. OZAWA & N. TANAKA: Urea treatment and pronase digestion of antitumor protein antibiotics, auromomycin and neocarzinostatin. J. Antibiotics 33: 1545~1550, 1980
12) KAPPEN, L. S.; M. A. NAPIER, I. H. GOLDBERG & T. S. A. SAMY: Requirement for reducing agents in deoxyribonucleic acid strand scission by the purified chromophore of auromomycin. Biochemistry 19: 4780~

4785, 1980

13) SUZUKI, H.; K. MIURA, Y. KUMADA, T. TAKEUCHI & N. TANAKA: Biological activities of non-protein chromophores of antitumor protein antibiotics: auromomycin and neocarzinostatin. Biochem. Biophys. Res. Commun. 94: 255~261, 1980

14) SUZUKI, H.; K. MIURA & N. TANAKA: DNA-cleaving potentials of macromomycin and auromomycin: a comparative study. Biochem. Biophys. Res. Commun. 89: 1281~1286, 1979

15) NAOI, N.; Y. KUMADA, T. YAMASHITA, T. TAKEUCHI & H. UMEZAWA: DNA strand scission of methanol-extracted chromophores of macromomycin and auromomycin. J. Antibiotics 35: 934~936, 1982

16) SAWYER, T. H. & K. GUETZOW: Amino terminal amino acid sequence of macromomycin, a protein antitumor antibiotic. Biochem. Biophys. Res. Commun. 86: 1133~1138, 1979

17) MAEDA, H.; C. B. GLASER, K. KUROMIZU & J. MEIENHOFFER: Structure of the antitumor protein neocarzinostatin. Arch. Biochem. Biophys. 164: 379~385, 1974

18) ALBERS-SCHÖNBERG, G.; R. S. DUVEY, O. D. HENSENS & J. M. LIESCH: Neocarzinostatin: chemical characterization and partial structure of the non-protein chromophore. Biochem. Biophys. Res. Commun. 95: 1351~1356, 1980

19) NAPIER, M. & I. H. GOLDBERG: Neocarzinostatin chromophore: presence of cyclic carbonate subunit and its modification in the structure of other biological active forms. Biochem. Biophys. Res. Commun. 100: 1703~1712, 1981

20) EDO, K. & N. ISHIDA: Chemistry and biological activity of neocarzinostatin. Kagaku To Seibutsu (in Japanese) 20: 213~215, 1982