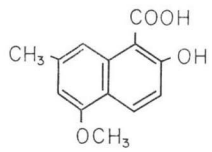
PEROXY ACID AS A PARTIAL STRUCTURE OF NEOCARZINOSTATIN (NCS) CHROMOPHORE

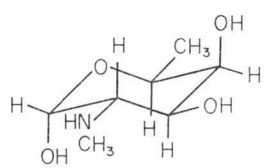
Sir:

Neocarzinostatin (NCS) chromophore was obtained as a separable component from polypeptide antitumor antibiotic NCS by us[1,2] and GOLDBERG *et al.*[3,4], independently. This chromophore, which was demonstrated to have biological activities such as growth inhibition of bacterial and tumor cells[1], inhibition of DNA synthesis in cultured mammalian cells, strand scission of DNA *in vitro* and *in vivo*[5,6] and inhibition of protein kinase[7,8], is thought to be the active center of NCS.

Considerable evidence shows that bacterial and tumor cellular DNA is the most important target in the action of NCS and the chromophore. The NCS chromophore, as well as NCS, is inactivated irreversibly under irradiation with ultraviolet light, exposure to heat or incubation with mercaptans prior to the introduction to DNA. The chromophore exhibited an optical rotation of $[\alpha]_D^{20}$ $-171°$[1], the molecular weight of 661 and the molecular formula of $C_{35}H_{35}NO_{12}$, by mass spectrometry[9]. Two compounds, 2-hydroxy-5-methoxy-7-methyl-1-naphthalenecarboxylic acid (**I**)[2] and *N*-methylfucosamine (**II**)[9] have been proposed as components of this compound. Compounds **I** and **II** have no antibacterial activity. Therefore, it has been suggested that the residual part of unknown structure is responsible for biological activities[9].



(I)



(II)

Recently, we defined the correlation between DNA strand scission with NCS chromophore and a free peroxyl radical generated from NCS chromophore on the basis of the results of bioassay and electron spin resonance (ESR) spectrometry[10]. But it has been remained as one of the most important questions what functional group of NCS chromophore generates a peroxyl radical. The insufficient informations on the structure of NCS chromophore hampered the finding of a plausible explanation. Thus a study of the total chemical structure and this function of NCS chromophore has been attempted. In this study we propose the presence of a peroxy acid moiety in NCS chromophore, on the basis of infrared (IR) spectrum, electrochemical (polarograph), and chemical data. Also, we discuss the role of this peroxyl radical in the generation of a free peroxyl radical from NCS chromophore and in its biological activities.

Purified NCS powder (clinical grade, 1,500 units/mg) produced in Kayaku Antibiotics Research Laboratory was provided for this investigation. NCS chromophore and apoprotein were separated according to a modification of a method previously reported[1,2]. The IR spectrum of NCS chromophore (5% KBr, 50 mg tablet) was measured by a JASCO IRA-1 spectrometer (one run spend 4 minutes). Yanagimoto pen-recording polarograph, model PA-102 was used to obtain the current-voltage curves. The values of m and t were 1.34 mg per second and 4.07 seconds, respectively. These values were obtained by using an open circuit with capillary immersed in non-aqueous electrolyte solution at room temperature. The electrolytic solution was 0.1 M tetraethylammonium perchlorate in equal volume of absolute methanol and benzene (1:1). Solutions of NCS chromophore, the concentration of which was set as 0.6 to 30×10^{-3} M were prepared, and aliquots were transferred to H-cells and polarograms were obtained. The content of the peroxy acid in the NCS chromophore was determined by iodometric titration. Two grams of sodium iodide were dissolved in a mixture of 40 ml of distilled water, 5 ml of gracial acetic acid and 5 ml of chloroform. To this mixture the sample was added followed by vigorous shaking for 30 minutes. The iodide liberated was titrated with 0.01 N sodium thiosulfate. Near the end of point, 5 ml of starch indicator (1%, in distilled water) was added and the titration continued until the blue color disappeared. Bioassay of antibacterial activity was carried out according to the previously reported method[1].

In the IR spectrum (Fig. 1) of NCS chromophore, carbonyl absorption bands were found at 1811 and 1783 cm^{-1} with a splitting of 28 cm^{-1}. The band at 1783 cm^{-1} was more intense. IR spectra of a series of acyl, aroyl and asymmetrical peroxides were investigated by DAVISON[11] and

Fig. 1. IR spectrum of NCS chromophore.

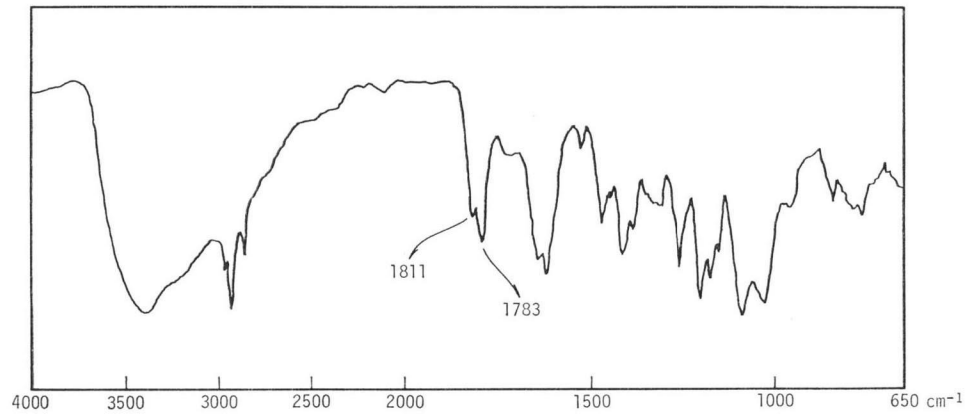
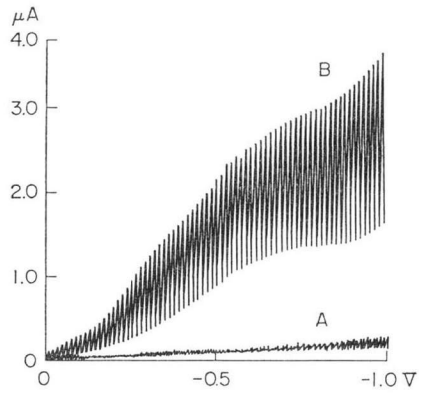


Table 1. IR spectral data of NCS chromophore, peroxides and anhydrides.

	(C=O) cm^{-1}		Split cm^{-1}
	A	B	
NCS chromophore	1811 (w)[a]	1783 (s)[b]	28
Diacetyl peroxide[c]	1820 (w)	1796 (s)	24
Dibenzoyl peroxide[c]	1792 (w)	1772 (s)	20
Acetyl benzoyl peroxide[c]	1811 (w)	1779 (s)	32
Benzoyl stearoyl peroxide[c]	1811 (w)	1786 (s)	25
Acetic anhydride[d]	1824 (s)	1748 (w)	76
Benzoic anhydride[d]	1789 (s)	1727 (w)	62

[a] w=weak intensity, [b] s=strong intensity, [c] W. H. T. DAVISON, J. Chem. Soc., 1951, 2456~2461, [d] L. J. BELLAMY, The Infra-red Spectra of Complex Molecules, p. 110, J. Wiley and Sons., Inc., New York, 1956.

Fig. 2. Polarograms of NCS chromophore in non-aqueous solvent [0.1 M TEAP/MeOH - benzene (1:1)]. A: Control, B: NCS chromophore (10^{-3} M).



those of anhydrides by BELLAMY[12] (Table 1). The paired carbonyl bands are different with respect to relative intensities, and this was used for further characterization. In acyclic aliphatic anhydrides and benzoic anhydride, the bands of the higher carbonyl frequency have higher intensity, while in acyclic aliphatic peroxides and dibenzoyl peroxide the bands of a lower carbonyl frequency are more intense. The splitting in anhydrides is about 60 cm^{-1}. Aliphatic diacyl peroxides have a pair of carbonyl bands in the regions of 1820~1811 and 1796~1784 cm^{-1}, with an average split of 25 cm^{-1}. The introduction of two aryl groups in the peroxide made a shift to lower band frequencies and the average split diminished to 22 cm^{-1}. Carbonyl absorptions of aryl peroxides were found in the range of 1805~1780 and 1783~1758 cm^{-1}. Asymmetric peroxides (RCO·OO·COR′: R≠R′) display larger shifts than usual (ranging from 24~33 cm^{-1}) because of the effect of aryl conjugation on the carbonyl group. Comparison of IR spectral data of these peroxides and anhydrides with those of NCS chromophore suggests the presence of an aliphatic aromatic diacyl peroxide (ArCO·OO·COR).

Secondarily, the electrochemical behavior of NCS chromophore was investigated by polarography (Fig. 2). The half-wave potential ($E_{1/2}$) value of −0.45 volt *vs.* the saturated calomel electrode (S.C.E.) and I value of 1.67 for NCS chromophore were obtained. SWERN postulated in his review that in non-aqueous solution such as

methanol - benzene (1 : 1), diacyl peroxides show $E_{1/2}$ values ranging from -0.4 to 0 volt. These results also support the presence of a diacyl peroxide in the NCS chromophore.

Thirdly, we tried to identify NCS chromophore by several chemical reactions, including iodometric analysis. Aqueous NCS chromophore (1mg/ml) reacted with ferrous sulfate (2.5 mg/ml) to give the precipitate of ferric hydroxide, whereas it did not react with lead tetraacetate in chloroform. These color reactions suggest the presence of a peroxide, particularly disubstituted peroxide in the molecule of NCS chromophore. Futher quantitation of peroxide in the chromophore molecule has been attempted by means of iodometric analysis. As shown in Table 2, the active oxygen content in both NCS and NCS chromophore was related to the concentration of chromophore. NCS chromophore and NCS had 7.1×10^{-4} and 6×10^{-5} mM equivalent active oxygen per mg, respectively. These results are compatible with the observation of GOLDBERG *et al.*[3] that NCS contained 6% of the chromophore. On the other hand, NCS apoprotein (75 mg), which has no activities of DNA degradation and the growth inhibition of microorganisms, did not contain active oxygen. Result of the examination of iodometric analysis of the NCS chromophore under various conditions are summarized in Table 3. No active oxygen was released after treatment of the chromophore by heat, UV irradiation, high pH or 2-mercaptoethanol. The decrease of the active oxygen content paralleled to the decrease of antibacterial activity against *Micrococcus luteus* during these inactivation processes (data not shown). These findings strongly suggest that the diacyl peroxide is responsible for the active function of DNA degradation.

Table 2. Iodometric analysis of NCS chromophore, NCS and NCS apoprotein.

Drugs	Weight (mg)	Active oxygen content (ml)*
NCS chromophore	3.5	0.48
	7.0	1.00
NCS	25	0.31
	50	0.63
	75	1.18
	100	1.32
NCS apoprotein	75	0.00
Control		0.00

* Titration volume of 0.01 N $Na_2S_2O_3$.

Table 3. Effect of inactivation conditions on NCS activity.

	Active oxygen content (ml)[a]	NCS Activity (units/mg)[b]
NCS chromophore[c]	0.70	10,400
+heating[d]	0.00	0
+ultraviolet irradiation[e]	0.00	50
+high pH[f]	0.00	0
+2-mercaptoethanol[g]	0.00	50

[a] Titration volume of 0.01 N $Na_2S_2O_3$. [b] Growth inhibition of *Micrococcus luteus* in the presence of NCS apoprotein (10 mcg/ml). [c] 5 mg in distilled water (5 ml). [d] In boiling water for 10 minutes. [e] Irradiation by germicidal lamp (500 μW/cm^2) for 1 hour. [f] pH 11.6 for 10 minutes. [g] 2-Mercaptoethanol (0.05 ml) at 37° for 1.5 hours.

It has been known that peroxides such as peroxy acids and diacyl peroxides can be used in catalytic polymerization, in autooxidation and degradation of rubber[14]. Furthermore, peroxides have been known in biosynthetic intermediates such as prostaglandin. Few papers dealing with synthetic peroxy acids on the antibacterial activity have been reported[15,16]. However, the presence of a peroxy acid moiety in the antibiotic was first established in this study.

Certain anticancer drugs induce DNA strand scission by free radicals. Bleomycin, adriamycin, daunomycin induce DNA strand scission through mechanisms involving the generation of free radicals such as superoxide and hydroxyl radicals[17,18,19]. Compared with these antibiotics, NCS chromophore generates a free peroxyl radicals[10,20]. The absence of ultraviolet absorption at 425 nm reveals that the NCS chromophore does not have a quinone skeleton like adriamycin, daunomycin, and carboquinone. Furthermore, NCS was independent of the influence of metal anions such as Fe^{2+}, Cu^{2+} and Zn^{2+}, which was observed in contrary to result obtained with bleomycin. The DNA scission is induced by a free peroxyl radical generated from NCS chromophore or NCS in the presence of both oxygen and reducing agents such as 2-mercaptoethanol or dithiothreitol[21,22,23]. When the thiol group reacts with oxygen it generates radicals. These radicals may induce the formation of a free

peroxyl radical from the diacyl peroxide of NCS chromophore. This free peroxyl radical degradates DNA.

Through the study, an aliphatic aromatic diacyl peroxide is proposed as the partial structure of NCS chromophore, and it is suggested that this peroxide is responsible for the biological activities of NCS chromophore.

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