# UNSTABILITY OF NEOCARZINOSTATIN-CHROMOPHORE†

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The unstability of neocarzinostatin (NCS), apo-NCS and NCS-chromophore (NCS-chr) has been investigated by using an extra-weak chemiluminescence (CL) analyzer. A significantly high emission intensity (10,840 counts/10 seconds) was detected from NCS under dark conditions at 20°C, while no significant emission was observed in other antitumor antibiotics, such as, mitomycin C and pepleomycin. This high emission intensity of NCS was due to NCS-chr I (epoxide form) but not apo-NCS.

The functional group generating the high extra-weak CL of NCS-chr I is probably the epoxide in the molecule, since the emission intensity of NCS-chr I (epoxide form) is much higher than that of NCS-chr II (hydrochloride adduct form). The extra-weak CL emission of NCS decreased under a nitrogen atmosphere and it was greatly enhanced under an oxygen atmosphere. The spectral analysis of NCS showed emission peaks around 460 and 570 nm. These observations strongly suggest that one of the emission species of NCS-chr may be due to singlet oxygen.

Neocarzinostatin (NCS), an antitumor antibiotic, isolated from a culture filtrate of *Streptomyces carzinostaticus* var. F-41[1], is composed of protein moiety (apo-NCS, MW 11,000) and a non-protein chromophore (NCS-chr I, MW 659) at a molar ratio of 1 : 1[2,3]. DNA is an important target in the antitumor action of NCS and the chromophore possesses full cytotoxic and DNA damaging properties of the parent drug[4~6]. The primary structure of apo-NCS was proposed by MEIENHOFER *et al.*[7] and revised by GIBSON *et al.*[8]. On the other hand, the chemical structure of NCS-chr I was proposed by us[9] to be a novel bicyclo[7,3,0]dodecadienediyne system having a naphthalenecarboxylic acid, *N*-methylfucosamine, ethylene carbonate and a highly strained epoxide moieties (Fig. 1). NCS-chr is very labile to heating, exposure to ultraviolet light and treatment at high pH, and is essentially responsible for the biological activity of NCS[4,5]. But it is not clear why NCS-chr is very labile and which moiety among the highly strained epoxide, 2-cyclopentene-l-ylidene, and strained cyclic diacetylenes, destabilizes the NCS-chr molecule. If the functional groups responsible for its activity and its instability are different, it would be possible to obtain a stable derivative of NCS-chr having the antitumor activity.

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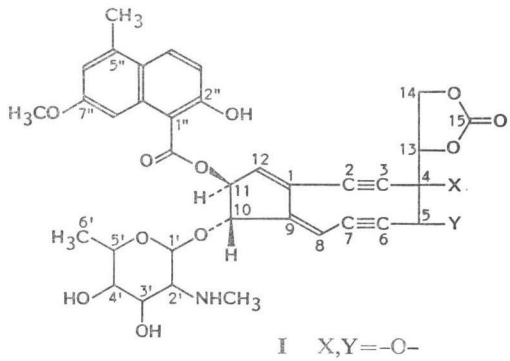
Recently, we have introduced an analytical method using a chemiluminescence analyzer for the evaluation of drug-stability. By the measurement of extra-weak chemiluminescence (CL) we observed strong emission of extra-weak light from relatively unstable drugs[10~12].

In this paper, we describe a highly strained epoxide moiety as one of the unstable functional group of NCS-chr because the emission intensity of NCS-chr I is much higher than that of NCS-chr hydrochloride adduct (NCS-chr II) and demonstrate by emission spectral analysis that the species of light emission is mainly correlated with the generation of singlet oxygen.

Fig. 1. The chemical structure of neocarzinostatin-chromophore (NCS-chr).

NCS-chr I (epoxide form) is native NCS-chr and NCS-chr II is hydrochloride adduct of native NCS-chr I.



I X,Y=-O-
II X=OH, Y=Cl

## Materials and Methods

### Chemicals

NCS and apo-NCS, mitomycin C (MMC) and pepleomycin (PLM) were gifts from Kayaku Antibiotics Research Co., Ltd., Kyowa Hakko Kogyo Co., Ltd., and Nippon Kayaku Co., Ltd., respectively. NCS-chr I and NCS-chr II were prepared by the previously reported methods[9,13]. All other chemicals, newly purchased from commercial sources, were of analytical grades.

### Apparatus

Quantitative detection of the extra-weak CL was performed with single photon counting of extra-weak sensitivity using a Chemiluminescence Analyzer OX-70 (Tohoku Electronic Industrial Co., Ltd., Sendai, Japan), which was equipped with a low noise photo-multiplier (HTV R878) and a 50-mm diameter photo-cathode (Hamamatsu Photonic Co., Hamamatsu, Japan). The photo-cathode was cooled by a thermoelectric cooler to minimize noise. Antibiotics, which were weighed at least 3 hours prior to counting and sheltered from the air in the dark condition at room temperature, were placed in a stainless dish type cell of 50-mm diameter, and single photo-electron pulses were counted in the atmosphere at 20°C, 50°C and 80°C, respectively. The results are arbitrarily expressed by the mean values of observed photo-electrons for 10 measurements of 10 seconds each after subtracting dark counts (about 670 counts/10 seconds).

### Determination of Emission Spectrum

The spectrum of chemiluminescence was recorded by a spectrometer with a filter-type spectral analyzer according to the method described by INABA *et al.*[14] for 10 times 10 seconds each with various wavelength in range of 420 to 610 nm (20 filters). The scanning time in this range was about 30 minutes. The spectral resolution was approximately 10 nm in the region between 420 and 610 nm.

## Results

### Extra-weak Chemiluminescence Generated from Neocarzinostatin, Mitomycin C and Pepleomycin

The extra-weak CL generated from NCS (300 mg), MMC (230 mg) and PLM (300 mg) were measured in the dark condition at 20°C, 50°C and 80°C, as are shown in Table 1. The emission intensity of NCS at 20°C and 50°C was 10,840 and 119,691 counts/10 seconds, respectively. At 80°C, and ex-

Table 1. Chemiluminescence of antitumor antibiotics.

| Temperature (°C) | Chemiluminescent intensity (counts/10 seconds)[a] | | |
|---|---|---|---|
| | Neocarzinostatin (300 mg) | Pepleomycin (300 mg) | Mitomycin C (230 mg) |
| 20 | 10,840±321 | 41 | 29 |
| 50 | 119,691±9,055 | 230 | 16 |
| 80 | >1,000,000 | 1,252±199 | 36 |

[a] Quantitative detection of the extra-weak CL arising from antibiotics was performed by the method described in Materials and Methods. CL intensities were expressed as the mean values of photon-electrons for 10 measurements of 10 seconds after subtracting dark counts (about 670 counts/10 seconds).

Table 2. Chemiluminescence of NCS and its related compounds.

| Temperature (°C) | Chemiluminescent intensity (counts/10 seconds)[a] | | | |
|---|---|---|---|---|
| | Neocarzinostatin (300 mg) | Neocarzinostatin-chromophore (I) (16.8 mg) | Neocarzinostatin-chromophore (II) (35.1 mg) | Apo-neocarzinostatin (300 mg) |
| 20 | 10,840±321 | 22,511±811 | 165±37 | 186±86 |
| 50 | 119,691±9,055 | 16,839±479 | 473±29 | 2,285±54 |
| 80 | >1,000,000 | 53,146±1,515 | 2,466±200 | 9,608±2,739 |

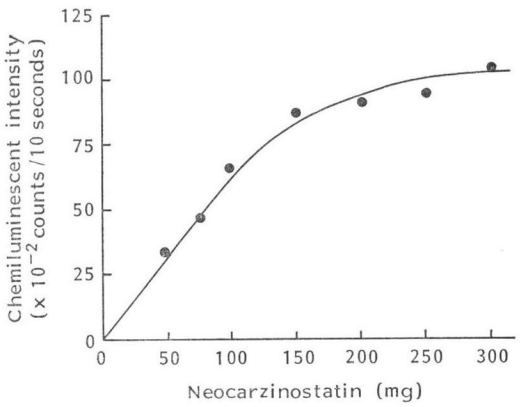
[a] See the footnote of Table 1.

tremely high extra-weak CL intensity of NCS was observed. In contrast, the extra-weak CL intensity of MMC was not temperature-dependent and its value was only 36 counts/10 seconds at 80°C. The extra-weak CL intensity of PLM was increased with temperature and was 1,252 counts/10 seconds at 80°C.

The dose response curve of extra-weak CL generated from NCS at 20°C is shown in Fig. 2. The extra-weak CL intensity of NCS increased linearly up to 150 mg and reached a plateau (about 10,000 counts/10 seconds) at 200 mg.

Fig. 2. Dose response of the extra-weak chemiluminescence of neocarzinostatin at 20°C.

The extra-weak CL of NCS samples (50, 75, 100, 150, 200, 250 and 300 mg) were counted in the dark condition at 20°C, respectively.




### Extra-weak Chemiluminescence of NCS and NCS Components

The extra-weak CL of NCS, apo-NCS and NCS-chr I were measured and compared with each other. The results are summarized in Table 2. The emission intensities of NCS-chr I (16.8 mg) at 20°C, 50°C and 80°C, were 22,511, 16,839 and 53,146 counts/10 seconds, respectively, whereas the emission intensities of apo-NCS (300 mg) at 20°C, 50°C and 80°C, were 186, 2,285 and 9,608 counts/10 seconds, respectively. The emission intensity of NCS-chr II, the hydrochloride adduct of NCS-chr I, was 2,466 counts/10 seconds even at 80°C.

### Extra-weak Chemiluminescence of Model Compounds of NCS-chromophore

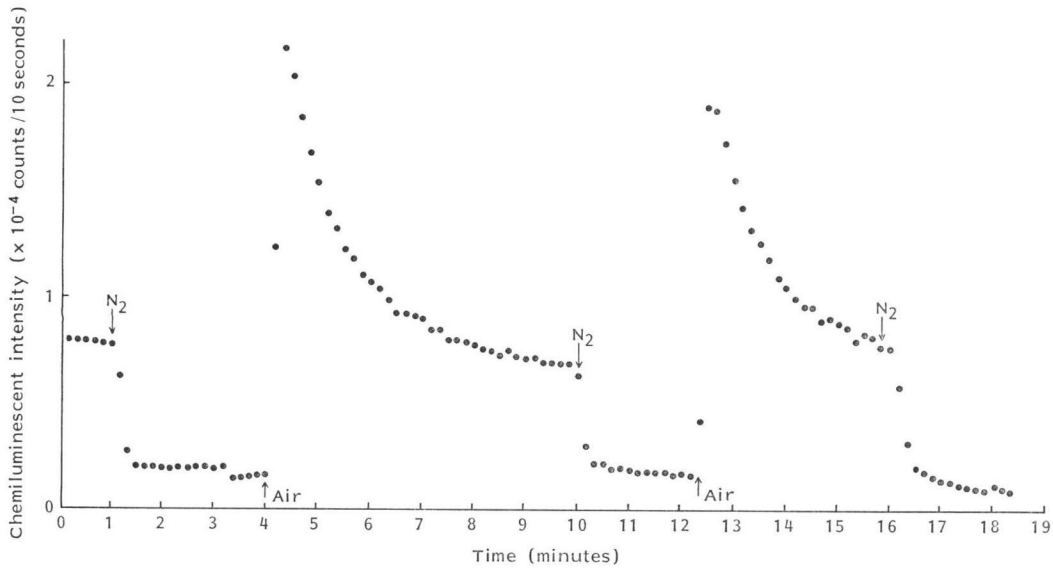
Seven model compounds, NCS-chr, 1-naphthalenecarboxylic acid, 2-naphthol, *trans*-stilbene, diphenylacetylene, D-glucosamine hydrochloride, propylene carbonate and styrene oxide, were tested

Table 3. Extra-weak chemiluminescence of model compounds of NCS-chr.

| | Emission intensity (counts/10 seconds)[a] | | |
|---|---|---|---|
| | 20°C | 50°C | 80°C |
| 1-Naphthalenecarboxylic acid | 176 | 312 | 789 |
| 2-Naphthol | 0 | 29 | 279 |
| *trans*-Stilbene | 49 | 80 | 143 |
| Diphenylacetylene | 71 | 137 | 1,144 |
| D-Glucosamine HCl | 5 | 52 | 97 |
| Propylene carbonate | 29 | 189 | 596 |
| Styrene oxide | 337 | 9,933 | 263,289 |
| Simple organic compounds (116) | 32 | 93 | 569 |

[a] The data of model compounds of NCS-chr are selected from the data of 116 kinds of simple organic compounds described in the reference 11. The results were expressed by the mean value of ten 3-continuous measurements of 10 seconds after the subtraction dark of counts (about 670 counts/10 seconds).

Fig. 3. Change of emission intensity of NCS bubbling with nitrogen stream at 20°C.
↓ Start of bubbling with nitrogen stream. ↑ Start of bubbling with air stream.

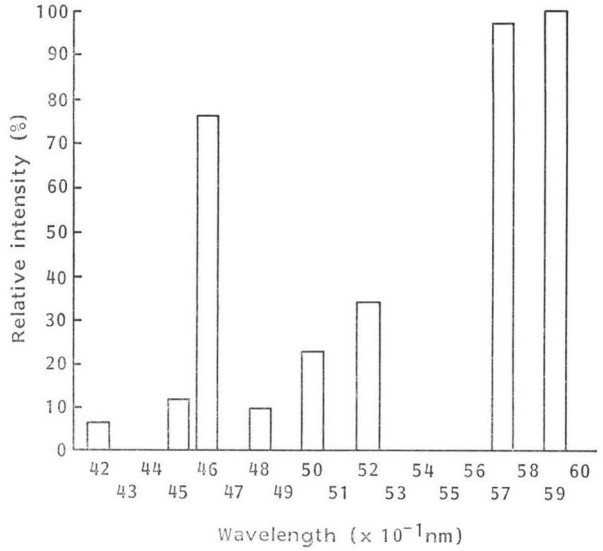



(Table 3). Styrene oxide, as the model of the highly strained epoxide, generated the highest extra-weak CL, 337, 9,933 and 263,289 counts/10 seconds at 20°C, 50°C and 80°C, respectively. The extra-weak CL intensities of 1-naphthalenecarboxylic acid and 2-naphthol, as the models of the 2-hydroxy-7-methoxy-5-methyl-1-naphthalenecarboxylic acid moiety, *trans*-stilbene and diphenylacetylene as the models of the bicyclo[7,3,0]dodecadienediyne moiety, glucosamine hydrochloride as the model of *N*-methylfucosamine moiety, propylene carbonate as the model of the ethylene carbonate moiety were not significant with less than 1,200 counts/10 seconds even at 80°C.

## Enhancing Effect of Air on the Emission Intensity of NCS Powder

Fig. 3 shows the change of emission intensity of NCS powder in the air and in nitrogen gas. Extra-weak CL of NCS reached a plateau after 5 minutes. Introduction of nitrogen decreased its intensity to about 2,000 counts/10 seconds. When air was re-introduced, the intensity rapidly increased again

Fig. 4. Emission spectrum of neocarzinostatin powder.
Spectrum of NCS powder 300 mg at 20°C was recorded as described under Materials and Methods. The spectrum represent is a typical one of five experiments.




to a level of 20,000 counts/10 seconds and then gradually decreased to the original level (8,000 counts/10 seconds) after 10 minutes. Rapid enhancing of the emission intensity of NCS by air and decreasing by nitrogen was observed (Fig. 3). In addition, similar results were obtained by introducing oxygen gas instead of air (data not shown).

### Spectral Analysis of Extra-weak Chemiluminescence Generated from NCS

To elucidate the active species which caused the light emission from NCS, the spectral analysis of extra-weak CL generating from NCS was performed in terms of a filter spectral analyzer as described under Materials and Methods. Fig. 4 shows that the emission intensities of 460, 570 and 590 nm were high in comparison with other ranges. The emission intensities at 460 and 570 nm of NCS suggest that the species which generates the CL emission from NCS is, at least in part, due to singlet oxygen.

## Discussion

Recently, we reported that unstable organic compounds such as epoxides, aliphatic aldehydes, aliphatic amines including clinical drugs generate high extra-weak CL by air-oxidation[10~12]. Previously, we found that among the 500 compounds so far tested in our laboratory NCS generated the highest extra-weak CL at room temperature with about 10,000 counts/10 seconds (Table 1).

Comparative studies on the measurements of the emission intensity of NCS, apo-NCS and native NCS-chr I (epoxide form) show that NCS-chr is responsible for the high emission CL intensity in NCS (Table 2). This is attributed to the fact that NCS-chr is very labile whereas apo-NCS is relatively stable. This labile property of NCS-chr was confirmed by the binding affinity to apo-NCS because the kinetics of binding ability depend upon the NCS-chr stability[15]. Moreover, in our study the emission intensity of NCS-chr I (epoxide form) was much higher than that of NCS-chr II (hydrochloride adduct form). Accordingly, the epoxide moiety of NCS-chr I may generate the CL intensity (Table 2), as supported by the following observations. Styrene oxide showed high emission CL intensity (Table 3). An epoxide, especially, linked directly with an aromatic ring, is one of the highest emission CL intensity among the organic compounds which have various functional groups[11]. Epoxide is an

alkylating agent, and alkylating agents are well known to be antitumor drugs; for example, chloroethylamine, ethyleneimine, nitrosourea *etc.* On the other hand, NCS-chr II (hydrochloride adduct form) as well as NCS-chr I (epoxide form) damaged DNA and showed cytotoxic acitivities against tumor and bacterial cells (unpublished data). These observations suggest that the epoxide moiety of NCS-chr may not be responsible for the antitumor activity and that the other moiety (possibly bicyclooctadienediyne) may be responsible for its antitumor activity (in preparation).

The generation of extra-weak CL intensity from NCS was decreased by the introduction of a nitrogen stream and it was highly enhanced by the introduction of air or an oxygen stream (Fig. 3). Spectral analysis of NCS revealed emissions at 460 and 570 nm (Fig. 4). Considering the relative intensities and center wavelength of lines, the emission at 460 nm corresponds to the $[^1\Delta g]+[^1\sum g^+]\rightarrow 2[^3\sum g^-]$ transition and the emission at 570 nm to the $2[^1\Delta g]\rightarrow 2[^3\sum g^-]$ with vibrational quantum numbers of $1\rightarrow 0$[16]. On the basis of the present observations, singlet oxygen seems responsible for the generation of extraweak CL from NCS.

Further experiments to determine the detailed molecular mechanism of auto-oxidation on the epoxide of NCS-chr I are currently under way.

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