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Effect of Sodium Copper Chlorophyllin on Lipid Peroxidation. IX.[1] On the Antioxidative Components in Commercial Preparations of Sodium Copper Chlorophyllin[2]

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Two antioxidative components in commercial preparations of sodium copper chlorophyllin were isolated as their methyl esters. Through the identification of the methyl esters by comparison with authentic samples and the saponification of the methyl esters, disodium copper isochlorin-e_4 and trisodium copper chlorin-e_6 were demonstrated to be included in sodium copper chlorophyllin as constituents. The antioxidative activities of both sodium copper chlorins on Fe^{2+} and ascorbic acid-induced lipid peroxidation in rat liver homogenates were about 8-fold greater than that of sodium copper chlorophyllin. The two components were concluded to play a principal role in the antioxidative action of sodium copper chlorophyllin.

Keywords——copper chlorophyllin; antioxidative component; copper isochlorin-e_4; copper chlorin-e_6; lipid peroxidation; silica gel column chromatography

The commercially available sodium copper chlorophyllin (Cu–Chl–Na) is a mixture of copper complexes of chlorophyll derivatives.[3] In the previous papers of this series,[1,4] we have reported that Cu–Chl–Na has an antioxidative effect on the peroxidation of lipids in rat liver homogenates and on that of a mixture of linoleic and linolenic acids, possibly due to its action as a radical scavenger. We also showed that in i.p. Cu–Chl–Na-treated rats, Cu–Chl–Na or some substance(s) derived from Cu–Chl–Na is distributed among the hepatic subcellular organelles such as lysosomes and microsomes in a form capable of exerting an antioxidative activity, thereby preventing the peroxidative deterioration of their membranes. Further, Cu–Chl–Na pretreatment affords protection against the rat liver injury induced *in vivo* by carbon tetrachloride, probably through an inhibition of hepatic lipid peroxidation. These findings strongly suggest the usefulness of the antioxidative component(s) present in Cu–Chl–Na as an *in vivo* lipid antioxidant.

Thus, this study was commenced in an attempt to search the antioxidative principle(s) in Cu–Chl–Na. In the present paper, we describe the isolation and identification of two copper chlorin compounds as antioxidants from Cu–Chl–Na.

Results and Discussion

Cu–Chl–Na, dissolved in water, was fractionated according to the method summarized in Chart 1, yielding the ether-soluble yellow fraction, the ether-soluble Cu–Chl–E fraction and the methyl ethyl ketone-soluble Cu–Chl–K fraction. The antioxidative effects of Cu–Chl–Na and its three fractions were examined by assaying the degree of $FeSO_4(Fe^{2+})$ and ascorbic(AsA)-induced, non-enzymatic lipid peroxidation in rat liver homogenates; the peroxidation was determined by the thiobarbituric acid (TBA) method as described pre-

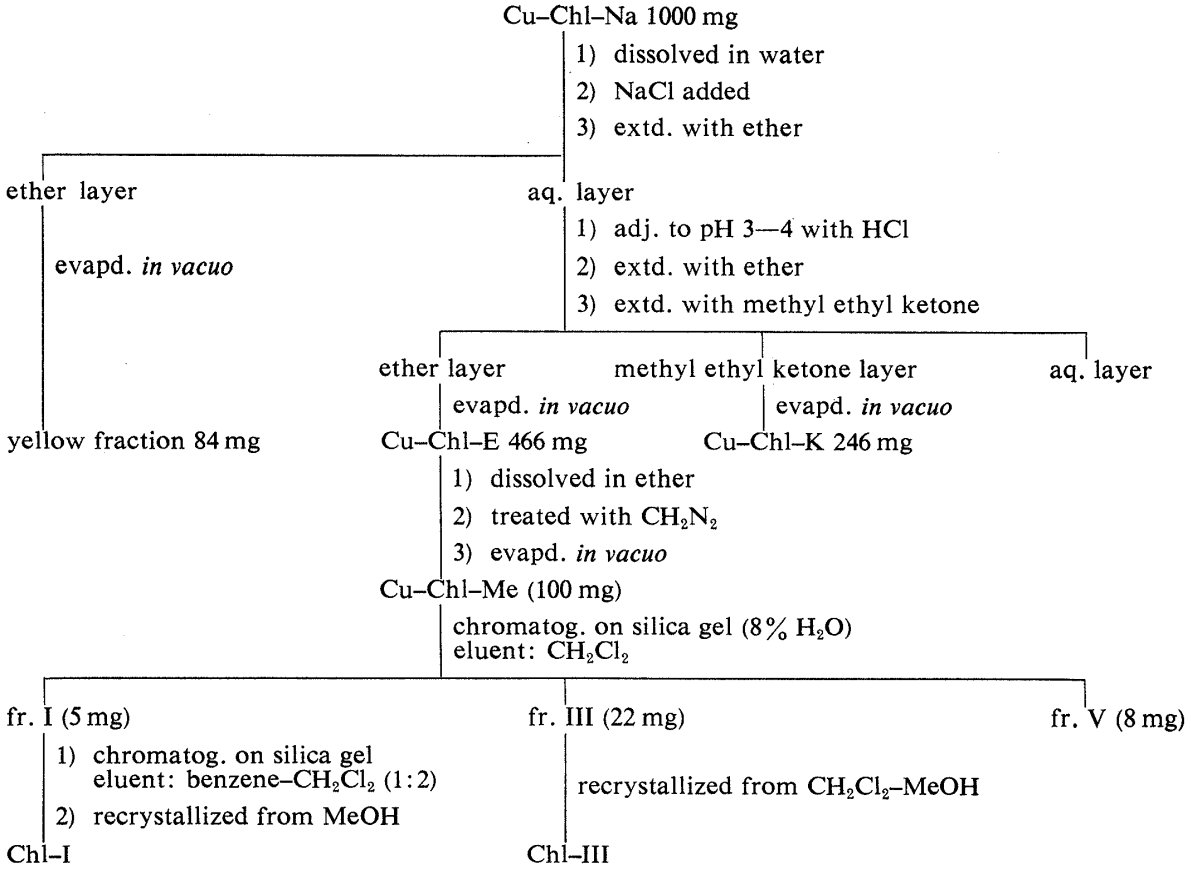


Chart 1. Isolation Procedures

viously[5] and expressed as TBA value. The results are shown in Fig. 1. The yellow fraction and Cu–Chl–K had no effect. Cu–Chl–E, as well as Cu–Chl–Na, showed a concentration-dependent decreasing effect on Fe^{2+} and AsA-elevated TBA value. From a comparison of the concentration (IC_{50}) required for 50% inhibition of lipid peroxide formation (calculated from the results), the antioxidative effect of Cu–Chl–E was estimated to be 2-fold stronger than that of Cu–Chl–Na. The yield of Cu–Chl–E was about 50% (Chart 1), so it appears that this fraction contains the bulk of the antioxidative components present in Cu–Chl–Na.

As shown in Fig. 2, thin-layer chromatography (TLC) of Cu–Chl–Na and each fraction revealed that Cu–Chl–Na comprised a number of components, and Cu–Chl–E contained most of the components in Cu–Chl–Na. For fractions 4, 7, 9 and 10 obtained by the preparative TLC of Cu–Chl–E (Fig. 2), which showed visually a deep color on the TLC plate, the inhibitory effects on the peroxidation of lipids in rat liver homogenates were studied. The results are given in Table I. All the fractions possessed antioxidative activity, and fractions 4 and 9 at a concentration of 2×10^{-4}% inhibited the reaction almost completely.

To isolate the antioxidative components in fractions 4 and 9 as their methyl esters, methylation of Cu–Chl–E with diazomethane was performed and the resulting material (Cu–Chl–Me) was fractionated by column chromatography on silica gel containing 8% water with dichloromethane as the eluent at the ambient temperature of 27—30 °C (Chart 1). The separation was monitored by TLC. There were three fractions visually showing a single spot on the TLC plate, *i.e.*, fractions I (*Rf* 0.52), III (*Rf* 0.28) and V (*Rf* 0.23). Fraction I or III gave the same color and *Rf* value in TLC as the methylation product of fraction 4 or 9 in Cu–Chl–E, respectively. Purification of fractions I and III was achieved by silica gel column chromatography followed by recrystallization from methanol, and by recrystallization from

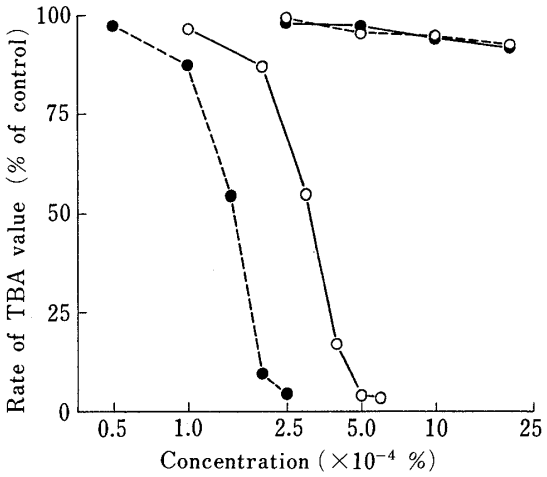


Fig. 1. Effect of Each Fraction in Cu–Chl–Na on Fe^{2+} and AsA-Stimulated Lipid Peroxidation in Rat Liver Homogenates

The control TBA values (OD_{532}/ml of reaction mixture) were as follows: 1.972 for Cu–Chl–Na, Cu–Chl–E and Cu–Chl–K; 1.893 for yellow fraction. Each point represents the mean for 3—6 experiments. —○—, Cu–Chl–Na; ---○---, yellow fraction; ---●---, Cu–Chl–E; —●—, Cu–Chl–K.
IC_{50} ($\times 10^{-4}$%): Cu–Chl–Na, 3.10; Cu–Chl–E, 1.53.

Table I. Effects of Fractions 4, 7, 9 and 10 of Cu–Chl–E on Fe^{2+} and AsA-Stimulated Lipid Peroxidation in Rat Liver Homogenates

Concentration (%, w/v)	Rate of TBA value (% of control)			
	Fr. 4	Fr. 7	Fr. 9	Fr. 10
Nil	100	100	100	100
2×10^{-4}	3.5	96.8	3.6	100.2
5×10^{-4}	2.0	8.6	1.8	65.6
1×10^{-3}	0.8	1.6	0.6	1.8

The control TBA value (OD_{532}/ml of reaction mixture) in the absence of any fraction was 1.941. Each value represents the mean for 3 experiments.

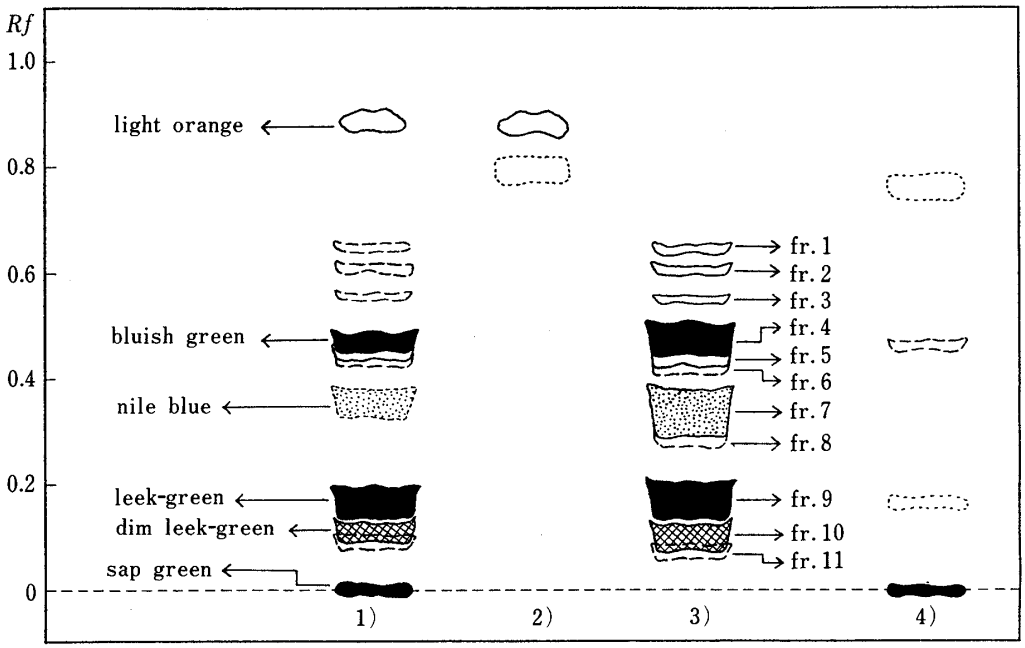


Fig. 2. Thin-Layer Chromatogram of Each Fraction of Cu–Chl–Na

1) Cu–Chl–Na; 2) yellow fraction; 3) Cu–Chl–E; 4) Cu–Chl–K.
Plate: Silica gel G. Solvent system: *n*-BuOH–EtOH–NH_4OH (2:1:1).

dichloromethane and methanol to afford crystalline materials, Chl-I and Chl-III, respectively.

Chl-I (purple plates, mp 179.5—180.5 °C) was assigned the molecular formula $C_{35}H_{38}CuN_4O_4$ on the basis of elementary analysis and the mass spectrum (MS) (*m/z*: 641 [M^+, 50.4%], 643 [$M^+ + 2$, 25.9%]). In the visible absorption spectrum, it showed Soret and red absorption bands with peaks at 410 and 631 nm, respectively, the latter band being

TABLE II. Diagnostic Ions in Mass Spectrum of Chl-I

MS (*m/z*)	Molecular and fragment ions
641 (50.4)	M^+
494 (8.7)	$M^+ - (2CH_2COOCH_3 + H$, or $COOCH_3 + CH_2CH_2COOCH_3 + H)$
481 (14.9)	$M^+ - (CH_2COOCH_3 + CH_2CH_2COOCH_3)$
466 (8.3)	$M^+ - (CH_3 + CH_2COOCH_3 + CH_2CH_2COOCH_3)$
59 (10.0)	$COOCH_3^+$
29 (15.5)	$C_2H_5^+$
27 (38.7)	$C_2H_3^+$
15 (4.5)	CH_3^+

Figures in parentheses represent relative abundance (%; base peak, *m/z* 55).

TABLE III. Diagnostic Ions in Mass Spectrum of Chl-III

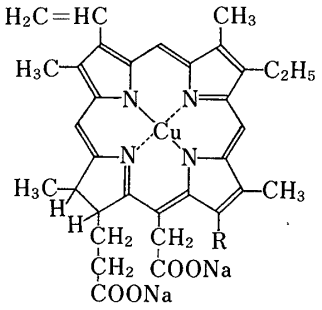
MS (*m/z*)	Molecular and fragment ions
699 (23.3)	M^+
640 (1.7)	$M^+ - COOCH_3$
552 (5.9)	$M^+ - (2CH_2COOCH_3 + H$, or $COOCH_3 + CH_2CH_2COOCH_3 + H)$
539 (3.9)	$M^+ - (CH_2COOCH_3 + CH_2CH_2COOCH_3)$
494 (2.2)	$M^+ - (COOCH_3 + 2CH_2COOCH_3$, or $2COOCH_3 + CH_2CH_2COOCH_3)$
73 (3.3)	$CH_2COOCH_3^+$
29 (11.6)	$C_2H_5^+$
27 (5.4)	$C_2H_3^+$
15 (2.6)	CH_3^+

Figures in parentheses represent relative abundance (%; base peak, *m/z* 44).

probably attributable to the chemical structure of copper chlorins.[6] It also exhibited infrared (IR) absorption bands at the wave numbers given in the experimental section. In the MS, peaks due to fragment ions at the mass numbers listed in Table II were observed. Treatment of Chl-I with acetic acid and conc. HCl gave a copper-free compound (deep violet plates, mp 171—172 °C), and elemental analysis indicated a molecular formula of $C_{35}H_{40}N_4O_4$. In the proton nuclear magnetic resonance (^{1}H-NMR) spectrum, the copper-free compound showed a two-proton singlet at δ 5.28 ppm, suggesting the presence of a methylene group adjacent to the $-COOCH_3$ group. Based on these results, Chl-I was inferred to be copper isochlorin-e_4 dimethyl ester, and this was confirmed by direct comparison with an authentic sample.

Chl-III (deep green needles, mp 218.5—219.5 °C) had the molecular formula $C_{37}H_{40}CuN_4O_6$ as determined from elementary analysis and the MS (*m/z*: 699 [M^+, 23.3%], 701 [$M^+ + 2$, 12.7%]). Its visible spectrum showed Soret and red absorption maxima at 412 and 638 nm, respectively. In the IR spectrum, Chl-III had the absorption bands at the wave numbers listed in the experimental section. Its MS revealed fragment ion peaks at the mass numbers indicated in Table III. From these experimental data, the sample appeared to be copper chlorin-e_6 trimethyl ester. This conclusion was confirmed by direct comparison of Chl-III with an authentic sample.

Chl-I and Chl-III were saponified and then acidified as described in the experimental section to give the free acids Chl-I-A and Chl-III-A, respectively, and treatment of the acids with NaOH dissolved in methanol yielded the corresponding sodium salts. In TLC on silica gel, each of the free acids and the sodium salts was detected as a single band on the plate,



disodium copper isochlorin-e_4: R=H
trisodium copper chlorin-e_6: R=COONa

Chart 2

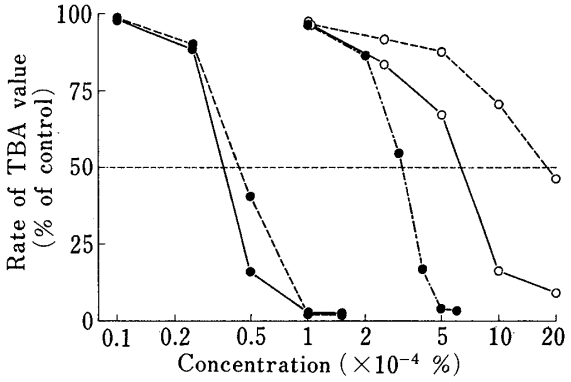


Fig. 3. Comparison of Inhibitory Effects of Cu–Chl–Na, Chl-I, Chl-III, Chl-I-A and Chl-III-A on Fe^{2+} and AsA-Stimulated Lipid Peroxidation in Rat Liver Homogenates

The control TBA values (OD_{532}/ml of reaction mixture) were as follows: 1.972 for Cu–Chl–Na, Chl-I-A and Chl-III-A; 1.927 for Chl-I and Chl-III. Each point represents the mean for 4—6 experiments. ---●---, Cu–Chl–Na; —○—, Chl-I; ---○---, Chl-III; —●—, Chl-I-A; ---●---, Chl-III-A.
IC_{50} ($\times 10^{-4}$%): Cu–Chl–Na, 3.10; Chl-I, 6.32; Chl-III, 17.90; Chl-I-A, 0.36; Chl-III-A, 0.44.

namely, Chl-I-A and its sodium salt, and Chl-III-A and its sodium salt exhibited colors and *Rf* values identical to those of Cu–Chl–Na's bluish green band (*Rf* 0.47, corresponding to fraction 4 of Cu–Chl–E) and Cu–Chl–Na's leek-green band (*Rf* 0.17, corresponding to fraction 9 of Cu–Chl–E), respectively. Chl-I-A and Chl-III-A gave elemental analytical values coinciding with the molecular formulae of the dicarboxylic acid ($C_{33}H_{34}CuN_4O_4$) and the tricarboxylic acid ($C_{34}H_{34}CuN_4O_6$), respectively. The IR spectra of both sodium salts showed strong absorption bands at *ca.* 1570 and 1400 cm^{-1} due to the carboxylate groups. Methylation of the two free acids with diazomethane gave the respective methyl esters, *i.e.*, Chl-I and Chl-III. On the basis of these results, Cu–Chl–Na was proved to contain disodium copper isochlorin-e_4 and trisodium copper chlorin-e_6 as constituents (Chart 2).

The inhibitory effects of Chl-I, Chl-III, Chl-I-A and Chl-III-A on Fe^{2+} and AsA-stimulated lipid peroxide formation in rat liver homogenates were examined in comparison with that of Cu–Chl–Na. As shown in Fig. 3, all the samples exerted dose-dependent antioxidative effects. The effects of Chl-I and Chl-III were weaker than that of Cu–Chl–Na, as shown by the IC_{50} values listed in the legend to the figure. The IC_{50} values of Chl-I-A and Chl-III-A were 0.36×10^{-4} and 0.44×10^{-4}%, respectively. Taking the IC_{50} value of Cu–Chl–Na, 3.10×10^{-4}%, into consideration, Chl-I-A and Chl-III-A possess approximately 8 times greater antioxidative effects than Cu–Chl–Na. In addition, the yields of the sodium salts of copper isochlorin-e_4 and copper chlorin-e_6, which were calculated by using the yields of Cu–Chl–E from Cu–Chl–Na and of fractions I and III from Cu–Chl–Me (Chart 1), were 2.5 and 10.1%, respectively. Taken together, about 92% of the antioxidative effect of Cu–Chl–Na is accounted for by the two copper chlorin compounds. Consequently, it follows that disodium copper isochlorin-e_4 and trisodium copper chlorin-e_6 are the main antioxidative principles in Cu–Chl–Na. The present finding is of significance, since there has been no previous report concerning the antioxidant action of copper chlorins.

Experimental

All melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. The

visible spectra were recorded with a Hitachi model 556 spectrophotometer, the IR spectra with a Nihon Bunko IRA-2 infrared spectrophotometer and the MS with a Shimadzu LKB-9000B spectrometer. The ^{1}H-NMR spectrum (in $CDCl_3$) was taken on a Hitachi R-24 nuclear magnetic resonance spectrometer with tetramethylsilane as an internal standard.

Assay of Lipid Peroxidation in Rat Liver Homogenates——Both Fe^{2+} and AsA were employed as stimulators of lipid peroxidation. The reaction mixture consisted of 0.5 ml of 4% (w/v) liver homogenate, 90 mM KCl, 10 μM Fe^{2+}, 0.5 mM AsA and 50 mM Tris–HCl buffer (pH 7.4) in a final volume of 2.0 ml. After incubation of this mixture at 37 °C for 30 min, a 0.5 ml aliquot was removed for the assay of lipid peroxide formation, which was performed by means of the TBA reaction as described in the previous paper.[5] Samples were dissolved in the following solvents: ethanol for the yellow fraction, 5 mM NaOH for Cu–Chl–E, fractions 4, 7, 9 and 10 of Cu–Chl–E, Cu–Chl–K, Chl-I-A and Chl-III-A, and 5% (v/v) pyridine in ethanol for Chl-I and Chl-III. A 0.1 ml aliquot of solution was added to the reaction mixture.

Fractionation of Cu–Chl–Na——Cu–Chl–Na (1000 mg), purchased from Wako Pure Chemical Ind., Ltd., Tokyo, was dissolved in water. NaCl (5 g) was added, and the solution was extracted with 400 ml of ether. The ether layer was washed with water, dried over Na_2SO_4 and evaporated to dryness under reduced pressure, yielding 84 mg of yellow fraction. The water layer was adjusted to pH 3—4 with dilute HCl and extracted twice with ether (500 ml), then twice with methyl ethyl ketone (500 ml). The former and latter extracts were each washed with water, dried over Na_2SO_4 and concentrated to dryness *in vacuo* to give Cu–Chl–E fraction (466 mg) and Cu–Chl–K fraction (246 mg), respectively.

TLC of Cu–Chl–Na and the above fractions was conducted on silica gel (Silica gel G, E. Merck; thickness, 0.25 mm) using *n*-BuOH–EtOH–NH_4OH (2 : 1 : 1) as the developing solvent. For the separation of fractions 4, 7, 9 and 10 of Cu–Chl–E, preparative TLC was carried out as described above except that the layer thickness was 0.5 mm. The bands on the chromatogram were extracted with acetone–50 mM Na_2CO_3 (1 : 1), and the extracts were adjusted to pH 3—4 by adding dilute HCl, then extracted with ether. The ether layers were each evaporated to dryness under reduced pressure to yield the four fractions.

Isolation of Chl-I and Chl-III from Cu–Chl–Me——Cu–Chl–Me was prepared by treating the ether solution of Cu–Chl–E with diazomethane followed by removal of the solvent *in vacuo*. Column chromatography of Cu–Chl–Me (100 mg) was carried out repeatedly on silica gel (Wakogel C-200, Wako Pure Chemical Ind., Ltd.; column, 1.4 × 20 cm) containing 8% water with dichloromethane as the eluent at the ambient temperature of 27—30 °C. This fractionation was monitored by TLC (Silica gel 60, E. Merck; developing solvent, chloroform). Fractions I, III and V visually gave a single spot each on the chromatogram, and the mean yields of these fractions from Cu–Chl–Me were 5, 22 and 8%, respectively. Fraction I was purified by silica gel column chromatograhy (Wakogel C-200; eluent, benzene–dichloromethane (1 : 2)) and then recrystallized from methanol to give a crystalline substance, Chl-I. The recrystallization of fraction III from dichloromethane and methanol afforded a crystalline substance, Chl-III.

Identification of Chl-I and Chl-III——Chl-I and Chl-III have the following properties. Chl-I: purple plates. mp 179.5—180.5 °C. *Anal.* Calcd for $C_{35}H_{38}CuN_4O_4$: C, 65.45; H, 5.96; N, 8.72. Found: C, 65.44; H, 5.94; N, 8.77. Visible spectrum $\lambda_{max}^{CHCl_3}$ nm (log ε): 410 (5.11), 500 (3.75), 631 (4.65). IR spectrum ν_{max}^{KBr} cm^{-1}: 2960, 1735, 1635, 1595, 1565, 1435, 1335, 1220, 1200, 1165, 1080, 995, 955, 930. MS: Table II. Chl-III: deep green needles. mp 218.5—219.5 °C. *Anal.* Calcd for $C_{37}H_{40}CuN_4O_6$: C, 63.46; H, 5.76; N, 8.00. Found: C, 63.51; H, 5.78; N, 8.00. Visible spectrum $\lambda_{max}^{CHCl_3}$ nm (log ε): 412 (5.06), 501 (3.70), 638 (4.68). IR spectrum ν_{max}^{KBr} cm^{-1}: 2920, 1718, 1590, 1435, 1245, 1195, 1155, 1075, 810. MS: Table III.

Chl-I and Chl-III were identified as copper isochlorin-e_4 dimethyl ester and copper chlorin-e_6 trimethyl ester, respectively, by mixed melting point determination and comparisons of TLC, and the visible and IR spectra with those of authentic samples.

Copper-Free Compound from Chl-I——The elimination of copper from Chl-I was performed according to the method of Strell and Zuther[7] with a slight modification. An acetic acid solution (40 ml) of Chl-I (200 mg) was treated with 40 ml of conc. HCl, and the mixture was allowed to stand for 2 min at room temperature. Water (1000 ml) was added to the mixture, followed by extraction with 600 ml of ether. The ether layer was dried over Na_2SO_4 and evaporated to dryness under reduced pressure, and the residue was subjected to silica gel column chromatography (Wakogel C-200; eluent, benzene–dichloromethane (1 : 2)). A fraction, whose copper complex prepared by the method of Conant and Armstrong[8] gave an *Rf* value equal to that of Chl-I on a TLC plate, was purified again by column chromatography and recrystallized from acetone and methanol to yield a copper-free compound (16 mg). Deep violet plates. mp 171—172 °C. *Anal.* Calcd for $C_{35}H_{40}N_4O_4$: C, 72.39; H, 6.94; N, 9.65. Found: C, 72.28; H, 6.92; N, 9.65. The compound was identified as isochlorin-e_4 dimethyl ester by comparing the TLC behavior, and the visible, IR and ^{1}H-NMR spectra with those of an authentic sample.

Preparation of Copper Isochlorin-e_4 Dimethyl Ester and Copper Chlorin-e_6 Trimethyl Ester——Sodium chlorin (2000 mg), a product of Nihon Yohryokuso Co., Ltd., Tokyo, in which sodium chlorin-e_6 is contained as a main constituent, was dissolved in water, adjusted to pH 3—4 with dilute HCl and extracted with 1200 ml of ether. The ether layer was then extracted three times with 500 ml of 3% HCl (chlorin-e_6 has a hydrochloric acid number of 3[9]), and the combined aqueous layer was washed with ether, followed by neutralization with 5 N NaOH and re-extraction

with ether. The extract was treated with diazomethane and the solvent was removed by evaporation *in vacuo*. The residue was purified by column chromatography on silica gel (Wakogel C-200, containing 8% water) with dichloromethane as the eluent and then by recrystallization from dichloromethane and hexane to afford chlorin-e_6 trimethyl ester (356 mg). Greenish-brown needles. mp 208.5—209.5 °C (lit.,[8] 209—210 °C). *Anal.* Calcd for $C_{37}H_{42}N_4O_6$: C, 69.57; H, 6.63; N, 8.77. Found: C, 69.59; H, 6.60; N, 8.76. MS *m/z*: 638 (M^+).

Isochlorin-e_4 dimethyl ester was prepared according to the method of Fischer and Kellermann[10] using chlorin-e_6 obtained by the saponification of chlorin-e_6 trimethyl ester.[11] The final product was purified by silica gel column chromatography (Wakogel C-200; eluent, dichloromethane) and recrystallized from acetone and methanol to give isochlorin-e_4 dimethyl ester. Deep violet plates. mp 170.5—172.0 °C (lit.,[10] 170 °C). *Anal.* Calcd for $C_{35}H_{40}N_4O_4$: C, 72.39; H, 6.94; N, 9.65. Found: C, 72.20; H, 6.95; N, 9.63. MS *m/z*: 580 (M^+).

Subsequently, the copper chelates of isochlorin-e_4 dimethyl ester and chlorin-e_6 trimethyl ester were prepared as described by Conant and Armstrong,[8] yielding copper isochlorin-e_4 dimethyl ester and copper chlorin-e_6 trimethyl ester, respectively. Copper isochlorin-e_4 dimethyl ester: purple plates. mp 179.0—180.5 °C. *Anal.* Calcd for $C_{35}H_{38}CuN_4O_4$: C, 65.45; H, 5.96; N, 8.72. Found: C, 65.36; H, 5.99; N, 8.84. MS *m/z*: 641 (M^+). Copper chlorin-e_6 trimethyl ester: deep green needles. mp 218—219 °C (lit.,[8] 217—219 °C). *Anal.* Calcd for $C_{37}H_{40}CuN_4O_6$: C, 63.46; H, 5.76; N, 8.00. Found: C, 63.43; H, 5.74; N, 8.00. MS *m/z*: 699 (M^+).

Saponification of Chl-I and Chl-III——Pyridine solution (1.2 ml) of Chl-I (100 mg) or Chl-III (100 mg) was treated with 12 ml of 25% KOH in methanol, and the mixture was heated at 80 °C for 2 min then poured into ice-cold water. After adjusting the pH to 2—3 with 10% HCl, each mixture was extracted with ether, and the extract was dried over Na_2So_4 then evaporated to dryness *in vacuo*. The residue was dissolved in acetone–methanol (2 : 1), and hexane in the case of Chl-I or benzene in the case of Chl-III was added to the solution. The resulting precipitate was collected by centrifugation. This process was repeated three times. Finally, the precipitate was dried under reduced pressure to afford the free acid Chl-I-A (77 mg) or Chl-III-A (63 mg). Chl-I-A: *Anal.* Calcd for $C_{33}H_{34}CuN_4O_4$: C, 64.53; H, 5.58; N, 9.12. Found: C, 64.83; H, 5.71; N, 9.14. Chl-III-A: *Anal.* Calcd for $C_{34}H_{34}CuN_4O_6$: C, 62.04; H, 5.21; N, 8.51. Found: C, 61.32; H, 5.24; N, 8.40.

Chl-I-A (50 mg) and Chl-III-A (50 mg) were dissolved in 8 ml of 0.04 N NaOH in methanol and 18 ml of 0.1 N NaOH in methanol, respectively, and ether was added slowly to each solution. The precipitates formed were collected by centrifugation, washed with ether–methanol (9 : 1) and dried under reduced pressure, yielding the respective sodium salts.

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