ZINCPHYRIN, A NOVEL COPROPORPHYRIN III WITH ZINC FROM *Streptomyces* sp.

MINORU TORIYA, SATOSHI YAGINUMA, SAKAE MUROFUSHI, KIYOSHI OGAWA, NAOKI MUTO, MITSUO HAYASHI and KAZUHIKO MATSUMOTO

Institute for Life Science Research, Asahi Chemical industry Co., Ltd. Ohito, Shizuoka 410-23, Japan

(Received for publication August 26, 1992)

Low molecular weight physiologically active substance are useful at least as medicines or reagents for research. During our screening for new inhibitors, an actinomycetes culture produced inhibitor against histamine-release from rat mast cells. This inhibitor was isolated and named zincphyrin.

The producing organism was isolated from a soil sample collected at Ooi-cho, Fukui Prefecture, Japan, and designated as AC8007 (FERM BP-10537).

It was classified as *Streptomyces* from cultural characteristic or microscopic observation[1]. Zincphyrin is a member of the porphyrin series with zinc elucidated by chemical analysis (Fig. 1). Zincphyrin was primarily isolated as histamine-release inhibitor from mast cells. On extensive pharmacological studies, zincphyrin was found to have a potent photosensitizer activity.

In this paper, we report the isolation, characterization and biological properties of zincphyrin.

A slant culture of the strain, AC8007 was inoculated into 100 ml of the seed medium cosisting of glucose 1%, dextrin 1%, yeast extract 0.5%, casein hydrolysate 0.5% and $CaCO_3$ 0.1% (adjusted to pH 6.5 before sterilization) and incubated at 28°C for 3 days on a rotary shaker (180 rpm). For production of zincphyrin, 100 ml of the seed culture was transferred to 20 liters of the production medium consisting of glucose 2%, soluble starch 2%, yeast extract 0.5%, meat extract 0.5%, NaCl 0.25%, $CaCO_3$ 0.35%, $ZnSO_4 \cdot 7H_2O$ 0.005% and FS anti-foam 028 (Dow Corning K. K., Japan) 0.02% (adjusted to pH 6.5 before sterilization) in 30-liter jar fermenter and cultured at 28°C for 3 days under agitation at 200 rpm and aeration at 20 liters per minute. Almost the inhibitor was secreted into medium.

Inhibitory activity against histamine-release from mast cells was assayed as follows[2]: Anti dinitrophenyl-ovalbumin (DNP-OVA) serum was approximately 1 : 125 as estimated by 48 hours PCA reaction[3]. Rats were passively sensitized by intraperitoneal injection of 2 ml of rat anti DNP-OVA serum. After 24 hours, rats were injected intraperitoneally with 15 ml of a solution containing 137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and gelatin 0.1% was added and the pH adjusted to 7.2 with 10% of 0.1 M phosphate buffer. The peritoneal exudate cells (PEC) collected into siliconized glassware, and was washed once with the same buffer. Number of mast cell was usually around 2×10^5 cells/ml. To the sensitized PEC (890 μl) kept at 37°C for 5 minutes were added 10 μl of the test solution and 100 μl of the DNP-OVA (40 μg/ml) solution. The mixture was further incubated at 37°C for 20 minutes. The reaction was then stopped by immersion of the tubes in melting ice. After centrifugation ($700 \times g$ for 10 minutes at 4°C), the supernatant was assayed for released histamine according to the method for SHORE *et al.*[4].

The culture broth (20 liters) was centrifuged and the supernatant (18 liters) was extracted with ethyl acetate (9 liters, at pH 2.0) and was transferred to an ammonium solution (pH 9.0, 4 liters). The ammonium solution was concentrated *in vacuo* to about 500 ml, and the concentrate was passed through a column of Diaion HP-20 (400 ml, Mitsubishi Kasei Corp.). After the column was washed with water, zincphyrin was eluated with gradient solution of 0 to 80% acetone in H_2O (6 liters). The active eluates were combined and concentrated under reduced pressure to give zincphyrin as crude powder. This powder was dissolved in small amount of 3 N NH_4OH, and was

Fig. 1. The structure of zincphyrin.

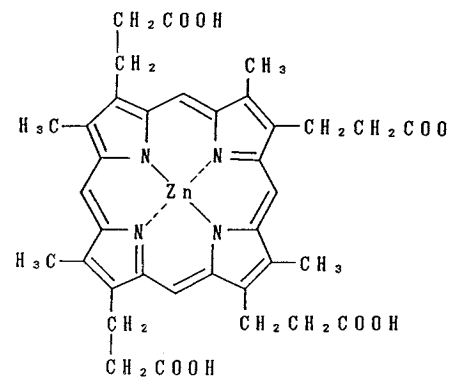
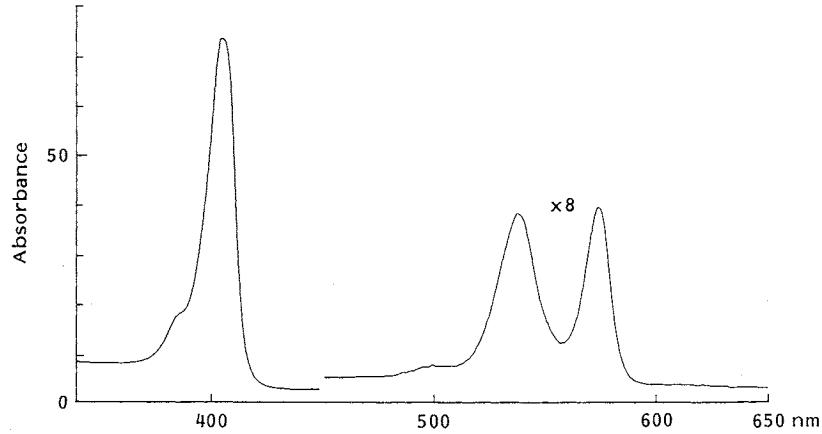
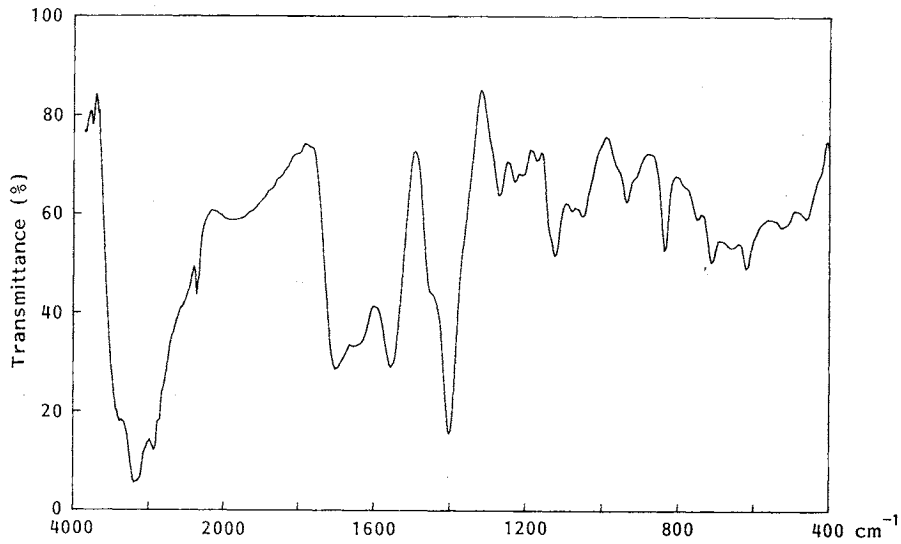


Fig. 2. Visible spectrum of zincphyrin in methanol.



The visible peaks have been amplified by a factor of 8 with respect to the Soret band (~400 nm).

Fig. 3. IR spectrum of zincphyrin (KBr).



charged on a silica gel column (350 ml) packed with *n*-BuOH - EtOH - $CHCl_3$ - NH_4OH (4:5:2:3) and the column was developed with the same solvent. The active eluate was concentrated under reduced pressure to give a crude powder and subjected to reverse phase HPLC (YMC-gel ODS, 660 ml). It was eluted with MeOH - 50% NH_4OAc (55:45). After concentration of the active fraction, the resultant dark-reddish solution was applied on Diaion HP-20 column. The column was washed with water, and eluted with 80% acetone. The fractions containing zincphyrin was collected and concentrated under reduced pressure. The concentrate held at room temperature gave pure zincphyrin (200 mg) as a dark-reddish powder. Zincphyrin is soluble in MeOH, DMSO and alkaline water, but insoluble in benzene, hexane and water. It gave positive color reactions to potassium permanganate and iodine. Zincphyrin showed Rf 0.37 on silica gel TLC developed with *n*-BuOH - EtOH - $CHCl_3$ - NH_4OH (4:5:2:4). The molecular formula of zincphyrin was determined to be $C_{36}H_{36}O_8N_4Zn$ from an elemental analysis, HRFAB-MS and atomic absorption. Zincphyrin has a characteristic visible absorption spectrum as shown in Fig. 2. λ^{MeOH} nm ($E^{1\%}$) 386 sh (750), 406 (3,525), 538 (185), 574 (190).

Fig. 4. ^{1}H NMR spectrum of zincphyrin (DMSO-d_6).

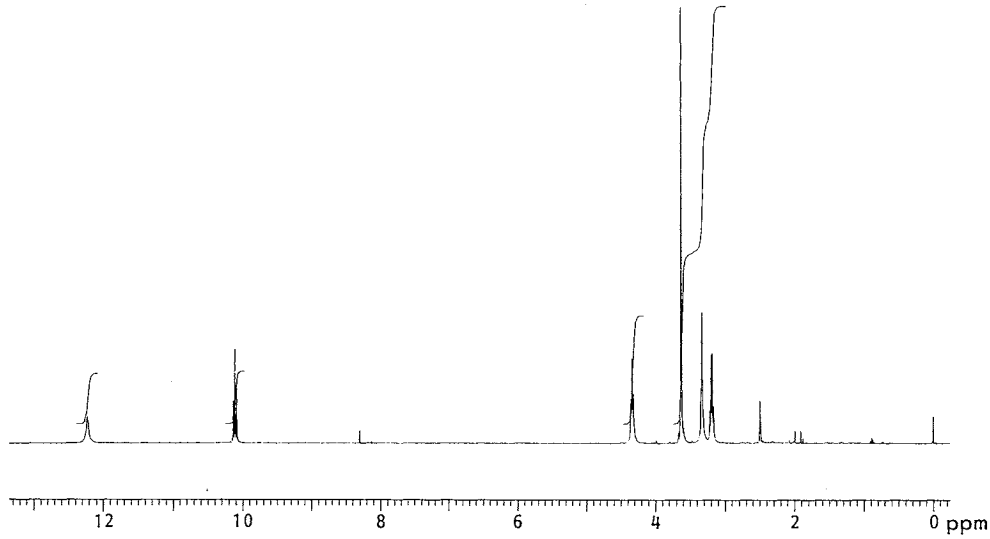
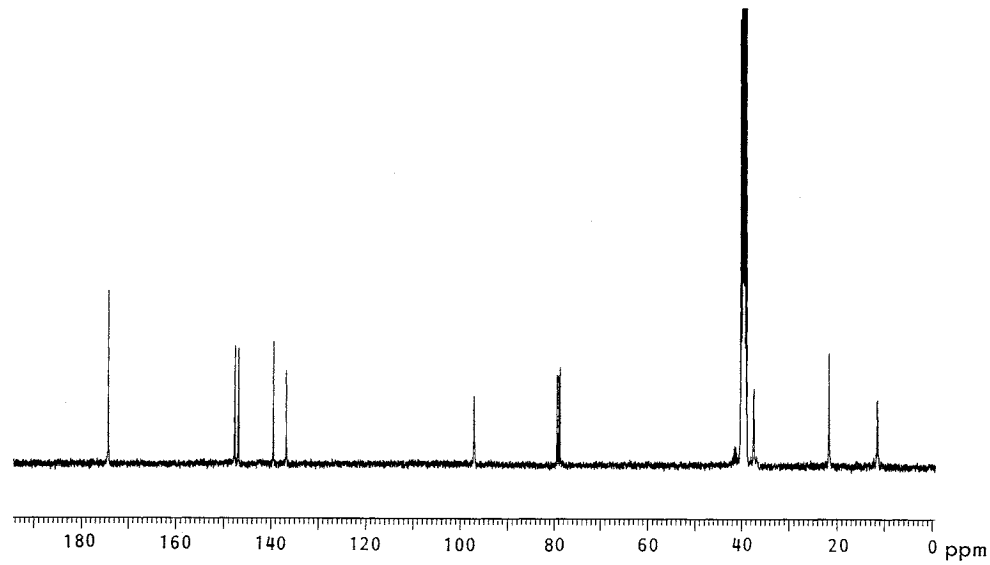


Fig. 5. ^{13}C NMR spectrum of zincphyrin (DMSO-d_6).



Main absorption bands of IR spectrum (KBr) occur at the following approximate wavelength: 3420, 2920, 1705, 1400, 1275, 1130, 940, 835 cm^{-1} (Fig. 3). ^{1}H and ^{13}C NMR spectra in DMSO-d_6 are shown in Figs. 4 and 5, respectively. It was observed that zinc is lost from zincphyrin in acidic methanol solution. The demetalized compound was transferred to ammonium methanol solution. It showed the typical porphyrin spectra[5] 393, 497, 532, 565 and 618 nm (data not shown) and the typical coproporphyrin Rf value by the fluorescence on paper chromatogram method[6]. However, the separation of coproporphyrins I and III could not be clearly confirmed with Rf value on this chromatography. So, zincphyrin was derivatized to tetramethyl ester with trimethylsilyldiazomethane (Tokyo Kasei Kogyo Inc.) and then demetalized with 6 N HCl. Also, coproporphyrins I and III (Porphyrin Product Inc.) were substituted for tetramethyl ester form by the same method. The structure identification was accomplished with HPLC, FAB-MS and NMR spectra. These results

Table 1. Effect of zincphyrin and disodium cromoglycate (DSCG) on histamine release from the passively sensitized rat peritoneal cells.

Drug	Conc (μg/ml)	Inhibition of histamine release (%)
Zincphyrin	10	80.1
	2.5	27.3
	0.63	7.4
DSCG	10	83.9
	2.5	50.1
	0.63	23.5

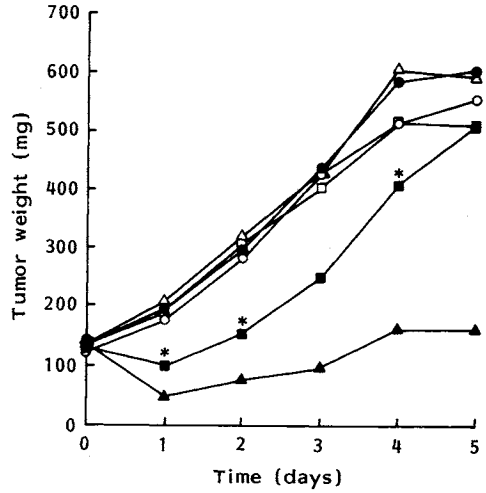
Control histamine release was 244 ng/2×10^5 mast cells.

of demetalized zincphyrin tetramethyl ester was corresponding to coproporphyrin III tetramethyl ester, but was different from coproporphyrin I tetramethyl ester. Therefore, zincphyrin was identified as Zn coproporphyrin III. After the completion of this work, we became aware of paper reported by HORIUCHI *et al.*[7]. This paper describes a porphyrin of the same structure as that described here for zincphyrin. Although, zincphyrin was discovered in the fermentation broth of *Streptomyces* sp. AC8007, this compound has also been isolated from meconium[7,8].

As the biological activity, zincphyrin significantly inhibited the histamine release from sensitized rat mast cells at concentrations of 2.5 to 10 μg/ml (Table 1). Clinical efficacy of photodynamic therapy (PDT) depends on the selective photosensitization of tumor tissue[9]. Photofrin II, which is the putative active component of hematoporphyrin derivative (HpD) extracted from blood, is the photosensitizer currently undergoing clinical evolution[10]. However, the purity of photofrin II is very low as well as HpD, which consists of numerous components and their active principle is still unknown[11]. Zincphyrin was compared to HpD, a photosensitizer commonly used in the PDT of cancer. PDT was carried out with sarcoma 180 (S-180) bearing mice. S-180 were transplanted into the shaven back of male ICR mice (22~24 g) by subcutanous injection of approximately 5×10^6 cells per mouse. The tumor grew about 130 mg in 2 days after implantation. Zincphyrin and HpD (50 mg/kg ip) which were dissolved in physiological saline containing 0.1 M Tris HCl buffer (pH 7.4) were given to mice in groups of five. Ten minutes later, the mice were anesthetized with Nenbutal (Dainabot Ltd.). Ten minutes later, the tumors irradiated with 75.48 mW/cm^2 white light derived from halogen

Fig. 6. Effect of zincphyrin and HpD on growth of sarcoma 180.

○ Control, ● irradiation, △ zincphyrin, ▲ zincphyrin + irradiation, □ HpD, ■ HpD + irradiation. Each point represents the mean of five mice. * One mouse died.



lamp (JCR 15V 150WB: Lumina Ace, Hayashi Watch Co. Ltd.) for 10 minutes. The light was passed through a transparent glass of ice water to keep the low temperature of the tumor. Tumor weight in mg was estimated by using the formula, $W = (a^2 \times b)/2$ where a is the width of the tumor in mm and b is the length of the tumor in mm[12]. Zincphyrin, HpD or light alone did not affect the rate of tumor growth. Zincphyrin was found to be effective as a photosensitizer to control tumor growth and less toxicity than HpD in mortality of mice (Fig. 6). Zincphyrin is considered to be a photosensitizer having high purity and low toxicity.

Addendum in Proof

Compound AC8007[1] has been identified as zincphyrin.

References

1) YAGINUMA, S.; M. TORIYA, N. MUTO & M. HAYASHI (Toyo Jozo): Histamine release inhibitor AC8007 and its preparation. Jpn Kokai: 234688 ('90), Sept. 17, 1990

2) KUSNER, E. J.; B. DUBNIK & D. J. HERZIG: The inhibition by disodium gromoglycate in vitro of anaphylactically induced histamine release from rat peritoneal mast cells. J Pharmacol. Exp. Ther. 184: 41~46, 1973

3) TADA, T. & K. OKUMURA: Regulation of homo-

cytotropic antibody formation in the rat (I). J. Immunol. 106: 1002~1011, 1971

4) SHORE, P. A.; A. BURKHALTER & V. A. COHN: A method for the fluorometric assay of histamine in tissues. J. Pharmacol. Exp. Ther. 127: 182~186, 1959

5) SMITH, KEVIN M.: Structure and chemistry of porphyrin compounds. *In* Porphyrin and Metalloporphyrins. *Ed.*, KEVIN M. SMITH, pp. 20~27, Elsevier scientific publishing company, 1975

6) FUHRHOP, J. H. & K. M. SMITH: Laboratory methods. *In* Porphyrin and Metalloporphyrins. *Ed.*, KEVIN M. SMITH, pp. 839~851, Elsevier scientific publishing company, 1975

7) HORIUCHI, K.; K. ADACHI, Y. FUJISE, H. NARUSE, K. SUMIMOTO, N. KANAYAMA & T. TERAO: Isolation and characterization of zinc coproporphyrin I: a majar fluorescent component in meconium. Clin. Chem. 37: 1173~1177, 1991

8) GOURLEY, R. G.; B. KREAMER & R. AREND: Excremental studies in human neonates. Identification of zinc coproporphyrin as amarker for meconium. Gastroenterology 99: 1705~1709, 1990

9) DOUGHERTY, T. J.: Therapy and detection of malignant tumors. Photochem. Photobiol. 45: 879~889, 1987

10) PANDEY, R. K.; T. J. DOUGHERTY & K. M. SMITH: Syntheses of hematoporphyrin dimers and trimers with ether linkages. Tetrahedron Lett. 29: 4657~4660, 1988

11) KORBELIK, M. & J. HUNG: Cellular delivery and retention of photofrin II: The effects of interaction with human plasma protein. Photochem. Photobiol. 53: 501~510, 1991

12) OVEJERA, A. A.; D. P. HOUCHENS & A. D. BARKER: Chemotherapy of human tumor xenografts in genetically athymic mice. Ann. Clin. Lab. Sci. 8: 50~56, 1978