NEW ANTIBIOTIC, ISOHEMATINIC ACID

II. PHYSICO-CHEMICAL PROPERTIES, STRUCTURAL ELUCIDATION AND BIOLOGICAL ACTIVITIES

YASUHIRO ITOH, MICHIKO TAKEUCHI, KEIKO SHIMIZU, SHUJI TAKAHASHI, AKIRA TERAHARA and TATSUO HANEISHI

Fermentation Research Laboratories, Sankyo Co., Ltd.
2-58, 1-Chome, Hiromachi, Shinagawa-ku, Tokyo 140, Japan

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Physico-chemical characterization of isohematinic acid revealed that this antibiotic has a succinimide nucleus. From elemental analysis, the molecular formula of isohematinic acid was determined to be $C_8H_9NO_4$.

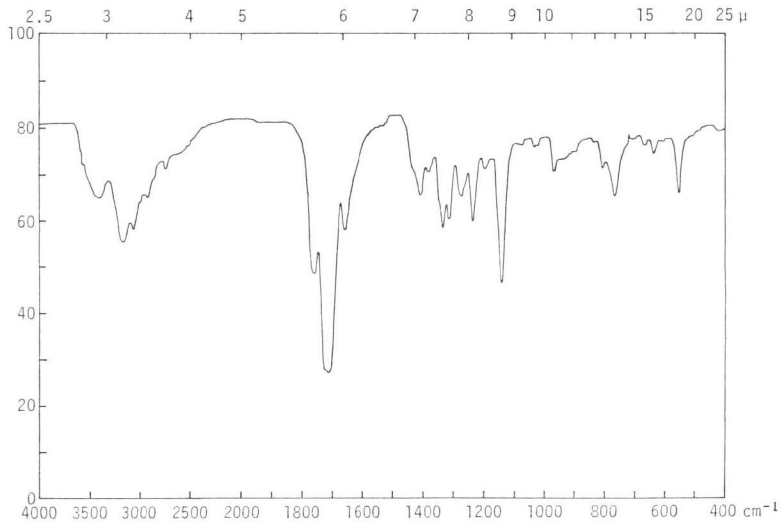
Isohematinic acid showed weak antimicrobial activities against anaerobic bacteria, such as *Bacteroides fragilis* and *Propionibacterium acnes*.

As reported in the previous paper[1], isohematinic acid was isolated from the culture broth of *Actinoplanes philippinensis* SANK 61681. Structural elucidation revealed that the antibiotic is an isomer of hematinic acid[2], an oxidation product of chlorophyll, and it was named isohematinic acid. In the present report, the physico-chemical properties, structure elucidation and biological activity of the antibiotic are described.

Physico-chemical Properties

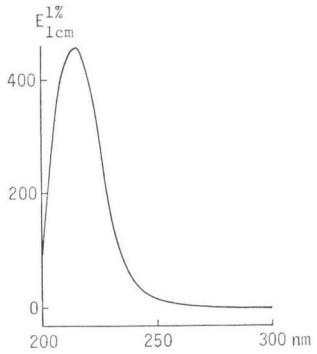
Isohematinic acid was obtained as colorless crystals, soluble in methanol, acetone and alkaline water, and slightly soluble in ethyl acetate. The antibiotic was visualized with iodine and bromocresol green on TLC plates of silica gel. It behaved as an acidic substance on high voltage paper electrophoresis (55 volt/cm, 0.8 mA/cm) carried out in 0.1 M tris-HCl buffer at pH 7.5 for 30 minutes. The relative

Fig. 1. Infrared absorption spectrum of isohematinic acid (KBr).



mobility of the antibiotic was 1.3 when compared with bromophenol blue (defined as 1.0). The molecular formula was derived from the elemental analysis. The IR, UV, ^{1}H NMR and ^{13}C NMR spectra of the antibiotic are shown in Figs. 1, 2, 3 and 4, respectively. The IR spectrum strongly suggested the existence of 5-membered ring imide at 1760 and 1730 cm^{-1}, and carboxylic acid at 3000~2500 and 1710 cm^{-1}. The ^{13}C NMR spectrum of isohematinic acid also revealed the presence of eight carbons in the molecule of the antibiotic. These results as well as other physical and chemical properties are summarized in Table 1. These data coupled with its biological properties suggested that isohematinic acid is a new antibiotic.

Fig. 2. Ultraviolet absorption spectrum of isohematinic acid in MeOH.



Structural Elucidation

The molecular formula of isohematinic acid, $C_8H_9NO_4$, was established from the elementary analysis. The IR spectrum of the antibiotic indicated the presence of carboxylic acid and 5-membered ring imide. The ^{13}C NMR spectrum indicated the presence of three carbonyl carbons including carboxylic acid at δ 179.4, 176.0 and 171.4, two olefinic carbons at δ 140.7 and 120.7, two methylene carbons at δ 31.0 and 26.8 and one methine carbon at δ 44.6. Two olefinic carbons showed singlet and triplet signals in the off resonance spectrum. Thus these two carbons are assigned to endomethylene carbons. The ^{1}H NMR

Fig. 3. ^{1}H NMR spectrum of isohematinic acid (100 MHz, Pyridine-d_5).

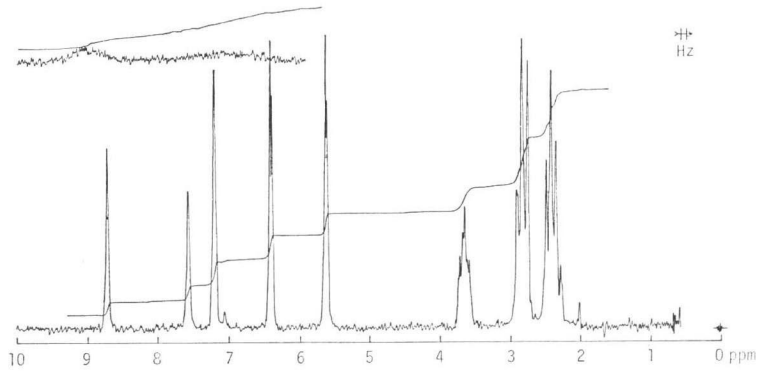


Fig. 4. ^{13}C NMR spectrum of isohematinic acid (25 MHz, CD_3OD).

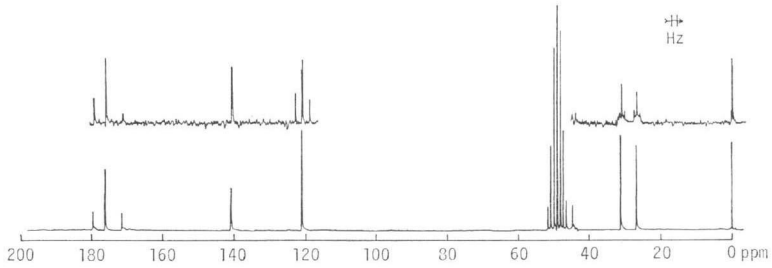


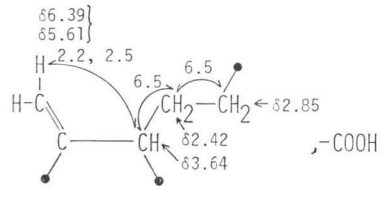
Table 1. Physico-chemical properties of isohematinic acid.

Nature	Acidic, colorless crystals
$[\alpha]_D^{20}$	0° (*c* 1, CH_3OH)
Elementary analysis (%)	Calcd. for $C_8H_9NO_4$: C 52.46, H 4.95, N 7.65
	Found: C 52.22, H 4.86, N 7.57
UV λ_{max}^{MeOH}	225 nm
IR ν cm^{-1} (KBr)	3000~2500, 1760, 1730~1710
^1H NMR δ ppm (Pyridine-d_5)	6.39 (1H, d, J=2.5 Hz)
	5.61 (1H, d, J=2.2 Hz)
	3.64 (1H, ddt, J=2.5, 2.2, 6.5 Hz)
	2.85 (2H, t, J=6.5 Hz)
	2.42 (2H, tt, J=6.5, 6.5 Hz)
^{13}C NMR δ ppm (CD_3OD)	179.4 (s), 176.0 (s), 171.4 (s), 140.7 (s), 120.7 (t), 44.6 (d), 31.0 (t), 26.8 (t)
Rf*	0.5
Color reaction	Positive: I_2, bromocresol green.

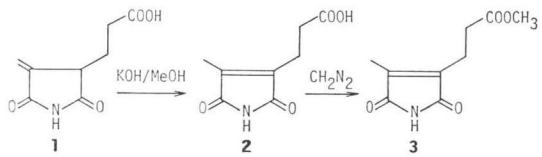
* Merck silica gel plate F_{254}, Art 5715 (benzene - EtOAc - AcOH, 10: 10: 1).

spectrum showed two methylene signals at δ 2.42 and 2.85, one methine proton at δ 3.64 and endomethylene protons at δ 5.61 and 6.39. When one of the methylene protons at δ 2.42 was irradiated, another methylene proton at δ 2.85 changed to a singlet but the endomethylene proton signals did not. When the methine proton at δ 3.64 was irradiated, the doublet signal of the endomethylene protons changed to a singlet and the multiplet signal of the methylene protons at δ 2.42 changed to a triplet. These ^1H NMR and ^{13}C NMR spectral analyses of the antibiotic afforded the partial structures as shown in Fig. 5. The IR spectrum of isohematinic acid strongly suggested the presence of a succinimide moiety, thus the structure of isohematinic acid was assumed to be the structure **1** in Scheme 1. Treatment of isohematinic acid with alkaline solution gave isomeric compound **2**, mp 112~113°C, $C_8H_9NO_4$, *m/z* 183. In the ^1H NMR spectrum of **2**, signals due to endomethylene proton at δ 5.61 and 6.39, and methine proton at δ 3.64 observed in that of **1** disappeared and a singlet methyl signal newly appeared at δ 1.99 and the methylene proton signal at δ 2.42 in **1** shifted downfield to δ 2.62. The IR spectrum of **2** also indicated the presence of an imide moiety. These results suggested the occurrence of a double-bond migration as shown in Scheme 1. When isohematinic acid was dissolved in CD_3OD and allowed to stand at room temperature for 18 hours, the methine proton at δ 3.64 disappeared and the doublet signal of the olefinic proton changed to a singlet. This result showed that the methine proton is active in causing the isomerization of isohematinic acid to hematinic acid[2] in alkaline solution as shown in Scheme 1. In the mass spectrum, the fragmentation pattern of **2** was the same as that of hematinic acid[2]. Treatment of **2** with diazomethane in ethereal solution gave the methyl ester **3**, $C_9H_{11}NO_4$, *m/z* 197. In the mass spectrum of **3**, the fragmentation pattern was also closely related to that of hematinic acid methyl ester[3]. Thus the

Fig. 5. Partial structure of isohematinic acid.



Scheme 1.



structure of the antibiotic was determined as **1**. All of the reaction procedures mentioned above are summarized in Scheme 1.

Biological Activity

The minimal inhibitory concentrations (MICs) of isohematinic acid against bacteria were determined by a serial two-fold agar dilution method. The result is shown in Table 2. The MICs were determined after 24 and 48 hours of incubation at 37°C. Isohematinic acid was weakly active against some species of anaerobic bacteria such as *Bacteroides fragilis* and *Propionibacterium acnes*. Isohematinic acid caused morphological change (swelling to spheroplast) of *Escherichia coli* and *Proteus mirabilis* at concentrations of 250~1,000 μg/ml but MIC values against these bacteria was over 800 μg/ml. Isohematinic acid also has the ability to enhance host defence mechanisms to protect against an experimental infection with *E. coli*. The most effective protection was observed when the antibiotic was given prior to infection. Details of the results will be reported elsewhere. The acute toxicity (LD_{50}, i.v.) of isohematinic acid in mice was 300 mg/kg. While isohematinic acid indicated some biological activity, hematinic acid did not exhibit any biological activity in the same tests including stimulation of protective effect against *E. coli* infection.

Table 2. Antimicrobial spectrum of isohematinic acid.

Test organism	Medium*	MIC (μg/ml)
Staphylococcus aureus FDA 209P JC-1	1	800
Streptococcus faecalis S-299	1	800
Escherichia coli NIHJ JC-2	1	>800
Proteus mirabilis SANK 71873	1	>800
P. vulgaris OX19	1	>800
Bacteroides fragilis SANK 71176	2	200
B. fragilis SANK 70478	2	200
B. fragilis SANK 70578	2	200
B. fragilis SANK 70678	2	200
Fusobacterium aerofaciens SANK 72276	2	>800
Propionibacterium acnes SANK 71976	2	400
Streptococcus faecalis S-299	2	>800

* 1. Nutrient agar
2. GAM agar

Experimental

Melting points were taken in a Yamato micro-melting point apparatus and were uncorrected. The IR spectra were run on a Hitachi infrared spectrophotometer. The NMR spectra were run on a Varian model NMR or Hitachi R-24 and chemical shifts were based on an internal tetramethylsilane standard and recorded as δ values.

Compound 2

Isohematinic acid (65 mg) in 4 ml of 1% potassium hydroxide in methanol was allowed to stand at room temperature for one hour and the reaction mixture was evaporated to dryness after acidification to pH 3.0 with 1 N hydrochloric acid. The residue was purified by column chromatography on Sephadex LH-20 developed with a mixture of chloroform - ethyl acetate (1 : 1). Compound **2** was obtained as colorless crystals (25 mg): mp 112~113°C; *m/z* 183; IR ν_{max}^{KBr} (cm^{-1}) 3260, 3000~2500, 1770, 1720, 1710; ^{1}H NMR $\delta_{ppm}^{CD_3OD}$ 2.62 (4H, m), 1.99 (3H, s); ^{13}C NMR $\delta_{ppm}^{CD_3OD}$ 175.5 (s), 174.2 (s), 174.0 (s), 140.6 (s), 140.0 (s), 32.7 (t), 20.2 (t), 8.4 (q);

Anal. Calcd. for $C_8H_9NO_4$: C 52.46, H 4.95, N 7.65.
Found: C 52.46, H 4.98, N 7.55.

Compound 3

The reaction mixture of **2** (162 mg) in 1 ml methanol with excess amount of diazomethane in ethereal solution was allowed to stand at room temperature for 30 minutes and then was evaporated to dryness. The residue was applied on the preparative thin-layers of silica gel (Merck Kieselgel F_{254}, 0.5 mm thick,

4 plates). Those were developed with a mixture of benzene - ethyl acetate (4: 1). **3** was obtained as colorless oil (92 mg): *m/z* 197; IR ν_{max}^{Liquid} (cm^{-1}) 2950, 1770, 1740, 1710; ^1H NMR $\delta_{ppm}^{CDCl_3}$ 8.1 (1H, bs, NH), 3.67 (3H, s, CO_2CH_3), 2.65 (4H, m), 1.99 (3H, s); ^{13}C NMR $\delta_{ppm}^{CDCl_3}$ 172.4 (s), 171.9 (s), 171.7 (s), 139.5 (s), 51.8 (q), 31.7 (t), 19.3 (t), 8.6 (q).

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