ANTIBIOTIC YC 73 OF PSEUDOMONAS ORIGIN. II*

STRUCTURE AND SYNTHESIS OF THIOFORMIN AND ITS CUPRIC COMPLEX (YC 73)

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The structure of thioformin, a new acidic antibiotic substance obtained by removing cupric ion from antibiotic YC 73, was shown to be N-methyl-N-thioformylhydroxylamine by degradative and synthetic studies. Numerous metal complexes of thioformin, including the cupric (antibiotic YC 73 itself) and ferric complexes, were also synthesized and characterized.

The purification, isolation and properties of a new antibiotic, YC 73 (I) from the culture broth of a pseudomonad were reported previously[1]. The antibiotic was a cupric complex of a low molecular weight, acidic antibiotic named thioformin (II). As reported in the present paper, thioformin has been shown to be N-methyl-N-thioformylhydroxylamine. During the past decade, a substantial number of hydroxamic acid antibiotics have been found from a variety of microorganisms[2]. However, thioformin is the first antibiotic that should be grouped as a thiohydroxamic acid.

The present paper is concerned with the structure and synthesis of II and its cupric complex, namely YC 73 itself. The paper also deals with other metal complexes of II together with O-acyl derivatives. The synthesis of thioformin analogues will be reported in the succeeding paper.

By treating I with H_2S gas in chloroform, cupric ion was removed from I and II recovered from the solvent as a light yellow oil, b.p. 66°C at 5 mm, which, at low temperature solidified. Physicochemical properties of II are described in the experimental part, and IR, UV and NMR spectra are shown in Figs. 1, 2 and 3, respectively. II gave a strong prositive color reaction with $FeCl_3$ suggesting the presence of a hydroxyl group (aci-form). II was further characterized by conversion into several O-acyl and 2,4-dinitrophenyl derivatives, of which the physicochemical properties are summarized in Table 1. The molecular formula, C_2H_5NOS, was deduced from the elementary analysis and mass spectrometry of II and its derivatives.

For the purpose of structure elucidation, I was first hydrolyzed with 6 N hydrochloric acid. Column chromatography of the acid hydrolysate on Sephadex LH-20 gave one ninhydrin-positive degradation product as colorless prisms, m.p. 225~226°C,

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Fig. 1. Infrared spectrum of thioformin (Nujol).

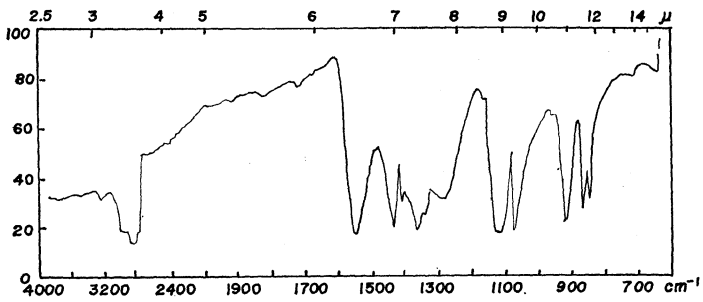


Fig. 2. Ultraviolet spectrum of thioformin in methanol.

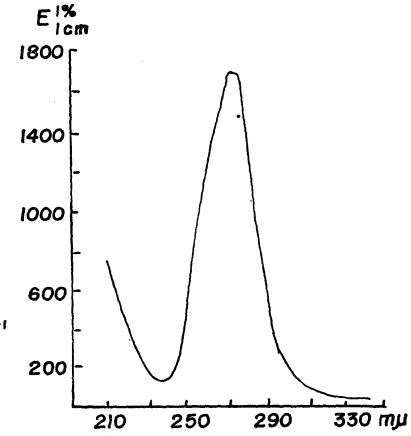
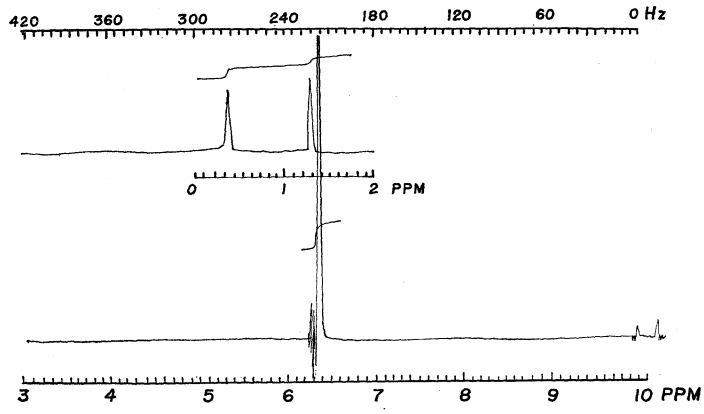


Fig. 3. NMR spectrum of thioformin (60 M Hz, in $CDCl_3$).



having Rf value 0.44 on a cellulose powder thin-layer plate (solvent system: *n*-BuOH - pyridine - water - AcOH, 6 : 4 : 3 : 1). The product was identified as N-methylamine hydrochloride by direct comparison with the authentic sample. The O-acetate of II was then hydrolyzed under the same conditions, and a crystalline degradation product was isolated as the hydrochloride which was different from N-methylamine hydrochloride. The product was identified as N-methylhydroxylamine hydrochloride by direct comparison with an authentic specimen. These results suggested that II contains a CH_3-$\overset{|}{N}$-OH moiety. The presence of this moiety was also supported by NMR spectra (Fig. 3): τ 6.4 (3H, singlet) for N-CH_3 and τ 1.5 (1H, singlet) for OH, the latter being absent from the NMR spectrum of the O-acetate of II.

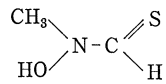
In view of the molecular formula of II, the rest of the molecule should be -CHS. In the NMR spectrum of II, one proton singlet which appeared at τ 0.3 was reasonably ascribed to the proton of S=$\overset{|}{C}$-H group. Since the presence of C-N and N-C=S was suggested by the IR (Fig. 1, 1560 cm^{-1}) and UV spectra (λ_{max}^{MeOH} 272 mμ, $E_{1cm}^{1\%}$ 1700), it

Table 1. Physicochemical and antimicrobial properties of O-acyl and 2,4-dinitrophenyl derivatives of thioformin

Compound	Acetate		*p*-Nitrobenzoate		*p*-Bromobenzoate		2,4-Dinitrophenyl derivative	
Appearance	light yellow oil		light yellow plates		colorless needles		orange plates	
M. p. or b. p.	85°C/3 mm		80°C		98°C		112°C	
Formula and elemental analysis (%)	$C_4H_7NO_2S$		$C_9H_8N_2O_4S$		$C_9H_8NO_2SBr$		$C_8H_7N_3O_5S$	
	Calcd.	Found	Calcd.	Found	Calcd.	Found	Calcd.	Found
C	36.09	35.52	45.00	45.08	39.96	39.42	37.35	37.32
H	5.26	5.24	3.33	3.44	3.00	2.92	2.72	2.73
N	10.56	10.25	11.67	11.43	5.04	5.11	16.34	16.07
S	24.06	24.04	13.33	13.13			12.45	12.51
M. W. (mass spectrometry)								
Found	133		240		273, 275		—	
Calcd.	133		240		274		257	
IR: ν_{max}^{Nujol} cm^{-1}	1787, 1505, 1173, 1134, 1007, 847 (liquid)		1754, 1523, 1349, 1232, 1097, 1041, 867, 722		1750, 1585, 1511, 1242, 1039, 1007, 850, 749		1590, 1523, 1340, 1242, 1170, 1049, 948, 921, 840, 741	
Antimicrobial activity*								
S. aureus FDA 209P	<0.195		3.12		12.5		12.5	
B. subtilis PCI-219	<0.195		0.39		6.25		0.78	

* Determined by agar dilution method (M.I.C. : μg/ml)

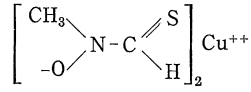
was assumed that II was N-methyl-N-thioformylhydroxylamine.



(II)

The above structure was finally confirmed by chemical synthesis of II. An aqueous solution of N-methylhydroxylamine hydrochloride was treated with excess aqueous solution of potassium dithioformate at room temperature. The reaction mixture was extracted with AcOEt, and the extract was purified by vacuum distillation. Thus, N-methyl-N-thioformylhydroxylamine was obtained as a light yellow oil, b.p. 70°C at 6 mm. The synthetic N-methyl-N-thioformylhydroxylamine was proved to be identical with natural thioformin (II) by direct comparison of its IR, UV and NMR spectra. Furthermore, the synthetic thioformin was converted to the cupric complex by treating with cupric sulfate and the cupric complex thus obtained was also identical with the natural I in all respects.

From elementary analysis and copper content, YC 73 (I) was found to be a chelate compound which consisted of one mole of copper and two moles of ligand as shown in formula (III).



(III)

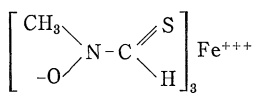
Since the compound II had a strong chelating ability, several metal complexes were prepared by adding an aqueous solution of metal salts to an alcoholic solution of II. The properties of typical metal complexes of II are summarized in Table 2. Among these metal complexes, the structure of the iron complex was further investi-

Table 2. Physicochemical and antimicrobial properties of thioformin metal complexes

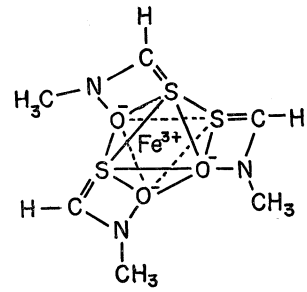
Metal complex	Appearance	Melting point (°C)	Formula	Elemental analysis (%)		IR: ν_{max}^{Nujol} cm^{-1}	Antimicrobial activity** M.I.C. (μg/ml)	
				Calcd.	Found		*S. aureus* FDA 209P	*B. subtilis* PCI-219
Fe	Black needles	>250 (184)*	$C_6H_{12}N_3O_3S_3Fe$	C 22.09 H 3.68 N 12.88 Fe 17.12	22.22 3.72 12.69 16.90	1557, 1153, 920, 897, 864	0.39	3.12
Ni	Dark red needles	227~228	$C_4H_8N_2O_2S_2Ni$	C 20.11 H 3.35 N 11.73	20.39 3.46 11.49	1590, 1153, 910, 887	0.39	0.39
Sn	Pale brown powder	176 (dec.)	$C_2H_4NOS\cdot SnCl$	C 9.84 H 3.35 N 5.74	9.91 3.46 5.82	1573, 1143, 910, 885, 850	0.78	1.56
Zn	Colorless prisms	222 (dec.)	$C_4H_8N_2O_2S_2Zn$	C 19.56 H 3.26 N 11.41	19.68 3.37 11.34	1563, 1145, 920, 888	0.78	1.56
Na	Colorless needles	243 (dec.)	$C_2H_4NOSNa\cdot H_2O$	C 19.83 H 4.97 N 11.57	19.52 4.58 11.28	1553, 1150, 933, 843	0.39	0.78
Ca	Colorless needles	>250	$C_4H_8N_2O_2S_2Ca$	C 21.81 H 3.63 N 12.72	21.94 3.37 12.42	1553, 1157, 933, 895	0.39	0.78

* Sintering point ** Determined by agar dilution method

gated by physical methods, because the complex was obtained by treating II with either ferric chloride or ferrous sulfate under aerobic conditions. Magnetic measurements of the complex showed a molar magnetic susceptibility χ_M: $13{,}920\times10^{-6}$ emu and, by calculation, a magnetic moment μ_{eff}: 5.86 B.M. (293°K) was obtained. Since the calculated value for Fe^{+++} with five unshared electrons is $\mu=\sqrt{5(5+2)}=5.92$, the complex was recognized as an ionic complex and iron in the complex was expected to be as Fe^{+++} in high spin state[3]. To support this observation, the MÖSSBAUER spectrum of the complex was measured and the following parameters were obtained: isomer-shift for stainless steel +0.467 mm/sec and ΔE=0.32 mm/sec. The observed MÖSSBAUER parameters fall in the range expected for typical high spin Fe^{+++} compounds[4]. From these values together with the elemental analysis and iron content determined by polarography, the iron complex was found to be a typical



(IV)



(V)

chelate compound consisting of one mole of iron and three moles of ligand* as shown in formula (IV).

The stereochemistry of IV has not yet been established, but the structure (V) would be the most probable representation of IV at present time.

Careful experiments showed that white precipitates which deposited on addition of ferrous sulfate solution to an aqueous solution of the sodium salt of II turned black as soon as they contacted air. This observation would suggest that the ferrous complex of II was formed first and then oxidized to the ferric complex by air[5].

As illustrated in Tables 1 and 2, O-acyl, 2,4-dinitrophenyl derivatives and metal complexes of II showed antimicrobial activities in preliminary *in vitro* test, and details of the biological properties will be reported later.

Experimental

Removal of copper from YC 73 (Preparation of thioformin)

After YC 73 (120 mg in 20 ml of chloroform) was saturated with H_2S gas at room temperature, the reaction mixture was filtered from the insoluble CuS. The colorless filtrate was concentrated *in vacuo* and the remaining substance was distilled *in vacuo*. Thus, thioformin (60 mg) was obtained, which at low temperature solidified, b.p. 66°C at 5 mm (Yield: 67 %).

Anal. Found: C 26.01, H 5.13, N 14.90, S 34.87 %. M.W. 91 (mass spectrometry)
Calcd. for C_2H_5NOS: C 26.37, H 5.49, N 15.38, S 35.16 %. M.W. 91.14

Thioformin gave the following color reactions. FEHLING, brown precipitate on heating; TOLLENS, colorless prisms precipitate; $FeCl_3$ in MACILVEIN buffer solution, black coloration at pH 5.0, violet coloration at pH 7.0 and colorless at pH 8.0; $KMnO_4$, positive; MOLISCH, negative; BENEDICT, green on heating; LEGAL, positive, turning to green on addition of acetic acid; SAKAGUCHI, biuret and EHRLICH, negative.

Rf value of 0.55 was obtained on thin-layer chromatography on Kieselgel GF_{254} (Solvent system: $CHCl_3$-MeOH (100 : 1). Detection by UV lamp and bioautography).

Preparation of YC 73 from thioformin

Cupric sulfate (2 g in 10 ml of water) was added to a suspension of thioformin (70 mg in 3 ml of EtOH) at room temperature. The dark green precipitate resulting in the reaction mixture was extracted with AcOEt. The organic layer was evaporated *in vacuo*. Thus, YC 73 (56 mg) was obtained as dark green needles, m.p. 201°C (dec.). The product was found to be identical with natural YC 73 (m.p. 199°C (dec.)) by a mixed melting point determination, and comparison of UV and IR spectra.

Preparation of O-acyl and 2,4-dinitrophenyl derivatives of thioformin

1. Thioformin acetate: Thioformin (611 mg) was acetylated with acetic anhydride and pyridine at room temperature, and the crude acetate was purified by vacuum distillation to give purea cetate (640 mg), b. p. 85°C at 3 mm (Yield: 72 %). UV: λ_{max}^{MeOH} 272 mμ ($E_{1cm}^{1\%}$ 1110), NMR (τ values in $CDCl_3$) 7.60 (3H, s), 6.35 (3H, s), 0.75 (1H, d).

2. Thioformin *p*-nitrobenzoate: Thioformin (300 mg) was acylated with *p*-nitrobenzoyl chloride in benzene–pyridine (2 : 1) solution at room temperature overnight. After the solvent had been removed by evaporation under reduced pressure, the residue was treated with water to give a crude product which was recrystallized from hot EtOH. Thus, the pure sample (540 mg) was obtained as light yellow plates, m.p. 80°C. NMR (τ values in $CDCl_3$), 6.20 (3H, s), 1.65 (4H, s), 0.70 (1H, d).

3. Thioformin *p*-bromobenzoate: The *p*-bromobenzoylation of thioformin (230 mg)

* According to Dr. K. MASUKAWA, a polarographic study on IV supported the present conclusion. Details will be reported by him elsewhere.

was prepared as described for thioformin *p*-nitrobenzoate. Recrystallization from MeOH gave the pure sample (420 mg) as colorless needles, m.p. 98°C (Yield: 61 %).

4. 2,4-Dinitrophenyl thioformin: Thioformin (278 mg in 5 ml of EtOH and 20 ml of water) was adjusted to pH 7.5 with solid $NaHCO_3$. Into the solution, 2,4-dinitrofluorobenzene (1.5 g) was gradually added with stirring and controlling the pH to 7.5 and the reaction was allowed to stand overnight at room temperature. There was deposited a yellow precipitate of the 2,4-dinitrophenyl derivative. After filtration, the crude product was recrystallized from AcOEt to afford a pure sample (380 mg) as orange plates, m.p. 112°C (dec.) (Yield: 49 %).

Hydrolysis of YC 73

YC 73 (680 mg) was hydrolyzed with 6 N HCl (30 ml) for 12 hours at 100°C in a sealed tube. The insoluble part was removed by filtration, and the solution was concentrated *in vacuo* to give a syrup. The syrup was dissolved in MeOH (2 ml), poured onto a column containing Sephadex LH-20 (200 ml), and eluted with MeOH. Ninhydrin positive eluates were gathered and the solvent was removed to give the crude product. The crude product was recrystallized from iso-PrOH - ether (2 : 1), and the pure sample (146 mg) was obtained as colorless plates, m.p. 225~226°C. The crystals were identical in all respects with N-methylamine hydrochloride.

Anal. Found: C 17.93, H 8.71, N 20.53, Cl 52.10 %.
Calcd. for $CH_3NH_2 \cdot HCl$: C 17.79, H 8.96, N 20.75, Cl 52.50 %.

Hydrolysis of thioformin acetate

Thioformin acetate (1 g) was hydrolysed as above. The hydrolysate was concentrated *in vacuo* to afford a syrup, out of which, on standing in the refrigerator overnight, colorless needles of crude hydrolysate were deposited. Recrystallization from iso-PrOH gave the pure sample (170 mg), m.p. 79~82°C. The degradation product thus obtained was found to be identical with N-methylhydroxylamine hydrochloride.

Anal. Found: C 14.50, H 7.50, N 16.66, Cl 42.00 %.
Calcd. for $CH_3NHOH \cdot HCl$: C 14.38, H 7.19, N 16.77, Cl 42.49 %.

Synthesis of thioformin

N-Methylhydroxylamine hydrochloride (1.16 g (1.4 mmol) in 10 ml of water) was reacted with stirring with excess aqueous red solution of potassium dithioformate[6] (5 ml (16.5 mmol)) at room temperature for about 1 hour. The reaction mixture turned with foaming of gas into colorless solution. It was extracted with AcOEt (20 ml) four times and the extract was concentrated *in vacuo*. The remaining syrup was distilled under reduced pressure to give thioformin (380 mg), b.p. 52°C at 3 mm (Yield: 30 %). The product was found to be identical with natural thioformin by direct comparison of its IR, UV and NMR spectra.

Preparation of thioformin metal complexes

1. General procedure: To EtOH solution of thioformin or aqueous solution of the sodium salt of thioformin, an aqueous solution of a metal salt was added at room temperature to give metal complex precipitation. The purification of the crude product was carried out by recrystallization. The properties of typical metal complexes of thioformin are summarized in Table 2.

2. Preparation of thioformin ferric complex: Thioformin (404 mg) was dissolved in EtOH (1 ml), and an aqueous solution saturated with ferrous sulfate was added drop by drop at room temperature. The crude complex thus obtained was recrystallized from $CHCl_3$ - MeOH (1 : 1) to give the pure sample (310 mg) as black prisms, m.p. >250°C (sinter at 184°C).

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