A NEW MICROBIAL METABOLITE, SPHYDROFURAN. I
ISOLATION AND THE STRUCTURE OF A HYDROLYSIS PRODUCT

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A new streptomycete metabolite sphydrofuran was isolated from the culture filtrate of the strain MC41-M1 and MC340-A1 by a chemical screening method using EHRLICH reagent. Sphydrofuran was an acid-unstable substance and the structure of an acid-hydrolysis product was determined as 2-methyl-4-(1-glyceryl) furan.

The screening study based on chemical color reactions of microbial metabolites was first described in the isolation of dienomycins[1,2]. This screening appears worthy of further continuation to find new metabolites which may reveal biosynthetic sequences in microorganisms. This paper describes the isolation and characterization of a new metabolite sphydrofuran which was found by a screening with the EHRLICH color reaction in culture filtrates of two strains of *Streptomyces*.

Screening Method

The screening method was essentially the same as that described in a previous paper[1] except for the color reaction. The test materials including culture filtrates were subjected to thin-layer chromatography on the plates coated with microcrystalline cellulose powder and developed with butanol-acetic acid-water (12:3:5). EHRLICH reagent is known to be sensitive to a variety of biologically important substances having such molecular moieties as indole (for example, tryptophan), urea (citrulline), furan or pyrrole. The detection method was as follows: the developed plate was sprayed with a mixture (1:4) of 10% *p*-dimethylaminobenzaldehyde in concentrated hydrochloric acid and acetone, prepared just before use. Pink, pink-violet or yellow color appeared immediately in most cases.

Among the 140 strains tested, the culture filtrates of approximately 15 strains of *Streptomyces* showed the color reactions, and these were proved not to originate from the nutrients. Two strains among the 15 strains showed strong pinkish spots immediately at Rf 0.70 and the laboratory numbers MC41-M1 and MC340-A1 were given to these strains. Both strains produced sphydrofuran.

Characters of Sphydrofuran-producing Strains

The strain No. MC41-M1 was isolated from a soil sample collected at Nepal in 1968, the strain MC340-A1 from Rome, Italy in 1969. The characteristics of the strain MC41-M1 can be summarized as follows: no whorls; open or compact spirals; spiny structure on surface of spore; pale yellow to pale yellowish brown or pale brown growth; white to light bluish gray to light gray aerial mycelium; a chromogenic type which produces a brownish soluble pigment in organic media; medium to strong proteolytic activity; strong hydrolysis of starch. Among known species, strain No. MC41-M1 has many characteristics in common with the group of *Streptomyces viridochromogenes*[3,4] (KRAINSKY, 1914) WAKSMAN *et* HENRICI, 1948. The characteristics of the strain MC340-A1 can be summarized as follows: it belongs to a nonchromogenic type of streptomyces; it forms neither whorls nor spirals; the surface of the spore is smooth; the growth is pale pink, or light reddish orange to reddish orange [61c Coral] in various media; aerial mycelium is pale pink [6ca Flesh Pink, 7ca Baby Pink] in various media; the soluble pigment is colorless or pinkish; reduces nitrate to nitrite; it has medium to strong proteolytic activity and strong hydrolytic activity towards starch (The description in parenthesis follows the color standard published by Container Corporation of America). Among known species, the strain MC340-A1 is most closely related to *Streptomyces termitum*[5] Duché, Heim and Lavoureur.

Production and Isolation

For laboratory production, 125 ml of a medium consisting of soy-bean meal (Ajinomoto Co.) 0.2 %, glucose 0.5 %, starch 0.5 %, K_2HPO_4 0.05 % and $MgSO_4 \cdot 7H_2O$ 0.05 % in a 500 ml SAKAGUCHI-flask was inoculated with the strain MC41-M1 from KRAINSKY's glucose asparagine agar slant and shake-cultured at 27°C for several days. The concentration of sphydrofuran in the shake-cultured broth was determined by the following method: a filtered broth was diluted 20 times with water, and to 3 ml of the solution 1 ml of 3 N sulfuric acid containing *p*-dimethylaminobenzaldehyde (60 mg) was added. After allowing to stand for 30 minutes, the absorption intensity of the solution was measured at 518 mμ in a 1-cm spectrophotometer cell[6].

The diluted filtrate (4.7 liters) was adjusted to pH 7.1 with 1 N sodium hydroxide. Active charcoal (180 g, Shirasagi, Wako Pure Chemicals Co.) was added and after agitation for a while, the mixture was filtered and the cake was washed with water (300 ml × 2). To the combined solution of the filtrate and the washings, charcoal (80 g) was again added and the solution filtered. The cake was washed once with water (150 ml). The cakes were combined, spread on a sheet of paper and lightly dried under vacuum for 1 hour. The cake was suspended in acetone (3 liters) and the suspension was agitated at 40~50°C for 30 minutes, filtered, and the residue was washed with acetone (300 ml). The residue was again suspended in acetone (1 liter) containing water (80 ml) and treated similarly. To the combined aqueous acetone extracts, powdered sodium bicarbonate (5 g) was added and after agitation for 30 minutes, the mixture was filtered and the filtrate was evaporated. During the procedure, when the pH of the solution rose above 9, the solution was neutralized with a small amount

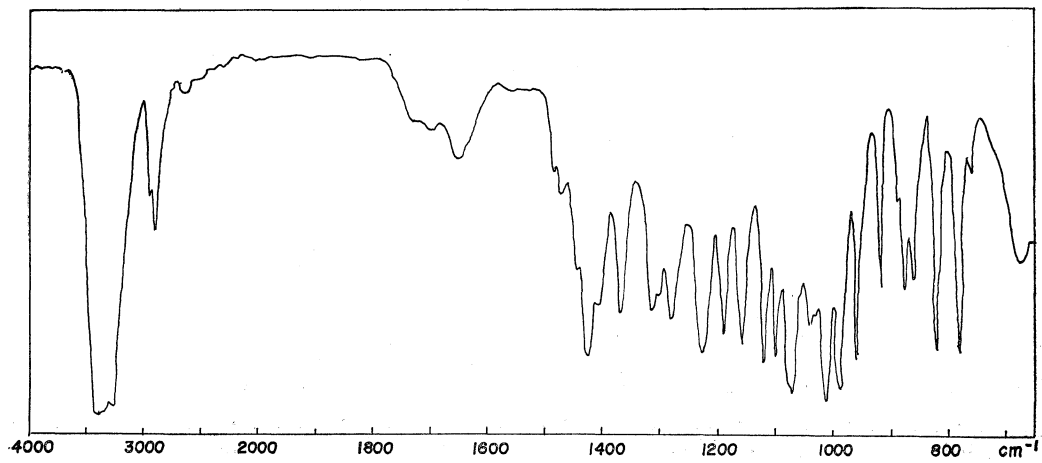
of Amberlite CG 50 (H form). Thus, a brown-yellow thick syrup (3.52 g) was obtained. The syrup was dissolved in a mixture of methanol - acetone - water (6 : 1 : 1) and chromatographed on a column of alumina (Woelm neutral, 300 g, 2.9×44 cm) with the same solvent system. The eluate was cut into 10-ml fractions and an EHRLICH-positive substance appeared in fractions Nos. 21~56, which were combined and concentrated to approximately 50 ml. This was treated with Amberlite CG 4B, and concentrated to give a pale yellow syrup (2.13 g), which crystallized on seeding; m.p. 90~92°C. When checked on TLC (silica gel, developed with chloroform - methanol, 7 : 1, detected with EHRLICH reagent or sulfuric acid), the product (Rf 0.23) was almost homogeneous, though a minor impurity (~Rf 0.40) was observed. The amount of impurity was increased when the pH of the culture broth was lower than 6.5 or when the solution became acidic during purification steps. The impurity was removed by recrystallization from acetone yielding pure sphydrofuran (1.35 g): m. p. 99.5~101°C, $[\alpha]_D^{22}$ +18° (*c* 1, water).

In the case of the strain MC340-A1, it was inoculated into a medium consisting of starch 1.0 %, glucose 1.0 %, soy-bean meal 1.5 %, K_2HPO_4 0.1 %, $MgSO_4 \cdot 7H_2O$ 0.1 %, NaCl 0.3 % and a solution (1 ml for 1 liter of the above medium) of metalic salts ($CuSO_4 \cdot 5H_2O$ 700 mg/liter, $FeSO_4 \cdot 7H_2O$ 100 mg/liter, $MnCl_2 \cdot 4H_2O$ 800 mg/liter and $ZnSO_4 \cdot 7H_2O$ 200 mg/liter) and shake-cultured at 27°C for several days. Then the broth (900 ml) was treated as described in the case of MC41-M1 and crystals (0.23 g, m.p. 90~92°C) were obtained. Recrystallization from acetone gave pure sphydrofuran; its IR and NMR spectra were superimposable with those of the sample obtained from the strain MC41-M1.

Chemical and Physical Properties

Sphydrofuran is a neutral substance giving positive reaction with EHRLICH reagent and antimony pentachloride but negative reaction with WOOD[7], ninhydrin, SAKAGUCHI (or diacetyl), triphenyltetrazolium chloride, and RYDON-SMITH reagents. It is also negative under the ultra-violet light.

Fig. 1. Infrared spectrum of sphydrofuran in KBr disk.



Sphydrofuran is soluble in water, methanol, ethanol, acetone, dioxane, pyridine and dimethyl sulfoxide and inosoluble in ether, benzene, chloroform and ethyl acetate.

An aqueous solution of sphydrofuran is stable in the pH range 7~9 and extremely unstable in acidic media.

Elemental analysis: found: C 50.61, H 7.41, O 41.79
calculated for $C_8H_{14}O_5$: C 50.52, H 7.42, O 42.06 %

The infrared spectrum of sphydrofuran is shown in Fig. 1. In the spectrum, only weak absorptions were observed near 1715 cm^{-1} (C=O).

These data indicate that sphydrofuran does not contain indole, urea or pyrrole group in its molecule. The presence of a furan group was also excluded because of the elemental analysis.

Fig. 2. Infrared spectrum of 2-methyl-4-(1-glyceryl)furan (I) in KBr disk.

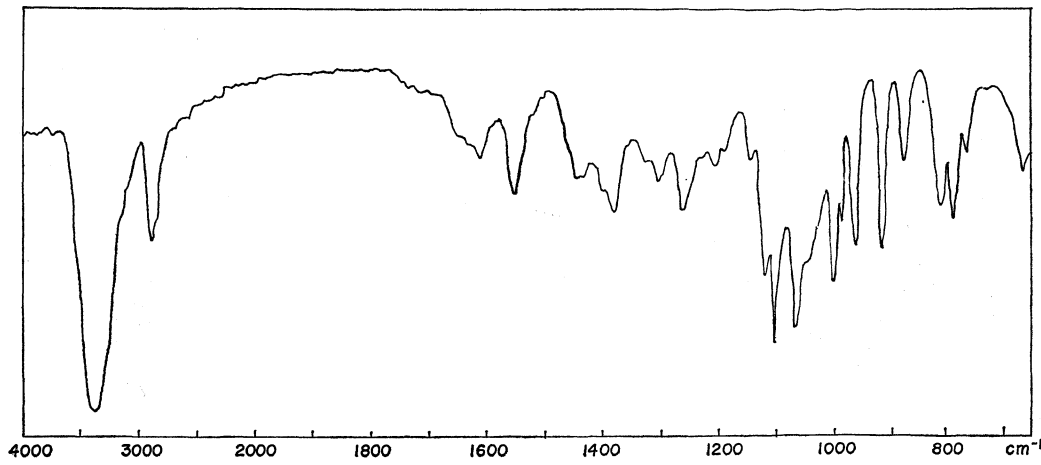
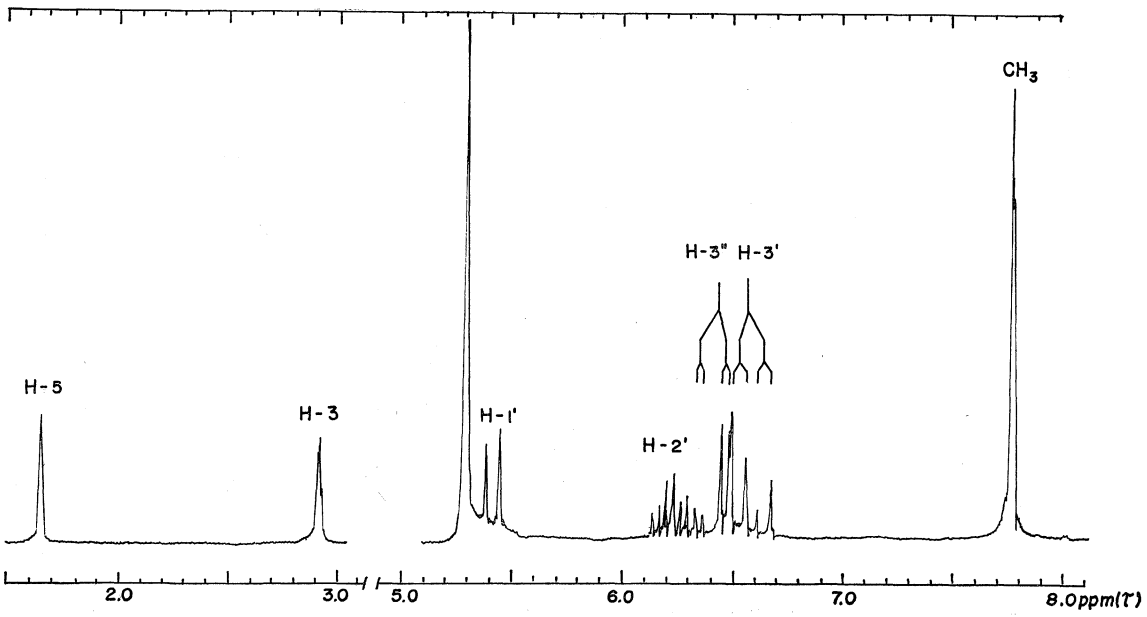


Fig. 3. NMR spectrum of 2-methyl-4-(1-glyceryl)furan (I) in D_2O at 100 MHz.



Acidic Hydrolysis of Sphydrofuran

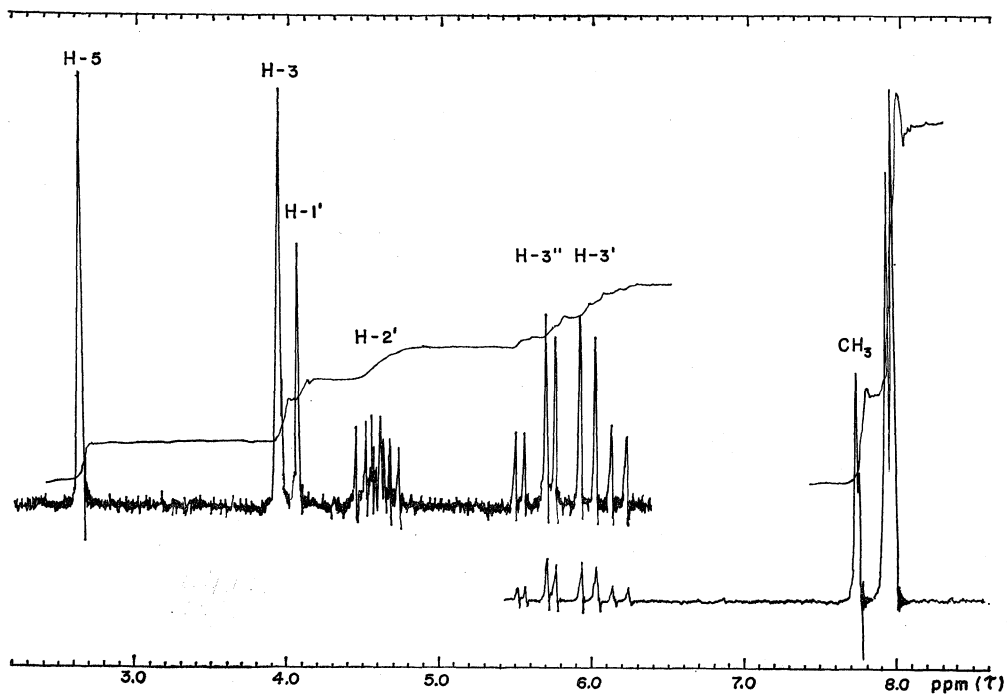
On hydrolysis with 0.1 N hydrochloric acid at room temperature for 2 hours sphydrofuran was changed completely into a product (I), a colorless syrup, $[\alpha]_D^{22}$ −16° (*c* 1, water) which was chromatographically homogeneous and gave the same pink color with EHRLICH reagent as does sphydrofuran.

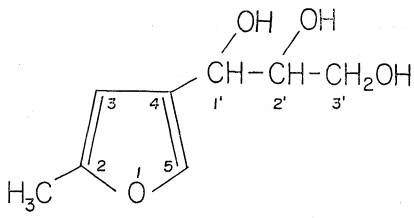
The infrared and NMR spectra of I are shown in Figs. 2 and 3, respectively.

Acetylation of I with acetic anhydride in pyridine gave a triacetate in 88 % yield; syrup, $[\alpha]_D^{22}$ −30° (*c* 1, methanol). In the mass spectrum, the molecular ion appears at m/e 298. The NMR spectrum of II is shown in Fig. 4.

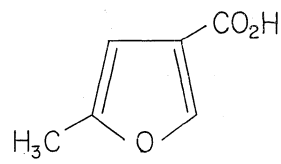
From these NMR spectra, it is suggested that I has a methyl group attached to an aromatic nucleus, two aromatic hydrogens and an α-glyceryl side chain. The details are as follows: the methyl protons of I and II resonate at τ 7.7~7.9 (in deuterium oxide, deuteriochloroform or pyridine-d_5) and show long-range coupling (~1 Hz) with a proton at τ ~3.8. This suggests that the methyl group is attached to an aromatic nucleus. The methine proton (H-2′) of I at τ 6.21 (in D_2O) doubly couples with vicinal methine (H-1′, τ 5.4) and methylene (H-3′, 3″, τ 6.58 and 6.45) protons, and indicates the presence of a sequence $CH_{1'}$-$CH_{2'}$-$CH_{3'}H_{3''}$. Moreover, the down-field shifts of H-1′ and 2′ of the tri-O-acetylated product (II) from that of I indicate that H-1′ and 2′ both bear a hydroxyl group. Considering these data, three hydroxyl groups on I (from the NMR spectrum of II), marked difference between τ values of two aromatic protons, and the chemical formula $C_8H_{12}O_4$ (from the analytical and mass spectral data), the structure I can be deduced.

Fig. 4. NMR spectrum of triacetate (II) of I in $CDCl_3$ at 60 MHz.





(I)



(III)

The structure I was further confirmed by the determination of a product III, obtained by periodate and silver nitrate oxidation of I. I rapidly consumed two equivalents of periodate indicating the presence of a glyceryl side-chain. In the preparative procedure, the periodate oxidation followed by oxidation with alkaline silver nitrate afforded 5-methyl-3-furoic acid (III). The melting point (115°C) accorded with that reported by JONES[8] and the IR and NMR spectra were in good agreement with the structure for III. Moreover, the reported melting points[9] for the other methyl-furoic acids, 4-methyl-2-furoic acid (129°C), 5-methyl-2-furoic acid (109°C), 2-methyl-3-furoic acid (101°C) and 4-methyl-3-furoic acid (139°C) were different from the melting point of III.

Experimental

Thin-layer chromatography (TLC) was performed on silica gel and the chromatograms were visualized by spraying with EHRLICH reagent or sulfuric acid. The NMR spectra were recorded with Varian A-60D and HA-100D spectrometers. Tetramethylsilane (τ 10.00; for the solutions of deuterochloroform, dimethylsulfoxide-d_6 and pyridine-d_5) and sodium 4, 4'-dimethyl-4-silapentane-1-sulfonate (τ 10.00; for the solution of deuterium oxide) were used as the internal standards.

2-Methyl-4-(1-glyceryl)furan (I) A solution of sphydrofuran (628 mg) in 0.1 N hydrochloric acid (3 ml) was kept at room temperature (28°C) for 2 hours. After addition of a small volume of Amberlite IR 45 (OH form), the solution was filtered and evaporated to give a pale yellow syrup (474 mg, 83 %). TLC: Rf 0.40 (chloroform - methanol, 7 : 1, visualized by EHRLICH reagent and sulfuric acid), $[\alpha]_D^{25}$ −16° (*c* 1, water). IR spectrum (KBr disk): 3370, 3100 (w, shoulder, ν CH of furan nucleus), 2900, 1610 (w), 1550 (ν C=C of furan), 1383, 1262, 1103 (ν HCOH), 1068 (ν H_2COH), 1000, 963, 918, 880 (furan), 810, 788 cm^{-1}.

NMR spectrum (in D_2O at 100 MHz): τ 7.78 (3H d, $J_{CH_3,3}$ ~1 Hz, CH_3), τ 6.58 (H-3') and 6.45 (H-3'') (eight-line signals with the inner four being most intense, typical for the AB part of an ABX system), $J_{3',3''}$ 12 Hz, $J_{3',2'}$ 6.6 Hz, $J_{3'',2'}$ 3.6 Hz, τ 6.21 (1H sextet, H-2'), $J_{2',3''}$ 3.6 Hz, $J_{2',3'}$~$J_{2',1'}$~6.5 Hz, τ 5.42 (1H doublet, which sharpened on irradiation at H-5, $J_{1',2'}$ 6.4 Hz, H-1'), τ 5.3 (moved with temperature, OH), τ 3.92 (1H short-range multiplet, which collapsed to a singlet on irradiation at τ 7.78, H-3), τ 2.65 (1H singlet, which sharpened on irradiation at H-1'; H-5). In pyridine-d_5 at 100 MHz: τ 7.85 (3H d, CH_3), 5.58~6.02 (3H ABC multiplet, H-2', 3', 3''), 4.77 (H-1'), 3.64 (H-3), 2.34 (H-5).

Found: C 55.59, H 7.26, O 37.24. Calcd for $C_8H_{12}O_4$: C 55.80, H 7.03, O 37.17 %.

Triacetate (II) of I To an ice-cold solution of I (363 mg) in pyridine (3.5 ml) a cold mixture of acetic anhydride and pyridine (1 : 2, 3.9 ml) were added and the solution was allowed to stand at room temperature for 20 hours. After addition of a little water, the solution was evaporated and the residue was dissolved in chloroform. The solution

was washed successively with water, potassium bisulfate solution, water, sodium bicarbonate solution and water, and dried over sodium sulfate. Evoporation gave a syrup which contained a small amount of EHRLICH-negative by-product(s) (TLC : Rf 0~0.3 with benzene - ethyl acetate 5 : 1). The by-product was removed from II (Rf 0.4, active to EHRLICH reagent) by column chromatography on silica gel (Mallinckrodt CC-4, 200~330 mesh, 1.7 ×20 cm) with benzene - ethyl acetate (5 : 1). The fraction containing II (40~140 ml) was evaporated to give a colorless syrup (552 mg, 88 %), $[\alpha]_D^{20}$ −30° (*c* 1, methanol).

Found : C 56.14, H 6.04, O 37.55. Calcd for $C_{14}H_{18}O_7$: C 56.37, H 6.08, O 37.55 %.

Mass spectrum (m/e) : 298 (molecular ion), 238, 196, 154, 153, 149, 136, 111. IR spectrum (KBr disk) : 3100 (w), 2900, 1745 (ester), 1610 (w), 1550, 1435, 1370, 1220, 1250 (ester), 1125, 1050, 955, 918, 865 (w), 810, 780 cm^{-1}.

NMR spectrum (in $CDCl_3$ at 60 MHz) : τ 7.91 (6H s, CH_3CO), 7.89 (3H s, CH_3CO), 7.72 (3H d, J ~1 Hz, CH_3), 6.03 (H-3′) and 5.69 (H-3″) (eight line signals typical for the AB part of an ABX system), $J_{3',3''}$ 12 Hz, $J_{3',2'}$ 6.0 Hz, $J_{3'',2'}$ 3.5 Hz, 4.58 (1H double quartets, J 6.0, 3.5 and 7.2 Hz, H-2′), 4.02 (1H d, $J_{1',2'}$ 7.2 Hz, H-1′), 3.95 (1H short-range multiplet, H-3), 2.63 (1H s, H-5). On irradiation at H-2′, H-3′ and 3″ collapsed to an AB quartet centered at τ 5.86. On simultaneous irradiation at τ 6.03 and 5.69, H-2′ collapsed to a doublet (J 7 Hz). In pyridine-d_5 at 100 MHz : τ 8.02, 7.95 and 7.91 (each 3H s, CH_3CO), 7.86 (CH_3), 5.78 (H-3′), 5.42 (H-3″), 4.23 (H-2′), 3.80 (H-3), 3.65 (H-1′), 2.37 (H-5).

Periodate oxidation of I

a) Analytical : To a solution of I (16.65 mg, 0.097 mmole) in water (7.75 ml), 0.1 M sodium metaperiodate in acetate buffer (0.05 M acetic acid and 0.05 M sodium acetate, 7.75 ml) was added and the solution was allowed to stand at room temperature in the dark. Aliquots were removed at intervals and the periodate consumption was determined by the arsenite method[10]. After 30 minutes the consumption of the periodate per mole of I was 1.85 moles and after 30 hours, 2.05 moles.

b) Preparative : To a solution of I (425 mg) in water (10.5 ml), 0.4 M sodium metaperiodate (10.5 ml) was added and the solution was allowed to stand for 3 hours. After addition of ethylene glycol (0.1 ml), the solution was neutralized with sodium bicarbonate and the resulting mixture was extracted with ethyl ether (15 ml). By TLC, a sole product (Rf 0.55 with chloroform) was recognized. The ethereal solution was concentrated to approximately 1 ml, ethanol (15 ml) was added, and then a solution of silver nitrate (850 mg) in water (9 ml) was added. The intermediary furyl aldehyde was oxidized to furyl acid (III) by gradual addition of 1 N sodium hydroxide (11 ml) under stirring. The reaction mixture was filtered, concentrated to approximately 10 ml, adjusted to pH 1 with 50 % sulfuric acid and the solution was extracted with chloroform (15 ml). The solution was washed with water, dried over sodium sulfate, and evaporated to give a solid (154 mg, 49 %). Recrystallization from water gave crystals, m.p. 114.5~115.5°C (lit[8] 114~115°C).

Mass spectrum (m/e) : 126 (molecular ion), 109, 81, 80. IR spectrum (KBr disk) : 1685 (CO_2H), 1550 (furan), 1445, 1305, 1220, 1145, 970, 910, 840, 760 (furan) cm^{-1}. NMR spectrum (in $CDCl_3$) : τ 7.67 (3H s, CH_3), 3.60 (1H short-range multiplet, H-4), 2.00 (1H s, H-2), −0.8 (1H, CO_2H).

References

1) UMEZAWA, S.; T. TSUCHIYA, K. TATSUTA, Y. HORIUCHI, T. USUI, H. UMEZAWA, M. HAMADA & A. YAGI : A new antibiotic, dienomycin. I. J. Antibiotics 23 : 20~27, 1970
2) UMEZAWA, S.; K. TATSUTA, Y. HORIUCHI, T. TSUCHIYA & H. UMEZAWA : Studies on dienomycins. II. J. Antibiotics 23 : 28~34, 1970
3) WAKSMAN, S. A. : The Actinomycetes, Vol. II, pp. 287~288, The Williams & Wilkins Co., Baltimore, 1961
4) TREJO, W. H. & R. E. BENNETT : *Streptomyces* species comprising the blue-spore series. J. Bact. 85 : 676~690, 1963

5) SHIRLING, E. B. & D. GOTTLIEB : Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from the second, third and fourth studies. Internat. J. Systematic Bacteriol. 19 : 483, 1969
6) KUPFER, D. & D. E. ATKINSON : Quantitative method for determination of indole, tryptophan and anthranillic acid in the same aliquot. Anal. Biochem. 8 : 82～94, 1964
7) WOOD, T. : A reagent for the detection of chloride and of certain purines and pyrimidines on paper chromatograms. Nature 176 : 175～176, 1955
8) JONES, R. G. : 2, 3-Furan, pyrrole and thiophenedicarboxylic acids. J. Amer. Chem. Soc. 77 : 4069～4074, 1955
9) GILMAN, H.; R. R. BURTNER & E. W. SMITH : Orientation in furan nucleus. III. 5-Methyl-3-furoic acid. J. Amer. Chem. Soc. 55 : 403～406, 1933
10) GUTHRIE, R. D. : Methods Carbohydrate Chem., Vol. I, pp. 435～441, Academic Press, New York, 1962