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Antitumor Activity of *Bacillus natto*. V.[1] Isolation and Characterization of Surfactin in the Culture Medium of *Bacillus natto* KMD 2311[2]

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For the purpose of finding out a strain which has stronger cytolytic activity on Ehrlich ascites carcinoma cells, the authors isolated 113 strains of *Bacillus natto* from straws, which were collected at various areas in Japan, and measured the cytolytic activities by cylinder plate method. As the results, the authors found out a strain of *Bacillus natto* (tentatively called KMD 2311) which has the strongest cytolytic activity in the 113 strains. There were at least two kind of cytolytic substances on Ehrlich ascites carcinoma cells in the culture medium of *Bacillus natto* KMD 2311. One was extracted with AcOEt from the culture medium, which constitute approximately 20% of the cytolytic activity in the culture medium and it was stable. This cytolytic substance was purified and colorless crystalline compound (mp 247—249°) was obtained by recrystallization from acetone–petr. ether. Chemical structure of this compound was examined by elementary analysis, infrared, nuclear magnetic resonance, and mass spectroscopy and study of decomposed products. As the results, this compound was proved to be identical with surfactin (mp 140°), which was obtained from culture medium of *Bacillus subtilis* by Kakinuma, *et al.* and from *Bacillus natto* KMD 1126 by the authors. Melting point of this compound (mp 249°) was higher about 100° than that of surfactin (mp 140°), but high mp compound was obtained from surfactin by recrystallization from acetone–petr. ether. From these results, it was indicated that the cytolytic substance was identified with surfactin and dimorphic.

In a previous paper of this series,[4] the authors reported that a strain of *Bacillus natto* (tentatively called KMD 1126), which was newly isolated from "Natto" (fermented beans), has a contact antitumor effect on solid type Ehrlich carcinoma cells. Then, it was reported[5] that there were at least two kinds of cytolytic substances in the culture medium of *Bacillus natto* KMD 1126 and the stable one was proved to be identical with surfactin which was potent clotting inhibitor in the thrombin fibrinogen system obtained from culture fluids of *Bacillus subtilis* by Kakinuma, *et al.*[6] As shown previously,[7] there were several strains in *Bacillus natto* which have different antitumor activities by *in vivo* test. For the purpose of finding out a strain which has stronger cytolytic activity, the authors isolated 113 strains of *Bacillus natto* from straws, which were collected at various areas in Japan, and measured cytolytic activities on Ehrlich ascites carcinoma cells by screening method of Yamazaki, *et al.*[8] As

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2) This work was reported at 93rd Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April 1973.
3) Location: *13-1, Takaramachi, Kanazawa.*
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the results, the authors found out a strain of *Bacillus natto* (tentatively called KMD 2311) which has the strongest cytolytic activity in the 113 strains of *Bacillus natto*. Then, the cytolytic activity in the culture medium was measured according to the method of Shimizu, *et al.*[9] and it was shown that there are more amounts of cytolytic substances in the culture medium than the medium of *Bacillus natto* KMD 1126.

Then purification of the cytolytic substances in the medium was carried out in order to see what compounds have the cytolytic activity. It was observed that there are at least two kinds of cytolytic substances in the culture medium. That is, one substance was extracted with AcOEt at acidic pH from the culture medium, which constitute approximately 20% of the cytolytic activity in the culture medium and the other was not extracted with AcOEt. The former substance was relatively stable. The purpose of the present paper is to describe the isolation and characterization of this stable cytolytic substance in the culture medium of *Bacillus natto* KMD 2311.

Isolation of the Cytolytic Substance

Bacillus natto KMD 2311 was grown in bouillon at 37° for 24 hr on a reciprocal shaker. After the elimination of cells by centrifugation, the culture medium was acidified with HCl, and the resulting precipitate was collected by centrifugation. The precipitate was extracted with AcOEt. The AcOEt extract was evaporated to dryness and the residue was dissolved in a buffer. This solution was poured over a column of Sephadex G-25 and eluted with the buffer. Active fractions were collected, acidified with HCl, and extracted with AcOEt. The AcOEt extract was evaporated to dryness and the residue was dissolved in acetone, and applied to a column of Sephadex LH-20, which was washed with acetone. The eluate containing active fraction was concentrated to dryness, recrystallized from acetone–petr. ether, and 0.86 g of a colorless crystalline compound I, mp 247—249°, $[\alpha]_D$: +39° (c=1, $CHCl_3$), $[\alpha]_D$: −39° (c=1, MeOH) was obtained from 10 liters of the culture medium. A typical results of purification of the cytolytic substance are summarized in Table I.

TABLE I. Purification of the Cytolytic Substance in the Culture Medium of *Bacillus natto* KMD 2311

Purification step	Weight mg	Specific Act. U/mg	Total Act. U	Recovery %
Crude extract	2540	2.5	6350	100
Sephadex G-25	1410	3.8	5370	84.5
Sephadex LH-20	1170	4.3	5030	79.3
Crystalline compound	860	4.8	4130	65.0

Characterization of Compound (I)

1) Amino Acid Composition——The infrared (IR) spectrum of I exhibits absorption bands due to peptide bonds. When I was subjected to acid hydrolysis in a sealed tube at 110° for 20 hr, aspartic acid (Asp), glutamic acid (Glu), valine (Val), and leucine (Leu) were detected by amino acid analyzer in the molecular ratio of 1:1:1:4. Simultaneously, yellowish brown colored HCl-insoluble oily material was liberated with the recovery of about 20% of original substance. This oily material did not contain N and showed in IR spectrum typical features characteristic to fatty acids. From these data, I is confirmed to be a peptide lipid composed of amino acids and fatty acids.

9) R. Shimizu, N. Nishita, K. Banto, S. Koshimura, A. Hayashi, and T. Kobayashi, *Annual Report of the Research Institute of Tuberculosis Kanazawa University*, **22**, 27 (1964); M. Hatano, R. Shimizu, O. Morita, and T. Yamagishi, *Medicine and Biology*, **74**, 293 (1967).

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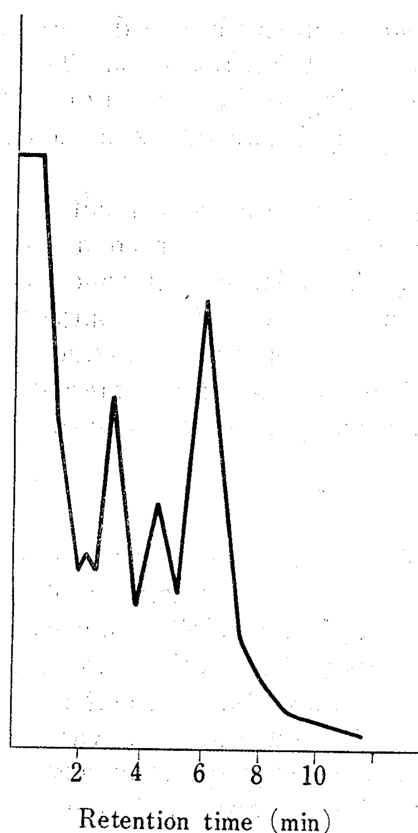

Fig. 1. Gas Chromatogram of Fatty Acid Methyl Ester on SE-30

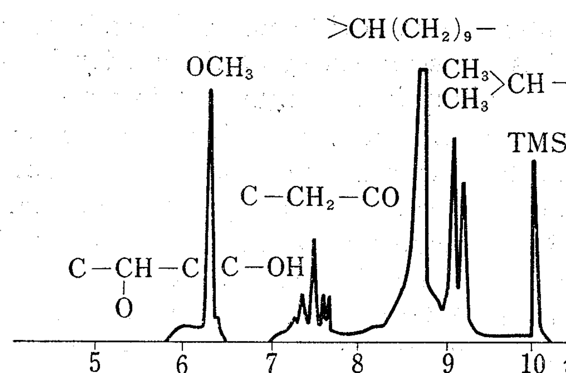

Fig. 2. NMR Spectrum of Fatty Acid Methyl Ester ($CDCl_3$)

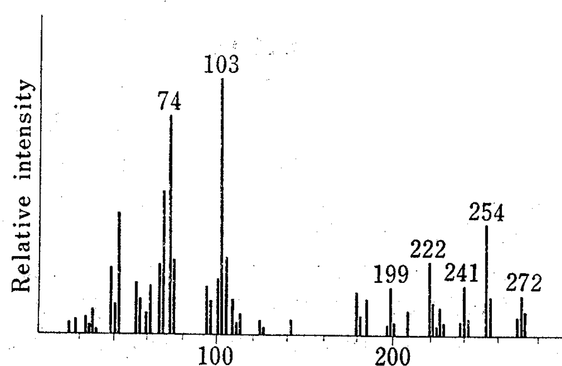

Fig. 3. Mass Spectrum of Fatty Acid Methyl Ester

2) **Structure of Fatty Acid**——I was dissolved in 30% HCl–MeOH and heated at 90° for 14 hr in a sealed tube. After methanolysis, HCl–MeOH was removed by evaporation and the residue was extracted with petr. ether. Separation of fatty acid methyl ester and acyl amino acid methyl ester from the petr. ether extract was accomplished by silicic acid column chromatography, using chloroform and chloroform–MeOH (4: 1) as developing solvents. The fatty acid methyl ester, which was obtained from chloroform eluate, was consisting of 3 components on gas chromatogram (Fig. 1). That is, main peak which had the longest retention time and two minor peaks were present on gas chromatogram. The main peak which was isolated by preparative gas chromatography, was determined to be methyl 3-hydroxy-13-methyltetradecanoate by the following nuclear magnetic resonance (NMR) and mass spectrum. NMR spectrum (Fig. 2). Doublet at 9.2 τ (6H, J=6.0 cps) is regarded to indicate the presence of CH_3-ÇH-CH_3 in this methyl ester. The presence of OH was further verified from the fact that the signal at 7.35 τ (1H, singlet) corresponding to OH disappeared after treatment of this preparation in D_2O. Mass spectrum (Fig. 3). From the parent peak (*m/e*, 272) and the dehydration peak thereof (*m/e*, 254), the molecular weight of this methyl ester was determined to be 272. The base peak at *m/e*: 103 due to the fragment -CH(OH)-CH_2-$COOCH_3$ together with the peak at *m/e*: 199 due to $(CH_3)_2$CH-$(CH_2)_9$-CH(OH)-, shows clearly the attachment of OH to the C_3 carbon. These data was completely identical to the data of surfactin reported by Kakinuma, *et al.*[6] and the authors.[5] Two minor peaks were proved to be C_{13}- and C_{14}-3-hydroxy acids from comparison of retention time with the authentic normal 3-hydroxy acids of C_{11}-C_{14}. That is, the cytolytic substance composing fatty acid was determined to be 3-hydroxy-13-methyltetradecanoic acid (C_{15}-iso-β-OH acid) and C_{13}- and C_{14}-β-OH iso acids were also detected by gas chromatography as minor components. On the other hand, the acyl amino acid methyl ester, which was isolated by silicic acid chromatography after methanolysis was subjected to acid hydrolysis, and Glu was detected by amino acid

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analyzer. This results suggested that there was amide linkage between carboxyl group of the fatty acid and amino group of Glu.

3) Amino Acid Sequence——Amino acid sequence of I was determined by mass spectrometry.[10] When I was dissolved in EtOH, and treated with alkali, at room temperature, II was obtained as colorless crystalline product, mp 189—191°, $[\alpha]_D$: +12° (c=2, MeOH). II was permethylated according to the method of Hakomori,[11] and measured mass spectrum (Fig. 4). From the parent peak (*m/e*: 1207) and the demethoxy peak thereof (*m/e*: 1176), the molecular weight of this material was determined to be 1207. The next peak at *m/e* 1144 is due to the further loss of MeOH from the fatty acid moiety of II. The mass difference of 127 between *m/e* 1176 and *m/e* 1049 corresponds to the loss of N-methylated leucine (Me-Leu) indicated that the C-terminal amino acid in II is Leu. The peaks at 922, 779, 666, 539, and 412 can be regarded to have derived by the further successive elimination of Me-Leu, Me-Asp-OMe, Me-Val, Me-Leu, and Me-Leu from *m/e* 1049, respectively. The remaining amino acid, Glu is therefore directly linked to the fatty acid moiety. From these result, II was proved to be identical amino acid sequence with surfactin.

4) Location of Lactone Ring——As mentioned above, fatty acid moiety and amino acid sequence of I was identical with that of surfactin, but melting point of I (mp 249°) was higher

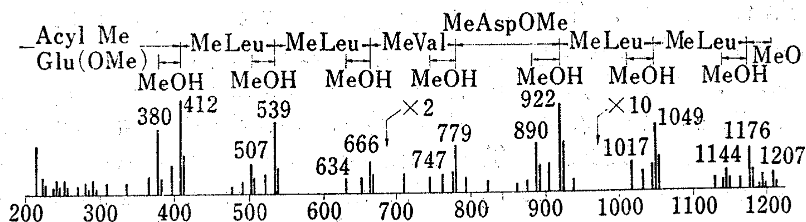

Fig. 4. Mass Spectrum of the Permethylated Derivative of Alkali Treated Cytolytic Substance (II)

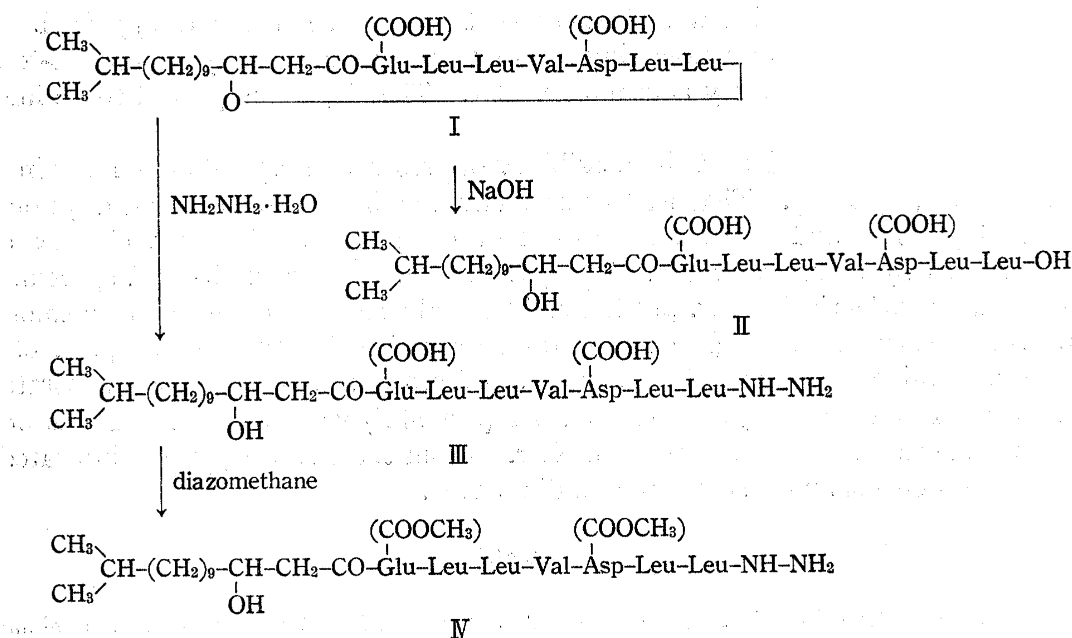

Chart 1

10) E. Lederer and B.C. Das, "Peptides," Proceeding of the Eighth European Peptide Symposium (Noordwijk, Netherlands), ed. by H.C. Beyerman, A. van de Linde, and W. Massen van den Brink, North Holland Publishing Company, Amsterdam, p. 131 (1967); M.M. Shemyakin, Yu. A. Ovchinnikov, A.A. Kiryushkin, E.I. Vinogradova, A.I. Miroshnikov, Yu.B. Alakhov, V.M. Lipkin, Yu.B. Shvetsov, N.S. Wulfson, B.V. Rosinov, V.N. Bochkarev, and V.M. Burikov, *Nature*, **211**, 361 (1967).

11) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).

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Table II. Determination of the Location of Lactone Ring in the Cytolytic Substance

Treatment				Relative amounts of amino acids in hydrolysate			
Hydrazine	Diazo-methane	$LiBH_4$	Curtius	Asp	Glu	Val	Leu
−	−	−	−	1.0	1.0	1.0	4.2
−	−	+	−	0.9	0.8	1.0	3.2
+	+	+	−	0.0	0.0	1.0	3.8
+	−	−	+	1.0	0.9	1.0	3.4

hydrazine: converted to hydrazide (III) by treatment with hydrazine hydrate
diazomethane: esterification by treatment with diazomethane
$LiBH_4$: reduction with $LiBH_4$
Curtius: Curtius degradation of hydrazide(III) with $NaNO_2$ and HCl

about 100° than that of surfactin (mp 140°). Therefore, the authors studied the location of lactone ring. That is, lactone group in I was reduced with $LiBH_4$ to alcohol, hydrolyzed with HCl, and measured amino acid composition. As shown in Table II, 1 mole of Leu was disappeared by this treatment. This result suggest that carboxyl group of C-terminal Leu formed lactone ring with β-hydroxy group of fatty acid moiety. Also, this result was proved from next experiments. That is, lactone group in I was treated with hydrazine hydrate to the hydrazide (III). The resulting hydrazide (III) was esterified with diazomethane to methyl ester (IV) and reduced with $LiBH_4$. The reduced product was hydrolyzed with HCl and amino acid composition was measured by amino acid analyzer. As shown in Table II, Asp and Glu were disappeared by these treatment. This result suggest that carboxyl groups of Asp and Glu in I were free. The hydrazide (III) was treated with $NaNO_2$, and the resulting azide was converted to amine by Curtius degradation. After HCl hydrolysis, amino acid composition was measured and it was observed that 1 mole of Leu was disappeared. From these results, it was proved that lactone ring was formed between carboxyl group of C-terminal Leu and β-hydroxy group of fatty acid moiety of I. That is, I was proved to be identical with surfactin.

Then, the authors studied that the possibility of presence of crystalline solvent in I (mp 249°) or surfactin (mp 140°). That is, I or surfactin was heated at 90° for 4 days on P_2O_5 under reduced pressure and measured mp of these compounds. However, changes of mp were not observed by these treatment. Then, the authors observed that IR spectrum of I was different with surfactin in KBr, but it was identical with that of surfactin in chloroform solution. Also, when surfactin (mp 140°) was recrystallized from acetone–petr. ether, I (mp 249°) was obtained, and when I (mp 249°) was dissolved in acetone and evaporated the solvent under reduced pressure, colorless powder (mp 148°) was obtained and this product showed same IR spectrum with surfactin in KBr. From these results, it was indicated that I (mp 249°) was identified with surfactin and dimorphic.

Experimental

Animal——Female ICR-SLC mice, weighing 18—22 g, were used for all experiments. All animals were fed on standard laboratory diet and given water *ad libitum.*

Tumor——Ehrlich ascites carcinoma was maintained by weekly intraperitoneal transplantation in ICR-SLC mice.

Buffer——Buffer, containing 8 g of NaCl, 0.2 g of KCl, 2.9 g of $Na_2HPO_4 \cdot 12H_2O$, 0.4 g of KH_2PO_4 in 1000 ml of H_2O and adjusted to pH 7.2, was used for all experiments.

Bacterial Strain and Culture——*Bacillus natto* KMD 2311 was newly isolated from straw. That is, 113 strains of *Bacillus natto* were isolated from straws, which were collected at various areas in Japan and measured cytolytic activity on Ehrlich ascites carcinoma cells by screening method of Yamazaki, *et al.*[8] A strain which has the strongest cytolytic activity in the 113 strains of *Bacillus natto* was tentatively called

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KMD 2311 and kept in this laboratory on nutrient agar slants. Culture was carried out by the following manner. To 3 liters shaking flasks, 800 ml of bouillon (pH 7.2) were added, sterilized in an autoclave under 1.0 kg/cm^2 pressure for 20 min, and inoculated with 50 ml of seed culture of the bacteria, which was prepared by shaking culture of the organisms at 37° for 15 hr in the same medium. The culture was carried out at 37° with reciprocal shaking (130 c/min, stroke 7 cm) for 24 hr.

Preparation of Tumor Cell Suspension——The ascites fluid, aspirated from mice bearing 6—9 days old Ehrlich ascites carcinoma by sterile syringe, was suspended in chilled buffer and centrifuged at 700 rpm for 3 min. The sedimented tumor cells were washed twice with chilled buffer and resuspended in appropriate volume of the buffer and then the tumor cell count was made in a hemocytometer by the standard method for blood leucocytes. The tumor cell suspension was further diluted, if necessary, to give a desired cell concentration (5×10^6 cells/ml for cylinder plate method, 2.5—3.5×10^7/ml for determination of *in vitro* cytolytic activity).

Cylinder Plate Method of Screening of the Cytolytic Activity by Yamazaki, *et al.*[8)]——Hanks agar: 8 g of NaCl, 0.4 g of KCl, 0.2 g of $MgSO_4\cdot7H_2O$, 0.14 g of $CaCl_2$, 0.15 g of $Na_2HPO_4\cdot12H_2O$, 0.06 g of KH_2PO_4, 1 g of dextrose, 0.02 g of phenolred were dissolved in 1000 ml of H_2O. Powdered agar was added at 2% to this solution and it was sterilized under 0.5 kg/cm^2 for 10 min.

Hanks agar was heated and melted, and made to 40°. To this solution, the same volume of the Ehrlich ascites carcinoma cells suspension, which was warmed to 40° was added, well mixed, and 10 ml of the mixture was placed in Petri dish. Penicylinders were placed on the solidified agar, and the cylinder was filled with the culture broth of test bacteria. The plate was placed in the incubator at 37° for 18 hr. The cylinders were removed, and 4 ml of dye solution (32 mg of 2,6-dichlorophenol-indophenol sodium was dissolved in 100 ml of the buffer. Before use, the same volume of horse serum was added to the dye solution) was added to the plate. The plate was placed in the incubator for 2 hr and the diameter of the blue inhibition zone was measured.

Cytolytic Activity by Shimizu, *el al.*[9)]——The reaction mixture, containing 0.2 ml of tumor cell suspension and 0.2 ml of appropriately diluted samples, were incubated in water bath at 37° for 2 hr. After incubation, they were diluted with 3.6 ml of the buffer, and centrifuged at 3000 rpm for 10 min. Absorbance of the supernatant was measured at 260 nm after 10 fold dilution (HITACHI Model 101 Spectrophotometer). At the same time, 2 control experiments were run using following 2 mixtures and absorbance of the controls were deducted from that of sample. 1) A mixture of 0.2 ml of the tumor cell suspension and 0.2 ml of the buffer. 2) A mixture of 0.2 ml of the sample and 0.2 ml of the buffer. One unit of the cytolytic activity was defined as the amount of cytolytic substance which gave an E_{260} reading of 1 under above conditions. Therefore, total activity was expressed as follows.

Total Activity (unit) $=E_{260}\times\text{Dilution}\times5\times\text{Volume}$

Purification of the Cytolytic Substance——*Bacillus natto* KMD 2311 was cultured at 37° for 24 hr in 12 flasks (dispense volume in a flask: 800 ml) by a reciprocal shaker. The culture broth were collected and continuously centrifuged at 15000 rpm for 200 ml/min. The supernatant was acidified with HCl and the resulting precipitate was separated by centrifugation at 10000 rpm for 20 min. The precipitate was extracted with AcOEt and the AcOEt extract was evaporated under reduced pressure. The residue was washed with petr. ether and the residue was dissolved in the buffer. The solution (50 ml) was poured over a column of Sephadex G-25 (3.6×50 cm) equilibrated with the buffer. The effluents were fractionated in 20 ml portion at a rate of approximately 150 ml/hr. Absorbance at 280 nm and cytolytic activity of the fractions were measured. The active fractions were collected, acidified with HCl, and extracted with AcOEt. The AcOEt extract was evaporated under reduced pressure and the residue was dissolved in small amount of acetone. This solution was poured over a column of Sephadex LH-20 (3×40 cm) equilibrated with acetone, and filtration through the gel was carried out with the same solvent. The effluents were fractionated in 20 ml portion at a rate of approximately 100 ml/hr. The solvent was evaporated under reduced pressure and the weight of residual material and cytolytic activities in each fractions were measured. The active fractions were collected, concentrated to dryness, and recrystallized from acetone–petr. ether. Colorless crystalline compound (0.86 g), mp 247—249°, $[\alpha]_D$: +39° (c=1, $CHCl_3$), $[\alpha]_D$: −39° (c=1, MeOH) was obtained from 10 liters of the culture medium. *Anal.* Calcd. for $C_{53}H_{93}O_{13}N_7$: C, 61.41; H, 9.05; N, 9.46. Found: C, 60.79; H, 9.05; N, 9.33.

Determination of Amino Acid Composition of the Cytolytic Substance (I)——Sample (10 mg) and 6 N HCl (1 ml) were added to a sealed tube and heated at 110° for 20 hr. After cooling, a content of the tube was taken out and extracted with ether. The aqueous layer was evaporated under reduced pressure to dryness. The residue was dissolved in a small amount of H_2O and applied to amino acid analyzer (HITACHI Amino Acid Analyzer Type KLA-2).

Isolation of Fatty Acid from the Cytolytic Substance (I)——Sample (0.5 g) was dissolved in 50 ml of 30% HCl–MeOH and heated at 90° in a sealed tube for 14 hr. After methanolysis, the HCl–MeOH was removed by evaporation and the residue was extracted with petr. ether. The extract was evaporated under reduced pressure. The oily material thus obtained, was dissolved in chloroform and poured over a column of silicic acid (2×30 cm) and the column was developed with chloroform. The chloroform eluate was concentrated by evaporation and obtained about 93 mg of fatty acid methyl ester. After that, the column was eluted

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with chloroform–MeOH (4: 1). The chloroform–MeOH eluate was concentrated to dryness and obtained 10 mg of oily material. The structure of fatty acid methyl ester was examined by gas chromatography, NMR, and mass spectroscopy. Gas chromatography of the methyl ester was carried out using a 2 m column of 1.5% SE-30 chromosorb w at 180°. The flow rate of the He carrier gas was 60 ml/min. (SHIMADZU Gas Chromatograph GC-4B). NMR spectra were recorded on NIHONDENSHI JNM C-60H spectrometer using TMS as the internal standard. Mass spectra were registered on NIHONDENSHI JMS-01SG spectrometer. The chloroform–MeOH eluate was examined amino acid composition after acid hydrolysis by amino acid analyzer.

Alkali Treatment of the Cytolytic Substance (I)——I (0.1 g) was dissolved in 2 ml of EtOH and added 0.2 ml of 2 N NaOH. After standing overnight at room temperature, the resulting precipitate was filtered off, dissolved in H_2O, acidified with HCl, and extracted with AcOEt. The extract was evaporated under reduced pressure and the residue was recrystallized from acetone–petr. ether. Fifty mg of II was obtained as colorless powder, mp 189—191°: $[\alpha]_D$: +12° (c=2, MeOH). *Anal.* Calcd. for $C_{53}H_{95}O_{14}N_7$: C, 60.37; H, 9.08; N, 9.30. Found: C, 59.82; H, 8.95; N, 9.43.

Determination of Amino Acid Sequence of II——Permethylation of II was carried out by the procedure of Hakomori.[11] Sodium hydride oil suspension (30 mg) was rinsed 3 times with dry ether. Dimethyl sulfoxide (0.3 ml) was added and heated at 100° under a nitrogen stream, until H_2 evolution ceased, and cooled to room temperature. To this solution, 3 mg of II was added and allowed to stand under a nitrogen stream for 10 min at room temperature. Then, 0.3 ml of methyl iodide was added and further allowed to stand for 1.5 hr at room temperature. The reaction mixture was diluted with H_2O and was followed by extraction of the methylated products with chloroform. The chloroform extract was evaporated under reduced pressure. The residue was subjected to measurement of mass spectrum without further purification.

Preparation of the Hydrazide (III) from I——To a solution of 220 mg of I in 5 ml of EtOH, 1.5 ml of 80% hydrazine hydrate was added and allowed to stand overnight at room temperature. The resulting precipitate was collected by filtration and recrystallized from MeOH–H_2O. The hydrazide III (130 mg) was obtained as colorless needles, mp 260—263°. *Anal.* Calcd. for $C_{53}H_{97}O_{13}N_9$: C, 59.58; H, 9.15; N, 11.80. Found: C, 59.22; H, 9.10; N, 11.86.

Determination of Location of the Lactone Ring——1) To a solution of 32 mg of I in 15 ml of dry tetrahydrofuran (THF), 30 mg of $LiBH_4$ was added and heated for 6 hr under reflux with stirring. After cooling, 10 ml of 60% MeOH was added with stirring and concentrated to dryness under reduced pressure. The residue was dissolved in H_2O, acidified with HCl, and extracted with AcOEt. The AcOEt extract was evaporated to dryness under reduced pressure. The remaining substance (15 mg) and 1 ml of 6 N HCl were added to a sealed tube and heated at 110° for 20 hr. After cooling, a content of the tube was taken out and extracted with ether. The aqueous layer was evaporated under reduced pressure to dryness. The residue was dissolved in a small amount of H_2O and applied to amino acid analyzer.

2) To a solution of 104 mg of III in 20 ml of MeOH, an excess amount of diazomethane solution was added and allowed to stand overnight at room temperature. The solution was evaporated to dryness under reduced pressure and the remaining product was recrystallized from EtOH–H_2O. A colorless crystalline IV (45 mg) was obtained, mp 218—220°. *Anal.* Calcd. for $C_{55}H_{101}O_{13}N_9$: C, 60.25; H, 9.28; N, 11.50. Found: C, 60.66; H, 9.02; N, 10.87.

To a solution of 20 mg of IV in 20 ml of dry THF, 70 mg of $LiBH_4$ was added and heated for 6 hr under reflux with stirring. After cooling, 10 ml of 60% MeOH was added with stirring and concentrated under reduced pressure. The liberated oily material was extracted with AcOEt and the extract was evaporated to dryness under reduced pressure. The residue was hydrolyzed with HCl at 110° for 20 hr and applied to amino acid analyzer.

3) To a solution of 14 mg of the hydrazide (III) in 1 ml of AcOH, 60 mg of $NaNO_2$ in 1 ml of H_2O solution was added with cooling and stirring. After standing for 1 hr, 2 drops of 18% HCl was added, warmed over a micro burner until foaming ceased, and then 2 drops of conc. HCl was added. The solution was evaporated to dryness and the resulting residue was hydrolyzed with HCl at 110° for 20 hr and applied to amino acid analyzer.

Preparation of I (mp 249°) from Surfactin (mp 140°)——Surfactin (550 mg) was dissolved in acetone (1 ml), and added petr. ether (10 ml). The resulting crystalline compound (490 mg) was filtered off and measured melting point, mp 245—249°.

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