Lipids of *Streptomyces sioyaensis*

Part II. Lipid Composition of *Streptomyces sioyaensis*

By Akira KIMURA, Jun'ichi KAWANAMI and Hideo OTSUKA

Shionogi Research Laboratory, Shionogi & Co., Ltd.,

Fukushima-ku, Osaka

Received June 28, 1967

Lipids were extracted with chloroform-methanol from *Streptomyces sioyaensis* and fractionated on a silicic acid column. Lipids of *Streptomyces sioyaensis* were mainly composed of neutral lipids, cardiolipin, phosphatidylethanolamines, phosphatidylinositolmonomannoside and a new lysine-containing lipid.

INTRODUCTION

In a previous report,¹⁾ the pattern of ninhydrin-positive lipids of *Streptomyces sioyaensis* was shown to be quite different from those of the other strains of *Actinomycetales*. The lipids of *St. sioyaensis* contained three ninhydrin-positive lipids which were designated as substances A, B and C in the decreasing order of R_F values on thin-layer chromatography. Substance C was proved to be a new lysine-containing lipid. The present paper deals with the isolation and identification of lipids from *Streptomyces sioyaensis*.

MATERIALS AND METHODS

Organism. *Streptomyces sioyaensis* H-690-3 was used throughout this study. This strain produces a chromopeptide antibiotic called siomycin.²⁾ It was precultured in Bennett's medium containing 1.0% of glucose, 0.1% of yeast extract, 0.1% of beef extract and 0.2% of NZ-amine (the pH was adjusted to 7.2 with 10% NaOH aqueous solution). Five ml of precultured broth was inoculated into Sakaguchi flasks containing the same medium. The flasks were incubated at 28°C for 4 to 5 days on a reciprocal shaker (115 reciprocations per minute, 7.0 cm ampli-

tude). A jar fermenter was operated as follows: temperature, 28°C; aeration, 1.0 v/v/m (20 liters per minute); agitation, 150 r.p.m.; inner pressure, 0.5 kg/cm².

Materials. Silicic acid (Mallinckrodt, 100 mesh) for column chromatography was processed as described by Rouser.³⁾ Diethylaminoethyl (DEAE)-cellulose (Serva 0.76 meq./g) was used in the acetate form according to Rouser's method.⁴⁾ Silica gel G and H (E. Merck, AG, Darmstadt, Germany) for thin-layer chromatography were used without pretreatment. Phosphatidylethanolamine and cardiolipin were prepared from tumor tissues⁵⁾ and bovine heart,⁶⁾ respectively. Phosphatidylglycerol was prepared from spinach leaves according to the method of van Deenen.⁷⁾ Methyl esters of branched chain fatty acids used as standards for gas chromatography were obtained from Applied Science Lab. Inc., State College, Pa., U. S. A.

Preparation of lipids. After harvest by centrifugation, the mycelia were washed with deionized water, dehydrated with acetone, and extracted with

1) A. Kimura, J. Kawanami and H. Otsuka, This Journal, **31**, 441 (1967).

2) H. Nishimura, S. Okamoto, M. Mayama, H. Otsuka, K. Nakajima, K. Tawara, M. Shimohira and N. Shimaoka, *J. Antib.*, **14**, 255 (1961).

3) G. Rouser, G. Kritchevsky, D. Heller and E. Lieber, *J. Am. Oil Chemists' Soc.*, **40**, 425 (1963); G. Rouser, G. Galli and E. Lieber, *J. Am. Oil Chemists' Soc.*, **41**, 836 (1964).

4) G. Rouser, J. O'Brien and D. Heller, *J. Am. Oil Chemists' Soc.*, **38**, 14 (1961).

5) J. Kawanami, T. Tsuji and H. Otsuka, *J. Biochem.*, **59**, 151 (1966).

6) M. Faure and M. J. Coulon-Morelec, *Ann. Inst. Pasteur*, **95**, 180 (1958).

7) F. Haverkate and L. L. M. van Deenen, *Biochim. Biophys. Acta*, **106**, 78 (1965).

chloroform-methanol (CM 2:1, v/v). Extraction was repeated several times at room temperature until no more lipids were detected in the solvent layer. The mycelia were finally extracted at 70°C. Extracts were dried in vacuo, and subjected to Folch's partition dialysis⁸⁾ to remove water-soluble substances. Per cent recovery by Folch's procedure was almost 100%. The chloroform layer was concentrated to 100 ml and treated with 20 ml of an aqueous solution of 1% CaCl₂ to prepare the calcium salt. After standing overnight at room temperature, the chloroform layer was separated and evaporated to dryness.

Detection of individual lipids. The extracted lipid fraction was applied to a thin-layer plate of silica gel G and developed with chloroform-methanol-water (65:25:4 or 70:22:2, v/v/v) as solvent system. Individual lipids were detected by Dittmer reagent⁹⁾ and the ninhydrin reagent.

Mild alkaline hydrolysis. The deacylated product of lipid was obtained by Dawson's method.¹⁰⁾ The hydrolysis products thus obtained were compared with those of authentic lipids by paper chromatography (phenol/water/acetic acid/ethanol=80:20:10:12, v/v/v/v; *i*-propanol/NH₄OH=3:1, v/v).

Silicic acid column chromatography. Total phospholipid (300 to 350 mg) was applied to a silicic acid column (20 g) and elution was carried out with chloroform-methanol. The concentration of methanol in chloroform was increased stepwise. One-ml aliquots were removed from each fraction (10 g) and used for the ninhydrin reaction and for phosphorus determination.

Gas-liquid chromatography. The methyl esters of fatty acids were prepared by transesterification of lipids with boron trifluoride-methanol.¹¹⁾

The phosphatidylinositolmannoside fraction (Fraction IV) was methanolized in a sealed tube with 5% HCl in methanol at 105°C overnight. The glycerol, inositol and mannose in the methanolizate were converted to trimethylsilyl derivatives and detected by gas-liquid chromatography with a Shimadzu Gas Chromatograph IB equipped with a hydrogen flame ionization detector. Separation was made under the following conditions: (a) on a 3-m stainless steel

column packed with 15% diethyleneglycol succinate polyester on Chromosorb W (acid washed, siliconized) at 190°C, for the methyl esters of fatty acids; and (b) on a 1.5-m stainless steel column packed with 5% SE-52 on Chamelite CS* at 214°C according to the methods of Sweeley¹²⁾ for trimethylsilyl derivatives of methylglycosides.

Analytical methods. Melting point was determined on a Kofler block (Yanagimoto micro-melting point apparatus) without correction. Optical rotation was measured in chloroform with a Rudolf Photo-electronic Polarimeter, model 200, and infrared absorption spectrum was taken in chloroform and on KBr pellets with a Koken IR spectrophotometer, model 301. Phosphorus was estimated by the method of Allen.¹³⁾ Ninhydrin-positive materials, ethanolamine and lysine, were determined by paper chromatography (*n*-butanol/acetic acid/water=4:1:2, v/v/v) and by an automatic amino acid analyzer (Hitachi KLA-2). Amino-nitrogen was estimated by the method of Moore and Stein.¹⁴⁾ Fatty acids were estimated as esters by the method of Rapport,¹⁵⁾ in which egg-yolk lecithin was used as a standard. Siomycin was estimated by pulp disc diffusion method against *Bacillus subtilis* PCI-219.

EXPERIMENTAL

Separation of lipids on silicic acid column.

The lipid fraction of *St. sioyaensis* prepared as described above was subjected to silicic acid column chromatography, and separated into four fractions (Fig. 1, Table I).

Fraction I. Neutral lipids. Fraction I was eluted with chloroform from the silicic acid column. It gave main spot with an *R_F* value identical to that of tristearin and other minor spots on a thin-layer chromatogram (*n*-hexane/diethyl ether/acetic acid=6:4:0.1, v/v/v), as shown in Fig. 2.

Fraction II. Substance Y (cardiolipin) and Substance Z. Substances Y, Z and siomycin²⁾ were eluted with CM (95:5) from the silicic acid column. They were separated by thin-layer chromatography,

8) J. Folch and M. Lees, *J. Biol. Chem.*, **191**, 807 (1951).

9) J. C. Dittmer and R. L. Lester, *J. Lipid Res.*, **5**, 126 (1964).

10) R. M. C. Dawson, *Biochem. J.*, **75**, 45 (1960).

11) W. R. Morrison and L. M. Smith, *J. Lipid Res.*, **5**, 600 (1964).

* Nishio Kogyo Co., 62 Azuma-cho, Meguro-ku, Tokyo.

12) C. C. Sweeley, R. Bentley, M. Makita and W. W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).

13) R. J. L. Allen, *Biochem. J.*, **34**, 858 (1940).

14) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

15) M. M. Rapport and N. Alonzo, *ibid.*, **217**, 193 (1955).

FIG. 1. Separation of Lipids on Silicic Acid Column Chromatography.

FIG. 2. Neutral Lipids of *St. siayaensis*.

TLC: Hexane/Ether/AcOH=6:4:0.1

The plate was sprayed with 50% sulfuric acid and charred.

FIG. 3. Paper Chromatogram of Deacylated Product of Phospholipids.

PPC: Phenol/H₂O/AcOH/EtOH=80:20:10:12

CL=Cardiolipin, Y=Substance Y.

Spots were detected with the Hanes-Isherwood reagent for phosphorus.

and each of them was eluted from the plate. Although substance Z could not be identified, substance Y was identified to be cardiolipin. The ratio of fatty acid to phosphorus in substance Y was 2.0 (Table II). The deacylated product of substance Y gave a spot with the same R_F value as deacylated cardiolipin by paper chromatography (Fig. 3). Substance Z was found only in trace and appeared to be a decomposed substance of cardiolipin. Its de-

TABLE I. SEPARATION OF LIPIDS ON SILICIC ACID COLUMN CHROMATOGRAPHY

Fraction	Weight (mg)	Phosphorus (μ g)	Ninhydrin	Hexose	Antibacterial activity	Components
I	2105.5	—	—	—	—	{Neutral lipids {(triglyceride)
II	159.6	2500	—	—	+	{Cardiolipin {Substance Z {Siomycin
III	42.6	750	+	—	—	{PE* (A and B)
IV	42.6	460	—	+	—	{Substance C PIM**

* PE=Phosphatidylethanolamine, ** PIM=Phosphatidylinositolmannoside.

TABLE II. MOLAR RATIO OF FATTY ACID AND PHOSPHORUS

Substances	Fatty acid/phosphorus
Lecithin	2.0 (as standard)
Substance Y (cardiolipin)	2.0
Substance Z (unidentified)	1.9
Substance A (PE*)	2.1
Substance B (PE*)	2.0
Substance C (a new lipid)	—
PIM**	1.9
Cardiolipin (bovine heart)	1.8

* PE=phosphatidylethanolamine.

** PIM=phosphatidylinositolmannoside.

acylated product behaved like phosphatide II of Coulon-Morelec¹⁶⁾ on paper chromatography. The ratio of fatty acid to phosphorus in substance Z was 1.9 (Table II).

Fraction III. Three ninhydrin-positive lipids.

Fraction III eluted with CM (80:20) from the silicic acid column contained three ninhydrin-positive lipids designated as substances A, B and C. Substance C was rechromatographed and successfully separated from a mixture of A, B and C by elution with different concentrations of methanol in chloroform. Substance C was eluted with CM (87:13), whereas substances A and B were eluted with CM (90:10). Substance C fraction was chromatographed again over Florisil column to remove concomitant substance B (phosphatidylethanolamine). Substance C was eluted with CM (50:50 to 20:80) from the Florisil column, which decomposed some amount of substance C. Fraction of substance C thus obtained was again chromatographed on silicic acid column to remove the decomposed substance. The substance C thus purified was submitted to chemical analyses. Substances A and B were separated from each other on

preparative thin-layer chromatography with silica gel H and extracted with CM (2:1) from the area where they were detected by iodine vapor. The separated substances A and B were purified by repeated chromatography on silicic acid column, respectively. When acid hydrolysis with 6N HCl was performed at 105°C for 12 hours, paper chromatography and analysis with an automatic amino acid analyzer revealed that substances A and B contained only ethanolamine as the ninhydrin-positive component (Fig. 4), whereas substance C contained only lysine (Fig. 4). Thin-layer chromatography showed that authentic phosphatidylethanolamine was quite similar to substance A from *St. sioyaensis* (Fig. 5). Substances A and B were subjected to mild alkaline hydrolysis.

FIG. 4. Analysis of a Hydrolysate of Ninhydrin-positive Substance with Amino Acid Analyzer.

16) M. J. Coulon-Morelec, M. Faure et J. Marechal, *Bull. Soc. Chim. Biol.*, **42**, 867 (1960).

FIG. 5. Thin-layer Chromatogram of Ninhydrin-positive Lipids from *St. siogaensis*.

TLC: $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}=65:25:4$

PS=Phosphatidylserine, PE=Phosphatidylethanolamine.

Spots were detected with ninhydrin reagent.

The deacylated products of purified substance A, purified substance B, and a mixed sample of substances A and B gave one spot, respectively, on paper chromatography, the R_F values of which were identical with that of deacylated phosphatidylethanolamine (Fig. 6). The ratio of fatty acid to phosphorus was 2.1 for substance A, and 2.0 for substance B (Table II). These results suggested that substances A and B had the same skeleton, and that they were two kinds of phosphatidylethanolamine with a different fatty acid composition (Figs. 9-a, 9-b).

Substance C contained lysine as described above (Fig. 4), but it did not possess phosphorus in its molecule. Its physicochemical properties are summarized in Table III. The infrared absorption spectrum of the substance is shown in Fig. 7. It

FIG. 6. Paper Chromatogram of Deacylated Products of Phospholipids.

PPC: Phenol/ H_2O /AcOH/EtOH=80:20:10:12

PE=Phosphatidylethanolamine, A=Substance A.

B=Substance B, AB=A mixed sample of substances A and B.

Spots were detected with the Hanes-Isherwood reagent for phosphorus.

TABLE III. PHYSICOCHEMICAL PROPERTIES OF SUBSTANCE C
(A New Lysine-containing lipid)

1. M.p. $132\sim 134^\circ\text{C}$
2. Elemental analysis
Found: C, 67.64; H, 11.47; N, 4.09; P, 0
Calcd. for $\text{C}_{38}\text{H}_{78}\text{N}_2\text{O}_7$: C, 67.66; H, 11.57; N, 4.15%
3. Molecular weight (vapour pressure method)
Found 630
Calcd. 674
4. Optical rotation
 $[\alpha]_{23.5}^D = +57. \pm 0.3$ (c 1.150 in chloroform)
5. IR-spectrum (Fig. 7)
3426, 1734, 1643, 1596, 1470, 1386, 1265, 1250, 1135 cm^{-1}

FIG. 7. Infrared Absorption Spectrum of Substance C (KBr).

has characteristic absorptions of the amide group at 1643, and of the primary amine at 1596 cm^{-1} which were not found in the IR-spectrum of lysyl-phosphatidyl glycerol reported by MacFarlane.¹⁷⁾

Fraction IV. Phosphatidylinositolmonomannoside (PIM). A lipid was separated from fraction IV. It was hydrolyzed with 5% HCl in methanol at 105°C overnight. After removal of the fatty acid methyl esters, the hydrolyzates were led to trimethylsilyl derivatives and analysed by gas-liquid chromatography as shown in Fig. 8, in which inositol and mannose were found in the ratio of 1:1. The ratio of fatty acid/phosphorus/hexose (as mannose) was 2:1:1. Therefore, it contained fatty acid, phosphorus, inositol and mannose in the molar ratio of 2:1:1:1.

FIG. 8. Gas-Chromatographic Analysis of Phosphatidylinositolmannoside from *Streptomyces sioyaensis*.

Condition: 5% SE-52 on Chamelite CS, at 180°C.

Fatty acid composition. The fatty acid composition of each lipid was determined by gasliquid chromatography and the results are shown in Figs. 9-a and 9-b. The patterns were similar with each other. Hydrogenation of these fatty acids did not cause any remarkable changes in their patterns, indicating that these fatty acids were saturated. Analysis revealed that these fatty acids were branched ones, so-called iso- and anteiso-acids which were generally found in the lipids of microorganisms. The fatty acid compositions of substances A and B were quantitatively different from each other. Individual fatty acid methyl esters from the neutral lipids were separated by an Aerograph Autoprep A-700 (25% DEGS on chromosorb W, at 190°C, flow rate 100 ml H₂/min.). They were then subjected to an identification of structure with a Hitachi RMU-

FIG. 9-a. Fatty Acid Composition of Lipids from *St. sioyaensis*.

i=iso-fatty acid
a=anteiso-fatty acid

FIG. 9-b. Fatty Acid Composition of Lipids from *St. sioyaensis*.

i=iso-fatty acid

a=anteiso-fatty acid

*PIM=phosphatidylinositolmonomannoside.

17) M. G. MacFarlane, *Nature (London)*, **196**, 136 (1962).

6 Mass Spectrometer (ionizing voltage; 80 V) which gave M minus 15($M - CH_3$) and M minus 65($M - CH_3OH + H_2O + CH_3$) peaks characteristic of iso-fatty acids (performed by Dr. Y. Naya).¹⁸⁾ As a result, it was verified that fatty acid methyl esters from the neutral lipids consisted of an iso-14, and iso-15 and an iso-16 fatty acid. Besides them anteiso-17 fatty acid was contained, though it was not subjected to Mass Spectrometer.

RESULTS AND DISCUSSION

Cardiolipin, phosphatidylethanolamine and phosphatidylinositolmonomannoside are commonly found in the pattern of phospholipids of *Actinomycetales*.¹⁹⁾ On the other hand, *St. sioyaensis* contained cardiolipin, two kinds of phosphatidylethanolamine, phosphatidylinositolmonomannoside and a new lysine-containing lipid.

St. sioyaensis contained three ninhydrin-positive substances (substances A, B and C), whereas many other strains contained only one ninhydrin-positive substance,¹⁾ i.e., phosphatidylethanolamine. The interest in the lipid composition of this microorganism led us to the separation of each lipid. The lipid of *St. sioyaensis* was separated by silicic acid column chromatography into four fractions.

Fraction I contained neutral lipids. Fraction II contained cardiolipin, substance Z, and siomycin which is a chromopeptide antibiotic produced by this microorganism. Fraction III gave three ninhydrin-positive spots by thin-layer chromatography, two of which (substances A and B) were proved to be phosphatidylethanolamines and another one (substance C) was shown to be a new lysine-containing lipid. Fraction IV contained phosphatidylinositolmonomannoside.

Substances A and B were found to be two types of phosphatidylethanolamine with dif-

ferent fatty acid composition.

Substance C containing lysine appeared, at first, to be similar to lysyl-phosphatidyl glycerol reported by MacFarlane¹⁷⁾ and other workers,^{20,21)} but it did not contain phosphorus (Table III). The IR-spectrum of substance C showed the presence of amide linkage (Fig. 7) which was not found in the spectrum of lysyl-phosphatidyl glycerol.¹⁷⁾

Of particular interest is this lysine-containing lipid. The preparation is under way to provide the large amounts of this lipid necessary for a more detailed study.

Recently Depinto reported about ornithine-containing lipid in *Rhodospirillum rubrum*,²²⁾ which did not contain phosphorus. Though physico-chemical property of this ornithine-containing lipid has not been reported R_F value on thin-layer chromatogram and other results suggest the possibility that the lipid has similar structure to ours (though α -amino group of lysine of our lipid is protected...to be published).

Though the physiological role of these lipids are not yet clear, according to Depinto, this ornithine-lipid might be a component of cells,²²⁾ since turn over rate of this lipid was quite low.

Acknowledgement. The authors are indebted to Dr. M. Ebata for his kind support throughout this experiment and to Dr. Y. Naya (The Institute of Food Chemistry) for mass spectral measurement of iso-fatty acids. They are also grateful to the members of the analytical room of this laboratory for various analyses.

20) E. F. Gale and J. P. Folkes, *Biochem. J.*, **94**, 390 (1965).

21) U. M. T. Houtsmuller and L. L. M. van Deenen, *Biochim. Biophys. Acta*, **106**, 564 (1965).

22) J. A. Depinto, *ibid.*, **144**, 113 (1967).

Addendum. Recently Dr. Asselineau suggested, in his private letter, that our lysine-containing lipid is quite similar to the ornithine-containing lipid found in a strain of *Mycobacterium* [*Biochim. Biophys. Acta*, **70**, 99 (1963)].

18) Y. Naya and M. Kotake, *Nippon Kagaku Zasshi* **87**, 482 (1966).

19) K. Kataoka and S. Nojima, The 8th Meeting of Japanese Conferences on the Biochemistry of Lipids, June 4th, 1966.