Structural Composition of Polar Lipid-Amino Acid Complex in Pseudomonas aeruginosa*

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(Received for publication, May 18, 1964)

Previous studies by Silberman and Gaby (1) have shown that alanine and other amino acids are incorporated in the lipids of metabolizing cells of the gram-negative bacillus Pseudomonas aeruginosa. They found a positive correlation between the utilization of alanine as determined by oxygen uptake and the corresponding incorporation of 14C-labeled alanine into the lipid fraction of the cell. This evidence, as well as the results of other investigations (1-4), suggested that phospholipid may be an important intermediate in metabolism of amino acids. The chemical composition of such a lipid-amino acid complex was virtually unknown until Macfarlane (5) reported glyceryl-phosphorylglycerol alanine complex formation in the gram-positive bacillus Clostridium welchii. Additional interest has been placed in this problem since the lipids of the gram-negative bacilli have been reported to contain no phosphatidic acids (6). The present investigation was carried out to elucidate the chemical nature of the lipid-amino acid complex extracted from the gram-negative bacillus Pseudomonas aeruginosa.

EXPERIMENTAL PROCEDURE

Preparation of Cells—P. aeruginosa, strain 83 (Hahnemann stock culture collection), was grown at 37°C on Trypticase soy agar which contained 0.5% dextrose. The addition of dextrose was found to increase the yield of cells. The cells were harvested after incubation for 18 hours by washing the agar with 15 ml of cold NaCl by gently shaking until all visible growth was removed. The cells were washed twice in cold NaCl and their wet weight was determined. Approximately 65 g (wet weight) of cells were harvested per batch.

Incorporation of 14C Labeled Amino Acid into Lipids—The packed cells obtained after centrifugation were diluted with 0.1 M phosphate buffer at pH 7.0 and distributed into Erlenmeyer flasks in proportions of 1 g of cells, wet weight, per 2.5 ml of total incubation mixture. The incubation mixture consisted of phosphate buffer which contained 2 μmoles per ml of DL-alanine-1.14C (5.1 × 10^4 c.p.m. per μmole) (New England Nuclear Corporation). Incubation was carried out at 37°C for 1 hour on a rotary shaker. The cells were centrifuged, washed with cold NaCl until no radioactivity could be detected in the supernatant, and transferred to an Erlenmeyer flask for extraction of lipids.

Extraction and Purification of Lipids—Lipids were extracted from the cells with chloroform-methanol (2:1, v/v) by a modified version of the Folch technique (7). The ratio of extraction solvent to wet weight of cells was 20:1. Extraction was carried out for 8 hours on a rotary shaker at 25°C, after which the solvent mixture was separated from the cellular residue by filtration through glass wool. The chloroform-methanol mixture containing the total lipids was placed in a beaker, overlaid with twice its volume of 0.9% sodium chloride solution, and allowed to stand at room temperature for 18 hours to remove water soluble impurities. The NaCl layer was discarded; the chloroform layer was washed at least 4 times with 0.2 volume of NaCl until no radioactivity could be detected in the NaCl wash and, finally, was washed once with an equal volume of distilled water. The chloroform was dehydrated with anhydrous sodium sulfate, filtered, and evaporated in a vacuum at 40°C to a volume of approximately 5 to 6 ml. The extract was stored at −20°C in the dark until used.

Column Chromatography—Silicic acid (Malinckrodt, 100 mesh) was washed thoroughly with a solvent mixture of acetone and anhydrous ether (1:1, v/v), anhydrous ether, and finally with chloroform to ensure the removal of water as well as nonpolar substances. The conditioned silicic acid was resuspended in chloroform and poured (5 g) into a glass column (1.1 × 55 cm). Three or four such columns were used in each experiment. Chloroform, under a constant pressure of nitrogen, was passed through the columns to facilitate packing of the silicic acid. The lipids (10 mg per g of silicic acid) in 2 to 3 ml of chloroform were applied to each of the columns, and four fractions were collected, consisting of 100 ml each of chloroform (nonpolar fraction), chloroform-methanol, 80:20 (v/v) (Fraction I), chloroform-methanol, 60:40 (v/v) (Fraction II), and absolute methanol (Fraction III). The solvents were passed through the column by nitrogen pressure to give a flow rate of 1 ml per minute. Model experiments, with known mixtures of standard lipids, were run prior to the fractionation of the bacterial lipid extracts.

Thin Layer Chromatography—Thin layer chromatography was performed on all fractions collected from silicic acid columns for the separation and identification of lipid components. The identification of the lipids of Pseudomonas was based on their comparative R_F values with known lipid standards. Synthetic phosphatidyethanolamine (DL-β,γ-dipalmitoylphosphatidylethanolamine) and phosphatidylserine (phosphatidyl-L-serine) were obtained from Mann Research Laboratories, Inc. Traces of other lipid contaminants were removed from the latter by elution from silicic acid columns. Chromatographically pure lysophosphatidylcholine (L-lysolecithin) was obtained from General Biochemicals, Inc. Phosphatidylcholine (t-lecithin)
methanol (1:4, v/v).

plate, scraping off the silica gel from specific areas of duplicate

Radioactivity was determined from thin

solvent under an infrared lamp, and by weighing and counting

detected by pipetting aliquots of the lipid samples into metal

hydrin (0.2% in acetone) and heated at 100° for 1 minute to

not touching. One paper was developed in I-butanol-acetic

components of the lipid-amino acid complexes. Strong acid

used to study the chemical nature of the amino acid-lipid

bonding as well as to isolate and identify the water-soluble

lipids (RF 0.94), non-choline-containing lipids (RF 0.5), and

identified (by comparison with known compounds) as neutral

revealed three distinct components which were tentatively

shown to be identical under these conditions. Total

amino nitrogen (present as primary amines) associated with the

unhydrolyzed lipids was determined by the method of Lea and

Rhodes (18). All the solvents used were of reagent grade (from

Baker and Company, Inc., Mallinckrodt Chemical Works, and

Fisher Scientific Company) and redistilled. To determine the

presence of free hydroxyl groups in phospholipid glycerol, aliquots

of unhydrolyzed lipid fractions (equivalent to 2 to 6 μg of glyce-

col) were treated with periodate-chromotropic acid reagents. The

procedure followed in this method was virtually the same

as the method used for glycerol analysis without the step of pre-
saponification.

RESULTS

The lipids extracted from P. aeruginosa with chloroform-

methanol represented 6.8% of the dry weight of cells. Chroma-
tography of the total lipid extract on thin layer silica gel plates

revealed three distinct components which were tentatively

identified (by comparison with known compounds) as neutral

lipids (RF 0.94), non-choline-containing lipids (RF 0.5), and

choline-containing lipids (RF 0.3). Isolation of the lipid frac-
tions on silicic acid columns was attempted by elution with

chloroform, methanol, and various mixtures of these solvents,
as previously determined in preliminary model experiments, to

separate known mixtures of lipids. The results in Table I

illustrate the separation obtained in each of the four column

fractions. The neutral lipids (phosphorus-free) represent 3 to

4% of the total lipids whereas the polar lipids comprise 95% of

the total lipids of P. aeruginosa. The major portion of the lipids,

water-soluble, deacylated products were identified by ascending

paper chromatography with 98% methanol-formic acid-H2O

(80:13:7, v/v) as the developing solvent.

It has been reported that LiBH4, generally used for the reduc-
tion of aldehydes and ketones, will reduce simple methyl ester

bonds (13). Crawhall and Elliott (14) reported the reduction of

the methyl ester of benzylglycylalanine by LiBH4 under

selected conditions under which the amide linkage was not

affected. This method was used in conjunction with mild

alkaline hydrolysis to compare the reactions of the synthetic

fatty acid amide, palmitoylalanine, with those of the lipid-amino

acid complex extracted from P. aeruginosa. In this procedure,

approximately 20 mg of lipid were added to 10 ml of anhydrous
tetrahydrofuran containing 1 mg of LiBH4. The mixture was

stirred for 20 hours at room temperature, after which methanolic

HCl was added to destroy the excess LiBH4 and, concomitantly,

to neutralize the reaction mixture. After addition of 10 ml of

ether and 5 ml of water, the solvent system separated into two

phases. Both the ether and water phase were evaporated to
dryness and their radioactivity was determined.

Chemical Determinations—Prior to chemical analysis, different

fractions were further purified by rechromatography on silicic

columns and repurification several times in acetone.

Lipid phosphorus was determined by the method of Chen,

Toribara, and Warner (15). However, the digestion mixture

was slightly modified by the use of 1 ml of 60% HClO4 instead

of concentrated H2SO4 and 2 drops of 72% HClO4. Glycerol

determined by the micromethod of Van Handel and Zilver-

smitt (16). Carboxylic esters were analyzed by the micromethod

of Skidmore and Entenman (17). Recrystallized tristearin

(m.p. 69°) was used as a standard. No correction was necessary

since the molar absorbance values of different carboxylic esters

were considered to be identical under these conditions. Total

amino nitrogen (present as primary amines) associated with the

unhydrolyzed lipids was determined by the method of Lea and

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fractions. The neutral lipids (phosphorus-free) represent 3 to

4% of the total lipids whereas the polar lipids comprise 95% of

the total lipids of P. aeruginosa. The major portion of the lipids,
Composition of fractionated lipid extract from P. aeruginosa and distribution of labeled amino acid in different fractions

Cells were incubated with labeled alanine (2 µmoles per ml of D3-alanine-1-14C, 5.1 × 106 c.p.m. per µmole) in phosphate buffer, pH 7, for 1 hour at 37°C. After extraction with chloroform-methanol, the total lipid was fractionated on silicic acid columns with chloroform, methanol, and their different mixtures as eluting solvent. Percentage recovery of lipid-phosphorus was 96%.

<table>
<thead>
<tr>
<th>Major nitrogenous bases*</th>
<th>Total lipid</th>
<th>Total phosphorus</th>
<th>Total radioactivity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>c.p.m. × 1000 mg lip</td>
<td></td>
</tr>
<tr>
<td>Nonpolar</td>
<td>3.2</td>
<td>0</td>
<td>trace</td>
<td>6.6</td>
</tr>
<tr>
<td>Polar lipids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction I: ethanolamine,†</td>
<td>81</td>
<td>84</td>
<td>7</td>
<td>2.9</td>
</tr>
<tr>
<td>serine, several ninhydrin spots</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction II: ethanolamine, serine, several ninhydrin spots</td>
<td>11.8</td>
<td>11.5</td>
<td>17</td>
<td>4.7</td>
</tr>
<tr>
<td>Fraction III: choline, several ninhydrin spots</td>
<td>4</td>
<td>4.5</td>
<td>3</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* Identified chromatographically.
† Estimated on basis of weight.
‡ Traces of sugar and glucosamine were detected chromatographically with aniline-hydrogen phthalate.

FIG. 1. Thin layer chromatography of four fractions on a silicic acid column. The solvent system was chloroform-methanol-water (80:25:3, v/v/v). The lipids were identified by spraying the chromatograms with dichlorofluorescein and examination under ultraviolet light. Amino-N was detected by ninhydrin spray, and phosphacon by Dawson's (0) method. Different areas were scraped, eluted with CHCl3-CH2OH (1:4, v/v), filtered, and analysed for choline and radioactivity. Spot 1, Original lipid extract; 2, nonpolar fraction (absolute chloroform); 3, Fraction I (20% methanol in chloroform); 4, Fraction II (40% methanol in chloroform); 5, Fraction III (absolute chloroform); 6, 7, and 8, authentic samples of phosphatidylyethanolamine, -serine and -choline; 9, pure palmitic acid; 10, palmitoylalanine; horizontal hatching, ninhydrin-positive; vertical hatching, phosphorus-positive; cross-hatching, ninhydrin- and phosphorus-positive; C, choline-positive; radioactivity is represented by broken lines.

as well as the majority of radioactivity, was eluted from the column in the 80:20 chloroform-methanol solvent mixture (Fraction I). Each of the column fractions was further characterized (Fig. 1) by thin layer chromatography. Although the Rf values of the lipids on thin layer plates varied slightly, consistent patterns were obtained and reference compounds were included as controls on all plates. When aliquots from column Fractions I, II, and III were subjected to mild alkaline or acid hydrolysis, paper chromatography of the hydrolysates revealed glycerophosphorylserine as the major component in Fractions I and II with glycerophosphorylethanolamine as present only as a minor component. Fraction III contained glycerophosphorylcholine. Traces of other phosphorus-containing spots, probably resulting from the formation of glycerophosphoric acid during the hydrolytic process, could also be detected. Each of the column fractions was further subjected to complete hydrolysis in 6 N HCl for 12 hours. It is evident from the results shown in Fig. 2 that all three fractions contained ninhydrin-positive compounds other than the native nitrogenous bases of phospholipids. Choline was also present in Fraction III, but was not included in Fig. 2. Alanine was detected in the hydrolysates of all three column fractions. To determine whether the amino group of the associated amino acid was free, as well as to obtain additional evidence for identification of the native nitrogenous bases of the lipid component, an aliquot of Fraction I (containing the bulk of lipids) was treated with fluorodinitrobenzene. Water-soluble derivatives of DNP alanine and DNP-ethanolamine were isolated. However, it can be noted from Fig. 3 that the DNP-alanine derivative was isolated from the lipid component before acid hydrolysis, presumably because of the mild alkaline condition of the experiment. The DNP-ethanolamine derivative could be separated after prolonged hydrolysis by 6 N HCl for 20 hours at 100°C.

Since Fukui and Axelrod (19) identified a fatty acid amide, oleoylphenylalanine, from rat liver, and Ohno, Tajima, and Toki (20) isolated a peptide in amide linkage with a fatty acid from a Pseudomonas species, the possible existence of such compounds in the lipids of P. aeruginosa was investigated. The model compound, N-palmitoyl-β-alanine, synthesized by the method of Hopwood and Weizmann (11), was found to be eluted from silicic acid columns in the 80:20 chloroform-methanol fraction.
ratio of phosphorus to amino nitrogen in Fraction III was considerably lower than the non-choline-containing phospholipids but was 35% higher than the standard phosphatidylcholine. However, such difference could not be detected in the phosphorus to glycerol ratios in any of these fractions. All of the fractions were susceptible to mild alkaline hydrolysis as were the majority of the lipids of *P. aeruginosa*. However, palmitoylalanine was resistant to mild alkaline hydrolysis as well as to reduction by LiBH₄. On the other hand, the lipid-alanine-¹⁴C complex extracted from *P. aeruginosa* was found to be susceptible to mild alkaline hydrolysis (Table II) and to reduction by LiBH₄ as indicated by the appearance of 80 to 90% of the radioactivity in the aqueous phase (Table III). Both compounds were sensitive to strong acid hydrolysis. It was concluded, therefore, that this type of compound (fatty acid amides) did not account for the uptake of W-labeled amino acids by the lipids of *P. aeruginosa*. Additional evidence that the bonding is not that of a peptide has been indicated by the susceptibility of the lipid-amino acid complex to enzymatic hydrolysis by carboxypeptidase A, trypsin, or phospholipase C.²

A comparison of the chemical composition of each of the three polar lipid fractions with that of the standard reference compounds is shown in Table IV. It is evident that although the chemical composition of each lipid fraction is similar to known phospholipids, the molar ratio of phosphorus to amino nitrogen was 14 to 15% higher, and phosphorus to acyl ester was 10 to 30% higher, in both lipid Fractions I and II than the standard phosphatidylethanolamine and phosphatidylserine. The molar

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**Table II**

Percentage distribution of radioactivity after mild alkaline hydrolysis of different fractions

<table>
<thead>
<tr>
<th>Samples</th>
<th>Ether phase</th>
<th>Water phase</th>
<th>Ether phase</th>
<th>Water phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction I</td>
<td>10</td>
<td>90</td>
<td>16.8</td>
<td>83.2</td>
</tr>
<tr>
<td>Fraction II</td>
<td>8.5</td>
<td>91.5</td>
<td>9.2</td>
<td>90.4</td>
</tr>
<tr>
<td>Fraction III</td>
<td>16.5</td>
<td>83.5</td>
<td>7.1</td>
<td>92.8</td>
</tr>
</tbody>
</table>

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**Table III**

Reaction of LiBH₄ with labeled lipid of *Pseudomonas aeruginosa*

Total unfractionated labeled lipid (34 mg; total count, 14,900 c.p.m.) was dissolved in 10 ml of tetrahydrofuran and allowed to react for 20 hours at 25°C with 1 mg of LiBH₄. The hydrolysate was extracted with ether.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Total radioactivity (c.p.m.)</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ether</td>
<td>2,160</td>
<td>14.6</td>
</tr>
<tr>
<td>Water</td>
<td>12,650</td>
<td>85.4</td>
</tr>
</tbody>
</table>

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**Table IV**

Chemical composition of lipid-amino acid complex eluted with different polar-nonpolar solvent mixtures from silicic acid columns

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Phosphorus</th>
<th>Acyl esters</th>
<th>Amino N</th>
<th>Molar ratio of phosphorus to amino nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylethanolamine</td>
<td>1.10</td>
<td>2.43</td>
<td>1.31</td>
<td>1:1.04:2.2:1.19</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>1.17</td>
<td>2.51</td>
<td>1.35</td>
<td>1:1.03:2.2:1.20</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>1.13</td>
<td>2.17</td>
<td>1.20</td>
<td>1:1.04:1.9:1.06</td>
</tr>
</tbody>
</table>

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1 W. L. Gaby, unpublished observations.

2 *Serine and several ninhydrin-positive spots were detected only after strong acid hydrolysis (see Fig. 2).*
solvents. All of these lipids contained radioactive material (7, 90, and 3%), and, as shown in Fig. 4, the major component was phosphatidyl-

column fractions was developed on thin layer silica gel plates, and the known reference phospholipid. Each of the silicic acid to amino nitrogen to acyl ester ratios between these fractions

Chemical analysis revealed significant differences in phosphorus in the lipid complex.

The total lipids extracted from metabolizing P. aeruginosa cells, incubated in the presence of D- alanine-1-14C prior to lipid extraction, separated into three distinct major components (neutral, non-choline-containing, and choline-containing lipids) when chromatographed on thin layer silica gel plates. Since all of these lipids contained radioactive material (7, 90, and 3%, respectively), it is probable that more than one lipid complex is involved in amino acid uptake. Phosphatidylethanolamine was the major component (90 to 95%) of the bacterial lipids, whereas phosphatidylcholine comprised only 3% of the total lipids. Phosphatidylycerine was present only as a minor constituent, and the presence of serine could be detected only after hydrolysis or after formation of 2,4-dinitrophenyl derivatives. All of the lipid fractions were shown to contain several ninhydrin-positive compounds, in addition to ethanolamine, serine, and alanine, following acid hydrolysis. However, traces of glucosamine and reducing sugar could also be detected in the unhydrolyzed lipids of the 80:20 chloroform-methanol fraction.

Chemical analysis of each of the fractions revealed a phosphorus to amino nitrogen ratio of more than 1, confirming the presence of other amino acids in addition to the native nitrogenous bases. The presence of free amino groups and the absence of hydroxyl groups, as well as the absence of amide linkages, in these lipids suggest that alanine is bound by ester linkage to the lipid complex. Analysis of the experimental data permits us to postulate the structure shown in Scheme 1 for the lipid amino acid complex.

The existence of such a labile phospholipid complex containing phosphate triester groups was first reported by Collins and Shotlander (21, 22) in animal, plant, and virus phospholipids. The proposed complex consists of 2 phospholipid molecules linked by glycerol to which the amino acid is bound by an O-ester linkage. If such a compound is cleaved at either position A or B, 1 mole of the lipid-amino acid complex and 1 mole of phospholipid results. The chromatographic fraction would either gain or lose 1 mole of aminoglycerol moiety attached to the phospholipid molecule. Data obtained from chemical analysis as well as specific radioactivity of the lipid fractions support such an assumption.

The ratios of phosphorus to amino nitrogen to acetyl ester found in the lipid fractions were somewhat lower than the theoretical values of the assumed structure; i.e. the ratios of phosphorus to amino nitrogen and phosphorus to acetyl ester of the non-choline-containing phospholipid should be 1:1.5 and 1:2.5, respectively, whereas the actual values found were 1:1.9 and 1:2.2 in the 80:20 fraction and 1:1.2 and 1:2.2 in the 40:60 chloroform-methanol fraction. The ratio of phosphorus to amino nitrogen in the choline-containing fraction was 1:0.35, evidently because of the presence of choline instead of a primary amine as the nitrogenous base of the phospholipid. These lower values could be explained by the presence of both diester- and triester-type phospholipids in the fractions. By simple calculation, 39 to 40% of the phospholipid complex of the 80:20 and 60:40 chloroform-methanol fractions should give rise to the above phosphorus to amino nitrogen ratio. Similarly, phosphorus to acetyl ester values will not be more than 1:2.2.

Variations in the specific radioactivity of the different lipid fractions can also be explained on the basis of the proposed structure. Following rechromatography of Fraction I (in chloroform-methanol, 80:20) on silicic acid columns, the specific radioactivity of Fraction IA (90:10 chloroform-methanol eluate), identified as phosphatidylethanolamine, was 83% higher than Fraction IB (80:20 eluate). Assuming that the cleavage occurs either at position A or B during rechromatography, the amino glycerol moiety will migrate with the less polar phospholipid fraction, resulting in an increase in specific activity. The less polar phospholipid is considered here as the part of phosphatidyl-

DISCUSSION

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ethanolamine that contains relatively more of the unsaturated fatty acid components (23). The extreme lability of the lipid-amino acid complex to alkaline hydrolysis may be explained by the presence of a phosphate group associated with the adjacent carbon atom (24). Kelemen and Baddiley (25) also accounted for the lability of ester-bound alanine in glycerolteichoic acid because of the presence of neighboring phosphate groups.

Working with gram-positive bacteria, Macfarlane (5) described a glycerylphosphorylglycerol-amino acid complex in C. welchii, and Houtsamuller and Van Deenen (26) described a glycerylphosphorylglycerol-ornithine complex in the lipids of Bacillus cereus. Such a complex was not found in the lipids of P. aeruginosa. These findings are in agreement with those of Kaneshiro and Marr (6), who reported only phosphatidylethanolamine, phosphatidyleholine, and fatty acids as the major lipid components of gram-negative bacteria, whereas the major lipid component of gram-positive bacteria is reported to be phosphatidylglycerol (5). We are aware of the possibility that an acid anhydride type of linkage as proposed by DeKoning (27) might exist in complex phospholipids. However, the results obtained in this laboratory can best be explained by a lipid-amino acid complex which contains a phosphate triester group.

SUMMARY

The lipids extracted from Pseudomonas aeruginosa, incubated with labeled alanine, have been separated into three major fractions, the nonpolar lipids, non-choline-containing phospholipids, and choline-containing phospholipids, by silicic acid column chromatography. The lipid-amino acid complex, which is associated with the non-choline-containing lipid fraction, was composed primarily of phosphatidylethanolamine and small amounts of phosphatidylserine.

The chemical composition of these fractions differed significantly in their molar ratios of phosphorus to acyl ester to amino nitrogen than those of the reference phospholipid standards. The lipid-amino acid complex was further characterized by the absence of free glycerol hydroxyl group, by the formation of dinitrophenyl derivatives, by lability to mild alkaline hydrolysis, and by susceptibility to reduction by LiBHI. Based on these findings, a chemical structure of the phospholipid-amino acid complex is presented, in which the carboxyl group of the amino acid residue is attached to the glycerol moiety of the complex phospholipid with an O-ester linkage. Such complexes were found to be associated with the diester-type phospholipids of P. aeruginosa.

REFERENCES

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