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Ornithine-Containing Lipid of *Bordetella pertussis* That Carries Hemagglutinating Activity

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The proposed structure of the ornithine-containing lipid of *Bordetella pertussis* is 3-hydroxyhexadecanoic acid amide-linked to ornithine and esterified to the second hexadecanoic acid. The aminolipid strongly agglutinates type A and B human erythrocytes.

As described in our previous paper (4), an ornithine-containing lipid is present in common in *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica*. This aminolipid is characteristic of the lipids of the genus *Bordetella*. In 1973, Thiele and Schwinn (10) reported on the ethylene glycol-containing structure of the aminolipid of one strain of *B. pertussis*. We propose in this paper a structure containing 3-hydroxyhexadecanoic acid for the aminolipid of two strains of *B. pertussis*.

An ornithine-containing lipid may act as an important membrane constituent with a characteristic structure not hydrolyzed with phospholipase, but its physiological function is not clear. This paper reports on hemagglutinating activity of the ornithine-containing lipid of *B. pertussis*.

The ornithine-containing lipid of *B. pertussis* was purified from the total extractable cellular lipid (5) of strains ATCC 8467 (phase 1) and Sakurayashiki (phase 3) as described previously (4). Silica gel thin-layer chromatography and snake venom phospholipase A were used in this process. Aminolipid (3.3 mg) was obtained from 120 mg of total extractable cellular lipid prepared from 1 g of cells (dry weight).

The ornithine-containing lipid was hydrolyzed with 6 N hydrochloric acid at 110°C for 24 h and yielded 1 mol of hexadecanoic acid, 1 mol of 3-hydroxyhexadecanoic acid, and 1 mol of ornithine. Mild alkaline hydrolysis with 0.3 N potassium hydroxide at 37°C for 4 h (9) indicated that hexadecanoic acid was linked by an ester bond to 3-hydroxyhexadecanoic acid. When the residual moiety was methanolyzed with 5% hydrochloric acid in methanol, 3-hydroxyhexadecanoic acid and ornithine were obtained. Ornithine was analyzed with an amino acid analyzer, and fatty acids were analyzed by combined gas-liquid chromatography and mass spectrometry. Other components were not detected in this sample.

The infrared absorption spectrum of the ornithine-containing lipid is shown in Fig. 1. Ester linkage and secondary amide linkage are indicated by a sharp carbonyl stretching band at 1,730 cm^{-1}, an amide 1 band at 1,640 cm^{-1}, and an amide 2 band at 1,540 cm^{-1}. Free amino and carboxyl groups were also identified in this spectrum. The infrared absorption spectrum of the deacylated aminolipid was compared with that of intact aminolipid, demonstrating the loss of ester carbonyl (1,730 cm^{-1}) and the appearance of an absorption band characteristic of hydroxyl groups.

Mass spectrometry was utilized to determine directly the structure of the ornithine-containing lipid (Fig. 2). Trimethylsilylation (TMS) was carried out with *N,O*-bistrimethylsilyltrifluoroacetamide at 150°C for 3 h. A molecular ion was detected at *m/e* 606 (Fig. 2, M-90; loss of TMS derivative from the mono-TMS derivative of the aminolipid). Although it was presumed that cleavage is apt to occur around the hydroxyl group of 3-hydroxyhexadecanoic acid (11), characteristic fragment ions were detected at *m/e* 350 (Fig. 2, M-346; loss of TMS derivative and hexadecanoic acid) and at *m/e* 257 [Fig. 2, M-439; loss of TMS derivative, hexadecanoic acid, and - $(CH_2)_{12}CH_3$]. High-intensity ions at *m/e* 77 and 72 were presumed to be derived from the structure around the ester linkage since deacylated aminolipid did not show these ions. It was concluded that other major ions at *m/e* 141, 113, and 97 were based on a piperidone derivative by cyclization of ornithine (2, 12) and hydrocarbon chains of fatty acids. 3-(3-Hydroxybutyryl-amino)-2-piperidone has been synthesized, and its fragment ions were investigated by Clarke and Waight (2). On the basis of characteristic

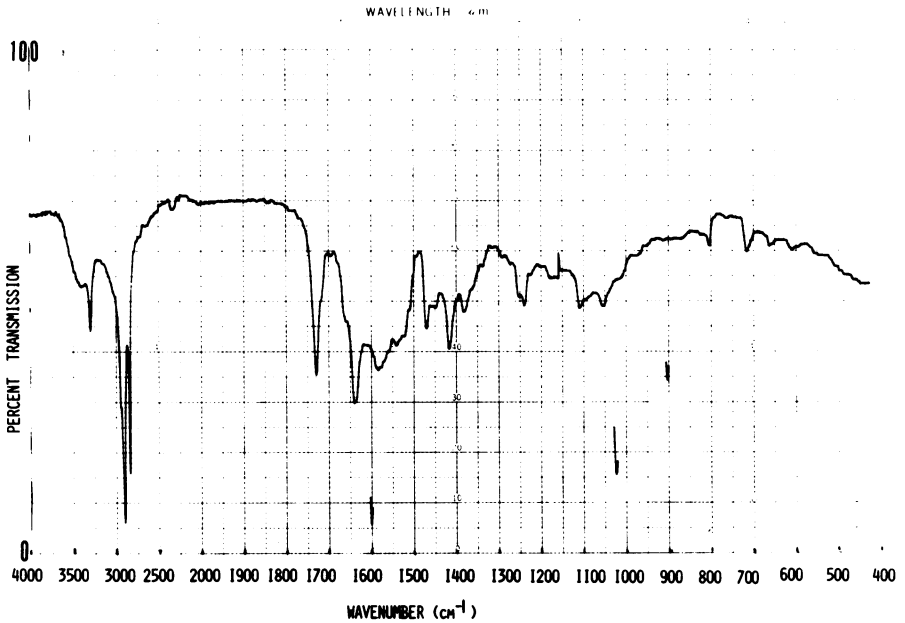

FIG. 1. Infrared absorption spectrum of the ornithine-containing lipid of *B. pertussis*. The spectrum was obtained with a potassium bromide tablet on a Nihonbunko model IR-G apparatus.

ions of this substance such as those at *m/e* 185, 156, and 141, the structure of an ornithine-containing lipid of *Rhodopseudomonas sphaeroides*, in which 3-hydroxy fatty acid is amide linked with ornithine at the α position, was finally determined by Gorchein in 1973 (3). Because the mass spectrum of the deacylated ornithine-containing lipid of *B. pertussis* that exhibits its characteristic fragment ions was very similar to that of the model substance above, 3-hydroxyhexadecanoic acid in the ornithine-containing lipid of *B. pertussis* was presumed to be also amide linked to ornithine at the α position.

From this analysis, the structure indicated in Fig. 3 is proposed for the ornithine-containing lipid of the two strains of *B. pertussis*.

The aminolipid of *B. pertussis* is the first ornithine-containing lipid to exhibit hemagglutinating activity. Hemagglutinating activity of the aminolipid was examined with erythrocytes of humans and of various animals. Good figures for hemagglutination with human erythrocytes were obtained at an erythrocyte concentration of 0.25% at 4 to 16°C overnight in 0.01 M phosphate buffer–0.85% NaCl at about pH 6.0. The minimum hemagglutinating concentration of the ornithine-containing lipid was 1 to 2 μg/ml for type A and B human and rabbit erythrocytes (Table 1). Hemagglutination was weak for the erythrocytes of chickens, horses, sheep, and guinea pigs. Inhibition tests for hemagglutination were carried out with 10 mM concentrations of L-fucose, D-galactose, D-glucose, D-mannose, lactose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, and sialic acid. Hemagglutination with human type A and B erythrocytes was strongly inhibited by *N*-acetyl-D-galactosamine, D-galactose, and lactose. Sialic acid did not inhibit this hemagglutination, and further hemagglutination was not affected, even when the

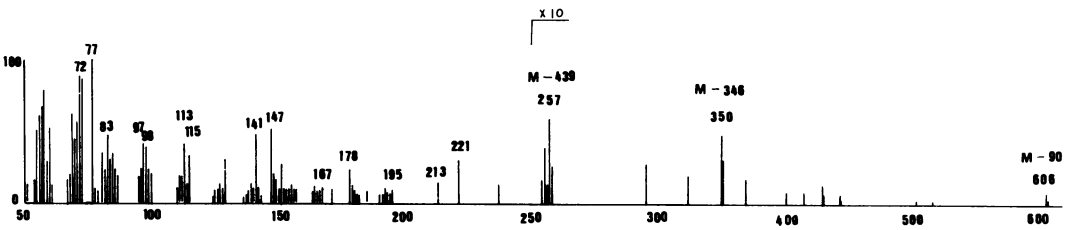

FIG. 2. Mass spectrum of the trimethylsilylated ornithine-containing lipid of *B. pertussis*. Mass spectrometry was carried out on a Hitachi type M-60 apparatus at 20 electron volts of ionization energy at temperatures of 330°C (molecular separator) and 250°C (ion source).

erythrocytes were treated with crude neuraminidase. Hemagglutinating activity was examined among phosphatidylethanolamine, cardiolipin, phosphatidylglycerol, sphingomyelin, and erythrosphingosine, but no lipid exhibited hemagglutinating activity.

From these results, the ornithine-containing lipid was determined to be a relatively specific hemagglutinin for type A and B human erythrocytes and rabbit erythrocytes.

When hexadecanoic acid was removed from the aminolipid by base hydrolysis, the aminolipid lost all of its hemagglutinating activity and gained hemolytic activity. We can easily understand this phenomenon, considering that some hydroxy fatty acids carry hemolytic activity. For example, it is known that hemolytic activity is exhibited by 3-hydroxymyristic acid derived from *Salmonella* lipopolysaccharides (O. Lüderitz, personal communication), 2- and 3-hydroxy fatty acids from siolipin (7), and rhamnolipid from *Pseudomonas aeruginosa* which contains 3-hydroxy fatty acid (8). To determine hemolytic activity, 2.5% type B and O human erythrocytes were mixed with an equal volume of the deacylated aminolipid at a concentration of 10 to 1,000 μg/ml in 0.85% NaCl, and the mixture was incubated at 37°C for 30 min. After centrifugation at 2,500 rpm for 10 min, the optical density of the supernatant at 580 nm was measured. The concentration of deacylated aminolipid that caused 50% hemolysis was 30 μg/ml in type B and O human erythrocytes.

It has been reported that siolipin, an ornithine-containing lipid of *Streptomyces sioyaensis* whose structure is ethylene glycol esterified with 2-hydroxy fatty acid and ornithine amide linked to 3-hydroxy fatty acid (6), exhibits hemolytic activity (7). When the structure of siolipin was compared with the deacylated aminolipid of *B. pertussis*, it was considered that hemolysis was caused by the hydroxy fatty acids of these lipids.

TABLE 1. Minimum hemagglutinating concentrations of the ornithine-containing lipid of *B. pertussis*

Erythrocyte	Aminolipid concn (μg/ml)
Human	
Type A	1–2
Type B	1–2
Type AB	4
Type O	8
Rabbit	1
Horse	62
Sheep	125
Guinea pig	125–250
Chicken	62–125

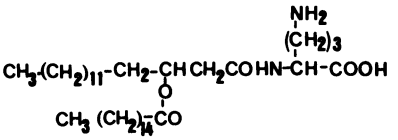

FIG. 3. Proposed structure of the ornithine-containing lipid from *B. pertussis*.

Hexadecanoic acid was indispensable for hemagglutinating activity in the ornithine-containing lipid of *B. pertussis*. This aminolipid strongly agglutinated type A and B human and rabbit erythrocytes. It has been reported that the phospholipid composition of human erythrocytes is similar to that of rabbit erythrocytes (1). Hemagglutination inhibition tests with some carbohydrates suggested that the ornithine-containing lipid interacted with type A and B human erythrocytes through their type-specific carbohydrates. Because of this, both the hydrophilic ornithine and hydrophobic fatty acid moieties of this aminolipid were presumed to be responsible for the hemagglutinating activity.

It is of interest that the activity of the aminolipid changes from hemagglutination to hemolysis after the removal of hexadecanoic acid. Further, the ornithine-containing lipid of *B. pertussis* may represent the first hemagglutinin that does not have polypeptide.

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