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Novel alk-1-enyl ether lipids isolated from *Clostridium acetobutylicum*

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Abstract. Alkenyl ether analogues of phosphatidylglycerol (plasmenylglycerol), bisphosphatidylglycerol (cardiolipin) (plasmenylglycerolphosphatidic acid), monoglycosyldiglyceride and diglycosyldiglyceride were isolated from the polar lipids of *Clostridium acetobutylicum* and characterized by chemical analyses and degradation. The position of the alkenyl ether bond (at C-1) and of the acyl ester bond (at C-2) as well as the configuration at C-2 of the phospholipids are the same as of the alkenyl ether phospholipids known so far. The alkenyl ether analogue of monoglycosyldiglyceride contains a galactosyl residue, that of diglycosyldiglyceride a glucosyl-galactosyl residue, glucosyl forming the terminal unit.

Key words: *Clostridium acetobutylicum* – Alkenyl ether lipids – Plasmalogens – Alkenyl-acyl-glycerolglycosides

Alk-1-enyl (vinyl, enol) glyceryl ether lipids are characterized by an $-O-CH=CH-$bond at C-1 of the glycerol moiety of a phospholipid or a triglyceride instead of an acyl ester bond. This linkage is characteristic in that it is easily split under very mild acid conditions yielding a fatty aldehyde (called plasmal) – in contrast to the saturated alkyl glyceryl ether bond which is comparatively unreactive. The alk-1-enyl glyceryl ethers exist mainly as phospholipids (plasmalogens) and to a smaller extent as glycerides (neutral plasmalogens). For the sake of conciseness, the alk-1-enyl group is termed alkenyl group throughout this paper – irrespective of eventual additional unsaturation along the hydrocarbon chain.

Most bacterial lipids are free of alkenyl ether lipids. Such lipids seem to occur only in strictly anaerobic bacteria while alkenyl ether lipids are missing in aerobic or facultative species (Goldfine and Hagen 1972). This paper reports on the occurrence in *Clostridium acetobutylicum* of novel plasmalogens (besides known ones) and of novel glycolipids containing alkenyl glyceryl ether bonds.

Materials and methods

Bacteria. The organism used was *Clostridium acetobutylicum* strain DSM 1731. Lyophilized cells were derived from a continuous culture that was run under phosphate limitation and produced acetone and n-butanol as the main fermentation products. The growth medium and the growth conditions were described by Bahl et al. (1982).

Extraction of lipids. Total lipids were extracted from bacteria and purified by passing them through a column of Sephadex G-25 fine as described by Thiele et al. (1980).

Chromatographic and degradation methods were described in detail elsewhere (Thiele et al. 1985). Briefly, lipids were separated by column and thin-layer chromatographic procedures. Schiff's reagent was used to detect alkenyl ethers or free aldehydes. Degradation of alkenyl ether lipids was carried out in different ways (Thiele 1979): 1) Mild acid hydrolysis is known to cleave only the alkenyl ether bonds releasing fatty aldehydes. 2) Phospholipase A_2 which ist present in snake venom catalyzes the hydrolysis of the fatty acid ester bond located in 2-position of phospholipids only. 3) Mild alkaline methanolysis with lithium hydroxide splits all acyl ester bonds, but no phosphate ester bonds and no alkenyl ethers. 4) Reductive cleavage of all ester bonds is achieved by the action of lithium aluminum hydride. In addition, vigorous acid hydrolysis was performed in order to release the sugars from glycolipids. Various glycosidases were also used to split the glycosidic linkages in glycolipids.

Results and discussion

Due to similar physical properties, intact alkenyl ether lipids cannot be separated from their analogous alkyl ether and acyl ester lipids. By this fact, on the other hand, the alkenyl ether lipids cochromatograph on thin-layer plates with the analogous acyl ester lipids (which are available as reference samples in contrast to alkenyl ether lipids).

The polar lipids of *Clostridium acetobutylicum* showed strong reactions with Schiff's reagent subsequent to mild acid cleavage. This indicated the presence of (fatty) aldehydes which had been released from alkenyl ether lipids. The molar ratio of alkenyl group/lipid phosphorus differs in bacteria grown at pH 7.0 from those grown at pH 4.3 (Table 1). The ratio of 1.26 suggests the presence of phosphorus-free alkenyl ether lipids. Thin-layer chromatograms of polar lipids, after spraying with Schiff's reagent, revealed almost all major lipid types to contain alkenyl ether bonds. The predominant types of polar lipids were phosphatidylglycerol, bisphosphatidylglycerol (cardiolipin), monoglycosyl-diglyceride and diglycosyl-diglyceride, while smaller amounts of phosphatidylserine, traces of

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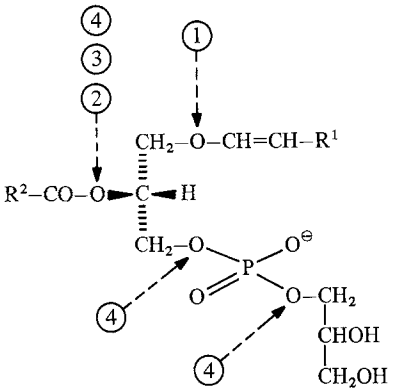
Table 1. Lipid content of *Clostridium acetobutylicum* strain DSM 173

	Grown at pH 7.0	Grown at pH 4.3
Total lipids (% of bacterial dry wt)	5.1	5.6
Polar lipids (% of total lipids)	83.4	80.4
Phospholipids (% of polar lipids)	51.8	50.3
Alkenyl group/Lipid P (molar ratios) in polar lipids	1.26	0.57



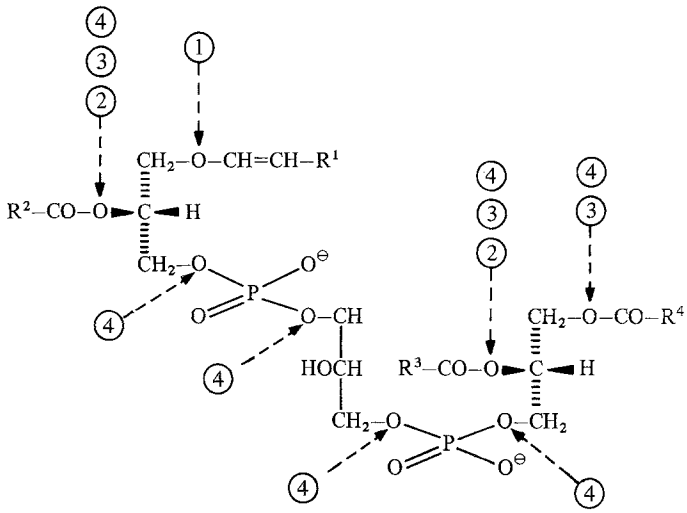
Scheme 1. 1-Alkenyl-2-acyl-*sn*-glycerol-3-phosphoryl-glycerol (plasmenylglycerol).

Key to all schemes:

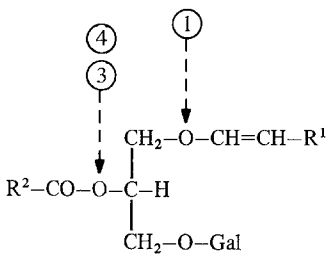
Sites of scission: ① by mild acid hydrolysis; ② by enzymatic hydrolysis with phospholipase A_2 (EC 3.1.1.4); ③ by mild alkaline methanolysis with LiOH; ④ by reductive ester cleavage with $LiAlH_4$

phosphatidylethanolamine and traces of unknown lipids were detected. The major Schiff positive phospholipids and glycosylglycerides were characterized by degradation to products of expected structures. Palmitic and stearic acids prevailed among the total fatty acids; no unusual (hydroxy, cyclopropane, branched) fatty acids or fatty aldehydes were found.

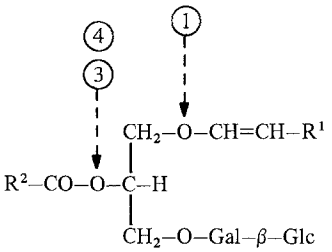
Alkenyl ether phospholipids (plasmalogens). The major phospholipids (phosphatidylglycerol and cardiolipin types) were studied in more detail. The structure of the phosphatidylglycerol type plasmalogen is given in Scheme 1. Acid hydrolysis under very mild conditions leads to a cleavage of the alkenyl ether bond (① in Scheme 1) releasing fatty aldehydes. All alkenyl ether bonds were readily split since, after removal of the fatty aldehydes, no trace of Schiff positive substance was left. Phospholipase A_2 has been demonstrated (Van Deenen and De Haas 1963) to catalyze the hydrolysis of the fatty acid ester bond located only adjacent to the phosphoryl-glycerol linkage; only the configuration of 2-acyl-*sn*-glycerol-3-phosphate is susceptible to phospholipase A_2 (② in Scheme 1). The degradation product of the action of lithium hydroxide (③ in Scheme 1) is the same as that of the action of phospholipase A_2. All three degradation procedures lead to a lysophospholipid with a



Scheme 2. 1-Alkenyl-2-acyl-*sn*-glycerol-3-phosphoryl-1′-glycerol-3′-phosphoryl-1″-2″-diacyl-*sn*-glycerol (plasmenylglycerol-phosphatidate)



Scheme 3. Alkenyl-acyl-glycerol-galactoside



Scheme 4. Alkenyl-acyl-glycerol-galacto-β-glucoside

long-chain group either at C-1 (carrying an alkenyl ether bond, therefore Schiff positive) or at C-2 (carrying an acyl ester bond, Schiff negative). The increase in polarity of those lysophospholipids was shown by thin-layer chromatography. The lysophospholipid was further degraded to yield a water-soluble product (glyceryl-phosphoryl-glycerol, identifiable by thin-layer chromatography) either by mild acid hydrolysis or by deacylation with lithium hydroxide, respectively. In addition, reductive cleavage with lithium aluminum hydride (④ in Scheme 1) of plasmalogens of every kind yields alkenyl ether glycerol. This can be separated from alkyl ether glycerol by thin-layer chromatography thus indicating the presence of saturated alkyl ether phospholipids.

The phosphatidylglycerol type plasmalogen of *Clostridium acetobutylicum* turned out to consist nearly entirely of the alkenyl ether analogue; there was only a trace

of the saturated alkyl ether, if any. As the phospholipid was entirely susceptible to phospholipase A_2, the configuration of a 1-alkenyl-2-acyl-*sn*-glycerol-3-phosphoglycerol became evident. A plasmalogen of the same structure has previously been found by Baumann et al. (1965) as constituent of *Clostridium butyricum*.

Degradation of the cardiolipin type plasmalogen was expected to be more complex because of two phosphate groups and three glycerol moieties in one cardiolipin molecule. Therefore, three forms of lysocompounds are possible which are characterized by the loss of one, two or three long-chain residues. The substance mentioned first may also be called a semilyso-compound; it showed a molar ratio acyl ester/phosphorus of 3:2. Only after the release of all four long-chain residues, was the most hydrophilic (water-soluble) degradation product obtained: glyceryl-phosphoryl-glyceryl-phosphoryl-glycerol. It became evident by chemical analyses and thin-layer chromatographic characterization of the degradation products that only one alkenyl ether bond was present in the cardiolipin type plasmalogen. By the effectiveness of phospholipase A_2 it became clear that the position of the alkenyl ether bond was at C-1 and that the configuration of C-2 and C-2″ was as indicated in Scheme 2, while the configuration at C-2′ remained open.

As a reproducible finding, the Schiff negative semilyso-cardiolipin was also present as a major substance in the polar lipids of the bacteria grown at pH 4.3 without preceding hydrolytic measures. This is obviously the reason why the ratio of alkenyl group/lipid phosphorus is much lower as compared to the ratio in bacteria grown at pH 7.0 (Table 1).

Alkenyl ether glycerolglycosides. The presumed alkenyl-acyl-glycerolglycosides were resistant to the action of phospholipase A_2, but were converted to more polar products by deacylation with lithium hydroxide, by reductive cleavage with lithium aluminum hydride or by mild acid hydrolysis (Schemes 3 and 4). All these degradation products virtually showed the same R_f values on thin-layer chromatography; only the last product did not react with Schiff's reagent. Further degradation yielded water-soluble mono- and diglycosylglycerol. Galactose was identified as a degradation product of alkenyl-acyl-glycerol-monoglycoside, equal amounts of galactose and glucose were found by analysis of the degradation products of alkenyl-acyl-glycerol-diglycoside. Application of various glycosidases showed that only β-glucosidase was able to degrade the alkenyl-glycerol-diglycoside prepared from alkenyl-acyl-glycerol-diglycoside. Hence, the diglycosyl group was probably a galacto-β-glucosyl residue. There was no evidence of corresponding diacyl or of saturated alkyl ether compounds besides the alkenyl ether compounds. The position of the alkenyl ether and of the acyl ester groups as well as the position of the glycosidic bonds remained open. In analogy to the well-known glycosyl-diglycerides, we suggest the structures as indicated in Scheme 3 and 4.

Recently, the lipids of *Clostridium acetobutylicum* ATCC 824 were investigated by different authors, but without regard to the occurrence of ether lipids (Vollherbst-Schneck et al. 1984) or to the nature of the alkenyl ether lipids (Johnston and Goldfine 1983).

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