Quantitative conversion of diether or tetraether phospholipids to glycerophosphoesters by dealkylation with boron trichloride: a tool for structural analysis of archaebacterial lipids

Masateru Nishihara[1] and Yosuke Koga

Department of Chemistry, University of Occupational and Environmental Health, Japan, Kitakyushu 807, Japan

Summary A method for preparing glycerophosphoesters from ether phospholipids by dealkylation with boron trichloride (BCl_3) is described. Treatment of ether phospholipids in chloroform with BCl_3 for 30 min at room temperature yielded almost quantitatively the corresponding glycerophosphoesters retaining the intact polar head group of the ether phospholipids. Thus, glycerophosphocholine, glycerophosphoinositol, glycerophosphoglycerol, glycerophosphoserine, glycerophosphate, and glycerophosphoethanolamine were prepared from the diether analogs of phosphatidylcholine, phosphatidylinositol, phosphatidylglycerol, phosphatidylserine, and phosphatidic acid, and the tetraether analog of phosphatidylethanolamine, respectively. BCl_3 also cleaved diacyl, alkyl-acyl, and alk-1-enyl-acyl forms of phospholipids to yield corresponding glycerophosphoesters. The glycerophosphoesters were separated more rapidly by cellulose thin-layer chromatography with the same solvent system as in paper chromatography. This method is of great use for structure determination of glycerophosphoester backbones of ether phospholipids, analogous to the mild alkaline methanolysis of diacyl form of phospholipids, as well as for the analysis of alkyl chains. It is, however, not applicable to glycolipids because of cleavage of glycosidic bonds by BCl_3. —**Nishihara, M., and Y. Koga.** Quantitative conversion of diether or tetraether phospholipids to glycerophosphoesters by dealkylation with boron trichloride: a tool for structural analysis of archaebacterial lipids. *J. Lipid Res.* 1988. **29:** 384–388.

Supplementary key words archaebacteria • ether lipids • ether cleavage

Mild alkaline methanolysis, which provides glycerophosphoester or glycosylglycerol, is one of the most convenient and useful methods for structural analysis of diester phospholipids or glycolipids. Polar lipids of archaebacteria are resistant to the mild alkaline methanolysis because glycerol and alkyl chains are linked by ether bonds instead of ester bonds. Recent surveys of lipid constituents of methanogens (1–7) have shown that the archaebacteria have a wide variety of polar lipids, many of which have not been structurally determined. Methods such as strong alkaline hydrolysis (1, 8), acid methanolysis (8), and HF hydrolysis (3) have been used for the identi-

Abbreviations: TLC, thin-layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PS, phosphatidylserine; PA, phosphatidic acid; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; GPI, glycerophosphoinositol; GPG, glycerophosphoglycerol; GPS, glycerophosphoserine; GP, glycerophosphate.

[1]To whom reprint requests should be addressed at: Department of Chemistry, University of Occupational and Environmental Health, Japan, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807, Japan.

fication of polar head groups of archaebacterial ether lipids. The two former methods require a free hydroxyl group next to the phosphodiester in the lipid molecule to cleave a phohsphodiester linkage. A number of phospholipids of the methanogens were found to be resistant to acid methanolysis (3, 5, 9), probably because these lipids lack a free hydroxyl group on a carbon atom (for example, the position 2 of *sn*-glycerol or the position 2 of myo-inositol) adjacent to the carbon atom which is linked to the phosphate group (10). Although HF treatment can hydrolyze phospholipids resistant to acid methanolysis, it is accompanied to some extent by degradation of the polar head group (3, 6). None of these methods can cleave the ether bond, and a procedure for obtaining glycerophosphoesters with retention of an intact polar head group from an ether phospholipid has not been previously available. Recently, it was shown that BCl_3 treatment of caldarchaetidylethanolamine (a tetraether analog of phosphatidylethanolamine) liberated glycerophosphoethanolamine with a yield of 99% (6). The cleavage reaction of ether with BCl_3 was described by Gerrard and Lappert (11) and this reaction has been used for preparing alkyl chlorides from ether lipids for over 20 years (12). This observation suggests that the method is also generally useful for the preparation of glycerophosphoesters from ether phospholipids as well as for alkyl chain analysis. This communication reports that BCl_3 treatment can be of general use in obtaining glycerophosphoesters from ether phospholipids. A systematic nomenclature of archaebacterial polar lipids proposed by us (6) is used in this text.

MATERIALS AND METHODS

Materials

Liquefied BCl_3 in a 25-g ampule was purchased from Nakarai Chemicals (Kyoto, Japan) and stored at −20°C. Standard diester (diacyl) phospholipids were those commercially available. Synthetic dioleoyl phosphatidylcholine (PC), beef heart phosphatidylethanolamine (PE), soybean phosphatidylinositol (PI), and synthetic dioleoyl phosphatidic acid (PA) were purchased from Serdary Research Laboratories Inc. Bovine brain phosphatidylserine (PS) was a product of Avanti Polar Lipids Inc. Semi-synthetic phosphatidylglycerol (PG) prepared from egg yolk PC was obtained from Sigma. Standard glycerophosphocholine (GPC), glycerophosphoethanolamine (GPE), glycerophosphoinositol (GPI), glycerophosphoglycerol (GPG), glycerophosphoserine (GPS), and glycerophosphate (GP) were prepared from the corresponding diester phospholipids by mild alkaline methanolysis. Phosphoethanolamine was a product of Nakarai Chemicals (Japan) and α-glycerophosphate disodium salt was purchased from Tokyo Kasei Chemicals (Japan). *sn*-1,2-Dipalmityl glycerophosphocholine (an ether analog of PC) was purchased from Serdary. 1-Alkyl-2-acyl GPC and 1-alk-1'-enyl-2-acyl GPC, which were prepared from bovine heart, were generous gifts from K. Saito and K. Satouchi. Archaetidylglycerol (a diether analog of PG) was purified from total lipid of *Halobacterium cutirubrum* by TLC using chloroform–methanol–acetic acid–water 85:30:15:5 (by volume). The total lipid was extracted and provided by M. Kamekura. The structural determination of archaetidylglycerol has been reported by Kates (13). Caldarchaetidylethanolamine, archaetidylinositol (a diether analog of PI), archaetidylserine (a diether analog of PS), archaetidic acid (a diether analog of PA), and gentiobiosyl caldarchaeol (diglucosylated dibiphytanyl diglycerol tetraether) were purified from total lipid extracted from the cells of *Methanobacterium thermoautotrophicum* ΔH (DSM 1053). The purification and structure determinations of caldarchaetidylethanolamine, gentiobiosyl caldarchaeol, and archaetidylserine have been previously described (3, 6, 7). The purification procedure and structures of the other lipids from the methanogens elucidated in our laboratory will be published elsewhere.

BCl_3 treatment

All the experiments were carried out at room temperature (25°C). The procedure is a modification of those described by Gerrard and Lappert (11) and Kates, Yengoyan, and Sastry (12). After opening an ampule of BCl_3 liquefied at −20°C, the liquid was poured as soon as possible into 1 ml of chloroform solution of phospholipid (10–131 μg of lipid-phosphorus) or glycolipid (1538 μg of lipid-glucose) in a graduated test tube to the 3-ml line. After mixing gently, the test tube was capped with a marble and was allowed to react for either 30 min or 4 hr. After the reaction mixture was dried under a stream of nitrogen, borate was removed by repeated addition of methanol and evaporation. The residue was partitioned by adding chloroform–methanol–water 1:1:0.9 (by volume). The upper aqueous methanol phase was evaporated under nitrogen. The small amount of borate was, if present, completely removed as described above because the presence of borate interfered with subsequent TLC.

Thin-layer chromatography of water-soluble products

Water-soluble dealkylated or deacylated products were dissolved in a small volume of water–methanol 1:1 (by volume) and spotted on a cellulose TLC plate (Merck Art 5716, 0.25 mm thick) as a 1.5-cm band and developed with a solvent of phenol–water 100:38 (by volume). The R_f values of various glycerophosphates in this thin-layer chromatography system were almost identical to the R_f values of paper chromatography using the same solvent

described by Kates (14). After spots on the plate were visualized with salicylsulfonic acid/$FeCl_3$ reagent (15) or ninhydrin, each spot was scraped from the plate and the organic phosphate ester along with the cellulose powder was digested with 0.6 ml of 70% perchloric acid + 1 ml of water at 170°C for 6 hr, followed by phosphorus determination. The blank TLC plate showed no phosphorus at all.

Other analytical methods

Organic and inorganic phosphorus were determined by the method of Bartlett (16). Total phosphorus was mea-

TABLE 1. Degradation of various forms of phospholipids by BCl_3 treatment or mild alkaline methanolysis

PL[a] Form	Reagent	Time	N[b]	Phosphorus: Original Lipid	Phosphorus: After BCl_3 Treatment: $CHCl_3$-Soluble	Phosphorus: After BCl_3 Treatment: Aqueous-Methanol-Soluble	Aqueous-Methanol-Soluble Products	
		hr		*μg*	*% of original lipid P*[c]			*% P*
PC								
Dialkyl	BCl_3	0.5	5	131	–[d]	101 ± 2	GPC[e] (98 ± 1)	GP (2 ± 1)
Diacyl	NaOH		1				GPC (94)	cyclic-GP (6)
	BCl_3	0.5	5	60	27 ± 3	77 ± 8	GPC (99 ± 1)	GP (1 ± 1)
	BCl_3	4	5	60	–	98 ± 3		
Alkyl-acyl	BCl_3	0.5	2	10	20	80	GPC (100)	
	BCl_3	4	2	10	–	100		
Alk-1-*enyl*-acyl	BCl_3	0.5	2	12	42	58	GPC (100)	
	BCl_3	4	2	12	8	92		
PE								
Tetraether	BCl_3	0.5	5	64	–	98 ± 2	GPE (98 ± 1)	phosphoethanolamine (2 ± 1)
Diacyl	NaOH		1				GPE (99)	phosphoethanolamine (1)
	BCl_3	0.5	5	64	31 ± 6	66 ± 6	GPE (98 ± 1)	phosphoethanolamine (2 ± 1)
	BCl_3	4	5	64	5 ± 2	97 ± 3		
PI								
Dialkyl	BCl_3	0.5	5	119	–	102 ± 3	GPI (98 ± 1)	inositol monophosphate (2 ± 1)
Diacyl	NaOH		1				GPI (94)	inositol monophosphate (6)
	BCl_3	0.5	5	90	12 ± 1	89 ± 2	GPI (95 ± 3)	inositol monophosphate (2 ± 1) unidentified[f] (3 ± 2)
	BCl_3	4	5	90	3 ± 1	98 ± 2		
PG								
Dialkyl	BCl_3	0.5	5	34	–	100 ± 3	GPG (94 ± 2)	GP (1 ± 0) unidentified (5 ± 2)
Diacyl	NaOH		1				GPG (99)	cyclic-GP (1)
	BCl_3	0.5	5	83	11 ± 4	86 ± 4	GPG (92 ± 2)	GP (1 ± 1) *unidentified* (7 ± 2)
	BCl_3	4	5	83	–	98 ± 5		
PS								
Dialkyl	BCl_3	0.5	5	66	–	98 ± 2	GPS (88 ± 4)	GPE (1 ± 1) unidentified (11 ± 4)
Diacyl	NaOH		1				GPS (100)	
	BCl_3	0.5	5	118	21 ± 3	78 ± 3	GPS (92 ± 3)	GPE (2 ± 1) unidentified (6 ± 3)
	BCl_3	4	5	118	3 ± 1	96 ± 3		
PA								
Dialkyl	BCl_3	0.5	5	25	–	100 ± 4	GP-Na[g] (100 ± 0)	
Diacyl	NaOH		1				GP-Na (100)	
	BCl_3	0.5	5	104	22 ± 3	77 ± 3	GP-Na (100 ± 0)	
	BCl_3	4	5	104	3 ± 3	96 ± 3		

After BCl_3 treatment of phospholipids for 30 min or 4 hr, the products were partitioned between aqueous-methanol and chloroform phases. Phosphorus contents in both fractions were determined. The aqueous-methanol-soluble products obtained by BCl_3 treatment or mild alkaline methanolysis were separated by cellulose TLC. After visualization of spots using salicylsulfonic acid/$FeCl_3$ reagent or ninhydrin, the spots were scraped from the TLC plate and their phosphorus contents were determined.

[a] PL, phospholipid. PC, PI, PG, PS, and PA signify various forms of phospholipids as listed.

[b] N, number of experiments.

[c] Values are expressed as mean value ± SD (n = 5), mean value (n = 2), or value itself (n = 1).

[d] –, Less than detection limit (0.2 μg phosphorus per sample).

[e] R_f values of the major products are described in the text.

[f] All the unidentified were ninhydrin-negative. R_f values of the unidentified products from PI, PG, and PS were 0.10, 0.56, and 0.18, respectively.

[g] GP-Na, Na salt of GP. Solutions of the products were alkalinized by the addition of 0.1 M NaOH before TLC development. R_f value of free form of GP was 0.29–0.36.

sured after digestion of the samples with perchloric acid. Mild alkaline methanolysis was performed as described by Kates (14). Glycerol was measured by the method of Lambert and Neish (17).

RESULTS AND DISCUSSION

After BCl_3 treatment of phospholipids for either 30 min or 4 hr, the products were partitioned between the aqueous-methanol and chloroform phases. The phosphorus contents in both fractions were determined (**Table 1**). In the case of the ether phospholipids, the results show that dealkylation of ether phospholipids was complete after 30 min. On the other hand, diester phospholipids were less readily cleaved by BCl_3, i.e., longer (4 hr) treatment was required for approximately 95% or greater deacylation of the lipids. No inorganic phosphate was found in the aqueous-methanol phase after the treatment of any kinds of phospholipids tested.

The aqueous-methanol-soluble phosphorus-containing products obtained by BCl_3 treatment were analyzed by cellulose TLC and compared with the products prepared by mild alkaline methanolysis of standard diacyl phospholipids (Table 1). Treatment with BCl_3 converted almost quantitatively diester and di- or tetraether analogs of PC, PE, PI, PG, PS, and PA to GPC, GPE, GPI, GPG, GPS, and GP, respectively. The yield was 88–100%. R_f values were 0.93 (GPC), 0.57 (GPE), 0.14 (GPI), 0.46 (GPG), 0.28 (GPS), and 0.13 (Na salt of GP). The minor products such as cyclic GP, phosphoethanolamine, inositol monophosphate, and GP were identified based on the comparison of their R_f values with those described in reference 14 and responses to the spray reagents.

The compositions of the products obtained by the BCl_3 treatment of the ether or diester phospholipids were identical to the phospholipids. That is, BCl_3 treatment is equivalent to mild alkaline methanolysis in yielding glycerophosphoesters. Prolonged treatment (4 hr) with BCl_3 of ether phospholipids did not cause further degradation of the products except for PS or archaetidylserine. Almost half of GPS was further degraded during 4-hr treatment (data not shown). It is necessary to stop the treatment at 30 min in the case of PS. 1-Alkyl-2-acyl GPC and 1-alk-1'-enyl-2-acyl GPC were also cleaved to yield GPC (Table 1) by 4-hr treatment with BCl_3.

It is concluded that 30-min treatment with BCl_3 liberates quantitatively glycerophosphoesters retaining the original polar head group from di- or tetraether phospholipids. This method can be used to determine total structure of ether phospholipids or phosphoglycolipids as follows: *1*) it yields glycerophosphoester for identification of the glycerol-linked polar head group; *2*) strong alkaline hydrolysis of the glycerophosphoesters obtained yields GP which can be used for determination of stereochemical configuration of the GP moiety in the ether lipids by using GP dehydrogenase (18); *3*) alkyl chlorides obtained in the chloroform layer can be used for the determination of hydrocarbon chains (12); and *4*) total glycerol content of the lipids and glycerol:phosphorus ratio can be determined after strong acid hydrolysis (12).

BCl_3 treatment for 30 min cleaved gentiobiosyl caldarchaeol completely into glucose and glycerol. Gas–liquid chromatographic analysis showed that the glucose was present as a monomer (data not shown). This indicates that BCl_3 cleavage cannot be used to obtain glycosylglycerol from glyceroglycolipids.

The authors wish to express their thanks to Drs. Kunihiko Saito and Kiyoshi Satouchi, Department of Medical Chemistry, Kansai Medical University, for the generous gift of alkyl-acyl and alkenyl-acyl PC. The authors are grateful to Dr. Masahiro Kamekura, Noda Institute for Scientific Research, for providing them with a total lipid extract of *Halobacterium cutirubrum*.

Manuscript received 23 June 1987 and in revised form 25 September 1987.

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