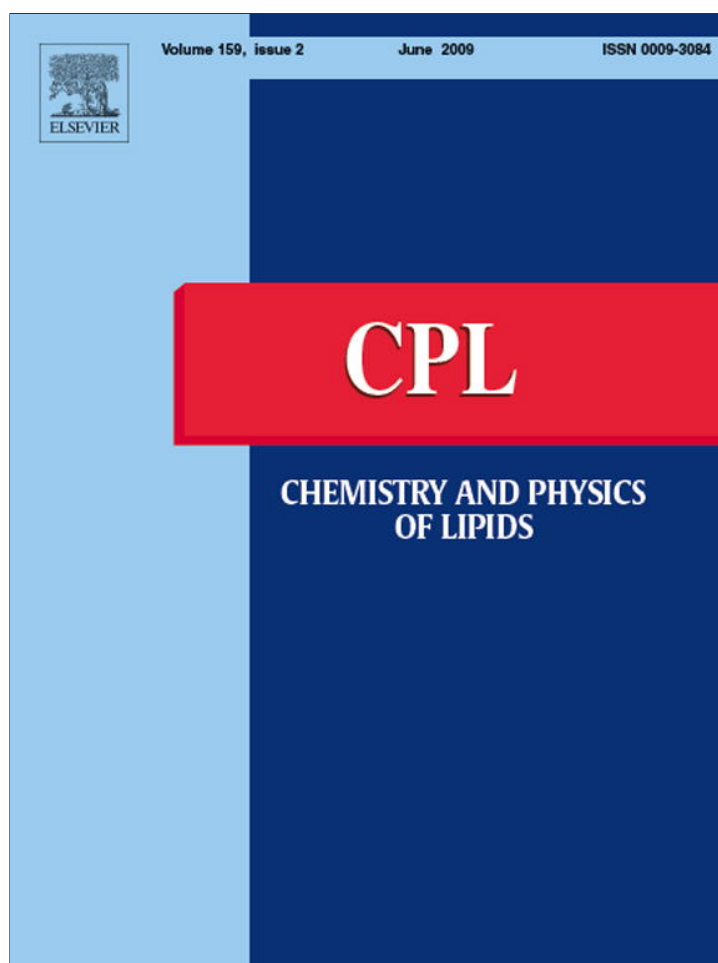




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Identification of (S)-11-cycloheptyl-4-methylundecanoic acid in acylphosphatidylglycerol from *Alicyclobacillus acidoterrestris*

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ABSTRACT

A method is described for the identification of molecular species of acylphosphatidylglycerols containing a branched cyclo fatty acid ((S)-11-cycloheptyl-4-methylundecanoic acid; brc19:0) from *Alicyclobacillus acidoterrestris* and its identification as picolinyl ester by means of GC-MS. The combination of TLC, negative RP-HPLC-ESI-MS/MS, enzymatic hydrolysis, and GC-MS was used to identify unusual molecular species of acylphosphatidylglycerols with cyclic and branched FA. The acid, brc19:0, was also synthesized to unambiguously confirm its structure. According to feeding experiments with ¹³C-labeled propionate, the C₃ internal unit (branched methyl) of brc19:0 is assembled from propionate and not from methionine.

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1. Introduction

Phospholipids are the major components of bacterial cell membranes. These lipid compounds possess closely related and complex structures classed according to the polar head group linked to the phosphate moiety. The diversity of the polar head groups is important, and as far as prokaryotic organisms are concerned, the main classes of phospholipids are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (cardiolipin, CL or DPG), phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidic acid (PA). All of them are considered to be characteristic of bacterial cell membranes (O'Leary and Wilkinson, 1988). Each phospholipid class is made up of several molecular species. The diversity of these molecular species depends on the two or more fatty acyl chains linked to the glycerol(s). Moreover, the study of the fatty acid (FA) distribution (*sn*-1 or *sn*-2 position) is of interest for determining the positional isomers of the molecular species. The most recent and convenient approach is based on detection and identification by electrospray ionization mass spectrometry (MS/ESI). Most authors have used either normal-phase columns or reversed-phase columns (Mazzella et al., 2004) coupled with an interface equipped with an ion trap or a triple quadrupole mass spectrometer (Hsu and Turk, 2005; Larsen and Hvattum, 2005). These techniques are the most efficient ones

for achieving qualitative and quantitative analyses of both classes and molecular species.

Acylphosphatidylglycerol (1,2-diacyl-*sn*-glycero-3-phospho-(3'-acyl)-1'-*sn*-glycerol) (acyl-PG), a polar lipid containing three fatty acyl substituents, has been identified in mycoplasma (Plackett et al., 1970), bacteria *Salmonella typhimurium* (Hsu et al., 2004; Olsen and Ballou, 1971), *Acinetobacter* sp. (Makula et al., 1978), *Escherichia coli* (Cho et al., 1976; Homma and Nojima, 1982), molds (*Dictyostelium discoideum*) (Ellingson, 1980), and trichomonads (*Trichomonas vaginalis* and *T. foetus*) (Beach et al., 1991).

HPLC-MS/ESI was used by Holmback et al. (2001) to analyze intact acyl-PG molecules. Collisional-induced dissociation (CID) tandem sector mass spectrometric methods with fast-atom bombardment were also used to identify acyl-PGs isolated from *Corynebacterium* spp. (Niepel et al., 1998) and *Trichomonas* (Costello et al., 2001). A systematic approach towards the structural characterization of acyl-PGs using low-energy or high-energy CID tandem mass spectrometry with electrospray ionization has been described by Hsu et al. (2004).

Members of the genus *Alicyclobacillus* (former misnomer *Bacillus*) (Wisotzkey et al., 1992) are thermo-acidophilic, strictly aerobic, heterotrophic, endospore-forming bacteria. The major membrane lipid components are ω -alicyclic FAs (ω -cyclohexane or ω -cycloheptane FAs) (De Rosa et al., 1974; Goto et al., 2003; Oshima and Ariga, 1975). The FAs of the extreme thermoacidophile *Bacillus acidocaldarius* (currently *Alicyclobacillus*) comprised primarily branched heptadecanoic, 11-cyclohexylundecanoic and 13-cyclohexyltridecanoic acids (Langworthy et al., 1976). The acid

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Table 1

Fatty acid composition of total acyl-PG.

Fatty acids	Full name of acid	% ^a
ai17:0	14-Methylhexadecanoic	20.4
c17:0	11-Cyclohexylundecanoic	41.7
cbr19:0	(S)-11-Cycloheptyl-4-methylundecanoic	3.4
c19:0	13-Cyclohexyltridecanoic	34.5

^a Minority fatty acids up to 0.1% of total fatty acids were omitted.

lipids were composed of a sulfonoglycosyldiacylglycerol, CL, lyso-CL, PA and PG.

Two major glycolipids with higher chromatographic mobility, a sulfonoglycosyldiacylglycerol, two phospholipids, and one glycolipid with a smaller R_f were the main components of the four strains belonging to the genus *Alicyclobacillus* (Nicolaus et al., 2001).

The shikimate pathway is an essential metabolic route by which microorganisms synthesize a number of aromatic and/or cyclohexyl compounds which are critical to sustaining the primary functions of living organisms. The shikimic acid origin of the ω -cyclohexyl FAs in their membrane lipids has been demonstrated, particularly when grown at high temperature and low pH (Floss, 1997).

The ω -cyclohexyl FAs of various *Alicyclobacillus* species are uniquely replaced by the homologous ω -cycloheptyl FAs in *A. cycloheptanicus* (Moore et al., 1995). The biosynthesis of the cycloheptyl moiety again originates from shikimic acid. FA biosynthesis arises from cyclohexylcarbonyl CoA (cycloheptylcarbonyl CoA) as starter units, which undergo chain elongation by addition of four to six acetate units.

Individual mid-methyl-branched FAs (MMBFAs) have been observed in several pure cultured prokaryotes, for example 10-methylhexadecanoic acid in *Desulfobacter* sp. (Dowling et al., 1986) or 10-methyloctadecanoic acid (tuberculostearic acid) as a typical constituent of mycobacteria (Fowler and Douglas, 1987). MMBFAs are generally synthesized from unsaturated FA and S-adenosylmethionine (Akamatsu, 1968; Jaureguiberry et al., 1966). On the other hand, the number of biosynthetic pathways in

which the methyl group in the middle of the chain derives from propionate (methyl-malonate) is low. The production of C₂₃–C₂₆ methyl-branched FAs (Fernandes and Kolattukudy, 1998) was determined by means of radio-GLC of methyl esters synthesized by the purified short chain mycocerosic acid synthase using [methyl-¹⁴C]methylmalonyl-CoA and C₂₀-CoA as primer. Further, the biosynthesis of multimethyl-branched FAs in *Mycobacterium tuberculosis* involves methylmalonyl-CoA as the chain-extending substrate (Minnikin et al., 2002).

Six novel FAs with methyl substituents at carbons 2 and 4 were identified in a halophilic *Bacillus* sp. isolated from the salt pans (Carballeira et al., 2001). These methyl-branched FAs probably originated from the selective incorporation of methylmalonyl-CoA by the FA synthase of the *Bacillus*.

We present tandem mass spectrometric methods using a triple quadrupole mass spectrometer to characterize acyl-PG in detail. We developed an optimized phospholipid detection method by HPLC-MS/ESI and performed an original synthetic study concerning the formation of the brc19:0 including the measurement of NMR and mass spectra. With the aid of labeled [3-¹³C] propionate we demonstrated the biosynthesis of a branched FA, including its absolute configuration determined by total chiral synthesis.

2. Results and discussion

Alicyclobacillus acidoterrestris was cultivated in a 5 L fermentor and cells were harvested in log phase when the optical density of the culture was maximal. The time of cultivation was 24 h. The yield of cells (wet weight) was about 11.7 g of bacteria. Lyophilized cells (2.65 g dry weight) were obtained from 3 L of culture.

The lipids were obtained by the Bligh–Dyer extraction (Bligh and Dyer, 1959) of lyophilized cells with a yield of 331.3 mg (12.5%). Total lipids were fractionated by means of cartridges with aminopropyl silica-based polar bonded phase, which were rinsed in a chloroform–methanol mixture and phospholipids were further eluted by a mixture of chloroform–methanol–concentrated aqueous ammonia. Phospholipids (68% of total lipids, i.e. 225.3 mg) were

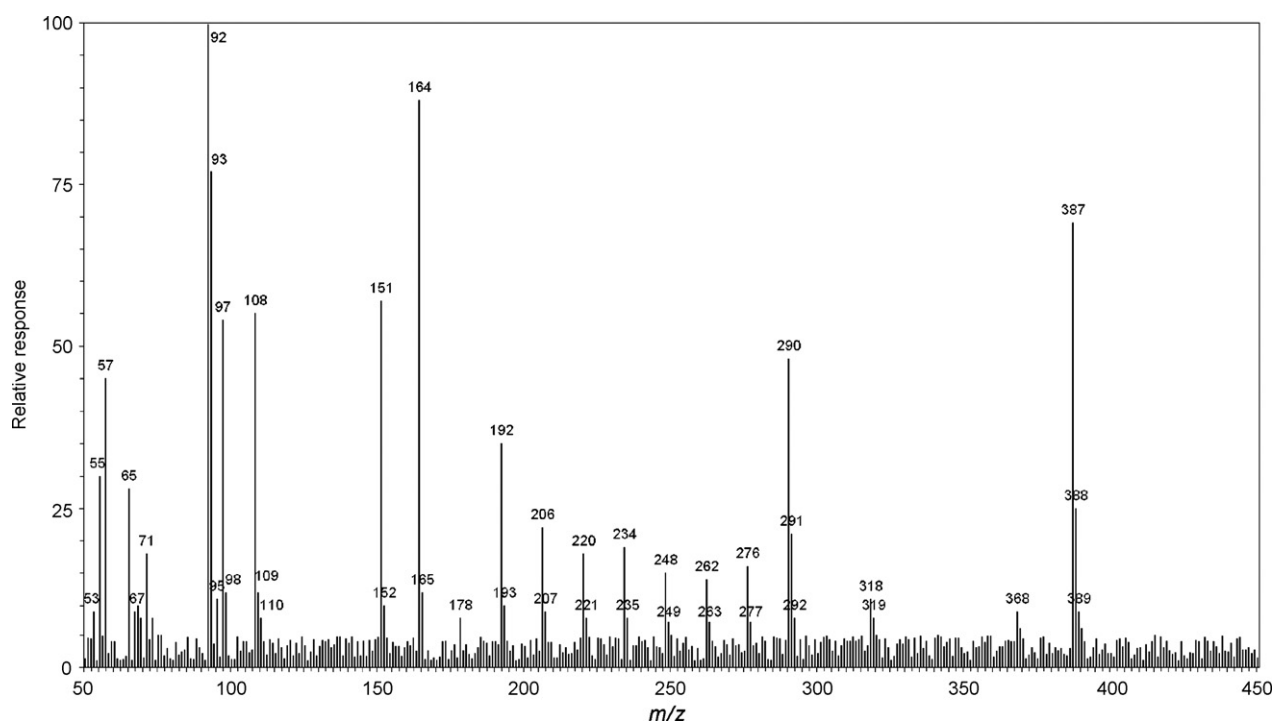


Fig. 1. Electron ionization mass spectrum of (S)-11-cycloheptyl-4-methylundecanoic (brc19:0) picolinyl ester of acyl-PG from *Alicyclobacillus acidoterrestris*.

Table 2
NMR chemical shifts of acyl-PG in CDCl₃–CD₃OD (1:1, v/v).

Compound	¹ H	¹³ C
C(ω)	0.85	14.3
C(ω-1)	1.20–1.40	23.2
C(ω-2)	1.20–1.40	32.3
C, methylenes	1.20–1.40	29.0–30.5
C1	–	174.0, 174.4, 174.8
C2	2.25	34.5
C3	1.60	25.4
sn-1	4.16, 4.36	63.0
sn-2	5.21	70.9
sn-3	3.92	63.9
sn-1'	3.80, 3.84	67.2
sn-2'	3.88	69.4
sn-3'	4.07	65.3

further separated by two-dimensional thin layer chromatography with chloroform–methanol–7 M ammonia in the first dimension and chloroform–methanol–glacial acetic acid in the second dimension. The major phospholipids were PE, PG, and CL; PA was present in minor amounts; PS and PC were not recovered.

UV-light detection after TLC showed a spot having *R_f* of 0.8–0.9 in both solvent systems. The spot gave negative responses to the ninhydrin spray for amine detection, Dragendorff stain for choline, and the vicinal glycol detection spray (periodate–Schiff stain), but a positive response to phosphate spray (Vaskovsky et al., 1975). This spot, denoted lipid X, was scraped from the plates and eluted, with a yield of 17% (38.3 mg) of total phospholipids. GC–MS analysis of FAME revealed that it contained 3.4% (1.3 mg) of the unknown FA—see also Table 1 for FAME composition from lipid X. The relative content of this acid in total FAs obtained by acid hydrolysis of total lipids was 0.45%, i.e. 1.5 mg. Taking into account the losses during isolation, nearly all of the unknown acid can be said to be contained in lipid X. It should be noted that only fatty acids above 0.1% of total fatty acids are included in Table 1.

The structure of the unknown acid, denoted provisionally as a branched alicyclic FA on the basis of GC–MS of methyl esters, was determined by means of mass spectrometry of picolinyl esters (Harvey, 1998). In the high mass region, the molecular ion (at *m/z*

387) is followed by a clear gap of 97 Da to an ion at *m/z* 290, representing the loss of cycloheptane ring. Thereafter, there is a regular series of ions 14 Da apart for cleavage at the successive methylene groups. With picolinate, the gap is between *m/z* 164 and 192. The ion for the charged cycloheptane ring (*m/z* 97) is also distinctive, see Fig. 1. On the basis of all the above data, the tentative structure was suggested as methyl 11-cycloheptyl-4-methylundecanoate (1). Full confirmation of the structure including the configuration on carbon 4 was carried out after its isolation and total synthesis (see below).

The ¹H NMR data (Table 2) of lipid X display a pattern of signals in the regions typical for polar glycerolipids, e.g., signals from the methyl, methylene and methine groups at δ 0.85–2.25 and from protons next to oxygen at δ 3.80–5.21 ppm. In the ¹³C NMR spectrum signals (Table 2) from the acyl moiety appear in the δ 14.3–34.5 ppm region and carbonyls at δ 174.0–174.8 ppm. Three distinct carbonyl signals are visible, showing that three acyl groups are present. Signals from carbons attached to hydroxyl or carboxylate groups appear at δ 63.0–70.9 ppm in the ¹³C NMR spectrum. By running DEPT experiments these carbons can be classified as methylene (CH₂) groups (δ 63.0, 63.9, 67.2, and 65.3 ppm) and/or methine (CH) groups (δ 70.9 and 69.4 ppm).

The TOCSY experiment allows the identification of one spin system for the proton signals at δ 4.16, 4.36, 5.21, and 3.92 ppm and another one at δ 3.80, 3.84, 3.88, and 4.07 ppm. For the first spin system, similar reasoning as above implies that we have a diacylglycerophosphate moiety present. The second spin system also resembles the glycerol moieties in the PG with the exception of the signal at the *sn*-3' position, which has been shifted upfield. This effect could be explained by the substitution of a carboxylate for a hydroxyl group in this position, which leads to the conclusion that lipid X is an acyl-PG.

The structure of acyl-PG was deduced on the basis of knowledge of negative mass spectra published previously by several authors (Hsu and Turk, 2005; Larsen and Hvattum, 2005; Olsen and Ballou, 1971). The identification can be based on four basic groups of ions, i.e. [M–H][–]; ions reflecting the losses of the fatty acyl substituent as a free acid ([M–H–RCOOH][–]) and as a ketene ([M–H–R'CH=C=O][–]) at *sn*-1 or *sn*-2, respectively; the [M–H–R_y'CH=C=O–R_xCOOH–74][–] ions; and carboxylate anions RCOO[–].

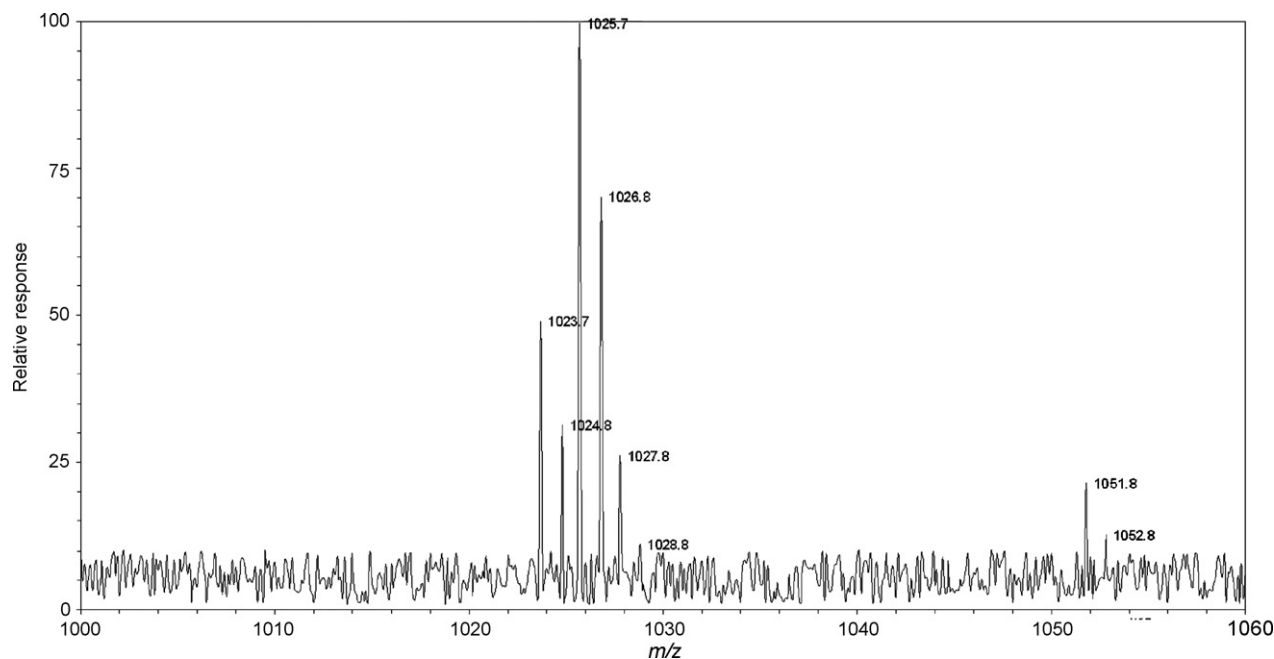


Fig. 2. Negative ion electrospray mass spectrum of deprotonated molecular ions of acyl-PG from *A. acidoterrestris*.

Table 3

Fatty acid composition of acyl-PG molecular species.

Mol. species	[M–H] [–]	%
c19:0-c17:0/c17:0	1023	30
c19:0-ai17:0/c17:0	1025	60
brc19:0-c19:0/c17:0	1051	10

Hsu et al. (2004) described a rule which, on the basis of observation of abundances of $R_2\text{COO}^- > R_3'\text{COO}^- \gg R_1\text{COO}^-$, provides a simple method leading to the assignment of the structure of an acyl-PG. Unfortunately, this strategy is not applicable to molecules having two identical fatty acyl substituents (brc19:0 and c19:0 have the same summary formula) at two different positions, or comprising more than one isomeric structure. We therefore made use of the results in papers (Mazzella et al., 2004, 2005) and attempted to separate the molecular species of acyl-PGs. Detailed analysis of the compounds separated by RP-HPLC is described below.

The negative ion mass spectrum of the total acyl-PGs, obtained from TLC, is reproduced in Fig. 2. We observed several $[M-H]^-$ ions from m/z 1000–1060, which were related to the molecular species. The relative amounts of the intact molecular species were determined by MS/ESI. Semiquantitative results based on the abundance of monoisotopic peaks (i.e. $[M-H]^-$ ions at m/z 1023, 1025 and 1051) are reported in Table 3.

Structural identification of acyl-PGs with identical fatty acyl substituents at *sn*-1 and *sn*-3' (i.e. in our case the compound with m.w. 1052 Da) is shown in Fig. 3. The product-ion spectrum of the $[M-H]^-$ ion at m/z 1051, which contains carboxylate anions at m/z 267 and 295, reflecting the c17:0- and c19:0-acyl constituents, is given in Fig. 3. The structural identification is achieved by the observation of the greater abundance of m/z 801 ($[M-H-C_{15}H_{29}CH=CO]^-$) relative to m/z 783 ($[M-H-C_{16}H_{31}COOH]^-$), signifying a c17:0-acyl residing at *sn*-2, along with the observation of the preferential formation of m/z 755 ($[M-H-C_{18}H_{35}COOH]^-$) over m/z 773 ($[M-H-C_{17}H_{33}CH=CO]^-$), signifying the presence of a c19:0-fatty acyl at *sn*-1 and/or *sn*-3'. Since the prominent ions at m/z 431 together with m/z 449 and 505 (for structures see paper

Hsu et al., 2004) are also present in the spectrum, the assignment of acyl ($C_{18}H_{35}CO$) at *sn*-3' can be easily confirmed.

Analysis of this acyl-PG revealed the presence of brc19:0 acid, whose position in acyl-PG, however, cannot be identified in this way. We therefore used semipreparative RP-HPLC for the separation of total acyl-PG. Despite using two columns in series, and achieving thereby the number of theoretical plates $\sim 30,000/30$ cm, we succeeded in separating only a peak of m.w. 1052 Da from two other unseparated peaks (m.w. 1024 and 1026 Da). The study of Mazzella et al. (2004) cited above also did not separate all molecular species, e.g. $16:1^*2 \times 18:1$ and $16:0^*2 \times 18:1$, i.e. the situation was the same as in our sample. In both cases the molecular species of acyl-PG differ by a mere 2 Da. However, this separation was sufficient for our purposes because it confirmed unambiguously the structure of acyl-PG having m.w. 1052 Da, which was separated by RP-HPLC in a semipreparative mode and further hydrolyzed by phospholipase C (Virto and Adlercreutz, 2000). The resulting monoacyl- and diacylglycerol were then separated by TLC (Kates, 1986). After hydrolysis, only the monoacylglycerol contained brc19:0 while the diacylglycerol contained only the two remaining c19:0 and c17:0 acids. All these data imply that the molecular species with m.w. 1052 Da has the structure depicted in Fig. 4, i.e. brc19:0-c19:0/c17:0 acyl-PG.

The two molecular species at m.w. 1024 and 1026 Da contained in the unresolved peak that could not be separated by semipreparative RP-HPLC were fully identified by means of their mass spectra.

The CID spectrum of acyl-PG at m/z 1025 exhibits three dominant $RCOO^-$ ions having m/z 267, 269, and 295, respectively, which belong to c17:0, ai17:0 and c19:0 acids. Abundances of carboxylate anions are of the order ai17:0 > c19:0 > c17:0, the ratio of abundances of ions at m/z 267–269 being more than 2. The structure of this acyl-PG was confirmed by the fragment ions at m/z 747 ($[M-H-C_{17}H_{33}CH=CO]^-$), m/z 773 ($[M-H-C_{15}H_{29}CH=CO]^-$), and m/z 775 ($[M-H-C_{15}H_{31}CH=CO]^-$), arising from losses of the c19:0, c17:0, and ai17:0 fatty acyl ketenes from m/z 1025, respectively. Similarly, ions at m/z 729, 755, and 757 arise from losses of the c19:0, c17:0, and ai17:0 FAs, respectively. Based on the abundance of ions $[M-H-FA]$ and $[M-H-fatty\ acyl\ ketenes]$ and using previously described rules (Hsu and Turk, 2005; Hsu et al., 2004) we could determine

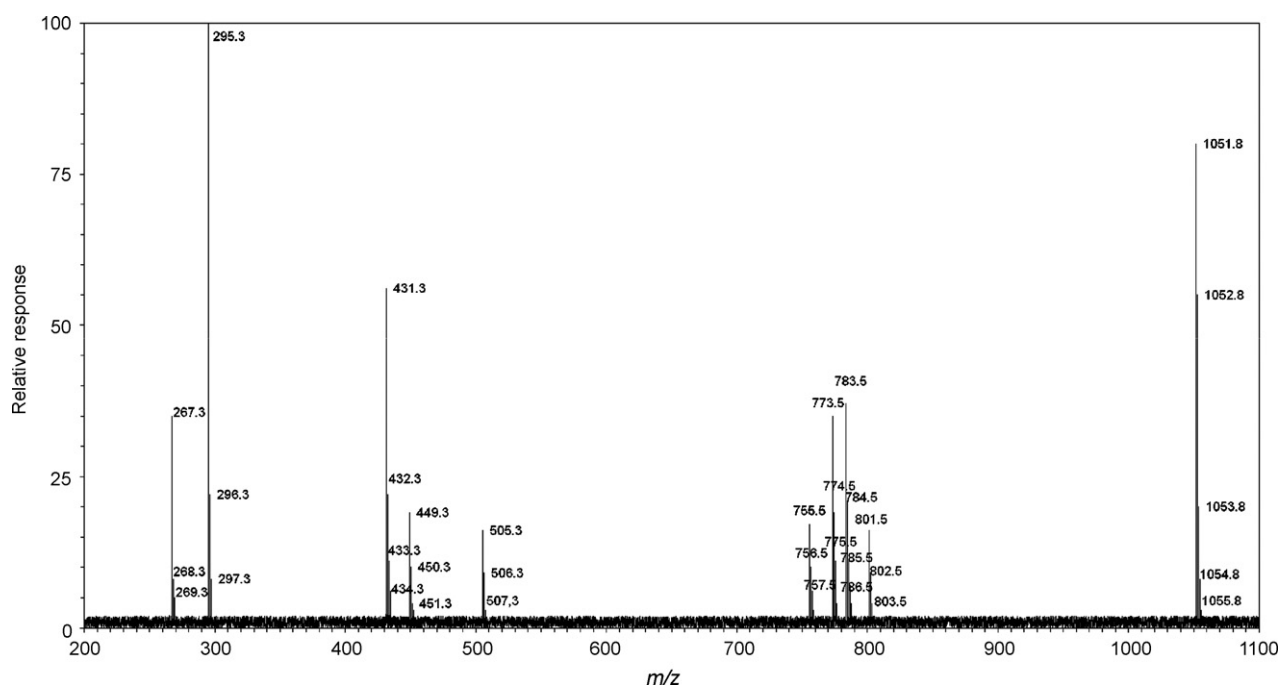


Fig. 3. The tandem quadrupole ESI product-ion spectra of the $[M-H]^-$ ion of brc19:0-c19:0/c17:0-PG at m/z 1051.8.

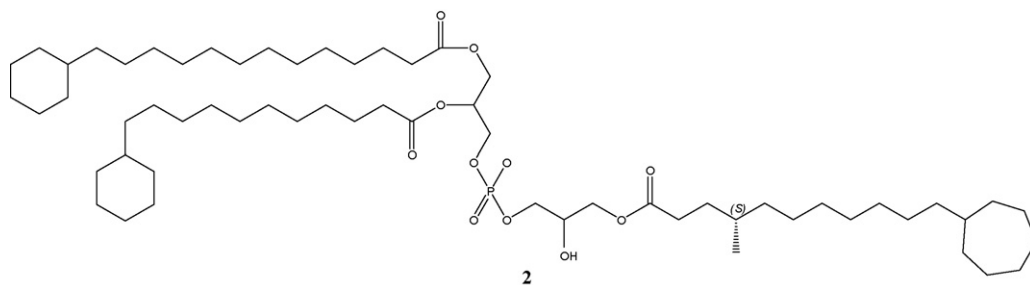


Fig. 4. Structure of acylphosphatidylglycerol [1-(13-cyclohexyltridecanoyl)-2-((S)-11-cyclohexylundecanoyl)-sn-glycero-3-phospho-(3'-(11''-cycloheptyl-4''-methylundecanoyl))-1'-sn-glycerol]; brc19:0-c19:0/c17:0-PG] (**2**).

that ai17:0 and c17:0 are at *sn*-2 and at *sn*-1 of the glycerol backbone, respectively. This was further confirmed by the presence of an ion at *m/z* 449, which belongs to acyl glycerophosphate and signifies a c19:0 fatty acid at *sn*-3'. The structure of acyl-PG can thus be determined as c19:0-ai17:0/c17:0.

The CID spectrum anion at *m/z* 1023 contains two RCOO[−] ions at *m/z* 267 and 295, which document the presence of c17:0 and c19:0 acids, respectively. The presence of c17:0 in position *sn*-2 can be determined from the higher abundance of ion at *m/z* 745, formed by the loss of c19:0 ketene, relative to the abundance of ion at *m/z* 755 arising by the loss of c17:0 acid. These values imply that c19:0 is either in position *sn*-1 or *sn*-3'. A c17:0 acylglycerophosphate anion at *m/z* 421, together with ion at *m/z* 403 and the absence of ion at *m/z* 449 further confirms the proposed structure and the fact that c19:0 acid is in position *sn*-3'. The ultimate structure of this acyl-PG (with m.w. 1024) is c19:0-c17:0/c17:0.

The brc19:0 acid was isolated by TLC, semipreparative RP-HPLC, and enzymatic hydrolysis. The starting material was a mixture obtained after a three-step separation–purification of hydrolysate of monoacylglycerols (see above). Its optical rotation was -0.7 . To confirm its structure, brc19:0 was also synthesized since the literature data (Levene and Harris, 1935; Rothen and Legend, 1939) obtained with model compounds did not

allow us to determine if it is the *R* or *S* isomer. The data obtained for three model compounds— ω -cyclohexyl FAs ($[\alpha]_D$ -1.2 for (*S*)-5-cyclohexyl-3-methyl-pentanoic acid, $[\alpha]_D$ -0.6 for (*S*)-6-cyclohexyl-4-methyl-hexanoic acid and $[\alpha]_D$ -1.34 for (*R*)-7-cyclohexyl-5-methyl-heptanoic acid) indicate that, irrespective of the compound being an *R* or *S* isomer, the optical rotation values are always negative. The full elucidation of the structure, including the $[\alpha]_D$ value, was therefore achieved by synthesizing chiral brc19:0.

Since optical rotations were very similar and near zero, we performed, for a complete evidence of the absolute configuration, in addition gas chromatography of both the natural and the synthetic methyl ester of (*S*)-11-cycloheptyl-4-methylundecanoic acid on the chiral phase, see Section 4. The retention times of both methyl esters were identical and equal to 69.3 min.

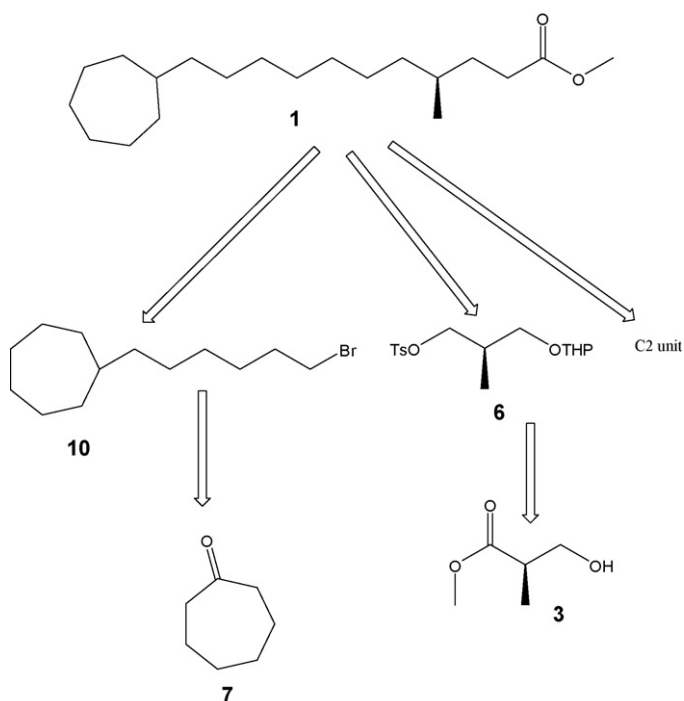
Scheme 1 shows our synthetic plan. Commercially obtained chiral ester **3** serves as the starting material for compound **6**. The key step is the alkylation of the tosylate **4** with the bromide **10**, which is prepared from cycloheptanone **7** by aldol condensation and shortening of the appropriate acid by one carbon atom.

The commercially available chiral starting material **3** was first protected as a THP **4**. After reduction of the ester moiety, the resulting primary alcohol **5** was derivatized to tosylate **6** (Mori and Takikawa, 1991; Mori, 1983).

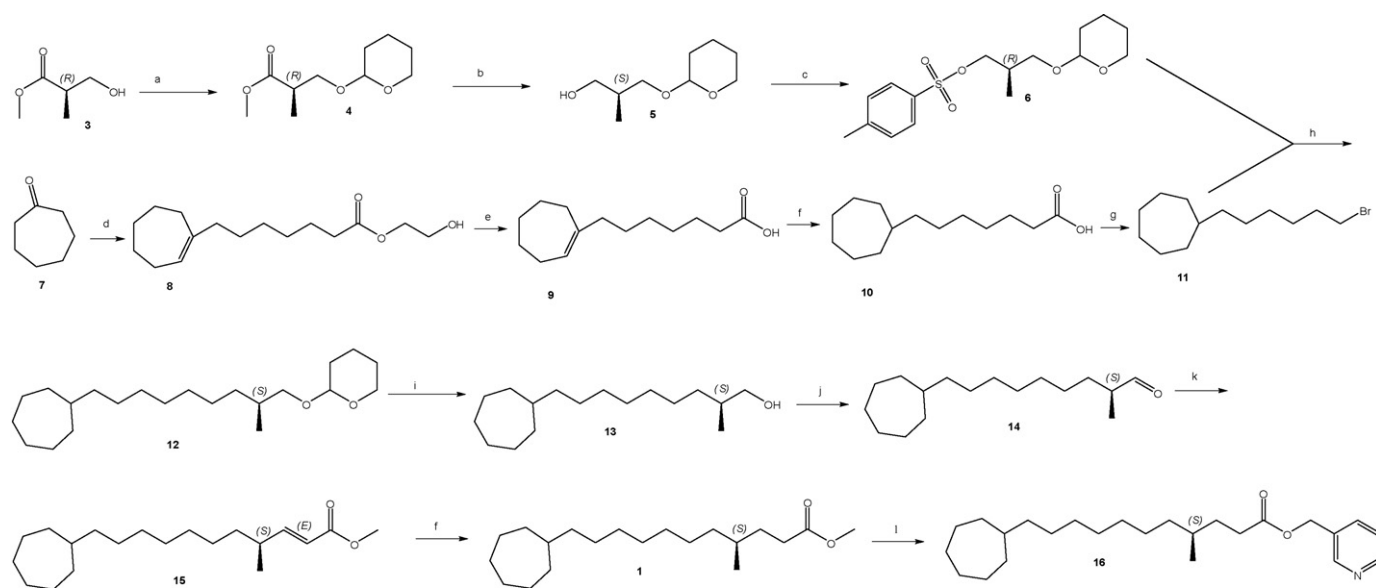
Ring cleavage and reconstruction via crossed intramolecular aldol condensation followed by Grob fragmentation under acetalization conditions (BF₃/ethylene glycol) was used for building the basic backbone of ω -cycloheptyl FA (Nagumo et al., 1993). In this reaction, two molecules of cycloheptanone **7** give in a 47% yield ester **8**, which provides acid **9** after hydrolysis. Reduction of this acid by Pd/C yields fully saturated 7-cycloheptylheptanoic acid (**10**). Another key reaction was a one-carbon shortening of acid **10**. This was performed using a bromodecarboxylation reaction (Hunsdiecker-type bromodecarboxylation of carboxylic acids) (Camps et al., 2000) with iodosobenzene diacetate and bromine under irradiation with a tungsten lamp, which yielded bromide **11**. Coupling of **6** with (6-cycloheptylhexyl)magnesium bromide **11** in the presence of a catalytic amount of Li₂CuCl₄ leads to **12**.

As shown in **Scheme 2**, the synthesis proceeded by removal of the protecting group and oxidation of alcohol **13** by Swern oxidation, the product of which was immediately subjected to Horner–Wadsworth–Emmons reaction, affording ene ester **15** as an isomeric mixture (*E*:*Z* = 85:15). Ene ester **15** was hydrogenated (H₂, Pd/C) to give the required saturated ester **1**. Picolinate **16** was prepared according to the literature (Rezanka, 1990).

Optical rotation of synthesized methyl ester of (*S*)-11-cycloheptyl-4-methylundecanoic acid was determined as the value $[\alpha]_D^{24}$ -0.75 , see Section 4. On the basis of both values (from synthetic and from natural compound **1**), we confirmed that configuration at C-4 is *S* and full structure of acyl-PG with m.w. 1052 Da is 1-(13-cyclohexyltridecanoyl)-2-((*S*)-11-cyclohexylundecanoyl)-sn-glycero-3-phospho-(3'-(11''-cycloheptyl-4''-methylundecanoyl))-1'-sn-glycerol).



Scheme 1. Structure of the acid [(*S*)-11-cycloheptyl-4-methylundecanoic (brc19:0)], and its retrosynthetic analysis.



- a: DHP, p-TsOH, Et₂O, (99%);
 b: LiAlH₄, Et₂O (95%);
 c: p-TsCl, C₅H₅N (97%);
 d: BF₃-etherate, ethylene glycol, 0 °C → rt, 24 h, (47%);
 e: KOH, water-MeOH, rt (90%);
 f: MeOH-Hex, Pd/C, H₂, rt, 12 h, (98%);
 g: (diacetoxyiodo)benzene, Br₂, CH₂Br₂, hv, Δ, (75%);
 h: Mg, Li₂CuCl₄, THF (91%);
 i: p-TsOH, MeOH (93%);
 j: DMSO, (COCl)₂, CH₂Cl₂, -70 °C; Et₃N, -70 → rt (95%);
 k: (EtO)₂P(=O)CH₂COOMe, NaH, THF, -78 → rt (92%);
 l: t-BuOK, THF, nicotinyl alcohol, 40 °C (85%);

Scheme 2. Enantioselective synthesis of **1** (brc19:0).

The biosynthetic origin of brc19:0 was studied by performing a feeding experiment with ¹³C-labeled precursor, i.e. sodium propionate (¹³CH₃CH₂COONa). After feeding [3-¹³C]propionate only one carbon, i.e. C4 methyl, was found with a very high ¹³C-enrichment—about 47.8% above the natural ¹³C content. This feeding experiment also confirmed that none of the other carbons were labeled by propionate, i.e. no α-oxidation of propionate to acetate and its incorporation into the molecule of brc19:0 takes place. We therefore assume that the rest of the molecule is biosynthesized by shikimate and acetate pathways, as published previously (Floss, 1997). The biosynthesis of brc19:0 was thus fully confirmed. Recently, a biosynthesis of the methyl-branched aliphatic chains from propionate unit(s) was described in the *Mycobacterium* sp. (Fernandes and Kolattukudy, 1998; Minnikin et al., 2002; Scaife et al., 1978). Natural mycosanoic, mycolipanoic, mycolipanoic, and phthioceranoic acids also have the S-configuration (Scaife et al., 1978) in agreement with our brc19:0 acid. Tuberculostearic acid has the R-configuration because it is biosynthesized by a different pathway (Akamatsu, 1968; Jaureguiberry et al., 1966).

3. Conclusion

While acyl phosphatidylglycerol has been identified in the lipids of many organisms, as described in Section 1, e.g. mycoplasma (Plackett et al., 1970), bacteria (Hsu et al., 2004; Olsen and Ballou, 1971) *Acinetobacter* sp. (Makula et al., 1978), *E. coli* (Cho et al., 1976; Homma and Nojima, 1982), molds (Ellingson, 1980), and trichomonads (Beach et al., 1991), the present paper represents the first complete structural characterization of this lipid in the thermophilic bacteria. The biosynthesis of acyl-PG could be visualized as involving the acylation of phosphatidylglycerol or the deacylation of a hypothetical bis-phosphatidic acid or phosphatidyl-diacylglycerol. Minor components of cellular extracts are often found to possess unusual metabolic activity. The difference

in the FA composition of the di- and monoesterified glycerol parts and the fact that the extra acyl group is located on the 3' position of the second glycerol suggest that the lipid is a natural cell constituent. In addition, a rapid incorporation of labeled propionate into this compound by growing cells has been observed with ¹³CH₃CH₂COONa.

The experimental data prove without doubt that the methyl moiety of the brc19:0 is generated by this strain of *A. alicyclobacillus* de novo from a propionate intermediate, and not via methylene-substituted acid. The hypothetical mechanism of propionate incorporation involves methylmalonyl-CoA and further condensation steps with malonyl-CoA. Similar to our results, feeding experiments with cycloheptanecarboxylic and acetic acids indicated (Floss, 1997) that the ω-cycloheptyl FAs are formed from a shikimate pathway intermediate and further via elongation by acetate units. These findings are in contrast with the commonly accepted biosynthesis of MMBFAs via unsaturated- and methylene-substituted acids, in which the methylene group is hydrogenated in the last step to methyl group.

Although 2-methyl-branched ω-cyclohexyl FA has been described (Nicolaus et al., 1998), its identification was not too convincing. We therefore assume that we are the first to describe not only the structure of the new branched alicyclic FA but also its total chiral synthesis and biosynthesis by using labeled propionate, as well as the structure of the acidic phospholipid–acyl-PG, which is the only complex lipid containing this acid.

4. Experimental

HPLC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Hewlett Packard, USA) and two Ascentis® Express HILIC HPLC column 2.7 μm particle size, L × I.D. 15 cm × 2.1 mm (Supelco, Prague) in series were used. This setup provided us with a high-efficiency column—approximately 30,000 plates/30 cm. LC was performed at

a flow rate of $300 \mu\text{L min}^{-1}$ with a linear gradient from mobile phase containing methanol/acetonitrile/aqueous 1 mM ammonium acetate (60:20:20, v/v/v) to methanol/acetonitrile/aqueous 1 mM ammonium acetate (20:60:20, v/v/v) for 40 min. The whole HPLC flow (0.37 mL min^{-1}) was introduced into the ESI source without any splitting.

The detector was an Applied Biosystems Sciex API 4000 mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) using electrospray mass spectra. The ionization mode was negative, the nebulizing gas (N_2) pressure was 345 kPa and the drying-gas (N_2) flow and temperature were 9 L min^{-1} and 300°C , respectively. The electrospray needle was at ground potential, whereas the capillary tension was held at 4000 V. The cone voltage was kept at 250 V. The mass resolution was 0.1 Da and the peak width was set to 6 s. For an analysis, total ion currents (full scan) were acquired from 200 to 1600 Da.

CID ions mass spectra were acquired by colliding the Q1 selected precursor ions with Ar gas at a collision target gas and applying collision energy of 50 eV in Q2. Scanning range of Q3 was m/z 200–1100 with a step size of m/z 0.1 and a dwell time of 1 ms. A peak threshold of 0.3% intensity was applied to the mass spectra. The instrument was interfaced to a computer running Applied Biosystems Analyst version 1.4.1 software.

In the semipreparative mode all conditions and instrumentation were identical, with the exception of mass spectrometer which was replaced by UV detection at 210 nm (HP 1040 M Diode Array Detector).

Gas chromatography–mass spectrometry of FAME was done on a GC–MS system consisting of Varian 450-GC, Varian 240-MS iontrap detector with external ionization (EI), and Combi-Pal autosampler (CTC, USA). The sample was injected onto a $25 \text{ m} \times 0.25 \text{ mm} \times 0.1 \mu\text{m}$ Ultra-1 capillary column (Supelco, Czech Republic) under a temperature program: 5 min at 50°C , increasing at $10^\circ\text{C min}^{-1}$ to 320°C and 15 min at 320°C . Helium was the carrier gas at a flow of 0.52 mL min^{-1} . All spectra were scanned within the range m/z 50–600.

A FA picolinyl ester mixture was analyzed on the instrument described above. Injection temperature (splitless injection) was 100°C , and a fused-silica capillary column (Supelcowax 10; $60 \text{ m} \times 0.25 \text{ mm i.d.}$, $0.25 \mu\text{m}$ film thickness; Supelco, PA) was used. The temperature program was as follows: 100°C for 1 min, subsequently increasing at $20^\circ\text{C min}^{-1}$ to 180°C and at 2°C min^{-1} to 280°C , which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm/s . All spectra were scanned within the range m/z 70–650.

Chiral chromatography. The resulting methyl esters of (S)-11-cycloheptyl-4-methylundecanoic acid (both synthetic and natural) were injected into the chiral fused silica capillary column HYDRODEX β -3P (i.d. 0.25 mm , length 25 m) with the stationary phase [heptakis-(2,6-di-O-methyl-3-O-pentyl)- β -cyclodextrine] from Macherey-Nagel GmbH & Co. KG, Duren, Germany. Oven temperature: 50 to 150°C at 2°C min^{-1} , then to 240°C at 5°C min^{-1} , carrier gas helium, 20 cm/s , detector FID, 300°C , injection of $1 \mu\text{L}$ mixture in methylene chloride (containing 0.5 mg/mL of each analyte), split ($100:1$), 300°C .

NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^1H) and 125.7 MHz (^{13}C). Optical rotations were measured with a Perkin-Elmer 243 B polarimeter.

A. acidoterrestris was cultivated in an alicyclobacillus medium (Siristova et al., 2009) containing (g L^{-1}): yeast extract 6.0, glucose 5.0, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.25, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5, $(\text{NH}_4)_2\text{SO}_4$ 0.2, KH_2PO_4 3.0 and 1 mL L^{-1} of trace element solution ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g L^{-1} , $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 0.03 g L^{-1} , H_3BO_3 0.3 g L^{-1} , $\text{CuCl}_2 \cdot 6\text{H}_2\text{O}$ 0.2 g L^{-1} , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.01 g L^{-1} , $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ 0.02 g L^{-1} , $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 0.03 g L^{-1}), pH was adjusted to 4. The inoculum (400 mL) was pre-

pared in 500-mL round-bottom flasks on a temperature controlled shaker at 45°C and 200 rpm .

The strain cultivation was carried out in a 5 L mechanically stirred fermentor for 24 h , under aerobic conditions with aeration rate 0.8 VVM . The stirrer frequency was 300 rpm and the cultivation temperature was 45°C . Cells were harvested in log phase when the optical density of the culture was maximal. Lyophilized cells (2.65 g) were obtained from 3 L of culture.

The extraction procedure was based on the method of Bligh and Dyer (1959), except that 2-propanol was substituted for methanol, since isopropanol does not serve as a substrate for phospholipases (Kates, 1986). The alcohol–water mixture was cooled and one part chloroform was added and the lipids were extracted for 30 min . Insoluble material was sedimented by centrifugation and the supernatant was separated into two phases. The aqueous phase was aspirated off and the chloroform phase was washed three times with two parts 1 M KCl each. The resulting chloroform phase was evaporated to dryness under reduced pressure.

First, total lipid extracts were applied to Sep-Pak Cartridge Vac 35 cc (Waters; with 10 g of amino propyl silica-based polar bonded phase), and from the cartridge were subsequently eluted nonacidic lipids with 40 mL of chloroform–methanol ($7:3$), and acidic lipids with 30 mL of chloroform–methanol–concentrated aqueous ammonia ($70:30:2$) containing 0.4% (w/v) ammonium acetate. The elute was reduced in volume and subjected to two-dimensional preparative TLC (PLC silica gel 60 F_{254} , $2 \text{ mm} \times 20 \text{ cm} \times 20 \text{ cm}$). The first solvent was chloroform–methanol– 7 M ammonia ($12:7:1$); the second solvent was chloroform–methanol–glacial acetic acid ($65:25:8$). Identification was made based upon cochromatography with commercial standards or by R_f described in the literature and a specific reagent, see below. In preparative mode, visualized spots under UV were scraped off and the lipid was eluted with 2 mL of chloroform–methanol ($1:1$, v/v). Specific reagents for lipids (7% , w/v, molybdophosphoric acid in ethanol), sugars (0.1% , w/v, orcinol in 40% , v/v, sulfuric acid), phosphorus (Dittmer–Lester spray) and amino groups (0.2% , w/v, ninhydrin in acetone) were used (Minnikin et al., 1977), together with authentic standards of CL (DPG), PG, PE, and PI.

The procedure for hydrolysis and identification of acyl-PG was performed (Virto and Adlercreutz, 2000). Briefly, acyl-PGs were dissolved in 1 mL diethyl ether. To the solution, 1 mL HEPES buffer, pH 7.5 containing 50 mM CaCl_2 , was added. The hydrolysis was started by the addition of phospholipase C (EC 3.1.4.3 from *Bacillus cereus*) and reaction medium was incubated in an orbital shaker (100 rpm) at 25°C for 12 h . The reaction mixture was mixed with 5 mL of 1 N HCl and 50 mL of chloroform–methanol ($2:1$). The mixtures were thoroughly vortexed and centrifuged at 6000 rpm for 10 min . The lower chloroform part was collected and evaporated and then analyzed by TLC with ethyl acetate–hexane–acetic acid–water ($13:13:4:10$) as mobile phase. The lipids were eluted from the adsorbent with chloroform–methanol ($1:1$). The relative R_f of reaction products after phospholipase C hydrolysis were: 1-acyl-phosphatidylglycerols, 0.13 ; 1-monoacylglycerols, 0.50 ; and diacylglycerols, 0.77 . During the reaction, hydrolysis of 1-acyl-phosphatidylglycerols to 1-monoacylglycerols also takes place as previously described (Ellingson, 1980). The monoacylglycerols (and 1-acyl-phosphatidylglycerols) and the diacylglycerols were reacted with 3% conc. HCl in methanol at 80°C for 1 h . FAMES were extracted with 2 mL of hexane, dried, and analyzed.

4.1. (2R)-Methyl 2-methyl-3-(tetrahydro-2H-pyran-2-yl)oxy propionate (4) (Mori and Takikawa, 1991).

p-Toluenesulfonic acid (3 mg) was added to a solution of **3** ((R)-methyl 3-hydroxy-2-methylpropanoate) (59 mg , 0.50 mmol , and dihydropyran (56 mg , 0.67 mmol) in ether (10 mL) and the mix-

ture was left to stand overnight at room temperature. The solution was washed with NaHCO₃, dried and concentrated in vacuo. The yield of **4** was 100 mg (99%), [α]_D²⁵ –16.1° (*c* = 0.09, ether) (Mori, 1983) [α]_D²¹ –16.3° (*c* = 1.39, ether); ¹H NMR δ (CHCl₃) 1.13 (3H, *d*, *J* = 7.1 Hz), 1.20–1.85 (6H, *m*), 2.65 (1H, *m*), 3.63 (3H, *s*), 3.10–3.95 (4H, *m*), 4.55 (1H, *brs*); HRMS: calcd. for C₁₀H₁₈O₄ 202.1205 [M]⁺; found 202.1201 (Δ = 0.4 ppm).

4.2. (2*S*)-2-Methyl-3-(tetrahydro-2*H*-pyran-2-yl)oxy)propan-1-ol (**5**) (Mori and Takikawa, 1991).

A solution of **4** (95 mg, 0.47 mmol) in dry ether (10 mL) was added dropwise to a stirred suspension of LiAlH₄ (25 mg, 0.66 mmol) in dry ether (10 mL) at 0 °C. The stirring was continued for 4 h at room temperature. The excess LiAlH₄ was decomposed by the successive addition of 5% NaOH (10 mL) to the stirred and ice-cooled mixture. After stirring for 1.5 h, the mixture was filtered and the solid was washed with ether. The combined filtrates were washed with water, dried, and concentrated in vacuo. The residue gave 78 mg (95%) of **5**, [α]_D²⁴ –1.1° (*c* = 0.12, ether) (Mori, 1983) [α]_D²¹ –1.2° (*c* = 1.471, ether); ¹H NMR δ (CHCl₃) 0.89 (3H, *d*, *J* = 7.1 Hz), 1.20–2.20 (7H, *m*), 2.65 (1H, *m*), 3.10–4.10 (6H, *m*), 4.61 (1H, *brs*); HRMS: calcd. for C₉H₁₈O₃ 174.1256 [M]⁺; found 174.1253 (Δ = 0.3 ppm).

4.3. (2*R*)-2-Methyl-3-(tetrahydro-2*H*-pyran-2-yl)oxy)propyl 4-methylbenzenesulfonate (**6**) (Mori and Takikawa, 1991).

p-Toluenesulfonyl chloride (114 mg, 0.55 mmol) was added to a stirred solution of **5** (71 mg, 0.41 mmol) in dry pyridine (10 mL) at 0 °C. The mixture was left to stand overnight in a refrigerator, and then poured into ice-cooled water and extracted with ether. The ether solution was washed with water, CuSO₄ solution, NaHCO₃ solution and brine, dried and concentrated in vacuo to give 131 mg (97%) of **6**. HRMS: calcd. for C₁₆H₂₄O₅S 328.1344 [M]⁺; found 328.1339 (Δ = 0.5 ppm).

4.4. (Z)-2'-Hydroxyethyl-7-cycloheptylheptanoate (**8**) (Nagumo et al., 1993).

To a solution of cycloheptanone **7** (448 mg, 4 mmol) in CH₂Cl₂ (10 mL), was added BF₃·etherate (1.8 mL, 14 mmol) at 0 °C. After 1 h, ethylene glycol (620 mg, 10 mmol) was added to the solution. The mixture was stirred at room temperature for 24 h, then diluted with saturated aqueous NaHCO₃, and extracted with ether. The extract was washed with brine, then dried and concentrated in vacuo. The crude product was purified by TLC (hexane–AcOEt 3:1) to give the hydroxyethyl ester **8** as colorless oil. The yield was 252 mg (47%). ¹H NMR (CDCl₃) δ 1.20–2.10 (20H, *m*), 2.36 (2H, *t*, *J* = 6.7), 3.63 (2H, *t*, *J* = 6.8), 4.21 (2H, *t*, *J* = 6.8), 5.30 (1H, *t*, *J* = 6.6); HRMS: calcd. for C₁₆H₂₈O₃ 268.2038 [M]⁺; found 268.2032 (Δ = 0.6 ppm).

4.5. (Z)-7-Cycloheptylheptanoic acid (**9**).

To a solution of ester **8** (244 mg, 0.91 mmol) in a water–methanol mixture (10 mL, 1:1) was added KOH (55 mg) at room temperature. After 5 h, the methanol was evaporated under reduced pressure, the residual oil was acidified with aqueous 5% HCl, and extracted with ether. The extract was washed with brine, then dried and evaporated. The crude product was purified by TLC (AcOEt–hexane 5:95). The yield was 184 mg (90%) of the acid **9** as colorless oil ¹H NMR (CDCl₃) δ 1.10–2.10 (20H, *m*), 2.36 (2H, *t*, *J* = 6.6), 5.20 (1H, *t*, *J* = 6.6); ¹³C NMR (CDCl₃) δ 24.5 (t), 26.7 (t), 27.3 (t), 27.5 (t), 28.3 (t), 28.9 (t), 29.4 (t), 32.0 (t), 33.8 (t), 34.7 (t), 37.1 (t), 127.6 (d), 143.9 (s),

178.8 (s); HRMS: calcd. for C₁₄H₂₄O₂ 224.1776 [M]⁺; found 224.1772 (Δ = 0.4 ppm).

4.6. 7-Cycloheptylheptanoic acid (**10**).

To a solution of **9** (150 mg, 0.67 mmol) in methanol–hexane (10 mL, 3:7) was added 10% Pd/C catalyst (10 mg), according to the procedure described for different compounds (Crlye et al., 2003). The solution was stirred under 1 atm hydrogen atmosphere and left to stir for 12 h. The solution was filtered to remove the catalyst before being concentrated in vacuo. The crude product was purified by TLC (AcOEt–hexane, 5:95) to afford **10** as a white solid with the yield 149 mg (98%). ¹H NMR (CDCl₃) δ 1.10–1.57 (23H, *m*), 2.33 (2H, *t*, *J* = 6.6); ¹³C NMR (CDCl₃) δ 24.6 (t), 26.8 (t), 28.3 (t), 28.4 (t), 28.9 (t), 29.1 (t), 30.1 (t), 30.3 (t), 33.1 (t), 33.6 (t), 34.2 (t), 35.6 (t), 37.7 (d), 177.6 (s); HRMS: calcd. for C₁₄H₂₆O₂ 226.1933 [M]⁺; found 226.1941 (Δ = 0.8 ppm).

4.7. (6-Bromohexyl)cycloheptane (**11**).

A mixture of the carboxylic acid **10** (145 mg, 0.64 mmol), iodosobenzene diacetate (483 mg, 1.5 mmol) and bromine (240 mg, 1.5 mmol) in CH₂Br₂ (10 mL) was irradiated under reflux with magnetic stirring in an argon atmosphere with two 100 W-tungsten lamps for 4 h, according to the procedure described for different compounds (Camps et al., 2000). The solution thus obtained was allowed to cool to room temperature, more iodosobenzene diacetate (483 mg, 1.5 mmol) and bromine (240 mg, 1.5 mmol) were added and irradiation under the same conditions was continued for 18 h more. The solution was allowed to cool to room temperature, washed with a 10% aqueous solution of Na₂S₂O₃ (3 × 10 mL), saturated aqueous solution of NaHCO₃ (3 × 10 mL), brine (2 × 10 mL), and the organic phase was concentrated in vacuo to give a residue which was purified by TLC (hexane–ether, 90:10). The yield of **11** was 125 mg (75%). ¹H NMR (CDCl₃) δ 1.10–2.10 (22H, *m*), 3.31 (2H, *t*, *J* = 6.6); ¹³C NMR (CDCl₃) δ 26.8 (t), 27.6 (t), 28.1 (t), 28.4 (t), 28.5 (t), 29.5 (t), 29.9 (t), 30.6 (t), 31.2 (t), 33.6 (t), 33.6 (t), 35.3 (t), 38.9 (d); HRMS: calcd. for C₁₃H₂₅⁷⁹Br 260.1140 [M]⁺; found 260.1144 (Δ = 0.4 ppm).

4.8. 2-((*S*)-9-((*Z*)-Cycloheptyl)-2-methylnonyloxy)tetrahydro-2*H*-pyran (**12**).

A catalytic amount of 1,2-dibromoethane was added under argon to a mixture of Mg (14.6 mg, 0.60 mmol) and a small amount of iodine. Bromide **11** (125 mg, 0.48 mmol) in THF (5 mL) was then added dropwise to this mixture during the course of 30 min. After the addition was complete, the mixture was stirred for 10 min. To a solution of **4** (118 mg, 0.36 mmol) in THF (10 mL) was added dropwise the Grignard reagent at –70 °C under argon, this being followed by the addition of a 0.1 M THF solution of Li₂CuCl₄ (4 mL, 0.4 mmol). The mixture was stirred at room temperature for 15 h, and the resulting mixture was poured into ice-cooled NH₄Cl. The mixture was extracted with ether, and the organic layer was washed with water, saturated NaHCO₃ and brine, dried, and concentrated in vacuo. The residue was chromatographed over TLC (hexane–AcOEt, 15:1) to give oil **12** with the yield 113 mg (93%). ¹H NMR δ (CHCl₃) 0.89 (3H, *d*, *J* = 6.8 Hz), 0.97–2.08 (33H, *m*), 3.14–3.33 (2H, *m*), 3.61 (2H, *m*), 4.35 (2H, *m*); ¹³C NMR (CDCl₃) δ 17.3 (q), 20.9 (t), 25.2 (t), 26.4 (t), 26.8 (t), 28.4 (t), 28.5 (t), 29.0 (t), 29.1 (t), 29.3 (t), 29.6 (t), 30.4 (t), 31.3 (t), 33.0 (t), 33.5 (t), 33.7 (t), 34.0 (d), 35.4 (t), 37.7 (d), 62.8 (t), 75.4 (t), 96.8 (d); HRMS: calcd. for C₂₂H₄₂O₂ 338.3185 [M]⁺; found 338.3190 (Δ = 0.5 ppm).

4.9. (S)-9-Cycloheptyl-2-methylnonan-1-ol (**13**).

p-Toluenesulfonic acid monohydrate (0.03 g, catalytic amount) was added to a solution of **12** (112 mg, 0.33 mmol) in methanol (10 mL), and stirring was continued for 4 h under reflux. After neutralization with potassium carbonate, the mixture was poured into brine and extracted with ether. The ethereal extract was washed with brine, dried, and concentrated in vacuo. The residue was chromatographed on TLC with hexane–ether (7:1) with a yield of 75 mg of **13** (89%). ^1H NMR (CDCl_3) δ 0.88 (3H, d, $J=6.9$ Hz), 1.10–2.10 (28H, m), 3.47 (2H, m); ^{13}C NMR (CDCl_3) δ 17.2 (q), 26.4 (t), 26.8 (t), 28.4 (t), 28.5 (t), 29.0 (t), 29.1 (t), 29.3 (t), 30.1 (t), 30.4 (t), 33.1 (d), 33.5 (t), 33.6 (t), 34.0 (t), 35.4 (t), 37.7 (d), 67.6 (t); HRMS: calcd. for $\text{C}_{17}\text{H}_{34}\text{O}$ 254.2610 $[\text{M}]^+$; found 254.2611 ($\Delta=0.1$ ppm).

4.10. (S,E)-Methyl 11-cycloheptyl-4-methylundec-2-enoate (**15**).

To a solution of 50 μL (0.57 mmol) of oxalyl chloride in 5 mL of CH_2Cl_2 at -70°C was added 51 μL (0.72 mmol) of dimethyl sulfoxide in 1 mL of CH_2Cl_2 , according to the procedure described for different compounds (Oikawa et al., 1995). The mixture was stirred for 10 min, 71 mg (0.28 mmol) of **13** in 5 mL of CH_2Cl_2 was introduced and the resulting mixture stirred for 30 min. To this was added 0.28 mL (2 mmol) of triethylamine. The mixture was further stirred at ambient temperature for 30 min and quenched with 30 mL of 5% aqueous NaHSO_4 . The aqueous layer was separated and extracted with ether (2×10 mL). The combined organic extracts were washed with 10 mL of brine, dried, filtered, and concentrated in vacuo. The crude aldehyde **14** thus obtained was used without further purification.

Sodium hydride (60% in mineral oil, 13.2 mg, 0.33 mmol) was washed with THF (3×1 mL) and suspended in 5 mL of THF. To this was added at 0°C 69 mg (0.33 mmol) of methyl diethylphosphonoacetate in 5 mL of THF, and the solution was stirred at ambient temperature for 20 min. The mixture was cooled to -78°C , followed by the addition of the above freshly prepared aldehyde **14** in 5 mL of THF. After being stirred for 30 min, this was allowed to warm to ambient temperature. The mixture was stirred for another 1 h and was then quenched by the addition of 10 mL of water. The aqueous layer was separated and extracted with ether (3×10 mL). The combined organic extracts were washed with 5 mL of brine, dried, filtered, and concentrated in vacuo. Purification by TLC (ether–hexane, 15:85) gave 85 mg (98%) of two geometrical isomers (E:Z=85:15) of esters **15** as a colorless oil. ^1H NMR (CDCl_3) δ 0.98 (3H, d, $J=6.8$ Hz), 1.04–2.15 (28H, m), 3.45 (3H, s), 5.76 (1H, d, $J=15.4$; E-isomer), 6.84 (1H, dd, $J=15.4, 6.2$); ^{13}C NMR (CDCl_3) δ 20.6 (q), 26.7 (t), 27.2 (t), 28.4 (t), 28.5 (t), 28.8 (t), 29.0 (t), 29.3 (t), 30.1 (t), 30.4 (t), 33.6 (t), 33.7 (t), 34.7 (d), 35.4 (t), 36.1 (d), 38.1 (t), 51.9 (q), 121.8 (d), 153.4 (d), 166.2 (s); HRMS: calcd. for $\text{C}_{20}\text{H}_{36}\text{O}_2$ 308.2715 $[\text{M}]^+$; found 308.2721 ($\Delta=0.6$ ppm).

4.11. (S)-Methyl 11-cycloheptyl-4-methylundecanoate (**1**).

The methyl ester **1** was prepared in a similar manner as compound **8**. From 80 mg (0.26 mmol) of **15** was obtained 79 mg (98%) of **1**. The final product was purified by TLC (EtOAc–hexane, 5:95). $[\alpha]_D^{24} -0.75$; IR (neat): 1729 (C=O); ^1H NMR (CDCl_3) δ 0.88 (3H, d, $J=6.8$ Hz), 1.04–1.75 (30H, m), 2.44 (t, $J=7.6$, H-2, 2H), 3.61 (s, 3H); ^{13}C NMR (CDCl_3) δ 174.2 (C1), 51.2 (OMe), 32.1 (C2), 31.6 (C3), 36.7 (C4), 37.0 (C5), 27.4 (C6), 28.3–29.6 (C7,8,9,4',5'), 27.4 (C10), 38.1 (C11), 21.4 (C12), 39.5 (C1'), 34.7 (C2',7'), 26.6 (C3',6'); HRMS: calcd. for $\text{C}_{20}\text{H}_{38}\text{O}_2$ 310.2872 $[\text{M}]^+$; found 310.2875 ($\Delta=0.3$ ppm).

4.12. (S)-Picolinyl 11-cycloheptyl-4-methylundecanoate (**16**) (Rezanka, 1990).

A solution of potassium tert-butoxide in THF (0.5 mL, 1.0 M) was added to nicotinyl alcohol (1 mL). After mixing, the appropriate methyl ester **1** (30 mg) in dry dichloromethane (1 mL) was added, and the mixture was held at 40°C for 30 min in a closed vial. After cooling to room temperature, water and hexane were added, and the organic phase was collected, dried over anhydrous sodium sulfate, and evaporated.

Feeding experiment was carried out in fermentor culture as described above. The labeled precursor ($^{13}\text{CH}_3\text{CH}_2\text{COONa}$) (485 mg, i.e. 5 mM) was added to 10 mL sterile aqueous solutions adjusted to pH 5.0 as three equal parts at intervals of 4 h starting at 0 h. Cells were harvested after 24 h. Because of the high ^{13}C content of the precursors (^{13}C : 99%) the enrichment in the labeled products is given and not converted into incorporation rates.

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