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Synthesis and surface activity of diether-linked phosphoglycerols: Potential applications for exogenous lung surfactants

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Abstract—The synthesis of three phosphoglycerols is described, one of which contains the previously unknown phosphonoglycerol headgroup. The surface tension-lowering capabilities of synthetic lung surfactant mixtures containing the PG analogs were measured on the pulsating bubble surfactometer and compared to known controls. The PG-containing mixtures exhibited superior surface tension-lowering properties indicating the significant potential of these analogs as components in synthetic exogenous lung surfactants.

A life-threatening deficiency of pulmonary surfactant is the cause of the neonatal respiratory distress syndrome (RDS), a common disease among preterm infants less than 32 weeks gestation. In addition, dysfunction (inactivation) of pulmonary surfactant is a major contributor to the pathophysiology of clinical acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS), which affect patients of all ages and have substantial mortality rates of 30–50 percent despite sophisticated medical intensive care.[1,2] Exogenous surfactant replacement therapy is currently being used with tremendous success to improve survival and minimize morbidity in premature infants with RDS, and there is great interest in extending this therapy to patients suffering from ALI/ARDS.[1–3]

Surfactant therapy for ALI/ARDS is complicated by the presence of inhibitory substances in injured inflamed lungs, and requires that exogenous surfactants having maximal surface activity and resistance to inactivation be delivered effectively to the alveoli. In addition to biophysical inhibitors such as plasma proteins, lytic enzymes including phospholipases in injured lung tissue can also detrimentally affect endogenous and exogenous pulmonary surfactants. Phospholipases are known to be released into the pulmonary interstitium during inflammatory injury in vivo.[4–7] Phospholipase A_2 (PLA_2) is also present in meconium,[8] a fetal product that can be aspirated to cause lethal lung injury in newborns. Several studies have shown that phospholipases are directly inhibitory to lung surfactant activity.[5,8–11] These enzymes not only degrade glycerophospholipids, but also produce reaction products such as free fatty acids and lysophosphatidylcholines that biophysically inhibit lung surfactant function[12–14] and damage the integrity of the alveolocapillary membrane.[15,16] The current paper deals with novel ether-linked phosphatidylglycerol (PG)-related phospholipids or phosphonolipids that are designed to be structurally resistant to degradation by phospholipases A_1, A_2 (and also phospholipase D in the case of PG phosphonolipids).

Despite the vulnerability of glycerophospholipids to phospholipase activity, almost all current mainstream exogenous surfactants depend on 1,2-dipalmitoyl-sn-3-phosphatidylcholine (DPPC) as a predominant surface tension-lowering lipid component.[1,17] A promising alternative to DPPC is its diether phosphonolipid analog **1**, designated DEPN-8, whose synthesis and high surface activity have been reported previously.[11,18–24] DEPN-8 reaches minimum surface tensions of <1 mN/m in dynamically compressed surface films, and has superior adsorption and respreading capabilities compared to DPPC.[20,21,23,24] When combined with purified lung surfactant proteins (SP)-B/C, DEPN-8 forms exogenous

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surfactants of very high activity that are resistant to degradation or inhibition if exposed to PLA_2.[11,19] The present study reports new PG-related analogs that further increase the activity of synthetic lung surfactants containing DEPN-8 (Fig. 1).

Although zwitterionic phospholipids like DPPC are key tensoactive components in endogenous lung surfactant, anionic lipids including PGs also play important roles in surface-active behavior. In particular, PG compounds have been shown to selectively interact with SP-B, the most biophysically active apoprotein in endogenous surfactant.[1,25–30] Accordingly, we have targeted surface-active phospholipase-resistant PG analogs to combine with DEPN-8 in novel lung surfactants. In this communication, we report the synthesis of three diether PG targets and provide preliminary results indicating the high surface activity of mixtures containing these compounds.

Based on available 1,2-disubstituted glycerols,[11,19] the laboratory synthesis of lipids **2** and **3** proceeded smoothly using well-established protocols.[31–33] Phosphorylation with $POCl_3$ was followed by treatment with solketal to complete the installation of the PG headgroup. Deacetalization using 70% HOAc in water and then flash chromatography offered lipids **2** and **3** in 48% and 43% yields, respectively (see footnote to data and experiment).[34] The double bond of **3** was not perturbed during its synthesis, as indicated by ^1H and ^{13}C NMR spectroscopy. Unsaturated lipid **3** is a new entity, and although lipid **2** has been prepared in the past,[35–38] the chemistry herein represents the first fully chemical synthesis of the acid form of **2**. Prior syntheses of **2** have largely been based on the work of Comfurius,[39] who demonstrated the introduction of the glycerol unit by the enzymatic action of phospholipase D (Fig. 2).

The preparation of phosphonoglycerol **4** proved more challenging than for compounds **2** or **3**. It was initially believed that phosphonate **5**, prepared by the Michaelis–Arbuzov reaction of triethyl phosphite with 4-(2-iodoethyl)-2,2-dimethyl-1,3-dioxolane,[40] could be used as a source of the phosphonoglycerol headgroup, and that eventual 1,2-disubstituted glycerol attachment would form the basis of the preparation. However, in accord with literature precedent,[41,42] phosphonate **5** could not be selectively dealkylated to the phosphonic acid with TMSBr or related reagents without competitive deacetalization.

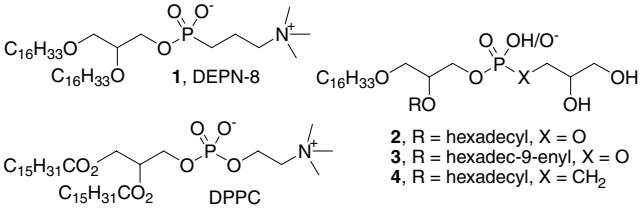


Figure 1. Schematic diagram of the previously synthesized diether phosphonolipid compound DEPN-8 (compound **1**) and its relation to DPPC and to the phosphoglycerol analogs (compounds **2**–**4**) of the current paper.

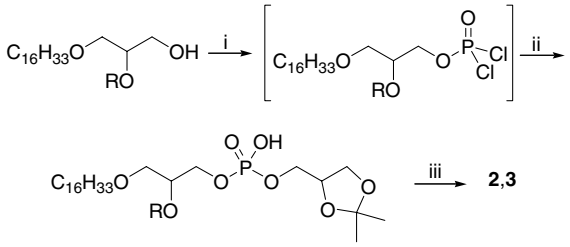
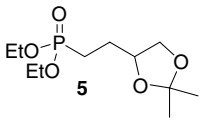


Figure 2. Reagents and conditions for synthesis of PG analogs **2** and **3**. (i) $POCl_3$, Et_3N, THF; (ii) 1—*rac*-solketal, Et_3N, THF, 2—1 M Na_2CO_3 (aq); (iii) 70% HOAc in H_2O, 48% over three steps for **2**; 43% for **3**.



Recognizing that dealkylation of the phosphonate must not perturb the protecting group of the 3,4-diol, we turned to benzoate groups for the latter. To this end, lithium dimethyl methanephosphonate was reacted with glycidyl benzoate (**6**) in the presence of $BF_3{\cdot}OEt_2$, using a protocol already described for glycidyl ethers.[43] The free hydroxyl group of **7** was readily converted to the second benzoate, giving **8**. The phosphonate was then converted to the bis(trimethylsilyl) ester (**9**) using TMSBr for immediate conversion to **10**.[44] Dichloride **10** was then captured with 1,2-bis(hexadecyl)glycerol affording protected lipid **11**. Phosphonoglycerol **4** was obtained through a methanolysis reaction and was isolated in 65% yield after chromatography and recrystallization. Lipid **4** represents the first known phosphonoglycerol bearing the 3,4-dihydroxybutyl hydrogen phosphonate headgroup, regardless of the functional groups linking the fatty alkyl chains to the glycerol backbone (Fig. 3).[45]

The overall dynamic surface tension-lowering ability of synthetic lung surfactants containing 9:1 (molar ratio) DEPN-8/PG-**2**, DEPN-8/PG-**3**, or DEPN-8/PG-**4** with and without added 1.5% (by wt.) of bovine SP-B/C were measured with a pulsating bubble surfactometer (Table 1).[46] Assessments of overall surface activity on

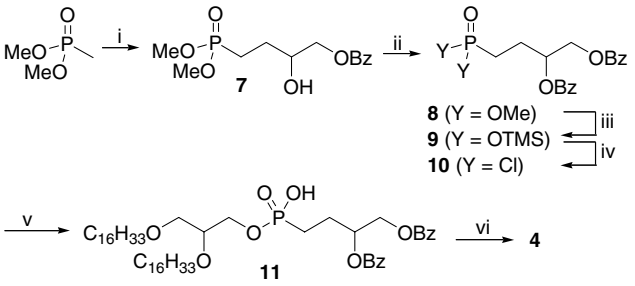


Figure 3. Reagents and conditions for synthesis of PG phosphonolipid **4**. (i) 1—*n*BuLi, THF, then $BF_3{\cdot}OEt_2$ and **6**, −78 °C to rt, 75%; (ii) BzCl, Et_3N, DMAP, CH_2Cl_2, 92%; (iii) 2.2 equiv TMSBr, CH_2Cl_2; (iv) 3 equiv $(COCl)_2$, cat DMF, CH_2Cl_2; (v) 1—*rac*-1,2-bis(hexadecyl)glycerol, Et_3N, $CHCl_3$, 2—Amberlite® resin, 65% from **7**; (vi) 1—MeOH/CH_2Cl_2 (1:2), K_2CO_3 (anhyd), 2—H_3O^+, 65%.

Table 1. Dynamic surface activities of synthetic lung surfactants containing DEPN-8 and a PG analog (PG-**2**, -**3** or -**4**) with and without 1.5% bovine SP-B/C

Surfactant mixture	Surface tension (mN/m) at minimum bubble radius at time (min)							
	0.25	0.5	1	2	5	10	15	20
DEPN-8	32 ± 2	27 ± 2	21 ± 2	14 ± 2	6 ± 1	2 ± 1	<1	
9:1 DEPN-8/PG-**2**	28 ± 1	26 ± 1	19 ± 1	12 ± 2	5 ± 2	<1		
9:1 DEPN-8/PG-**3**	25 ± 1	24 ± 1	17 ± 2	10 ± 2	2 ± 1	<1		
9:1 DEPN-8/PG-**4**	29 ± 1	28 ± 1	19 ± 1	11 ± 1	4 ± 1	<1		
DEPN-8 + 1.5% SP	13 ± 1	7 ± 1	4 ± 1	3 ± 0	<1			
9:1 DEPN-8/PG-**2** + 1.5% SP	10 ± 1	3 ± 1	2 ± 1	<1				
9:1 DEPN-8/PG-**3** + 1.5% SP	8 ± 0	3 ± 0	2 ± 0	<1				
9:1 DEPN-8/PG-**4** + 1.5% SP	11 ± 1	4 ± 1	2 ± 1	<1				
DPPC	66 ± 1	64 ± 1	60 ± 1	41 ± 1	29 ± 1	25 ± 1	23 ± 0	21 ± 0
9:1 DPPC/POPG	45 ± 2	36 ± 2	28 ± 2	25 ± 2	22 ± 1	21 ± 1	19 ± 1	18 ± 1
9:1 DPPC/POPG + 1.5% SP	17 ± 2	15 ± 2	11 ± 2	5 ± 1	2 ± 1	<1		

Data are means ± SEM for $n = 3$–5. All surfactants were studied at a uniform total lipid concentration of 2.5 mg/ml on a pulsating bubble surfactometer (General Transco, Largo, FL) at 37 °C, 20 cycles/min, and 50% area compression. Surfactants were dispersed in 0.15 M NaCl + 2 mM $CaCl_2$, and a tiny air bubble was formed and pulsated between maximum and minimum radii of 0.55 and 0.4 mm while the pressure drop across the air–water interface of the bubble was measured with a precision transducer. Surface tension at minimum bubble radius (minimum surface tension) was calculated from the measured pressure drop using the Laplace equation.[46] *Abbreviations:* DPPC, dipalmitoyl phosphatidylcholine; POPG, palmitoyl-oleoyl phosphatidylglycerol; SP, mixture of surfactant proteins B/C isolated chromatographically from lavaged calf lung surfactant.[11]

this instrument reflect the combined effects of adsorption and dynamic film compression at a cycling rate (20 cycles/min), temperature (37 °C), and area compression (50% from maximum to minimum area) relevant for the mammalian lungs in vivo.[1,46,47] The mixed bovine SP-B/C used in activity studies was isolated on an LH-20 column from extracted calf lung surfactant, as described previously by Wang et al.[11] The presence of a PG analog (PG-**2**, -**3**, or -**4**) was beneficial for overall surface tension lowering in synthetic lung surfactants with DEPN-8. The overall surface activities of 9:1 mixtures of DEPN-8 with PG-**2**, -**3**, or -**4** were increased slightly but consistently compared to DEPN-8 alone (Table 1). Similarly, the overall surface activities of synthetic lung surfactants containing 9:1 DEPN-8/PG-**2**, -**3**, or -**4** + 1.5% bovine SP-B/C all showed a small but consistent increase relative to DEPN-8 + 1.5% bovine SP-B/C. The high surface activities of mixtures of 9:1 DEPN-8/PG-**2**, -**3**, or -**4** with and without 1.5% bovine SP-B/C also greatly exceeded the activity of corresponding control mixtures containing the ester-linked glycerophospholipids DPPC and 1-palmitoyl-2-oleoyl-PG (POPG). In particular, the surface activities of mixtures of 9:1 DEPN-8/PG-**2**, -**3**, or -**4** on the pulsating bubble were all much greater than the activity of 9:1 DPPC/POPG, and the surface activities of 9:1 DEPN-8/PG-**2**, -**3**, or -**4** + 1.5% bovine SP-B/C were also all substantially greater than the activity of 9:1 DPPC/POPG + 1.5% bovine SP-B/C (Table 1).

To summarize, this paper has reported the synthesis of three ether-linked PG lipids designed to have structural resistance to cleavage by phospholipase A_1 and A_2 (PG-**2**, -**3**, and -**4**) as well as to phospholipase D (PG-**4**). The anionic nature of these compounds at neutral pH affords the potential for enhanced intermolecular interactions with zwitterionic phosphonolipids and phospholipids in synthetic lung surfactants. These new phospholipase-resistant PG compounds also have the potential to interact with amphipathic lung surfactant proteins or related synthetic peptides in exogenous surfactants. Anionic PG glycerophospholipids in native lung surfactant have previously been reported to have specific interactions with surfactant apoproteins or synthetic peptides.[1,25–30] Preliminary biophysical assessments of model lung surfactants containing 9:1 DEPN-8/PG-**2**, 9:1 DEPN-8/PG-**3**, or 9:1 DEPN-8/PG-**4** with or without added bovine SP-B/C indicated that the presence of the PG component enhanced the already high surface activity of corresponding surfactant mixtures containing DEPN-8. Moreover, the surface activities of mixtures containing 9:1 DEPN-8/PG-**2**, -**3**, or -**4** were greater than corresponding mixtures containing the ester-linked glycerophospholipids 9:1 DPPC/POPG. These results indicate that mixtures of ether-linked PG analogs combined with DEPN-8 or related zwitterionic phosphonolipids plus surfactant proteins/peptides have significant potential utility as synthetic exogenous lung surfactants. Further research detailing the interfacial biophysics and physiological activity of synthetic lung surfactants of this kind is clearly warranted. Because of the high surface activity and molecular structural phospholipase-resistance of phosphonolipids, synthetic surfactants containing these compounds may be particularly applicable for use in clinical syndromes like ALI/ARDS where inflammatory pulmonary injury is present.

In terms of significance relating to organic chemistry, the preparation of the diether PG compounds reported in this paper includes novel contributions to phospholipid synthesis. This is particularly true for the synthesis of the phosphonoglycerol analog **4**, which is noteworthy in its potential to incorporate chirality in both the 3-carbon glycerol-based fragment and the 4-carbon butanol-based fragment. The preparation of additional phosphonoglycerol compounds for use in synthetic lung surfactants is currently underway.

Acknowledgments

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Supplementary data

Supplementary data is available showing copies of ^{1}H and ^{13}C NMR spectra of lipids **2**, **3**, **4** (6 pages). Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.09.083.

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34. *Preparative procedures and characterization data for PG's **2** and **3**. rac-1,2-Dihexadecyl-3 phosphoglycerol* (**2**). Freshly distilled $POCl_3$ (410 mg, 2.64 mmol) was cooled in an ice-bath. A solution of 1,2-dihexadecylglycerol (1.19 g, 2.20 mmol) in 15 mL THF and Et_3N (334 mg, 3.3 mmol) was added dropwise to the phosphorus oxychloride while stirring continuously. Stirring was continued at rt for 2 h. After recooling to 0 °C, a solution of solketol (349 mg, 2.64 mmol) and Et_3N (668 mg, 6.6 mmol) in 10 mL THF was added dropwise. After 2 h at rt, the reaction mixture was filtered by suction and 1 M Na_2CO_3 (aq) was added to the filtrate and stirring continued for 15 h at rt. Acetone (30 mL) was added to the upper THF phase and the precipitate was removed by filtration. The filtrate was evaporated to dryness. The residue was dissolved in a solution of $HOAc/H_2O$ (70:30, v/v) and was stirred for 2 h. The lipid was extracted with $CHCl_3$ and purified by flash chromatography (eluent $CHCl_3$:MeOH = 10:1) to yield 730 mg of **2**, 48% yield. Characterization data for *rac-1,2-dihexadecyl-3 phosphoglycerol* (**2**): mp 157–159 °C; IR (neat, ν_{max}): 3396, 2917, 2850, 1467, 1384, 1226, 1116, and 1060 cm^{-1}; ^{1}H NMR ($CDCl_3/CD_3OD$ = 4:1, 400 MHz): 3.92 (m, 4H), 3.80 (m, 1H), 3.54–3.62 (m, 6H), 3.35–3.50 (m, 3H), 1.56 (m, 4H), 1.26–1.36 (m, 52H), 0.88 (t, J = 6.8 Hz, 6H) ppm; ^{13}C NMR ($CDCl_3/CD_3OD$ = 4:1, 100 MHz): 77.4, 71.6, 70.7, 70.4, 70.2, 66.3, 64.7, 62.2, 31.7, 29.7, 29.5, 29.3, 29.1, 25.8 (d, J = 6.1 Hz), 22.4, 13.8 ppm; ^{31}P NMR ($CDCl_3/CD_3OD$ = 4:1, 162 MHz): 5.8 ppm; HRMS, ESI TOF (−ve), *m/z*: calcd for $C_{38}H_{78}O_8P$ $[M-H]^-$: 693.5434; found: 693.5395.Characterization data for *rac*-1-hexadecyl-2-hexadec-9-encyl-3-phosphoglycerol (**3**): IR (neat, ν_{max}): cm^{-1}: 3420, 3195, 3010, 2921, 2851, 1467, 1256, 1240, 1132, 1101, 1058 cm^{-1}; ^{1}H NMR ($CDCl_3/CD_3OD$ = 4:1, 400 MHz): 5.30 (m, 2H), 3.90–3.84 (m, 4H), 3.74 (pent, J = 4.9 Hz, 1H), 3.63–3.49 (m, 6H), 3.45–3.38 (m, 3H), 1.98 (m, 4H), 1.51 (m, 4H), 1.23 (s, 44H), 0.84 (m, 6H), ppm; ^{13}C NMR ($CDCl_3/CD_3OD$ = 4:1, 100 MHz): 129.6, 129.4, 77.6 (d, J = 8.2 Hz), 71.4, 70.9 (d, J = 5.1 Hz), 70.3, 70.2, 66.1 (d, J = 5.7 Hz), 64.7, 62.2, 57.2, 31.6, 31.4, 29.7, 29.4, 29.3, 29.2, 29.0, 28.9, 28.6, 26.8, 25.7 (d,J = 3.2 Hz), 22.3 (d, J = 3.1 Hz), 13.2 ppm; ^{31}P NMR ($CDCl_3/CD_3OD$ = 4:1, 162 MHz): 5.3 ppm; HRMS, ESI TOF (+ve), *m/z*: calcd for $C_{38}H_{78}O_8P$ $[M+H]^+$: 693.5434; found: 693.5430.
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45. *Key experimental procedures leading to PG* ***4***. Preparation of dibenzoyl protected lipid **11**. Neat TMSBr (0.39 mL, 3.06 mmol) was added dropwise to a solution of compound **8** (546 mg, 1.39 mmol) dissolved in CH_2Cl_2 (4 mL) stirring at −30 °C. After 40 min., the mixture was stirred at rt for 3 h. Removal of the solvent under anhydrous conditions, afforded a thick oil (**9**). To this oil at rt were added CH_2Cl_2 (5 mL), DMF (two drops), and oxalyl chloride (0.36 mL, 4.17 mmol). The solution was stirred at rt for 30 min. during which time gas evolved. The solvent was removed under anhydrous conditions and residue was heated to 50 °C under vacuum for 1 h. The resulting oil (**10**) was diluted with $CHCl_3$ (30 mL) and stirred at 0 °C. To this solution was added dropwise and slowly a solution of Et_3N (0.19 mL, 1.39 mmol) and 1,2-dihexadecylglycerol (376 mg, 0.695 mmol) in $CHCl_3$ (30 mL). The mixture was allowed to warm to rt and was stirred for 48 h. After addition of water (0.7 mL), the mixture was stirred for 1 h. The majority of solvent removed under vacuum and to the residue was added 15 mL of the $CHCl_3$/MeOH/H_2O = 10:10:1 solvent system and 18 mL of Amberlite® ion exchange resin. The mixture was stirred for 1 h, was filtered, and the resin washed with the same solvent system. The combined solvents were removed under vacuum. The resulting residue was dissolved in CH_2Cl_2, washed with brine, and dried over $MgSO_4$. After filtration and concentration, the residue was triturated into hot hexanes (3×). The hexanes were removed under vacuum and crude compound **11** was purified by flash chromatography (eluent with MeOH/$CHCl_3$ = 1:20) to get 399 mg of the protected phosphonolipid (**11**), 65% yield. Data for **11**: ^{1}H NMR ($CDCl_3$, 400 MHz): 7.92 (m, 4H), 7.42 (m, 2H), 7.31 (m, 4H), 4.50–4.47 (m, 1H), 4.35 (m, 1H), 3.97–3.91 (m, 2H), 3.48-3.28 (m, 6H), 2.07 (m, 2H), 1.8 (m, 2H), 1.42 (m, 4H), 1.16 (m, 54H), 0.79 (t, J = 6.6 Hz, 6H) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz): 166.0, 165.9, 133.1, 132.9, 129.7, 129.6, 128.3, 77.5, 72.0, 71.7, 70.6, 70.1, 65.2, 64.2, 60.3, 31.9, 29.9, 29.7, 29.6, 29.5, 29.3, 26.0 (d, J = 4.3 Hz), 22.6, 14.1 ppm; ^{31}P NMR ($CDCl_3$, 162 MHz): 31.4 ppm; IR (neat, ν_{max}): 3300, 3063, 2917, 2850, 1723, 1265, 1071 cm^{-1}.*rac-2,3-Bis(hexadecyloxy)propyl hydrogen 3,4-dihydroxybutylphosphonate* (***4***). Phosphonate **11** (364 mg, 0.40 mmol) was dissolved in MeOH/CH_2Cl_2 (1:2, 20 mL). K_2CO_3 (anhyd, 223 mg, 1.62 mmol) was added and the mixture was stirred at rt for 18 h. Water (5 mL) was added and the pH was adjusted to 2–3 with6 M HCl. The mixture was extracted with CH_2Cl_2/MeOH = 2:1 (2 × 20 mL) and the organic extracts were washed with brine, dried over $MgSO_4$, and filtered through Celite®. After removal of the solvent, the residue was purified by flash chromatography (eluent from MeOH/$CHCl_3$ = 1:10 to MeOH/$CHCl_3$ = 1:4) to get 169 mg of phosphonolipid **4**, 65% yield.Characterization data for **4**: IR (neat, ν_{max}): 3392, 2917, 2850, 1457, 1180, 1072 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz): 3.90 (m, 2H), 3.75 (m, 1H), 3.63–3.56 (m, 5H), 3.51–3.46 (m, 4H), 1.75–1.69 (m, 4H), 1.57 (m, 4H), 1.29 (m, 52H), 0.89 (t, J = 6.6 Hz, 6H) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz): 77.7 (d, J = 6.0 Hz), 71.7 (d, J = 9.1 Hz), 71.3, 70.2, 69.9, 65.1, 63.1, 31.4, 29.5, 29.2, 29.1, 29.0, 28.9, 25.6 (d, J = 4.4 Hz), 22.2, 13.4 ppm; ^{31}P NMR ($CDCl_3$, 162 MHz): 29.6 ppm; HRMS, ESI TOF (+ve), *m/z*: calcd for $C_{39}H_{82}O_7P$ $[M+H]^+$: 693.5798; found: 693.5753.
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