Synthesis of mono- and di-sialophospholipids via the H-phosphonate approach

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Abstract: To overcome their inherent instability, stable modified mono- and di-sialophospholipids of the group C meningococcal polysaccharide were synthesized. Stability was achieved by introducing a spacer between the sialic acid residue and the phospholipid component, and by replacing the native ester linkages to the lipid by ether linkages. Mono- and di-hydroxylethylénésialosides were coupled to phosphoglyceroildiethelipid using H-phosphonate chemistry to give the anomerically pure sialophospholipids in good yields.

Key words: polysialic acid, glycolipid, H-phosphonate, meningococcus.

Résumé : Afin de circonvenir à leur instabilité inhérente, on a synthétisé des mono- et des di-sialophospholipides modifiés et stables du polysaccharide meningoque du groupe C. On a atteint la stabilité en introduisant un groupe d’espacement entre le résidu d’acide sialique et le composant phospholipide et en remplaçant les liaisons esters naturelles vers le lipide par des liaisons éthers. On a réalisé le couplage des mono- et di-hydroxyéthylénésialosides au phosphoglycéroléthérlipide en se basant sur la chimie des H-phosphonates afin d’obtenir des sialophospholipides anomériquement purs avec de bons rendements.

Mots clés : acide polysialique, glycolipide, H-phosphonate, méningocoque.

Introduction

Sialic acid (N-acetyl neuraminic acid) is a naturally occurring keto sugar that is involved in a wide range of biological processes. The capsular polysaccharides of various highly pathogenic bacteria, including those of Escherichia coli K1 and groups B and C Neisseria meningitidis are known to be homopolymers of sialic acid (1, 2), and both the group C polysaccharide and more recently, its improved protein conjugates (3, 4), have been shown to be effective human vaccines. Based on previous assays (5, 6), it was demonstrated that groups B and C meningococcal and E. coli K1 polysaccharides bear glycerol phospholipid anchors, and the proposed structure of the group C phospholipid anchor is as shown in 1 (5). Although small in size, this lipoidal group is responsible for the aggregation of the polysaccharides, and it has been proposed that it is the entity by which the polysaccharide is attached to the outer membrane of the above bacteria (5). While aggregation improves the immunogenicity of the group C meningococcal polysaccharide in humans (7), it has little or no effect on that of the group B meningococcal and E. coli K1 polysaccharides (8), because of the fact that their basic structure (α(2–8)-polysialic acid) mimics a human tissue antigen (9). However, highly aggregated forms of these lipitated polysaccharides do contain a protective epitope distinct from those of α(2–8) polysialic acid, but nevertheless having this latter molecule as a component (3, 10). This protective epitope is mimicked by the N-propionylated form of α(2–8) polysialic acid, which in its conjugated form is a potential human vaccine (3, 11, 12). To study the roles of α(2–8) polysialic acid and lipid anchor in the formation of this epitope, we proposed first to synthesize the truncated mono- and di-sialophospholipids, and then at a later date to use them as precursors to elongate the sialic acid chain at their nonreducing ends, using polysialyl transferases (13).

Results and discussion

Due to the miniscule content of phospholipid in the native lipitated polysaccharides (5) and its reported inherent instability (6), it has never been isolated. This latter property is probably also responsible for our inability to synthesize it using conventional synthetic methods (unpublished results). Therefore, we opted to synthesize more stable modified ver-
sions of the mono- and di-sialophospholipids as model compounds. In an attempt to stabilize the lipid anchor of the monosialophospholipid, we first introduced a spacer between the sialic acid and the phospholipid as shown in 2a. Assembly of 2a was effected by coupling 4 (14), previously prepared from chlorosugar 3 (15), to phospholipid 5, using H-phosphonate chemistry (see the following). However, removal of the acetyl protecting groups from 2a in the presence of the palmitoyl esters proved to be problematic, 2b only being isolated in very poor yields. Having also had limited success in the synthesis of an alternate donor to 3, the fully benzylated 1-chlorodeoxy derivative of sialic acid (16), which could have possibly avoided this problem, we decided to additionally replace the palmitoyl ester groups of the glycerol moiety with ether groups (as shown in 2c). Because the long-chain ether groups are stable under the conditions under which O-acetates are removed by methoxide ion, the unblocking of the fully protected sialophospholipid 2c should furnish 2d.

To synthesize phosphoether lipid 2d we found that H-phosphonate chemistry, which has been widely used in oligonucleotide synthesis (17), can be readily employed (Scheme 1). Thus, 1,2-dihexadecylglycerol (18) was readily phosphonylated by ammonium 4-methylphenyl H-phosphonate (19) to afford H-phosphonate 5. Benzyl (glycolyl 5-acetamido-4,7,8,9-tetraacetyl-3,5-dideoxy- D-glycero-α-D-galacto-nonulopyranosid)onate 4 (14), which was obtained in anomerically pure α form, was then coupled with 5 in the presence of pivaloyl chloride to yield the H-phosphonate diester 6 in good yield. Subsequent oxidation with iodine furnished phosphodiester 2c, which on deesterification gave a good yield of the target compound (2d).

Synthesis of the disialophospholipid derivative (12), shown in Scheme 2, was achieved using similar methods to those employed in the synthesis of monosialophospholipid 2d. The α-2,8-linked sialic acid dimer was obtained in a pure form by controlled acid hydrolysis of colominic acid, followed by fractionation of the hydrolysate on a Bio-gel P-10 column as previously described (20). More conveniently, the hydrolysate was transformed in situ (Scheme 2) to afford the peracetylated lactone disialoside methyl ester (7) in moderate yield (21). Thus, 7 was converted to its 2-chloro derivative (8) by treatment with a mixture of acetyl chloride, dichloromethane, and a trace amount of water. Glycosylation of chloride 8 with ethylene glycol was effected with silver triflate to afford α-anomer 9. Coupling of 9 with H-phosphonate 5, followed by oxidation of the H-phosphonate intermediate 10 gave the partially protected disialophosphate diester 11 in good yield. Subsequent deprotection of 11 with
methoxide ion also gave a good yield of the target compound (12).

$^1$H NMR spectroscopic analysis of mono- (2d) and disialophospholipids (12) showed that they were both $\alpha$-sialosides because the chemical shifts of the $H_{3\alpha}$ signals of their reducing end sialic acid residues were found to be 2.81 and 2.83 ppm, respectively (Fig. 1), which is indicative of $\alpha$-sialosides (22). The presence of the disialic acid moiety in compound 12 is also clearly indicated by the resonances at 2.83 and 2.65 ppm, which represent the $H_{3\alpha}$ protons of both

Scheme 2. Reagents and conditions: (i) CH$_2$Cl$_2$, CH$_3$COCl, H$_2$O; (ii) HOCH$_2$CH$_2$OH, AgOTf, 2,6-di-tert-butylpyridine, MeCN, CaCO$_3$, –20 °C to room temp.; (iii) 5, (CH$_3$)$_2$COCl, C$_6$H$_5$N, 0 °C, 10 min; (iv) I$_2$, C$_6$H$_5$N–H$_2$O, room temp.; (v) MeOH, NaOMe; (vi) MeOH, H$_2$O, NaOH.
the reducing and nonreducing sialic acid residues of 12, respectively.

**Experimental**

$^1$H and COSY NMR spectra were measured at 400 MHz with a Varian 400 spectrometer; tetramethylsilane was used as an internal standard and $J$ values are given in Hz. $^{31}$P NMR spectra were measured at 80.2 MHz with a Varian 200 spectrometer spectrometer; 85% orthophosphoric acid was used as an internal standard. The FAB and ESI mass spectra were measured with a Jeol AX 505H and an Applied Biosystems Q-Star quadrupole/time-of-flight (Qq-TOF) mass spectrometer, respectively. Merck silica gel 60 Art 7729 and Art 7734-3 were used for short column chromatography and flash column chromatography, respectively. Sialic acid was purchased from Rose Scientific (Edmonton, Alberta). Colominic acid was purchased from Nacalai Tesque (Kyoto, Japan). Anhydrous dichloromethane, methanol, pyridine, and tetrahydrafuran were purchased from Sigma-Aldrich. Ethylene glycol was distilled under reduced pressure. The other chemicals were purchased from Sigma-Aldrich and were used without further purification.

**Triethylammonium 1,2-dihexadecyl-glycerol-3-H-phosphonate (5)**

Ammonium 4-methylphenyl H-phosphonate (19) (3.16 g, 16.71 mmol), triethylamine (5.0 mL, 35.8 mmol), and methanol (5.0 mL) were coevaporated under reduced pressure. To the residue was added (±)-1,2-dihexadecyl glycerol (18) (3.00 g, 5.566 mmol) and the mixture was azeotroped with pyridine (2 × 5 mL). The residue was redissolved in pyridine (30 mL) and cooled to –10 °C. Pivotal chloride (2.0 mL, 16.2 mmol) was added over a period of 5 min. After 1 h, water (5 mL) was added and the reactants were allowed to warm up to room temperature over a period of 1 h. The products were then partitioned between dichloromethane (100 mL) and saturated aqueous sodium hydrogen carbonate (2 × 80 mL). The layers were separated and the combined aqueous layers were back extracted with dichloromethane (2 × 20 mL). The combined organic layers were further extracted with triethylammonium phosphate buffer (0.5 mol/L, pH 7.0, 50 mL). The layers were separated and the aqueous layer was back extracted with dichloromethane (15 mL). The combined dried (MgSO$_4$) organic layers were concentrated under reduced pressure. The residue was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (9:1 v/v) were pooled and concentrated under reduced pressure to give the title compound (5) as a colourless froth (3.53 g, 89.9%). $^1$H NMR (CDCl$_3$–CD$_3$OD, 1:1 v/v) include the following peaks δH: 0.88 (6H, t, $J = 6.6$ Hz), 1.20–1.32 (52H, br), 1.50–1.58 (4H, br), 5.99 (0.5H, s), 7.56 (0.5H, s).

$^{31}$P NMR (CDCl$_3$–CD$_3$OD, 1:1 v/v) δP: 5.66 ($^{1}{/}_{p}$ = 628.3 Hz). FAB–HRMS calcd. for C$_{35}$H$_{72}$O$_5$P–: 603.51174; found: 603.5117.

**[Benzyl (glycolyl-5-acetamido-4,7,8,9-tetraacetyl-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosyl)]-1,2-dihexadecyl-glycerol-3-H-phosphonate (6)**

Benzyl (glycolyl-5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-β-D-manno-2-nonulopyranosid)onate (4) (0.50 g, 0.818 mmol) in triethylammonium 1,2-dihexadecyl-glycerol-3-H-phosphonate (5) (0.693 g, 0.981 mmol) were evaporated with dry pyridine (2 × 5 mL) and the residue was redissolved in anhydrous pyridine (10 mL) and cooled (ice-water bath). Pivaloyl chloride (0.26 mL, 2.11 mmol) was added. After a period of 10 min, the reaction was quenched by addition of water (0.5 mL). After a further period of 10 min, the products were partitioned between dichloromethane (80 mL) and saturated aqueous sodium hydrogen carbonate (2 × 50 mL). The layers were separated and the F254 TLC plates were developed in solvent systems A (dichloromethane–methanol (95:5 v/v)) and B (chloroform – methanol – water – acetic acid (50:50:7:0.2 v/v)). Merck silica gel 60 Art 7729 and Art 7734-3 were used for short column chromatography and flash column chromatography, respectively. Sialic acid was purchased from Rose Scientific (Edmonton, Alberta). Colominic acid was purchased from Nacalai Tesque (Kyoto, Japan). Anhydrous dichloromethane, methanol, pyridine, and tetrahydrafuran were purchased from Sigma-Aldrich. Ethylene glycol was distilled under reduced pressure. The other chemicals were purchased from Sigma-Aldrich and were used without further purification.
aqueous layer was back extracted with dichloromethane (2 × 10 mL). The combined organic layers were further washed with triethylammonium phosphate buffer (0.5 mol/L, pH 7.0, 50 mL). The layers were separated and the aqueous layer was back extracted with dichloromethane (10 mL). The combined dried (MgSO₄) organic layers were concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel. The appropriate fractions, which were eluted with ethyl acetate, were pooled and evaporated under reduced pressure to give the title compound 6 as a colourless froth (0.85 g, 86.7%). ¹H NMR (CDCl₃–CD3OD–D2O, 1:1:0.04 v/v) include the following peaks: δH: 0.88 (6H, t, J = 6.6 Hz), 1.22–1.32 (52H, br), 1.50–1.58 (4H, br), 1.87 (3H, s), 2.02 (3H, s), 2.03 (3H, s), 2.38 (3H, t), 2.40 (3H, t), 2.65 (1H, dt, J = 12.8, 4.0 Hz), 5.97 (0.5H), 7.37 (5H, m), 7.76 (0.5H). ³¹P NMR (CDCl₃) δp: 10.50, 10.36, 10.33, 10.18 (J\textsubscript{P-H} = 713.2 Hz). ESI⁺ calcd. for C₆₃H₁₀₈NO₁₈PNa+: 1220.7; found: 1221.0.

Methyl [glycolyl-5-acetamido-4,7,8,9-tetraacetyl-3,5-dideoxy-8-0-(5-acetamido-4,7,8,9-O-tetraacetyl-3,5-dideoxy-α-galacto-α-galacto-2-nonulopyranosylono-1′,9-lactone)-d-glycero-n-galacto-2-nonulopyranosid]onate (9)

The fully protected disialo-lacton peracetylated 7 (0.600 g, 0.67 mmol) was dissolved in dichloromethane (20.0 mL) and cooled to −20 °C. Acetyl chloride (8.0 mL) and water (0.1 mL) were then added. After 2 h, the reaction mixture was warmed up to room temperature, and stirring was continued for 16 h. The products were then concentrated under reduced pressure. The residue was dissolved in dichloromethane (40 mL) and washed with ice water (15 mL). The organic layer was separated and evaporated under reduced pressure. The residue was dissolved in acetonitrile (15 mL) and cooled to −20 °C. Calcium carbonate (0.48 g, 4.8 mmol), 2,6-di-tert-butylpyridine (0.30 mL, 1.34 mmol), ethylene glycol (2.0 mL, 35.86 mmol), and silver triflate (0.30 g, 1.17 mmol) were added. The reaction mixture was kept at −20 °C for 2 h and then allowed to warm up to room temperature. After 18 h, dichloromethane (10 mL) was added and the products were filtered. The filtrate was evaporated under reduced pressure, and the residue was dissolved in ethyl acetate (20 mL) and extracted with saturated aqueous sodium hydrogen carbonate (15 mL). The layers were separated and the dried (MgSO₄) organic layer was evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (85:15 v/v), were combined and concentrated under reduced pressure to give the title compound 2c as a colourless waxy solid (0.75 g, 85.3%). ¹H NMR (CDCl₃–CD3OD–D₂O, 1:1:0.04 v/v) include the following peaks: δH: 0.89 (6H, t, J = 6.6 Hz), 1.23–1.34 (52H, br), 1.49–1.57 (4H, br), 1.86 (3H, s), 2.02 (3H, s), 2.04 (3H, s), 2.13 (6H, s), 2.69 (1H, dd, J = 12.8, 4.4 Hz), 5.83 (5H, m). ³¹P NMR (CDCl₃–CD₃OD–D₂O, 1:1:0.04 v/v) δp: 13.9. FAB⁺ calcd. for C₆₃H₁₀₈NO₁₈PNa⁺: 1221.7; found: 1221.6.

**Methyl [glycolyl-5-acetamido-2,4,7-O-triacetyl-3,5-dideoxy-8-O-(5-acetamido-4,7,8,9-O-tetraacetyl-3,5-dideoxy-α-galacto-α-galacto-2-nonulopyranosylono-1′,9-lactone)-d-glycero-n-galacto-2-nonulopyranosid]onate (10)**

Methyl [glycolyl 5-acetamido-2,4,7-O-triacetyl-3,5-dideoxy-8-O-(5-acetamido-4,7,8,9-O-tetraacetyl-3,5-dideoxy-α-galacto-α-galacto-2-nonulopyranosylono-1′,9-lactone)-d-glycero-n-galacto-2-nonulopyranosid]onate (9) (0.100 g, 0.112 mmol) was added. After a period of 10 min, the reaction was quenched by addition of water (0.5 mL). After a further period of
10 min, the products were partitioned between dichloromethane (15 mL) and saturated aqueous sodium hydrogen carbonate (2 × 15 mL). The layers were separated and the aqueous layer was back extracted with dichloromethane (2 × 5 mL). The combined organic layers were further washed with triethylammonium phosphate buffer (0.5 mol/L, pH 7.0, 10 mL). The layers were separated and the aqueous layer was back extracted with dichloromethane (5 mL). The combined dried (MgSO4) organic layers were concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (10 mL) and aqueous sodium sulfite (0.1 mol/L, 0.5 mL), followed by addition of iodine (8.0 mg). After 10 min, the products were partitioned between dichloromethane (15 mL) and saturated aqueous sodium hydrogen carbonate (2 × 15 mL). The layers were separated and the aqueous layer was back extracted with dichloromethane (5 mL). The combined dried (MgSO4) organic layers were concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (85:15 v/v), were pooled and evaporated under reduced pressure to give the title compound 11 as a colourless froth (0.130 g, 77.6%). Rf (system A) 0.32. 1H NMR (CDCl3) include the following peaks δH: 0.88 (6H, t, J = 6.6 Hz), 1.22–1.32 (52H, br), 1.52–1.59 (4H, br), 2.46 (1H, dd, J = 13.6, 5.6 Hz), 2.68 (1H, dd, J = 12.8, 5.2 Hz), 6.03 (0.5H), 7.82 (0.5H). 31P NMR (CDCl3) δP: 10.50, 10.41, 10.23, 10.13 (1JpH = 720 Hz). FAB− calcd. for C72H122N2O27P−: 1477.8; found: 1477.9.

[Glycolyl 5-acetamido-3,5-dideoxy-8-O-(5-acetamido-3,5-dideoxy-o-glycero-a-o-galacto-2-nonulopyranosylono)-o-glycero-a-galacto-2-nonulopyranosyl]-1,2-dihexadecylglycero-3-phosphatet (12)

The H-phosphonate diester 10 (65 mg, 43.9 µmol) was dissolved in a mixture of pyridine (3.0 mL) and water (0.5 mL), followed by addition of iodine (8.0 mg). After 30 min, the products were partitioned between dichloromethane (10 mL) and aqueous sodium sulfite (0.1 mol/L, 3.0 mL). The layers were separated and the aqueous layer was back extracted with dichloromethane (5 mL). The combined organic layers were further extracted with triethylammonium phosphate buffer (50 mol/L, pH 7.0, 10 mL). The layers were separated and the aqueous layer was back extracted with dichloromethane (5 mL). The combined organic layers were dried (MgSO4) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (85:15 v/v), were pooled and evaporated under reduced pressure to give phosphate 11 as a colourless froth (52 mg). A portion of this froth (40 mg, 27 µmol) was dissolved in anhydrous methanol (10.0 mL) followed by addition of a solution of sodium methoxide in methanol (8.0 mL) followed by the addition of water (10.0 mL). After 16 h, the products were evaporated under reduced pressure. The residue was taken up in dichloromethane (3.0 mL) and saturated aqueous sodium hydrogen carbonate (15 mL) and passed through a Sephadex G-25 column (1.6 cm × 35 cm), eluted with water. The appropriate fractions were pooled and lyophilized to give the title compound 12 as a colourless froth (30.0 mg, 66% over two steps). Rf (system B) 0.16. 1H NMR (CDCl3–CD3OD–D2O, 1:1:0.03 v/v) include the following peaks δH: 0.84 (6H), 1.23 (52H), 1.52 (4H), 1.99 (3H, s), 2.00 (3H, s), 2.56 (1H), 2.83 (1H). 31P NMR (CDCl3–CD3OD–D2O, 1:1:0.03 v/v) δP: 4.27. FAB− calcd. for C58H110N2O25P: 1245.7; found: 1245.6.

Acknowledgements

The authors thank Dr. Jianjun Li, Ms. Lisa Morrison, and Mr. Ken Chan at the Institute for Biological Sciences of the National Research Council of Canada, Ottawa Ontario, for mass spectrometry analysis.

References