Synthetic Methods

DOI: 10.1002/ange.200502779

The Chemical Synthesis of Bioactive Glycosylphosphatidylinositols from *Trypanosoma cruzi* Containing an Unsaturated Fatty Acid in the Lipid**

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Dedicated to Professor Nikolay K. Kochetkov on the occasion of his 90th birthday

Glycosylphosphatidylinositols (GPIs) are a class of natural glycosylphospholipids that anchor proteins and glycoproteins (through their C terminus) as well as phosphoglycans (through the reducing end of the chain) to the membrane of eukaryotic cells. Since the first full assignment of a GPI structure in 1988,[1] a number of GPI anchors have been characterized.[2] The function of the compounds, in addition to the clear one of linking the above biopolymers to membranes, has been extensively discussed.[2,3] There is also evidence that GPIs and/or their metabolites can act as secondary messengers, which modulate biological events including insulin production, insulin-mediated signal transduction, cellular proliferation, and cell–cell recognition. The discovered role as mediators of regulatory processes makes the chemical preparation of the compounds and their analogues of great interest. To date, a number of syntheses of GPIs (yeast,[4] rat brain Thy-1,[5] *Trypanosoma brucei*,[6] *Leishmania*,[7] *T. gondii*,[8] *Plasmodium falciparum*,[9] and *T. cruzi* 1G7 antigen[10]) have been reported.

The protozoan parasite *Trypanosoma cruzi* is a causative agent of Chagas' disease, which affects about 18 million individuals in South and Central America.[11] It is transmitted to mammals in the feces of a biting insect vector (hematophagous triatomine bug) and has four distinct developmental stages. Throughout the life cycle, *T. cruzi* produces both common and stage-specific GPI-anchored cell-surface macromolecules.[12–15] Local release of GPI-anchored mucins by the bloodstream trypomastigote stage of the parasite is believed to be responsible for the development of parasite-elicited inflammation, which causes cardiac and other pathologies associated with the acute and chronic phases of Chagas' disease.[15]

It has recently been discovered[13] that a purified GPI fraction of *T. cruzi* trypomastigote mucins (trypomastigote GPI, or tGPI) revealed extraordinary proinflammatory activities, comparable to those of bacterial lipopolysaccharide. An ability to trigger the induction of tumor necrosis factor-α, interleukin-12, and nitric oxide at the 2–30 pM level (when presented to macrophages) showed that tGPI is one of the most potent microbial proinflammatory agents known. The structure of the cytokine- and NO-inducing tGPI anchor has been defined,[13] and the extreme biological activity was allegedly associated with the presence of unsaturated fatty acids in the *sn*2 position of the alkylacylglycerophosphate moiety[*] (**1** and **2**, Scheme 1) and/or with D-galactose branches along the glycan core (nonstoichiometric, not shown). As the issues regarding the structural features responsible for the activity can only be resolved through synthesis, a multidisciplinary program has been launched in our laboratory aimed at the chemical preparation of various *T. cruzi* trypomastigote mucin GPIs (including those containing D-galactose branches) and the meticulous elucidation of their structure–activity relationships. Herein, we report the first chemical syntheses of tGPIs from *T. cruzi* bearing oleic (compound **1**) and linoleic (compound **2**) acid moieties.

There are two major structural features that make compounds **1** and **2** different from the GPIs synthesized previously:[4–10] 1) the presence of unsaturated fatty acids in the lipid moiety instead of saturated ones; and 2) the presence of 2-aminoethylphosphonate at the O6 position of the D-glucosamine moiety, which is a parasite-specific substituent

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[**] This work, D.V.Y., and V.S.B. were supported by a Wellcome Trust Grant 067089/Z/02/Z. Prof. A. S. Shashkov (Moscow, Russia) is greatly acknowledged for NMR spectral characterization of the final compounds. We are grateful to Dr. A. Mehlert (Dundee) and Dr. V. Oleynikov (Moscow, Russia) for mass spectroscopic analyses and to Dr. A. J. Ross (Dundee) for the purification of compounds **1** and **2**.

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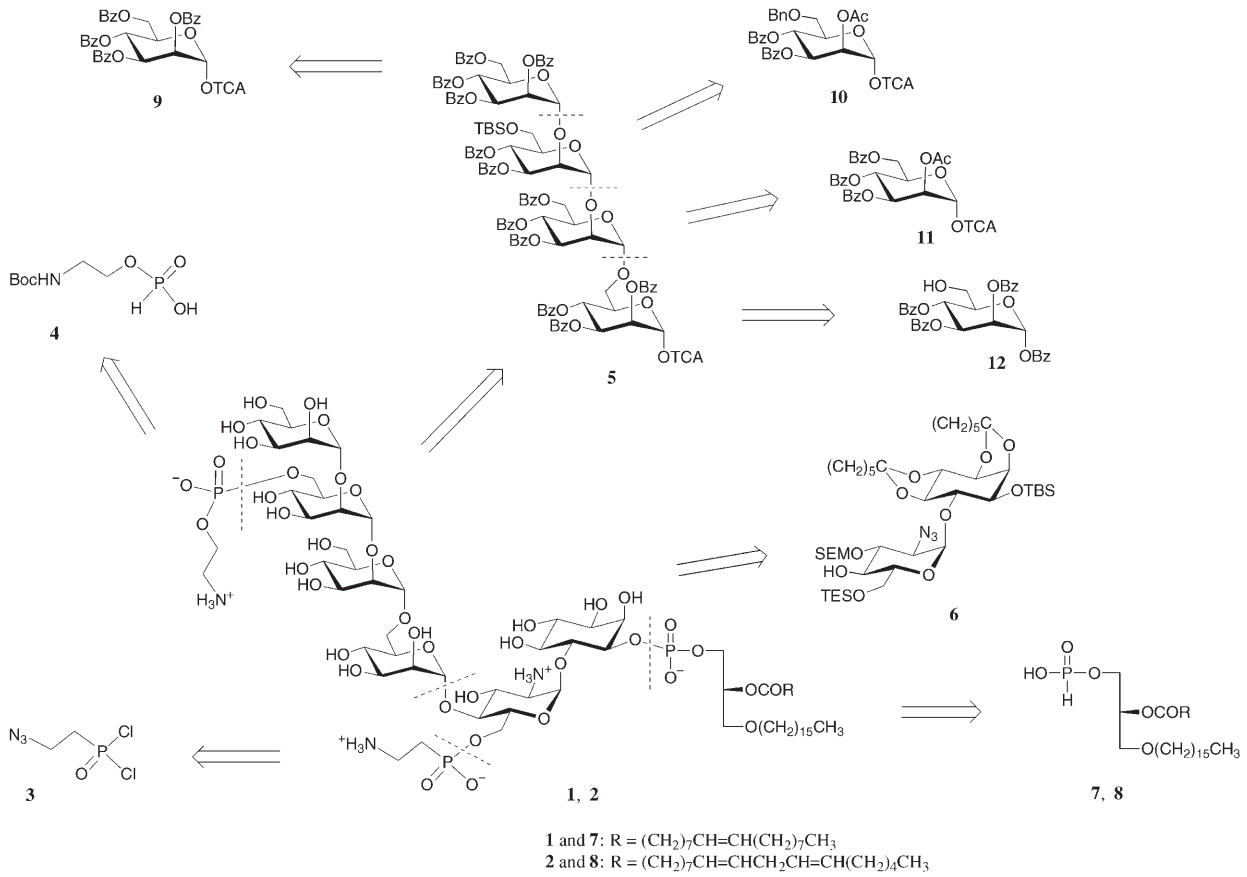
[*] The content of fatty acid components in the biologically active tGPI anchor fraction was: oleic acid, C18:1, 31%; linoleic acid, C18:2, 21%; and palmitic acid, C16:0, 37%.[13]



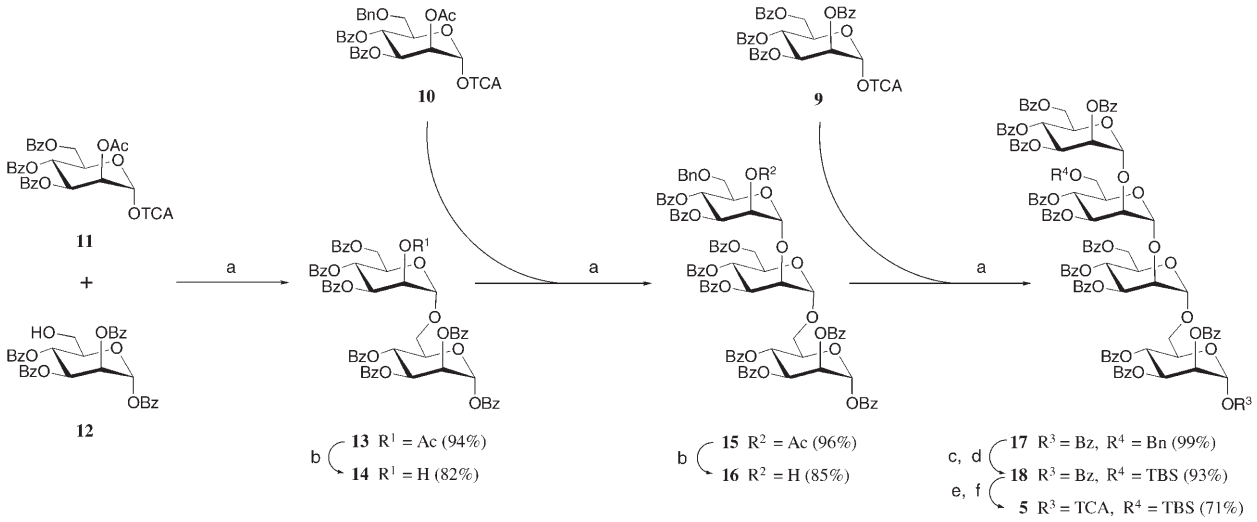
Scheme 1. Retrosynthetic scheme showing key building blocks and D-mannose monosaccharide intermediates. Boc = *tert*-butoxycarbonyl, Bz = benzoyl, SEM = 2-trimethylsilylethoxymethyl, TBS = *tert*-butyldimethylsilyl, TCA = C(NH)CCl_3, trichloroacetimidyl, TES = triethylsilyl.

for *T. cruzi* only. As the presence of double bonds was not compatible with the use of benzyl ethers (widely used before[4–10]) as permanent O-protecting groups, a novel strategy was developed which implied exploration of benzoic esters and acid-labile (acetals and *N*-Boc) groups for O,N protection. Various silyl ethers were employed as orthogonal blocking groups for the O6 of D-glucosamine (TES), O6 of D-mannose-3 (primary TBS), and O1 of *myo*-inositol (secondary TBS) to ensure further introduction of the P-containing esters. For the final deprotection, we expected that mild basic treatment in polar solvent would preferentially cleave the benzoates and leave the fatty ester of the lipid mostly intact because of micelle formation.

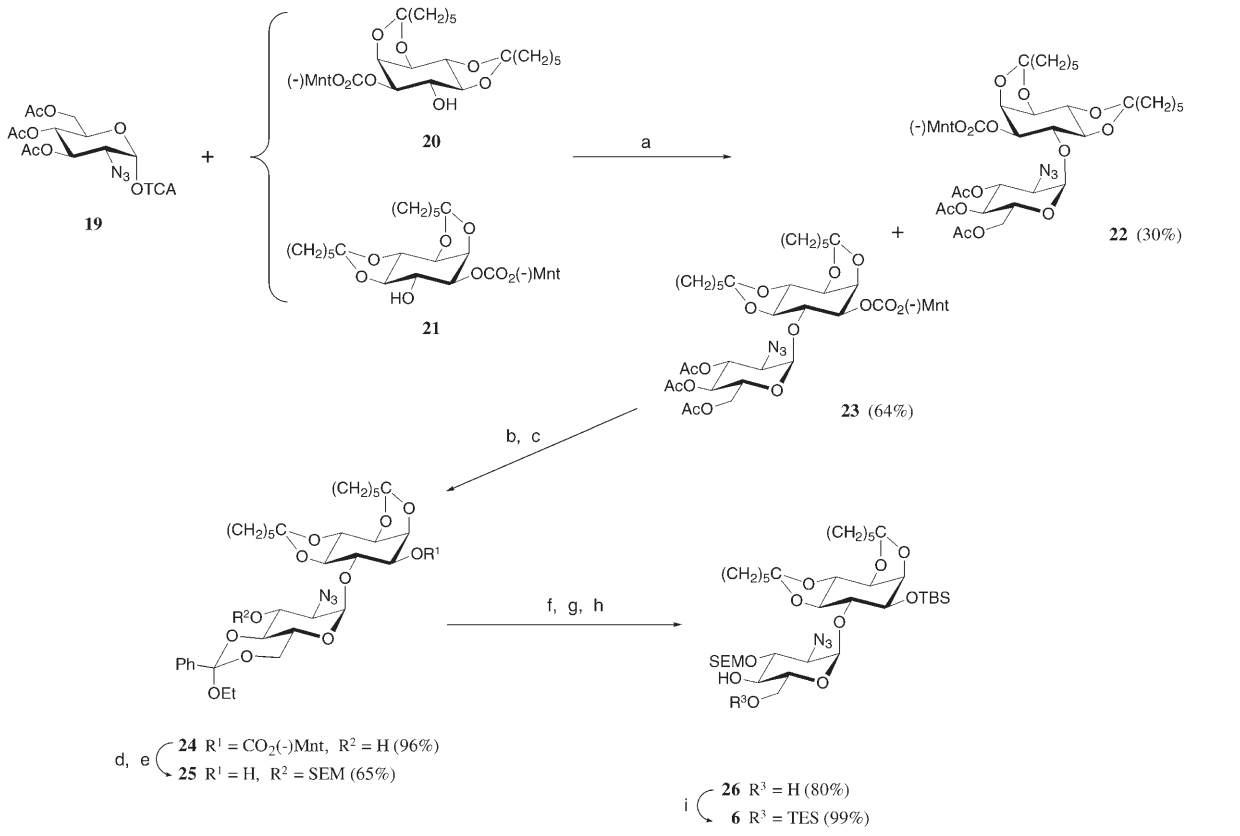
By following the retrosynthetic disconnection shown in Scheme 1, the GPIs **1** and **2** were assembled from the mannotetraose building block **5**, the azidoglucose–inositol block **6**, and P-containing derivatives phosphonodichloridate **3** and the hydrogenphosphonates **4** and **7** (or **8**), which were used for consecutive introduction of the 2-aminoethylphosphonate, ethanolamine phosphate, and acylalkylglycerophosphate fragments, respectively.

The tetrasaccharide block **5** was prepared from the monosaccharide derivatives **9**–**12**, which were assembled in a step-by-step manner (Scheme 2). Compounds **9** and **12**, in turn, were synthesized from D-mannose as well as the derivatives **10** and **11**, which progressed via the common intermediate 3,4,6-tri-*O*-acetyl-1,2-*O*-(1-methoxyethylidene)-β-D-mannose.[16] The disaccharide **13** (94%) was prepared first by coupling of the glycosyl acceptor **12** and the trichloroacetimidate **11** in the presence of trimethylsilyl triflate (TMSOTf). It was then deacetylated[17] with HCl in MeOH (→**14**) followed by reaction with glycosyl donor **10** and TMSOTf to produce the trisaccharide **15** (96%). Subsequent deacetylation (→**16**) and one more glycosylation with the trichloroacetimidate **9** and TMSOTf provided the tetrasaccharide **17** (99%). The α configuration of the newly created D-mannoside bonds was secured by the structure of mannosyl donors **9**–**11**, which contained participating protecting groups at the O2 position. Compound **17** was then converted to the mannotetraose glycosyl donor **5** (66% yield) by consecutive reprotection at the O6″ position (hydrogenation over Pd catalyst followed by silylation with TBSOTf/Et_3N; →**18**), anomeric debenzoylation with ethylenediamine, and reaction with CCl_3CN in the presence of Cs_2CO_3.

The azidoglucose–inositol block **6** was synthesized (Scheme 3) from the pseudo-disaccharide **23**, which was described by Schmidt and co-workers[4,18] as a glycosylation product of the optically pure D-*myo*-inositol derivative **21**[19] with the azidoglucose trichloroacetimidate **19**. We found that the resolution of diastereomeric (−)-menthyloxycarbonyl-



Scheme 2. Reagents: a) $TMSOSO_2CF_3$, CH_2Cl_2; b) 2% HCl, $MeOH/CH_2Cl_2$; c) H_2, $Pd(OH)_2/C$, THF; d) $TBSOSO_2CF_3$, Et_3N, CH_2Cl_2; e) $NH_2CH_2CH_2NH_2{\cdot}HOAc$, THF; f) CCl_3CN, Cs_2CO_3, CH_2Cl_2. Bn = benzyl, THF = tetrahydrofuran, TMS = trimethylsilyl.



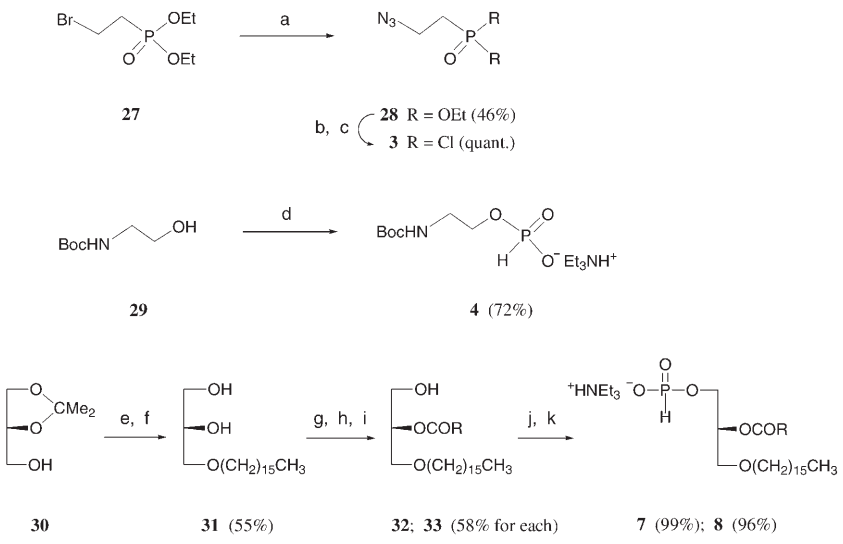
Scheme 3. Reagents: a) $TMSOSO_2CF_3$, MS4A, Et_2O/CH_2Cl_2; b) 0.02 M NaOMe, $MeOH/CH_2Cl_2$; c) $PhC(OEt)_3$, CSA, CH_2Cl_2; d) SEMCl, iPr_2NEt, CH_2Cl_2; e) 1 M NaOMe, $MeOH/CH_2Cl_2$; f) $TBSOSO_2CF_3$, Et_3N, CH_2Cl_2; g) CH_2Cl_2/CF_3COOH/water (1000:1:0.1); h) 0.1 M NaOMe, $MeOH/CH_2Cl_2$; i) TESCl, pyridine, CH_2Cl_2, −20 °C. CSA = camphor-10-sulfonic acid, Mnt = menthyl, MS4A = 4-Å molecular sieves.

myo-inositols **21** (D product) and **20** (L product) could be avoided, but it was possible to perform glycosylation of the whole mixture and then isolate the required derivative **23** by standard flash column chromatography on SiO_2. Coupling of the mixture **21** + **20** (7:3, according to ^{1}H NMR data; prepared as described in reference [19]) with the glycosyl donor **19**[20] in the presence of TMSOTf and 4-Å molecular sieves proceeded smoothly and gave the easily separable (R_f difference of 0.1) diastereomers **23** and **22**, which were isolated in 64 and 30% yield, respectively (that is, 94% total yield of the glycosylation based on the mixture **21** + **20**). Both compounds had an α configuration of the D-glucoside bond,

which was confirmed by the characteristic values (3.5 Hz for **23** and 3.6 Hz for **22**) for $J_{1',2'}$ coupling constants. Clearly, the 2-azido-2-glucose-α-D-glucosyl moiety worked as an additional powerful chiral auxiliary and facilitated the separation of D- and L-*myo*-inositol derivatives.

For the transformation **23**→**6**, first, the introduction of the acid-labile 2-trimethylsilylethoxymethyl (SEM) permanent protecting group at the O3′ position was needed. This was performed through the mild basic deacetylation of **23** followed by orthoesterification with $PhC(OEt)_3$ in mild acidic conditions (to form the 4′,6′-orthoester derivative **24**) and reaction with SEM chloride in the presence of *N*,*N*-diisopropylethylamine. Basic cleavage of the (−)-menthylcarbonate gave the 1-hydroxy derivative **25**, which was then successively silylated with TBSOTf/Et_3N, hydrolyzed with 0.1% trifluoroacetic acid (TFA)/water in CH_2Cl_2 (10 min) to open the orthoester (thus forming a mixture of 4′- and 6′-acetates), deacetylated (with MeONa in MeOH; →**26**), and silylated at the O6′ position with Et_3SiCl in pyridine/CH_2Cl_2. Thus, the azidoglucose–inositol block **6** was prepared from compound **23** in seven steps in approximately 50% overall yield.

The 2-azidoethylphosphonodichloridate **3** was prepared (Scheme 4) from diethyl 2-bromoethylphosphonate **27** through the azidation reaction with NaN_3 (→**28**) followed by de-esterification with Me_3SiBr and chlorination with oxalyl chloride in the presence of *N*,*N*-dimethylformamide. The 2-(*N*-Boc)-aminoethyl hydrogenphosphonate **4** was prepared by the reaction of *N*-Boc-ethanolamine **29** with H_3PO_3 in the presence of pivaloyl chloride.[21] The 2-*O*-acyl-1-*O*-hexadecyl-*sn*-glyceryl hydrogenphosphonates **7** and **8** were synthesized starting from commercially available 2,3-*O*-isopropylidene-*sn*-glycerol **30**, which was first alkylated with *n*-hexadecyl iodide in the presence of NaH followed by acid hydrolysis to produce 1-*O*-hexadecyl-*sn*-glycerol **31** (55%). This compound was then successively silylated at the 3-hydroxy group with Et_3SiCl in pyridine, esterified with oleoyl or linoleoyl chloride, and desilylated with 3HF·Et_3N, thus providing the 2-*O*-acylated glycerol derivatives **32** and **33**, respectively. Each of them was converted (almost quantitatively) to the corresponding hydrogenphosphonate derivative (**7** and **8**, respectively) by reaction with triimidazolylphosphine[22] followed by hydrolysis.



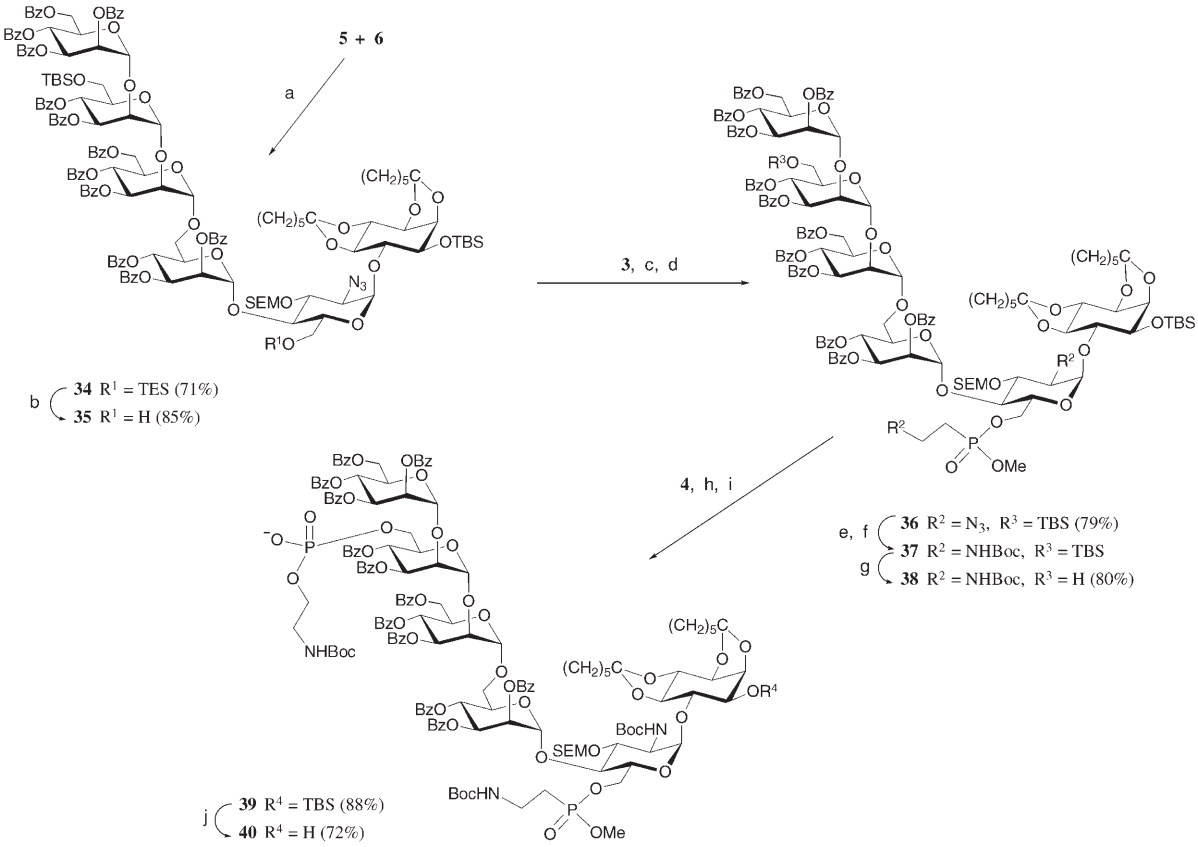
7 and **32** R = $(CH_2)_7CH{=}CH(CH_2)_7CH_3$
8 and **33** R = $(CH_2)_7CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$

Scheme 4. Reagents: a) NaN_3, nBu_4NHSO_4 cat., toluene; b) TMSBr, MeCN; c) $(COCl)_2$, DMF cat., CH_2Cl_2; d) H_3PO_3, pivaloyl chloride, pyridine; e) $CH_3(CH_2)_{15}I$, NaH, DMF/THF; f) CF_3COOH/water (9:1); g) TESCl, pyridine, CH_2Cl_2; h) oleoyl chloride for **32** (or linoleoyl chloride for **33**), Et_3N, DMAP, pyridine; i) 3HF·Et_3N, MeCN/CH_2Cl_2; j) triimidazolylphosphine, MeCN/CH_2Cl_2; k) Et_3NHHCO_3, water (pH 7). DMAP = 4-dimethylaminopyridine, DMF = *N*,*N*-dimethylformamide.

With all the principal building blocks in hand, we pursued the preparation of the target GPIs **1** and **2**. First, the glycan–inositol backbone **34** was prepared (Scheme 5) by the glycosylation of the glycosyl acceptor **6** with the mannotetraose trichloroacetimidate **5** in the presence of TMSOTf and 4-Å molecular sieves. Subsequent cleavage of the TES group (the "weakest" of the three silyl protecting groups) with acetic acid-buffered tetrabutylammonium fluoride (TBAF) smoothly gave the 6′-hydroxy pseudo-hexasaccharide derivative **35** (60% from **5**), ready to turn to the "P-decoration" procedures. 1*H*-Tetrazole-assisted esterification of **35** with the phosphonodichloridate **3** followed by methanolysis afforded the phosphonic diester **36** (79%) as a diastereomeric mixture[*] ($\delta_P = 28.5, 28.8$). It was then a subject of successive reduction of the azido groups with Ph_3P, N protection with Boc anhydride (→**37**), and selective cleavage of the primary TBS ether with 3HF·Et_3N, thus cleanly producing the 6′′′′-hydroxy glycan–inositol–phosphonate compound **38** (80%). Furthermore, the introduction of the ethanolamine phosphate moiety was performed by the condensation of **38** with the hydrogenphosphonate derivative **4** (activated by pivaloyl chloride)[23] followed by in situ oxidation with iodine in aqueous pyridine. The phosphonate–phosphate block **39** was isolated in 88% yield prior to the final desilylation with TBAF/AcOH (at 55 °C), which gave the 1-hydroxy glycoconjugated derivative **40**.

We reported earlier[24] that the presence of phosphodiester units in a molecule still allows the next O-phosphorylation step to be performed effectively by the hydrogenphosphonate method (that is, P protection for phosphodiesters is not required). Indeed, compound **40** (containing a phosphodiester moiety at the O6′′′′ position) was successfully phospholipi-

[*] The P protection was required at this stage to avoid undesired modifications of the phosphonate moiety during further transformations. Each of the methyl phosphonate derivatives **36**–**42** was formed as a mixture of diastereomers at the phosphorus atom (in a ratio of 1:1), as clearly indicated by the ^{31}P NMR spectra (see Supporting Information).



Scheme 5. Reagents: a) $TMSOSO_2CF_3$, MS4A, CH_2Cl_2; b) nBu_4NF, AcOH, THF (20 °C, 1.5 h); c) 1*H*-tetrazole, iPr_2NEt, toluene; d) MeOH; e) Ph_3P, water, THF; f) Boc_2O, Et_3N, MeOH; g) 3HF·Et_3N, MeCN/THF; h) pivaloyl chloride, pyridine; i) I_2, pyridine/water; j) nBu_4NF, AcOH, THF (55 °C, 60 h).

dated (Scheme 6) by a pivaloyl chloride assisted reaction with the acylalkylglyceryl hydrogenphosphonate **7**, followed by in situ oxidation with iodine to provide the fully protected oleic ester GPI **41** in 95% yield. Similarly, the protected linoleic ester GPI **42** (85%) was prepared from **40** and the hydrogenphosphonate **8**. Both compounds **41** and **42** were immediately demethylated at the aminoethylphosphonate moiety with $PhSH/Et_3N$[25] to form GPI derivatives **43** and **44**, respectively.

Global deprotection of **43** was performed in two steps. First, controlled O-debenzoylation with 0.05 M methanolic sodium methoxide (3 h) gave the partly protected GPI **45**, which was isolated in 40% yield by flash column chromatography on SiO_2. The presence of the fatty ester in the molecule was clearly indicated by MALDI-TOF mass spectrometry (MS) data.[26] Subsequent cleavage of *O*-acetal and *N*-Boc protecting groups with aqueous 90% TFA followed by purification by reversed-phase chromatography (on a C4 silica column with gradient elution with propan-1-ol/water/TFA, 10:90:0.05→95:5:0.05) provided the targeted oleic ester GPI **1**. The protected derivative **44** was converted to the linoleic ester GPI **2** in a similar manner.

The structures of the glycosylphospholipids **1** and **2** were supported by NMR spectroscopy and MS data. The ^1H, ^{13}C, and ^{31}P NMR spectra for **1** were almost identical to those for **2**. Full structural assignment of **2** was performed by a combination of ^1H and ^{13}C NMR, COSY, ROESY, TOCSY, and heteronuclear single-quantum correlation (HSQC) spectroscopy (see Table 1). The ^{31}P NMR signals were assigned with the $^1H,^{31}P$ heteronuclear multiple-quantum correlation (HMQC) technique. The molecular masses for the GPIs **1** and **2** were confirmed by MALDI-TOF and electrospray (ES) MS.[26]

The synthetic GPIs **1** and **2** revealed biological activity: preliminary experiments using Toll-like receptor (TLR) transfected Chinese hamster ovary (CHO) cell lines showed they stimulated TLR2-transfected cells and not TLR4-transfected cells,[27] like naturally occurring tGPI.[28] A detailed biological evaluation of the compounds is currently in progress and will be published elsewhere in due course.

In summary, a novel approach for the chemical synthesis of glycosylphosphatidylinositols, which exploits the use of non-benzyl-type protecting groups, was designed. The method showed its utility in the first syntheses of *T. cruzi* trypomastigote GPIs containing unsaturated fatty acids in the lipid moiety.

Received: August 5, 2005
Revised: October 3, 2005
Published online: December 8, 2005

Table 1: Correlation table of **2**: ^{1}H, ^{13}C NMR and ^{31}P chemical shifts at 500, 125, and 202 MHz, respectively. Measurements in $[D_6]$DMSO at 30 °C; δ values [ppm] are given relative to Me_4Si (for ^{1}H and ^{13}C) and external aqueous 85 % H_3PO_4 (for ^{31}P).

Residue[a]	Atom							
	H1	H2	H3	H4	H5	H6		P
	C1	C2	C3	C4	C5	C6		
Ino[b]	4.03	3.81	3.48	3.46	3.16	3.75		−0.6
	75.7	70.45	73.65	72.65	73.1	78.5		
$GlcNH_2$[c]	5.48	2.96	3.82	3.49	4.27	4.07 + 3.94		18.7
	94.3	53.8	70.1	76.25	69.2	63.1		
Man-1	5.11	3.86	3.48	3.60	3.19	3.85 + 3.62		
	101.25	71.8	70.65	66.1	70.8	65.6		
Man-2	4.85	3.78	3.70	3.45	3.62	3.67 + 3.42		
	98.2	77.05	70.1	67.1	72.4	61.2		
Man-3[d]	5.08	3.88	3.61	3.39	3.37	4.10 + 3.75		−1.5
	100.9	77.05	69.9	67.1	70.6	65.4		
Man-4	4.86	3.80	3.75	3.71	3.30	3.68 + 3.49		
	101.85	71.8	69.9	66.6	71.8	60.9		

[a] Important inter-residue correlation peaks in the ROESY spectrum: H1 ($GlcNH_2$)/H6 (Ino); H1 ($GlcNH_2$)/H1,5 (Ino); H1 (Man-1)/H4 ($GlcNH_2$); H1 (Man-2)/H6,6′ (Man-1); H1 (Man-3)/H2 (Man-2); H1 (Man-4)/H2 (Man-3). Important correlation peaks in the $^{1}H,^{31}P$ HMQC spectrum: P^{I} ($\delta_P = 18.7$ ppm) with H6,6′ ($GlcNH_2$) and P-CH_2-CH_2-NH_3^+; P^{II} ($\delta_P = -0.6$ ppm) with H1 (Ino) and H3 (glycerol); P^{III} ($\delta_P = -1.5$ ppm) with H6,6′ (Man-3) and P-O-CH_2-CH_2-NH_3^+. [b] Additional signals of glycerol were present: CH_2-O-P, $\delta_H = 3.80$ ppm, $\delta_C = 63.1$ ppm; CH-O-acyl, $\delta_H = 5.02$ ppm, $\delta_C = 71.0$ ppm; CH_2-O-alkyl, $\delta_H = 3.52$ and 3.47 ppm, $\delta_C = 68.9$ ppm. [c] Additional signals of the aminoethylphosphonate P-CH_2-CH_2-NH_3^+ were present: PCH_2, $\delta_H = 2.01$ ppm, $\delta_C = 26.5$ ppm; NCH_2, $\delta_H = 3.06$ and 3.00 ppm, $\delta_C = 35.0$ ppm. [d] Additional signals of the ethanolamine phosphate P-O-CH_2-CH_2-NH_3^+ were present: $POCH_2$, $\delta_H = 4.05$ and 3.83 ppm, $\delta_C = 65.7$ ppm; NCH_2, $\delta_H = 3.06$ and 3.00 ppm, $\delta_C = 40.0$ ppm.

Keywords: carbohydrates · glycosides · phospholipids · protozoa · synthetic methods

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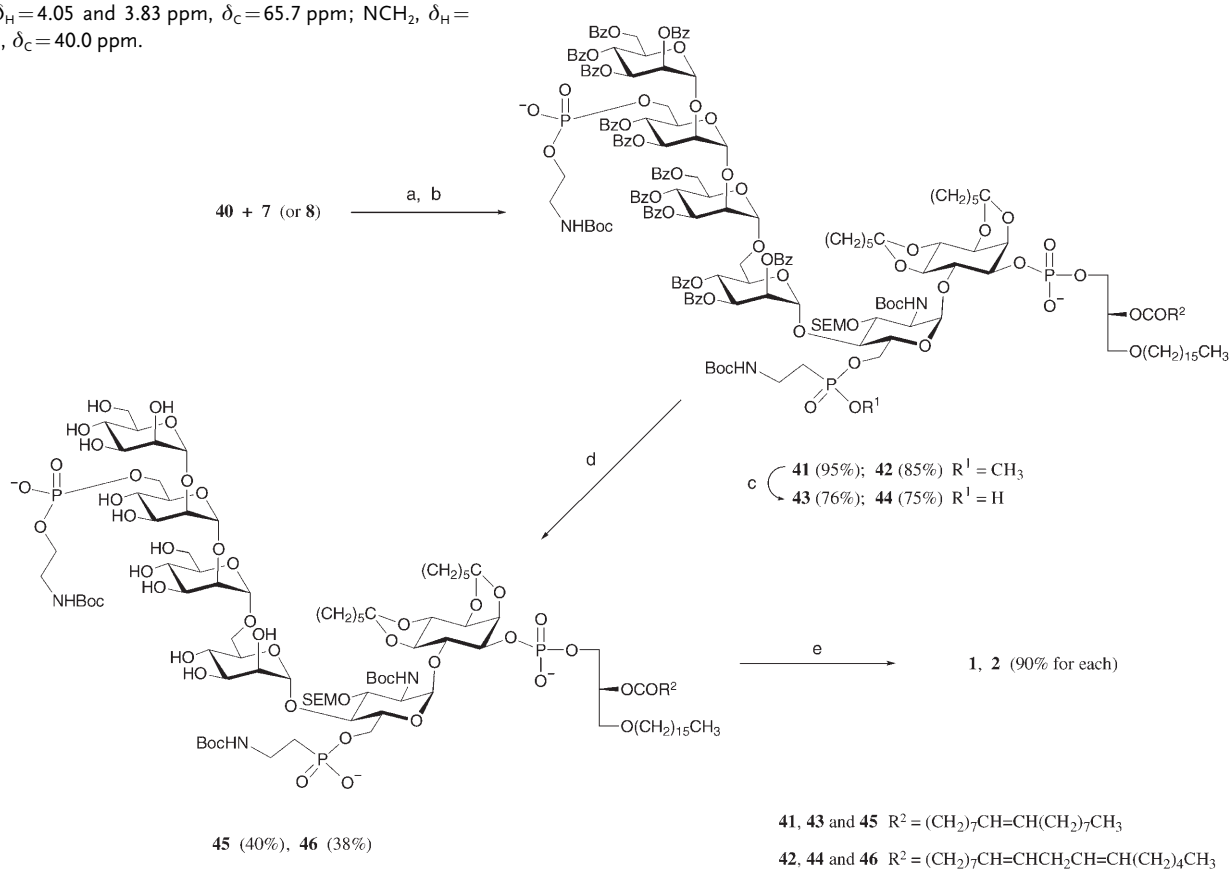

Scheme 6. Reagents: a) pivaloyl chloride, pyridine; b) I_2, pyridine/water; c) PhSH, Et_3N, DMF; d) 0.05 M NaOMe, MeOH; e) CF_3COOH/water (9:1).

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