Probing the substrate specificity of *Trypanosoma brucei* GlcNAc-PI de-\(N\)-acetylase with synthetic substrate analogues†

Amy S. Capes,† Arthur Crossman,‡ Michael D. Urbaniak, Sophie H. Gilbert, Michael A. J. Ferguson* and Ian H. Gilbert*

A series of synthetic analogues of 1-\(d\)-(2-amino-2-deoxy-\(\alpha\)-\(d\)-glucopyranosyl)-myo-inositol 1-(1,2-di-O-hexadecanoyl-sn-glycero-3-phosphate), consisting of 7 variants of either the \(d\)-myo-inositol, \(d\)-GlcP, or the phospholipid components, were prepared and tested as substrates and inhibitors of GlcNAc-PI de-\(N\)-acetylase, a genetically validated drug target enzyme responsible for the second step in the glycosyl-phosphatidylinositol (GPI) biosynthetic pathway of *Trypanosoma brucei*. The \(d\)-myo-inositol in the physiological substrate was successfully replaced by cyclohexanediol and is still a substrate for *T. brucei* GlcNAc-PI de-\(N\)-acetylase. However, this compound became sensitive to the stereochemistry of the glycoside linkage (the \(\beta\)-anomer was neither substrate or inhibitor) and the structure of the lipid moiety (the hexadecyl derivatives were inhibitors). Chemistry was successfully developed to replace the phosphate with a sulphonamide, but the compound was neither a substrate or an inhibitor, confirming the importance of the phosphate for molecular recognition. We also replaced the glucosamine by an acyclic analogue, but this also was inactive, both as a substrate and inhibitor. These findings add significantly to our understanding of substrate and inhibitor binding to the GlcNAc-PI de-\(N\)-acetylase enzyme and will have a bearing on the design of future inhibitors.

Introduction

The enzymes of the glycosylphosphatidylinositol (GPI) biosynthetic pathway are located in the endoplasmic reticulum, contain between one and thirteen predicted trans-membrane domains and are mostly present as components of multisubunit complexes.1 No high-resolution structural data are available on any of these enzymes and our research group has been probing the specificities of several of the enzymes in the GPI pathway of the protozoan parasite *Trypanosoma brucei*, the causative agent of African sleeping sickness in humans and the related disease Nagana in cattle, using synthetic substrate analogues in *vivo*.2–8 One of the key enzymes of interest is an amidase, the GlcNAc-PI de-\(N\)-acetylase (EC 3.5.1.89) that de-\(N\)-acylates 1-\(d\)-(2-acetamido-2-deoxy-\(\alpha\)-\(d\)-glucopyranosyl)-myo-inositol 1-(1,2-di-O-hexadecanoyl-sn-glycero-3-phosphate) (1, \(\alpha\)-\(d\)-GlcPNAc-PI) to 1-\(d\)-(2-amino-2-deoxy-\(\alpha\)-\(d\)-glucopyranosyl)-myo-inositol 1-(1,2-di-O-hexadecanoyl-sn-glycero-3-phosphate) (2, \(\alpha\)-\(d\)-GlcPn-PI), Fig. 1.

This enzyme catalyses the second step in the *T. brucei* GPI biosynthetic pathway, which is a prerequisite for all subsequent steps in the pathway.9 In earlier studies, we showed that *T. brucei* GlcNAc-PI de-\(N\)-acetylase is a zinc-dependent metalloenzyme10 and demonstrated, by construction of a condition-null mutant cell line, that it is essential for the bloodstream form of the parasite and, therefore, a genetically validated drug target.11 Previous studies with other substrate analogues showed that the phosphate, 2'-NHAc and 3'-OH groups of the natural substrate \(\alpha\)-\(d\)-GlcPNAc-PI (1) are critical for recognition by the *T. brucei* GlcNAc-PI de-\(N\)-acetylase.2–4 In contrast, the diacylglycerol moiety is not strictly required and may be efficiently replaced with an octadecyl chain,3 as shown in analogues 3 and 4. In the case of the *T. brucei* enzyme, we had hypothesised that one or more of the inositol 2, 3, 4 and 5-OH groups is/are not required.2–4 We came to this hypothesis from the ability of the enzyme to recognise and process both \(\alpha\)-\(d\)-GlcPNAc-\(L\)-PI (5) and \(\beta\)-\(d\)-GlcPNAc-PI (6). Molecular dynamics simulations, showed that \(\alpha\) and \(\beta\) anomers can adopt conformations in which the phosphate, the 2'-amide
and the 3′-OH overlay. Given the 2′-amide is where the reaction occurs, and evidence suggests that the 3′-OH and phosphate are important for recognition of the substrate with the enzyme, these conformations are likely to be the active enzyme-bound conformations. In these conformations the inositol 2-, 3-, 4- and 5-hydroxys are in different orientations for the two α and β anomers, implying the hydroxys are not critical for interaction with the enzyme. Further evidence was obtained from a compound in which the inositol 2-hydroxyl was alkylated. This was also a substrate for the T. brucei enzyme. One of the goals of the study described in this paper was to investigate the hypothesis that the inositol 2, 3, 4 and 5-OH groups are not required.

Bearing in mind these key structural features, we have synthesised a variety of analogues to further probe the requirements for substrate recognition by the T. brucei GlcNAc-PI de-N-acetylase and, specifically, to test:

1. The hypothesis that the inositol 2, 3, 4 and 5-OH groups are not required for enzyme recognition, a series of pseudodisaccharides (7–10, Fig. 2) containing a cyclohexanediol moiety in place of the inositol aglycone were prepared.
2. Whether the phosphate group can be replaced by more cell-permeable sulphonamide isosteres,12 compounds 11 and 12 (Fig. 2) were prepared.
3. Whether, given the essentiality of the 2′-NHR and 3′-OH groups but non-essentiality of the 4′- and 6′-OH groups for substrate recognition, the glucosamine residue might be simplified to a simple acyclic structure, as in compound 13.

The N-acetylated derivatives of the above analogues required for biological studies with the de-N-acetylase were prepared from the corresponding amines by standard procedures.13 All the analogues were examined for their recognition and processing by the T. brucei GlcNAc-PI de-N-acetylase.

Results and discussion

Synthesis of analogues 7–13

The synthesis of the required α and β-glucosaminyl (1′ → 1) cyclohexanediol building blocks 16 and 17, respectively, began by reacting the known14 trichloroacetimidate 14 and the commercially available 1R,2R-trans-cyclohexanediol 15, Scheme 1, with a catalytic amount of trimethylsilyl trifluoromethanesulfonylfluoride (TMSOTf). The separation of anomers was achievable at this stage and these anomers were vital in providing the glucosamine-phosphodiester target analogues discussed herein. For the sake of brevity, we have chosen to describe in the main text the formation of the α-anomers, while details for the corresponding β-anomers appears in the ESI.† Therefore, the pseudodisaccharide 16 was coupled to the hydrogen phosphonate 18,15 and the ensuing mixture of diastereoisomeric

Fig. 1 Some previously prepared GPI analogues.

Fig. 2 Target molecules.
phosphonic diesters, was oxidised with iodine in pyridine-water\textsuperscript{16} to give the corresponding phosphodiester \textsuperscript{19}. The diester was transformed into the triol \textsuperscript{20} after conventional \textit{O}-deacylation of the latter compound with 0.05 M methanolic NaOMe in CH\textsubscript{2}Cl\textsubscript{2}–MeOH.

Our first attempt at hydrogenolysis of the triethylammonium (TEA) salt \textsuperscript{20} over Pd(OH)\textsubscript{2}/C gave, surprisingly, the alkyl alcohol secondary amine \textsuperscript{21}. Initially, the azide of \textsuperscript{20} was reduced to the primary amine, however, if there are peroxides present in the THF then the formation of THF hydroperoxide is, apparently, possible which could then lead to an amine–THF coupling via a free-radical-based mechanism.\textsuperscript{17} This intermediate is susceptible to a Pd-mediated THF ring opening reaction that gives an imine which is then further hydrogenated to an aminobutanol.\textsuperscript{17} Consequently, after purchasing a fresh bottle of anhydrous stabilised THF, the second hydrogenolysis attempt at \textsuperscript{20} → \textsuperscript{7} proceeded without incident.

The preparation of the dipalmitoyl glycerol pseudodisaccharide \textsuperscript{9} was accomplished from the triacetate \textsuperscript{16}, Scheme 2. However, the acetate protecting groups in \textsuperscript{16} are unsuitable because if they were left in place and removed by base at the final step of the synthesis, then those requisite esters of the lipid fragment would likewise be saponified. Therefore, the acetates of \textsuperscript{16} needed to be swapped to a more appropriate protecting group but first, the temporary tert-butyldimethylsilyl (TBDMS) protection of the 2-OH, \textsuperscript{16} → \textsuperscript{22}, was performed and

---

\textbf{Scheme 1} Synthesis of \textsuperscript{7}.

\textbf{Scheme 2} Synthesis of \textsuperscript{9}.
then followed by conventional O-deacetylation, as previously described for 19 → 20, furnished the triol 23. The benzyl group was chosen as the 3', 4' and 6'-OH protecting group because, from our past experiences synthesising GPI analogues, the benzyl group has been a very reliable protecting group via ease of installation and removal. Thus, the triol 23 was benzylated with benzyl bromide in the presence of NaH, as the base, to afford compound 24. We next turned our attention towards the reduction of the azide in 24 and because of the issues with Pd(OH)$_2$ catalysed reduction in the presence of peroxodic THF discussed earlier, we chose to reduce the azide via the Staudinger reaction$^{18}$ to give the amine 25 which was subsequently tert-butyl carbamate (Boc) protected to furnish 26. Desilylation of 26 using HF–pyridine conditions afforded the alcohol 27. The known hydrogen phosphonate 28$^{19}$ was coupled, as already described, to the 2-OH of 27 when, after oxidation, it was isolated and characterised as the TEA salt 29. Hydrogenolysis of 29 over Pd(OH)$_2$/C gave the Boc protected derivative 30 and subsequent cleavage of the Boc group produced the deprotected target analogue 9.

The synthesis of the sulphonamides 11 and 12, Scheme 3, was accomplished by reacting commercially available 1R,2R-1-amino-2-benzyloxycyclohexane 31 with 1-octadecanesulfonyl chloride in the presence of triethylamine and CH$_2$Cl$_2$ to give the benzyl derivative 32. The benzyl group was removed by hydrogenolysis over Pd(OH)$_2$/C to furnish the alcohol 33. Coupling of 33 with the known trichloroacetimidate 34$^{20}$ resulted in an inseparable mixture of the α,β-anomers 35 in the ratio of ~1:1, as determined by $^{1}H$ NMR spectroscopy. Finally, hydrogenolysis of the aforementioned anomers 35 over Pd(OH)$_2$/C and subsequent silica gel column chromatography (9:1 CH$_2$Cl$_2$–MeOH) gave first the β-anomer 11 and then the α-anomer 12.

We were also interested in seeing if we could replace the glucose ring with an acyclic moiety. From our knowledge of the SAR, retaining the 2-amino and 3-hydroxy groups are important for activity. The synthesis of the amino-phosphate 13, Scheme 4, began by epoxide ring-opening of cyclohexene oxide 36 with p-methoxybenzyl alcohol (PMBOH), using Cu(BF$_4$)$_2$·nH$_2$O as a catalyst$^{21}$ to give the known racemic PMB monoprotected cyclohexanediol$^{12}$ contaminated with unreacted PMBOH. Chromatographic separation of this PMB cyclohexyl derivative from the excess of PMBOH was not achievable, in our hands, and so the entire PMB reaction residue was acetylated with acetic anhydride in the presence of pyridine and a catalytic amount of 4-(dimethylamino)pyridine DMAP to furnish the acetate 37, which was easily separated from the acetate of p-methoxybenzyl alcohol by silica gel column chromatography.$^{23}$ After deacetylation, the resulting alcohol$^{22}$ residue was alkylated using sodium hydride and allyl bromide to give the allyl derivative 38. The epoxide 39 was prepared upon reacting 38 with 3-chloroperbenzoic acid (mCPBA), and the subsequent hydrolysis of epoxide 39 with DMSO, H$_2$O and a catalytic amount of KOH$^{24}$ worked smoothly to furnish diol 40. The primary alcohol of 40 was protected using tert-butyl(chloro)diphenylsilane (TBDPSCI) and DMAP to give compound 41. The azido group of 43 was satisfactorily installed (51% yield over two steps) via the mesylate 42 obtained by reacting the secondary alcohol 41 with methanesulfonyl chloride in the presence of pyridine, followed by treatment of 42 with sodium azide under forcing conditions. The crude mesylate 42 was used directly in the displacement reaction but a small portion of 42 was purified for a full characterisation of this intermediate. The p-methoxybenzyl protecting group of 43 was removed with mild acid to give alcohol 44 and then it was phosphorylated, as previously described, to give the phosphoric diester 45. Thereafter, the removal of the silyl protecting group of 45 with 1.0 M tetrabutylammonium fluoride (TBAF) in THF proceeded smoothly to give 46 which, after hydrogenolysis over Pd(OH)$_2$/C, provided the TEA salt 13.

### Biological results

**Substrate analogues**

The ability of the T. brucei GlcNAc-PI de-N-acetylase to recognise and process the synthetic pseudodisaccharides 7–13 was tested in T. brucei cell-free system using an LC-MS/MS.
The N-acetylated analogues of these compounds were prepared as previously described to give compounds 47–53 (Fig. 3). Using LC-MS/MS, multiple reaction monitoring of characteristic transitions for the N-acetylated and corresponding amine form of each compound was used to directly measure the rate of conversion of the N-acetylated compound to the free amine (Table 1). As no suitable transition was identified for the amine form of 13, the enzymatic turnover was accessed by reacting any free amine formed with d$_6$-Ac$_2$O, and measuring the formation of the d$_3$-N-acetylated form by LC-MS/MS.

Over the range of enzyme concentration that gave a linear turnover for α-D-GlcNAc-PI (1) the substrate analogue α-D-GlcNAc-IPC$_{18}$ (3) was de-N-acetylated at 450% the rate of α-D-GlcNAc-PI (1). The increased turnover is most likely due to improved accessibility of the compound, conferred by the single alkyl chain, to the membranes that contain the de-N-acetylas enzyme in the cell-free system. Of the synthetic pseudodisaccharides (47–50) tested, only the α-anomer of the dipalmitoylated compound 49 showed any appreciable turnover at 22% the rate of α-D-GlcNAc-PI (1).

Inhibitors
Since the majority of the compounds were not processed by the T. brucei GlcNAc-PI de-N-acetylas, we tested their ability to inhibit the turnover of the α-D-GlcNAc-IPC$_{18}$ (3) substrate by the T. brucei GlcNAc-PI de-N-acetylas in the LC-MS/MS assay. Most compounds showed no inhibitory activity at 100 μM. However, compounds 47 and 48 showed significant inhibition, with IC$_{50}$ values of 11 ± 4 μM and 37 ± 20 μM, respectively.
indicating that they can be recognised but not processed by the *T. brucei* GlcNAc-PI de-N-acetylase. Interestingly, the potency of 47 and 48 is comparable to that observed with the hydroxamic acid pseudosaccharide analogue 54 (Fig. 4), where the N-acetyl group is replaced with the zinc-chelating hydroxamic acid (IC₅₀ = 19 ± 0.5 μM) and suggests that the zinc-chelating group may not be driving the potency of the latter compound.

The ability of 49 and 48 to act as a substrate and inhibitor, respectively, of the *T. brucei* GPI pathway was confirmed using the trypanosome cell-free system with [3H]-mannose labelling (Fig. 5). Priming the cell-free system with 49 produced three bands corresponding to the addition of 1–3 mannose residues (Fig. 5A), and, consistent with this assignment, the bands were sensitive to jackbean α-mannosidase. As these mannosylated compounds lack the inositol 2-OH group they cannot undergo inositol acylation, a prerequisite for the transfer of ethanolamine, and thus are not processed past the Man3-species.25 Priming the cell-free system with 3 (α- or β-GlcPN-ac-IPC₁₈) at 10 μM was efficiently prevented by incubation with 48 at 100 μM (Fig. 5B), confirming that inhibition of the GlcNAc-PI de-N-acetylase is sufficient to prevent the formation of downstream GPI precursors.

Previous studies (see the introduction for more detail) have shown that both α- and β-GlcPN-ac-PI (1) and β-GlcPN-ac-PI (6) are recognised and processed by the *T. brucei* GlcNAc-PI de-N-acetylase, leading to the hypothesis that one or more of the inositol 2, 3, 4, and 5-OH groups is/are not required. Our data supports this hypothesis with an important caveat; when these hydroxyl groups are removed, substrate recognition and turnover is dependent on both the stereochemistry of the glycosidic linkage and the lipid composition. With the diacylglycerol lipid containing compounds 49 and 50, only the natural α-anomer 49 is both recognised and processed, whereas the β-anomer 50 is neither a substrate nor an inhibitor. However, neither the α nor β-anomer of the octadecyl lipid containing compounds 47 and 48 is processed, although both appear to be recognised and act as inhibitors. These sets of diastereoisomers differ only in the identity of their lipid component, with the more flexible diacylglycerol moiety allowing the glycans to be recognised and processed. Thus, it appears that the requirement for the presence of inositol 2, 3, 4, and 5-OH groups for recognition by the *T. brucei* GlcNAc-PI de-N-acetylase is nuanced and may depend on the conformational flexibility of the substrate analogue.

The inability of the sulphonamide-containing compounds, 51 and 52, to act as substrates or inhibitors confirms the importance of the phosphate group in substrate recognition. It
may be that the presence of the negatively charged phosphate is essential for binding at the enzyme active site.

The inactivity of compound 53 is difficult to interpret. It may be that removal of the inositol 2, 3, 4, and 5-OH groups is not compatible with having a modified glucosamine moiety, or that the entire glucosamine ring is required. Having said this, the glucosamine ‘replacement’ in compound 53 is likely to have a considerable degree of conformational flexibility, which could allow it to take up multiple orientations within the active site.

Conclusions

In summary, we have prepared a series of compounds to probe the substrate specificity and inhibition of enzymes involved at an early stage of GPI biosynthesis. The enzyme of interest to us, GlcNAc-PI deacetylase, proved to be fastidious in its processing of variants of α-N-GlcPN-PI. We conclude that the glucosamine and the phospholipid moieties are essential for binding and that, while the β-myod-inositol residue is the preferred aglycone for recognition by the enzyme, the dispensing of it entirely with a cyclohexanediol group is tolerated but should be used with caution. Further work on this enzyme should focus on using the emerging structure activity relationship data to develop less synthetically complex, cell permeable analogues, which will be valuable chemical tools and may serve as leads for a drug discovery programme.

Experimental

Synthesis general methods

1H, 13C, 31P NMR spectra were recorded on a Bruker AVANCE 500 MHz spectrometer using tetramethylsilane or the residual solvent as the internal standard. High resolution electrospray ionisation mass spectra [HRMS (ESI)] were recorded with a Bruker microToF spectrometer. Melting points were determined on a Reichert hot-plate apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 343 polarimeter. Thin layer chromatography (TLC) was performed on Kieselgel 60 F254 (Merck) plates with various solvent systems as developers, followed by detection under UV light or by charring with sulfuric acid – H2SO4 systems as developers, followed by detection under UV light or.

Conclusions

In summary, we have prepared a series of compounds to probe the substrate specificity and inhibition of enzymes involved at an early stage of GPI biosynthesis. The enzyme of interest to us, GlcNAc-PI deacetylase, proved to be fastidious in its processing of variants of α-N-GlcPN-PI. We conclude that the glucosamine and the phospholipid moieties are essential for binding and that, while the β-myod-inositol residue is the preferred aglycone for recognition by the enzyme, the dispensing of it entirely with a cyclohexanediol group is tolerated but should be used with caution. Further work on this enzyme should focus on using the emerging structure activity relationship data to develop less synthetically complex, cell permeable analogues, which will be valuable chemical tools and may serve as leads for a drug discovery programme.

1R,2R-1-O-(2-Azido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranosyl)-cyclohexanediol 16 and the β-anomer 17

After drying overnight over P2O5 in a vacuum desiccator, the glycosyl donor14 14 (731 mg, 1.54 mmol) and the acceptor 15 (Sigma-Aldrich) were dissolved in 1:1 Et2O–CH2Cl2 (10 mL). To this solution was added activated 4 Å molecular sieves (1 g) and TMSOTf (5.4 μL, 0.03 mmol) at rt under argon. The reaction mixture was stirred at rt overnight, whereafter it was neutralised with TEA, percolated through a short column of silica gel (further elution with EtOAc) and the subsequent eluent was concentrated under reduced pressure. RBC [elution first with PE (40–60°) and then with 3:2 PE (40–60°)–EtOAc] of the residue gave first the α-linked pseudosadcharide 16 (255 mg, 39%) as a waxy solid; [α]D25 +83.7° (c 2.37, CHCl3); δH (500 MHz, CDCl3) 5.42 (dd, 1H, J4',5' = 10.3 Hz, H-3'), 5.09 (d, 1H, J1',2' = 3.7 Hz, H-1'), 4.95 (dd, 1H, J6',7' = 10.3 Hz, H-4'), 4.18 (dd, 1H, J5',6' = 4.8, J6',6'' = 12.2 Hz, H-6'), 4.08 (m, 1H, H-5'), 4.01 (dd, 1H, J5',6'' = 12.2, H-6',6''b = 12.2 Hz, H-6'b), 3.58 (dd, 1H, H-2'), 3.44 (m, 1H, H-2), 3.23 (m, 1H, H-1), 3.05 (s, 1H, 2-0H), 2.50–1.90 (m, 11H, 3 × CH3, H-3a and 6a), 1.64 (m, 2H, H-4a and 5a), 1.35–1.15 (m, 4H, H-3b, 5b and 6b); δC (125 MHz, CDCl3) 170.6–169.8 (3 × COCH3), 99.3 (C-1)', 87.5, 71.7, 68.4 (C-4'), 67.8, 67.5 (C-2'), 62.1 (C-2'), 61.9 (C-6'), 32.0, 31.7, 24.3, 23.8, 20.7 (COCH3); HRMS (ESI) calcd for C18H27N3O9 [M + Na]+ 452.1640, found 452.1622 and then the β-anomer 17 (202 mg, 31%) as a white solid; mp 120–122 °C; [α]D25 +21.5° (c 1.15, CHCl3); δH (500 MHz, CDCl3) 4.91 (m, 2H, H-3' and 4'), 4.43 (d, 1H, J4',5' = 8.1 Hz, H-1'), 4.12 (m, 2H, H6'a and 6'b), 3.67 (m, 1H, H-5'), 3.45 (m, 1H, H-2'), 3.40–3.30 (m, 2H, H-1 and 2), 2.50–1.93 (m, 11H, 3 × CH3 and 2H-cycloil), 1.70–1.60 (m, 2H, cycloil), 1.35 (m, 1H, cycloil), 1.17 (m, 3H, cycloil); δC (125 MHz, CDCl3) 170.6–169.6 (3 × COCH3), 102.1 (C-1'), 88.2, 73.2, 72.1, 71.8 (C-5'), 68.3, 67.3 (C-2'), 61.8 (C-6'), 32.2, 30.9, 24.3, 23.6, 20.7 (COCH3); HRMS (ESI) calcd for C18H23N3O9 [M + H]+ 430.1820, found 430.1831.

Triethylammonium 1R,2R-1-O-(2-Azido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranosyl)-cyclohexanediol 2-(α-octadecylphosphoryl) 19

Each of the compounds 16 (117 mg, 0.27 mmol) and 1815 (237 mg, 0.54 mmol) were dried overnight over P2O5 in a vacuum desiccator, whereafter anhyd pyridine was evaporated therefrom. They were then dissolved in dry pyridine (10 mL), pivaloyl chloride (216 μL, 1.76 mmol) was added and the resulting solution was stirred under argon at rt for 1 h. A freshly prepared solution of iodine (274 mg, 1.08 mmol) in 9:1 pyridine–water was then added and stirring of the reaction mixture was continued for 45 min. After the addition of CH2Cl2 (20 mL), the organic solution was washed successively with 5% aq. NaHSO3 (25 mL), water (25 mL), 1 M TEAB buffer solution (3 × 15 mL), dried (MgSO4) and concentrated under reduced pressure. RBC of the residue (elution first with CH2Cl2) and then with 1:1 CH2Cl2–MeOH afforded the TEA phosphate derivative 19 (140 mg, 60%); [α]D25 +68.6° (c 1.07, CHCl3); δH (500 MHz, CDCl3) 5.40 (dd, 1H, J4',5' = 9.2 Hz, H-3'), 5.27 (d, 1H, J1',2' = 3.7 Hz, H-1'), 4.94 (t, 1H, J5',6' = 9.6 Hz, H-4'), 4.24–4.13 (m, 2H, H6'a and H-1 or 2), 4.07–3.98 (m, 2H, H-5' and 6'b), 3.84–3.76 (m, 3H, OCH3 and H-1 or 2), 3.17 (dd, 1H, J5',6' = 10.6 Hz, H-2'), 2.83 (q, 6H, J = 6.8 Hz, 3 × CH3CH2), 2.05–1.95 (m, 10H, 3 × COCH3 and 1H-cycloil), 1.87 (m, 1H,
Triethylammonium 1R,2R-1-O-[2-azido-2-deoxy-α-D-glucopyranosyl]-cyclohexanediol 2-(n-octadecylphosphoryl) 20

A solution of the TEA salt 20 (52 mg, 0.07 mmol) in 1:1 stabilised THF-MeOH (5 mL) containing 10–20% Pd(OH)2 on carbon (5 mg) was stirred under a hydrogen atmosphere at rt for 1 h. Work-up as described for the derivative 21 gave, after column chromatography (5:1 CH2Cl2-MeOH), the amino compound 7 (33 mg, 77%); [α]D +57.5° (c 3.3, 1:1 CHCl3-MeOH); δH (500 MHz, 1:1 CDCl3-MeOD-d4), 5.42 (d, 1H, J = 3.9 Hz, H-1), 4.05 (m, 1H, H-1 or 2), 3.90–3.65 (6H, OCH2, H-3’, 5’, 6’a, b), 3.58 (3H, 1H, H-1 or 2), 3.32 (m, 3H, H-4’ and MeOD-d4), 3.05 (dd, 1H, J = 10.5 Hz, H-2’), 2.10 (2H, 2H-cyclo), 1.74–1.57 (4H, OCH2CH2 and 2H-cyclo), 1.44–1.21 (34H, CH2), 0.90 (34H, CH3), 0.54 (with heteronuclear decoupling); HRMS (ESI) calcd for C30H61NO9P [M + H]+ 610.4078, found 610.4050.

1R,2R-1-O-[2-Azido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranosyl]-2-O-(tert-butyldimethylsilyl)-cyclohexanediol 22

The alcohol 16 (284 mg, 0.66 mmol) was dried overnight in a desiccator over P2O5 under high vacuum and then dissolved in anhyd. CH2Cl2 (10 mL). To this solution, at room temperature, was added 2,6-lutidine (154 μL, 1.32 mmol) and tert-butyldimethylsilyl trifluoromethane sulfonate (228 μL, 0.99 mmol). After 30 min, CH2Cl2 (25 mL) and brine (25 mL) were added and the organic layer separated. The aqueous layer was re-extracted with CH2Cl2 (25 mL) and the combined organic layers were washed with brine (2 × 25 mL), dried (MgSO4) and concentrated under reduced pressure. RBC [elution first with PE (40–60°) and then with 1:1 PE(40–60°)-Et2O] of the residue gave the azide 22 (284 mg, 79%) as an oil; [α]D′′ +90.2° (c 1.52, CHCl3); δH (500 MHz, CDCl3), 5.40 (t, 1H, J = 9.7 Hz, H-3), 5.35 (d, 1H, J = 3.3 Hz, H-1), 5.20 (t, 1H, J = 9.7 Hz, H-4), 4.26 (dd, 1H, J = 4.7 Hz, J = 12.1 Hz, 6’a, b), 4.10 (2H, H-5’ and 6’b), 3.75 (m, 1H, H-1 or 2), 3.57 (3H, 1H, H-1 or 2), 3.17 (dd, 1H, H-2’), 2.10–0.03 (3 × s, 9H, 3 × COCH3), 1.89 (m, 2H-cyclo), 1.70–1.25 (6H-cyclo), 0.89 (s, 9H, 3 × CH2), 0.12–0.08 (2 × s, 6H, 2 × CH3); δC (125 MHz, CDCl3), 170.6–169.7 (3 × COCH3), 97.9 (C-1’), 79.7 (C-1 or 2), 72.6 (C-1 or 2), 70.4 (C-3’), 67.4 (C-5’), 62.1 (C-6’), 61.0 (C-2’), 32.5, 29.8, 25.9, 22.1, 20.7 (COCH3), 20.6 (COCH3), 18.0, -4.1, -4.9; HRMS (ESI) calcd for C42H64N5O15Si [M + H]+ 544.2685, found 544.2698.
1R,2R-1-O-(2-Azido-2-deoxy-α-D-glucopyranosyl)-2-O-(tert-butyl dimethylsilyl)-cyclohexanediol 23

To a solution of the triacetate 22 (185 mg, 0.34 mmol) in 1:1 CH₂Cl₂-MeOH (92 mL) was added 5.4 M NaOMe in MeOH (230 μL). The mixture was kept for 30 min at rt and was then neutralised with Amberlite IR-120 (H⁺) ion-exchange resin, filtered and the filtrate concentrated under reduced pressure. The residue, so obtained, was percolated through a short silica-gel column (further elution with EtOAc) and the eluent was concentrated under reduced pressure to afford the triol 23 (136 mg, 96%) as a white solid, mp 122–123 °C (from 10:1 hexane–Et₂O; [α]D⁰ 75 +65.6° (c 1.09, CHCl₃); δH (500 MHz, CDCl₃) 7.35–7.09 (15H, 3 × Ph), 4.98–4.44 (7H, H-1' and 3 × CH₂Ar), 3.87 (m, 1H, H-3'), 3.57 (dd, 1H, J₀₋₁ = 9.2 Hz, H-2'), 3.45 (m, 1H, H-1 or 2), 2.77 (dd, 1H, J₀₋₁ = 3.7, J₂₋₁ = 9.7 Hz, H-2'), 1.98 (m, 1H, cyclitol), 1.70 (m, 1H, cyclitol), 1.63–1.16 (6H, cyclitol), 0.84 (s, 9H, 3 × CH₃), 0.00 (2 × s, 6H, 2 × CH₃; δC (125 MHz, CDCl₃) 138.7–138.0 (Ph), 128.5–127.7 (Ph), 100.2 (C-1'), 84.1 (C-3'), 79.9 (C-1 or 2), 78.9, 75.6, 74.9, 73.5, 71.4 (C-5'), 71.0, 68.7 (C-6'), 56.4 (C-2'), 31.9, 29.4, 25.9, 22.4, 21.8, 18.0, 4.3; −4.4; HRMS (ESI) calced for C₁₉H₃₆NO₆Si [M + H⁺] 662.3874, found 662.3874.

1R,2R-1-O-(2-Azido-3,4,6-tri-O-benzyl-2-deoxy-α-D-glucopyranosyl)-2-O-(tert-butyl dimethylsilyl)-cyclohexanediol 26

The amine 25 (147 mg, 0.22 mmol) was dissolved in EtOAc (10 mL) at rt. Di-tert-butylcarbonate (58 mg, 0.26 mmol) was then added and the mixture was stirred overnight at rt. Afterwards, the reaction mixture was diluted with EtOAc (25 mL) and then washed successively with water (25 mL), brine (25 mL), dried (Na₂SO₄) and concentrated under reduced pressure. RBC [eluion gradient hexane → 7 : 1 → 5 : 1 hexane–EtOAc] of the residue afforded the Boc protected derivative 26 (132 mg, 79%); [α]D²⁰ +39.0° (c 1.06, CHCl₃); δH (500 MHz, CDCl₃) 7.30–7.05 (15H, 3 × Ph), 4.95 (d, 1H, J₀₋₁ = 3.3 Hz, H-1'), 4.76–4.38 (7H, NH and 3 × CH₂Ar), 3.90 (m, 1H, H-2') 3.83 (m, 1H, H-5'), 3.69 (dd, 1H, J₀₋₁ = 4.1, J₂₋₁ = 10.7 Hz, H-6'a), 3.61 (m, 3H, H-3', 4', and 6'b), 3.48 (m, 1H, H-1 or 2), 2.01 (m, 1H, cyclitol), 1.74 (m, 1H, cyclitol), 1.52 (m, 2H, cyclitol), 1.35 (s, 9H, 3 × BocCH₃), 1.30–1.10 (4H, cyclitol), 0.83 (s, 9H, 3 × CH₃), 0.00 (2 × s, 6H, 2 × CH₃; δC (125 MHz, CDCl₃) 155.3 (C=O), 138.6–138.2 (Ph), 128.4–127.5 (Ph), 99.1 (C-1'), 81.5, 81.3 (C-1 or 2), 79.5, 78.5, 75.3, 75.1, 73.4, 73.2 (C-1 or 2), 71.4 (C-5'), 68.8 (C-6'), 54.6 (C-2'), 33.4, 30.8, 28.5, 26.1, 23.3, 22.9, 18.1, 3.9, −4.3; HRMS (ESI) calced for C₃₉H₅₆NO₆Si [M + H⁺] 762.4396, found 762.4393.

1R,2R-1-O-[2-N-(t-Butoxycarbonyl)amino-3,4,6-tri-O-benzyl-2-deoxy-α-D-glucopyranosyl]-2-O-(tert-butyl dimethylsilyl)-cyclohexanediol 27

To a stirred solution of the silyl derivative 26 (114 mg, 0.15 mmol) in THF (10 mL) at 0 °C was added ~70% HF-pyridine (90 μL). The solution was stirred overnight at rt whereafter a further aliquot of ~70% HF-pyridine (90 μL) was added and the solution was left to stir overnight; this process was continued on day 3. On day 4, TLC revealed the complete disappearance of the starting material, whereafter said NaHCO₃ (1 mL) was added dropwise to quench the reaction and the resulting solution was poured into brine (25 mL) and extracted with EtOAc (3 × 25 mL). The EtOAc extracts were combined and
washed with brine (25 mL), dried (MgSO₄) and concentrated under reduced pressure. RBC [elution gradient hexane → 2:1 hexane–EtOAc] of the residue afforded the alcohol 27 (97 mg, 49%); [α]D²⁰ +27.2° (c 1.08, CHCl₃); δH (500 MHz, CDCl₃) 7.36–7.10 (15H, 3 × Ph), 5.12 (dd, 1H, J₂⁻,₂⁺ = 3.6 Hz, H-1′), 4.86–4.44 (7H, NH and 3 × CH₂Ar), 3.95 (dd, 1H, J₁⁻,₁⁺ = 9.7 Hz, H-4′), 3.86 (m, 1H, H-2′), 3.75–3.61 (4H, H-3′, 5′ and 6′a,b), 3.47 (m, 1H, H-1 or 2), 3.32 (m, 1H, H-1 or 2), 2.10–1.91 (2H, cyclitol), 1.65 (m, 2H, cyclitol), 1.43 (s, 9H, 3 × CH₃), 1.33–1.16 (4H, cyclitol); δC (125 MHz, CDCl₃) 155.6 (C = O), 138.4–138.0 (Ph), 128.4–127.7 (Ph), 99.5 (C-1′), 85.0 (C-1 or 2), 80.5, 79.7, 78.5, 75.0, 74.0 (C-1 or 2), 73.4, 71.2 (C-4′), 68.7 (C-6′), 54.9 (C-2′), 32.9, 31.7, 28.4, 24.3, 23.9; HRMS (ESI) calc'd for C₃₀H₆₀NO₇ [M + H⁺] + 648.3531, found 648.3527.

Triethylammonium 1R,2R-1-O-[2-N-(tert-butoxycarbonyl)-amino-3,4,6-tri-O-benzyl-2-deoxy-α-D-glucopyranosyl]-cyclohexanediol 2-(1,2-di-O-hexadecanoyl-sn-glycerol) 3-phosphate 29

This compound was obtained from the alcohol 27 (55.7 mg, 0.086 mmol) and 1,2-di-O-hexadecanoyl-sn-glycerol 3-hydrogenophosphate TEA salt 28³⁰ (126 mg, 0.17 mmol) in the presence of pivaloyl chloride (69 µL, 0.56 mmol) essentially as described for the 2-(n-octadecyl phosphate) 19. After the oxidation with iodine (87 mg, 0.34 mmol) in 9:1 pyridine–water followed by the same aqueous workup as described for 19, RBC (elution first with CH₂Cl₂ and then with 20:1 → 15:1 CH₂Cl₂–MeOH) afforded the TEA phosphate derivative 29 (57 mg, 52%) as an opaque oil; [α]D²⁰ +27.7° (c 1.08, CHCl₃); δH (500 MHz, CDCl₃) 12.5 (brs, 1H, NH TEA salt), 7.36–7.10 (15H, 3 × Ph), 5.24 (m, 1H, H-2-glycerol), 4.96 (s, 1H, H-1′), 4.84–4.44 (6H, 3 × CH₂Ar), 4.39 (m, 1H, 1- or 3-CH₃-glycerol), 4.17 (dd, 1H, J₁⁻,₁⁺ = 6.6, J₁⁻,₂⁺ = 12.0 Hz, 1- or 3-CH₃-glycerol), 4.13–3.97 (m, 4H, H-2′, 1 or 2 cyclitol and 1- or 3-CH₂-glycerol), 3.95 (m, 1H, H-4′), 3.85 (t, J₁⁻,₁⁺ = J₂⁻,₂⁺ = 9.9 Hz, H-3′), 3.74 (dd, 1H, J₂⁻,₃⁻,₂⁺ = 4.1, J₂⁻,₉⁻,₉⁺ = 10.7 Hz, H-6′a), 3.66 (m, 2H, H-5′ and 6′b), 3.50 (m, 1H, H-1 or 2), 2.97 (q, 6H, J = 7.1 Hz, 3 × CH₃CH₂), 2.27 (m, 4H, 2 × COCH₂Ph), 2.21–1.95 (2H, cyclitol), 1.72–1.50 (m, 6H, 2 × COCH₂CH₂ and 2H cyclitol), 1.44 (s, 9H, 3 × CH₃), 1.33–1.18 (6H, 3 × CH₂CH₂), 2 × CH₂[12] and 4H cyclitol, 0.88 (t, 6H, J = 7.3 Hz, 2 × CH₂CH₃); δC (125 MHz, CDCl₃) 172.4 (C = O), 171.9 (C = O), 155.4 (C = O), 138.9–137.4 (Ph), 127.3–126.3 (Ph), 98.9 (C-1), 80.0, 76.8, 73.7, 72.7, 72.3, 70.3, 69.8, 69.5, 68.1, 67.6, 62.4, 61.8, 61.4, 53.5, 44.2 [N(CH₂CH₃)]₃, 33.3, 33.1, 30.9, 30.2, 28.7–28.1, 27.5, 23.4, 21.7, 13.1, 7.40 [N(CH₂CH₃)]₂; δ (202 MHz, CDCl₃) 0.04 (with heteronuclear decoupling); HRMS (ESI) calc'd for C₃₂H₇₁NO₁₃P [M – NET₃ – H⁺] − 1276.8010, found 1276.8015.

1R,2R-1-O-[2-N-(tert-Butoxycarbonyl)-amino-2-deoxy-α-D-glucopyranosyl]-cyclohexanediol 2-(1,2-di-O-hexadecanoyl-sn-glycerol) 3-phosphate 30

A solution of the benzylated compound 29 (57 mg, 0.041 mmol) in 1:1 THF–n-propanol (10 mL) containing 10–20% Pd(OH)₂ on carbon (15 mg) was stirred under 3 atm of hydrogen for 3 h before it was percolated through a short column of Chelex 100 on a bed of Celite (further elution with 1:1 THF–n-propanol). The eluent was concentrated under reduced pressure and the ensuing residue was purified by column chromatography [elution gradient 7:1 → 4:1 CH₂Cl₂–MeOH] to give the Boc protected derivative 30 (30 mg, 73%); [α]D²⁰ +39.1° (c 3.00, 1:1 CH₂Cl₂–MeOH); δH (500 MHz, 1:1 CDCl₃–MeOH-d₄) 5.25 (m, 1H, H-2 glycerol), 4.90 (s, 1H, H-1′), 4.45 (m, 1H, 1- or 3-CH₃-glycerol), 4.20 (dd, 1H, J = 6.7, J = 11.7 Hz, 1- or 3-CH₂-glycerol), 4.00 (m, 2H, 1- or 3-CH₃-glycerol), 3.75 (m, 1H, H-3′ and 4′), 3.65 (m, 1H, H-1 or 2), 3.55 (dd, 1H, J₁⁻,₂⁺ = 3.0, J₂⁻,₃⁻,₂⁺ = 10.5 Hz, H-2′), 3.47 (m, 1H, H-1 or 2), 3.40 (m, 3H, H-5′ and 6′a,b), 2.40–2.00 (m, 6H, 2 × COCH₂ and 2H cyclitol), 1.74–1.57 (m, 6H, 2 × COCH₂CH₂ and 2H cyclitol), 1.47 (s, 9H, 3 × CH₃), 1.40–1.20 (52H, 2 × [CH₂]₁₂ and 4H cyclitol), 0.89 (t, 6H, J = 7.1 Hz, 2 × CH₂CH₃); δC (125 MHz, 1:1 CDCl₃–MeOH-d₄) 174.6 (C = O), 174.1 (C = O), 158.2 (C = O), 98.9 (C-1′), 82.0, 78.0, 73.3, 73.0, 72.0, 71.1, 64.0, 63.1, 62.0, 57.1, 56.4, 34.7, 34.5, 33.0–29.5, 28.5, 25.4, 24.3, 23.5, 21.1, 14.3; δ (202 MHz, 1:1 CDCl₃–MeOH- d₄) 0.28 (with heteronuclear decoupling); HRMS (ESI) calc'd for C₃₅H₇₅NO₁₅P [M – H⁻] + 1006.6601, found 1006.6635.

N'-[1R,2R]-2-(Benzyloxy)cyclohexyl)octadecane-1-sulphonamide 32

To a solution of CH₂Cl₂ (10 mL) and triethylamine (2.1 mL) under argon was added (1R, 2R)-1-amino-2-benzylxycyclohexane 31 (1.0 g, 4.87 mmol) and 1-octadecanoylsulfonil...
NH₂(αOH)₂ on carbon (50 mg) was stirred under a slight over pressure of hydrogen at room temperature for 2 h before it was filtered through a bed of Celite. The catalyst was further washed with 1:1 THF-MeOH (2 × 10 mL) and the washings were combined and concentrated under reduced pressure. Column chromatography (9:1 CH₂Cl₂-MeOH) gave first the β anomer 11 (9.8 mg, 14%) as a waxy solid; [α]D²⁵ +3.7 (c 0.98, MeOH); δH (500 MHz, MeOH-d₄) 4.44 (d, 1H, J₁,₂ = 8.1 Hz, H₁'), 3.94 (dd, 1H, J₁,₅a = 2.1, J₅a,c = 11.7 Hz, H₆a'); 3.65-3.50 (m, 2H, H₁ or 2 and 6'b), 3.34 (m, 2H, H₃' and 5'), 3.22 (q, 1H, J₁,₅a = 9.2 Hz, H₄'), 3.09 (m, 3H, H₁ or 2 and SO₂CH₃); 2.63 (t, 1H, J₁,₂ = 9.0 Hz, H₂'), 2.13 (m, 2H, cyclohexyl), 1.90-1.20 (38H, SO₂CH₂CH₂, CH₂₃', and 6H cyclohexyl), 0.89 (t, 1H, J = 7.0 Hz, CH₃CH₂₃'); δC (125 MHz, MeOH-d₄) 100.7 (C1'), 80.5 (C1 or 2'), 78.5 (C₃' or 5'), 72.2 (C₄'), 62.9 (C₆'), 58.1 (C1 or 2 or 5'), 54.1 (SO₂CH₃), 35.4, 32.2, 30.8, 30.6, 30.5, 25.4, 25.0, 14.5 (CH₃CH₂); HRMS (ESI) calcd for C₄₃H₇₅N₂O₇S [M + H]⁺ 817.4732, found 817.4709. Continued elution gave the α anomer 12 (14.5 mg, 20%) as an oil; [α]D²⁵ +66.5 (c 1.45, MeOH); δH (500 MHz, MeOH-d₄) 5.28 (d, 1H, J₁,₂ = 3.7 Hz, H₁'), 3.81 (m, 1H, H₆a'), 3.71 (m, 3H, H₃', 5' and 6'b), 3.42 (m, 1H, H₁ or 2), 3.34 (m, 1H, H₄'), 3.23 (m, H₁ or 2), 3.08 (m, 1H, H₂' and SO₂CH₃), 2.24 (m, 1H, cyclohexyl), 1.96 (m, 1H, cyclohexyl), 1.84-1.67 (46H, SO₂CH₂CH₂ and 2H cyclohexyl), 1.52-1.21 (34H, CH₂₃'; and 4H cyclohexyl), 0.90 (t, 3H, J = 7.0 Hz, CH₃CH₂₃'); δC (125 MHz, MeOH-d₄) 97.3 (C1'), 81.4 (C1 or 2'), 73.2 (C₃' or 5'), 70.4 (C₃' or 4' or 5'), 70.3 (C₃' or 4' or 5'), 60.8 (C₆'), 56.6 (C1 or 2'), 54.9 (C₂'), 52.6 (SO₂CH₃), 32.7, 32.3, 31.6, 29.4, 29.3, 29.2, 29.0, 28.8, 27.8, 24.2, 23.5, 23.3, 22.3, 13.0 (CH₃CH₂); HRMS (ESI) calcd for C₄₃H₇₅N₂O₇S [M + H]⁺ 893.4194, found 893.4178.

N-(1R,2R)-2-O-[2-Azido-3,4,6-tri-O-benzyl-2-deoxy-d-glucopyranosyl]-cyclohexyloctadecane-1-sulphonamide 35

A solution of the anomeric mixture 35 (108 mg, 0.12 mmol) in 1:1 THF-MeOH (6 mL) containing 10-20% Pd(OH)₂ on carbon (25 mg) was stirred under a slight over pressure of hydrogen at room temperature for 24 h before it was filtered through a bed of Celite. The catalyst was further washed with 1:1 THF-MeOH (2 × 10 mL) and the washings were combined and concentrated under reduced pressure. Column chromatography (9:1 CH₂Cl₂-MeOH) gave first the β anomer 11 (9.8 mg, 14%) as a waxy solid; [α]D²⁵ +3.7 (c 0.98, MeOH); δH (500 MHz, MeOH-d₄) 4.44 (d, 1H, J₁,₂ = 8.1 Hz, H₁'), 3.94 (dd, 1H, J₁,₅a = 2.1, J₅a,c = 11.7 Hz, H₆a'); 3.65-3.50 (m, 2H, H₁ or 2 and 6'b), 3.34 (m, 2H, H₃' and 5'), 3.22 (q, 1H, J₁,₅a = 9.2 Hz, H₄'), 3.09 (m, 3H, H₁ or 2 and SO₂CH₃); 2.63 (t, 1H, J₁,₂ = 9.0 Hz, H₂'), 2.13 (m, 2H, cyclohexyl), 1.90-1.20 (38H, SO₂CH₂CH₂, CH₂₃', and 6H cyclohexyl), 0.89 (t, 1H, J = 7.0 Hz, CH₃CH₂₃'); δC (125 MHz, MeOH-d₄) 100.7 (C1'), 80.5 (C1 or 2'), 78.5 (C₃' or 5'), 72.2 (C₄'), 62.9 (C₆'), 58.1 (C1 or 2 or 5'), 54.1 (SO₂CH₃), 35.4, 32.2, 30.8, 30.6, 30.5, 25.4, 25.0, 14.5 (CH₃CH₂); HRMS (ESI) calcd for C₄₃H₇₅N₂O₇S [M + H]⁺ 817.4732, found 817.4709. Continued elution gave the α anomer 12 (14.5 mg, 20%) as an oil; [α]D²⁵ +66.5 (c 1.45, MeOH); δH (500 MHz, MeOH-d₄) 5.28 (d, 1H, J₁,₂ = 3.7 Hz, H₁'), 3.81 (m, 1H, H₆a'), 3.71 (m, 3H, H₃', 5' and 6'b), 3.42 (m, 1H, H₁ or 2), 3.34 (m, 1H, H₄'), 3.23 (m, H₁ or 2), 3.08 (m, 1H, H₂' and SO₂CH₃), 2.24 (m, 1H, cyclohexyl), 1.96 (m, 1H, cyclohexyl), 1.84-1.67 (46H, SO₂CH₂CH₂ and 2H cyclohexyl), 1.52-1.21 (34H, CH₂₃'; and 4H cyclohexyl), 0.90 (t, 3H, J = 7.0 Hz, CH₃CH₂₃'); δC (125 MHz, MeOH-d₄) 97.3 (C1'), 81.4 (C1 or 2'), 73.2 (C₃' or 5'), 70.4 (C₃' or 4' or 5'), 70.3 (C₃' or 4' or 5'), 60.8 (C₆'), 56.6 (C1 or 2'), 54.9 (C₂'), 52.6 (SO₂CH₃), 32.7, 32.3, 31.6, 29.4, 29.3, 29.2, 29.0, 28.8, 27.8, 24.2, 23.5, 23.3, 22.3, 13.0 (CH₃CH₂); HRMS (ESI) calcd for C₄₃H₇₅N₂O₇S [M + H]⁺ 893.4194, found 893.4178.

trans-4-(Methoxybenzyl)oxycyclohexyl acetate 37

Cu(BF₄)₂·nH₂O (42 mg, 0.18 mmol) was dissolved in CH₂Cl₂ (20 mL) and cyclohexene oxide 36 (1.8 mL, 17.8 mmol) and 4-methoxybenzyl alcohol (10 mL, 80.2 mmol) were added. The reaction mixture was stirred for 24 h, diluted with water (20 mL), and the aqueous layer extracted with CH₂Cl₂ (3 × 30 mL). The combined organic extracts were washed with brine, dried over MgSO₄, filtered and the solvent was removed.
The reaction mixture was stirred overnight at rt, diluted with water (100 mL) and extracted with EtOAc (3 × 100 mL). The combined organic extracts were washed with water (100 mL), brine (100 mL), dried over MgSO₄, filtered and the solvent was removed under reduced pressure. The residue was passed down a short plug of silica gel (further elution with EtOAc) and evaporated to dryness under reduced pressure. RBC (elution gradient 1:1 → 2:1 EtOAc–hexane) furnished the epoxide 39 (1.50 g, 64%) as a clear oil; δH (500 MHz, CDCl₃) 7.20 (dd, 2H, J = 8.7 Hz, Ph), 6.77 (dd, 2H, J = 8.7 Hz, Ph), 4.50 (s, 2H, CH₂Ar) 3.76–3.40 (5H, OCH₃ and 1- or 3-CH₂ propyl), 3.25 (m, 2H, H-1 and 2), 3.05 (m, 1H, H-2 propyl), 2.67–2.52 (2H, 1- or 3-CH₂ propyl), 1.90 (m, 2H, cyclitol), 1.57 (m, 2H, cyclitol), 1.22–1.06 (m, 4H, cyclitol); δC (125 MHz, CDCl₃) 159.0, 131.4, 131.3, 129.14, 129.08, 113.7, 82.3 (C-1 or 2), 82.1 (C-1 or 2), 80.8 (C-1 or 2), 71.5 (CH₂Ar), 71.2 (1- or 3-CH₂ propyl), 70.4 (1- or 3-CH₂ propyl), 55.2 (OCH₃), 51.3 (C-2 propyl), 44.4 (1- or 3-CH₂ propyl), 44.38 (1- or 3-CH₂ propyl), 30.3, 30.20, 30.16, 23.6, 23.5; HRMS (ESI) calcd for C₁₇H₂₆NaO₅ [M + Na]+ 315.1567, found 315.1553.

3-[trans-2-(4-Methoxybenzyl)oxy]cyclohexyl]oxy)propane-1,2-diol 40

To a solution of 39 (1.50 g, 5.13 mmol) in DMSO (56.4 mL) was added water (10.8 mL) and aq. 0.3 M KOH (2.4 mL). The reaction mixture was heated to 100 °C for 18 h, and then diluted with water (200 mL) followed by extraction with CH₂Cl₂ (3 × 200 mL). The combined organic extracts were washed with water (100 mL), brine (100 mL), dried over MgSO₄, filtered and the solvent was removed under reduced pressure. The residue, so obtained, was percolated through a short column of silica gel (further elution with EtOAc) and the subsequent eluent was concentrated under reduced pressure. RBC (elution first with hexane → 6:1 EtOAc–hexane) of the residue afforded the diol 40 (942 mg, 65) as a clear oil; δH (500 MHz, CDCl₃) 7.28 (d, 2H, J = 8.6 Hz, Ph), 6.88 (dd, 2H, CH, J = 8.6, Ph), 4.59–4.50 (2H, CH₂Ar), 3.82–3.47 (8H, OCH₃, H-2 propyl, 1- and 3-CH₂ propyl), 3.25 (m, 2H, H-1 and 2), 2.50 (bs, 1H, OH), 2.40 (bs, 1H, OH), 2.11–2.01 (m, 2H, cyclitol), 1.68 (m, 2H, cyclitol), 1.23 (m, 4H, cyclitol); δC (125 MHz, CDCl₃) 159.3, 159.2, 130.5, 130.4, 129.5, 129.4, 113.84, 113.83, 83.40 (C-1 or 2), 82.39 (CH, C-1 or 2), 80.86 (C-1 or 2), 80.78 (C-1 or 2), 72.31 (1- or 3-CH₂ propyl), 71.37 (C-2 propyl), 71.03 (1- or 3-CH₂ propyl), 70.81 (1- or 3-CH₂ propyl), 70.51 (C-2 propyl), 64.20 (1- or 3-CH₂ propyl), 63.83 (1- or 3-CH₂ propyl), 55.24 (OCH₃), 30.75, 30.54, 29.99, 29.93, 23.78, 23.71, 23.69; HRMS (ESI) calcd for C₁₇H₂₆NaO₅ [M + Na]+ 333.1672, found 333.1678.
1-[(trans-Butyldiphenylsilyl)oxy]-3-[(trans-2-[(4-methoxybenzyl)-oxycyclohexyl]oxy)propan-2-ol 41

To a solution of the primary alcohol 40 (942 mg, 3.34 mmol) and DIPA (5.8 mL, 3.75 mmol) in CH2Cl2 (5 mL) was added TBDPSCl (1.04 mL, 4.00 mmol) dropwise followed by DMAP (5 mg, 0.038 mmol) and the reaction stirred for 24 h at rt. Afterwards, TLC revealed the presence of the starting material 40; thus an additional aliquot of TBDPSCl (0.521 mL, 2.00 mmol) was added and the reaction mixture was stirred for a further 3 h, whereafter it was quenched with water (60 mL) and then extracted with CH2Cl2 (3 × 60 mL). The combined organic extracts were washed successively with water (100 mL), brine (50 mL), filtered through cotton wool and the solvent was removed under reduced pressure. The residue so obtained was percolated through a short column of silica gel (further elution with EtOAc) and the subsequent eluent was concentrated under reduced pressure. RBC [elution first with hexane → 1:1 EtOAc–hexane] of the residue afforded the silyl protected product 41 (1.33 g, 76%) as a pale yellow oil; δH (500 MHz, CDCl3) 7.68-6.79 (14H, Ph), 4.56-4.44 (m, 2H, CH2Ar), 3.90-3.51 (8H, OCH3, 2- propyl, 1- and 3-CH2 propyl), 3.25 (m, 2H, H-1 and 2), 3.12 (bs, 1H, OH), 1.97 (m, 2H, cyclitol), 1.66 (m, 2H, cyclitol), 1.30-1.15 (m, 4H, cyclitol), 1.05 (s, 9H, 3 × CH3); δC (125 MHz, CDCl3) 159.1, 135.6, 135.6, 134.7, 133.5, 133.49, 133.42, 130.9, 130.8, 129.73, 129.72, 129.3, 129.2, 127.74, 127.71, 113.79, 113.77, 82.9 (C-1 or 2), 82.3 (C-1 or 2), 80.7 (C-1 or 2), 80.6 (C-1 or 2), 71.7, 71.6, 71.2, 71.1, 71.0, 70.4, 65.0, 64.8, 55.3, 55.2, 30.6, 30.4, 30.11, 30.06, 29.7, 26.9, 23.69, 23.66, 19.29, 19.28; HRMS (ESI) calcd for C33H43N3NaO4Si [M + Na]+ 571.2856, found 571.2860.

2-Azido-3-[[trans-2-[(4-methoxybenzyl)oxycyclohexyl]oxy]propoxy] (trans-butyl)diphenylsiline 43

A solution of the mesylate 42 in DMF (10 mL) containing sodium azide (496 mg, 7.64 mmol) was heated and stirred at 125 °C for 24 h, cooled and then poured into water (40 mL). The resulting aqueous solution was extracted with CH2Cl2 (5 × 40 mL) and the combined organic extracts were washed successively with water (100 mL), brine (100 mL), filtered through cotton wool and concentrated under reduced pressure. A solution of the residue in EtOAc was percolated through a short column of silica gel (elution with EtOAc) and the eluent concentrated under reduced pressure. RBC of the residue [elution first with hexane → 1:1 Et2O–hexane] gave the azide 43 (1.30 g, 51%) as a clear oil; δH (500 MHz, CDCl3) 7.62-6.73 (14H, Ph), 4.44 (s, 2H, CH2Ar), 3.71-3.32 (8H, OCH3, 2-propyl, 1- and 3-CH2 propyl), 3.22 (m, 2H, H-1 and 2), 1.86 (m, 2H, cyclitol), 1.55 (m, 2H, cyclitol), 1.25-1.04 (m, 4H, cyclitol), 0.99 (s, 9H, 3 × CH3); δC (125 MHz, CDCl3) 159.0, 135.9, 135.8, 135.6, 135.3, 134.8, 133.10, 133.06, 131.31, 131.29, 129.8, 129.7, 129.2, 129.12, 129.08, 129.0, 127.8, 127.73, 127.67, 113.74, 113.70, 82.2 (C-1 or 2), 82.0 (C-1 or 2), 80.5 (C-1 or 2), 80.4 (C-1 or 2), 71.5, 71.4, 71.19, 69.5, 69.0, 64.08, 64.05, 63.2, 63.0, 55.3 (OCH3), 30.0, 29.9, 26.9, 26.8, 26.6, 23.43, 23.40, 23.35, 19.2; HRMS (ESI) calcd for C33H42N3NaO4Si [M + Na]+ 596.2915, found 596.2926.
Triethylammonium trans-2-[2-azido-3-[(tert-butylphenylsilyl)-oxy]propoxy]cyclohexyl n-octadeyl phosphate 45

This compound was obtained from the alcohol 44 (280 mg, 0.62 mmol) and the hydrogenphosphonate TEA salt 18\textsuperscript{15} (537 mg, 1.23 mmol) in the presence of pivaloyl chloride (0.48 mL, 3.86 mmol) essentially as described for the TEA salt 19. After oxidation with iodine (623 mg, 2.47 mmol) in 9:1 pyridine–water followed by the same aqueous workup as described for 19, column chromatography (CH\textsubscript{2}Cl\textsubscript{2} → 8:1 CH\textsubscript{2}Cl\textsubscript{2}-MeOH) of the residue afforded the octadeyl phosphate TEA salt 45 (276 mg, 50%) as a yellow paste; \(\delta\text{H} (500 \text{ MHz, CDCl}_3) 7.63–7.30 (10H, Ph), 4.10 (m, 1H, H-1 or 2), 3.92–3.33 (7H, OCH\textsubscript{2}), 1.73 (s, 9H, 3 × CH\textsubscript{3} propyl), 0.80 (t, 3H, CH\textsubscript{3}, cyclitol), 1.95–1.73 (m, 2H, cyclitol), 1.58–1.46 (m, 4H, OCH\textsubscript{2}CH\textsubscript{2} and 2H cyclitol), 1.35–1.14 (43H, [CH\textsubscript{2}]\textsubscript{15}, 3 × CH\textsubscript{2}CH\textsubscript{3} and 4H cyclitol), 1.73 (s, 9H, 3 × CH\textsubscript{3}), 0.80 (t, 3H, CH\textsubscript{3}, J = 6.8 Hz, CH\textsubscript{2}CH\textsubscript{3}); \(\delta\text{C} (125 \text{ MHz, CDCl}_3) 134.9, 134.8, 134.6, 132.6, 132.4, 132.1, 132.0, 128.9, 128.8, 126.8, 126.7, 78.8 (C-1 or 2), 78.6 (C-1 or 2), 77.6 (C-1 or 2), 70.8, 70.1, 68.2, 67.7, 65.7, 65.6, 65.5, 63.0, 62.9, 44.5 [N(CH\textsubscript{2}CH\textsubscript{2})\textsubscript{2}], 30.9, 29.7, 29.6, 29.5, 29.0, 28.70, 28.65, 28.4, 27.2, 26.0, 25.9, 25.7, 24.8, 24.7, 21.7, 21.1, 21.0, 18.2, 14.3 (CH\textsubscript{2}CH\textsubscript{3}), 8.5 [N(CH\textsubscript{2}CH\textsubscript{2})\textsubscript{2}]; \(\delta\text{P} (202 \text{ MHz, CDCl}_3) −1.22 \text{(with heteronuclear decoupling); HRMS (ESI) calcd for C}_{143}\text{H}_{325}\text{N}_4\text{O}_6\text{P} [M − \text{NET}_3 − H^+] = 784.4885, found 784.4759.}

Triethylammonium trans-2-(2-azido-3-hydroxypropoxy)-cyclohexyl n-octadeyl phosphate 46

Compound 45 (68 mg, 0.077 mmol) was dissolved in THF (1 mL) and 1.0 M TBAF in THF (153 µL, 0.15 mmol) was added. The reaction mixture was stirred at rt for 16 h and then diluted with water (25 mL), extracted CH\textsubscript{2}Cl\textsubscript{2} (3 × 25 mL) and the combined organic extracts were washed withaq. 1.0 M TEAB (2 × 10 mL). The organic phase was filtered through cotton wool, the solvent was removed under reduced pressure and the resulting residue was purified by column chromatography (8:1 CH\textsubscript{2}Cl\textsubscript{2}-MeOH) to afford the alcohol 46 (50 mg, 100%) as a white paste; \(\delta\text{H} (500 \text{ MHz, CDCl}_3) 4.00–3.31 (8H, OCH\textsubscript{2}H\textsubscript{2}-1 or 2, H-2 propyl, 1- and 3-CH\textsubscript{2} propyl), 3.10 (m, 1H, H-1 or 2), 3.02 (q, 6H, J = 7.4 Hz, 3 × CH\textsubscript{2}CH\textsubscript{3}), 2.04–1.93 (m, 2H, cyclitol), 1.63–1.50 (m, 4H, OCH\textsubscript{2}CH\textsubscript{2} and 2H cyclitol), 1.33–1.05 (43H, [CH\textsubscript{2}]\textsubscript{15}, 3 × CH\textsubscript{2}CH\textsubscript{3} and 4H cyclitol), 0.81 (t, 3H, CH\textsubscript{3}, J = 6.7 Hz, CH\textsubscript{2}CH\textsubscript{3}); \(\delta\text{C} (125 \text{ MHz, CDCl}_3) 81.6 (C-1 or 2), 78.4 (C-1 or 2), 77.9 (C-1 or 2), 67.1, 65.9, 64.8, 53.2, 44.5 [N(CH\textsubscript{2}CH\textsubscript{2})\textsubscript{2}], 33.0, 31.8, 30.9, 29.7, 29.6, 29.3, 29.29, 29.0, 28.70, 28.65, 28.43, 28.35, 27.9, 24.9, 24.8, 24.8, 24.7, 23.2, 23.1, 22.8, 22.7, 13.1 (CH\textsubscript{2}CH\textsubscript{3}), 7.5 [N(CH\textsubscript{2}CH\textsubscript{2})\textsubscript{2}]; \(\delta\text{P} (202 \text{ MHz, CDCl}_3) −0.23 \text{(with heteronuclear decoupling); HRMS (ESI) calcd for C}_{143}\text{H}_{325}\text{N}_4\text{O}_6\text{P} [M − \text{NET}_3 − H^+] = 520.3772, found 520.3747.}

Biological assays

Materials

The synthesis of 1→6-O-(2-amino-2-deoxy-α-D-glucopyranosyl)-myo-inositol 1-octadeyl phosphate, 4, \(\alpha\text{-Glc}p\text{NH}_2\text{-IPC}_{18}\),\textsuperscript{15} has been described previously. The corresponding N-acetyl derivate \(\alpha\text{-Glc}p\text{NAc}\text{-IPC}_{18}\) (3) was prepared by treatment with acetic anhydride,\textsuperscript{13} and the concentration of stock solutions determined by measurement of the inositol content by selected ion-monitoring GC-MS.\textsuperscript{5} Bloodstream form Trypanosoma brucei (variant M1a1.4) were isolated and membranes (cell-free system) prepared as described previously and stored at −80 °C.\textsuperscript{26}

Activity assays

Substrate recognition assays were performed using 500 pmol of \(\alpha\text{-Glc}p\text{NAc}\text{-Pl}\) (1) in incorporation buffer (25 mM Tris pH 8.0, 50 mM KCl, 50 mM MnCl\textsubscript{2}) and varying amounts of trypansome cell-free system (0–15 × 10\textsuperscript{6} cell equivalents per assay) in 96-well plates containing 100 µL final volume, and incubated at 37 °C for 1 h. The reaction was quenched and the glycolipids enriched and analyzed by LC-MS/MS as described below.

Inhibition assays were performed in 96-well plates in 100 µL final volume, with 1% v/v DMSO with or without inhibitor. Trypanosome cell-free system (2.5 × 10\textsuperscript{8} cell equivalents per assay) in incorporation buffer were added to wells containing 500 pmol \(\alpha\text{-Glc}p\text{NAc}\text{-IPC}_{18}\) (3) with or without inhibitor and incubated at 37 °C for 1 h. The reaction was quenched and the glycolipids enriched and analyzed by LC-MS/MS as described below.
Glycolipid enrichment

Enrichment of glycolipids was performed in a 96-well plate format. Reactions were quenched by addition of 200 μL of 5% propan-1-ol, 5 mM NH₄OAc, and the glycolipids were bound to C₁₈ resin (50 mg Isolute Array cartridge), washed three times with 200 μL 5% propan-1-ol, 5 mM NH₄OAc and eluted with 100 μL 40% propan-1-ol, 5 mM NH₄OAc into a 96-well collection plate.

Prior to subsequent analysis, compound 13 was dried under nitrogen, resuspended in MeOH (100 μL) and any free amine reacted with excess d₆-Ac₂O (1.5 μL) in the presence of pyridine (10 μL) for 15 min. The reaction was quenched with water (50 μL), dried under nitrogen and resuspended in 100 μL 40% propan-1-ol, 5 mM NH₄OAc.

Liquid chromatography – tandem mass spectrometry of glycolipids

Glycolipids were analyzed by liquid chromatography coupled to an electrospray tandem mass spectrometer (LC-MS/MS). Samples (40 μL) were injected directly from a 96-well plate onto a 10 × 1 mm C₁₈ column (ACE, 5 μM) using a binary gradient of 5% propan-1-ol, 5 mM NH₄OAc. The gradient consisted of 2 min 0% B, 2–4 min 0–100% B, 4–8 min 100%, 8–9 min 100–0% B, 9–10 min 0% B where buffer A consisted of 5% propan-1-ol, 5 mM NH₄OAc and buffer B 80% propan-1-ol, 5 mM NH₄OAc. The glycolipids were analysed on an electrospray triple quadrupole mass spectrometer (Micromass Quattro Ultima) in multiple reaction monitoring mode.

For each pseudodisaccharide analogue (7–12), standards of the N-acetylated compound and corresponding free amine were analyzed separately in order to identify unique transitions for use in subsequent multiple reaction monitoring experiments (Table 1). The ratio of the integrals for these transitions was used to calculate the percentage of substrate conversion to product in a given sample. For compound 13, standards of the N-acetylated compound and the d₆-N-acetylated form were analyzed separately and found to produce a common fragment for use in subsequent multiple reaction monitoring experiments.

For inhibition assays, the turnover of the substrate α-3-GlcNAc-IPC₁₈ (3) was used to calculate the percentage of substrate conversion to product in a given sample. Inhibitor IC₅₀ values were calculated using a four-parameter fit of eight-point potency curves derived from three independent experiments, and are quoted with a standard deviation.

Trypanosome cell-free system assays

The formation of GPI precursors is monitored by following the incorporation of [³H]-mannose and then they were analysed using high-performance liquid chromatography and fluorography as described previously.⁸

Acknowledgements

We would like to acknowledge the Wellcome Trust (programme grant 085622 and strategic awards 08348 and 100476) and the MRC (studentship to ASC) for financial support.

References