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Synthesis of archaeal glycolipid adjuvants—what is the optimum number of sugars?

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ABSTRACT

As part of a programme to optimize the use of archaeal-lipid liposomes (archaeosomes) as vaccine adjuvants, we present the synthesis and immunological testing of an oligomeric series of mannose glycolipids (Manp_{1-5}). To generate the parent archaeol alcohol precursor, the polar lipids extracted from the archaeon *Halobacterium salinarum* were hydrolyzed to remove polar head groups, and the archaeol so generated partitioned into diethyl ether. This alcohol was then iteratively glycosylated with the donor 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α/β-D-mannopyranosyl trichloroacetimidate to yield α-Man*p*-(1→2) oligomers. A starch-derived trimer was also synthesized as a control. To promote hydration and form stable archaeosomes, an archaeal anionic lipid archaetidylglycerol (AG) was included in a 4:1 molar ratio. Archaeosomes prepared from Manp_{1-2}–AG were recovered at only 34–37%, whereas Manp_{3-4}–AG recoveries were 72–77%. Lipid recovery following hydration of Manp_5–AG archaeosomes declined to 34%, indicating an optimum of 3–4 Man*p* units for bilayer formation. The $CD8^+$ T cell response in mice immunized with Manp_{3-5} archaeosomes containing ovalbumin was highest for Manp_4 and declined for Manp_3 and Manp_5, revealing an optimum length of four unbranched units. The starch-derived trimer was more active than the Man*p* oligomers, suggesting the involvement of either a general binding lectin on antigen-presenting cells with highest affinity for triglucose or multiple lectin receptors.

1. Introduction

The control of disease states through the use of vaccination with disease-associated antigens is a proven concept.[1] Recent developments include the use of proteomics and genetic data mining to determine candidate antigens from bacteria, viruses, parasites and cancer cells as the commonest diseases to be studied by these approaches.[2] However, the complexity of the host response to the introduction of such foreign antigens requires careful and detailed studies to allow for the preparation of vaccine formulations that lead to effective disease control. In most cases, antigen alone is insufficient, and adjuvants that boost specific components of the host's immune response are necessary.[3] This is an active area of biomedical research. Our contribution to this area arose out of the recognition that specialized liposomes named archaeosomes,[4] composed of the total polar lipids from *Archaea*, could entrap antigen and lead via both major histocompatibility complexes, MHC class I and MHC class II pathways to strong immunological responses in suitable animal models.[5]

Polar glycero-lipids of *Archaea* differ from those found in the *Bacteria* and *Eukarya* domains of life by sn-2,3 versus sn-1,2 stereochemistry, ether versus ester linkages from the glycerol backbone to the alkyl chains, and having isoprenoid chains typically fully saturated.[6] As well, some archaea biosynthesize C_{86} dimer species where the methyl groups of the isoprenoid side chains are joined such that the two glycerol head groups are at either end of the molecule, named caldarchaeol.[7] In most archaeal species these head groups are phosphodiester linked to small polar groups like serine, glycerol, ethanolamine or glycosidically linked to short oligosaccharides like the β-(1→6) glucose linked dimer gentiobiose. Recent experiments from our group using synthetic glycolipids based on the archaeol core lipid demonstrate that a major part of the $CD8^+$ cytotoxic thymic lymphocyte (CTL) adjuvant properties are related to the glyco-portion of such glycolipids.[8]

The combination of these newer studies and a larger number of earlier studies with total polar lipids such as those from the species *Methanobrevibacter smithii* have led to a model to describe the interaction of archaeosomes with antigen-presenting cells (APCs). APCs such as dendritic cells or macrophages can show adjuvant effects by at least five mechanisms.[8a,9] This can be via (1) an extracellular depot effect where the highly stable archaeosomes slowly dose out antigen, (2) a cell surface effect by targeting receptors on APC which also leads to specific delivery of the entrapped antigen, and cytostolic effects whereby antigens are sorted via endosomal compartments into (3) MHC class II or (4) MHC class I presentation

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pathways and (5) a co-stimulatory effect, probably receptor mediated, which leads to up-regulation of cytokines and other immunological regulators.

Previous studies had shown that APC targeting with *M. smithii* total polar lipids probably does not employ lipid A-like pathways, which are through toll receptors 2 or 4.[10] Nor does it appear to go through CD1 receptors like α-Gal glycolipids that activate natural killer (NK) cells.[11] Targeting of total polar lipid archaeosomes may proceed via archaetidyl serine (AS), the analogue of phosphatidylserine (PS).[12] PS is flipped from the inner leaflet of healthy cells to the outer bilayer surface in apoptotic cells where it binds to receptors on APCs as an integral part of the apoptosis process. Recent evidence suggests that PS mediated APC targeting leads to down-regulation instead of the required up-regulation of the immune response.[13] Therefore, we wondered if we could engineer targeting to APCs[14] via well-known receptors such as DC-sign[15] and the mannose receptor[16] by synthesizing glycolipids based on the archaeol lipid and mannose-containing oligosaccharide head groups. These mannose-containing lipids would be formulated as archaeosomes without AS, and possibly achieve both targeting and cytokine up-regulation as well as benefiting from the stability of archaeosomes.[4] Other groups have prepared mannosylated liposomes and presented evidence for targeting APCs but not with archaeal lipids.[17]

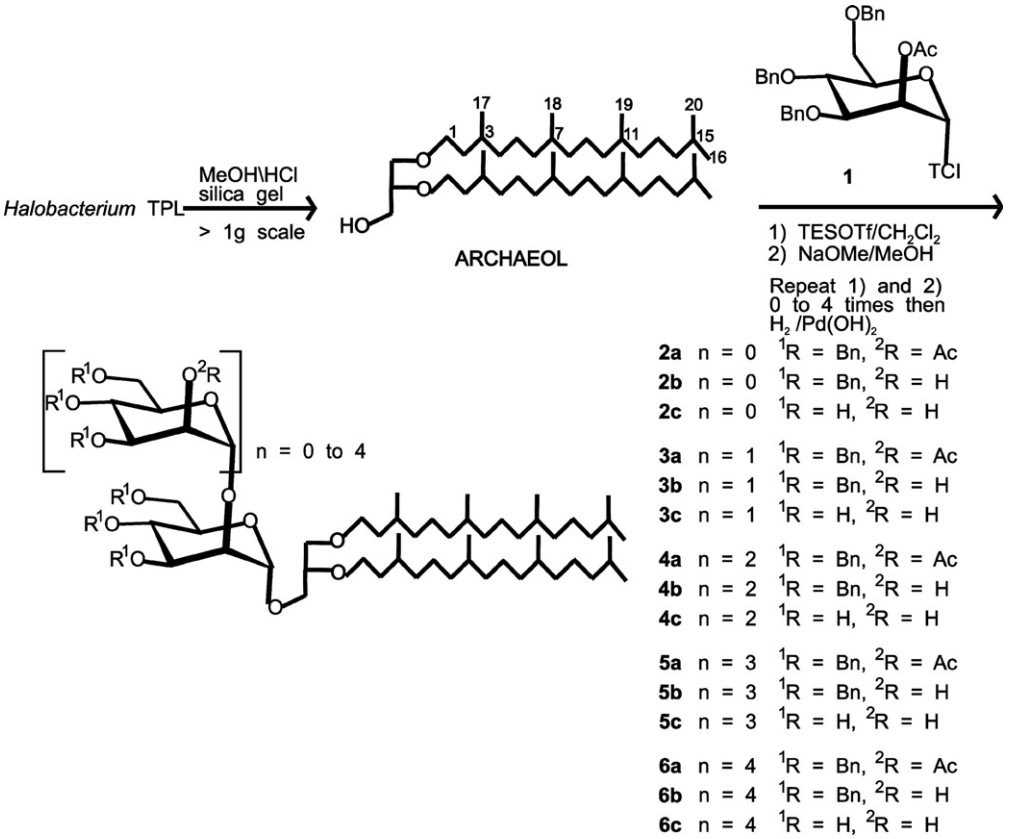
To this end we decided to synthesize α-Man*p*-(1→2)-α-Man*p* oligomers with 1–5 mannose residues attached glycosidically to archaeol. There have been a number of successful syntheses of oligosaccharides attached to archaeal lipids and related analogues but none to our knowledge containing oligomannosides or using our semi-synthetic approach.[18] The α-Man*p*-(1→2)-α-Man*p* disaccharide is frequently found at the non-reducing termini of glycoconjugates known to be recognized by receptors on APCs.[19] We present the synthesis and characterization of these oligomers as well as some biophysical and immunological data as adjuvants.

2. Results and discussion

2.1. Glycolipid synthesis

Archaeol is readily available in gram quantities from the biomass of *Halobacterium salinarum* grown in research scale reactors by a simple process of lipid extraction, head group hydrolysis and silica gel chromatography.[8a] The mannose donor (**1**) was chosen based on previous synthetic work, and is easily made in multigram quantities.[20] Donor **1** was coupled with archaeol under standard glycosylation conditions to give α-mannosylated lipid **2a** in 47% yield as the only detectable anomer. The anomericity was initially determined by the chemical shift of Man*p* H-5, which was in the range 3.7–3.8 ppm as expected for α-mannosides.[21] The acetyl group at O-2 is readily removed to give alcohol **2b** in almost quantitative yield. This can be used for chain extension or the benzyl groups removed by hydrogenation. Our first attempt to remove the benzyl groups with a Pd–C catalyst, which had been used successfully in the previous synthetic work with **1**, led to a complex mixture of products. By switching to Pearlman's catalyst ($Pd(OH)_2$–C), which has been recommended for other mannose oligomers,[22] the benzyl groups could be easily removed and the resulting tetraol **2c** easily purified. This process was repeated leading to Man*p* dimers **3a**–**c**, trimers **4a**–**c**, tetramers **5a**–**c** and pentamers **6a**–**c**, see Scheme 1.

Monomer **2c**, dimer **3c** and trimer **4c** readily dissolved in $CDCl_3$–CD_3OD mixtures (typically 1:1, v/v) and gave NMR spectra with sharp lines. Tetramer **5c** and especially pentamer **6c** on the other hand in spite of numerous attempts and experiments with different solvent mixtures gave broad lines, and definitive assignments were not possible. Analysis of the 1D ^1H or ^{13}C spectra was not particularly informative outside of identifying the anomeric resonances. Except that the ^{13}C pattern of the aliphatic



Scheme 1. Synthesis of Manp_1 to Manp_5 oligomers attached to archaeol.

lipid portion was nearly identical in all lipid containing species. The remaining ^{1}H and ^{13}C sugar resonances were assigned by 2D ^{1}H–^{1}H COSY, and 1D or 2D ^{1}H–^{1}H TOCSY correlation experiments. Subsequent ^{13}C–^{1}H correlation assigned the ^{13}C resonances. The glycerol resonances and the resonances of the ether methylenes of the side chains were almost always overlapped with each other and with some of the sugar resonances. Partial assignment could be achieved by ^{1}H–^{1}H COSY connectivities to the rest of the phytanyl side chain or more effectively from edited ^{13}C–^{1}H HSQC where methine connectivities can be distinguished from methylene connectivities by the sign of the crosspeaks. Finally inter-residue or sugar–lipid connectivities were established by NOE measurements in order to distinguish sugar residues from each other. The NOE also corroborated the α-anomericity of the linkages. A series of representative spectra are shown in Figure 1a and b for Man*p* dimer **3c**. Complete assignments are presented in Tables 2–5 in Section 4. The composition of all species was also confirmed by MALDI MS and compiled in Table 6. All lipid containing species gave characteristic $[M+Na]^{+}$ ions (Scheme 2).

In what was designed as a negative control experiment we also synthesized the α-Glc*p*-(1→4)-α-Glc*p*-(1→4)-β-Glc*p*-(1→O)-archaeol trimer derived from starch. A previous synthesis of O-2 glycosubstituted 1,3-di-phytanyl-glycerol with the same trisaccharide was used as a precedent for making the natural stereoisomer from archaeol.[23] Thus, the commercially available α-Glc*p*-(1→4)-α-Glc*p*-(1→4)-Glc*p* trimer was acetylated to give **7a**. Subsequent hemi-acetal formation with hydrazine and treatment with CCl_3CN and DBU led to trichloroacetimidate **7c** via **7b**. Glycosylation of archaeol and deprotection formed **8a** and **8b**. These species were also characterized as above, and the respective data are presented in Tables 2–6.

2.2. Archaeosome preparation and adjuvant properties

Manp_{1-5}-archaeols were dried, and hydration was attempted in water at 65 °C. The Manp_4 **5c** hydrated well, Manp_3 **4c** and Manp_5 **6c** hydrated moderately well with clumps occurring and the Manp_1 **2c** and Manp_2 **3c** hydrated with difficulty. Previously it was noted

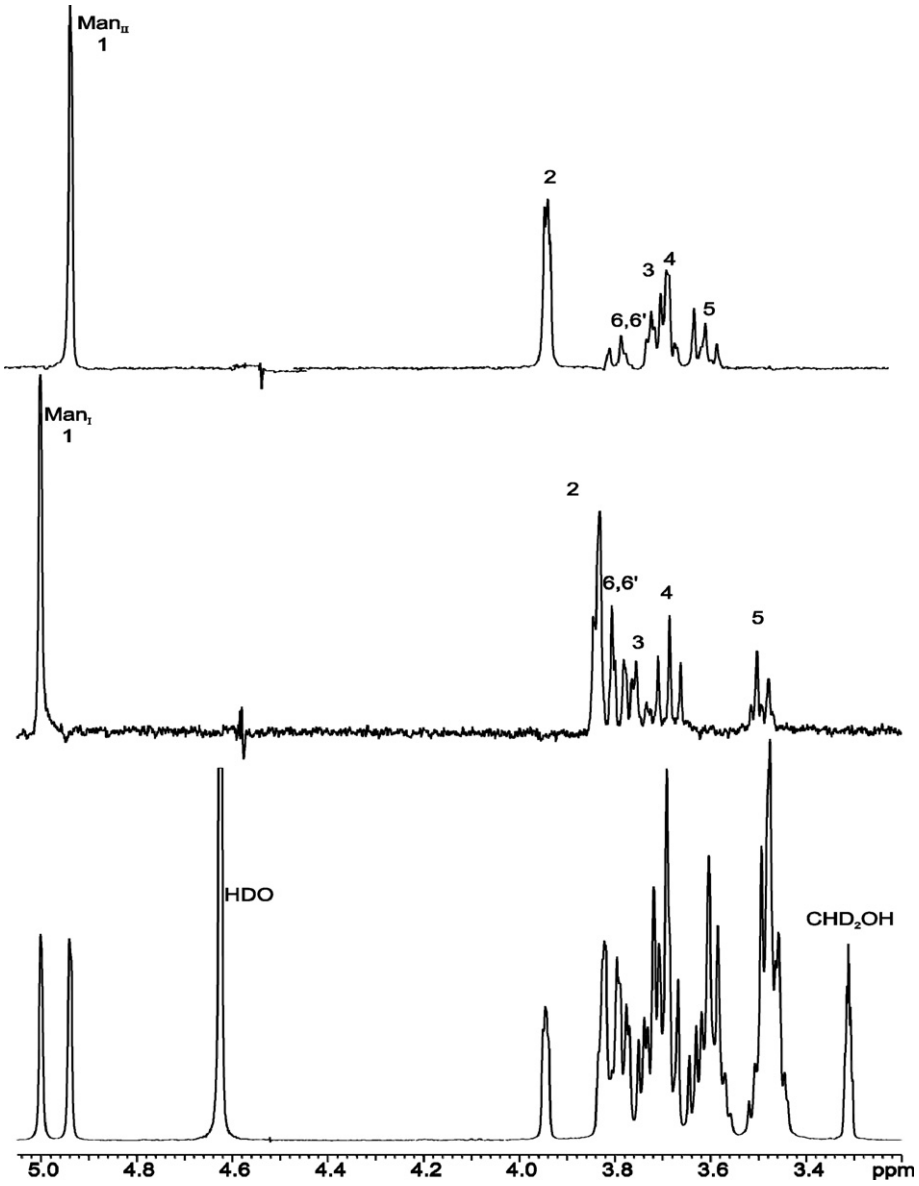


Figure 1a. 1D ^{1}H–^{1}H TOCSY correlation experiments for **3c**, Top, irradiate ManII H-1, middle, irradiate ManI H-1 and bottom, reference partial ^{1}H NMR spectra, both spectra with 135 ms mixing time.

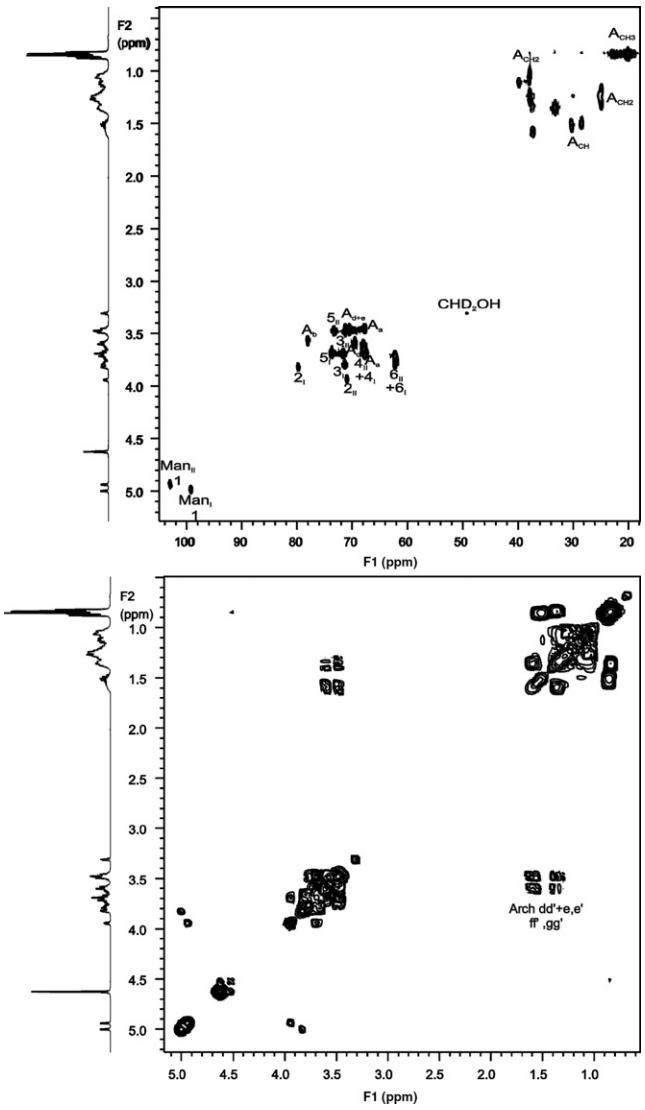


Figure 1b. ^{1}H–^{1}H COSY (bottom, positive contours only) and ^{13}C–^{1}H HSQC (top, both positive and negative contours plotted) spectra for **3c**. For labels A = archaeol.

Table 1
Archaeosome characteristics following 0.45 μm sterilizing filtration

Lipids (mol %)	Size (nm ± SD)	% Lipids recovered
Manp_2–AG (80:20)	78 ± 36	34
Manp_3–AG (80:20)	101 ± 57	77
Manp_4–AG (80:20)	85 ± 52	72
Manp_5–AG (80:20)	105 ± 50	34
AG	91 ± 53	88

that vesicles composed of 1,3-di-*O*-phytanyl-2-*O*-(β-D-maltotriosyl)glycerol aggregated in the presence of salts, but with incorporation of the anionic glycolipid sulfonoquinovosyl diacylglycerol vesicle stability was improved.[24] By introducing an anionic phospholipid, archaetidylglycerol at 20 mol %, the recovery of archaeosomes was greatly improved, with the exception of Manp_1 **2c** (Table 1). To achieve some yield of archaeosomes from Manp_1, it was necessary to prepare the vesicles by a detergent dialysis method using the non-ionic detergent octyl β-D-glucopyranoside.[25] Because archaeosome yields were comparably high for Manp_3–AG (80:20 mol %) and Manp_4–AG (80:20 mol %), and low for the others only these compositions were used in subsequent animal trials. The archaeosomes formed from Manp_3 **4c**, Manp_4 **5c** and Glcp_3 **8b** were stable for weeks in buffer at 4 °C.

Ovalbumin used as a test protein antigen in which the T cell epitope is known[26] was entrapped within the archaeosomes. Upon subcutaneous injection at zero and 3 weeks, measuring antigen-specific $CD8^{+}$ CTL activity in splenic cells[8b] and titrating anti-OVA antibody in mouse sera[27] assessed adjuvant activity for both MHC class I and class II pathways. Results are compared with archaeosomes consisting of total polar lipid extracts from *M. smithii*, as these are known to promote high CTL activity to an entrapped antigen.[28] These pure glycolipid adjuvants all showed CTL activity that was only slightly less than the TPL from *M. smithii*, that is, the adjuvant activity is predominantly from the glycolipids. This is indicated by only low CTL activity for AG archaeosomes (Fig. 2e).

The results of a CTL assay depicted in Figure 2 clearly show that archaeosomes made from Manp_{3-4} **4c** and **5c** are potent. However, the supposed negative control Glcp_3 **8c** was indeed more active than **4c** and **5c**. Antibody responses for the same compounds demonstrated that in addition to MHC class I, the Th2 arm of the MHC class II pathway is also activated by these archaeosomes (data not shown). While means of anti-OVA antibody titres were high for **4c**, **5c** and **8c** compared to TPL from *M. smithii*, they were not significantly higher (p >0.05). If these species function via the expected pathways, then this result is surprising and contrasts sharply with other results with mannose-containing liposomes where the non-mannose-containing negative controls were indeed negative.[29] A separate communication also found that mannose residues on a glycoprotein did not enhance processing and presentation of antigens in dendritic cells. This failure was not attributed to failure of internalization but rather to an inability to enter late endosomes or lysosomal compartments.[30] Our result suggests that up-regulation of the CTL response by archaeosomes does not proceed through mannose receptors alone, but includes another mechanism.

3. Conclusion

We present a stepwise synthesis of α-Man*p*-(1→2)-α-Man*p* oligomers glycosidically attached to the lipid archaeol. Especially the trimer and tetramer **4c** and **5c** show strong adjuvant activity by both MHC class I and class II pathways. The observation that the starch-derived trimer linked to archaeol **8c** also shows good adjuvant activity suggests the presence of a novel immunostimulatory pathway for archaeal glycolipids. These novel lipids can be prepared by relatively inexpensive chemical synthesis at least in comparison to the more complex glycolipids like lipid A analogues,[31] α-Gal ceramides[32] and complex saponins like QS-21A.[33]

4. Experimental

4.1. General methods

Archaetidylglycerol was purified from *Haloferax volcanii*.[34]

4.2. Procedure A

4.2.1. (2*R*)-2,3-Bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (2a)

To a mixture of (2*R*)-2,3-bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-ol (archaeol) (720 mg, 1.1 mmol), 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl trichloroacetimidate (**1**, 1.4 g, 2.2 mmol) and molecular sieves 4 Å (3 g) was added

Table 2
^{1}H NMR data of mannose archaeol compounds—sugars and protecting groups

Compound residue	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5 ($J_{5,6}$)	H-6 ($J_{5,6'}$)	H-6′ ($J_{6,6'}$)	BnAr	$BnCH_2$ ($J_{H,H}$)	$AcCH_3$	OH ($J_{2,OH}$)
2a	4.82 br s	5.36 (2.9)	3.93 (9.2)	3.87 (9.4)	3.79 br t	3.78 br d	3.78 br d	7.3–7.1 m (15)	4.82 (12.0) 4.66 (12.0) 4.65 br s (2) 4.48 (12.0) 4.45 (12.0)	2.10 s (3)	
2b	4.92 (1.2)	4.06 br s	3.87 m	3.87 m	3.80 br t	3.77 br d	3.69 br d (11.2)	7.3–7.1 m (15)	4.82 (12.0) 4.68 abq (2) 4.66 (12.0) 4.52 d (2) (12.0)		2.4 br s
2c	4.76 br s	3.83 br s	3.70 m	3.68 m	3.51 m	3.76 br d	3.76 br d	—	—	—	
3a											
Man_I	4.86 br s	3.99 br s	3.89 m	3.85 m	3.90 m	3.7–3.8 m	3.7–3.8 m	7.3–7.1 m (30)	4.83 d 4.80 d 4.64 m (5) 4.54 (11.2) 4.46 (3) 4.35 (10.8)	2.10 s (3)	
Man_{II}	5.08 br s	5.53 br s	3.96 m	3.85 m	3.79 m	3.7–3.8 m	3.7–3.8 m	—	—	—	
3b											
Man_I	4.92 br s	3.99 br s	3.87 m	3.79 m	3.88 m	3.7–3.8 m	3.7–3.8 m	7.3–7.1 m (30)	4.78 (10.4) 4.75 (11.2) 4.61 m (4) 4.47 m (6)		—
Man_{II}	5.10 br s	4.07 br s	3.81 m	3.79 m	3.72 m	3.7–3.8 m	3.7–3.8 m				
3c											
Man_I	4.99 br s	3.81 br s	3.70 m	3.67 m	3.48 m	3.76 m	3.76 m				
Man_{II}	4.91 br s	3.92 br s	3.69 m	3.66 m	3.58 m	3.78 m	3.75 m				
4a											
Man_I	4.89 br s	3.98 br s	3.83 m	3.83 m	3.79 m	3.8–3.7 m	3.8–3.7 m	7.3–7.1 m (45)	4.81 (2) 4.7–4.38 (15) 4.28 (12.0)	2.10 s (3)	
Man_{II}	5.18 br s	4.08 br s	3.80 m	3.83 m	3.79 m	3.8–3.7 m	3.8–3.7 m				
Man_{III}	5.03 br s	5.52 br s	3.96 m	3.70 m	3.83 m	3.8–3.7 m	3.8–3.7 m				
4b											
Man_I	4.89 br s	3.97 m	3.75 m	3.67 m	3.74 m	3.7 m	3.64 m	7.3–7.1 m (45)	4.78 m (3) 4.65 m (2) 4.59–4.42 m (12) 4.30 (12.2)		2.35 br s
Man_{II}	5.19 br s	4.09 m	3.81 m	3.82	3.8 m	3.7 m	3.64 m				
Man_{III}	5.10 br s	4.09 m	3.81 m	3.82	3.8 m	3.7 m	3.64 m				
4c											
Man_I	4.94 br s	3.78 m	3.60 m	3.60 m	3.44 m	3.8–3.6 m	3.8–3.6 m				
Man_{II}	4.96 br s	3.99 br s	3.63 m	3.63 m	3.48 m	3.8–3.6 m	3.8–3.6 m				
Man_{III}	5.22 br s	3.93 br s	3.73 m	3.58 m	3.53 m	3.8–3.6 m	3.8–3.6 m				
5a											
Man_I	4.86 br s	4.00 m	3.82 m	3.66 m	3.67 m	3.7–3.4 m	3.7–3.4 m	7.3–7.1 m	4.83–4.30 m, 4.16 d (12.0)	2.12 s (3)	
Man_{II}	5.19 br s	4.10 m	3.77 m	3.75 m	3.76 m	3.7–3.4 m	3.7–3.4 m				
Man_{III}	5.22 br s	4.10 m	3.77 m	3.75 m	3.76 m	3.7–3.4 m	3.7–3.4 m				
Man_{IV}	5.04 br s	5.55 br dd	3.75 m	3.75 m	3.76 m	3.7–3.4 m	3.7–3.4 m				
5b											
Man_I	4.92 br s	3.98 m	3.92–3.78 m	3.74 m	3.90–3.75 m	3.74–3.48 m	3.74–3.48 m	7.3–7.1 m (60)	4.82–4.26 m (23) 4.13 d (12.4)		2.3 br
Man_{II}	5.20 m	4.11 m	3.92–3.78 m	3.90 m	3.90–3.75 m	3.74–3.48 m	3.74–3.48 m				
Man_{III}	5.20 m	4.11 m	3.92–3.78 m	3.90 m	3.90–3.75 m	3.74–3.48 m	3.74–3.48 m				
Man_{IV}	5.11 br s	4.08 m	3.92–3.78 m	3.90 m	3.90–3.75 m	3.74–3.48 m	3.74–3.48 m				
5c											
Man_I	4.97 br s	3.85 m	3.80 m	3.48 m	n.d.	n.d.	n.d.				
Man_{II}	5.24 br s	3.99 m	3.80 m	3.62 m	n.d.	n.d.	n.d.				
Man_{III}	5.27 br s	3.99 m	3.80 m	3.65 m	n.d.	n.d.	n.d.				
Man_{IV}	4.99 br s	3.95 m	3.70 m	3.70 m	n.d.	n.d.	n.d.				

(*continued on next page*)

Table 2 (*continued*)

Compound residue	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5 ($J_{5,6}$)	H-6 ($J_{5,6'}$)	H-6′ ($J_{6,6'}$)	BnAr	$BnCH_2$ ($J_{H,H}$)	$AcCH_3$	OH ($J_{2,OH}$)
6a											
Man_I	4.93 br s	3.97 m	n.d.	n.d.	n.d.	n.d.	n.d.	7.3–7.1 m (75)	4.84 m (3) 4.77 (11.6) 4.66–4.30 m (23) 4.25 (11.0) 4.17 m (2)	2.03 s (3)	
Man_{II}	5.17 br s	4.07 m	n.d.	n.d.	n.d.	n.d.	n.d.				
Man_{III}	5.21 br s	4.07 m	n.d.	n.d.	n.d.	n.d.	n.d.				
Man_{IV}	5.22 br s	4.07 m	n.d.	n.d.	n.d.	n.d.	n.d.				
Man_V	5.01 br s	5.51 br s	n.d.	n.d.	n.d.	n.d.	n.d.				
6b											
Man_I	4.93 br s	3.91 m	n.d.	n.d.	n.d.	n.d.	n.d.	7.3 7.0 m (75)	4.83–4.28 m (28) 4.20 (11.2) 4.08 m (1)		n.d.
Man_{II}	5.18 br s	4.08 m	n.d.	n.d.	n.d.	n.d.	n.d.				
Man_{III}	5.21 m	4.08 m	n.d.	n.d.	n.d.	n.d.	n.d.				
Man_{IV}	5.21 m	4.08 m	n.d.	n.d.	n.d.	n.d.	n.d.				
Man_V	5.10 br s	4.08 m	n.d.	n.d.	n.d.	n.d.	n.d.				
6c											
Man_I	4.92 br s	3.80 m	3.74 m	3.46 m	n.d.	n.d.	n.d.				
Man_{II}	5.20 br s	3.94 m	3.76 m	3.58 m	n.d.	n.d.	n.d.				
Man_{III}	5.24 br s	3.94 m	3.76 m	3.62 m	n.d.	n.d.	n.d.				
Man_{IV}	5.24 br s	3.94 m	3.76 m	3.49 m	n.d.	n.d.	n.d.				
Man_V	4.95 br s	3.92 m	3.65 m	3.52 m	n.d.	n.d.	n.d.				
8a											
Glc_I	4.57 d (8.0)	4.81 dd (9.4)	5.22 t (9.0)	3.93 m	3.68 ddd (3.7)	4.43 br d (4.1)	4.29 dd (12.1)			2.15, 2.13, 2.07, 2.04, 2.01, 2.00, 1.98 (3), 1.96	
Glc_{II}	5.25 d (4.1)	4.72 dd (10.3)	5.375 t (9.0)	3.93 m	3.93 m	4.43 br d (3.6)	4.16 dd (12.3)				
Glc_{III}	5.380 d (4.1)	4.83 dd (9.9)	5.33 t (9.5)	5.04 t (9.0)	3.93 m (3.6)	4.23 dd	4.03 br d (12.0)				
8b											
Glc_I	4.27 d (7.8)	3.30 dd (10.0)	3.61 t (9.0)	3.53 t (9.3)	3.32 ddd (2.1)	3.84 dd (3.9)	3.77 dd (12.0)				
Glc_{II}	5.09 d (3.7)	3.51–3.42 m	3.85 t (9.2)	3.51–3.42 m	3.7–3.58 m	3.81–3.75 m	3.7–3.58 m				
Glc_{III}	5.09 d (3.7)	3.51–3.42 m	3.73 m	3.26 t (9.8)	3.7–3.58 m	3.81–3.75 m	3.81–3.75 m				

Table 3
^{13}C NMR data of mannose archaeol compounds—sugars and protecting groups

Compound residue	C1	C2	C3	C4	C5	C6	Ac C=O	Ac CH_3	Bn_{ip}	Bn CH	Bn CH_2
2a	97.9	68.7	78.2	74.2	71.3	68.8	170.5	21.1	138.5, 138.2, 137.9	128.4–127.5	75.1, 73.4, 71.8
2b	99.4	68.3	80.2	74.2	71	68.8	–	–	138.4, 138.2, 137.9	128.5–127.5	75.0, 73.4, 72.0
2c	101.1	67.7	72.1	72.1	73.5	62.2					
3a											
Man_I	98.7	74.9	78.2	74.5	71.8	69.2	170.1	21.1	138.56, 138.52, 138.50, 138.4, 138.2, 138.0	128.3–127.3	75.0 (2), 73.4, 73.2, 72.1, 71.9
Man_{II}	99.6	68.7	79.7	74.3	71.8	69					
3b											
Man_I	98.8	74.6	79.7	74.3	71.5	69.2	–	–	138.6 (2), 138.4, 138.24, 123.2, 138.0	128.4–127.3	75.0 (2), 73.3, 73.2, 72.3, 72.1
Man_{II}	101.1	68.5	80	74.3	71.8	69					
3c											
Man_I	99.5	80.1	71.9	68.2	71.6	62.6	–	–	–	–	–
Man_{II}	103.4	71.3	74	68.2	73.7	62.3					
4a											
Man_I	98.8	74.7	79.4	71.9	74.7	69.2	170.2	21.2	138.6 (3), 138.4 (3), 138.3, 138.2, 138.1	128.3–127.5	75.0, 73.3, 72.1
Man_{II}	100.6	74.7	79.5	71.9	74.7	69.2					
Man_{III}	99.4	68.7	78.2	71.9	74.2	69.3					
4b											
Man_I	98.8	74.6	79.9	74.2	71.9	68.9	–	–	138.5, 138.3, 138.1, 138.0	128.4–127.4	75.0, 73.3, 73.2, 72.3, 72.0
Man_{II}	100.7	74.9	79.4	74.2	71.9	68.9					
Man_{III}	100.9	68.3	80.1	74.2	71.5	69.1					
4c											
Man_I	99.6	79.6	71.6	68.3	74.13	62.6	–	–	–	–	–
Man_{II}	101.6	79.4	71.7	68.5	74.05	62.7					
Man_{III}	103.1	71.2	71.9	67.9	73.6	62.2					
5a											
Man_I	98.8	75.2	78.3	71.8	74.7	69.2	170.1	21.2	138.62 (2), 138.58, 138.55, 138.5 (2), 138.4 (3), 138.3, 138.2, 138.1	128.4–127.4	75.0, 73.3, 73.2, 71.92, 71.86, 71.8
Man_{II}	100.7	75.4	79.3	71.8	74.7	69.35					
Man_{III}	101.1	75.5	79.3	71.7	74.6	69.35					
Man_{IV}	99.4	68.8	79.3	72.3	74.3	69.41					
5b											
Man_I	98.8	75.5	79.2	71.7	74.8	69.2	–	–	138.62 (3), 138.56 (2), 138.5(2), 138.4, 138.35, 138.31, 138.2, 138.1	128.4–127.4	74.9, 73.3, 73.2, 72.1, 71.8
Man_{II}	100.9	75.1	79.3	71.6	74.8	68.8					
Man_{III}	100.9	75.1	79.4	71.6	74.7	68.8					
Man_{IV}	101.1	68.5	80.1	72.4	74.3	69.4					
5c											
Man_I	99.9	80.1	71.6	69	74.6	62.9					
Man_{II}	102	79.93	72	68.8	74.6	63.1					
Man_{III}	101.9	79.87	72	68.6	74.5	63					
Man_{IV}	103.7	71.2	72.2	68.2	74.1	62.6					

(*continued on next page*)

Table 3 (*continued*)

Compound residue	C1	C2	C3	C4	C5	C6	Ac C=O	Ac CH_3	Bn_{ip}	Bn CH	Bn CH_2
6a											
Man_I	98.8	76.1	78.2	71.72	74.3	69.6	170.1	21.2	138.7, 138.5, 138.4, 138.3, 138.2, 138.1	128.4–127.2	75.2, 74.9, 73.3, 73.23, 73.19
Man_{II}	101.2	75.7	79	71.78	74.7	69.5					
Man_{III}	101.3	75.7	79.1	71.85	74.7	69.3					
Man_{IV}	101.3	75.5	79.2	71.9	74.7	69.2					
Man_V	99.4	75.0	79.3	71.9	74.3	67.3					
6b											
Man_I	98.8	75.7	79.1	71.8	75.1	69.5			138.7, 138.6, 138.42, 138.36, 138.2, 138.1	128.4–127.2	75.1, 75.0, 74.3, 73.2, 72.4, 72.1
Man_{II}	101.3	75.7	79.1	71.8	75.1	69.5					
Man_{III}	101.3	75.7	79.3	71.8	75	69.4					
Man_{IV}	100.9	75.7	79.7	71.8	75	69.3					
Man_V	100.9	68.8	80.1	71.8	75	67.3					
6c											
Man_I	99.6	79.5	71.4	68.4	74.3	62.8					
Man_{II}	101.6	79.6	71.5	68.4	74.3	62.8					
Man_{III}	101.6	79.6	71.5	68.4	74.3	62.8					
Man_{IV}	101.6	79.6	71.5	68.4	74.3	62.8					
Man_V	103.3	72	71.6	68.4	74.3	62.8					
8a											
Glc_I	100.5	72.2	75.4	72.4	71.7	63	170.59, 170.56, 170.51, 170.46, 170.3, 170.1, 169.8, 169.7, 169.5, 169.4	20.9, 20.8, 20.64, 20.61, 20.5			
Glc_{II}	95.7	70.5	71.9	73.8	68.4	62.3					
Glc_{III}	95.6	70.4	70.1	67.8	68.8	61.3					
8b											
Glc_I	104.2	73.9	76.9	83.4	76	61.6					
Glc_{II}	102.6	73.6	74.4	83.2	74.2	62.5					
Glc_{III}	102.5	73.1	72.8	71	74.6	61.6					

Table 4
^{1}H NMR data of lipid in archaeol compounds

Compound	aa′	b	cc′	dd′	ee′	ff′ + gg′	CH	CH_2	CH_3
2a	3.67 m, 3.50 m	3.53 m	3.55 m	3.55 m	3.42 m	1.52 m, 1.31 m	1.47 m, 1.30 m	1.4–1.0	0.8 m
2b	3.74 m, 3.53 m	3.53 m	3.57 m	3.55 m	3.45 m	1.52 m, 1.35 m	1.54 m, 1.36 m	1.4–1.0	0.85 m
2c	3.68 m, 3.47 m	3.54 m	3.56 m	3.45 m	3.44 m	1.50 m, 1.32 m	1.56 m, 1.33 m	1.4–1.0	0.8 m
3a	3.62 m, 3.37 m	3.47 m	3.50 m	3.40 m	3.38 m	1.54 m, 1.29 m	1.48 m, 1.44 m, 1.31 m	1.4–1.0	0.8 m
3b	3.65 m, 3.40 m	3.51 m	3.54 m	3.44 m	3.40 m	1.57 m, 1.32 m	1.52 m, 1.50 m, 1.26 m	1.4–1.0	0.85 m
3c	3.69 m, 3.43 m	3.55 m	3.57 m	3.46 m	3.46 m	1.55 m, 1.33 m	1.49 m, 1.47 m, 1.33 m	1.4–1.0	0.81 m
4a	3.63 m, 3.33 m	3.48 m	3.50 m	3.40 m	3.36 m	1.56 m, 1.31 m	1.52 m, 1.48 m, 1.36 m	1.4–1.0	0.85 m
4b	3.64 m, 3.35 m	3.53 m	3.52 m	3.43 m	3.39 m	1.53 m, 1.30 m	1.51 m, 1.46 m, 1.33 m	1.4–1.0	0.81 m
4c	3.65 m, 3.38 m	3.51 m	3.53 m	3.41 m	3.40 m	1.51 m, 1.29 m	1.44 m, 1.42 m, 1.30 m	1.4–1.0	0.79 m
5a	3.60 m, 3.40 m	3.50 m	3.51 m	3.40 m	3.38 m	1.55 m, 1.30 m	1.50 m, 1.45 m, 1.33 m	1.4–1.0	0.85 m
5b	3.60 m, 3.40 m	3.50 m	3.51 m	3.40 m	3.38 m	1.55 m, 1.30 m	1.50 m, 1.45 m, 1.33 m	1.4–1.0	0.85 m
5c	3.68 m, 3.42 m	3.56 m	3.54 m	3.46 m	3.45 m	1.57 m, 1.34 m	1.50 m, 1.49 m, 1.35 m	1.4–1.0	0.84 m
6a	3.59 m, 3.45 m	3.54 m	3.57 m, 3.50 m	3.50 m	3.49 m	1.60 m, 1.40 m	1.50 m, 1.40 m	1.4–1.0	0.85 m
6b	3.59 m, 3.38 m	3.46 m	3.71 m	3.38 m	3.34 m	1.54 m, 1.39 m	1.50 m, 1.40 m	1.4–1.0	0.9 m
6c	3.48 br m	3.57 m	3.58 m	3.47 m	3.43 m	1.54 m, 1.39 m	1.53 m	1.4–1.0	0.9 m
8a	3.86 m, 3.54 m	3.54 m	3.54 m	3.42 m	3.40 m	1.56 m, 1.32 m	1.48 m, 1.34 m	1.4–1.0	0.8 m
8b	3.92 br dd (10.0), (2.7) 3.59 m	3.63 m	3.62 m	3.53 m	3.48 m	1.59 m, 1.35 m	1.52 m, 1.36 m	1.4–1.0	0.85 m

Table 5
^{13}C NMR data of lipids in archaeol compounds

Compound	a	b	c	d	e	CH	CH_2	CH_3
2a	67.4	77.4	69	70.5	70.1	32.8, 29.9, 29.8, 28.0	39.4, 37.54, 37.47, 37.4, 37.3, 37.0, 36.7, 24.8, 24.5, 24.4	22.7, 22.6, 19.74, 19.70, 19.6
2b	67.1	77.5	68.9	70.6	70.1	32.8, 29.9, 29.8, 28.0	39.4, 37.53, 37.46, 37.4, 37.3, 37.0, 36.7, 24.8, 24.5, 24.4	22.7, 22.6, 19.74, 19.70, 19.6
2c	67.5	78.5	69.6	71.3	70.7	33.5,30.6, 30.4, 30.3	40.1, 38.1, 38.0, 37.7, 37.3, 25.5, 25.13, 25.08	23.1, 23.0, 20.2
3a	67.2	77.5	68.9	70.8	70.1	32.8, 29.9, 29.8, 28.0	39.4, 37.53, 37.46, 37.4, 37.3, 37.0, 36.7, 24.8, 24.5, 24.4	22.7, 22.6, 19.75, 19.71, 19.6
3b	67.2	77.5	69	70.8	70.1	32.8, 29.9, 29.8, 28.0	39.3, 37.5, 37.4, 37.3, 37.0, 36.6, 24.8, 24.5, 24.4	22.7, 22.6, 19.7, 19.6
3c	67.9	77.5	69.7	71.6	70.8	33.5, 30.6, 30.5, 28.7	40.1, 38.1, 38.0 37.7, 37.4, 25.5, 25.2, 25.1	23.14, 23.06, 20.2
4a	67.2	77.4	69	70.9	70	32.8, 30.0, 29.8, 28.0	39.4, 37.5, 37.3, 37.1, 36.7, 24.8, 24.5, 24.4	22.7, 22.6, 19.8
4b	67.1	77.6	69.3	71.2	70.3	33.1, 30.2, 30.0, 28.2	39.6, 37.7, 37.5,37.3, 36.9, 25.1, 24.7, 24.6	23.0, 22.9, 20.0
4c	67.9	77.7	68.5	71.2	69.7	33.5, 30.6, 30.4, 28.6	40.1, 38.1, 37.9, 37.6, 37.3, 25.5, 25.12, 25.07	23.1, 23.0, 20.2, 20.1
5a	67.3	78.3	69.2	71	70.1	32.8, 30.0, 29.8, 28.0	39.4, 375, 37.3, 37.1, 36.7, 24.8, 24.5, 24.4	22.7, 22.6, 19.74, 19.68, 19.6
5b	67.3	77.6	69	71.6	71	32.8, 30.0, 29.8, 28.0	39.4, 37.5, 37.3, 37.1, 36.7, 24.8, 24.5, 24.4	22.7, 22.6, 19.7, 19.6
5c	68.4	78.9	69.8	71.7	70.9	33.8, 30.8, 30.7, 28.9	40.3, 38.43, 38.36, 38.3, 38.2, 38.0, 37.7, 25.7, 25.3	23.2, 23.1, 20.4, 20.3
6a	68.7	77.6	70.1	71.9	71	32.8, 30.0, 29.8, 29.7	39.4, 37.6, 37.5, 37.3, 27.1, 36.7, 24.8, 24.5, 24.4	22.7, 22.6, 19.8, 19.7
6b	68.5	77.6	69	71	70.1	32.83, 32.81, 31.9, 30.0, 29.8, 29.7, 29.4	39.3, 37.5, 37.3, 37.1, 36.7, 24.8, 24.5, 24.4	22.7, 22.6, 19.8, 19.7, 19.6
6c	68	78.7	69.7	71.2	70.8	33.5, 30.6, 30.5, 30.3, 28.7	40.1, 39.6, 38.13, 38.09, 38.0, 37.7, 37.4, 25.5, 25.15, 25.10	23.1, 23.0, 20.23, 20.15
8a	70.4	77.7	69.3	70.4	70.1	32.8, 29.9, 29.8, 27.9	39.3, 37.41, 37.37, 37.2, 37.1, 36.5, 24.8, 24.4, 24.3,	22.7, 22.6, 19.71, 19.67
8b	69.8	78.4	69.4	71.2	70.8	33.5, 30.6, 30.5, 28.7	40.01, 38.13, 38.09, 38.0, 37.7, 37.3, 25.5, 25.15, 25.09	23.1, 23.0, 20.3 20.2

CH_2Cl_2 (15 mL). After stirring at rt under an argon atmosphere for 1 h, triethylsilyltrifluoromethanesulfonate (25 μL, 0.11 mmol) was added and the stirring continued for 40 min. The reaction was quenched with diisopropylethylamine (100 μL). The whole reaction was adsorbed on silica gel and then purified by silica gel chromatography eluting with 9:1 hexanes–EtOAc to yield pure product as a viscous oil (0.58 g, 47%) plus some mixed fractions.

4.3. Procedure B

4.3.1. (2*R*)-2,3-Bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl 3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (2b)

Compound **2a** (0.58 g, 0.51 mmol) was dissolved in a mixture of dry CH_3OH (10 mL) and CH_2Cl_2 (2 mL). Then, 1 M $NaOCH_3$ (0.5 mL) was added and the stirring continued for 4 h. The reaction mixture was diluted with CH_2Cl_2 (150 mL) and washed 2× with NH_4Cl_{aq} followed by saturated $NaCl_{aq}$. After drying with Na_2SO_4, filtration and evaporation the residue was purified by column chromatography on silica gel eluting with 5:1 hexanes–EtOAc to yield pure compound as a viscous oil (520 mg, 93%).

4.3.2. (2*R*)-2,3-Bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl 2-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (3a)

Compound **3a** (1.04 g, 63%) was prepared from **2b** (1.15 g, 0.206 mmol) using procedure A and purified by silica gel eluting with 9:1 hexanes–EtOAc followed by 85:15 hexanes–EtOAc.

4.3.3. (2*R*)-2,3-Bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl 2-*O*-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (3b)

Compound **3b** (0.98 g, 88%) was prepared from **3a** (1.14 g, 0.73 mmol) using procedure B and purified by silica gel eluting with 85:15 hexanes–EtOAc.

4.3.4. (2*R*)-2,3-Bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl 2-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-2-*O*-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-3,4,6-tri-*O*-benzyl α-D-mannopyranoside (4a)

Compound **4a** (440 mg, 38%) was prepared from **3b** (880 mg, 0.58 mmol) using procedure A and purified by silica gel eluting with 85:15 hexanes–EtOAc.

Table 6
MS data for archaeol compounds

Compound	Formula	MW calcd	MS MALDI	$[\alpha]_D$	*c* (solvent)
2a	$C_{72}H_{118}O_9$	1127.23	1149.69 $[M+Na]^+$ 1165.65 $[M+K]^+$	22	0.3 $CHCl_3$
2b	$C_{70}H_{116}O_8$	1085.7	1107.78 $[M+Na]^+$ 1123.71 $[M+K]^+$	21	1.1 $CHCl_3$
2c	$C_{49}H_{98}O_8$	814.73	837.52 $[M+Na]^+$ 853.38 $[M+K]^+$	19.5	0.8 $CHCl_3$
3a	$C_{99}H_{146}O_{14}$	1559.07	1583.17 $[M+Na]^+$ 1599.13 $[M+K]^+$	18.7	0.4 $CHCl_3$
3b	$C_{97}H_{144}O_{13}$	1518.21	1540.75 $[M+Na]^+$ 1556.71 $[M+K]^+$	23.3	0.3 $CHCl_3$
3c	$C_{55}H_{108}O_{13}$	976.78	999.83 $[M+Na]^+$ 1015.79 $[M+K]^+$	28.3	0.6 $CHCl_3$
4a	$C_{126}H_{174}O_{19}$	1991.27	2015.16 $[M+Na]^+$ 2031.12 $[M+K]^+$	33.5	0.2 $CHCl_3$
4b	$C_{124}H_{172}O_{18}$	1949.25	1972.89 $[M+Na]^+$ 1988.84 $(M+K)^+$	29.8	0.7 $CHCl_3$
4c	$C_{61}H_{118}O_{18}$	1139.61	1161.90 $[M+Na]^+$ 1173.83 $[M+K]^+$	42.3	0.6 $CHCl_3$
5a	$C_{153}H_{202}O_{24}$	2423.46	2447.60 $[M+Na]^+$ 2463.59 $(M+K)^+$	19.1	0.9 $CHCl_3$
5b	$C_{151}H_{200}O_{23}$	2383.26	2405.74 $[M+Na]^+$ 2421.71 $[M+K]^+$	20.5	1.4 $CHCl_3$
5c	$C_{67}H_{128}O_{23}$	1301.75	1324.11 $[M+Na]^+$ 1327.13 $[M+K]^+$	32.5	1.2 $CHCl_3$
6a	$C_{180}H_{230}O_{29}$	2855.65	2880.77 $[M+Na]^+$ 2896.70 $[M+K]^+$	20.5	1.2 $CHCl_3$
6b	$C_{178}H_{228}O_{28}$	2813.64	2838.57 $[M+Na]^+$ 2854.55 $[M+K]^+$	39.3	2.2 $CHCl_3$
6c	$C_{73}H_{138}O_{28}$	1462.94	1485.99 $[M+Na]^+$ 1501.86 $[M+K]^+$	20.8	0.4 CH_2Cl_2–CH_3OH 1:1 v:v
8a	$C_{81}H_{138}O_{28}$	1558.94	1582.0 $[M+Na]^+$		
8b	$C_{61}H_{118}O_{18}$	1139.61	1161.7 $[M+Na]^+$ 1177.6 $[M+K]^+$		

4.3.5. (2*R*)-2,3-Bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl 2-*O*-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-2-*O*-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (4b)

Compound **4b** (370 mg, 90%) was prepared from **4a** (420 mg, 0.21 mmol) using procedure B and purified by silica gel eluting with 85:15 hexanes–EtOAc.

4.3.6. (2*R*)-2,3-Bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl 2-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-2-*O*-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-2-*O*-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-3,4,6-tri-*O*-benzyl α-D-mannopyranoside (5a)

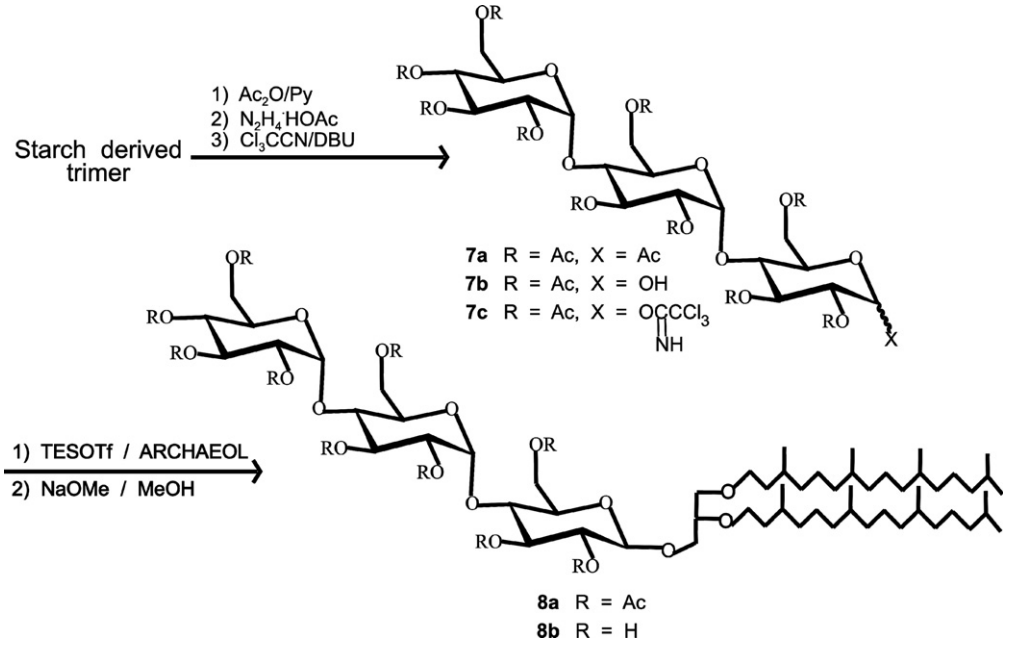
Compound **5a** (178 mg, 73%) was prepared from **4b** (190 mg, 0.11 mmol) using procedure A and purified by silica gel eluting with 85:15 hexanes–EtOAc.

4.3.7. (2*R*)-2,3-Bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl-2-*O*-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl-2-*O*-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-2-*O*-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (5b)

Compound **5b** (93 mg, 55%) was prepared from **5a** (172 mg, 0.61 mmol) using procedure B and purified by silica gel eluting with 85:15 hexanes–EtOAc.

4.3.8. (2*R*)-2,3-Bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl 2-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-2-*O*-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-2-*O*-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-2-*O*-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-3,4,6-tri-*O*-benzyl α-D-mannopyranoside (6a)

Compound **6a** (163 mg, 68%) was prepared from **5b** (200 mg, 0.084 mmol) using procedure A and purified by silica gel eluting with 85:15 hexanes–EtOAc.



Scheme 2. Synthesis of starch-derived trimer attached to archaeol.

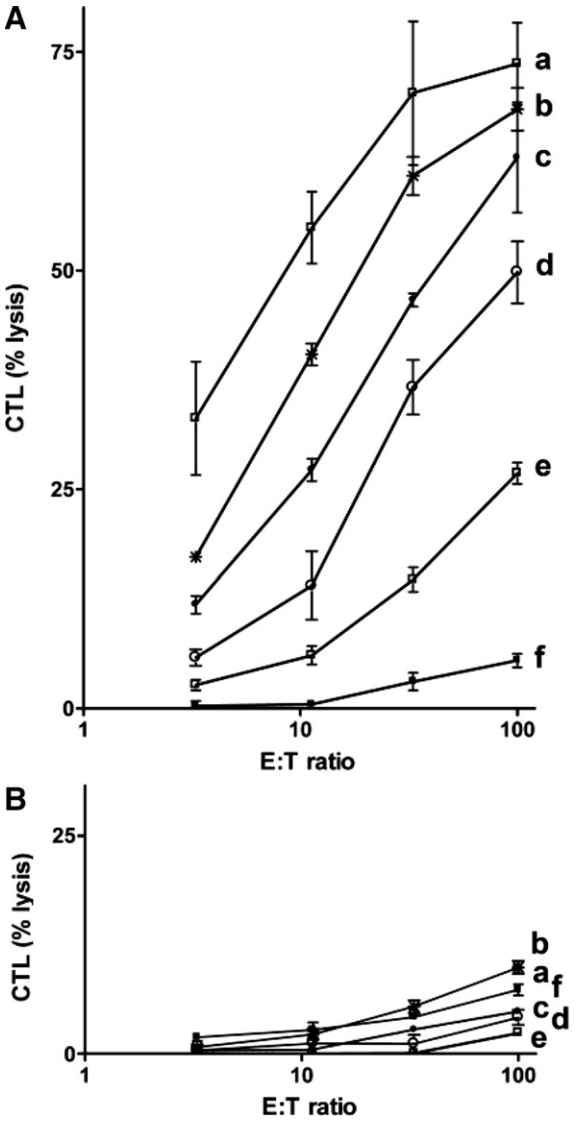


Figure 2. CTL MHC class I immune responses in mice immunized with various synthetic glycosylarchaeols. (A) CTL activity is presented as % lysis of antigen-specific EG.7 target cells. (B) Antigen specificity was confirmed by measuring CTL activity for non-specific EL-4 target cells. Vaccines used to immunize mice were OVA entrapped in (a), total polar lipid archaeosomes from *M. smithii* or in archaeosomes containing 20 mol % AG with 80 mol % of either (b), starch-derived trimer **8b**; (c) Man*p* tetramer **5c**; or (d) Man*p* trimer **4c**. (e) OVA entrapped in glycolipid-free archaeosomes consisting of AG–cholesterol (80:20 mol %); (f) naive.

4.3.9. (2*R*)-2,3-Bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl-2-*O*-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-2-*O*-(3,4,6-tri-*O*-benzyl-α-D-manno-pyranosyl)-2-*O*-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-2-*O*-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (6b)

Compound **6b** (88 mg, 90%) was prepared from **6a** (134 mg, 0.086 mmol) using procedure B and purified by silica gel eluting with 75:25 hexanes–EtOAc.

4.4. Procedure C

4.4.1. (2*R*)-2,3-Bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl α-D-mannopyranoside (2c)

Compound **2b** (100 mg, 0.051 mmol) was dissolved in EtOAc (10 mL) and, after purging with argon, $Pd(OH)_2$–C (Pearlman's catalyst) (150 mg) was added and the mixture hydrogenated using a Parr apparatus at 50 psi of H_2 with shaking for 64 h. The catalyst was removed by filtration through a bed of Celite, and was well washed with EtOAc and CH_3OH. The combined filtrates were evaporated and then purified by silica gel chromatography eluting with 7:1:1 EtOAc–CH_3OH–H_2O to yield a waxy solid (62 mg, 83%).

4.4.2. (2*R*)-2,3-Bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl 2-*O*-(α-D-mannopyranosyl) α-D-mannopyranoside (3c)

Compound **3c** (53 mg, 82%) was prepared from **3b** (100 mg, 0.066 mmol) using procedure C.

4.4.3. (2*R*)-2,3-Bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl 2-*O*-(α-D-mannopyranosyl)-2-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (4c)

Compound **4c** (41 mg, 70%) was prepared from **4b** (100 mg, 0.051 mmol) using procedure C.

4.4.4. (2*R*)-2,3-Bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl 2-*O*-(α-D-mannopyranosyl)-2-*O*-(α-D-mannopyranosyl)-2-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (5c)

Compound **5c** (43 mg, 66%) was prepared from **5b** (120 mg, 0.050 mmol) using procedure C.

4.4.5. (2*R*)-2,3-Bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl 2-*O*-(α-D-mannopyranosyl)-2-*O*-(α-D-mannopyranosyl)-2-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (6c)

Compound **6c** (28 mg, 65%) was prepared from **6b** (83 mg, 0.029 mmol) using procedure C.

4.4.6. (2*R*)-2,3-Bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl 4-*O*-(2,3,4,6-tetra-*O*-α-D-glucopyranosyl)-4-*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (8a)

To known[23] 4-*O*-(2,3,4,6-tetra-*O*-α-D-glucopyranosyl)-4-*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl trichloroacetimidate (280 mg, 0.29 mmol), 4 molecular sieves (300 mg) and archaeol (78 mg, 0.12 mmol) was added CH_2Cl_2 (3 mL), and the mixture stirred for 1 h at rt under an atmosphere of argon. To this was added triethylsilyltrifluoromethanesulfonate (3 μL, 0.013 mmol) and the mixture stirred for 40 min when TLC in 1:1 hexanes–EtOAc (R_f = 0.5) indicated the reaction was complete. The reaction was quenched with diisopropylethylamine (10 μL), filtered with rinsing with CH_2Cl_2. The combined filtrates were concentrated and the residue purified by silica gel chromatography eluting with 2:1 hexanes–EtOAc to yield pure **8a** as a waxy solid (99 mg, 53%).

4.5. (2*R*)-2,3-Bis[(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl 4-*O*-(α-D-glucopyranosyl)-4-*O*-(α-D-glucopyranosyl)-β-D-glucopyranoside (8b)

Compound **8a** (134 mg, 0.086 mmol) was deacetylated following procedure B and purified by silica gel chromatography eluting with $CHCl_3$–CH_3OH–H_2O 10:3:0.3 to yield pure **8b** (88 mg, 90%).

4.6. NMR and MS tables

^1H and ^{13}C NMR of **2a**, **2b**, **3a**, **3b**, **4a**, **4b**, **5a**, **5b**, **6a**, **6b** and **8a** were obtained in $CDCl_3$ solution (referenced to residual $CHCl_3$ at 7.26 ppm ^1H and 77.0 ppm central resonance ^{13}C), whereas those

of **2c**, **3c**, **4c**, **5c**, **6c** and **8b** were obtained in 1:1 (v:v) solutions of CD_3OD–$CDCl_3$ (referenced to residual CHD_2OD at 3.31 ppm ^1H and 49.15 ppm central resonance ^{13}C). Chemical shifts are in ppm and coupling constants in hertz. Subscript Roman Numerals refer to the residue number with I the reducing termini. NMR was performed on either a Varian 400 MHz or 200 MHz spectrometers.

4.7. Archaeosome preparation and characterization

Archaeosomes were prepared by drying lipids from $CHCl_3$–CH_3OH (2:1) and hydrating 20–30 mg lipids at 40 °C in 2 mL of pyrogen-free water containing the test antigen OVA dissolved at 10 mg/mL. In all cases AG was mixed in $CHCl_3$–CH_3OH with the synthetic glyco-archaeols. An AG–cholesterol (80:20 mol %) archaeosome with OVA entrapped was prepared as above. The size of the vesicles in the preparations was decreased by sonication in a sonic water bath at 40 °C. Antigen not entrapped was removed by centrifuging (200,000*g*, R_{max} for 30 min) and washing three times from 8 mL volumes of PBS. Archaeosome preparations were filter sterilized using 0.45 μm filters and stored at 4 °C. Just prior to immunizations, dilutions were made to achieve 15 μg OVA/0.1 mL of PBS buffer (10 mM sodium phosphate, 160 mM NaCl, pH 7.1). Quantification of antigen loading by SDS polyacrylamide gel electrophoresis was as described, where antigen loading was based on salt corrected dry weights.[35] Average diameters were determined by particle size analysis using a 5 mW He–Ne laser (Nicomp 370). All glassware was rendered pyrogen-free by heating for 6 h at 180 °C.

4.8. Animal usage

To determine adjuvant activity, OVA entrapped in archaeosomes (OVA-archaeosomes) was used to immunize female C57BL/6 mice on days 0 and 21 (8 weeks old on first injection). Injections were subcutaneous at the tail base with 0.1 mL PBS containing 15 μg OVA entrapped in 0.2–0.63 mg lipids. Blood samples were collected on week 7 from the tail vein for anti-OVA antibody titration done by Elisa.[27] Spleens were collected on week 7 from duplicate mice to determine CTL activity using the Cr^{51}-assay with specific and non-specific targets EG.7 and EL-4, respectively.[8b] All protocols were approved by the Institutional Animal Care Committee, and were in compliance with guidelines set by the Canadian Council on Animal Care.

4.9. Statistical analysis

Means are reported as means ± S.D., and significance between means ($P < 0.05$) compared by two-tailed *t*-test.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.06.021.

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