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Chemoenzymatic Synthesis of a Glycolipid Library and Elucidation of the Antigenic Epitope for Construction of a Vaccine Against Lyme Disease

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Abstract: Lyme disease (LD) is the most common tick-borne disease in Europe, North America, and Asia. The etiologic agents of LD are spirochetes of the group *Borrelia burgdorferi* sensu lato, which possess a lipid content of 25–30% of the dry weight. The major glycolipid cholesteryl 6-*O*-acyl-β-D-galactopyranoside (ACGal), present in *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*, is a specific and highly prevalent antigen frequently recognized by antibodies in late-stage LD. Here we report a convenient route for the chemical synthesis of ACGal by employing a combination of chemical synthesis steps with enzymatic transformations. This synthesized molecule was compared with bacterial extracts by immunoblots with patient sera, confirming the preserved antigenicity. Next, a glycolipid library derived from the native molecules with variations in the fatty acyl moiety and derivatives in which the cholesterol has been replaced was designed and synthesized. The chemical structures were confirmed by 1D and 2D NMR spectroscopy and mass spectrometry. The native and synthetic glycolipids were utilized in immunoblots to determine the epitope recognized by antibodies in patient sera. By this method we could demonstrate that galactose, cholesterol, and a fatty acid with a minimal chain length of four carbon atoms comprises the essential structure for recognition by antibodies. Finally, this finding allowed the synthesis of a functionalized ACGal with an ω-mercapto group at the fatty acid and a facile protection and deprotection strategy. This antigenic hapten can be conjugated to a carrier protein to effect immunization against Lyme disease.

Keywords: adaptive immunity · *Borrelia burgdorferi* · carbohydrates · glycolipids · Lyme disease

Introduction

Lyme disease (LD) is the most common tick-borne disease in North America, Europe, and Asia. In endemic areas in the US the incidence is 29 cases per 100000 per year,[1] whereas in Central and Northern Europe it is up to 155 cases per 100000 individuals.[2] LD is a multisystemic chronic inflammatory disorder divided into three different clinical stages. *Erythema migrans* characterizes the early localized stage, whereas in the early disseminated stage, mainly the cardiac and nervous system are affected. The late stage is characterized by Lyme Arthritis or *Acrodermatitis chronica atrophicans*.[3] The etiologic agent of LD is *Borrelia burgdorferi*,[4,5] which is transmitted by ticks. Currently, the *B. burgdorferi* sensu lato group comprises at least 16 species with *B. burgdorferi* sensu stricto, *B. garinii*,[6] and *B. afzelii*[7] being of highest clinical importance.

Borrelia are spirochetes and possess an inner and outer membrane,[8] and are known to exhibit an extraordinary high lipid content of 25–30% of the dry weight.[9,10] In previous studies, focusing on *Borrelia burgdorferi* glycolipids, we

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and others identified four glycolipids: mono-α-D-galactopyranosyl-diacylglycerol,[9] 6-*O*-acylated cholesteryl β-D-galactopyranoside (ACGal), cholesteryl β-D-galactopyranoside (CGal),[11,12] and cholesteryl β-D-glucopyranoside.[10] Of these ACGal, which is present in all three pathogenic species *B. burgdorferi* s.s., *B. afzelii*, and *B. garinii*,[10] is the most abundant one comprising 45% of the glycolipids. Despite of its presence in *Borrelia spp.*, ACGal has as of yet only been described in higher plants.[13] Furthermore, we have shown that ACGal is an antigen in LD patients especially in the late stages of the disease with over 80% prevalence,[10] making it a unique target for vaccination.

Today, the most prominent antigens in LD are the outer surface proteins (Osp), which are used as recombinant proteins in serodiagnosis and for a vaccine (OspA), which has been withdrawn from the market.[14] Development of a new LD vaccine is challenged by the heterogeneity of the surface proteins between[15] or even within the genospecies.[16] The advantages of the nonproteinacious antigen ACGal abundant in all relevant *B. burgdorferi* species, but yet specific for these bacteria, are evident and a vaccination with it could lead to protection against LD worldwide.

Borrelia do not synthesize cholesterol,[17] and, furthermore, have to be supplied with fatty acids[8] (FA) due to the limited genome.[18] If cultured in BSK-H medium,[19] they utilize palmitic and oleic acid for ACGal biosynthesis,[11] but it is still unknown which fatty acids are incorporated during growth in ticks or vertebrate hosts.

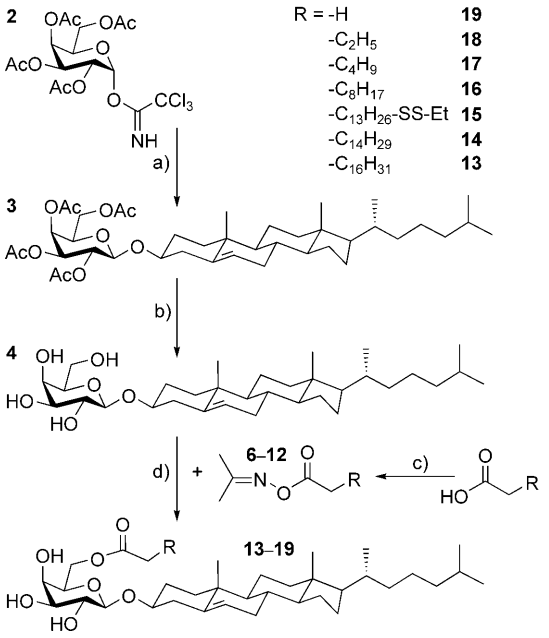
Published chemical syntheses for CGal and ACGal obtained the initial β-glycosylation by the Koenigs–Knorr reaction[20,21] or glycosyl iodide[22] and the subsequent regioselective acylation was performed with a sophisticated protecting-group strategy involving seven additional steps[21] or carbodiimide.[22] So far acylation is limited to palmitoyl and oleoyl groups and no evaluation of the recognition of the synthesized molecules by patient sera, potentially verifying the antigenicity, has been conducted. Moreover, the minimal recognized structure has not been defined yet.

The aim of this work was to develop a convenient synthesis route for ACGal. Based on our previous results in the synthesis of glycolipid libraries,[23,24] we intended to synthesize CGal first by the trichloroacetimidate β-glycosylation method and then perform the 6-*O*-acylation without protecting groups exploiting the increased nucleophilicity of the primary hydroxyl group. The first part was to synthesize ACGal containing palmitic and oleic acid as present in *B. burgdorferi* to confirm that this molecule is the major glycolipid antigen in LD. In a second step, a glycolipid library with variations in the FA chain length and the cholesterol moiety should be synthesized. This library should allow us to investigate the structural requirements of ACGal to be recognized by human antibodies and their specificity. With the information about the antigenic epitope gathered, we would then be able to introduce an additional functional group without affecting the antibody binding. Such functionalized ACGal derivatives will enable conjugation of this hapten to a carrier protein to elicit a full immune response upon vaccination.

Abstract in German: *Borreliose ist in Europa, Nordamerika und Asien die am häufigsten durch Zecken übertragene Krankheit. Die Erreger sind Spirochäten aus der Gruppe* Borrelia burgdorferi *sensu lato, die heute 16 bekannte Arten umfasst. Borrelien besitzen einen sehr hohen Lipidanteil von 25–30% des Trockengewichts. In den drei wichtigsten humanpathogenen Arten* B. burgdorferi *sensu stricto,* B. afzelii *und* B. garini *wurde das mengenmäßig häufigste Glykolipid—6-*O*-*acyliertes* *Cholesteryl-β-D-galactopyranosid—als ein spezifisches und weit verbreitetes Antigen identifiziert, gegen das in Spätstadien der Erkrankung Antikörper gebildet werden. In diesem Artikel beschreiben wir einen praktischen Syntheseweg für dieses Glykolipid, der aus einer Kombination von chemischen Syntheseschritten mit enzymatischen Transformationen besteht. Das aus Borrelien isolierte Glykolipid wurde mit dem synthetischen in Immunblots mit Patientenseren verglichen und seine Antigenität bestätigt. Anschließend haben wir basierend auf den natürlichen Leitstrukturen eine Glykolipidbibliothek konzipiert und synthetisiert, deren Derivate Veränderungen der Fettsäure und des Cholesterolanteils aufweisen. Die synthetisierten Strukturen wurden durch 1D und 2D NMR Spektroskopie und Massenspektrometrie charakterisiert. Sowohl die natürlichen als auch die synthetisierten Strukturen wurden verwendet, um in Immunblots die Reaktivität von Patientenseren damit zu untersuchen. Dadurch konnte die für eine Immunreaktion minimal erforderliche Strukur aufgeklärt werden. Für eine Erkennung durch Antikörper sind der β-Galactosylrest und das Cholesterol essentiell, der Fettsäureester in der 6-*O*-Position muß mindestens eine Butanoylgruppe sein. Schließlich erlaubte uns die Kenntnis des immunreaktiven Epitops eine funktionelle Gruppe einzufügen, die die Bindung der Antikörper nicht beeinflusst. Dazu haben wir eine ω-Mercaptopentadekansäure verwendet und eine einfache Schützung und Entschützung entwickelt. Das auf diese Weise erhaltene Glykolipid kann an ein Trägerprotein konjugiert und für die Immunisierung gegen die Borreliose eingesetzt werden.*

Results and Discussion

Facile synthesis of chlolesteryl-β-galactoside (CGal): The first step within the synthesis of acylated cholesteryl β-D-galactopyranoside was the glycosylation of cholesterol with galactose. Instead of the Koenigs–Knorr method,[25] as applied previously,[20,21] we chose the trichloroacetimidate approach[26] (Scheme 1). Initially the anomeric acetyl group of pentaacetylgalactose was removed chemoselectively with hydrazine acetate in DMF by heating at 50 °C for 2 h.[27] Following extraction and washing, 96% of **1** could be obtained. Next, hemiacetal **1** reacted with a threefold excess of trichloroacetonitrile in dichloromethane under basic catalysis (1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)) at RT for 2 h.[28]



Scheme 1. Synthesis of acylated cholesteryl galactosides: a) **2**, cholesterol in CH_2Cl_2 (0.85 equiv), cat. TMSOTf, 1.5 h at RT, triethylamine, flash chromatography, 77%; b) **3**, MeOH, sodium methylate, pH 9–10, 3 h at RT, amberlite IR-120(H^+), flash chromatography, 54%; c) fatty acid, acetone oxime (1 equiv), EDC (1.25 equiv), cat. DMAP in CH_2Cl_2, 2 h at RT, flash chromatography, 84–97%; d) **4**, fatty acid acetone oxime ester 6–12 (0.9 equiv), lipase Amano PS in pyridine, 10 days at 50°C, filtration, flash chromatography, 5–31%.

Due to the anomeric effect, the reaction proceeded diastereospecifically delivering exclusively the α-trichloroacetimidate **2**. The solvent was evaporated and the crude product was purified by flash chromatography in the presence of triethylamine yielding 88% of **2**.

Glycosylation of galactosyl-donor **2** with cholesterol was catalyzed by trimethylsilyl trifluoromethanesulfonate (TMSOTf) and performed in dichloromethane at RT for 1.5 h.[29] After quenching with triethylamine, the solvent was evaporated and chromatographed yielding **3** (77%). Whereas no formation of an orthoester byproduct was observed, traces of the β-elimination product of cholesterol were separated from the desired product. Next, the acetyl groups were deprotected under Zemplén conditions:[30] Compound **3** was dissolved in methanol and the deprotection was effected by a catalytic amount of sodium methoxide and stopped by neutralization with acidic amberlite resin. Although according to TLC the conversion was complete after 3 h, pure CGal **4** was recovered after column chromatography in a modest yield of 54%, which can be attributed to the weak solubility of the amphiphilic product. CGal dissolves well only in pyridine.

Regioselective acylation of CGal: It was our aim to acylate the primary 6-hydroxyl group in the last reaction step without the use of protecting groups to reduce the number of reactions and to facilitate the library synthesis. Our first attempts employed carbodiimides as coupling reagents to acylate the 6-*O*-position of CGal similar to published routes. Direct acylation of CGal with free palmitic acid by *N*,*N*-dicyclohexylcarbodiimide (DCC) at RT[31] in dioxane failed to lead to conversion. Activation of palmitic acid with 2,4-dinitrophenol by DCC in dichloromethane followed by transesterification of the dinitrophenyl active ester with CGal catalyzed by 4-dimethylaminopyridine (DMAP) in pyridine at RT[32] also failed to deliver the desired product. In contrast, direct acylation with palmitic acid, *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC), and catalytic amounts of DMAP in pyridine succeeded, but unfortunately included lots of multiple acylated byproducts diminishing the yield substantially. Reactions with different carbodiimides (DCC, EDC, or bis(trimethylsilyl) carbodiimid), different solvents (pyridine, dioxane, dichloromethane, dimethylsulfoxide, or sulfolan) at even reduced temperatures (RT down to −20°C) catalyzed by DMAP were not successful in the selective acylation of CGal at the 6-*O*-position.

Numerous reports on the enzymatic regioselective acylation of carbohydrates prompted us to pursue this approach. Although in most attempts, free fatty acids have been transferred to the primary hydroxyl group of glucose, it was reported to be more effective to use activated esters.[33] Therefore, we selected acetone oxime esters as acyl transfer agents and synthesized the esters with equimolar amounts of fatty acid and acetone oxime by using EDC and a catalytic amount of DMAP in dichloromethane. The reactions were carried out for 2 h at RT to yield 84–97% of the products after column chromatography (Scheme 1). Two lipases—Amano Lipase PS from *Burkholderia cepacia* and Amano Lipase from *Pseudomonas fluorescens*—were compared in analytical reactions. Reaction control by TLC revealed a slightly better performance for Amano Lipase PS and thus this enzyme was selected. The reactions were conducted as follows: CGal and FA acetone oxime ester were dissolved in dry pyridine and heated to 50°C. Amano lipase PS and molecular sieves were added and the reaction was carried out for five days. Then, fresh lipase and new molecular sieves were added and reacted for another five days. The lipase was filtered off and the crude product purified by column chromatography. The yield ranged for the non-cholesteryl lipids from 52 to 64% and for the cholesteryl lipids from 5 to 31%. This suggests that the cholesteryl moiety is either too hydrophobic or hinders the reaction sterically resulting in diminished conversion rates as described below. No diacylated compounds were observed, which indicates a regioselective acylation. Furthermore, lots of acetone oxime esters were recovered by the subsequent column chromatography, which shows that hydrolysis of these esters seems not to be a competing reaction. The advantages of the enzymatic reaction are its ease due to complete omission of protecting groups and the full regioselectivity, which we could not achieve by purely chemical methods. The disadvantages are the long reaction time and the partially rather low yields, although the educts are not expensive. The structures of all mentioned compounds were confirmed by complete 1D and

2D NMR spectroscopic assignment and substantiated by ESI- or MALDI-TOF-MS.

Confirmation that synthetic ACGal is an antigen: In our first reports, we demonstrated that antibodies against ACGal extracted from bacteria are abundant in patient sera in the late stages of disease,[10,11] but whether synthetic ACGal structures were also recognized by these antibodies remained to be elucidated. Hence, to verify the antigenicity of our compounds, we performed comparative immunoblottings with native and synthetic ACGal. Stripes of polyvinylidene difluoride (PVDF) membrane were dotted with equal amounts of 1) native ACGal extracted from *B. garinii* and comprising a mixture of the palmitoyl and oleoyl derivatives, 2) synthetic palmitoyl ACGal (**14**), and 3) synthetic CGal (**4**). The stripes were immunostained with the individual sera of four late-stage LD patients. Two patients had high titers against ACGal, one only a low titer, and the last one was negative, whereas all four did not recognize CGal, in line with previous results.[10,11] There were no apparent differences regarding intensity of chemiluminsecence between native and synthetic ACGal (Figure 1). This finding corroborates that ACGal is a major antigen in LD.

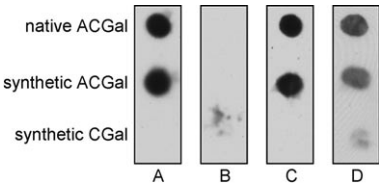
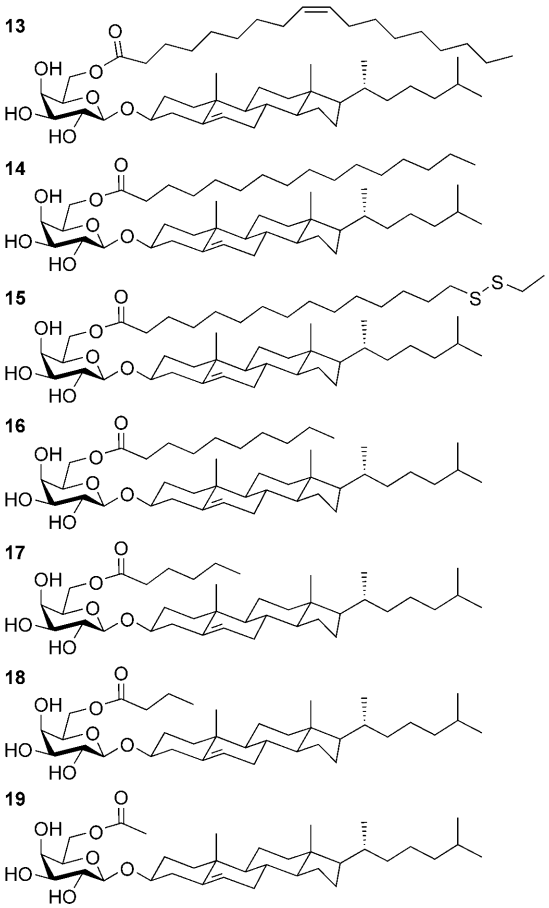


Figure 1. Comparative immunoblotting of native and synthetic ACGal with individual patient sera: Native ACGal extracted from *B. garinii*, synthetic ACGal (with palmitic acid, **14**), and synthetic CGal (**4**, 1 μg each) was pipetted on PVDF membrane stripes. The single stripes were incubated with the sera of four different patients diagnosed for late-stage LD (serum dilutions: A/C 1:8000, B/D 1:250) for 2.5 h and developed as described in the Experimental Section.

Design and synthesis of a glycolipid library to elucidate the immunogenic epitope: To elucidate the immunoreactive epitope, we designed a library of glycolipid derivatives. ACGal contains three different moieties—the carbohydrate, cholesterol, and a fatty acid ester—which all can be subject to modification. The fact that ACGlc from *B. hermsii* is not recognized by antibodies in patient sera[11] indicates that the configuration of the carbohydrate residue is crucial for antigenicity, and therefore we left this moiety unmodified. Thus, our main focus was on a variation of the fatty acyl residue (Scheme 2), which is facilitated by its coupling in the last step of the synthesis. Oleic acid (C 18:1), palmitic acid (C 16:0), decanoic (C 10), hexanoic (C 6), butyric (C 4), and acetic acid (C 2) were chosen as building blocks. Eventually, to assess the necessity of the cholesterol residue as well, we decided to replace this by methyl and phenyl groups (Scheme 3).

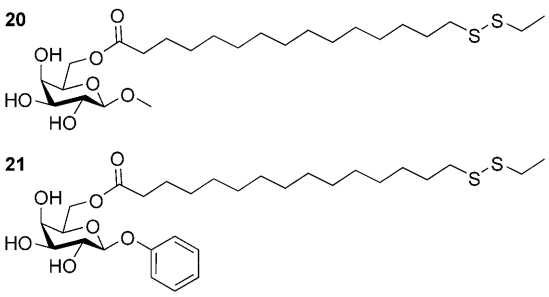
For the enzymatic acylation of cholesteryl galactoside with different FAs, the latter have to be activated as acetone



Scheme 2. Synthesized acylated cholesteryl galactosides: The glycolipid library consists of seven ACGal derivatives with a varying FA chain length from C 18:1 to C 2.

oxime active esters first. These reactions were performed by utilizing carbodiimides for oleic to butyric acid as described before. By flash chromatography, we gained highly purified oxime esters (**6**–**11**) in excellent yields of 84.5 to 97%.

The acetone oxime ester of acetic acid was made in pyridine with acetone oxime and a sixfold excess of acetic anhydride overnight. Evaporation yielded 92% of product **12**. The lipase driven transesterification from acetone oxime to the secondary hydroxyl group of CGal to gain ACGal was



Scheme 3. Synthesized acylated non-cholesteryl galactosides: Two 6-*O*-acylated galactosides were synthesized that contain methyl or phenyl groups as a replacement for cholesterol.

conducted as described. The yields of the reaction after column chromatography varied significantly without any clear correlation between structure and yield. The highest yields were achieved for C6-ACGal (**17**) and C4-ACGal (**18**) with 31 and 29% respectively. In medium yields of 16 and 14% C18:1-ACGal (**13**) and C10-ACGal (**16**) could be obtained, whereas C16-ACGal (**14**), C2-ACGal (**19**), and C15SSEt-ACGal (**15**) yielded only 8.5, 7.5, and 5.1%, respectively (Scheme 2).

For the synthesis of acylated non-cholesteryl galactopyranosides (Scheme 3), we used commercially available methyl and phenyl galactopyranoside and acylated these starting materials with **8** catalyzed by lipase. The yields for enzymatic acylations of non-cholesteryl containing galactosides were much higher than for the cholesteryl-containing derivatives—64% for the acylated methyl β-D-galactopyranoside (**20**) and 52% for the acylated phenyl β-D-galactopyranoside (**21**). Obviously, the more sterically demanding the acyl acceptor is, the lower the conversion rate. The diminished hydrophobicity of the synthesized glycolipids with decreasing chain length of the FA was demonstrated by TLC (Figure 2).

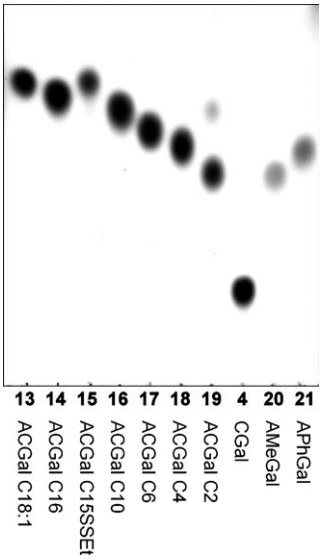


Figure 2. Thin-Layer chromatogram of the synthesized glycolipids: 2 μl of each of the cholesterol-containing lipids (**13**–**19** and **4**) and 4 μl of each of the cholesterol-free lipids (**20** and **21**) were pipetted on a TLC sheet and the run was performed in chloroform/methanol 85:15 (v/v). The spots were detected by using molybdenum stain. Depicted is the part from the start line to $R_f = 0.7$.

Elucidation of the minimal structure recognized by antibodies in LD patient sera: To determine the structural features essential for recognition by human antibodies, additional immunoblots were performed. Equal amounts of the 10 synthesized glycolipids, whole cell lysate and total lipids of *B. burgdorferi* s.s., the purified ACGal fractions of *B. burgdorferi* s.s., *B. afzelii*, *B. garinii*, purified ACGlc of *B. hermsii*, and free galactose and free cholsterol were blotted on a PVDF membrane and immunostained with the pooled sera of the four patients (Figure 3).

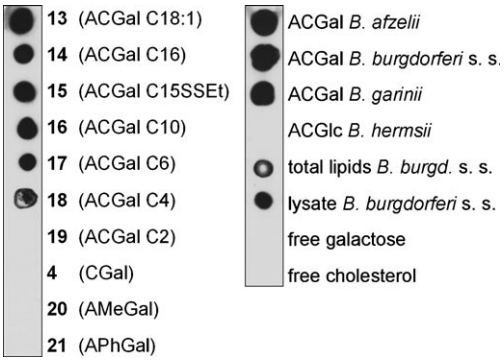
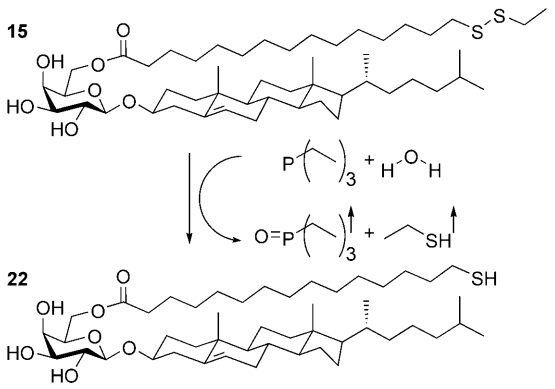


Figure 3. Immunoblotting of native and synthetic structures with pooled patient sera: Synthetic glycolipids, extracted ACGal fractions from *B. burgdorferi* s.s., *B. garinii*, and *B. afzelii*, extracted ACGlc from *B. hermsii*, total lipids, and a whole cell lysate from *B. burgdorferi* s.s. as well as free galactose and free cholesterol were pipetted on PVDF membrane stripes (1 μg each). The stripes were incubated with the pooled sera of four late-stage LD patients for 2.5 h and developed as described in the Experimental Section.

As expected, the native compounds containing ACGal (lysate, total lipids, ACGal fractions) were all recognized by the patient sera. The only structure with an altered carbohydrate moiety, ACGlc, was not recognized by the sera, in line with our previous results. Since D-galactose and D-glucose are epimers with a distinct configuration at C4, this indicates that the antibodies are highly specific for the galactose moiety, which is unambiguously essential for recognition. In contrast, neither free Gal nor cholesteryl galactopyranoside (CGal, **4**) are sufficient for recognition and an acyl residue seems indispensable. Of the 6-*O*-acylated CGal molecules, all those with long-chain fatty acyl moieties were recognized and apparently antibody binding is unaffected whether they are saturated (palmitoyl, 16:0, **14**), unsaturated (oleoyl, 18:1, **13**), or terminally substituted with a mercapto function (ω-(2-ethyldisulfanyl)pentadecanoyl, **15**). The derivatives with shorter FAs, such as decanoyl (10:0, **16**) or hexanoyl (6:0, **17**), react well too but already butanoyl (**18**) exhibits diminished reactivity, whereas acetyl (**19**) is not recognized at all. Eventually both cholesterol-free synthetic glycolipids (**20** and **21**) were not immunoreactive, which demonstrates the importance of the cholesteryl moiety for antibody binding. The acylated methyl galactopyranoside (**20**) possesses only a methyl group as aglycon replacing the cholesterol scaffold totally lacking the second hydrophobic arm of the molecule, which can lead to an altered conformation abrogating binding completely. In contrast, acylated phenyl galactopyranoside (**21**) contains an aromatic ring as aglycon replacing the aliphatic A-ring of cholesterol which does not suffice either for immunoreactivity (Figure 3). The minimal immunoreactive structure for the highly specific anti-ACGal antibodies would, therefore, comprise a cholesteryl β-D-galactopyranoside, which has to be 6-*O*-acylated with butyric acid.

Synthesis of a functionalized ACGal molecule for vaccination studies: The insight gained in the recognition of ACGal allowed us to design a modified ACGal for vaccination studies carrying a functional group at a variable position remote from the epitope. Facilitated by the coupling of the FA in the last step of the synthesis, we planned to introduce a terminally substituted FA—ω-mercaptopentadecanoic acid. To prevent a polymerization of this nucleophilic functional group with the carboxyl group during acetone oxime activation with EDC/DMAP the mercapto group had to be protected beforehand. Initially, dimethoxytrityl was selected as a protecting group;[34] however, the lipase failed to show any conversion of the dimethoxytrityl-protected acetone oxime active ester. In a second attempt, 2,4-dinitrothiophenyl (S-DNP) was employed for protection. The terminal fatty acyl mercaptane was converted with 2,4-dinitrophenylsulfenyl chloride followed by acetone oxime activation and enzymatic acylation. Upon deprotection of the DNP-S group with tributylphosphine, the released DNP-thiol was removed to avoid competition with the ACGal-SH. Contact with air, for instance during column chromatography, however, led to oxidation and the undesired formation of homodimers of ACGal-SH and ACGal-S-S-DNP heterodimers. Thus, ethanthiol was selected as the protecting group, allowing for the complete evaporation of the byproduct of deprotection. Unprotected 15-mercaptopentadecanoic acid was heated with diethyl disulfide in triethylamine for 17 h at 70 °C.[35] Evaporation delivered pure 15-(2-ethyldisulfanyl) pentadecanoic acid (**5**). Activation with acetone oxime in the described manner yielded 89 % of active ester **8**. After the subsequent enzymatic acylation of CGal with this FA and column chromatography, 180 mg (5.1 %) of the pure product could be obtained (Scheme 1).

Reductive cleavage of the mercaptoethyl group was first examined with the protected FA **5**. We found that pyridine/H_2O 9:1 (v/v) is the best solvent for this reaction and employed triethylphosphine as the reducing agent. Although tributylphosphine and tris(2-carboxyethyl) phosphine (TCEP) also worked well for the reduction, triethylphosphine had the advantage that the remaining reagent and its oxidation product, triethylphosphineoxide, were removed completely by evaporation under high vacuum (HV) as proven by NMR spectroscopy. In addition, the reduction could be monitored conveniently in the NMR tube and an equimolar amount of triethylphosphine was sufficient to complete the reaction within a few minutes. Analogously, the final deprotection of **15** (Scheme 4) to gain the unprotected 15-mercaptopentadecanoyl-CGal **22** in an elegant way without any further purification can be utilized for a conjugation of this ACGal to proteins or beads.



Scheme 4. Deprotection of the mercaptoethyl protecting group: **15** in pyridine/H_2O 9:1, triethylphosphine (1 equiv) in THF, 30 min at RT, evaporation, 99 %.

Conclusion

To summarize, we have established a straightforward, six-step synthesis of 6-*O*-acylated cholesteryl β-D-galactopyranosides and applied it to the preparation of a glycolipid library with 10 derivatives. The synthetic ACGal library was employed to confirm native ACGal as an antigen inducing the formation of antibodies in Lyme disease and to determine the minimal structure recognized by human antibodies as cholesteryl 6-*O*-butanoyl-β-D-galactopyranoside. Identification of the epitope enabled the rational design of a functionalized glycolipid that can be conjugated to a carrier protein for vaccination and could overcome the limitations of proteinacious antigens.

Experimental Section

General remarks: All solvents and reagents were purchased from Sigma–Aldrich, Fluka (Taufkirchen, Germany), Acros Organics (Geel, Belgium), or Roth (Karlsruhe, Germany). All solvents were reagent grade and used without further purification. Dry solvents were acquired and stored over molecular sieves. The applied lipase--Amano Lipase PS (Sigma)—is from the bacteria *Burkholderia cepacia* formerly classified as *Pseudomonas cepacia*. TLC was performed on silica gel 60 F_{254} aluminum sheets (Merck, Darmstadt, Germany) and the compounds detected by staining with molybdenum stain (dipped into 1 M H_2SO_4, 40 mM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 2.5 mM $Ce(SO_4)_2{\cdot}4H_2O$ and heated to 250 °C). Flash chromatography was conducted in glass columns packed with silica gel 60, particle size 40–63 μm (Merck) and the collected product fractions were filtered through a 0.2 μm PTFE membrane filter (Schleicher & Schuell, Dassel, Germany). The NMR samples were dissolved in [D]chloroform or [D_5]pyridine (Deutero, Kastellaun, Germany or Euriso-Top, Saarbrücken, Germany) and the spectra recorded in 5 mm tubes (Norell, Landisville, NJ, USA) at 300 K. The experiments were run on a Bruker Avance 300 spectrometer (Bruker, Karlsruhe, Germany) at 300.2 (^{1}H and all-proton-detected 2D spectra) or 75.5 MHz (^{13}C, DEPT 135, and all-carbon-detected 2D spectra) and processed by using TopSpin 2.1 (Bruker). The ^{1}H chemical shift, multiplicity, and coupling constants were taken from ^{1}H spectra and for the superimposed signals from HMQC spectra. The ^{1}H shifts are given in ppm referenced to internal TMS ($\delta_H = 0.00$ ppm) and the ^{13}C shifts to the solvent signal ($CDCl_3$: $\delta_C = 77.0$ ppm, pyridine: $\delta_C = 123.5$ ppm). In general, the signal assignments were made according to ^{1}H, ^{13}C, DEPT 135, and homo- and heteronuclear correlation experiments (DQF-Cosy, HMQC, HMBC). Those cholesterol signals, which could not be deduced from multidimensional spectra, were assigned according to reference data.[36]

The mass spectra were measured without a column run by using an Agilent 1100 series liquid chromatography system equipped with a single quadrupole mass spectrometer with electrospray ionization (Agilent, Bö-

blingen, Germany). The MALDI-TOF mass spectra of the glycolipids were measured on an AB 4700 MALDI-TOF/TOF (Applied Biosystems, Carlsbad, CA, USA). After internal calibration with two standards (717.405, 1479.012 Da) a device accuracy of 10 ppm could be achieved. As matrices 2,5-dihydroxybenzoic acid and 2,4,6-trihydroxyacetophenone were used and the given m/z is the mean of both signals.

The molecular modeling was conducted by the Sybyl 7.0 package (Tripos Inc., St. Louis, MO, 63144 USA).

The NMR spectroscopic assignments of **1** and **2** and the full experimental details for compounds **5**, **7**–**12**, **14**–**19**, and **21** are supplied in the Supporting Information.

Cholesteryl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (3):[29] Product **2** (0.2 g, 0.4 mmol) and cholesterol (0.13 g, 0.34 mmol) were dissolved in dry dichloromethane (4 mL) at RT and the reaction was started by the addition of catalytic TMSOTf (5 μL, 30 μmol). After stirring for 1.5 h, the reaction was quenched with triethylamine (5 μL). The solution was evaporated and the crude product purified by column chromatography (hexane/ethyl acetate 1:1) to yield **3** as a colorless, crystal-like solid (0.185 g, 77%). R_f=0.59 (hexane/ethyl acetate 3:1); ^{1}H NMR (300 MHz, $CDCl_3$, 300 K, TMS): δ=5.37 (Cho H-6), 5.37 (s, 1H; Gal H-4), 5.17 (t, $J_{2,3}$=10.2 Hz, 1H; Gal H-2), 5.03 (dd, $J_{3,4}$=3.0 Hz, 1H; Gal H-3), 4.56 (d, $J_{1,2}$=7.8 Hz, 1H; Gal H-1β), 4.18 (m, $J_{6b,5}$=4.5 Hz, 1H; Gal H-6b), 4.12 (m, $J_{6a,6b}$=11.1 Hz, 1H; Gal H-6α), 3.90 (t, $J_{5,6a}$=6.6 Hz, 1H; Gal H-5), 3.49 (Cho H-3), 2.22 (Cho H-4α), 2.18 (Cho H-4β), 2.15 (s, 3H; Ac4 H-2), 2.06 (s, 3H; Ac2 H-2), 2.04 (s, 3H; Ac6 H-2), 2.02 (Cho H-12β), 1.99 (s, 3H; Ac3 H-2), 1.97 (Cho H-7β), 1.88 (Cho H-2α), 1.86 (Cho H-1β), 1.61 (Cho H-2β), 1.56 (Cho H-15α), 1.52 (Cho H-11α), 1.51 (Cho H-7α), 1.51 (Cho H-25), 1.47 (Cho H-11β), 1.43 (Cho H-8), 1.36 (Cho H-20), 1.32 (Cho H-23R), 1.31 (Cho H-22R), 1.27 (Cho H-16β), 1.17 (Cho H-12α), 1.12 (Cho H-23S), 1.12 (Cho H-24), 1.08 (Cho H-17), 1.05 (Cho H-1α), 1.04 (Cho H-15β), 0.99 (Cho H-14), 0.99 (Cho H-19), 0.99 (Cho H-22S), 0.92 (Cho H-9), 0.91 (Cho H-21), 0.88 (Cho H-27), 0.85 (Cho H-26), 0.68 ppm (Cho H-18); ^{13}C NMR (75 MHz, $CDCl_3$, 300 K, $CDCl_3$): δ=170.6 (Ac6 C-1), 170.5 (Ac4 C-1), 170.3 (Ac3 C-1), 169.7 (Ac2 C-1), 139.9 (Cho C-5), 122.0 (Cho C-6), 99.9 (Gal C-1), 80.1 (Cho C-3), 70.9 (Gal C-3), 70.3 (Gal C-5), 69.0 (Gal C-2), 67.0 (Gal C-4), 61.1 (Gal C-6), 56.5 (Cho C-14), 55.9 (Cho C-17), 49.9 (Cho C-9), 42.1 (Cho C-13), 39.5 (Cho C-12), 39.2 (Cho C-24), 38.7 (Cho C-4), 36.9 (Cho C-1), 36.4 (Cho C-10), 35.9 (Cho C-22), 35.5 (Cho C-20), 31.67 (Cho C-7), 31.61 (Cho C-8), 29.2 (Cho C-2), 27.9 (Cho C-16), 27.7 (Cho C-25), 24.0 (Cho C-15), 23.5 (Cho C-23), 22.4 (Cho C-27), 22.2 (Cho C-26), 20.8 (Cho C-11), 20.43 (Ac2 C-2), 20.26 (Ac6 C-2), 20.23 (Ac4 C-2), 20.18 (Ac3 C-2), 19.0 (Cho C-19), 18.4 (Cho C-21), 11.5 ppm (Cho C-18); ESI-MS: m/z: calcd for $C_{41}H_{64}NaO_{10}$: 739.4 $[M+Na]^+$; found: 739.4.

Cholesteryl β-D-galactopyranoside (4):[30] Compound **3** (7.26 g, 10.1 mmol) was dissolved in dry methanol (100 mL). With 0.5 M sodium methylate in methanol the pH value was adjusted to 9–10 and the solution was stirred at RT for 3 h. The solution was neutralized with amberlite IR-120(H^+), filtered, and evaporated to give the crude product **4** as a colorless solid (5.48 g, 99%). After further purification by flash chromatography (chloroform/methanol 88:12), product **4** (2.99 g, 54%) was obtained. R_f=0.18 (chloroform/methanol 85:15); ^{1}H NMR (300 MHz, [D_5]pyridine, 300 K, TMS): δ=6.39 (s, 4H; Gal OH), 5.35 (d, $J_{6,7a}$=4.2 Hz, 1H; Cho H-6), 4.94 (d, $J_{1,2}$=7.6 Hz, 1H; Gal H-1β), 4.58 (d, $J_{4,5}$=3.0 Hz, 1H; Gal H-4), 4.46 (m, $J_{6b,5}$=4.8 Hz, 1H; Gal H-6b), 4.44 (t, $J_{2,3}$=10.6 Hz, 1H; Gal H-2), 4.42 (m, $J_{6a,6b}$=10.2 Hz, 1H; Gal H-6a), 4.19 (dd, $J_{3,4}$=3.2 Hz, 1H; Gal H-3), 4.08 (t, $J_{5,6a}$=5.9 Hz, 1H; Gal H-5), 3.95 (Cho H-3), 2.71 (Cho H-4α), 2.44 (Cho H-4β), 2.14 (Cho H-2α), 1.98 (Cho H-12β), 1.92 (Cho H-7β), 1.81 (Cho H-16α), 1.73 (Cho H-1β), 1.73 (Cho H-2β), 1.56 (Cho H-15α), 1.54 (Cho H-25), 1.54 (Cho H-7α), 1.47 (Cho H-11α), 1.42 (Cho H-20), 1.40 (Cho H-11β), 1.39 (Cho H-23R), 1.38 (Cho H-22R), 1.37 (Cho H-8), 1.26 (Cho H-16β), 1.19 (Cho H-23S), 1.17 (Cho H-24), 1.09 (Cho H-15β), 1.09 (Cho H-12α), 1.08 (Cho H-17), 1.07 (Cho H-22S), 0.99 (Cho H-1α), 0.94 (Cho H-14), 0.94 (Cho H-19), 0.91 (Cho H-9), 0.91 (Cho H-26), 0.90 (Cho H-27), 0.67 ppm (Cho H-18); ^{13}C NMR (75 MHz, [D_5]pyridine, 300 K, [D_5]pyridine): δ=141.0 (Cho C-5), 121.9 (Cho C-6), 103.2 (Gal C-1), 78.0 (Cho C-3), 76.9 (Gal C-5), 75.4 (Gal C-3), 72.7 (Gal C-2), 70.3 (Gal C-4), 62.5 (Gal C-6), 56.9 (Cho C-14), 56.4 (Cho C-17), 50.4 (Cho C-9), 42.5 (Cho C-13), 40.0 (Cho C-12), 39.8 (Cho C-24), 39.4 (Cho C-4), 37.5 (Cho C-1), 36.9 (Cho C-10), 36.5 (Cho C-22), 36.1 (Cho C-20), 32.21 (Cho C-7), 32.09 (Cho C-8), 30.3 (Cho C-2), 28.5 (Cho C-16), 28.3 (Cho C-25), 24.5 (Cho C-15), 24.2 (Cho C-23), 23.0 (Cho C-27), 22.7 (Cho C-26), 21.3 (Cho C-11), 19.4 (Cho C-19), 19.0 (Cho C-21), 12.0 ppm (Cho C-18); MALDI-MS: m/z: calcd for $C_{33}H_{56}NaO_6$: 571.398 $[M+Na]^+$; found: 571.385.

Acetone oxime oleate (6): Oleic acid (4.81 mL, 15 mmol), acetone oxime (1.1 g, 15 mmol), EDC (3.59 g, 18.8 mmol), and DMAP (183 mg, 1.5 mmol) were dissolved in dry CH_2Cl_2 (40 mL) and stirred at RT for 3 h. Silica gel was added and the solution evaporated. The crude product was purified by flash chromatography (hexane/acetone 10:1) to give **6** as a clear, colorless liquid (4.77 g, 94%). R_f=0.82 (toluene/acetone 3:1); ^{1}H NMR (300 MHz, $CDCl_3$, 300 K, TMS): δ=5.33 (t, J=4.4 Hz, 2H; FA H-9, H-10), 2.40 (t, J=7.5 Hz, 2H; FA H-2), 2.04 (s, 3H; oxime H-*trans*), 2.02 (4H; FA H-8, H-11), 1.99 (s, 3H; oxime H-*cis*), 1.69 (m, J=7.0 Hz, 2H; FA H-3), 1.32 (2H; FA H-4), 1.27 (m, 18H; FA H-5–H-7, H-12–H-17), 0.88 ppm (t, J=6.3 Hz, 3H; FA H-18); ^{13}C NMR (75 MHz, $CDCl_3$, 300 K, $CDCl_3$): δ=170.7 (FA C-1), 163.1 (oxime C-2), 129.6 (FA C-10), 129.3 (FA C-9), 32.6 (FA C-2), 31.6 (FA C-16), 29.42, 29.32, 29.18, 28.97 (2C), 28.79, 28.76 (FA C-5–C-7, C-12–C-15), 28.72 (FA C-4), 26.86 (FA C-11), 26.80 (FA C-8), 24.6 (FA C-3), 22.3 (FA C-17), 21.6 (oxime C-*trans*), 16.5 (oxime C-*cis*), 14.1 ppm (FA C-18); ESI-MS: m/z: calcd for $C_{21}H_{40}NO_2$: 338.3 $[M+H]^+$; found: 338.3.

Cholesteryl 6-*O*-oleoyl-β-D-galactopyranoside (13, Figure 4):[33] CGal **4** (2.5 g, 4.6 mmol), compound **6** (1.38 g, 4.1 mmol), lipase Amano PS (3 g), and molecular sieve were suspended under argon in dry pyridine (24 mL) and agitated at 50 °C for five days. Extra lipase (2.5 g) was added and the suspension was stirred for an additional 5 days. The lipase was filtered off and the solution supplied with silica gel evaporated. The yellow powder was purified by column chromatography by stepwise elution with chloroform, chloroform/methanol 98:2, 95:5, 90:10, and 50:50 to provide product **13** as a colorless, amorphous solid (0.53 g, 16%). R_f=0.56 (chloroform/methanol 85:15); ^{1}H NMR (300 MHz, $CDCl_3$, 300 K, TMS): δ=5.34 (FA H-9, H-10), 5.33 (Cho H-6), 4.38 (Gal H-6b), 4.31 (Gal H-1β), 4.24 (Gal H-6a), 3.90 (Gal H-4), 3.64 (Gal H-5), 3.62 (Gal H-2), 3.57 (Gal H-3), 3.53 (Cho H-3), 2.40 (Cho H-4α), 2.28 (FA H-2), 2.25 (Cho H-4β), 2.01 (Cho H-12β), 2.01 (FA H-8, H-11), 1.94 (Cho H-7β), 1.82 (Cho H-1β), 1.59 (Cho H-2α), 1.59 (FA H-3), 1.52 (Cho H-25), 1.47 (Cho H-7α), 1.47 (Cho H-11β), 1.38 (Cho H-8), 1.37 (Cho H-23R), 1.36 (Cho H-20),

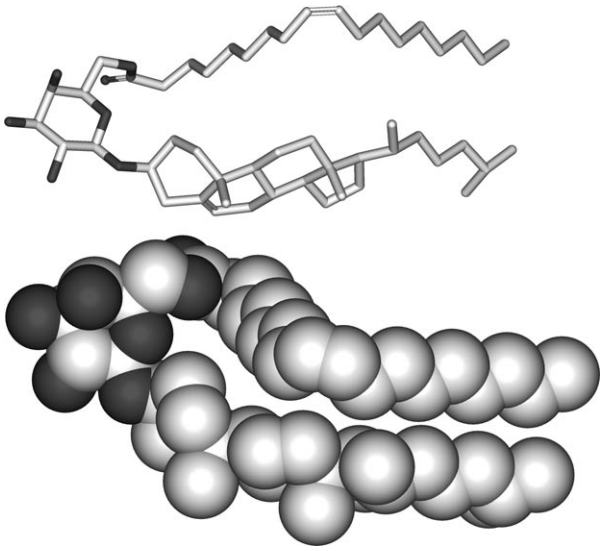


Figure 4. Structural depiction of ACGal C18:1: Representation as a capped sticks (top) and a space filled model (bottom). Built and minimized by using Sybyl 7.0. First Steepest descent then a conjugate gradient until a gradient of 0.05 kcal mol^{-1} Å^{-1}) was reached for each routine.

1.32 (Cho H-22R), 1.292, 1.271 (FA H-4…7, H-12…17), 1.26 (Cho H-2β), 1.13 (Cho H-16β), 1.12 (Cho H-24), 1.12 (Cho H-12α), 1.12 (Cho H-23S), 1.08 (Cho H-17), 1.06 (Cho H-24), 1.02 (Cho H-1α), 1.00 (Cho H-15β), 0.98 (Cho H-19), 0.96 (Cho H-14), 0.92 (Cho H-9), 0.91 (Cho H-21), 0.91 (Cho H-22S), 0.88 (Cho H-26), 0.88 (FA H-18), 0.84 (Cho H-27), 0.68 ppm (Cho H-18); ^{13}C NMR (75 MHz, $CDCl_3$, 300 K, $CDCl_3$): $\delta =$ 173.5 (FA C-1), 140.4 (Cho C-5), 130.0, 129.6 (FA C-9, C-10), 122.0 (Cho C-6), 101.8 (Gal C-1), 79.7 (Cho C-3), 73.5 (Gal C-3), 72.5 (Gal C-5), 71.0 (Gal C-2), 68.9 (Gal C-4), 63.2 (Gal C-6), 56.8 (Cho C-14), 56.2 (Cho C-17), 50.2 (Cho C-9), 42.3 (Cho C-13), 39.8 (Cho C-12), 39.5 (Cho C-24), 38.8 (Cho C-4), 37.3 (Cho C-1), 36.6 (Cho C-10), 36.2 (Cho C-22), 35.8 (Cho C-20), 34.2 (FA C-2), 31.88 (Cho C-7), 31.85 (Cho C-8), 29.79, 29.74, 29.52, 29.35, 29.29 (4C) (FA C-4–7, C-12–17), 29.63 (Cho C-2), 28.2 (Cho C-16), 28.0 (Cho C-25), 27.2 (2C) (FA C-8, C-11), 25.0 (FA C-3), 24.3 (Cho C-15), 23.9 (Cho C-23), 22.8 (Cho C-27), 22.7 (Cho C-11), 22.5 (Cho C-26), 19.4 (Cho C-19), 18.7 (Cho C-21), 14.1 (FA C-18), 11.8 ppm (Cho C-18); MALDI-MS: m/z: calcd for $C_{51}H_{88}NaO_7$: 835.643 $[M+Na]^+$; found: 835.634.

6-*O*-[15-(2-Ethyldisulfanyl)pentadecanoyl]methyl-β-D-galactopyranoside (20):[33] Methyl-β-D-galactopyranoside (0.61 g, 3.2 mmol), **8** (0.99 g, 2.5 mmol), and lipase (1.3 g) were suspended in dry pyridine (10 mL) and stirred at 50 °C for 8 days. The crude product was filtered and chromatographed by stepwise elution with chloroform, chloroform/methanol 98:2, 95:5, 90:10, and 80:20 to provide product **20** as a colorless powder (0.83 g, 64 %). $R_f = 0.39$ (chloroform/methanol 85:15); ^{1}H NMR (300 MHz, $CDCl_3$, 300 K, TMS): $\delta = 4.33$ (m, 1H; Gal H-6a), 4.26 (m, 1H; Gal H-6b), 4.15 (d, $J_{1,2} = 7.2$ Hz, 1H; Gal H-1β), 3.91 (s, 1H; Gal H-4), 3.66 (t, $J_{5,6a} = 6.8$ Hz, 1H; Gal H-5), 3.62 (d, $J_{2,3} = 8.4$ Hz, 1H; Gal H-2), 3.58 (1H; Gal H-3), 3.53 (s, 3H; Me), 2.70 (q, 1.9H; Et H-1), 2.68 (t, 1.9H; FA H-15), 2.31 (t, 2H; FA H-2), 1.67 (m, 2H; FA H-14), 1.60 (m, 2H; FA H-3), 1.37 (2H; FA H-13), 1.32 (t, 3H; Et H-2), 1.26 ppm (m, 18H; FA H-4–H-12); ^{13}C NMR (75 MHz, $CDCl_3$, 300 K, $CDCl_3$): $\delta =$ 173.7 (FA C-1), 103.9 (Gal C-1), 73.3 (Gal C-3), 72.5 (Gal C-5), 70.9 (Gal C-2), 68.8 (Gal C-4), 63.1 (Gal C-6), 56.9 (Me), 39.2 (FA C-15), 34.0 (FA C-2), 32.7 (Et C-1), 29.54 (3C), 29.49, 29.40 (2C), 29.22, 29.13 (2C) (FA C-4–C-12), 29.08 (FA C-14), 28.4 (FA C-13), 24.8 (FA C-3), 14.4 ppm (Et C-2); MALDI-MS: m/z: calcd for $C_{24}H_{46}NaO_7S_2$: 533.258 $[M+Na]^+$; found: 533.255.

Cholesteryl 6-*O*-(15-mercaptopentadecanoyl)-β-D-galactopyranoside (22): Compound **15** (24.6 mg, 28.4 μmol) was dissolved in pyridine/H_2O 9:1 (217 μL). Whilst stirring, a solution of triethylphosphine in THF (1.0 M, 28.4 μL, 3.36 mg, 28 μmol) was added to the resulting mixture. After 30 min, the solution was evaporated and the mixture dried under HV. The split protecting group, remaining triethylphosphine, and resulting triethylphosphineoxide were totally vaporized and only the pure, slightly yellow product remained (99 % according to NMR spectroscopy). However, the product is air sensitive and was immediately used further (Scheme 2). $R_f = 0.53$ (chloroform/methanol 85:15).

Immunoblotting of glycolipids: The 10 synthesized glycolipids (**4** and **13**–**21**), the isolated ACGals from *B. afzelii*, *B. burgdorferi* s.s., and *B. garinii*, isolated ACGlc from *B. hermsii* (described previously[10]) as well as free cholesterol were dissolved in *tert*-butanol/H_2O 5:1 (w/v) at 1 mg mL^{-1}. The total lipids from *B. burgdorferi* s.s. were dissolved in chloroform at 1 mg mL^{-1}. The lysate of *B. burgdorferi* s.s. (1.5 mg mL^{-1} protein content) and free galactose were dissolved in H_2O. A volume equivalent to 1 μg (usually 1 μL) of each substance was directly pipetted onto 1 cm stripes of a hydrophilized PVDF membrane (Millipore, Bedford, MA, USA). The lipid amount spotted is over 100-fold in excess over the quantity sufficient for antibody binding. Therefore, differences in antibody attachment are solely caused by distinct structural properties of the lipids. The membranes were blocked with PBS/0.05 % Tween 20/5 % skim milk (Fluka, Buchs, Switzerland) for 1 h at RT and washed with PBS/Tween. Membranes were incubated with the pooled sera of four late-stage LD patients (diagnosed by ELISA and confirmative Immunoblot) diluted 1:4000 fold in PBS/Tween/skim milk for 2.5 h at RT and washed three times with PBS/Tween. Next the membranes were incubated with a rabbit anti-human immunoglobuline G—horseradish peroxidase conjugate (Santa Cruz, Palo Alto, CA, USA) diluted 50000 fold in PBS/Tween/skim milk for 1.5 h at RT. The blots were washed four times with PBS and dots were detected with the ECL X-ray film system (ThermoScientific, Rockford, IL, USA) as recommended by the manufacturer's protocol. A signed consent for the use of the sera was obtained from the patients.

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