The total synthesis of supposedly immunoactive glycolipids from *S. pneumoniae* and a re-evaluation of their immunological activity

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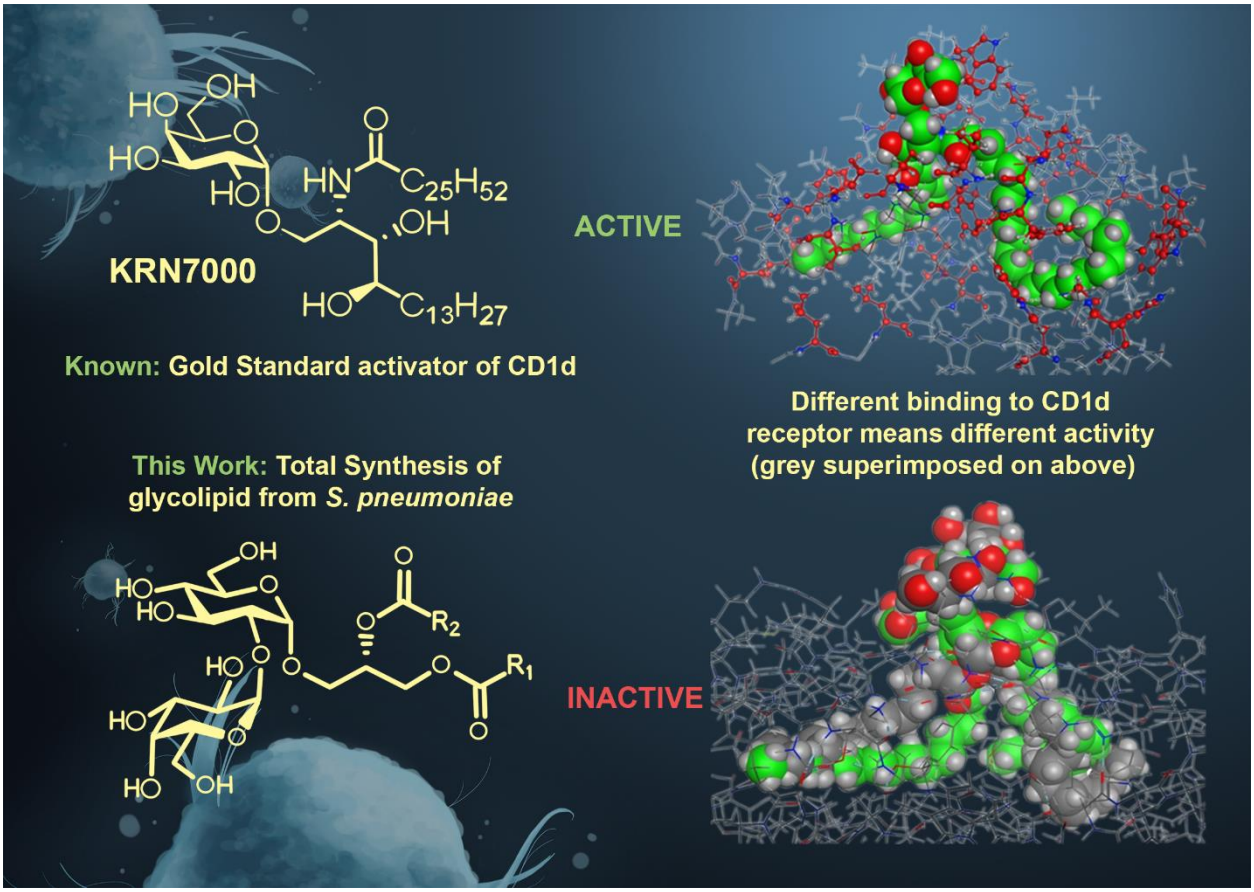
Abstract:

Invariant natural killer T cells (iNKT), a subclass of white blood cells, are responsible for the production of pro-inflammatory cytokines which induce a systemic immune response. They are distinctive in having an invariant T-cell receptor that recognizes glycolipid antigens presented by the class I major histocompatibility complex-related protein CD1d, which is conserved across multiple mammalian species in a class of proteins well-renowned for their high degree of polymorphism. This receptor's first identified antigen is the potent KRN7000, a glycosphingolipid isolated from bacteria that were found on a Japanese marine sponge. The corresponding terrestrial antigen remained unidentified until quite recently, when diacylglycerol-containing glycolipids, reported to activate iNKT cells, were isolated from *Streptococcus pneumoniae*. We report the total synthesis and immunological re-evaluation of these two glycolipids. The compounds are unable to activate iNKT cells. Computational modelling shows that these ligands, while being capable of interacting with the CD1d receptor, create a different surface for the binary complex that makes formation of the ternary complex with the iNKT T-cell receptor difficult. Together these results suggest that the reported activity might have been due to an impurity in the original isolated sample, and highlights the importance of taking care when reporting biological activity from isolated natural products.

Keywords: Invariant natural killer T cells, *S. pneumoniae*, immune system, KRN 7000, glycolipid, monosaccharide, disaccharide.


KRN7000
Known: Gold Standard activator of CD1d
ACTIVE
Different binding to CD1d
receptor means different activity
(grey superimposed on above)
This Work: Total Synthesis of
glycolipid from S. pneumoniae
INACTIVE

Introduction

The immune proteome is possibly the least conserved system between mammalian species, meaning that animal models are only of limited value in immunological research due to a high rate of failure in translation to human clinical populations.[1] However, there is one notable exception. Both human and mice invariant natural T killer (iNKT) cells express an invariant T cell receptor for the completely conserved nonpolymorphic major histocompatibility complex class I (MHC-I)-like molecule, CD1d. This conservation strongly implies that this protein is important for survival.[2] This unusual constancy is further emphasized by the ligand recognized by the receptor. Unlike other classical MHC-I molecules that recognize peptides, CD1d is specific for exogenous glycosphingolipids.[3] The first such ligand identified was KRN 7000, whose isolation from a Japanese sea sponge was reported in 1994 (**Fig 1**).[4] The molecule was purified from a fraction with highly potent anticancer activity, and upon binding to CD1d induces a potent innate immune system response through the activation of T cell, B cell, dendritic cell and macrophage functions.[5]

KRN 7000's unusual mode of action and incredibly potent activity, stimulated a burst of interest in analogues peaking in the mid-2010s;[6] the pathway's biological role clearly suggests it represents a final line of defense against an existential threat to survival; consequently, this system has been proposed as potentially manipulatable as a potent, if dangerous, defense against cancers, or systemic pathogenic infections.[7] iNKTs' specificity for these ligands, coupled with their evolutionary stability is unprecedented, they clearly play an essential role in immune defense. However, this research left the mystery as to why the mammalian immune system responds to a glycolipid antigen, isolated from a deep-sea sea sponge, unanswered. A potential solution arose in 2011, when Kinjo and coworkers reported the isolation of two new glycolipid antigens capable of binding to CD1d **(Figure 1)**.[8] The glycolipid antigens were isolated from *S. pneumoniae*, which is the leading cause of neonatal sepsis and meningitis,[9] and until the antibiotic modern era, one of the leading causes of death.[10] The first was identified as consisting of an α-D-glucosylpyranose O-linked to a diacylglycerol where the primary alcohol was esterified with palmitic acid, and the secondary alcohol with *cis*-vaccenic acid (**2**). The second fraction had the same core, but with a proposed additional α-D-galactosylpyranose attached at the 2-position of the glucose (**3**).[8] The glycolipid response appeared to be dependent on the inclusion of *cis*-vaccenic acid, which is highly unusual in mammalian cells, where the *trans* isomer dominates.[11]

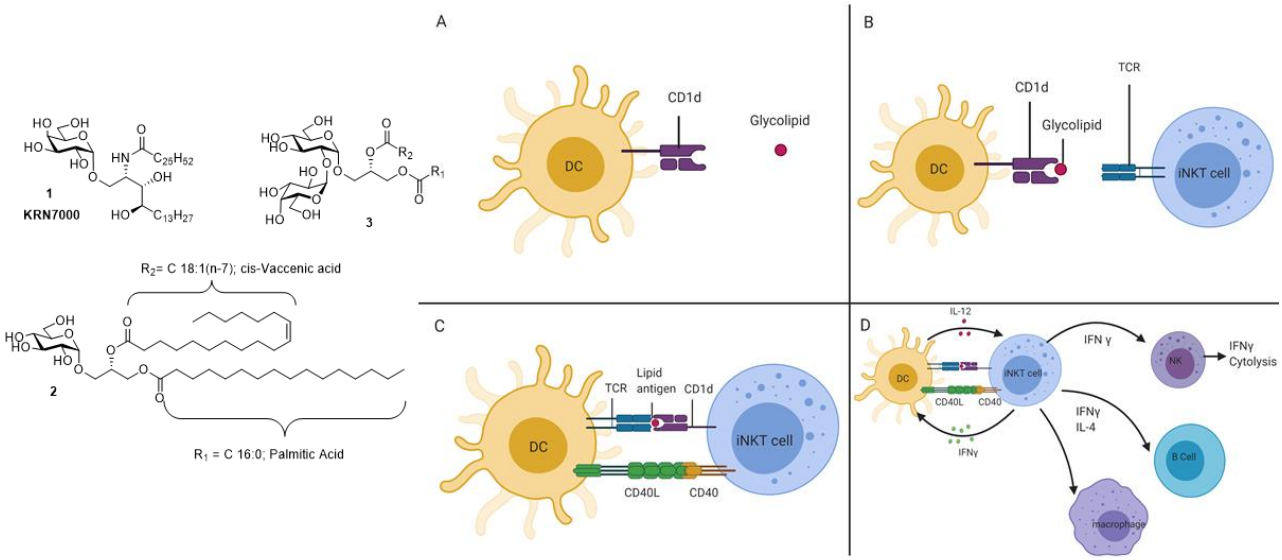


Figure 1. Structure of KRN 7000 (**1**), monosaccharidic glucose diacyl glycerol (**2),** and disaccharidic galactosyl-glucose diacylglycerol (**3).** Cartoon representation of the activation of iNKT cells by the glycolipid antigen presented by the CD1d molecule on a subclass of dendritic cells. A) The dendritic cells express CD1d on their surface that can bind the glycolipid antigen; B) This complex can then bind $V_{\alpha}14i$ on invariant NKT cells; C) This then activates the cell, and additional interactions between the cells validate the interaction; D) This leads to the production of cytokines by the iNKT that in turn recruits an immune response from NK cells, B-cells, and macrophages.

Both glycolipid antigen isolates activated iNKT cells *in vivo* and *in vitro*. However, these tests were carried out using very small amounts of isolated glycolipids,[9] making it impossible to conclusively ascertain whether the activity was due to these compounds or to some minor impurity. Kronenberg and coworkers synthesized **2**; however, activity was considerably less for the synthetic version than for the isolated material. This same sugar has since been resynthesized once since this original report, but it was not evaluated.[12] Disaccharide **3** was found to be far more active,[8] but has never been prepared. This is notable as the initial report did not provide incontrovertible proof for the unusual proposed structure. There is only a single other example of a 2-linked Gal-Glc disaccharide coupled to a diacylglycerol reported in the literature, isolated from *Listeria* and involved in cell wall structure.[13] The motif is also proposed as the terminal disaccharide in the

polysaccharide-containing Yuccoside C.[14] This particular Gal-Glc disaccharide has never, to the best of our knowledge, been synthesized, let alone conjugated to a diglyceride,

These glycoside diglycerides, incorporating unsaturated fatty acid chains are challenging synthetic targets due to the mutual sensitivity of protecting groups and the esters to standard reagents. The extant synthetic approaches to similar compounds commonly suffer from low efficiency, synthetic flexibility, and stereopurity.[15] Herein we report a simple total synthesis of both *S. pneumoniae* glycolipids, a reevaluation of their biological activity, and an *in silico* investigation of their functional structural biology. We find that they are incapable of activating iNKT cells. We conclude that some of the activity observed in the original report might be attributable to minor impurities present in the evaluated samples, perhaps a glycosphingolipid similar in structure to KRN7000.

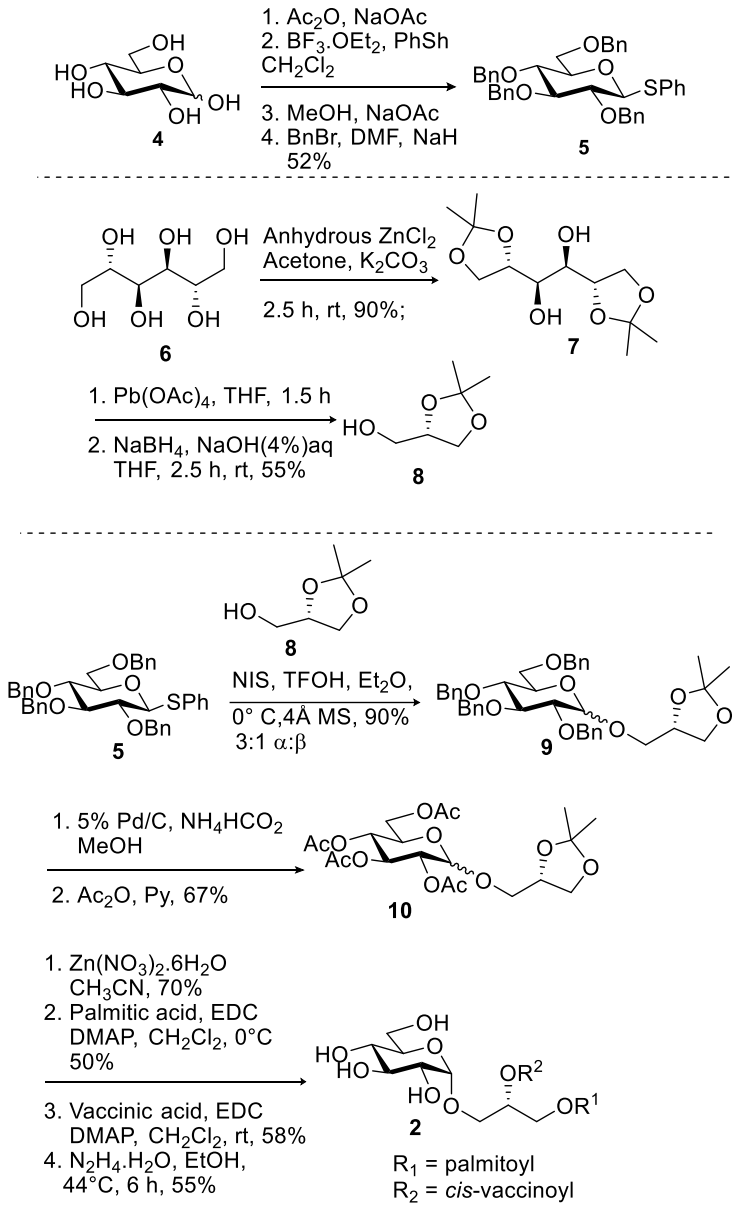
Result and Discussion

Before embarking on this campaign, we developed a synthetic route based on excellent literature precedent and high familiarity with the required reactivity to overcome these challenges. Like Thomas Jefferson, we believed that "the acquisition of the [molecules] this year (2017), as far as the [disaccharide glycolipid], will be a mere matter of marching."[16] Overconfidence in the face of the new but seemingly familiar is a recurrent and constant error in both military and synthetic chemistry campaigns; like Mr. Jefferson, we were gravely disappointed. However, unlike Mr. Jefferson and the American military, we were eventually successful.

Synthesis of monosaccharidic glucose diacyl glycerol, 2. The synthesis of **2** begins with glucose **4 (Scheme 1)**, which was converted using standard chemistry to the base-stable, non-anchiomeric-assistance active,[17] β- thiophenol glycoside **5.** The diglyceride acceptor, (*S*)-(+)-Solketal **8**, was synthesized through a modification of Terivedi's strategy to provide the appropriate protected chiral glycerol;[18] the change is the use of lead (IV) acetate for the oxidative cleavage of the mannitol di-acetonide **7** rather than the reported sodium periodate, which proved irreproducible in our hands.[19]

The glycosylation is superficially simple; however standard α-specific glycosylations failed to provide the desired anomer, all forming the undesired β diastereomer of **9** either exclusively or as the major product (See supporting information, **9a**).[15a, 20] This was highly

surprising as the glycosylation appears far simpler than most, but perhaps is affected by interference of Lewis acid co-ordination by the proximal ketal on the acceptor, and was especially problematic as the two anomers were completely inseparable by preparative column chromatography. The best ratio achieved was a disappointing 3:1 ratio of α:β anomers of **9** (diethyl ether) in 90% combined yield, and the mixture of anomers was carried forward with the eventual expectation that separation could be affected in the final step, if necessary, by semi-preparative HPLC.

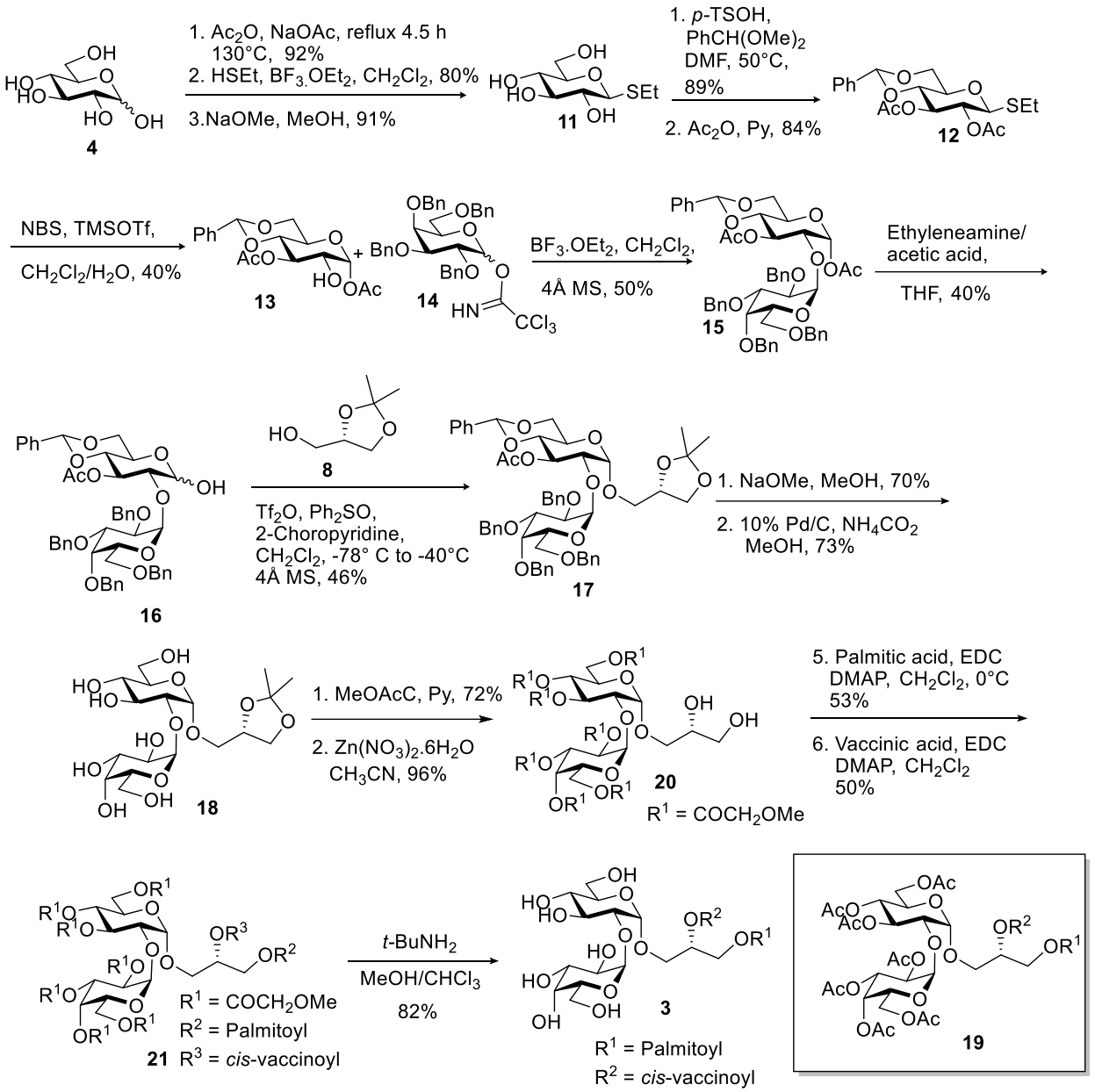


Scheme 1. Synthesis of monosaccharide **2**

Having performed their function, the benzyl groups were replaced with acetates, due to the incompatibility of hydrogenolysis with *cis*-vaccenic acid, to provide fully protected **10** in two steps. Zinc nitrate hexahydrate in acetonitrile selectively hydrolysed the isopropylidene group of the glyceride to provide the diol.[21] Sequential esterification, relying on the increased reactivity of the primary alcohol over the secondary alcohol, with palmitic acid (at 0 ºC) and freshly prepared *cis*-Vaccenic acid (at ambient temperature due to a more sluggish reaction)[22] provided the fully protected diglyceride. Following the second esterification, the two anomers surprisingly separated easily by column chromatography as the Rf values, for the first time since **9** diverged significantly. The reported yields up until the second esterification are for the combined mixture of the anomers; the second esterification, with a 58% yield, is reported for the recovery of the pure α-anomer from the mixture, yield from the α-precursor alone was 77%; the β-anomer, was also isolated and recovered at this point. Finally, the desired deprotected carbohydrate **2**, was obtained following global deacetylation using hydazine hydrate in 55% yield.[23] Byproducts in this final step ominously included limited desterification and a small amount of deglycosylation. The β-anomer was likewise deprotected to provide epitopically pure **2β.**

Synthesis of disaccharidic galactosyl-glucose diacylglycerol, 3. With the monosaccharide in hand, we believed the disaccharide would not prove overly challenging, with the only obstacle being to efficiently obtain the C-2 hydroxyl free precursor required to introduce the galactosyl substituent. The rest of the synthesis should be able to remain strategically unchanged.[24] Fortunately, we could readily solve this challenge (**Scheme 2**). As with **2**, glucose was transformed to a fully deprotected thioether, this case ethylthiol-derived **11.** Kinetic benzylidene formation was followed by acetylation provides **12**. Then, the C-2 hydroxyl was selectively unmasked through Ensley's method of concomitant oxocarbenium formation and acetyl migration to the anomeric position to provide glycosyl acceptor **13** in 40% yield.[25] Multiple glycosylations were investigated to prepare the disaccharide, but simple tricholoacedimidate (TCA) chemistry proved to be sufficiently reliable after a preliminary screen; notably, the reaction was highly sensitive to the stereochemistry of the donor and the Lewis acid. The reaction proceeds only from the α-anomer and using $BF_3{\cdot}OEt_2$. Although we were readily able to generate the β-anomer of the TCA agent in near analytical purity without purification, we were unable to obtain the α anomer in greater than a 1:1 ratio with the β isomer.[26] Freshly distilled TMSOTf did not catalyze the transformation with

either anomer even at elevated temperatures and extended reaction times. Test reactions showed that the reaction was facile with almost any other acceptor, and this recalcitrant reactivity is a function of both donor and acceptor.



Scheme 2. Synthesis of Disaccharide **3**

Anomeric acetates are themselves moderately reactive glycosylation agents;[27] however, continuing the trend of this campaign, **15**, proved to be a reluctant donor for glyceride acceptor **8**, and we were unable to convert the α acetate directly. The chemoselective removal of the acetyl

group at anomeric position was accomplished *via* a mixture of ethylene amine and acetic acid in THF at 24 hours,[28] with the mass balance being unreacted starting material that can be resubjected to reaction conditions (longer reaction times started to result in cleavage of the intersaccharidic bond). To limit the step count, we attempted Savage's glycosylation of the free hydroxyl, which was employed for the galactosyl-galactosyl analogue of this current target, using diphenylsulfoxide activated with triflic anhydride and tri-*t*-butylpyrimidine as base, but this failed to provide any product.[28] However, increasing the reactivity of the system by using 2-chloropyridine as base proved successful, providing **17**, the core of the glycolipid.[29] The protecting group strategy had been selected to be compatible with the chemistry developed for the monosaccharide. Global deprotection of the carbohydrate protecting groups (without touching the acetonide on the glycerol), proceeded smoothly to **18**.

We then conducted the same sequence used for the monosaccharide to complete the synthesis: peracetylation, acetonide removal, and selective esterifications to generate **19**. However, all attempts to cleave the acetates from the sugars without affecting the esters on the glyceride failed. Reagents variously either cleaved all esters, did not react, were unable to provide selectivity, or cleaved the apparently sensitive intersaccharidic bond. Despite a very significant effort, we were unable to remove the acetates in the presence of the esters without damaging the rest of the molecule. A new approach was required.

Consequently, we employed methoxyacetyl groups that can be cleaved by *tert*-butylamine.[30] This is an underappreciated protecting group as the deprotection conditions are highly compatible with a wide variety of different functional groups. A full description of an expansion of the potential of this system will be published in due course. Fully deprotected **18** was acylated, the acetonide removed as before, and the esterifications proceeded without difficulty. Fortunately, the tert-butylamine, unlike any of the other deacylation reagents tested, was gentle enough to evince the final deprotection to provide **3** in 82 %yield.[30c]

iNKT activation ability of the synthetic glycolipids. With the materials in hand, we evaluated their ability to induce production of cytokines by CD1d-restricted iNKT cells. We employed the mouse iNKT cell hybridoma DN32.D3, especially well designed for this experiment as it possesses both the CD1d and the unique conserved, invariant $V_{\alpha}14T$ cell receptor of iNKT cells, allowing the cells to present antigenic glycolipids and cross-activate each other. This cell line has been used

extensively in the past to measure iNKT cell responses, represented by the production of interleukin-2 (IL-2, other cytokines show similar results).[31] These cells produce IL-2 in response to KRN7000 treatment in a CD1d-dependent manner, making KRN7000 an excellent positive control. Several other glycolipid formulations have also been shown to induce an immune response from these cell types, including bacterial superantigens (SAgs),[31a] a series of glycolipids from pathogenic bacteria,[32] including the ones synthesized here,[8] and even a multivalent acetal-free,[33] dendron-supported carbohydrate previously reported by us.[31c] Kinjo and co-workers evaluated the immunogenicity of their isolated glycolipids using a similar system to the one used by our team: mouse $V_{\alpha}14i$NKT cell hybridomas were used.[8] The isolates showed good elicitation of IL-2 from $V_{\alpha}14i$NKT cell hybridomas in a CD1d-dependent manner, but no activation of CD1d-reactive non-$V_{\alpha}14i$NKT cell hybridomas. The team then synthesized the monosaccharide and evaluated the immunogenicity of that system (while also confirming the structure and regiochemistry of the ester groups) and demonstrated that it showed similar activity to the isolated monosaccharide.[8] This is not the result we observed here. We observe no meaningful IL-2 response from the DN32.D3 cells towards the synthesized glycolipids at either 100 ng/mL to 1 μg/mL, while KRN7000 showed its typical potent activity in inducing IL-2 production by these cells (Figure 2).

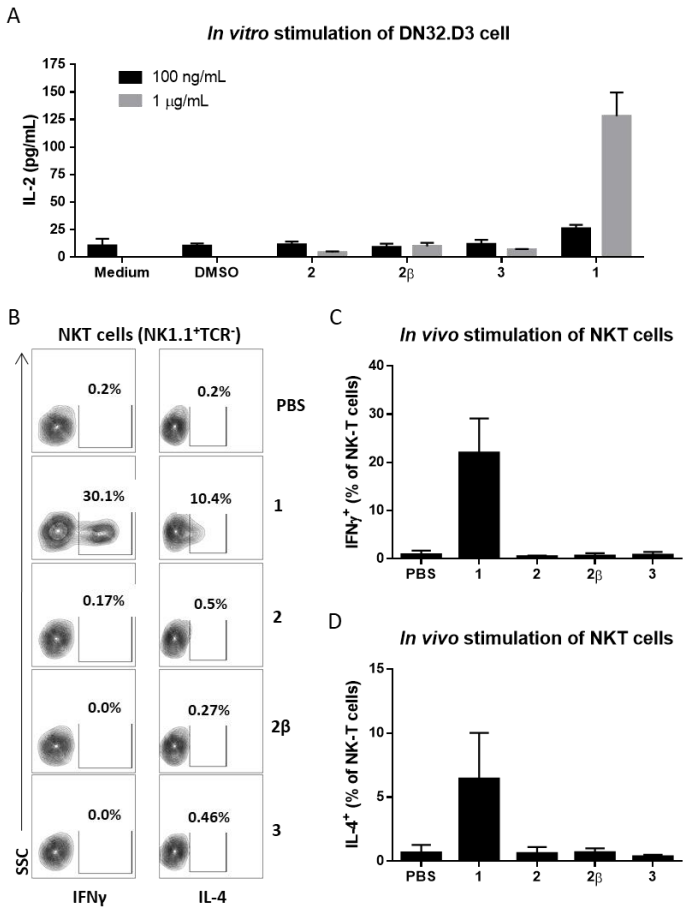


Figure 2. Cytokine production by iNKT cells in response to glycolipid stimulation. A) Elicited IL-2 levels obtained from exposing DN32.D3 cells to the synthesized glycosphingolipids measure by ELISA. Data represent mean ± standard deviation of triplicate samples; B) Flow cytometry analysis of intracellular IL-4 and IFNγ levels in NKT cells ($NK1.1^+TCR\beta^-$) in the spleens of 8-12 week-old C57BL/6 mice 2 hours after intraperitoneal injection with the indicated agents (4 μg in 200μL PBS/mouse). Percentage of $IL\text{-}4^+$ and $IFN\gamma^+$ cells are indicated for each treatment. Data represent one out of at least 6 mice in each of the treatment groups; C) Quantification of the $IFN\gamma^+$ results from B; D) Quantification of the $IL\text{-}4^+$ results from B. Data represent mean ± standard deviation of measurements from at least 4 mice in each treatment group. **1**, KRN7000; **2**, monosaccharidic glucose diacyl glycerol; **2β**, monosaccharidic glucose diacyl glycerol (β-anomer); **3**, disaccharidic galactosyl-glucose diacylglycerol.

This is in stark contrast to the results previously reported. Consequently, we resynthesized fresh batches of the glycolipids, repurified them, and re-evaluated the samples obtaining the same result.

We further evaluated the ability of the materials to induce iNKT cells stimulation *in vivo* in C57BL/6 mouse strain. We reasoned that the CD1d-restricted cell line may not provide the full required machinery needed to elicit an *i*NKT cell response to these modified glycolipids *in vitro*, that a fully intact immune system could provide. This hybridoma is sufficient for antigens that function directly through the CD1d- $V_{\alpha}14T$ interaction, but it is possible that the antigens function like superantigens and activate the iNKTs indirectly. To this end, we treated groups of mice with 4 μg dose of the glycolipid antigens and KRN7000 intraparitoneally. Two hours post injection, mice were sacrificed and the percentage of splenic NKT cells expressing IL-4 and IFNγ were determined by intracellular staining and flow cytometry analysis. In our experiments NKT cells were recognizes as $NK1.1^{+}TCR\beta^{-}$ lymphocytes. Again, KRN7000 proved a potent stimulator of NKT cells, but none of the synthetic glycolipids showed significant activity (Figure 2). Extended *in vivo* stimulation up to 48 h did not elicit any IL-4 and IFNγ response in iNKT cells against any of the synthetic glycolipids (not shown). These molecules failed to induce IL-4 and IFNγ production by NKT cells *in vivo*.

One of the differences between this study and the one by the Kronenberg group is in the iNKT cell hybridomas used to evaluate iNKT cell responses. Our cells (DN32.D3) express both CD1d and the invariant $V_{\alpha}14$ T cell receptor, allowing the cells to present antigens and activate each other, while the Kronenberg group used an iNKT cell hybridoma which did not express CD1d, therefore, requiring the use of dendritic cells (DC) or plate-bound CD1d molecules for presentation of antigenic glycolipids. Using *Il-12*$^{-/-}$ and *Myd88*$^{-/-}$ mice, the Kronenberg group demonstrated that iNKT cell responses in their assays were independent of IL-12 and activation of DC via Toll-like receptors, however, the contribution of other pro-inflammatory cytokines induced by their antigenic preparations cannot be completely ruled out. Alternatively, CD1d-dependant presentation of glycolipids to iNKT cells may require additional processing of the isolated glycolipids by DCs. Our in vitro assays are performed in the absence of DCs as antigen presenting cells, therefore, are not subjected to the same factors. Furthermore, our *in vivo* assay in the immunocompetent mice firmly demonstrate that, unlike KRN7000, which induced iNKT cell stimulation and cytokine production, our glycolipid preparations did not elicit such response under similar conditions. The contribution of glycolipid preparations in the activation of iNKT cells, if any, appears to be mechanistically different than that of KRN7000.

The difference between the cell lines used by our research group and that of the Kronenberg group is that our cells, had the murine MHC receptors removed, leaving only CD1d.[8] The Kronenberg group's cells still have the other receptors present. Glycolipids do not, as a rule, interact with other MHC receptors, but the possibility remains that this could explain this discrepancy; however, our mice had fully active murine immune systems that are stimulated by KRN7000, and would be sensitive to superantigen effects. Not seeing any response makes it less likely that this would be the mechanism of action.

There is also a difference in how the *in vivo* experiments were conducted by Kronenberg's team and ours in that they prepulsed the dendritic cells with the glycolipids before injecting the cells into the animal model, while we injected the glycolipids directly. We also sacrificed the mice at the 2 hour and 48 hour marks rather than the 14 hour mark. However, KRN7000, with a nearly identical solubility profile, and the same proposed mechanism of action, was a potent activator in our experiments, while these other glycolipids were not. This suggests that the *i*NKT system did not co-evolve to recognize the presence of these particular glycolipids, this difference in activity could be ascribed to the manner that they interact with the receptor.

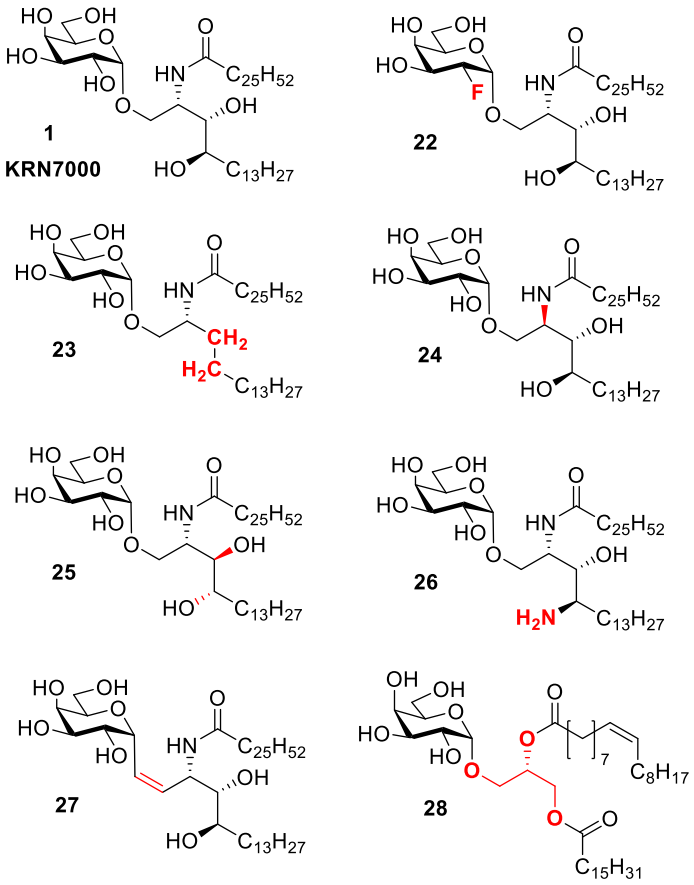


Figure 3. Examples of the modifications to KRN7000 not tolerated by CD1d..

We know that the CD1d-dependent response to glycolipids is exquisitely sensitive to the structure of the glycolipid antigens (Figure 3). The structure-activity relationship studies conducted on KRN7000 have been extensive and have repeatedly reiterated that modifications to the structure are often deleterious to its iNKT cell-activating ability.[6] Substitution at C2 as in **22**, is not tolerated except by a sugar; nor are the removal of the hydroxyl groups of the phytosphingosine (i.e. **23**), changes in stereochemistry of the phytosphingosine (**24** and **25**), or replacement of the hydroxyl group by an amine **26**. Introduction of a *Z*-double bond, **27**, also abrogates activity, although the *E* is tolerated. An important exception is that certain specific galactose diglycerides, such as **28**, do continue to activate the system. Likewise, the glucose derivative of KRN-7000 shows very moderate activity. Finally, C-2 substitution of KRN7000 with an α-D-galactoside is tolerated in the preliminary studies. Together these modifications suggest the glucose derivatives synthesized here could show activity, but perhaps the multiple modifications simply distort the CD1d antigen-

bind surface to too great a degree to allow for ternary complex formation with the T-cell receptor. To address this question, we conducted a series of computational analyses.

Computational Analysis Starting with the published crystal structure of the extracellular α1-α3 domains of the CD1d α-chain, and the β_2 microglobulin (β_2M) chain complexed to KRN7000, **KRN7000@CD1d** (*1ZT4*),[34] we solvated the system, then minimized the structure. The resulting conformation remains substantially the same as in the crystal, although we note that the sugar sinks a bit deeper into the pocket in the simulation (Figure 4A). We then deleted KRN7000 and redocked both **2** and **3**, then minimized the structure complexes followed by a 10ns molecular dynamic simulation to relax the geometry. The extracellular domain of CD1d consists of a *C*-terminal domain, containing two layers of β-sheets (blue and purple, Figure 4), linked to a more structurally complex *N*-terminal binding domain comprising two helices acting as a 'jaw' that hinges shut over a β-sheet floor. This jaw is widest in the centre, which determines the position of the carbohydrate of a bound glycolipid, while the long alkyl chains can sprawl into the two large hydrophobic cavities (pockets **A** and **C**) formed between the helices and the β-sheet floor, to the left and right of the jaw's centre.

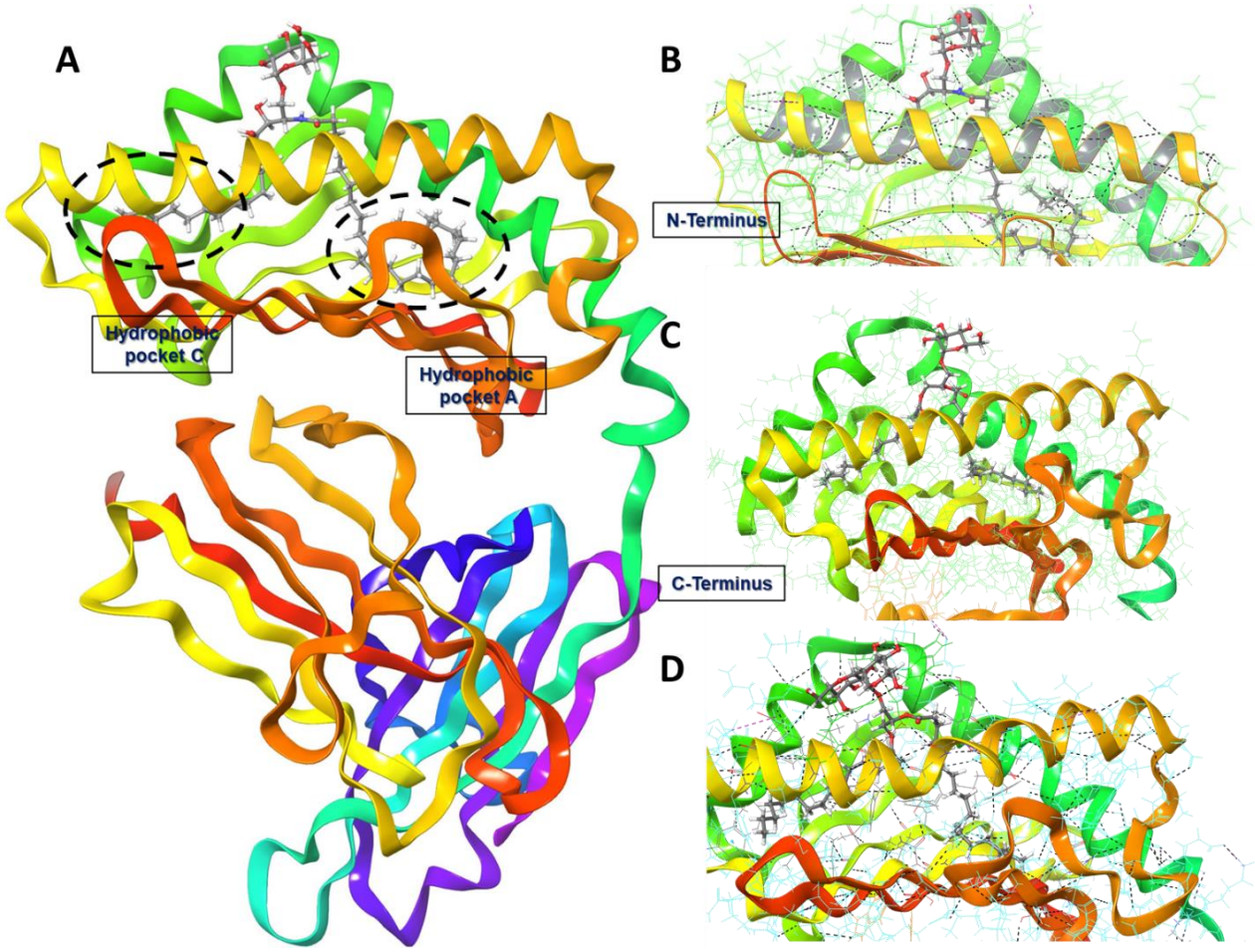


Figure 4. Comparison of the binding modes of the ligands to CD1d. A) Relaxed structure of the complete protein bound to KRN7000, **1**, (**KRN7000@CD1d**), highlighting the two hydrophobic pockets in the binding groove and the slightly folded down nature of the sugar relative to the crystal structure. B) Focus on the hydrogen bond interactions around the binding site with **1**; C and D) Focus on the hydrogen bond interactions around the binding site with **2** and **3** respectively.

A number of residues position the chains of the binding glycolipid into the pocket, but the polar head group is held in place through a series of hydrogen bond interactions, such as that between the amide of the sphingosine with Thr154 (as either H-bond donor with the glycosidic oxygen atom or a H-bond acceptor with the 2'-NH amide moiety) and Asp151 of the α2-helix (Figure S1); the simulation is consistent with previous examples in the literature.[35] The generated H-bond interactions originated from 2-OH, Thr154, Asp151, and 2'-NH have been shown to play important roles in localizing the polar head group. Similarly, the 3'-OH of the phytosphingosine interacts with Asp80 of the α1-helix, positioning it above the groove. These H-bonding networks,

of course, do not exist for the synthetic diglycerides. The main effect this has is that many of these interactions with these rim-residues are taken over by the carbohydrate instead of the phytosphingosine residues. This sinks the sugars deeper into the groove, decreasing their apparent surface (Figure 5), such that they don't extrude from the mouth as far as KRN7000.

With KRN7000, Trp153 of the α2-helix lies against the more hydrophobic bottom face of galactose, ensuring it orients in the right direction. It is able to do this with **3** as well but as this ligand sits deeper, the torsional bend in the angle is greater, twisting it from coplanar with the groove's floor, to a greater degree. This is further exacerbated as Asp151 then pulls down the sugar though interactions with the C-2 OH of the pendent galactose residue, greatly distorting the positioning of the helix relative to the polar head group compared to KRN7000. For **2** the effect is even more drastic as the hexose slides over so that the C3-OH can interact with Asp151, meaning that Trp153 can do little to position the sugar as it is more distant. Finally, Asp80, so central for guiding KRN7000 into the groove, is now located far from the glycolipids of the diglycerides, and instead of binding to the OH of phytosphingosine, interacts with an adjacent arginine on the α1-helix.

The alkyl chains of these pneumococcal glycolipids do extend down the binding grooves, but they occupy them differently. KRN7000 has the exceedingly long 25-carbon amide alkyl group that curls around, filling the end of the A-pocket, and sinking deeper into it. This is a large cavity, and it is not satisfied by the far shorter *cis*-vaccenic acid residues. This likely contributes to a lower binding affinity, that although not determined in this, or other, studies, could be partially responsible for the lower observed activity.

However, the bigger difference might be in the exposed surface for ternary complex formation of TCR∩KRN7000@CD1d (Figure 5). Plotting the surface shows that it is quite different for KRN7000 vs the other two glycolipids as they sit deeper in the groove as the key interactions are with the sugar itself and not the phytosphingosine residues. This would be partially compensated for if the sugar present was a galactoside as the C4-OH would project out towards solvent, but as this is glucose, we lack this extended volume, shrinking the surface significantly relative to KRN7000. We know that the phytosphingosine residues (specifically the 2'- and 3'-OH) are essential for activity *via* T-cell recognition (Figure 3),[36] and that modification of them weakens the key interactions resulting in loss of activity. We know that the invariant T-cell

receptor for CD1d is highly selective for the glycolipid guest in the CD1d receptor, and this selectivity could mean that it is not able to be activated by these residues (see Supporting Information).

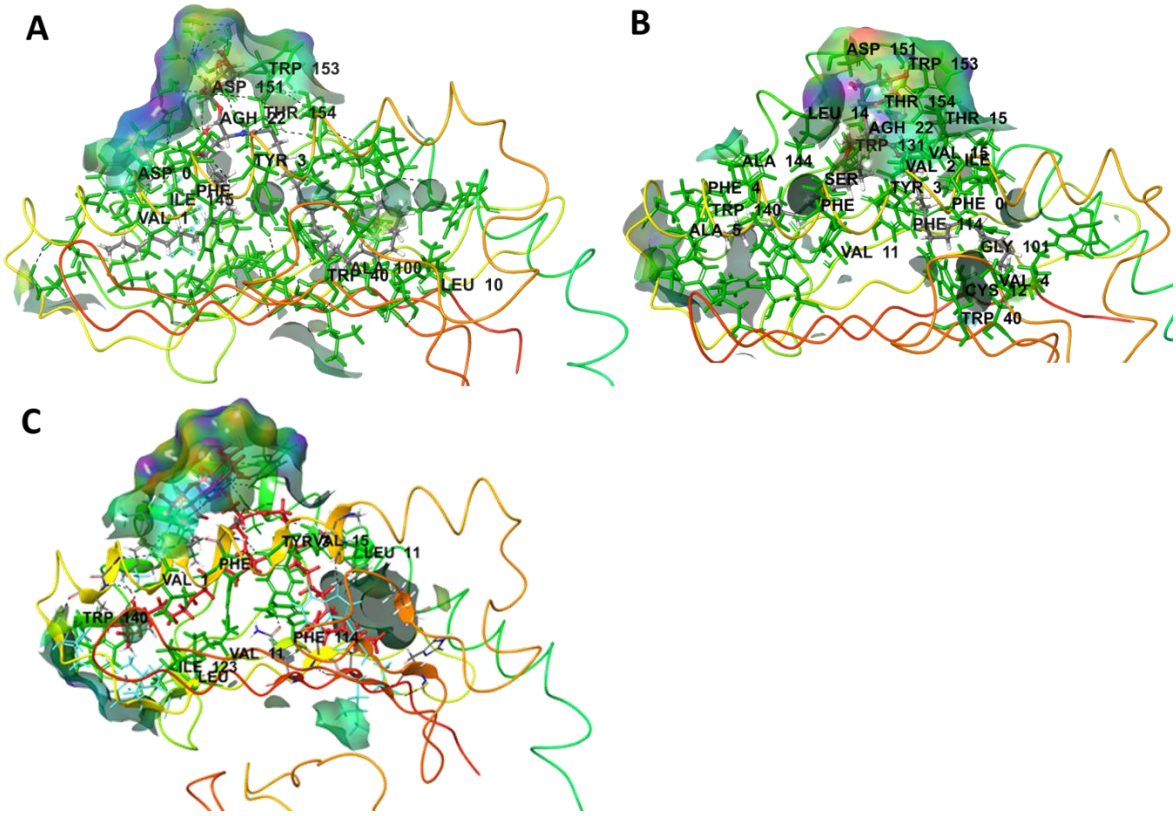


Figure 5. Plot of the electrostatic surface of the first hydration sphere around the three ligands bound to CD1d.

Conclusions CD1d is an essential component of our intact immune system. It is almost certainly activated by the presence of bacterial glycolipids present in existential threat infectious agents such as *S. pneumoniae*. However, the isolated glycolipids **2** and **3** are not responsible for the observed activity. Their efficient synthesis and re-evaluation in this study provides a significant example for the need for the evaluation of synthetic rather than isolated samples as even a very small amount of impurity-undetectable on NMR (<2%)[37] can be responsible for observed activity. The changes to the interaction surface between the **antigen@CD1d** dimer and the T-cell receptor are significant and would hinder the interaction. A re-analysis and repurification of these *S. pneumoniae* extracts searching for other known glycolipid activators of CD1d might prove very fruitful to explaining the existence of the invariant iNKT immune defense system.

Acknowledgements. The authors gratefully acknowledge financial support for the project from the Windsor Cancer Centre Foundation (2019-003), the Natural Sciences and Engineering Research Council of Canada (JFT: grant # 2018-06338), the Canadian Tricouncil (NFRFE-2018-00075). SMT and JFT wish to recognize that this work was made possible by the facilities of the Shared Hierarchical Academic Research Compu-ting Network (SHARCNET: www.sharcnet.ca) and Compute/Calcul Canada. The authors would like to thank Chelsea Ymana, and Science Meets Art UWindsor, for executing the TOC graphic. JFT would like to thank S. Mansour Haeryfar of the University of Western Ontario for initiating the idea behind this study.

Author Contributions. Conceptualization, JFT; Funding acquisition JFT, MR; Investigation, SIS, EI, GY, SM, SMT; Methodology, SIS, SMT, MR; Project administration, JFT, MR; Supervision, JFT, MR; Writing original draft, SIS, SMT, JFT; Writing – review and editing, All authors.

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