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α-*C*-Galactosylceramides: Synthesis and Immunology

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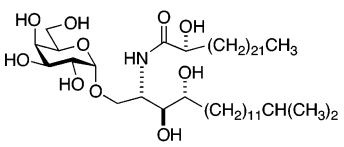
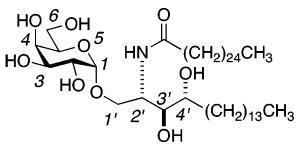
ABSTRACT

The immunostimulant activity of α-galactosylceramides provided the impetus for the research described here. The activity was first discovered via screening of extracts of a marine sponge. The active materials purified from the extracts were α-*O*-galactosylceramides. The work described herein focuses on syntheses of α-*C*-galactosylceramides. Crucial methodologies for the syntheses were (i) Ramberg−Bäcklund reaction, (ii) modified Julia olefination, (iii) olefin cross-metathesis, and (iv) Sharpless asymmetric epoxidation in four independent routes. The immunostimulant activity of the synthetic α-*C*-galactosylceramide far surpasses that of the *O*-galactosyl material. A discussion of the reasons for the difference in activity is presented.

Glycolipids and their interaction with the immune system serve as the strategic impetus for the research described in this Account. Our chemistry and immunology project has its beginnings in 1993 when researchers at Kirin Pharma reported the isolation of a small family of galactosylceramides from an Okinawan sponge, *Agelas mauritianus*.[1] Although sponges of this type had previously been examined for natural products,[2] this isolation was of interest because of the unusual α configuration of the ceramide residue, illustrated by agelasphin-9b (**1**), and



(1) Agelasphin-9b



(2) KRN7000
or α- galactosylceramide

because of the potent antitumor activity found in vivo at the organism level. In the ensuing years, parallel biological and synthetic studies revealed that the potent activity of the ceramide required neither the α-hydroxyl in the fatty amide residue nor the branching in the phytosphingosine chain; hence the candidate molecule for immunology researchers became the synthetic material **2**, known as KRN7000.[3] Shortly after the disclosure of KRN7000, it became clear that this marine-derived species acted as a powerful immunostimulant in mammals.[4] Through this observation, investigators were able to explain why early antitumor activity studies were only successful in organisms with immune systems and not with isolated tumor cells in culture. In the interest of providing a context for the discussion of the synthetic carbohydrate chemistry and the immune-active materials that are central to our story, a brief introduction to the immunology associated with our small molecules is warranted.

There are basically two components of the immune systems in mammals. One is named adaptive (or acquired) and the other is called innate. Two types of cells associated with the adaptive immune system are necessary for the recognition of foreign invaders such as bacteria, fungi, viruses, and parasites. One family is B cells (B for their origin in bone marrow), which directly recognize the invader (called an antigen) and secrete a soluble protein, called an antibody, which specifically binds the foreign surface protein. The second family, T cells (T for their genesis in the thymus gland), recognize fragments of foreign molecules that are presented by antigen-presenting cells (APCs) of the host. APCs include dendritic cells (DCs) and macrophages. Within the T cell family, there are three general types of antigen recognition. Two types involve peptide recognition and require MHC (major histocompatibility complex) class I and class II molecules, which are encoded by MHC genes and expressed by APCs. These MHC class I and class II molecules bind foreign peptide fragments and then present them to CD8+ T cells and CD4+ T cells, respectively. The T cells then respond to the MHC/foreign peptide complex. There is a third antigen-presentation pathway, key for the work described in this Account. This is an atypical pathway that involves

Richard W. Franck was born in Germany in 1936 and, with his parents, emigrated to the U.S. in 1938 to escape the Nazi persecution of the Jews. He received his A.B. from Amherst College in 1958 and, under the mentorship of William S. Johnson, received his M.S. (Wisconsin) followed by a Ph.D. (Stanford) in 1962. After a postdoctoral year at MIT with Herbert House, he assumed an assistant professorship at Fordham University in September 1963. In 1983, he moved to Hunter College of CUNY where he retired from teaching in 2003, having achieved the rank of Distinguished Professor. His research interests in the 1960s and 1970s included mitomycin synthesis and a crowded *tert*-butyl naphthalene study with the important collaboration of J. E. Anderson of University College, London. In the 1980s, his synthetic goals shifted to the aureolic acid antibiotics, which led to detailed cycloaddition studies, including the Bradsher cycloaddition of isoquinolinium salts. The aureolic acid work led to a synthetic carbohydrate chemistry program in the 1990s continung up to the present, which has included methods for 2-deoxyglycoside synthesis, cycloaddition methods for glycosyl transfer with the important participation of the Capozzi team at the University of Florence, and most recently *C*-glycoside synthesis. For the present work, the partnership with his coauthor Moriya Tsuji has been of inestimable value.

Moriya Tsuji was born in 1958 in Tokyo. He received his M.D. in 1983 from The Jikei University School of Medicine, Tokyo, Japan, and in 1987 earned his Ph.D. in Immunology from the University of Tokyo, Faculty of Medicine. After being a postdoctoral fellow from 1987 to 1990 in the Department of Medical and Molecular Parasitology, New York University School of Medicine, he began his independent career an instructor in the department, rising to associate professor with tenure in 1998. Since 2002, he has been at the Aaron Diamond AIDS Research Center, The Rockefeller University New York, where he is currently Associate Professor and Staff Investigator, HIV and Malaria Vaccine Program. His major research interests are (i) recombinant viral vaccines against microbial infections, (ii) identification of new glycolipid-based adjuvants for HIV and malaria vaccines, (iii) the protective role of T cells against HIV infection, and (iv) the protective role of CD1a/b/c molecules.

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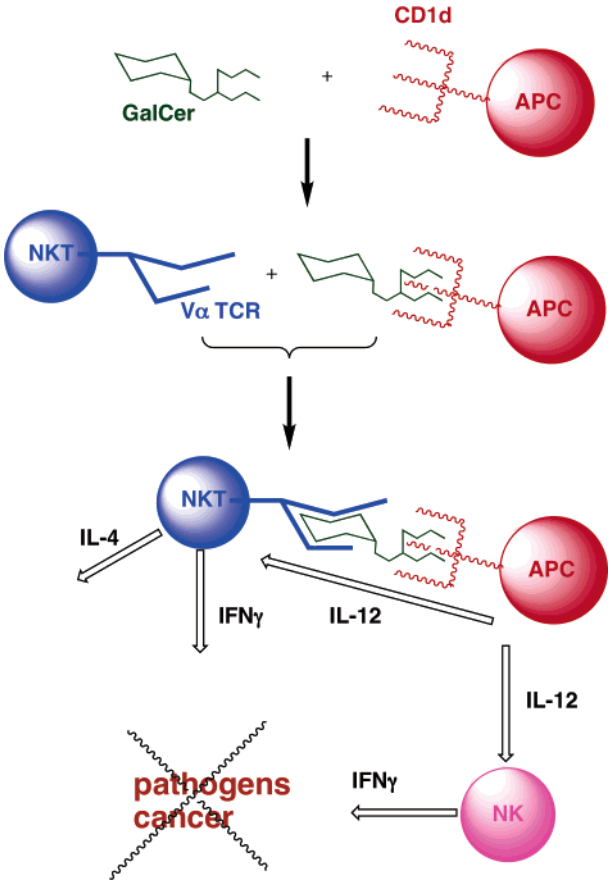


FIGURE 1. Schematic chart showing stepwise formation of the GalCer—CD1d complex followed by formation of the triplex of CD1d—GalCer—NKT and the resultant cytokine production.

CD1 (cluster of differentiation 1) molecules. CD1 molecules are encoded by the CD1 locus comprising a family of nonpolymorphic genes located on chromosome 1 outside the MHC locus. CD1 molecules, which noncovalently associate with β_2-microglobulin, are structurally related to MHC class I molecules and, therefore, are called nonclassical MHC class I molecules. In contrast to MHCs, which bind peptides, CD1 molecules bind lipids and glycolipids and then present them to the T cells to elicit an immune response. Upon recognition of foreign antigens, T cells then display two major types of response. Some T cells attack and kill the invader by direct cell—cell contact. Other T cells secrete soluble proteins, called cytokines, which can have inhibitory activities against pathogens and tumors by themselves, can recruit killer cells, or can promote the proliferation of killer cells. These recruited cells then also are able to attack the invader. It is this last T cell pathway of recognition and response that connects our chemistry research to immunology.

Mechanistic immunologic studies revealed that the α-galactosylceramide (α-GalCer) requires two distinct molecules on two different cell types to exhibit its effect. First, the α-GalCer is bound to the CD1d molecule of APCs (Figure 1). The CD1d molecule is among several CD1 molecules. Interestingly and of practical utility, the CD1d molecules in both mice and humans are quite similar in sequence and in recognition characteristics. The α-GalCer/CD1d complex is then recognized by a receptor, called the T cell receptor (TCR), on natural killer T (NKT) cells. A TCR consists of heterodimeric α and β chains. Normally TCRs expressed by T cells are diversified to be able to recognize a wide variety of fragments of foreign molecules. Different from conventional T cells, however, NKT cells express nonpolymorphic, invariant TCRs (*i*TCR). In mice, the α chain of the NKT cell receptor is identified as $V_\alpha 14$, and in humans, the very similar receptor is identified as the $V_\alpha 24$. NKT cells have phenotypic and functional properties of both natural killer (NK) cells and T cells, thus their nametag—NKT. Upon recognition of the α-GalCer/CD1d complex through the TCR, the NKT cells rapidly initiate the secretion of cytokines, such as interferon-γ (IFN-γ) and interleukin-4 (IL-4), while the CD1d-bearing APCs begin to secrete interleukin-12 (IL-12) (Figure 1). Continuing investigations have shown that the balance of cytokine levels is important for determining the target of the immune response. It is remarkable that α-GalCer is *not* an antigen presented by APCs as a fragment of a foreign pathogen. It simply marshals the immune system to attack the invader, which is not associated in any way with α-GalCer. That a glycolipid from a sponge, with an α-configuration unusual in mammals, has such a notable effect as a messenger for the mammalian immune system is fascinating and raises the question of the existence of an endogenous mammalian ligand that could bind the same molecules and have a similar effect. A synthetic trihexosylceramide (Gal α1,3 Gal β1,4 Glc β1,1 Cer called iGb3) with a β-ceramide linkage resembles known mammalian glycolipids and demonstrates similar, but significantly less potent activities toward NKT cells compared with the sponge α-ceramide. Although the iGb3 has not been detected in mouse or human, it is possible that a close structural analogue to iGb3 is the principal endogenous antigen.[5]

Recently, two groups reported X-ray crystal structures of α-GalCers bound to both murine and human CD1d molecules.[6] A third group described a structure of the protein bound to phosphatidyl choline, which has some elements in common with the GalCer-bound structure.[7] The murine CD1d receptor structure, without a description of bound lipids, had been determined in 1997. Several salient features of the complex had been deduced in advance because of the nature of the earlier protein structure and because of structure—activity data derived from analogues of KRN7000. For example, the structure of the complex reveals that the hydrocarbon chains are buried in the two grooves in the protein composed largely of hydrophobic amino acids. These hydrophobic channels were a characteristic of the 1997 structure. The galactose residue is largely exposed for subsequent recognition by the T cell receptor (TCR), but its 2 and 3 hydroxyls are hydrogen-bonded to acceptors in the protein. This was expected because 2 and 3 hydroxyls are required for the activity of α-GalCer's. Other hydrogen bonds between the protein and the ligand include those to the phytosphingosine hydroxyls, which were anticipated because the 3′,4′ deoxy species is devoid of activity and the 4′-deoxy

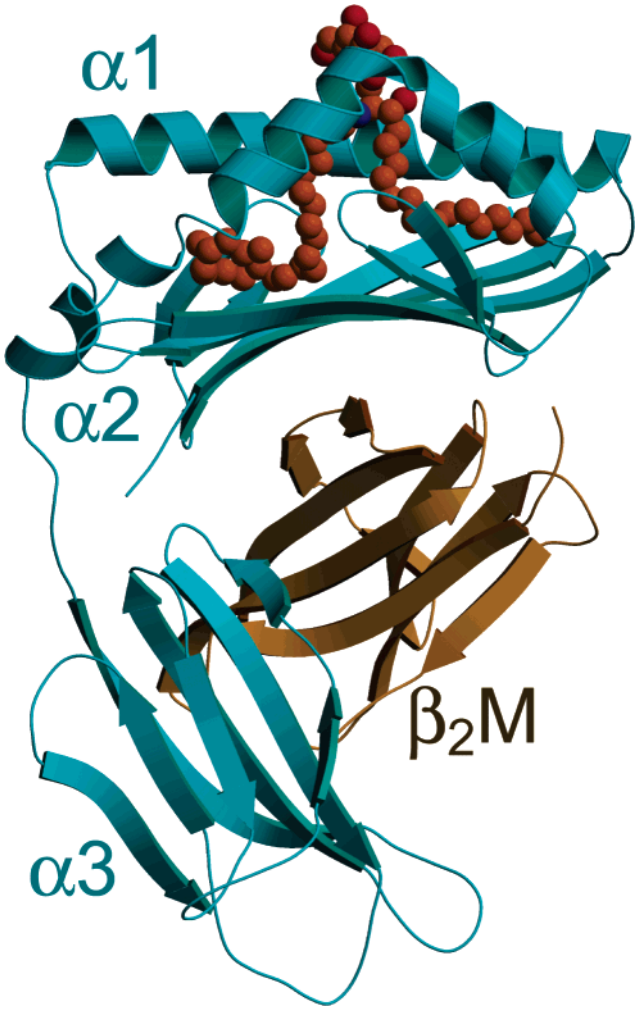


FIGURE 2. Side view of α-GalCer bound to human CD1d. Reprinted by permission from *Nature Immunology* (http://www.nature.com), ref 6a. Copyright 2005 Nature Publishing Group.

analogue, although active, is weaker than the parent. Also, there is a long hydrogen bond of 3.4 Å between Thr156-(murine) or Thr 58(human), and the glycosidic oxygen. Also a feature that may be significant is that the carbonyl of the fatty amide does not seem to be H-bonded to the protein. This may imply that it is left exposed, along with the 4 and 6 OH's of the galactose, for recognition by the NKT cell receptor. The gal-O5−gal-C1−O1−phyto-C1′ dihedral angle (or Φ_O) is 57° in one molecule of the unit cell and 83° in the other. These are typical for the exo anomeric effect influence and are found in many glycosides.The dihedral angle of gal-C1−O1−phyto-C1′−phyto-C2′ (or Θ_C) is 165° in one molecule and 177° in the second molecule, or almost anti, which is often found in glycosyl aglycones (Figure 2).

The largest number of analogues in the *O*-glycoside series derive from lipid chain variation. The fatty amide is easily subject to "libraries", and it is safe to conclude that bioactivity is not highly sensitive to chain length once a threshold of approximately 16 carbons is crossed. The introduction of unsaturation has a small effect in relative levels of cytokines induced, with a small bias toward IL-4 compared with the saturated fatty amides.[3,8] Fluorescent tags have been introduced at the terminus of the fatty amide chain by derivatizing the phytosphingosine amino function with a fatty acid bearing a terminal amino group. Then a second amidation is used to introduce the tag.[9]

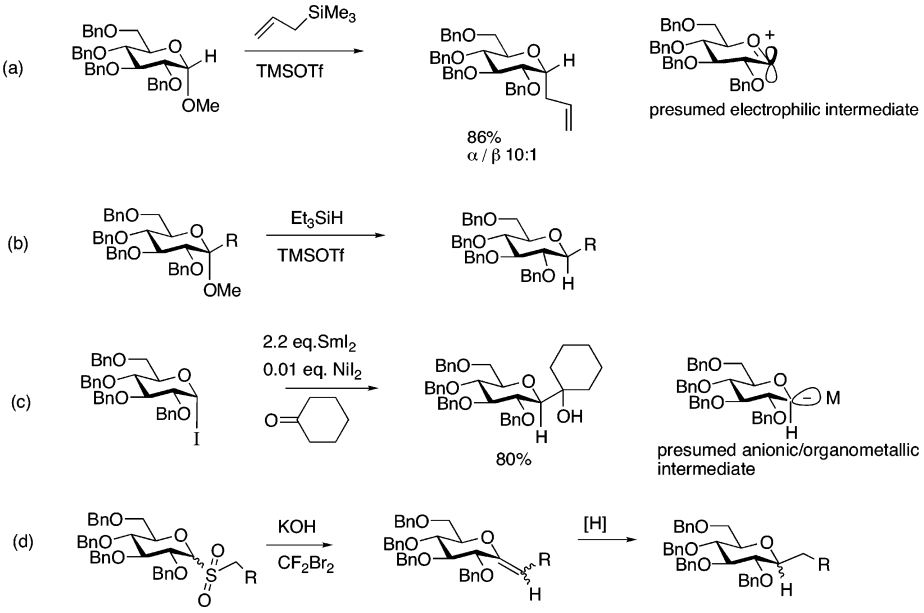
The most significant effect of lipid variation in the *O*-glycoside series is the truncation of the phytosphingosine lipid chain. The material, known as OCH, with a hydrocarbon chain length of 5, compared with the parent 14, induces a typical level of IL-4 but produces very little IFN-γ. This selectivity or polarization for IL-4 is identified as T_H2 (meaning the response of T helper cells of type 2) as opposed to T_H1, and the material has a superior effect on the murine model of encephalomyelitis.[10]

Only two modifications on the galactose are not fatally deleterious. The replacement of the 6-OH with NH_2, which is then further derivatized with fluoresecent tags, is reported to sustain activity.[11] The 3-sulfate (modeled after sulfatide) is also reported to be effective.[12] Significantly, the 6-deoxygal (fucosyl) analogue is devoid of activity.[13] This reinforces the idea that the 6-position, which is free of interactions with the CD1d molecule, is involved in a polar recognition event with the NKT cell receptor. Although various disaccharide ceramides had been described as active,[14] it has been shown that they must be enzymatically cleaved to the monosaccharide α-GalCer for activity to be observed.[15]

The more deep-seated modification, replacement of the anomeric oxygen of α-GalCer by CH_2, had not been described when we began our studies in 1999. The *C*-glycoside strategy has been an active subfield of research in carbohydrate chemistry for two decades. The operating assumption has been that the principal recognition and binding properties of the *C*-analogue would be similar to the *O*-glycoside save for the inertness of the *C*-analogue to glycosidase enzymes.[16] The persuasiveness of the "similarity" assumption is open to question. Thus, the replacement of glycosidic O by CH_2 deletes the anomeric effect, which is a controller of glycoside conformation. An axial alkyl destabilizes the $^4C_1$ chair by approximately 2 kcal/mol relative to an equatorial aglycone, while the axial *O*-glycoside stabilizes the $^4C_1$ conformer by approximately an equal amount. The exo-anomeric effect also stabilizes an aglycone conformation known as the exo syn form, whereas the *C*-glycoside really demonstrates a less strong preference between exo syn and the non-exo or anti form. Furthermore, the glycosidic O is a hydrogen-bond acceptor, and the CH_2 has no H-bond properties.

Despite these caveats about the suitability of CH_2 standing in for O, organic chemists have attacked the challenge of *C*-glycoside preparation with a wide selection of C−C bond-forming reactions taken from our synthetic methods canon.[17] A consideration specific to glycosides that is necessary to reaction planning has been compatibility with traditional protecting groups of carbohydrates or, ideally, compatibility with unprotected carbohydrates. A second factor has been the control of anomeric stereochemistry in a practical way. One widely applied method

Scheme 1. Representative Methods for *C*-Glycoside Synthesis



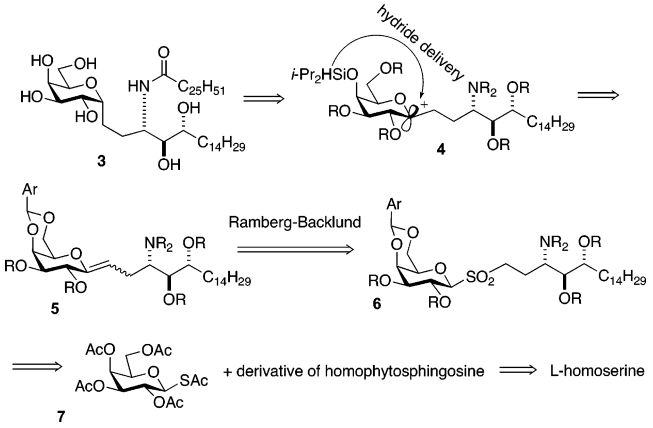
exploits the observation that anomeric carbonium ions are alkylated by allyl silanes to form axial (α) *C*-glycosides (Scheme 1a). A complementary axial hydride transfer to an alkylated anomeric carbonium ion provides an equatorial (*β*) *C*-glycoside (Scheme 1b).[18] Typically, simple chains are introduced initially and then are elaborated in a linear fashion to obtain the desired *C*-analogue of the glycoside of interest. Approaches applying the opposite polarity are also widely applicable and employ an organometallic, anionic anomeric carbon. One version of this strategy uses samarium as the metal and carbonyls as the electrophile (Scheme 1c).[19] A convergent and intramolecular variant of the nucleophilic anomeric carbon approach is the Ramberg−Bäcklund rearrangement of easily accessible carbohydrate sulfones, developed simultaneously by our group in New York and the Taylor lab in (old) York, England (Scheme 1d).[20]

In the late 1990s, our group had been educated on the importance of glycolipids by Prof. Robert Bittman at Queens College. We were thus inspired to apply our *C*-glycoside technology based on the Ramberg−Bäcklund reaction to prepare simple *C*-glycolipids of interest to the Bittman group.[21] This led us to consider other possible targets, and we turned our focus to the C-analogue of KRN7000. In addition to the synthetic challenge, we thought that this analogue afforded a structural change of a more fundamental kind than had been realized via lipid chain variation. After our initial reports, the Annoura group has described a *C*-analogue of OCH.[22]

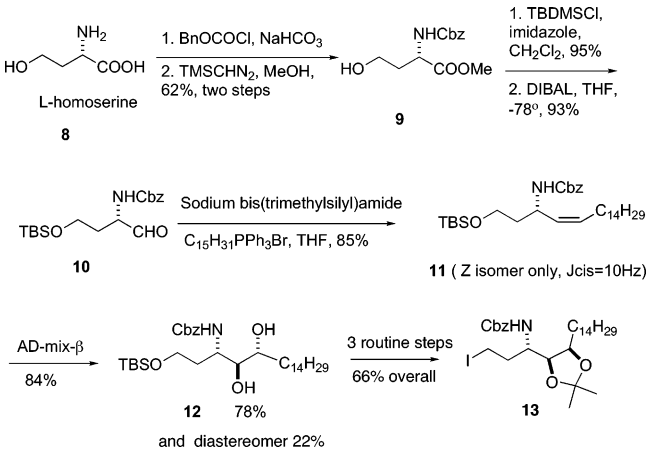
Our synthetic plan, using the Ramberg−Bäcklund reaction as the key C−C bond forming step, is outlined in Scheme 2. Aside from the creation of the *C*-glycosidic bond, there was required a stereoselective introduction of an anomeric hydrogen from the *β*-face of the exo glycal so as to form an α-glycoside (see intermediate **4** in the scheme).

Our material requirements were a suitably protected galactose and a phytosphingosine homologated by one

Scheme 2. Retrosynthesis of *C*-Glycoside Analog of KRN7000

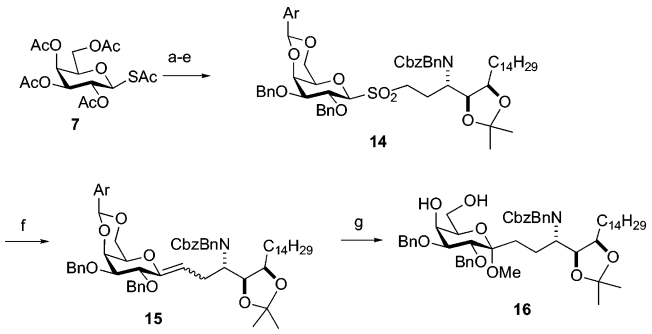


Scheme 3



carbon, which would serve as the replacement of the glycosidic O. In 1999, phytosphingosine was not an article of commerce (vide infra), so we patterned our synthesis on one reported by Nakanishi, which began with L-serine via Garner aldehyde (Scheme 3).[23] We simply started with L-homoserine. The synthesis was uneventful and not very

Scheme 4[a]



[a] (a) (i) NH_2NH_2/HOAc, DMF; (ii) **13**, Et_3N, 90%; (b) NaOMe, MeOH; (c) *p*-$MeOC_6H-CH(OMe)_2$, *p*-TsOH, DMF/CH_2Cl_2, 86% for two steps; (d) NaH, BnBr, THF, 83%; (e) MMPP, THF/H_2O/EtOH, 60 °C, 93%; (f) $C_2F_4Br_2$, *t*-BuOH, KOH/Al_2O_3, reflux, 70%; (g) TMSCI, MeOH, 0 °C, 66%.

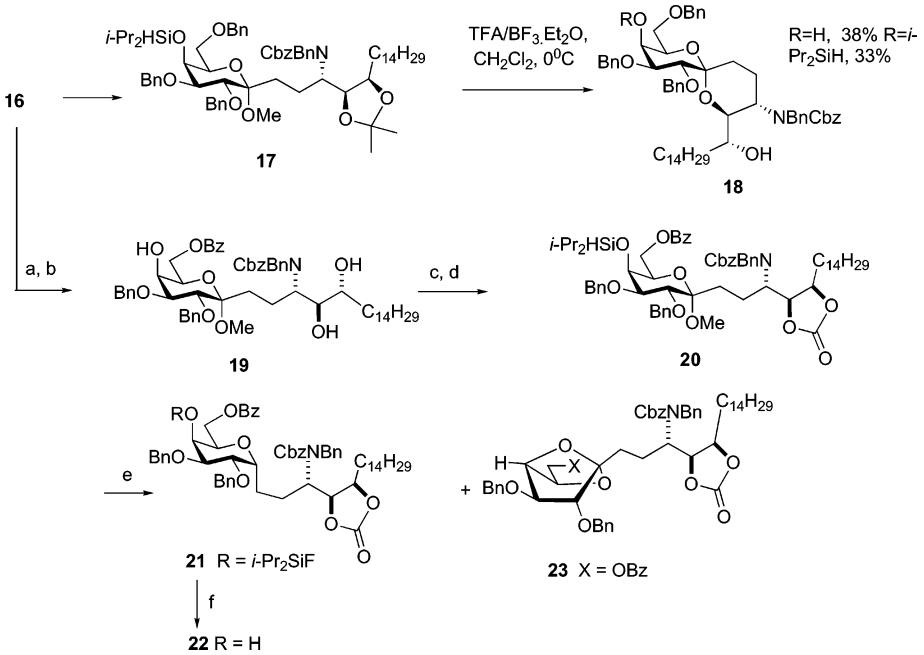
stereoselective (<70% ee) in the Sharpless dihydroxylation step (AD-mix-β) of the cis double bond obtained from our homoserinal derivative via Wittig olefination.

We were fortunate that an intermediate material obtained from the major Sharpless isomer was crystalline, and its X-ray structure revealed the anticipated stereochemistry of the OH's. After routine linkage of the phytosphingosine **13** to our protected galactose **7** and conversion of intermediate sulfide to sulfone **14**, the Chan modification of the Ramberg−Bäcklund reaction was carried out with $Br_2C_2F_4$ (bp 45°) as the brominating agent and KOH/alumina as the base.[24] The change from the standard Freon CBr_2F_2 (bp 23°) was necessary to be able to increase the reaction temperature to obtain useful yields in the Ramberg−Bäcklund step (Scheme 4). The two-carbon Freon is on the EPA "ozone" list and is no longer available; thus this modification of the Chan conditions is now defunct.

The exo glycal product **15** needed some manipulation to introduce a silane at the galactose 4-hydroxyl. This silane **17** was designed to serve as an internal hydride source to stereospecifically deliver the required β-face hydrogen to the carbonium ion intermediate at C-1 of galactose, a concept that we had elaborated in a simple alkyl galactoside. When the key hydride transfer step was attempted, we found that the carbonium ion was trapped completely by a phytosphingosine oxygen even though it was blocked as part of an acetonide. We therefore had to backtrack to convert the phytosphingosine oxygens to the cyclic carbonate **20** to reduce their nucleophilicity. Then the intramolecular hydride transfer process took place as desired to afford an α-*C*-galactoside (Scheme 5). The synthesis was completed by a series of deprotections, culminated by a hydrogenolysis performed in refluxing methanol with cyclohexene as the hydrogen transfer reagent (Scheme 6). This little-used method developed by Roush was not the first one we explored for this step.[25] Intermediate **24** was important because it provided convincing nuclear Overhauser effect (NOE) data for the desired stereochemistry at the anomeric carbon, which had been by the hydride transfer. Finally, amidation using the *p*-nitrophenyl ester of cerotic acid as the acyl transfer agent afforded the target *C*-glycoside analogue of KRN7000.[26]

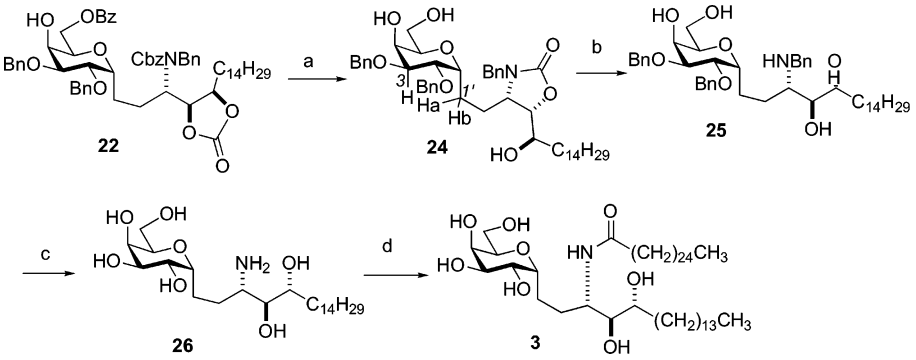
As we describe below, *C*-analogue **3** is a remarkably active immunostimulant. Clearly, our Ramberg−Bäcklund approach, although interesting, required too many manipulations to produce the required α-*C*-galactoside configuration. Thus, we began studies on syntheses where the α stereochemistry could be installed directly by the thoroughly developed methodology of silyl-stabilized axial

Scheme 5[a]



[a] (a) BzCl, Et_3N, CH_2Cl_2, 88%; (b) 1 N HCl/Et_2O, MeOH, 80%; (c) triphosgene, pyridine, CH_2Cl_2, 83%; (d) *i*-Pr_2SiHCl, imidazole, DMF, 96%; (e) 5 equiv of BF_3/Et_2O, CH_2Cl_2, 0 °C; (f) TBAF, THF.

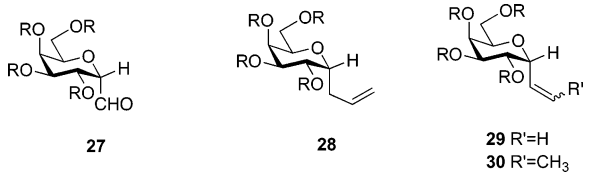
Scheme 6[a]



[a] (a) 1,4-dioxane/H_2O, NaOH, reflux, >90%; (b) KOH/EtOH, reflux, 80%; (c) 10% Pd/C, cyclohexane, 1 N HCl, MeOH, reflux, >90%; (d) *p*-nitrophenyl hexadecanoate, THF, DMAP, 49 h, rt, 60%; (e) Ac_2O, DMAP, 80%.

alkylation of anomeric carbonium ions. Our studies focused on four known materials that are easily prepared.

One synthesis scheme used the aldehyde **27** as a

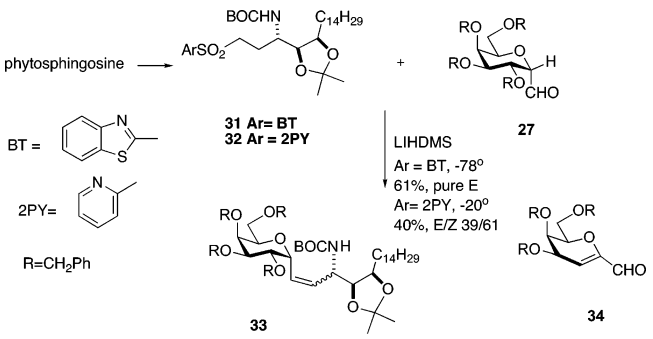


partner in the Julia−Lythgoe−Kocienski (JLK) olefination with a family of sulfones easily prepared from commercially available phytosphingosine, which came on the market after our Ramberg−Bäcklund had been completed. The JLK route was anticipated to be risky since any anomeric carbanion formed via proton transfer from aldehyde to sulfone anion would almost certainly afford a glycal via β-elimination. It was fortunate for our synthesis that the standard low-temperature conditions for the JLK favored C−C bond formation over elimination. However, when the reaction was attempted with the pyridyl sulfone reported by Charette to enhance formation of *Z* alkenes,[27] higher temperatures were required and significant levels of elimination to **34** were observed (Scheme 7).

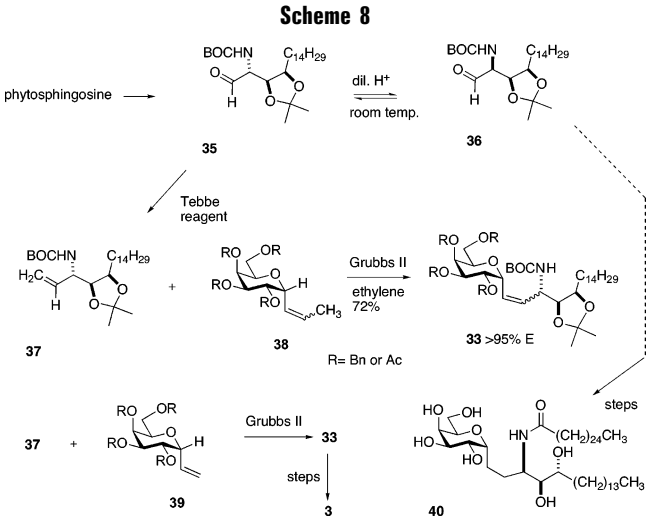
The alkene products of the JLK allowed us to prepare saturated analogues via hydrogenation/hydrogenolysis, whereas the use of Birch conditions for debenzylation afforded analogues where the 1,2 alkene linker was preserved.[28]

A direct and attractive route to the same trans alkene is olefin cross-metathesis. Thus, we discovered that in the

Scheme 7



Scheme 8

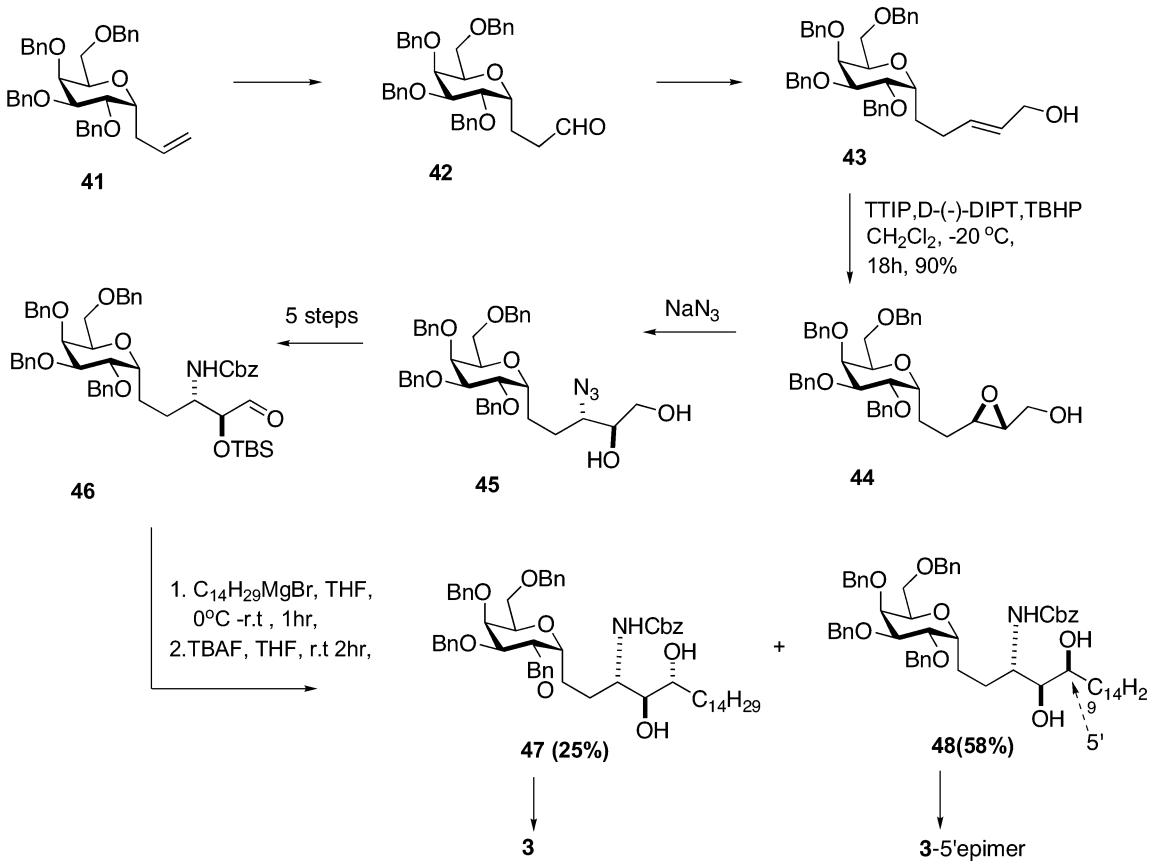


presence of ethylene, the alkene **38**, easily obtained as the tetraacetate in two steps from pentaacetyl galactose, underwent cross-metathesis with alkene **37** derived from aldehyde **35**, which itself is easily obtained in five steps from phytosphingosine. Cross-metathesis is also successful with galactoside **39**, but its preparation requires an extra step. The chemistry in this process is very clean but requires the use of excess sugar alkene, which is partly consumed by dimer formation. Of course the dimer can be reused since cross−metathesis with ethylene will convert it back to **39**. Overall, this scheme provides a synthesis of our potent immunostimulant in 11 steps.[29]

Aldehyde **35** is sensitive to both acidic and basic catalysis of an epimerization process to afford aldehyde **36**, which can eventually lead to the C-3 amido epimer **40** of the "exact" analogue (Scheme 8).

Our fourth successful synthetic scheme begins with the aldehyde **42** derived from alkene **41**. Homologation via Wittig chemistry followed by reduction to the alcohol afforded the allylic alcohol for treatment with the well-established Sharpless epoxidation, which established the stereocenters for the phytosphingosine sector. Then sodium azide opening with inversion eventually led to the protected amino hydroxy aldehyde. Then Grignard chemistry afforded two alcohols; the minor isomer of the pair, upon routine transformation, matched up with the original α-*C*-GalCer. Hence, the major isomer must be the C-4

Scheme 9



diastereomer of α-*C*-GalCer (Scheme 9).[30] To conclude the synthetic discussion, our group has accomplished a set of versatile syntheses of members of the α-*C*-galactosylceramide family, the immunostimulant activity of which will be summarized in the following section.

In addition to our research and the work of Annoura cited earlier, it is important to note that the groups of Mootoo, Postema, Dondoni, and Gurjar have reported related *C*-glycolipid work, differing from the present work either by stereochemistry, by carbohydrate, or by nature of the ceramide.[31]

The excitement for these synthetic materials stems from the very sizable improvement in the "curative" effects of the *C*-glycoside **3** over the *O*-glycoside **2**. Notably the increased efficacies of the *C*- vs *O*-glycoside observed on the disease states of mice are much greater than the changes in cytokine levels found in their serum (see Table 1, data abstracted from our published[29] graphical presentations describing cytokine levels vs time, the tabular mode conserves space). It is interesting that the switch from O to C does not cause a proportional change in cytokine levels or lifetimes. There is not a linear relationship between these cytokine levels and disease outcome. Also, there is not the same "cure ratio" of C/O with different diseases—1000/1 for malaria, 100/1 for melanoma, 2/1 for vaccine adjuvant effect—all in favor of our *C*-glycoside **3**. The following four figures summarize the effects of our synthetic **3** with *O*-glycoside **2** as positive control in mice.

The data needs little explanation. Figure 3a shows the protective effect of doses of glycolipid given to mice 24 h prior to challenge with a sporozoite stage of malaria parasites. The mice are then sacrificed 42 h after the challenge, when the parasites fully mature, and the level of the parasite burden in their livers is measured by an assay developed by the Tsuji group. It is shown that 1 ng of our *C*-glycolipid has a protective effect equivalent to that of 1 μg of *O*-glycolipid. Figure 3b shows a crude kinetic experiment; the interval between an inoculation of 100 ng of each glycolipid and malaria challenge was varied between 10 days and 1 day. It is clear that neither glycolipid has an effect at day −10 and day −7. However, the *C*-glycolipid is effective when the mouse is treated 4 days before challenge, whereas the O-lipid is only effective 1 day before challenge. This difference is consistent with a "hydrolytic stability of *C*-glycoside" argument. But, if one refers to the lifetime of cytokine production shown in Table 1, it may be seen that cytokine production is greatly reduced long before the 4-day (96 h) interval of a glycolipid inoculation versus malaria challenge. So the real explanation is more complicated than the classic *C*- vs *O*-glycoside argument.

Figure 3c shows that our *C*-glycoside can elicit complete protection from actually getting malaria infection in 9 out of 10 treated mice. Thus, in a nonsacrifice experiment, all but one mouse treated with *C*-glycolipid remained free of malaria infection in the blood. Note that all of the mice treated with *O*-glycoside did develop the

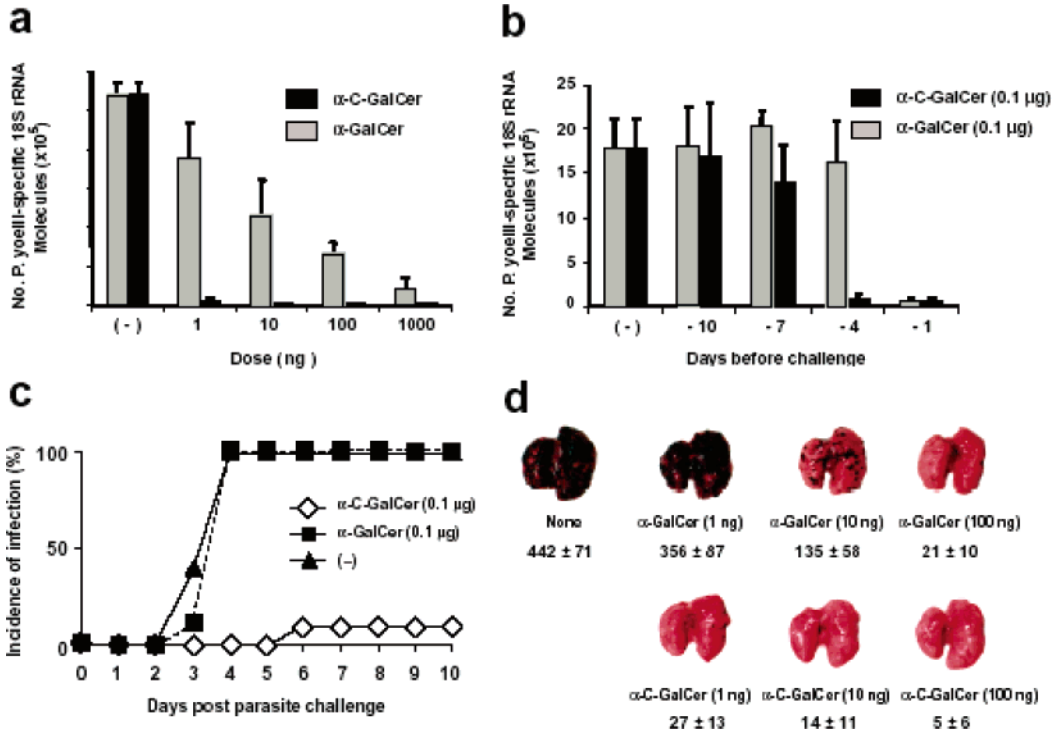


FIGURE 3. Panel a shows dose-response data as sporozoite levels vs pretreatment of mice with glycolipids. Panel b shows sporozoite level vs time of pretreatment with glycolipids prior to sporozoite challenge. Panel c shows postchallenge levels of blood parasite as a function of time. Panel d shows lungs of mice pretreated with glycolipids followed by melanoma challenge. Reprinted with permission from ref 32. Copyright 2003 Rockefeller University Press.

Table 1. Maximum Cytokine Levels in Murine Sera after 1 μg Injections of Ligands

ligand	IFN-γ, pg/mL	time (h) max IFN-γ	IL-4, pg/mL	time (h) max IL-4	ratio IFN-γ/IL-4
control	0		0		
KRN7000**2**	1800 ± 50	12	1100 ± 50	2	1.64 ± 0.13
C-gly **3**	2100 ± 50	24	475 ± 50	2	4.42 ± 0.55

blood stage of malaria. Figure 3d shows the protective effect of glycolipid in mice challenged with melanoma. In this experiment, the mice are first treated with glycolipid and then challenged with melanoma cells, which in these mice migrate to the lungs and grow. It can be seen that both glycolipids have a protective effect and that, again, the *C*-glycolipid is about 100-fold more effective than the O-analogue. Not shown are the control experiments using genetically engineered so-called "knock-out" mice that lack various molecules correlated to immune responses. These mice are deficient in either CD1d receptors or Vα14+ NKT cells or IFN-γ production ability. Using these "knock-out" mice, other investigators have published that the *O*-glycolipid, to display activity, requires these immune system components. We have reconfirmed the *O*-glycolipid results and furthermore have demonstrated that our *C*-glycoside similarly requires these molecules and cells to display its biological activity. In further probing of the system, we have found that IL-12, produced from the antigen-presenting cell component of the triple complex APC−GalCer−NKT, stimulates NK cells to produce IFN-γ. We have described these data in full in our *Journal of Experimental Medicine* paper.[32]

At this time, our explanation for the enhanced activity in mice of our *C*-analogue is based on differences in the behavior of the target NKT cells toward stimulation by the *O*-glycoside and the *C*-glycoside. It is known that the O-analogue causes a down regulation of activity of the NKT cell after its initial burst of cytokine secretion. We assume that our *C*-analogue, which perhaps binds the CD1d molecule or TCR of NKT cell a little differently, does not provoke down regulation so that the NKT cells and subsequently the NK cells continue to produce cytokines. In fact, the *C*-glycoside induces a prolonged production of IFN-γ in vivo compared to *O*-glycoside as shown in Table 1. This issue needs to be clarified by further studies.[33]

A second point of interest in the *C*-analogue series is the comparison of the *E* and *Z* materials available via the modified Julia chemistry described above. Examination of the X-ray data for the bound *O*-glycoside reveals an anti or transoid conformation in the linker region. Hence, it is easy to predict that the shape of the *E* analogue more resembles the shape of the bound O-analogue. In vivo assay for levels of IL-4, IL-12, and IFN-γ induced by the *E* and *Z* isomers in mice confirm the X-ray binding model. Thus, the *E* isomer is effective in stimulating cytokine production, while the *Z* isomer is essentially inactive. Figure 4 shows the levels of IL-12 production of parent *O*-glycoside, *C*-glycoside X, and the *E* and *Z* isomers. This preference of *E* over *Z* is a typical result for all cytokines examined.[28]

In conclusion, our discovery of the *C*-analogues of α-*O*-GalCer has heightened prospects for the production of a

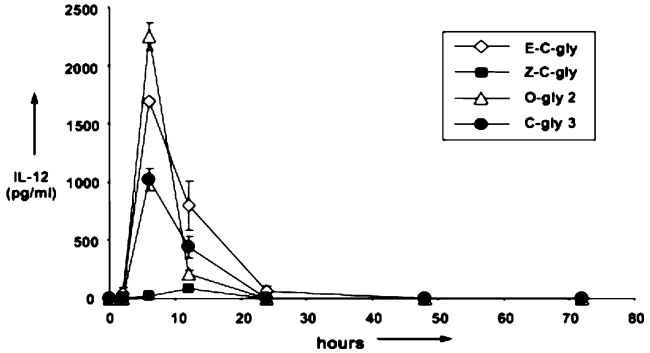


FIGURE 4. IL-12 levels in mouse sera from 0-72 hrs post-injection of glycolipids. Reprinted with permission from ref 28. Copyright 2006 Wiley-VCH.

potent immunostimulant and has provided suggestive information about the details of the interaction of this family of glycolipids with the CD1d molecule of antigen-presenting cells. Our analogues are the best confirmation to date of the notion that replacement of the linking O of a bioactive glycoside by C can provide a superior material.

Note Added in Proof

The reader is referred to a new review of the galactoceramide area recently published online: Savage, P. B.; Teyton, L.; Bendelac, A. *Chem. Soc. Rev.* 2006, DOI: 10.1039/b510638a.

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