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Synthesis of Fungal Glycolipid Asperamide B and Investigation of Its Ability to Stimulate Natural Killer T Cells

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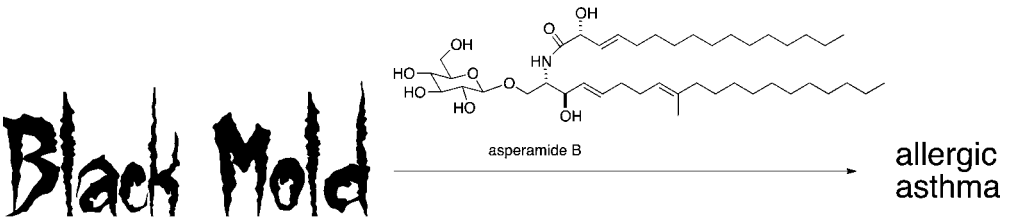
ABSTRACT



The relationship between mold and asthma has been recognized for decades, but the molecular triggers of asthma generated by molds have not been fully elucidated. A glycolipid generated by *Aspergillus* species has recently been identified that triggers airway hyperreactivity via natural killer T cell activation. The synthesis of this glycolipid and structural variants designed to allow identification of the features of this glycolipid required for recognition by natural killer T cells is described.

Aspergillus species (e.g., *A. niger* and *A. fumigatus*) are ubiquitous in nature, and humans are continuously exposed to these organisms. For example, *A. niger* comprises the black mold commonly found in food and which often grows indoors in moist environments. These species of fungus have been implicated in either triggering or perpetuating asthma,[1,2] a disease that affects nearly 300 million people worldwide.[3] Recently, we discovered a link between a glycolipid produced by these organisms, asperamide B (Figure 1), and natural killer T (NKT) cells that contribute to airway hyperreactivity in animal models.[4] Airway hyperreactivity is a cardinal feature of asthma,[3] and asperamide B appears to be a primary causal link between NKT cells, *Aspergillus*, and asthma.[4]

NKT cells are a subset of T cells and constitute an important aspect of innate immune function in most mammals.[5] These cells recognize glycolipids presented by the CD1d protein on antigen-presenting cells, and in response release cytokines, which impact both innate and adaptive immune functions. Responses of NKT cells to glycolipid antigens of bacterial origin have been shown to contribute to inflammation in the lung, leading to airway hyperreactivity.[6,7]

Considering the role that fungi can play in triggering asthma, it was anticipated that *Aspergillus* species might also generate antigens that stimulate cytokine production by NKT cells. Though multiple antigens from *Aspergillus* species are recognized by aspects of innate immunity,[8] antigens that directly activate NKT cells from these fungi, or from fungi in general, had not been identified. Through careful fractionation of *Aspergillus* glycolipids, guided by

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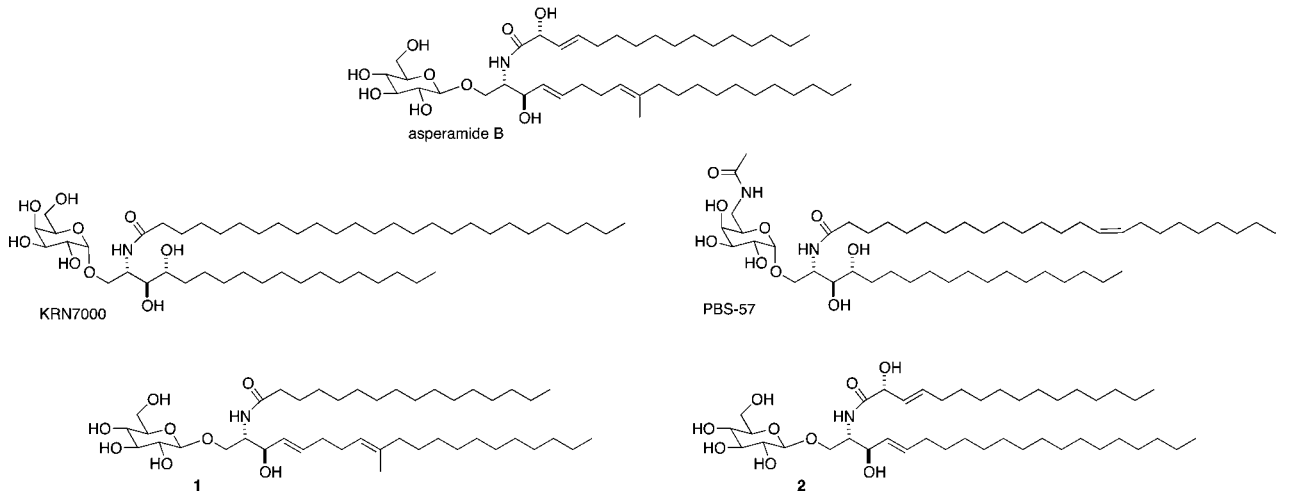

Figure 1. Structures of asperamide B, KRN7000, PBS-57, and asperamide B variants **1** and **2**.

the responses of NKT cell lines, we identified a glycolipid from *Aspergillus* that is effectively presented by CD1d, elicits cytokine release from NKT cells, and triggers airway hyperreactivity in animal models.[4]

This glycolipid, termed asperamide B, has been isolated from *A. fumigatus*[9] and *A. niger*,[10] and in these reports the structure of asperamide B was proposed but not confirmed via synthesis of the proposed structure. A closely related glycolipid was isolated from *Penicillium funiculosum* (termed Pen II),[11] and Pen II differes from asperamide B in the lengths of the lipid chains found in the glycolipids. Mori and Uenishi reported the synthesis of Pen II,[12] though no structure−activity studies were performed. At the time, the only known biological activity of Pen II was as a fruiting inducer with mushrooms.

Asperamide B includes a 9-methyl-4,8-sphingadine chain, which is more complex than the sphingosine chain typically found in mammals and an α-hydroxy, β-γ unsaturated acyl chain. Notably, the glucose-ceramide bond in asperamide B is β. In general, NKT cells respond most strongly to α-linked galactosylceramides (e.g, KRN7000 and PBS57, Figure 1).[13] And although endogenous β-glycosylceramides have been shown to be recognized by NKT cells,[14] it was unexptected that a β-linked glucosylceramide from a microorganism would elicite responses from NKT cells.

We obtained asperamide B via isolation from fungi and from total synthesis of the glycolipid. And to better understand the features of asperamide B required for recognition by NKT cells, we also prepared structural variants of the parent glycolipid (**1** and **2**, Figure 1) in which the acyl and sphingadiene chains in asperamide B were exchanged for simpler and more common acyl and sphingosine chains. Herein, we describe procedures used for the isolation of asperamide B and detail the synthesis of asperamide B and variants **1** and **2** and their abilities to stimulate cytokine release from NKT cells. These studies expand the number of organisms producing antigens for NKT cells and the types of glycolipids known to stimulate these cells.

To isolate asperamide B from *A. niger* (ATCC 1015) and *A. fumigatus* (ATCC 9197), the fungi were cultured in nutrient media and a crude lipid extract was generated using Folch's procedure.[15] Glycosphingolipids were separated from other polar lipids using an aminopropyl stationary phase,[16] and asperamide B was isolated as a white solid by silica gel chromatography. From equivalently sized samples of crude lipid extract from *A. niger* and *A. fumigatus*, we compared the relative amounts of asperamide B from each species and found that *A. fumigatus* generated approximately five times as much of the glycolipid as *A. niger*. Isolated asperamide B was used as a comparator for the synthetic glycolipid (vide infra). In the initial report of NKT cell stimulation by asperamide B, we showed that isolated and synthetic asperamide B elicited similar responses from NKT cells.[4]

Synthesis of asperamide B largely followed published procedures for the generation of the 9-methyl-4,8-sphingadiene and α-hydroxy, β-γ unsaturated acyl chains. To prepare the 9-methyl-4,8-sphingadiene portion (**5**, Scheme 1), we first followed the efficient procedure developed by

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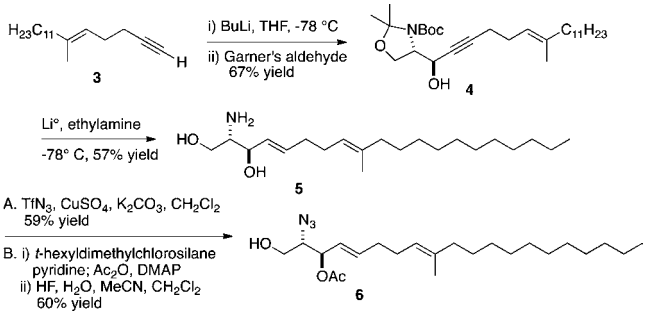
Murakami et al. to generate **3**.[17] They prepared this alkyne using Negishi's carbalumination protocol[18] with the corresponding alkyne, reaction with paraformaldehyde, conversion of the resulting alcohol to the chloride, and chain extension with lithiated TMS propyne. In Murakami's synthesis, hydrozirconation of **3** and reaction with Garner's aldehyde were used to generate the protected sphingadiene, which was subsequently deprotected. We found that it was simpler to deprotonate the alkyne, add it to Garner's aldehyde (giving **4** (the Felkin–Ahn product) with high stereoselectivity),[12] and reduce the alkyne and deprotect the amine and alcohol with lithium in ethylamine in a single step to give **5**. To prepare this sphingadiene for coupling to glucose and for ceramide formation, we converted the amine in **5** to the corresponding azide and through a sequence of protection and deprotection steps generated **6**.

Scheme 1. Preparation of Sphingadiene Derivative **6**



In an attempt to prepare asperamide B, we prepared protected ceramide **7** (Figure 2) but found that substantial epimerization occurred at the C2′ position in the process of glycoside bond formation. The fact that this is an allylic position apparently lowers the pK_a of the α proton sufficiently that deprotonation and epimerization compete with other processes in the glycosidic bond-forming reaction. Consequently, we elected to form the glycosidic bond prior to ceramide generation, and this required conversion of the amine in **5** to the corresponding azide in **6**.

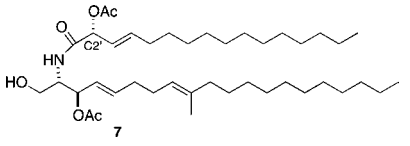
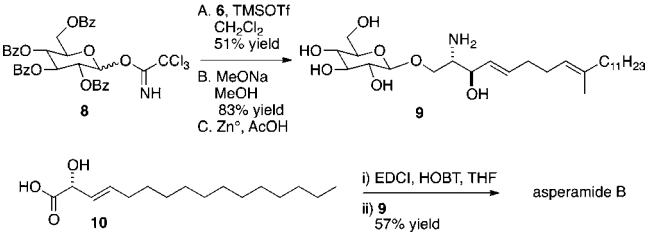


Figure 2. Diacetyl form of the ceramide found in asperamide B.

Reaction of Schmidt donor **8** with **6**, followed by deprotection and azide reduction, gave amine **9**. The acyl chain found in asperamide B (**10**, Scheme 2) was prepared using the method reported by Ratovelomanana-Vidal and co-workers in their synthesis of symbrioramide.[19] Due to the propensity of **10** to racemize, it was incorporated in the last step of the synthesis to minimize diastereomer formation. Any epimerization became apparent after amide formation and generation of diastereomeric forms of the asperamide B, which were readily observable via NMR spectroscopy. The NMR spectra of synthetic and isolated asperamide B matched well (see Supporting Information).

Scheme 2. Synthesis of Asperamide B



With asperamide B, in synthetic and isolated forms, in hand and with data indicating that asperamide B stimulates NKT cells, we sought to determine the structural features of asperamide B that were responsible for its activity. As noted above, asperamide B differs from glycolipids found in mammalian systems in both its 9-methyl-4,8-sphingadiene and α-hydroxy, β-γ acyl chains. To distinguish between the influences of each chain, we prepared asperamide B variants **1** and **2** (Figure 1), in which the more complex acyl chain is replaced by palmitate (giving **1**) and 9-methyl-4,8-sphingadiene is replaced by sphingosine (giving **2**). To prepare **1**, amine **9** (Scheme 2) was reacted with palmitic acid, EDCI, and HOBT to give the corresponding amide in 67% yield. To prepare **2**, C_{20} sphingosine was converted to **11** (Figure 3), which was coupled with glucose, and the resulting glucoside was deprotected and reduced as described in Scheme 2 to give amine **12**. Amine **12** was coupled with **10** to give the corresponding amide (**2**, Figure 1).

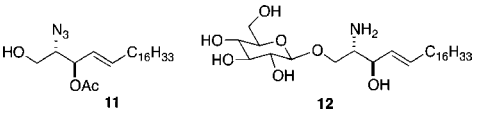


Figure 3. Intermediates used in the synthesis of asperamide B variant **2**.

NKT cell stimulation requires glycolipid presentation by CD1d on antigen presentation cells, and stimulation can be quantified by measuring cytokine production (e.g., IFN-γ and IL-4). To compare the stimulatory activity of

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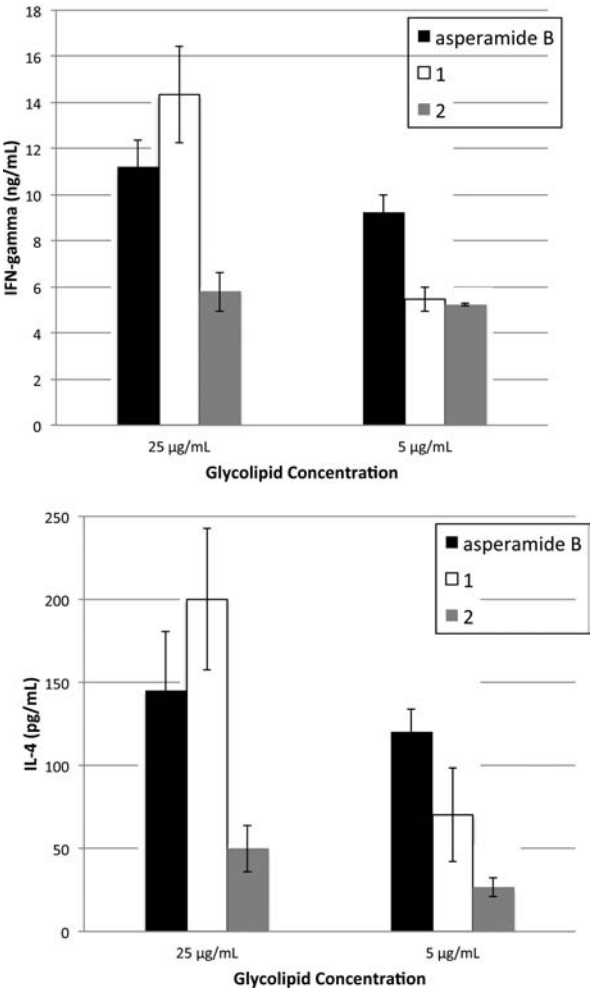

Figure 4. In vitro culture of murine NKT cells and BMDCs incubated with synthetic asperamide B, **1**, or **2**. Supernatant was harvested after 48 h, and IL-4 and IFN-γ concentrations were measured by ELISA.

asperamide B, **1**, and **2**, we used a mouse NKT cell line and bone marrow-derived dendritic cells (BMDCs) as antigen presenting cells. PBS57, a potent stimulator of NKT cells,[20] was used as a comparator. Cytokine concentrations were measured using ELISA and are shown in Figure 4. Asperamide B and **1** elicited similar responses from NKT cells with both IFN-γ and IL-4 release. Exposure of NKT cells to variant **2** resulted in the release of significantly less of both cytokines. This result suggests that the 9-methylsphingadinene portion of asperamide exerts a greater influence on cytokine release than the α-hydroxy, β-γ unsaturated acyl chain. The comparator, PBS57 (100 ng/mL), gave concentrations of IFN-γ and IL-4 of 23.4 ng/mL and 1780 pg/mL, respectively, in the same assay. It should be noted that, as an α-galactosylceramide, PBS57 stimulates NKT cells at much lower concentrations than the β-glucosylceramides asperamide B, **1**, and **2**.

NKT cells play a central role in responses to diseases ranging from infection to cancer to asthma, and this breadth of impact on immune function underlies the need to fully understand the repertoire of glycolipids that stimulate responses from these cells. This understanding applies to environmental sources of stimulatory glycolipids *and* to the development of potential therapeutic agents targeting NKT cells. By identifying asperamide B as an NKT cell antigen of fungal origin, we have expanded the number of known environmental sources of stimulatory glycolipids. In addition, by establishing the role of 9-methyl-4,8-sphingadiene in causing NKT cell stimulation, we have identified a structural motif that may prove useful in optimizing glycolipid structures for NKT cell stimulation.

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Supporting Information Available. Experimental proceedures for the synthesis of asperamide B and variants **1** and 2. Materials and methods for measuring NKT cell stimulation. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.