Original
Received January 18, 2006
Accepted for Publication August 24, 2006

Construction of Sphingolipids Libraries and their Utilities

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Sphingolipids such as ceramide and glycosylceramide have recently attracted intense research interests because of their roles as signalling molecules in many important physiological processes, such as apoptosis, inflammation and immune responses. Their well-defined modular structures are ideally amenable to library formation for medicinal chemistry investigations. We have developed practical, divergent synthetic routes to sphingosine and phytosphingosine isomers as well as to carba-sugar analogues of all D-aldohexopyranose isomers toward these goals. And then we have proceeded to prepare ceramide libraries, composed of more than 500 compounds each, based on these sphingosine and phytosphingosine isomers, and demonstrated their utility in cell-based bioassays involving activation of NF-κB and induction of apoptosis. We are also in the process of forming libraries of mono-glycosylceramide and mono-carba-glycosylceramide.

Key Words : sphingosine, phytosphingosine, carba-D-aldohexopyranose, ceramide library, cerebroside library

Lipids are water-insoluble, structurally heterogeneous group of substances, and they occur throughout the living systems and in all cell types. They contribute to cellular structure, provide stored biological fuel, and participate in many biological processes ranging from transcription of the genetic code to regulation of vital metabolic pathways and physiological responses. Although lipids' roles as structural component as well as energy sources have been long recognized, only recently have their important roles in the regulatory processes been appreciated. Glycerolipids, sterols and sphingolipids represent the three major structural types of lipid backbone. Sphingolipids are particularly abundant in nervous tissue and they tend to be concentrated in the outer leaflet of the plasma membrane. So far more than 300 different types of complex sphinglipids have been reported even without considering the length variation of the backbone chain of sphingosine. The basic structure of sphingolipids is shown in Figure 1.

Ceramide has long been recognized as a signaling molecule in the inflammatory response. It acts as a second messenger in the signal transduction pathway triggered by several agents of stress, including oxidative stress and ionizing radiation, and extracellular stimuli such as proinflammatory cytokines and lipopolysaccharide. Ceramide activates the transcription factors NF-κB and others,

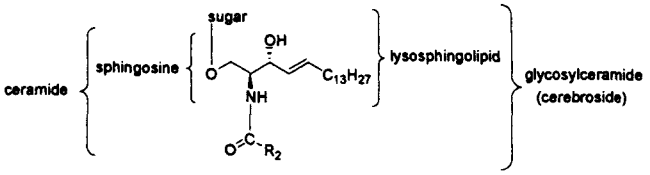


Figure 1 Sphingosine and sphingolipid

leading to the expression of multiple inflammatory proteins that amplify the inflammatory responses. Ceramide has also been well recognized as an important second messenger in triggering apoptotic/necrotic processes in normal and cancer cells. In response to several stimuli, ceramide induces apoptosis, apparently by activating caspases and inducing clustering of death receptors in the cell membrane. Naturally, ceramide has been implicated in several human diseases. For example, ceramide derivatives are markedly elevated in bronchoalveolar lavage of patients with acute respiratory distress (ARD) syndrome, an acute lung injury. Many mediators have been implicated in acute lung injury, including tumor necrosis factor-α (TNF-α), platelet activating factor (PAF), and prostaglandins. Recently ceramide has been suggested as a key molecule that integrates several inflammatory and injurious mechanisms.[1] Furthermore, it has been reported that cell growth was closely correlated with an increase of ceramide levels. A study on the effect of ceramide on the phosphorylation state of retinoblastoma (Rb) has revealed that addition of short chain ceramide to cells resulted in dephosphorylation of Rb and cell cycle arrest. Ceramide is also recognized as a differentiating agent in HL-60 cells. Further studies with neuronal cell lines have shown that ceramide mimics nerve growth factor function and is able to induce differentiation in T9 glioma cells (Figure 2).[2]

We have spent a considerable amount of efforts in developing practical methodologies for divergent and diastereo-controlled preparations of sphingosine[3] and phytosphingosine[4] (Figure 3, Scheme 1 and Scheme 2). As shown in Scheme 1, serine was efficiently converted to the β-ketophosphonate **3**. The Horner-Wadsworth-Emmons olefination of the β-ketophosphonate **3** with tetradecyl aldehyde provided the enone **4** in good yield, which was then reduced with $NaBH_4$ in the presence of $CeCl_3 \cdot 7H_2O$ to the *N,O*-diprotected L-*threo*-sphingosine **5** in 92% d.e.. On the other

hand, removal of the two protecting groups of **4**, followed by reduction with $Zn(BH_4)_2$ gave D-*erythro*-sphingosine **7** in 90% d.e.. Epoxidation of *syn*-sphingosine derivative **8** and *anti*-sphingosine derivative **15** with *m*-CPBA or DMD provided diastereomeric mixture of the corresponding epoxides (**9**, **10** and **16**, **17**), respectively. After separation, each diastereomer was reduced with DIBAL to yield 'inside alcohols' (**11**, **12**, **18** and **19**) which were deprotected to provide the corresponding phytosphingosines (**13**, **14**, **20** and **21**, Scheme 2). By employing the same procedures on D-serine, L-*erythro*- and D-*threo*-sphingosines, and the corresponding phytosphingosines were similarly synthesized.

In the construction of the ceramide library, the core structure (sphingosine or phytosphingosine) was derivatized in solution with a series of solid-phase acylating reagents. A number of reaction conditions were examined for the formation of ceramides using solid-phase activated esters as acyl donor with various resins, solvents, and activating ester groups. It was found that use of a nitrophenol ester on polystyrene in THF provided a very pure product, after a simple filtration step, without a trace of sphingosine or by-products containing *O*-acylation. Activated esters on solid support were prepared using either the acyl halide in pyridine or the isopropylcarbodiimide-activated acid. This way we have synthesized a library of more than 500 ceramide compounds by starting out with 4 sphingosine diastereomers and 12 sphingosine analogues, and 33 acyl chain donors (Scheme 3).[5] Recently we have also completed constructing another library of more than 500 ceramides based on phytosphingosine backbone (Figure 4).[6]

Some biological activities of the ceramide library based on the sphingosine backbone were tested, namely, two cell-based assays for NF-κB activation and the induction of apoptosis. Several cytokinines, icluding TNF-α and IL-1β, are known to induce the NF-κB activation with concomitant accumulation of ceramides in various cell types. However, it was not clear whether ceramide plays a direct role in the activation or not. When 10μM compound was added to the cells, a marked direct activation of NF-κB was observed only by the psychosine-based ceramides with β-galactosyl

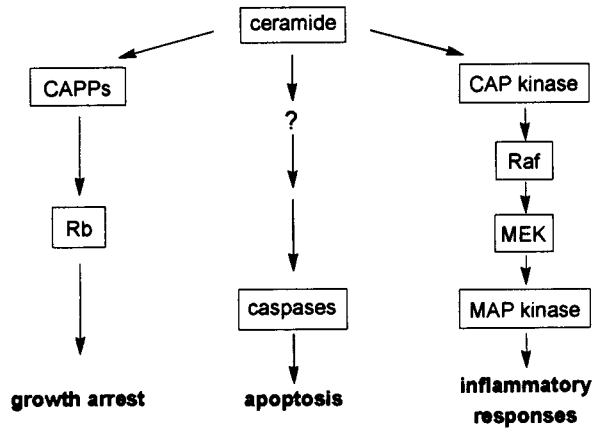


Figure 2 Physiological roles of ceramide

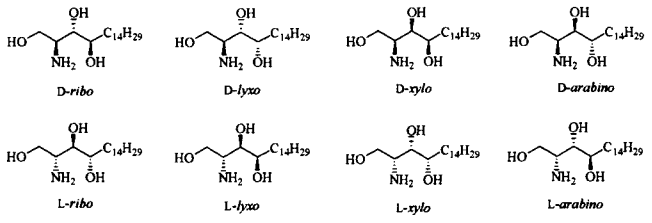
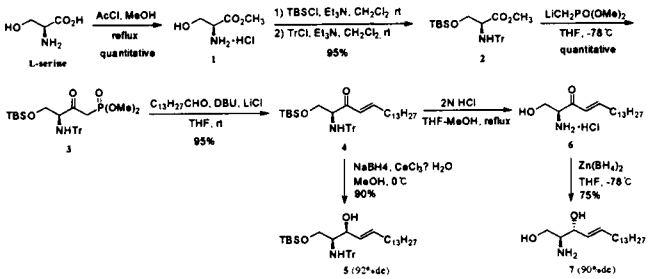
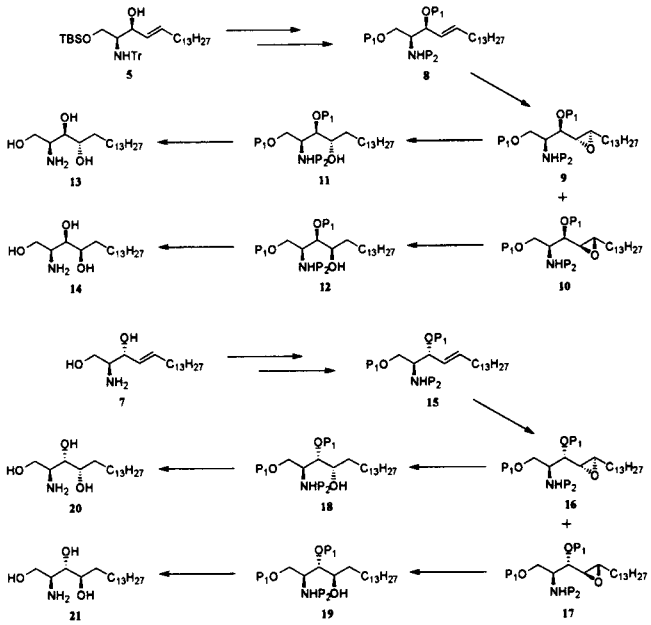


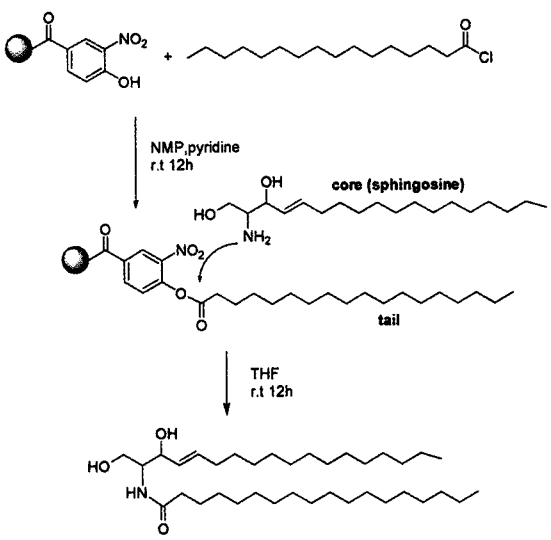
Figure 3 Structure of diastereomers of phytosphingosine



Scheme 1 Stereoselective synthesis of sphingosine



Scheme 2 Stereoselective synthesis of phytosphingosine



Scheme 3 A representative synthesis of ceramide with a solid phase acylating reagent

head group, and the ceramide with C9 chain was most potent with more than 8-fold increase of the NF-κB activity. It is most noteworthy that direct activation was sensitive to the sugar stereochemistry, as an analogue containing β-glucose showed no activity. We also screened the library compounds in the apoptosis assay with U937 leukemia cells, in which it was known that exogenous ceramide promotes apoptosis. The most active one was found to be the 12-carbon-(D)-*erythro*-sphingosine with C10 chain (IC50 of 4 μM). The activity vs. carbon chain length profile showed a Gausssian distribution with an optimum around 18 SCCL. These results suggest that the apoptotic activity depends on specific structural features, although the exact mechanism still remains to be elucidated (Figure 5).[5a]

Monoglycosylceramide, also known as cerebroside, occurs in all tissues, and the sugar moiety, in particular, depends largely on tissue types; for example, brain cerebrosides are mainly of galactoside types, while serum glycoceramides are of glucoside. In the early 1990s potent antitumor activity was located in the glycolipids (agelasphins) extracted from Okinawan sponge *Agelas mauritianus* by Kirin Pharmaceuticals, and α-galactosyl ceramide named KRN7000 was found to have the best activity in the SAR (structure-activity relationship) studies of related synthetic glycolipids (Figure 6).[7] The subsequent investigation of the biological activity of KRN7000 has revealed remarkable physiological activities against several diseases such as cancer, malaria, juvenile diabetes, hepatitis-B and autoimmune diseases such as encephalomyelitis. These findings have resulted in a proposed mechanism of action in which the glycosylceramide activates NKT cells via its binding to CD1d receptor of antigen presenting cells (APC), thus releasing a cascade of cytokines such as IFN-γ and IL-4 (Figure 7).[8] Little information is available about how KPN7000 structure is related to the cytokine release profiles of NKT cells. It has been reported that certain truncations of phytosphingosine chain and acyl chain results in release of greater amount of IL-4 by NKT cells[9], and that α-C-galactosyl ceramide derivative, which is not degraded by endogenous α-galactosidases (whereas the KRN7000 is) remains active *in vivo* for a longer period of time.[10] The structural feature of glycosylceramide represent an almost ideal situation for constructing combinatorial libraries. In addition, it would be highly desirable to have available well-focused combinatorial libraries of these molecules for a variety of cell-based bioassays in connection with developing and optimizing pharmacologically useful agents. The variable structural modular units are shown in Figure 8.

We are currently in the process of forming libraries of mono-glycosylceramide and mono-carba-glycosylceramide. We have already carried out the condensation between the β-ketophosphonate and a vatiety of alkyl aldehyde to give the desired *N*-protected α-amino enone derivatives, thus providing a reliable way to vary the hydrophobic chain length of phytosphingosine. Coupling of glycosyl donor with phytosphingosine derivatives gives glycosyl phytosphingosine derivatives. After the removal of the *N*-protecting group from glycosyl phytosphingosine derivatives, followed by acylation with varied carboxylic acids and deprotection provide glycosyl ceramide derivatives in which the lipid chain lengths have been varied (Scheme 4).[11] For the construction of non-hydrolyzable derivatives of glycosyl ceramide, we have designed and studied the synthesis of carba-glycosyl ceramide derivatives. Coupling of an aziridine derivative with the carba-sugar analogue,

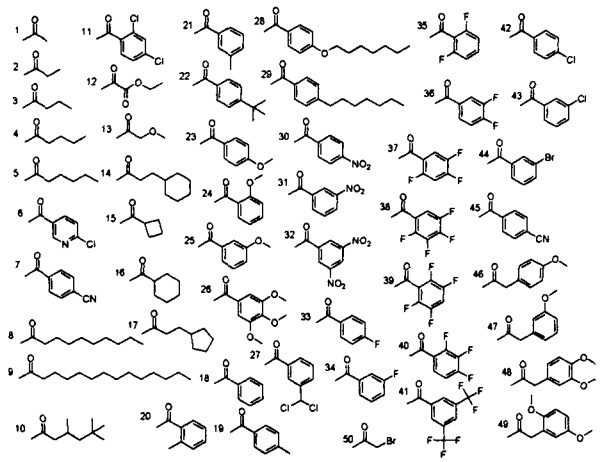


Figure 4 Tail structures of phytosphingosine-ceramide library

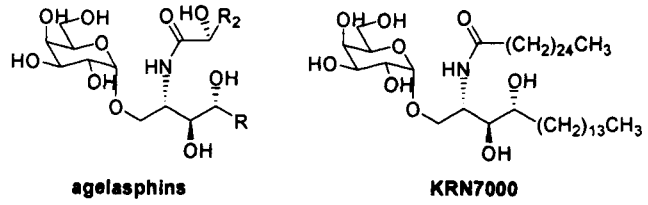


Figure 6 Structures of agelasphins and KRN7000

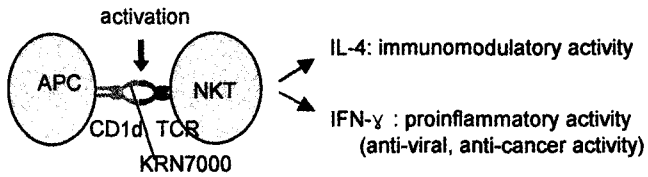


Figure 7 Role of KRN7000

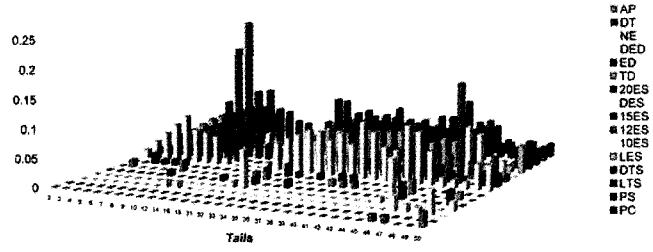


Figure 5 Apoptosis inducing effect of ceramide libraries

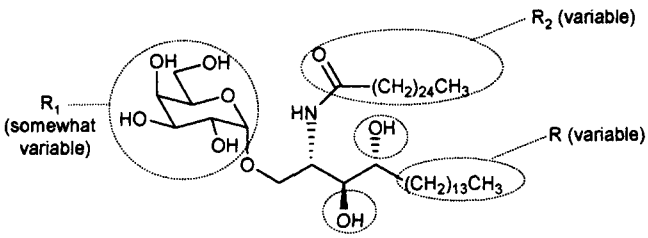
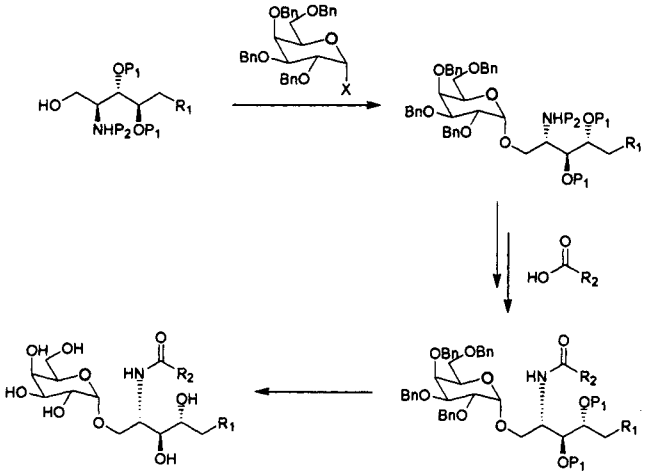


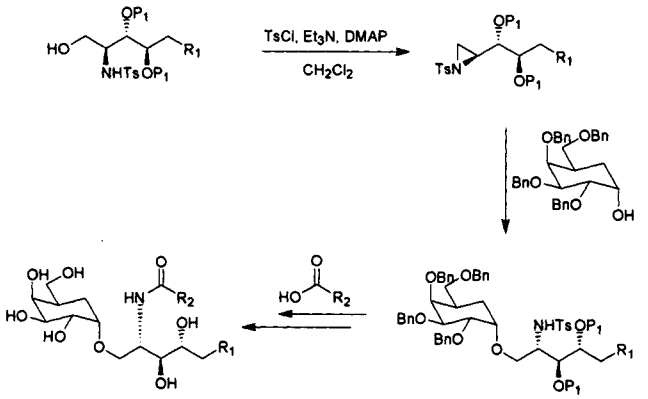
Figure 8 Various structural domains in KRN7000

followed by *N*-deprotection and acylation with varied carboxylic acids gives carba-glycosyl ceramide derivatives. The aziridine derivative, one of the key intermediates, was prepared by tosylation of primary alcohol of phytosphingosine derivative (Scheme 5). The other key intermediate, carba-sugar analogue was readily obtained by the method recently developed in our laboratory (Scheme 6).[12]

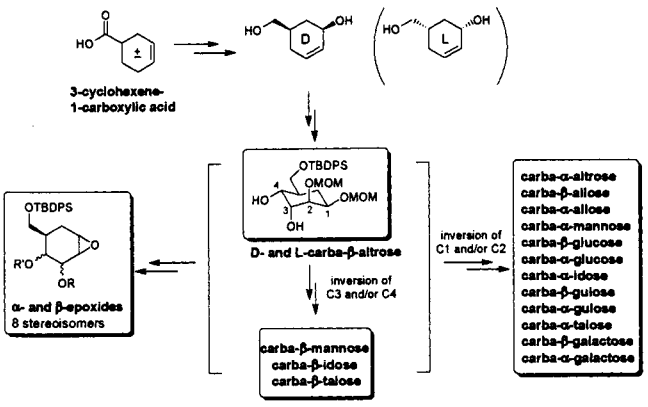
Acknowledgments – We thank all of our collaborators whose names appear in the references, in particular professor Y. T. Chang of New York University. This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2005-070-C00078).



Scheme 4 Synthesis of α-galactosyl ceramide derivatives



Scheme 5 Synthesis of carba-α-galactosyl ceramide derivatives



Scheme 6 Divergent syntheses of carba-sugar stereoisomers

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