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The Synthesis and Biological Characterization of a Ceramide Library

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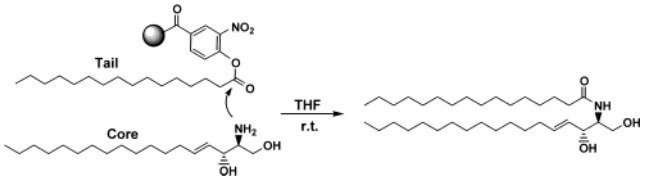
Fatty acids and their metabolites play key roles as second messengers in cellular signal transduction and as hormones that regulate an enormous array of physiological processes. For example, ceramides are sphingosine-based signaling molecules that regulate cell cycle arrest, proliferation, differentiation, and apoptosis.[1] Although many chemical libraries have been synthesized and screened for biologically active molecules over the past decade, combinatorial libraries of fatty acids, their derivatives, and analogues have not yet been extensively explored. Herein we report the synthesis of a combinatorial ceramide library, and present preliminary results obtained in cell-based screens of apoptosis and signal transduction that suggest libraries of this sort will be a rich source of biologically active synthetic molecules.

To synthesize the ceramide library, the core structure was derivatized in solution with a series of solid-phase reagents. A number of reaction conditions were screened for the formation of ceramides using sphingosine and solid-phase activated esters as acyl donor with various resins, solvents, and activated ester groups. Use of a nitrophenol ester on polystyrene in tetrahydrofuran was found to yield a very pure product after a simple filtration step, without a trace of sphingosine or byproducts containing O-acylation (Scheme 1).[2] With these optimized conditions, a library of 528 compounds was generated using 16 sphingosine-like core structures (Figure 1, Cores) and 33 acyl groups (**2**−**50** in Figure 1). Four stereoisomers of sphingosine, **DES**, **DTS**, **LES**, and **LTS**, were synthesized by a slightly modified literature procedure.[3]

Scheme 1. Representative Synthetic Scheme for Natural Ceramide



Activated esters on solid support were prepared using either the acyl halide in pyridine or the diisopropylcarbodiimide-activated acid. Completion of the reaction was confirmed by negative ninhydrin staining and thin-layer chromatography with phosphomolybdic acid staining. About 100 synthetic compounds among the 528 in the library were selected randomly and characterized by high-resolution mass spectrometry (MALDI-FTMS) and ^{1}H NMR to confirm their identity and purity.

The biological activities of members of this library were tested in two cell-based assays: NF-κB signaling and the induction of apoptosis. Several cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), are known to induce NF-κB activation, concomitant with ceramide accumulation in various cell types.[4] However, it is not clear whether ceramide plays a direct role in NF-κB activation.[5] Thus it was of interest to assay the agonistic effects of the library in an NF-κB reporter gene (luciferase) assay using C6 glioma cells.[6] When 10 μM compound was added to the cells, a marked direct activation of NF-κB was only observed for the **PC** ceramides with a β-galactose headgroup: **PC9** was the most potent (>8-fold increase of NF-κB activity). None of the

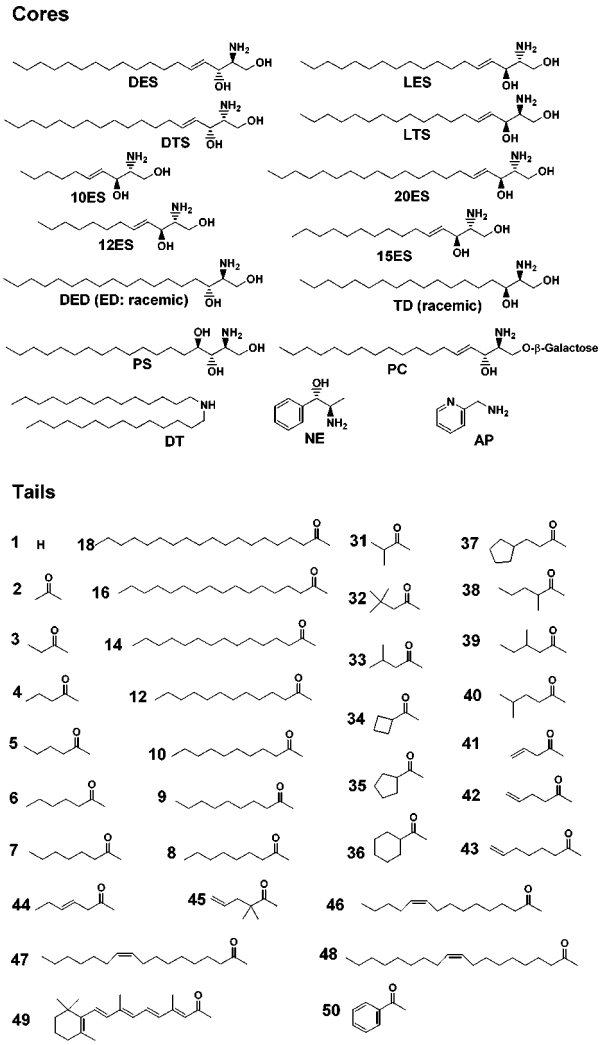


Figure 1. Structures of cores and tails of the ceramides.

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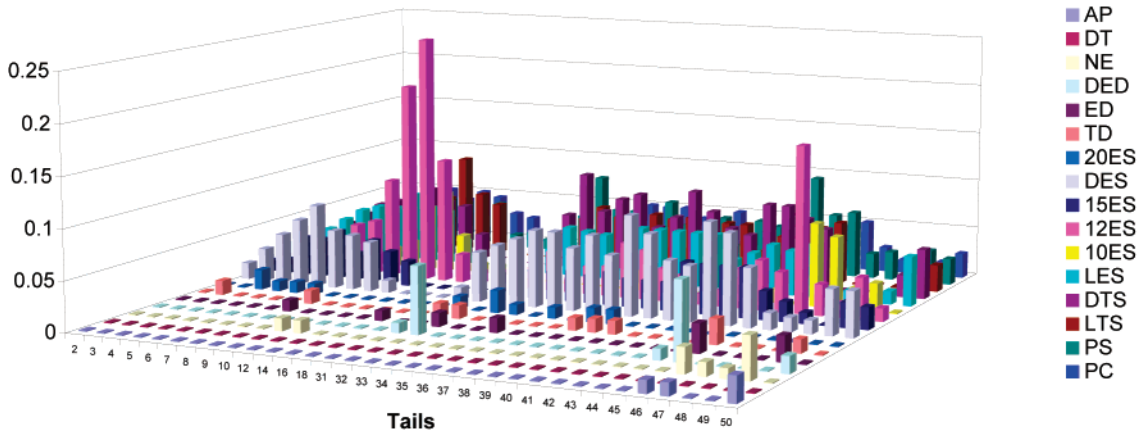

Figure 2. Apoptosis inducing effect of ceramide libraries. The *Z* axis is the reciprocal of IC_{50} (μM).

natural ceramides (**DES**) or their cell membrane permeable derivatives showed any effects in this assay. It is noteworthy that direct activation was sensitive to the sugar stereochemistry, as analogues containing β-glucose (**GP1**, **5**, **7**, **9**, **12**, and **32**) proved to be inactive in this system. This strict stereospecificity was not observed in a related cellular screen involving TNF-α transcription in a murine mast cell line.[7] While α-galactose-containing ceramides have already been demonstrated to activate the immune system,[8] this is the first example of such effects with β-sugar-containing ceramides.

We also screened the library molecules in a apoptosis screen with U937 leukemic cells, in which it is known that exogenous ceramide promotes apoptosis, and endogenous ceramide generation plays an essential role in activation of apoptosis under stress conditions (Figure 2).[9] In general, the most active members of the library had IC_{50} values in the micromolar range with **12ES10** as the most potent compound (IC_{50} 4 μM). The data revealed several important structural elements of ceramides that are required to induce apoptosis. The activity vs carbon length profile has a Gaussian shape with an optimum around a sum of carbon chain length (SCCL) of 18 [the most potent compounds from each core structure with their IC_{50} and SCCL were **10ES10** (IC_{50} 12 μM, SCCL = 16), **12ES10** (IC_{50} 4 μM, SCCL = 18), **15ES6** (IC_{50} 20 μM, SCCL = 17), **DES6** (IC_{50} 12 μM, SCCL = 20), and **20ES4** (IC_{50} 50 μM, SCCL = 20)]. The stereochemistry of the headgroup is not critical as the four streoisomers, **DES**, **DTS**, **LES**, and **LTS**, showed similar activities throughout. However, a C_4-C_5 double bond in the ceramide core structure does appear to be important for apoptotic activity, as both erythro (**DED**, **ED**) and threo (**TD**) dihydro-ceramides were generally inactive. This result contrasts with a previous study that indicated threo isomers are active whereas erythro isomers are not.[10] Activity is restored when a bulky tail is introduced with a preference for the D-configuration in **ED** (see **32** and **45**). This result suggests that a hydrophobic moiety may be required near the headgroup of the ceramide for biological activity. Finally, compounds based on a short chain core, i.e., **NE**, were not active with short carbon tails, but activity was restored with long unsaturated tails (see **46**, **47**, and **49**), consistent with the reported trend.[11] This study suggests that the apoptotic activity of these ceramide analogues depends on specific structural features; however, the exact mechanism of action is unclear at present.

In conclusion, we have developed a facile synthetic pathway to generate a library of ceramides and have demonstrated that members of this library have novel biological activities that may ultimately provide unique insights into cellular processes or possibly serve as leads for the development of therapeutic agents.

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Supporting Information Available: The full name for the abbreviation used, experimental procedures, and characterization data; NF-κB activation data for **PC**; and IC_{50} values of all available compounds in the U937 cell assay and the murine mast cell data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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