Synthesis of the First Selective Irreversible Inhibitor of Neutral Sphingomyelinase**

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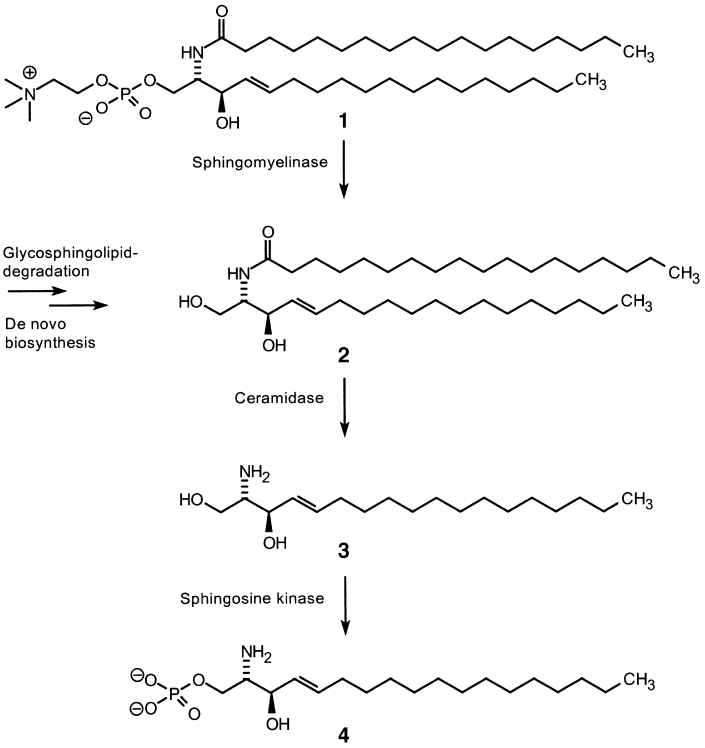
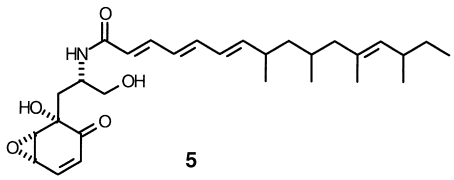
Sphingomyelin (**1**, Scheme 1) is a ubiquitous constituent of cell membranes.[1] In vertebrates, it accounts for up to 25 % of the total amount of membrane lipids, depending on the cell type. The catabolites of sphingomyelin, which are ceramide



Scheme 1. Sphingomyelin and catabolites.

(**2**), sphingosine (**3**), and sphingosine-1-phosphate (**4**), have become a focus of extensive research due to their possible role as secondary messengers.[1–4] The primary sphingomyelin catabolite, ceramide, is generated in the so-called sphingomyelin cycle[5, 6] through the action of either a lysosomal acid sphingomyelinase (A-SMase) or a membrane-bound neutral sphingomyelinase (N-SMase). Ceramide is believed to play a vital role in cell regulation, in modulation of inflammatory processes, and also in programmed cell death (apoptosis).[1, 2, 5–7] Many experiments suggest that various cytokines (such as TNF-α, Interleukin-1β, Interferon-γ), as well as radiation, heat, oxidative agents, and vitamin D_3 are all able to activate sphingomyelinases. Nonetheless, various aspects of ceramide-mediated signal transduction, particularly its role in apoptosis, are controversial.[8, 9] Certainly, the biological outcome of ceramide action is not uniform and depends on the cell-type, the topology of ceramide within the cell, and on the crosstalk (interaction) with other signals pathways. Additionally, the question regarding which of the sphingomyelinases is important for stimulus-induced ceramide production is still a point of controversy.[5, 8–11] The membrane-located N-SMase underlies physiological regulation through substances like glutathione or arachidonic acid[12] and is believed to play an important role in signal transduction.

Selective inhibitors of the different sphingomyelinase-types can contribute to a better understanding of the precise roles of these enzymes and ceramide in signal transduction. While several modest inhibitors are known for A-SMase,[1] the natural product scyphostatin (**5**)[13, 14] was only recently



isolated and found to be a potent competitive inhibitor of N-SMase ($IC_{50} = 1.0$ μM). However, this compound inhibits A-SMase as well ($IC_{50} = 49.3$ μM).[15]

Herein we report the synthesis of the first selective irreversible inhibitor of N-SMase. Preliminary studies in our laboratory revealed that short-chain ceramide analogues, such as 2-*N*-lauroylamido-1,3-propanediol, weakly inhibit N-SMase. Moreover we supposed that N-SMase—in analogy with other phosphodiesterases[16]—may contain nucleophilic groups (for example, amino acid side chains such as arginine, serine, or cysteine) in its active site, which are able to react with suitable electrophiles within the head group of prospective ceramide analogues. We expected that the compound **6** would fulfil these requirements; in comparison to scyphostatin, it contains a more reactive epoxide group.[17] Reaction of D-serine with decanoylchloride under Schotten–Baumann conditions afforded *N*-decanoyl-D-serine (**7**, Scheme 2). Coupling of 5-amino-2-hydroxybenzyl alcohol (**8**, obtained by a two-step reaction sequence) with **7**, which was activated with dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBT), afforded compound **9**. After subsequent reaction with sodium periodate, the spiroepoxide **6** was obtained as a 53:47 mixture of diastereomers.[18] In the enzyme assay, compound **6** was revealed to be a covalent inhibitor of N-SMase (Figure 1). Furthermore increased concentrations of sphingomyelin decreased the inhibitory effect of the epoxide **6** (Figure 2), suggesting that the inhibitor binds to the active site of the N-SMase. Under the assay conditions, the epoxide **6** showed no inhibition of A-SMase even at a concentration of 500 μmol L^{-1} (Figure 3). To examine the influence of the amino acid configuration, the enantiomeric derivative *ent*-**6** was synthesized from L-serine. This compound showed a similar time-dependent inactivation of N-SMase whereas the enzymatic activity of A-SMase was not affected (data not shown).

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[**] This work was supported by grants from the Fonds der Chemischen Industrie. C.A. is grateful to the Land of Baden-Württemberg for a scholarship from the Landesgraduiertenförderung.

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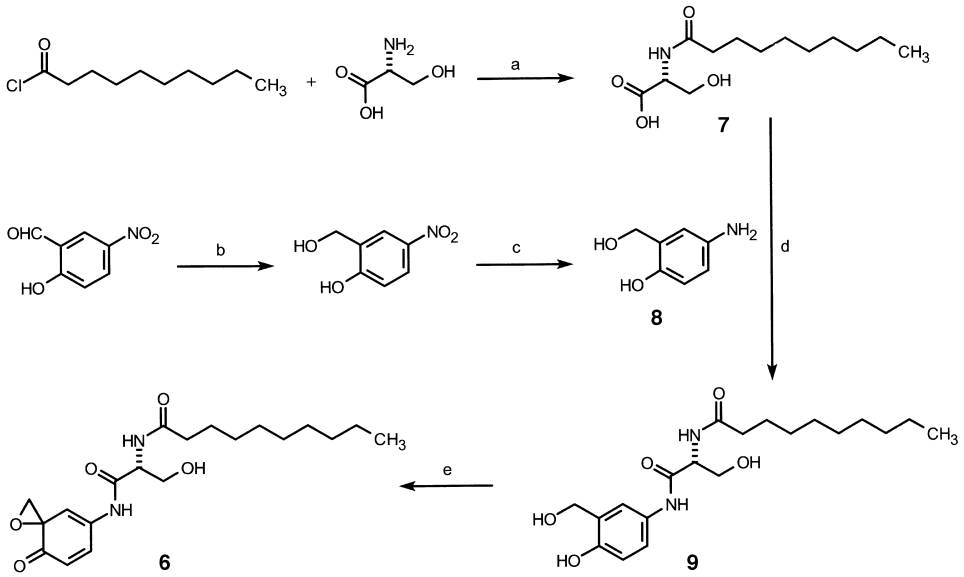

Scheme 2. Synthesis of the spiroepoxide **6**. Reagents and conditions: a) H_2O, THF, 3 equiv. Na_2CO_3, 3 h, 68 %; b) $NaBH_4$, EtOH, 1 d, 99 %; c) In, NH_4Cl, EtOH, 90 °C, 3 h, 91 %; d) DCC, HOBT, DMF, 16 h, 61 %; e) $NaIO_4$, $MeOH/H_2O$, 3 h, 20 °C, 70 %.

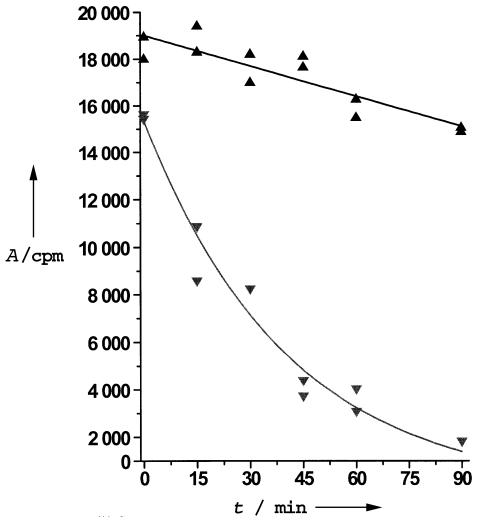

Figure 1. Time dependence of the inhibition of N-SMase by compound **6** (200 μM in preincubation buffer). With inhibitor **6**: ▼, control: ▲. A = activity, t = preincubation time.

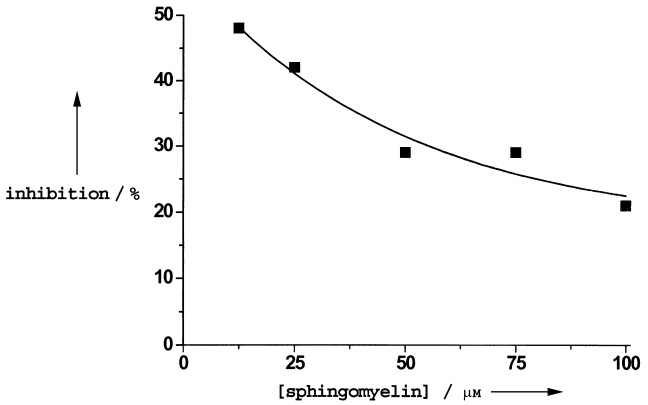

Figure 2. Inhibition of the N-SMase at different concentrations of sphingomyelin (average of two results, at a concentration of 100 μM of **6**, without preincubation).

Due to its interesting biochemical behaviour, compound **6** appears to be a valuable tool for the elucidation of the biological role of N-SMase and ceramide concerning signal transduction processes. Moreover, this compound could be used to label the active site of N-SMase and gain some insight into the enzyme mechanism which is currently unknown.[19] In addition, our results could be helpful for the development of further inhibitors of N-SMase, as the synthetic pathway allows the incorporation of various fatty acids, amino acids, and functionalized aniline derivatives. The fact that scyphostatin shows remarkable anti-inflammatory effects justifies the development of such inhibitors and makes N-SMase an interesting target for the experimental therapy of inflammatory diseases, including disorders of the central nervous system with an autoimmune component, such as multiple sclerosis.[20, 21]

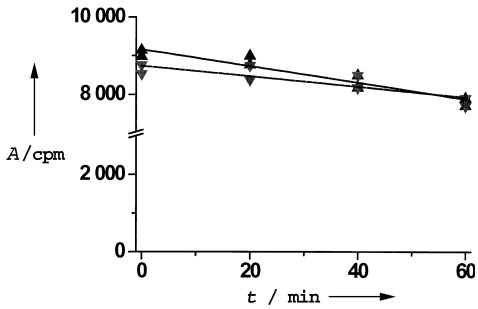

Figure 3. Effect of the compound **6** (200 μM in the preincubation buffer) on A-SMase. With inhibitor **6**: ▼, control: ▲. A = activity, t = preincubation time.

Experimental Section

Partial purification of sphingomyelinases was carried out following the method described by Hannun et al.[22] Four rats were decapitated and the brains were homogenised in a 25 mM tris(hydroxymethyl)aminomethane (TRIS)/HCl buffer (pH 7.4), which contained 20 mg L^{-1} each of leupeptine, chymostatin, antipain, and pestatin, as well as 1 mM phenylmethylsulfonylfluoride (PMSF), 2 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM 1,2-di(2-aminoethoxy)ethane-*N,N,N′N′*-tetraacetic acid (EGTA). The mixture was centrifuged for 1 h at 100 000 *g*, the supernatant was removed, and the pellet was dissolved in the above-mentioned buffer, containing an additional 1 % Triton X-100. The solution was loaded through a sterile filter onto an anion exchange column (POROS 20 HQ, PerSeptive Biosystems Inc.). After equlibration with a solution of 20 mM TRIS/HCl buffer (pH 7.4), which contained 1 mM each of EDTA, EGTA, PMSF, and a change to 1M sodium chloride in the same buffer, the sphingomyelinases were eluted with a gradient from 0 to 1 % Triton X-100 in the equlibration buffer.

For the determination of the N-Smase activity, the inhibitor (diastereomeric mixture) was dissolved in chloroform. An aliquot of 10 nmol was

dried under a nitrogen stream, redissolved in 40 μL 75 mM TRIS/HCl buffer (pH 7.4), which contained 0.05 % Triton X-100 and 2.5 mM $MgCl_2$, and mixed with 10 μL of enzyme preparation. Together with control experiments, the probes were preincubated for 90, 60, 45, 30 and 15 min at 37 °C. After addition of 10 nmol ^{14}C-sphingomyelin (ca. 40 000 cpm (counts per minute)) in 50 μL of the same buffer, the reaction was incubated for another 30 min. The reaction was stopped by the addition of 750 μL chloroform/methanol (1/1). After addition of 200 μL water, the lipids were extracted and the radioactivity of the polar upper phase, which contained ^{14}C-phosphorylcholine, was determined by scintillation counting.

The determination of A-SMase proceeded analogously, with use of an Mg^{2+}-free sodium acetate buffer (pH 4.5).

Received: October 6, 1999 [Z14115]

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[18] Physical Data of **6**: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.85$ (t, 3H, $J =$ 6.8 Hz, CH_3), 1.23 (m, 12H, 6 × CH_2), 1.57 (m, 2H, CH_2) 2.21 (t, 2H, $J = 7.5$ Hz, $COCH_2$), 3.65 (m, 1H, $CHH_{epox.}$), 3.86 (m, 2H, $COCH$-$H_{epox.}$, $CHHOH$), 4.31 – 4.49 (m, 3H, α-CH, OH, $CHHOH$), 6.29 (d, 0.47H, $J = 3.5$ Hz, H_{ring}), 6.32 (d, 0.53H, $J = 3.2$ Hz, H_{ring}), 6.77 (br., 0.47H, H_{ring}), 6.79 (br., 0.53H, H_{ring}), 6.93 – 7.05 (m, 2H, H_{ring}, NH), 8.00 (s, 0.53H, NH) 8.03 (s, 0.47H, NH); ^{13}C NMR: (100.6 MHz, $CDCl_3$, major diastereomer): $\delta = 14.1$, 22.6, 25.6, 29.2, 29.3, 29.4, 31.8, 36.3, 54.7, 59.4, 62.5, 80.1, 130.6, 138.7, 140.3, 144.6, 170.1, 174.7, 185.2; charactaristic signals of the minor diastereomer: $\delta = 54.8$, 59.3, 62.6, 80.0, 130.4, 138.4, 144.4, 170.2, 174.8, 185.1; high resolution EI-MS (155 °C): calculated for $C_{20}H_{30}N_2O_5$, 378.2139; found, 378.2194; $R_f =$ 0.49 (dichloromethane/methanol, 10/1).

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Structural Characterization of a Plutonium(IV) Siderophore Complex: Single-Crystal Structure of Pu-Desferrioxamine E**

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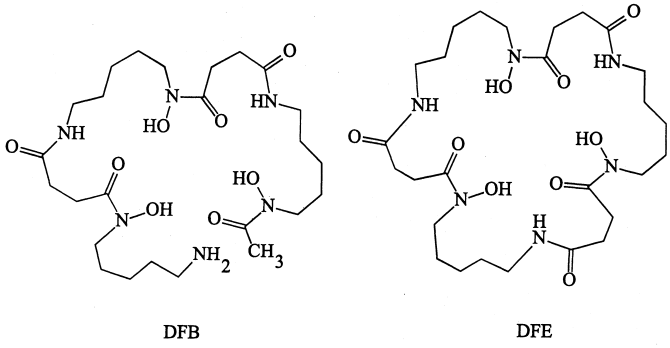
In order to acquire sufficient essential iron, most microorganisms produce very powerful, low molecular weight chelating agents that can deliver iron into cells via active transport systems. Ferrioxamine siderophores are linear or cyclic hydroxamates that contain 1-amino-5-hydroxyaminopentane as a building block.[1] Desferrioxamine E (DFE) and



DFB DFE

D_2 (DFD_2) are the cyclic compounds of this group; the linear compound desferrioxamine B (DFB)[2] has become the drug of choice for iron and aluminum overload by transfusion.[3] Desferrioxamines (DFOs) bind Fe^{III} over a wide pH range (1 – 12), while showing very little affinity for iron(II) (DFB-Fe^{III}: $\log\beta_{110} = 30.6$; DFB-Fe^{II}: $\log\beta_{110} = 7.2$).[4] The hexadentate, hard oxygen-donor DFO ligands also strongly bind other hard metal ions.

We are studying the complexation and solubilization of plutonium by siderophores. While plutonium does not occur

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[**] Support for this work is provided by the U.S. Department of Energy (Natural and Accelerated Bioremediation Research (NABIR) Program). We thank Professor G. Winkelmann, Universität Tübingen, for generously providing a sample of desferrioxamine E.

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