Sphingolactones

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Sphingolactones: Selective and Irreversible Inhibitors of Neutral Sphingomyelinase

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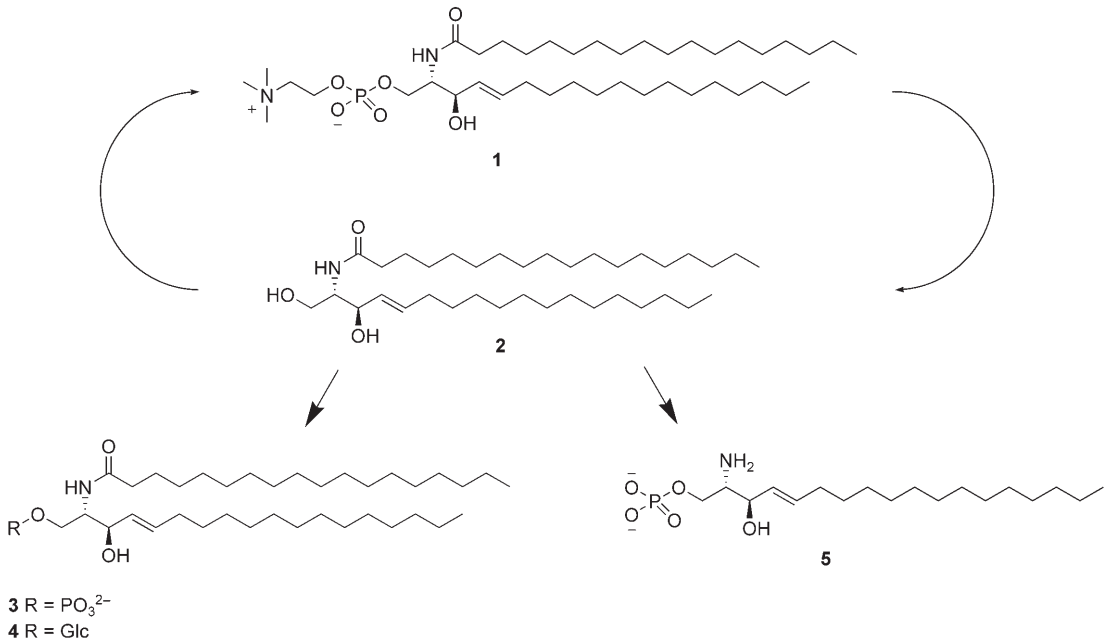
Sphingolipids are important components of the plasma membranes of eukaryotic cells. In the last few years increasing attention has been focussed on sphingolipid metabolites, because of their numerous biological effects and particularly because of their possible function as second messengers.

The primary catabolite of sphingomyelin (**1**) is ceramide (**2**), which is generated through the enzymatic activity of sphingomyelinases and is assumed to be involved in cell regulation, modulation of inflammatory processes, and also in programmed cell death (apoptosis).[1–5] Ceramide is also able to activate different signal-transduction cascades, both directly and indirectly.[1] Control of these biological effects is reached through regulation of the intracellular ceramide concentration by enzymes of the ceramide biosynthesis and by the formation of other sphingolipids, such as ceramide-1-phosphate (**3**), more complex glycosphingolipids (**4**), and sphingosine-1-phosphate (**5**).[1]

It has been assumed for a few years that the hydrolysis of sphingomyelin is the major process for the formation of biological active ceramide. In the tightly regulated sphingomyelin cycle[1–3,6] (Scheme 1) ceramide is generated through the action of either an acid sphingomyelinase (A-SMase) or a membrane-bound neutral sphingomyelinase (N-SMase). Different cytokines, such as TNF-α, interleukin-1β, interferon-γ, as well as radiation, heat, oxidative agents, vitamin D_3, and NO are all able to activate sphingomyelinases.[3,4]

However, various aspects of ceramide-mediated signal transduction, particularly its role in apoptosis, are controversial.[3–7] Additionally, the question as to which of the sphingomyelinases is important for stimulus-induced ceramide production is still a point of controversy.[3,8–11] The membrane-located neutral sphingomyelinase, which underlies physiological regulation through glutathione and arachidonic acid, is believed to play a relevant role in signal transduction.[1,3]

Potent, and above all, selective inhibitors are necessary to understand the biological function of ceramide and the sphingomyelinases. Although some inhibitors of N-SMase are known,[12,13] only scyphostatin (**6**),[12b,c,g] spiroepoxide **7**,[13a,b] and manumycin A (**8**),[12a] as well as its simple analogues have been used to examine the precise roles of these enzymes (Scheme 2).[14] However, all these compounds contain reactive epoxy groups, which are able to modify different cellular proteins through covalent interactions. Therefore, it is difficult to interpret the results of biological cell experiments. Here, we present the development of a new class of potent, selective and epoxy-free inhibitors of neutral sphingomyelinase, which we term sphingolactones.



Scheme 1. The sphingomyelin cycle. Ceramide **2** is generated from spingomyelin (**1**) through the action of sphingomyelinases. This competes in a metabolic equilibrium with ceramide-1-phosphate (**3**), more complex glycosphingolipids (e.g., **4**, Glc = glucose), and sphingosine-1-phosphate (**5**).

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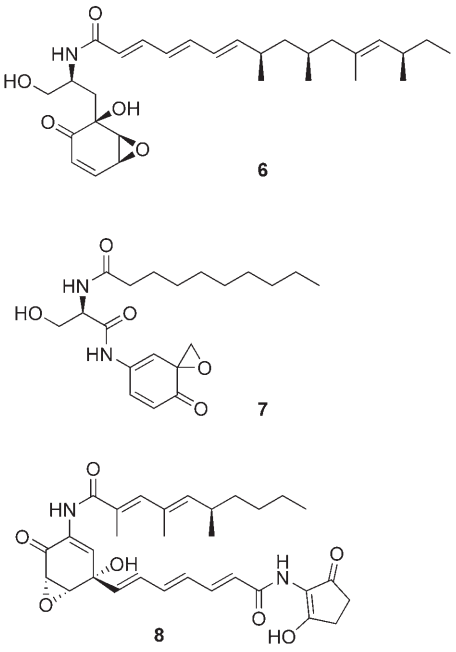


Scheme 2. Scyphostatin (**6**), spiroepoxide **7**, and manumycin (**8**).

The conception of the inhibitors is based on the following facts: Both, scyphostatin (**6**) as well as manumycin A (**8**) contain a polyunsaturated fatty acid residue as a common structural element. Furthermore, synthetic manumycin analogues with unsaturated carboxylic acids in the amide moiety possess a higher affinity to N-SMase relative to those with saturated fatty acid chains.[12a] Moreover, it is known from our own research, that the primary hydroxy group of **7** is essential for the inhibitory activity.[13c] As a replacement for the reactive epoxy groups we chose the γ-butyrolactone scaffold, a privileged structure, which is a recurrent structural motif[15] present in many biological active molecules. Figure 1 shows the structural features of the desired inhibitor.

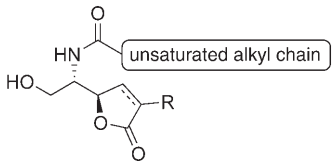
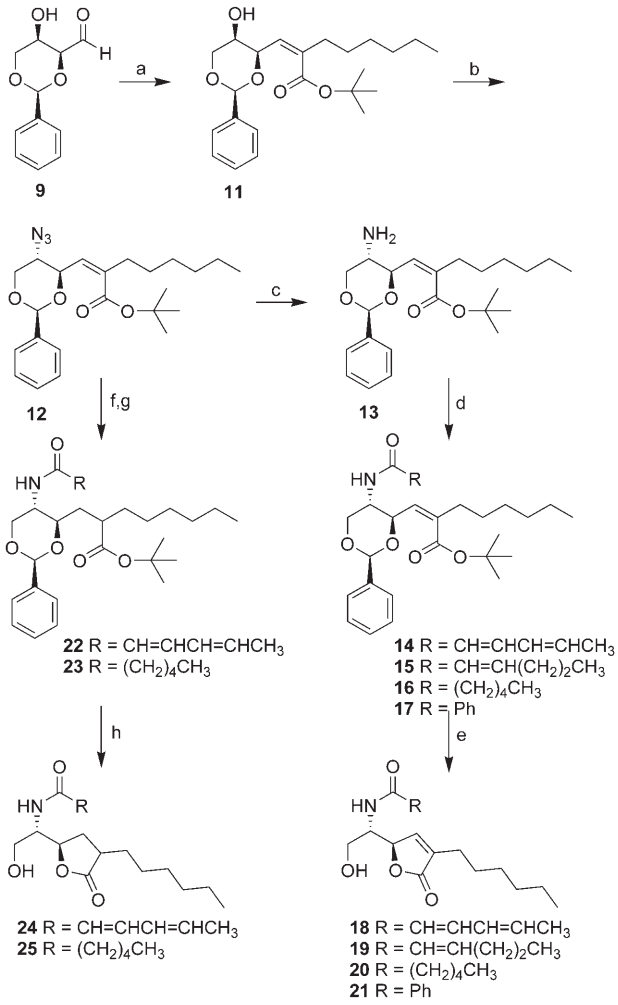


Figure 1. Structural features of the desired inhibitor.

The synthesis of the targeted inhibitor (Scheme 3) starts with the reaction of 2,4-*O*-benzylidene-D-threose (**9**), which is readily accessible by periodate cleavage of 4,6-*O*-benzylidene-D-galactose,[16,17] with the appropriate modified aryl phosphonate **10**[18] in an Ando variant[19] of the Horner–Wadsworth–Emmons (HWE) reaction[20] at −78 °C. The desired *Z* isomer **11** was obtained together with the corresponding *E* isomer in a ratio of 20:1. After azide exchange and reduction, amine **13** was modified with different carboxylic acid chlorides to obtain the amides **14**–**17**. Cleavage of the protecting groups with catalytic amounts of *para*-toluenesulfonic acid and in situ lactonization yielded the desired lactones **18**–**21**. The syntheses of **24** and **25** were completed by catalytic hydrogenolysis of compound **12** with Pd on charcoal (Scheme 3). The reaction results in an inseparable mixture of diastereomers in a ratio of 4:1. Further conversion into the desired compounds **24** and **25** was carried out in analogy to the described synthesis above.



Scheme 3. Synthesis of sphingolactones. a) 1.2 equiv LiCl, 1.2 equiv $(PhO)_2P(O)CH((CH_2)_5CH_3)COOtBu$ (**10**), 1.1 equiv DBU, THF, 67%; b) 1. 1.5 equiv Py, 1.3 equiv TfO_2, CH_2Cl_2, 2. 4 equiv NaN_3, DMF, 62%; c) 1.6 equiv Ph_3P, THF, H_2O, 60 °C, 94%; d) 1.5 equiv NEt_3, 1.1 equiv RCOCl, THF, 87–98%; e) cat. *p*-TsOH, DMF, 1,2-ethandiol, 76–85%; f) cat. Pd/C, H_2, ethanol, quant.; g) 1.5 equiv NEt_3, 1.1 equiv RCOCl, THF, 94–97%; h) cat. *p*-TsOH, DMF, 1,2-ethandiol, 72–79%. DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, Py = pyridine, Tf = trifluoromethanesulfonyl, Ts = toluene-4-sulfonyl.

The synthesized compounds were then evaluated regarding their inhibitory effect against N-SMase, for which a microsome preparation containing Mg^{2+}-dependent N-SMase from rat brain was used. Indeed, lactone **18**, as well as **24** and **25** proved to be time-dependent (irreversible) inhibitors of the N-SMase (Figure 2). Derivatives **19**–**21** also proved to be effective (irreversible inhibition, data not shown).

Subsequently, we investigated the inhibitors to determine which functional group is crucial for irreversible inhibition. In this context, the reduced derivative **24**, which contains a more

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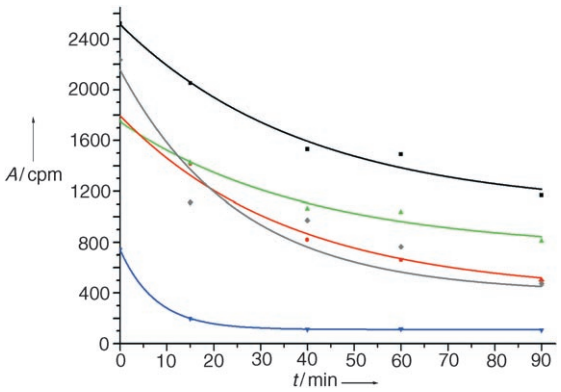

Figure 2. Time-dependence of the inhibition of the neutral sphingomyelinase by **18** (◆), **24** (▼), and **25** (●) (final concentration 350 μM). Hypothetical curve through a reversible competitive inhibition by **25**: ▲; control: ■; *A* = activity; cpm = counts per minute; *t* = preincubation time.

electrophilic lactone group, was identified as a better inhibitor than compound **18**. Therefore, it seems apparent that the inhibitory activity correlates directly with the lactone and is not accomplished by a 1,4-addition to the Michael system of compound **18**. This hypothesis is supported by the fact that N-SMase is also irreversibly inhibited by the saturated lactone **25**, as well as by derivative **21**. Moreover, increasing concentrations of sphingomyelin in the assay attenuate the inhibitory effect of **24** (Figure 3). This result indicates that inhibitor **24**

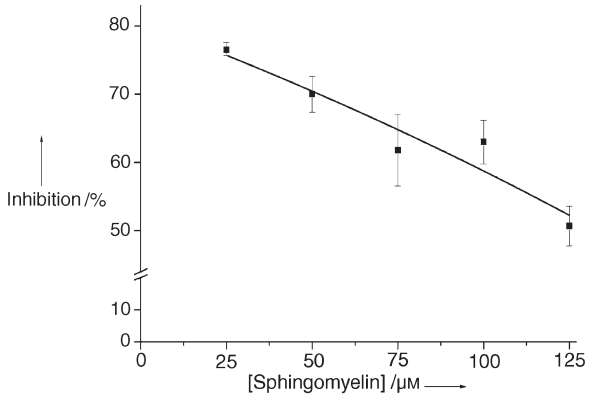

Figure 3. Inhibition of N-Smase by **24** at different concentrations of sphingomyelin. (final concentration 350 μM, no preincubation).

directly binds at the active site of N-SMase. The acid sphingomyelinase was only weakly competitive and only slightly dependent on the concentration of compound **24** (Figure 4).

The *N*-acyl chain exerts a decisive influence on the inhibitory effect of the synthesized lactones. In particular, the conjugated system of (2*E*,4*E*)-hexadienic acid seems to be able to adjust the inhibitor **24** at the active site of N-SMase, thus allowing an as yet unidentified nucleophile of the enzyme to attack the lactone.

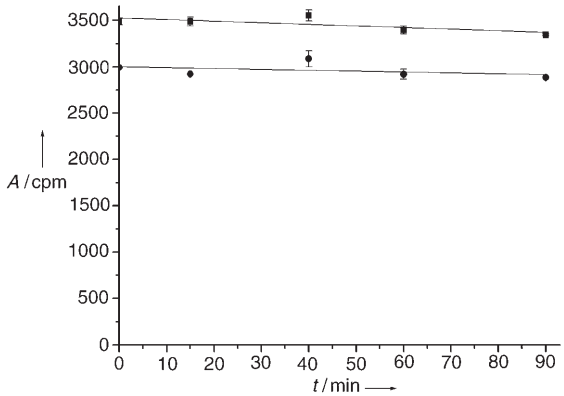

Figure 4. Effect of **24** (●) on A-SMase (final concentration 350 μM). control: ■.

The selective N-SMase inhibitors describe, which we term sphingolactones, are characterized by their high potency, with compound **24** possessing the highest inhibitory activity. In contrast to the previously described N-SMase inhibitors such as scyphostatin (**6**), spiroepoxide **7**, and manumycin A (**8**), the sphingolactones are stable and contain no epoxy function. Therefore, they represent useful chemical tools to explore the biological significance of ceramide and N-SMase in apoptosis, inflammation responses, malignant processes, and neurodegenerative diseases, such as Alzheimer's disease or multiple sclerosis.[1,21]

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