Synthesis of the First Selective Irreversible Inhibitor of Neutral Sphingomyelinase

Christoph Arenz[a] and Athanassios Giannis*[a]

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The sphingolipid ceramide is a candidate second messenger assumed to be involved in fundamental processes such as growth control, inflammation and apoptosis. Many aspects of ceramide-mediated processes remain to be clarified, including the question of which of the different sphingomyelinases is critical for the stimulus-induced ceramide production. Selective inhibitors of the sphingomyelinases are useful tools for clarifying the biological role of these enzymes and, moreover, appear to be interesting motives for the development of pharmacological agents for an experimental therapy of inflammatory diseases. The full synthesis of *N*-[2-hydroxy-1-(8-oxo-1-oxa-spiro[2.5]octa-4,6-diene-5-ylcarbamoyl)-ethyl] decanamide **(2),** the first selective irreversible inhibitor of neutral sphingomyelinase, is described and the relevant analytical data are given. The inhibitor **2** was obtained by a five-step synthesis starting from D-serine.

Introduction

Sphingomyelin is ubiquitously present in the membranes of eukaryotic cells. In vertebrates it accounts for up to 25 percent of the total amount of plasma membrane lipids, depending on the cell type.[1] Increasing attention has been focused on sphingomyelin and its primary catabolist ceramide, since ceramide is regarded as a lipid second messenger playing a vital role in cell regulation, modulation of inflammatory processes and also in programmed cell death (apoptosis).[2] Ceramide is generated in the sphingomyelin cycle[3] through the action of either a lysosomal acid sphingomyelinase (A-SMase) or a membrane-bound neutral sphingomyelinase (N-SMase). It is suggested that various cytokines (e.g. TNF-α, Interleukin-1β, Interferon-γ) as well as radiation, heat, oxidative agents and vitamin D_3 are all able to activate sphingomyelinases.[1,2,4,5] The released ceramide is believed to activate ceramide-activated protein kinases (CAPKs) or ceramide-activated protein phosphatases (CAPPs). Furthermore, there is evidence that ceramide is able to activate the signalling molecule Raf directly.[6] Nonetheless, various aspects of ceramide-mediated signal transduction, particularly its role for apoptosis, are controversial.[7,8] 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 with other signal pathways. Additionally, the question regarding which of the sphingomyelinases is important for stimulus-induced ceramide production is still a point of controversy. The membrane-located neutral sphingomyelinase underlies physiological regulation through substances such as glutathione[9,10] or arachidonic acid,[11] and is believed to play a relevant 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 of ceramide in signal transduction. Contrary to acid sphingomyelinase, for which some modest inhibitors are known,[1] only recently was a natural product, scyphostatin (**1**) (Figure 1), isolated and found to be a potent competitive inhibitor of N-SMase (IC_{50} = 1 μM).[12,13]

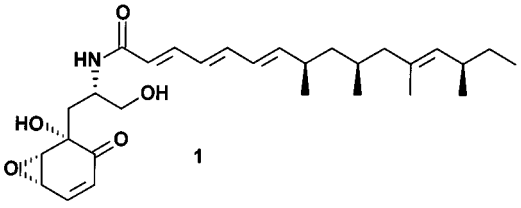

Figure 1

Results and Discussion

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[14] — may contain nucleophilic groups (e.g. amino acid side chains such as serine or cysteine) in its active site that are able to react with suitable electrophiles within the head group of prospective ceramide analogues. We expected that the compound **2** (Figure 2) would fulfil these settings because, in comparison with scyphostatin, it contains a more reactive epoxide group.[15–19]

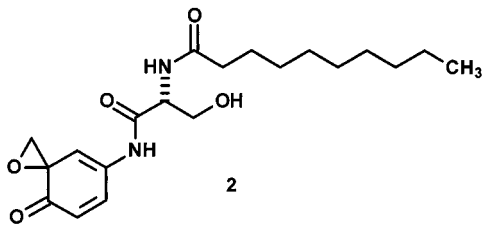

Figure 2

[a] Institut für Organische Chemie, Universität Karlsruhe, Richard-Willstätter Allee 2, 76128 Karlsruhe, Germany
Fax:(internat.) +49-721/608-7652
E-mail: giannis@ochhades.chemie.uni-karlsruhe.de

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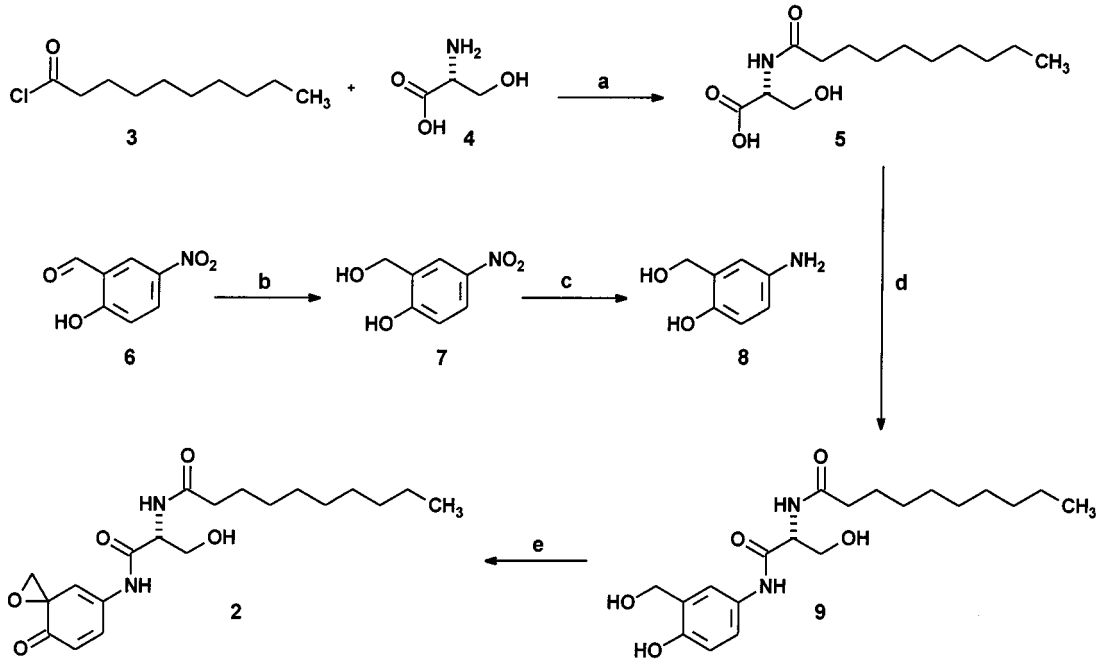
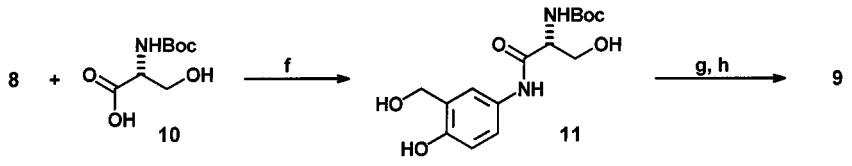


Scheme 1. Synthesis of the N-SMase inhibitor **2**; reagents and conditions: **a** H_2O, THF, 3 equiv. Na_2CO_3, 3 h, 68%; **b** $NaBH_4$, EtOH, 1 d, 99%; **c** H_2, Pd/C, methanol, 97% **d** DCC, HOBT, DMF, 16 h, 61%; **e** $NaIO_4$, $MeOH/H_2O$, 3 h, 20 °C, 70%



Scheme 2. Alternative synthesis of **9**; reagents and conditions: **f** DCC, HOBT, DMF, 16 h, 70%; **g** 5% TFA, CH_2Cl_2, 2 h; **h** $NaHCO_3$, H_2O, THF, 5 h, 86%

The synthesis was carried out starting with the acylation of D-serine **(4)** with decanoyl chloride **(3)**. The resulting *N*-decanoyl-D-serine **(5)** was coupled with 4-amino-2-hydroxymethyl phenol **(8)** by the active ester method using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT). The amino phenol **8** was obtained by reduction of 2-hydroxy-5-nitrobenzaldehyde **(6)** and subsequent hydrogenation of the nitro compound **7** with palladium on activated charcoal. After coupling of **5** and **8** the resulting amide **9** was treated with a sodium periodate solution[15] to yield the spiroepoxide **2** as a 53:47 mixture of diastereomers (Scheme 1).

In the enzyme assay, compound **2** was revealed to be an irreversible inhibitor of N-SMase, but under the assay conditions no inhibition of A-SMase was observed.[20]

In order to establish a synthetic pathway which allows a rapid synthesis of various inhibitors with different fatty acid side chains, we furthermore synthesized derivative **9** (Scheme 2), starting from Boc-D-serine **(10)** and the aniline derivative **8**. Treatment of **11** with TFA followed by acylation with decanoyl chloride afforded again compound **9**.

Conclusion

The spiroepoxide **2** represents the first selective irreversible inhibitor of neutral sphingomyelinase. Due to its interesting biochemical behaviour, this compound appears to be a valuable tool for the elucidation of the biological role of N-SMase and ceramide in signal transduction processes. Moreover, compound **2** could be used to label the active site of N-SMase and to gain some insight into the enzyme mechanism, which is currently unknown. In addition, the synthetic pathway allows the incorporation of various fatty acids, amino acids and functionalized aniline derivatives, making our results helpful for the development of further N-SMase inhibitors. The fact that scyphostatin shows remarkable anti-inflammatory effects justifies the development of such compounds and makes N-SMase an interesting target for the experimental therapy of inflammatory diseases.[21–23]

Experimental Section

General Remarks: Melting points were determined in open capillaries using a Büchi 535 apparatus and are uncorrected. ^1H and ^{13}C

NMR spectra were recorded on a Bruker AC 250, AM 400, or DRX 500 spectrometer at room temperature. Mass spectra and high-resolution mass spectra (HRMS) were measured on a Finnigan MAT MS70 spectrometer. Elemental analyses were performed on a Heraeus CHN-Rapid apparatus. Materials: Solvents were dried by standard methods and stored over molecular sieves. For column chromatography, silica gel (40−60 μm, Merck AG) was used. Commercial reagents were used without further purification.

***N*-Decanoyl-D-Serine (5):** D-Serine **4** (2.10 g 20.0 mmol) and sodium bicarbonate (3.18 g, 30.0 mmol) were suspended in 50 mL of water and 20 mL of THF. Under vigorous stirring, four portions of decanoyl chloride **3** (1.00 mL each) were added within four hours. After 16 h of additional stirring the organic layer was evaporated and the aqueous solution was quenched to a pH of 2 with conc. hydrochloric acid. The precipitated solid was filtered off and dried. Recrystallizing from diethyl ether gave *N*-decanoyl-D-serine (3.73 g, 14.4 mmol, 72%) as white crystals. R_f = 0.17 (dichloromethane/methanol 5:1); m.p. 80 °C {ref.[24] 80−81 °C}. − ^{1}H NMR ([D6]DMSO, 500 MHz): δ = 0.83 (t, *J* = 6.8 Hz, 3 H, CH_3), 1.18−1.25 (m, 12 H, 6 × CH_2), 1.46 (m, 2 H, CH_2), 2.12 (t, *J* = 7.5 Hz, 2 H, $CH_2C{=}O$), 3.59 (dd, *J* = 4.2 Hz and 11.0 Hz, 1 H, C*H*HOH), 3.65 (dd, *J* = 5.3 Hz and 11.0 Hz, 1 H, CH*H*OH), 4.24 (m, 1 H, CH_α), 7.89 (d, *J* = 7.8 Hz, 1 H, NH). − ^{13}C NMR DEPT ([D6]DMSO, 125 MHz): δ = 14.0 (CH_3), 22.2 (CH_2), 25.3 (CH_2), 28.7 (CH_2), 28.8 (CH_2), 28.9 (CH_2), 29.1 (CH_2), 35.2 (CH_2) 54.7 (CH), 61.59 (CH_2), 172.3 (C), 127.5 (C). − HRMS (70 eV, 140 °C) for $C_{13}H_{25}NO_4$ [M^+]: calcd. 259.1783; found 259.1794.

2-Hydroxymethyl-4-nitrophenol (7): 2-Hydroxy-5-nitrobenzaldehyde **6** (10.0 g, 59.8 mmol) was dissolved in ethanol (300 mL). After addition of sodium borohydride (2.00 g, 52.8 mmol) the mixture was stirred overnight. After the reaction was complete (TLC monitoring) water (5 mL) and conc. hydrochloric acid were added until the colour of the mixture changed from yellow to white. The precipitate was removed by filtration and the solvent was evaporated. Afterwards the residue was redissolved in methanol three times and the solvent was evaporated again. Recrystallizing from water gave pure 2-hydroxy-5-nitrobenzyl alcohol (10.1 g, 59.2 mmol, 99%) as a yellow powder. R_f = 0.54 (dichloromethane/methanol 10:1); m.p. 128 °C {ref.[25] 125−126 °C}. − ^{1}H NMR (CD_3OD, 500 MHz): δ = 4.66 (s, 2 H, CH_2OH), 6.85 (d, *J* = 8.6 Hz, 1 H, $H_{arom.}$), 8.01 (dd, *J* = 2.9 Hz and 8.6 Hz, 1 H, $H_{arom.}$), 8.25 (d, *J* = 2.9 Hz, 1 H, $H_{arom.}$). − ^{13}C NMR (CD_3OD, 125 MHz): δ = 59.7 (CH_2), 115.5 (CH), 124.5 (CH), 125.3 (CH), 130.4 (C), 141.7 (C), 162.0 (C). − HRMS (70 eV, 80 °C) for $C_7H_7NO_4$ [M^+]: calcd. 169.0375; found 169.0360. − $C_7H_7NO_4$: calcd. C 49.71, H 4.17, N 8.28; found C 49.39, H 4.17, N 8.28.

4-Amino-2-hydroxymethylphenol (8): 2-Hydroxymethyl-4-nitrophenol **(7)** (4.25 g, 25.0 mmol) was dissolved in methanol (150 mL). Palladium on activated charcoal was then added (150 mg approximately) and the solution was stirred for 5 h under an atmosphere of hydrogen. After the reaction was complete, the charcoal was removed by filtration over celite and the solvent was evaporated. The yellow solid residue was chromatographed (dichloromethane/methanol 7:1) to give the aminosalicyl alcohol **8** as a yellow powder (3.37 g, 24.2 mmol, 97%). The compound was stored under argon at room temperature. R_f = 0.31 (dichloromethane/methanol 7:1). − ^{1}H NMR (CD_3OD, 500 MHz): δ = 4.79 (s, 2 H, CH_2), 6.95 (d, *J* = 8.2 Hz, 1 H, $H_{arom.}$), 7.07 (dd, *J* = 2.4 Hz and 8.2 Hz, 1 H, $H_{arom.}$), 7.28 (d, *J* = 2.4 Hz, 1 H, $H_{arom.}$) ppm. − HRMS (70 eV, 110 °C): calcd. for $C_7H_9NO_2$(M^+): *m*/*z* = 139.0633 found *m*/*z* = 139.0629.

***N*-[2-Hydroxy-1-(4-hydroxy-3-hydroxymethylphenylcarbamoyl)-ethyl] Decanamide (9):** *N*-Decanoyl-D-serine **(5)** (777 mg, 3.00 mmol), 4-Amino-2-hydroxymethyl-phenol **(8)** (417 mg, 3.00 mmol) and HOBT (445 mg, 3.30 mmol) were dissolved in dry DMF (15 mL) and cooled to 0 °C. Then DCC (680 mg, 3.30 mmol) was added and the mixture was allowed to warm at room temperature. After stirring overnight the precipitated dicyclohexyl urea was filtered off and the solvent was removed. The residue was purified by column chromatography (dichloromethane/methanol 10:1). If further purification was necessary the product was recrystallized from dichloromethane to give a white powder (696 mg, 1.83 mmol, 61%). R_f (dichloromethane/methanol 10:1) = 0.27, m.p = 164 °C. − ^{1}H NMR ([D6]DMSO, 500 MHz): δ = 0.73 (t, *J* = 6.5 Hz, 3 H, CH_3), 1.08−1.15 (m, 12 H, 6 × CH_2), 1.35 (m, 2 H, CH_2), 2.03 (t, *J* = 7.3 Hz, 2 H, $CH_2C{=}O$), 3.47 (m, 2 H, $CHCH_2OH$), 4.31 (d, *J* = 5.5 Hz, 2 H, $PhCH_2OH$), 4.79 (m, 1 H, CH_α), 4.78−4.90 (m, 2 H, 2 × OH), 6.53 (d, *J* = 8.5 Hz, 1 H, $H_{arom.}$), 7.21 (dd, *J* = 2.1 Hz and 8.5 Hz, 1 H, $H_{arom.}$), 7.35 (dd, *J* = 1.6 Hz, 1 H, $H_{arom.}$), 7.73 (d, *J* = 7.9 Hz, 1 H, NH), 9.01 (s, 1 H, NH), 9.53 (s, 1 H, OH). − ^{13}C NMR ([D6]DMSO, 100.6 MHz): δ = 14.0 (CH_3), 22.1 (CH_2), 25.2 (CH_2), 28.7 (CH_2), 28.8 (CH_2), 28.9 (CH_2), 29.0 (CH_2), 31.3 (CH_2), 35.2 (CH_2), 55.6 (CH), 58.2 (CH_2), 61.9 (CH_2), 114.2 (CH), 118.8 (CH), 119.2 (CH), 128.6 (C), 130.6 (C), 150.1 (C), 168.5 (C), 172.4 (C). − HRMS (70 eV, 195 °C) for $C_{20}H_{32}N_2O_5$ [M^+]: calcd. 380.2311; found 380.2292.

***N*-[2-Hydroxy-1-(8-oxo-1-oxaspiro[2.5]octa-4,6-diene-5-ylcarbamoyl)ethyl] Decanamide (2):** Compound **9** (100 mg, 0.26 mmol) was dissolved in a mixture of dichloromethane and methanol (3:2, 10 mL). Then fresh 0.3 M sodium periodate solution was added (2.00 mL, 0.60 mmol) and the reaction mixture was stirred in the dark for 5 h at room temperature. Afterwards, the upper phase was shaken with dichloromethane three times. Then the combined dichloromethane layers were dried over sodium sulfate and the solvent was removed. The residue was chromatographed over silica gel (dichloromethane/methanol 12:1) to give **2** as a gum (69.8 mg, 0.18 mmol, 70%). R_f = 0.49 (dichloromethane/methanol 10:1). − ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 0.85 (t, *J* = 6.8 Hz, 3 H, CH_3), 1.23 (m, 12 H, 6 × CH_2), 1.57 (m, 2 H, CH_2), 2.21 (t, *J* = 7.5 Hz, 2 H $COCH_2$), 3.65 (m, 1 H, $CH H_{epox.}$), 3.86 (m, 2 H, $COCH H_{epox.}$, C*H*HOH), 4.31−4.49 (m, 3 H, *H*-α, O*H*, CH*H*OH), 6.29 (d, *J* = 3.5 Hz, 0.47 H, H_{ring}), 6.32 (d, *J* = 3.2 Hz, 0.47 H, H_{ring}), 6.77 (b, 0.47 H, H_{ring}), 6.79 (b, 0.53 H, H_{ring}), 6.93−7.05 (m, 2 H, H_{ring}, NH), 8.00 (s, 0.53 H, NH) 8.03 (s, 0.47 H, NH). − ^{13}C NMR ($CDCl_3$, 100.6 MHz, major diastereomer): δ = 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. Characteristic signals of the minor diastereomer: δ = 54.8, 59.3, 62.6, 80.0, 130.4, 138.4, 144.4, 170.2, 174.8, 185.1. − HRMS (70 eV, 155 °C) for $C_{20}H_{30}N_2O_5$ [M^+]: calcd. 378.2139; found 378.2194.

[2-Hydroxy-1-(4-hydroxy-3-hydroxymethylphenylcarbamoyl)ethyl]-carbamic Acid (11): 4-Amino-2-hydroxymethyl-phenol **(8)** (672 mg, 4.83 mmol), *N*-Boc-D-serine **10** (990 mg, 4.83 mmol) and HOBT (802 mg, 5.30 mmol) were dissolved in DMF (20 mL) and cooled to 0 °C. Then, DCC (1.09 g, 5.30 mmol) was added and the solution was allowed to warm up to room temperature. After stirring overnight, the precipitated dicyclohexyl urea was filtered off and the solvent was removed under reduced pressure. The resulting thick oil was loaded onto a chromatography column (silica gel 60) and was flash chromatographed with dichloromethane/methanol (10:1). The resulting solid was recrystallized once from dichloromethane to yield compound **11** as white crystals (1.10 g, 3.38 mmol, 70%). m.p. 154 °C. − R_f = 0.36 (dichloromethane/methanol 7:1).

− HRMS (70 eV, 220 °C) for $C_{15}H_{22}N_2O_6$ [M^+]: calcd. 326.1478; found 326.1492. − ^{1}H NMR ([D6]DMSO, 500 MHz): δ = 1.38 (s, 9 H, *t*Bu), 3.56 (m, 2 H, CHCH_2), 4.10 (m, 1 H, CH_α), 4.44 (m, 2 H, PhCH_2OH), 4.88 (br, 1 H, OH), 4.98 (br, 1 H, OH), 6.63−6.67 (m, 2 H, NH + $H_{arom.}$), 7.32 (dd, *J* = 2.8 Hz + 8.6 Hz, 1 H, $H_{arom.}$), 7.47 (d, *J* = 2.9 Hz, 1 H, $H_{arom.}$), 9.11 (br, 1 H, OH), 9.61 (s, 1 H, NH). − ^{13}C NMR DEPT ([D6]DMSO, 125 MHz): δ = 28.2 (3 × CH_3), 57.2 (CH), 58.1 (CH_2), 61.9 (CH_2), 78.2 (CH), 114.2 (CH), 118.7 (CH), 119.1 (CH), 128.6 (C), 130.6 (C), 150.0 (C), 155.2 (C), 168.5 (C).

***N*-[2-Hydroxy-1-(4-hydroxy-3-hydroxymethylphenylcarbamoyl)-ethyl] Decanamide (9):** Derivative **11** (1.10 g, 3.38 mmol) was dissolved in dry dichloromethane (100 mL) containing 5% (v/v) TFA. After stirring for 2 h the reaction was complete (TLC monitoring) and the solvent was removed. The residue was suspended in an aqueous sodium hydrogen carbonate solution (50 mL, 0.5 M) and THF (20 mL) was added. Decanoyl chloride (1.00 mL, 933 mg, 4.81 mmol) was added with vigorous stirring of the solution. After stirring for 5 h the THF was evaporated and the water fraction was filtered. The residue was then recrystallized twice from diethyl ether. The analytical data of the product (1.11 g, 2.91 mmol, 86%) were in agreement with those mentioned above.

Acknowledgments

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