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Synthesis and Biochemical Investigation of Scyphostatin Analogues as Inhibitors of Neutral Sphingomyelinase

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Abstract—The sphingolipid ceramide is considered to be an important intracellular mediator. However, many aspects of its action and the role of several different ceramide generating sphingomyelinases are still unclear. Recently, we reported on the synthesis of the first selective irreversible inhibitor of the neutral sphingomyelinase (N-SMase), as well as the identification of Manumycin A and some of its analogues as irreversible inhibitors of N-SMase. For the development of pharmacologically interesting competitive inhibitors of N-SMase, structure–activity studies are essential. Herein we show the synthesis and enzymatic investigation of two scyphostatin analogues **3a** and **3b**, revealing the importance of the primary hydroxy group in compound **2** for N-SMase inhibition.

Introduction

The sphingolipid ceramide is a putative second messenger and can be generated from sphingomyelin—an important constituent of eucaryotic membranes—through the action of various sphingomyelinases.[1,2] These enzymes can be activated by various cytokines, or by radiation, heat and oxidative stress leading to an increased ceramide production. It is suggested, that ceramide acts as a second messenger and triggers or modulates a number of fundamental processes like programmed cell death (apoptosis), the cell cycle or inflammatory processes in various tissues and cell lines.[3] Moreover, recent studies strongly suggest a vital role for ceramide as a key mediator in tumor suppression.[4,5] However, numerous aspects of ceramide mediated signal transduction, especially those related to apoptosis remain unclear.[6,7] Furthermore the question, whether the plasma membrane-bound Mg^{2+} dependent neutral sphingomyelinase (N-SMase), out of several described neutral sphingomyelinases[8–10] or the lysosomal acid sphingomyelinase (A-SMase) is most important for stimulus-induced ceramide production is discussed controversially.[1,6] Selective inhibitors of the different sphingomyelinases would be valuable tools for the investigation of the biological role of these enzymes and ceramide. Recently, we reported on the synthesis of the spiroepoxide **2**,[11,12] which was synthesized as an analogue of the natural product scyphostatin **1**.[13–15] The latter is known as a potent inhibitor of the membrane-bound neutral sphingomyelinase (N-SMase). In fact, analogue **2** inhibits N-SMase, but contrary to scyphostatin, it is an irreversible inhibitor of this enzyme. Moreover, we could identify the antibiotic Manumycin A and two of its analogues as potent irreversible N-SMase inhibitors[16] (Fig. 1).

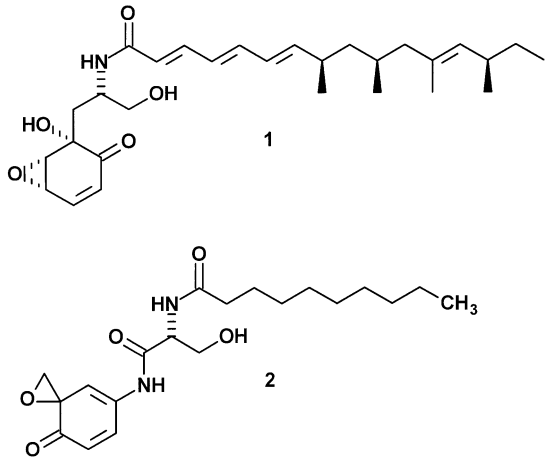

Figure 1.

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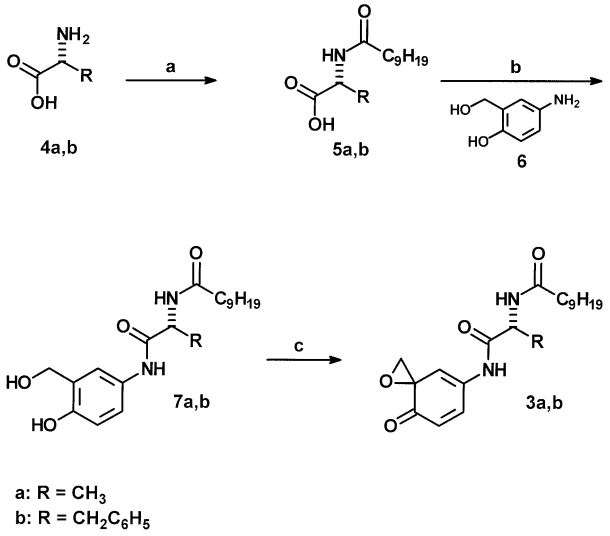
Regarding the variety of biological effects possibly triggered by ceramide, N-SMase emerges as an interesting target for the development of anti-inflammatory drugs.[13] Therefore, the synthesis of competitive inhibitors, which in comparison to irreversible inhibitors are more likely to fulfill the requirements of a 'drug', is well justified.

Our first aim is to gain more insights into the mode of N-SMase inhibition by the different inhibitors and to clarify the role of the different functional groups which are involved. Our investigations with analogues of Manumycin A revealed, that inhibitory potency of such compounds is strongly influenced by the nature of the hydrophobic side chain.[16]

In the present paper, we synthesized two analogues of **2** in which the primary hydroxy group is replaced by a hydrogen or a phenyl function (Scheme 1). Furthermore, the ability of both compounds to inhibit N-SMase was investigated.

Results and Discussion

The amino acids D-alanine **4a** and D-phenylalanine **4b** were acylated with decanoyl chloride under Schotten–Baumann conditions to give *N*-decanoyl-D-alanine **5a** and *N*-decanoyl-D-phenylalanine **5b**. Subsequently, the acylated amino acids **5 a,b** were coupled with the aniline derivative **6**, which was obtained by $LiAlH_4$-reduction of aminosalicylic acid methyl ester. Treatment of the resulting compounds **7a**,**b** with sodium periodate in a mixture of methanol/water and dichloromethane afforded the spiroepoxides **3a** and **3b** as mixtures of the corresponding diastereomeres.



Scheme 1. Synthesis of the scyphostatin analogues **3a**,**b**: (a) H_2O, THF, Na_2CO_3, 18 h, rt, 58%/63%; (b) DCC, HOBt, DMF, 18 h, 26%/39%; (c) $NaIO_4$, $MeOH/H_2O$, 3 h, rt, 26%/21%.

Table 1. Inhibition of N-SMase by different spiroepoxides **2**, **3a** and **3b** (200 μM in pre-incubation buffer, 100 μM final concentration)

	Inhibition without pre-incubation (%)	Inhibition with pre-incubation (%)
2	17	88
3a	12	33
3b	5	23

The pre-incubation time was 90 min.

Compounds **3a** and **3b** were tested upon N-SMase and A-SMase inhibition, as described,[11,16] using a raw preparation of rat brain microsomes. To determine the inhibitory potency towards N-SMase they were tested with and without pre-incubation for 90 min with the enzyme. The latter likely represents the ability of the inhibitor to compete with the substrate for binding at the active site of the enzyme, whereas the assay with pre-incubation of the inhibitor with the enzyme in the absence of substrate represents the kinetics of the irreversible inhibition. In fact both derivatives **3a,b** proved to be weak irreversible N-SMase inhibitors (Table 1). Compared to the epoxide **2**, which was derived from D-serine, these results clearly show the importance of the primary hydroxy group. Furthermore, no inhibition of A-SMase was observed (data not shown).

Conclusion

The inhibitory potency of the epoxides **3a** and **3b** towards N-SMase is drastically reduced, compared to the known inhibitor **2**, indicating that the primary hydroxy group is crucial for a enzyme inhibition.

These insights and findings will greatly contribute in the development of potent competitive inhibitors of N-SMase and may finally provide a means for the development of novel antiinflammatory therapeutic strategies. Now, we are concentrating our research interests on the synthesis of selective competitive inhibitors of N-SMase, which in our knowledge are not identified so far.

Experimental

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 (HR-MS) were measured on a Finnigan MAT MS70 spectrometer. 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-alanine (5a).** To a suspension of D-alanine (5.00 g, 56.1 mmol) and sodium bicarbonate (8.93 g, 84.2 mmol) in water (100 mL) and tetrahydrofuran (50 mL) decanoyl chloride (12.62 mL, 11.77 g, 61.7 mmol) was slowly added. After stirring for 18 h at room temperature tetrahydrofuran was carefully

removed under reduced pressure. The resulting solution was acidified to a pH of 1–2 with 6 N hydrochloric acid until a white solid was precipitated. After filtration and drying the crude product was recrystallized from diethyl ether. White solid 7.92 g (32.5 mmol, 58%). Mp 89 °C, R_f = 0.70 (dichloromethane/methanol 10:1). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.88 (t, 3H, ^3J = 7.0 Hz, CH_3), 1.26–1.33 (m, 12H, 6×CH_2), 1.46 (d, 3H, ^3J = 7.1 Hz, CH_3), 1.60–1.66 (m, 2H, CH_2), 2.24 (t, 2H, ^3J = 7.7 Hz, CH_2C=O), 4.55–4.61 (m, 1H, CH_α), 6.16 (m, 1H, NH), 9.37 (br, 1H, COOH) ppm. ^{13}C NMR ($CDCl_3$, 125.8 MHz): δ 14.09 (CH_3), 18.01 (CH_3), 22.66 (CH_2), 25.55 (CH_2), 29.18 (CH_2), 29.25 (CH_2), 29.30 (CH_2), 29.42 (CH_2), 31.85 (CH_2), 36.45 (CH_2), 48.29 (CH), 174.04 (C), 176.01 (C) ppm, HR-MS (70 eV, 100 °C): calcd for $C_{13}H_{25}NO_3(M^+)$: *m*/*z* 243.1834; found: *m*/*z* 243.1838.

***N*-Decanoyl-D-phenylalanine (5b).** Preparation was taken out analogously as described for (**5a**). White solid (63% yield). Mp 118 °C, R_f = 0.70 (dichloromethane/methanol 12:1). ^{1}H NMR (CD_3OD, 500 MHz): δ 0.80 (t, 3H, ^3J = 7.0 Hz, CH_3), 1.16–1.23 (m, 12H, 6×CH_2), 1.42–1.47 (m, 2H, CH_2), 2.06 (t, 2H, ^3J = 7.6 Hz, CH_2C=O), 2.95 (dd, 1H, ^2J = 13.9 Hz, ^3J = 7.1 Hz, C*H*HPh), 3.12 (dd, 1H, ^2J = 13.9 Hz, ^3J = 5.3 Hz, CH*H*Ph), 4.57–4.60 (m, 1H, CH_α), 7.09–7.20 (m, 5H, $CH_{arom.}$) ppm. ^{13}C NMR (CD_3OD, 125.8 MHz): δ 13.91 (CH_3), 22.52 (CH_2), 25.51 (CH_2), 29.05 (CH_2), 29.15 (CH_2), 29.21 (CH_2), 29.30 (CH_2), 31.74 (CH_2), 36.28 (CH_2), 37.39 (CH_2), 53.97 (CH), 126.65 (CH), 128.24 (2×CH), 129.19 (2×CH), 136.69 (C), 173.85 (C), 175.23 (C) ppm. HR-MS (70 eV, 140 °C): calcd for $C_{19}H_{29}NO_3(M^+)$: *m*/*z* 319.2147; found: *m*/*z* 319.2137.

5-Amino-2-hydroxy-benzylalcohol (6). A solution of 5-amino-2-hydroxy-benzoic acid methyl ester (10.03 g, 60.0 mmol) in absolute tetrahydrofuran (50 mL) was added dropwise to an ice-cooled and well-stirred suspension of lithium aluminium hydride (4.55 g, 120.0 mmol) in absolute tetrahydrofuran (150 mL). Then the yellow reaction mixture was allowed to warm up at room temperature and stirred for 3 h. After the reaction was complete (TLC control) the mixture was cooled again to 0 °C and quenched with isopropanol. The resulting suspension was filtered under nitrogen and the solid residue was washed two times with dry tetrahydrofuran. The solvent was removed in vacuo and the benzyl alcohol **5** was obtained as a yellow air-sensitive solid (7.71 g, 55.4 mmol, 92%), which was stored under argon atmosphere. R_f = 0.30 (dichloromethane/methanol 7:1). ^{1}H NMR (CD_3OD, 250 MHz): δ 4.62 (s, 2H, CH_2OH), 6.61–6.62 (m, 2H, $CH_{arom.}$), 6.79–6.80 (m, 1H, $CH_{arom.}$) ppm. HR-MS (70 eV, 110 °C): calcd for $C_7H_9NO_2(M^+)$: *m*/*z* 139.0633; found: *m*/*z* 139.0629.

Decanoic acid [1-(*R*)-(4-hydroxy-3-hydroxymethylphenylcarbamoyl)-ethyl]-amide (7a). To an ice-cooled solution of *N*-decanoyl-D-alanine **5a** (4.50 g, 18.5 mmol), 5-amino-2-hydroxy-benzylalkohol **2** (3.86 g, 27.8 mmol) and HOBt (3.40 g, 22.2 mmol) in dry DMF (100 mL) DCC (4.58 g, 22.2 mmol) was added. After stirring for 1 h at 0 °C, the mixture was allowed to warm up at room temperature and stirred for additional 18 h. The precipitate was filtered off and washed twice with ice-cold THF. Then the solvent was removed under reduced pressure and the residue was chromatographed (silica gel 40–60, dichloromethane/methanol 12:1). Chromatography yielded a white solid (1.77 g, 4.9 mmol, 26%). Mp 178 °C, R_f = 0.25 (dichloromethane/methanol 12:1). ^{1}H NMR DMSO-d_6, 500 MHz): δ 0.84 (t, 3H, ^3J = 6.8 Hz, CH_3), 1.22–1.27 (m, 15H, 6×CH_2 and 1×CH_3), 1.45–1.48 (m, 2H, CH_2), 2.10 (t, 2H, ^3J = 7.4 Hz, CH_2C=O), 4.37 (m, 1H, CH_α), 4.43 (d, 2H, ^3J = 5.4 Hz, C*H*$_2$OH), 4.96 (t, 1H, ^3J = 5.4 Hz, CH_2O*H*); 6.65 (d, 1H, ^3J = 8.6 Hz, NH), 7.29 (dd, 1H, J = 8.6 Hz and J = 2.4 Hz, $CH_{arom.}$), 7.44 (d, 1H, J = 2.0 Hz, $CH_{arom.}$), 7.98 (d, 1H, J = 7.4 Hz, $CH_{arom.}$), 9.10 (s, 1H, NH), 9.64 (s, 1H, OH) ppm. ^{13}C NMR (125.8 MHz, (DMSO-d_6): δ 13.94 (CH_3), 18.29 (CH_3), 22.09 (CH_2), 25.20 (CH_2), 28.65 (CH_2), 28.75 (CH_2), 28.81 (CH_2), 28.90 (CH_2), 31.27 (CH_2), 35.05 (CH_2), 48.69 (CH), 58.11 (CH_2), 114.20 (CH), 118.72 (CH), 119.11 (CH), 128.66 (C), 130.59 (C), 149.98 (C), 170.64 (C), 172.04 (C) ppm. HR-MS (70 eV, 180 °C): calcd for $C_{20}H_{32}N_2O_4(M^+)$: *m*/*z* 364.2362; found: *m*/*z* 364.2356.

Decanoic acid [2-phenyl-1-(*R*)-(4-hydroxy-3-hydroxymethylphenylcarbamoyl)-ethyl]-amide (7b). Preparation was taken out analogously as described for (**7a**). White solid (39% yield). Mp 116 °C, R_f = 0.40 (dichloromethane/methanol 10:1). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 0.84 (t, 3H, ^3J = 7.0 Hz, CH_3), 1.08–1.09 (m, 2H, CH_2), 1.16–1.25 (m, 10H, 5×CH_2), 1.33–1.39 (m, 2H, CH_2), 2.03 (t, 2H, ^3J = 7.3 Hz, CH_2C=O), 2.81 (dd, 1H, ^3J = 13.7 Hz, ^3J = 9.8 Hz, C*H*HPh), 3.00 (dd, 1H, ^2J = 13.7 Hz, ^3J = 4.9 Hz, CH*H*Ph), 4.44 (d, 2H, ^3J = 4.1 Hz, C*H*$_2$OH), 4.60–4.65 (m, 1H, CH_α), 4.96–4.97 (m, 1H, CH_2O*H*), 6.66 (d, 1H, J = 8.6 Hz, $CH_{arom.}$), 7.15–7.17 (m, 1H, $CH_{arom.}$), 7.22–7.31 (m, 5H, $CH_{arom.}$ (Phe)), 7.42–7.43 (m, 1H, $CH_{arom.}$), 8.07 (d, 1H, ^3J = 8.4 Hz, NH (Phe)), 9.11 (s, 1H, NH), 9.77 (s, 1H, OH) ppm. ^{13}C NMR (125.8 MHz, DMSO-d_6): δ 13.94 (CH_3), 22.08 (CH_2), 25.19 (CH_2), 28.45 (CH_2), 28.65 (CH_2), 28.78 (CH_2), 28.85 (CH_2), 31.27 (CH_2), 35.15 (CH_2), 37.86 (CH_2), 54.52 (CH), 58.10 (CH_2), 114.21 (CH), 118.84 (CH), 119.20 (CH), 126.18 (CH), 127.94 (2×CH), 128.69 (C), 129.16 (2×CH), 130.44 (C), 137.94 (C), 150.08 (C), 169.57 (C), 172.14 (C) ppm. HR-MS (70 eV, 220 °C): calcd for $C_{26}H_{36}N_2O_4(M^+)$: *m*/*z* 440.2675; found: *m*/*z* 440.2651.

Decanoic acid [1-(*R*)-(8-oxo-1-oxaspiro[2,5]octa-4,6-diene-5-ylcarbamoyl)-ethyl]-amide (3a). Compound **7a** (300 mg, 0.82 mmol) was dissolved in a mixture of dichloromethane/methanol 3:2 (30 mL) and treated with a 0.3 M aqueous solution of sodium periodate (6.8 mL, 2.05 mmol). After stirring for 3 h in the dark, the organic layer was separated and the water phase was extracted three times with dichloromethane. Then the combined organic layers were dried over sodium sulfate and the solvent was removed. The crude product was chromatographed on silica gel (dichloromethane/methanol 12:1) to afford the product as an inseparable mixture of diastereomeres in the ratio of 59:41 (77 mg, 0.21 mmol, 26%). R_f = 0.35 (dichloromethane/methanol

12:1). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.86 (t, 3H, $^3J = 7.0$ Hz, CH_3), 1.23–1.47 (m, 15H, 6×CH_2, 1×CH_3), 1.52–1.59 (m, 2H, CH_2), 2.09–2.28 (m, 2H, $CH_2C{=}O$), 3.20–3.21 (m, 2H, $CH_{2\ epox.}$), 4.49–4.55 (m, 1H, CH_α), 6.29–6.30 (m, 0.59H, CH_{ring}), 6.31–6.32 (m, 0.41H, CH_{ring}), 6.49 (d, 0.59H, $^3J = 7.4$ Hz, NH (Ala)), 6.54 (d, 0.41H, $^3J = 7.4$ Hz, NH, (Ala)), 6.70 (br, 0.59H, CH_{ring}), 6.83 (br, 0.41H, CH_{ring}), 7.04 (dd, 0.59H, $J = 10.2$ Hz and $J = 3.0$ Hz, CH_{ring}), 7.10 (dd, 0.41H, $J = 10.2$ Hz and $J = 3.0$ Hz, CH_{ring}), 7.85 (br, 0.59H, NH), 7.95 (br, 0.41H, NH) ppm. ^{13}C NMR ($CDCl_3$, 125.8 MHz, major diastereomer): δ 14.08 (CH_3), 18.18 (CH_3), 22.63 (CH_2), 25.59 (CH_2), 29.20 (CH_2), 29.24 (CH_2), 29.30 (CH_2), 29.42 (CH_2), 31.82 (CH_2), 36.44 (CH_2), 49.06 (CH), 59.95 (CH_2), 79.90 (C), 130.24 (CH), 138.81 (CH), 139.88 (C), 144.58 (CH), 172.09 (C), 173.82 (C), 185.38 (C) ppm. Minor diastereomer: δ 14.08 (CH_3), 18.18 (CH_3), 22.63 (CH_2), 25.59 (CH_2), 29.20 (CH_2), 29.24 (CH_2), 29.30 (CH_2), 29.42 (CH_2), 31.82 (CH_2), 36.44 (CH_2), 49.11 (CH), 59.53 (CH_2), 79.87 (C), 129.99 (CH), 138.35 (CH), 140.11 (C), 144.47 (CH), 172.34 (C), 173.94 (C), 185.20 (C) ppm. HR-MS (70 eV, 160 °C): calcd for $C_{20}H_{30}N_2O_4(M^+)$: m/z 362.2206; found m/z 362.2213.

Decanoic acid [2-phenyl-1-(*R*)-(8-oxo-1-oxaspiro[2,5]octa-4,6-diene-5-ylcarbamoyl)-ethyl]-amide (3b). Preparation was taken out analogously as described for **3a**. Product was obtained as an inseparable mixture of corresponding diastereomeres in the ratio of 63:37 (21% yield). $R_f = 0.40$ (dichloromethane/methanol 12:1). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.85–0.88 (m, 3H, CH_3), 1.04–1.36 (m, 12H, 6×CH_2), 1.39–1.58 (m, 2H, CH_2), 2.01–2.25 (m, 2H, CH_2), 2.93–3.08 (m, 2H, CH_2–Ph), 3.11–3.14 (m, 2H, $CH_{2epox.}$), 4.70–4.85 (m, 1H, CH_α), 6.21–6.24 (m, 0.37H, CH_{ring}), 6.26–6.28 (m, 0.63H, CH_{ring}), 6.61 (br, 0.63H, CH_{ring}), 6.66 (br, 0.37H, CH_{ring}), 6.95–7.00 (m, 1H, CH_{ring}), 7.12–7.31 (m, 6H, $CH_{arom.}$ (Phe)), 7.58 (br, 0.63H, NH), 7.85 (br, 0.37H, NH) ppm. ^{13}C NMR ($CDCl_3$, 125.8 MHz, major-diastereomer): δ 14.10 (CH_3), 22.65 (CH_2), 25.56 (CH_2), 29.15 (CH_2), 29.27 (CH_2), 29.34 (CH_2), 29.43 (CH_2), 31.85 (CH_2), 36.42 (CH_2), 38.37 (CH_2), 54.55 (CH), 59.86 (CH_2), 79.83 (C), 127.05 (CH), 128.55 (2×CH), 129.34 (2×CH), 130.12 (CH), 136.16 (C), 138.63 (CH), 139.85 (C), 144.53 (CH), 170.92 (C), 173.79 (C), 185.36 (C) ppm. Minor-diastereomer: δ 14.10 (CH_3), 22.65 (CH_2), 25.56 (CH_2), 29.15 (CH_2), 29.27 (CH_2), 29.34 (CH_2), 29.43 (CH_2), 31.85 (CH_2), 36.42 (CH_2), 38.44 (CH_2), 54.87 (CH), 59.45 (CH_2), 79.83 (C), 127.05 (CH), 128.55 (2×CH), 129.31 (2×CH), 129.85 (CH), 136.16 (C), 138.23 (CH), 140.04 (C), 144.28 (CH), 171.38 (C), 174.04 (C), 185.17 (C) ppm. HR-MS (70 eV, 200 °C): calcd for $C_{26}H_{34}N_2O_4(M^+)$: m/z 438.2519; found: m/z 438.2548.

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