Synthesis and Evaluation of Three Novel Scyphostatin Analogues as Neutral Sphingomyelinase Inhibitors

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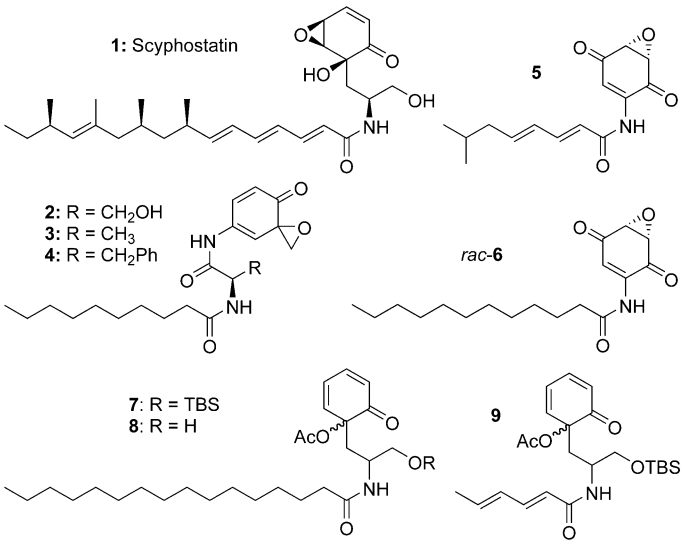
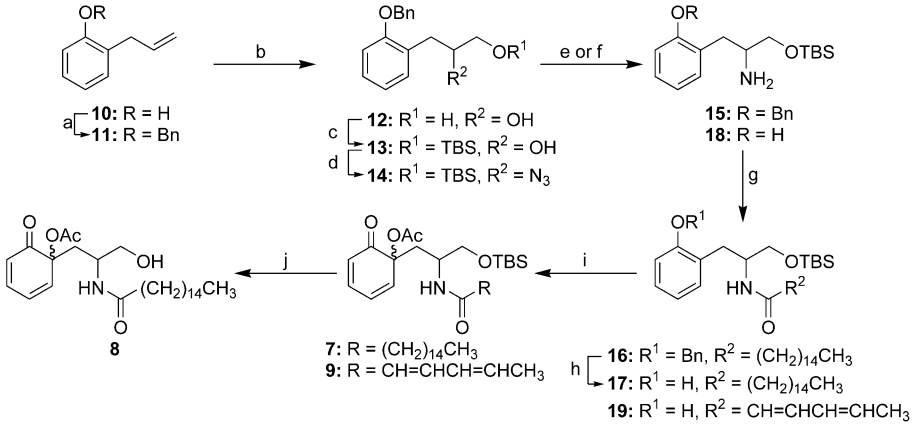
Low-molecular-weight inhibitors of sphingomyelinases have attracted considerable attention as molecular tools for the investigation of the enzymatic mechanism of the various isoforms of these enzymes and for clarification of their biological role, as well as the role of the product of their action on sphingomyelin (ceramide), in apoptosis and signal-transduction processes. In addition, such molecules might prove valuable for the development of new therapies for various inflammatory and autoimmune diseases.[1]

To date, several natural products have been identified in the quest for such inhibitors.[2] Of these, scyphostatin (**1**, Scheme 1) inhibits the neutral (N-SMase; $IC_{50} = 1\ \mu M$) and the acidic (A-SMase; $IC_{50} = 49.3\ \mu M$) plasma-membrane-bound isoforms in a competitive manner.[3] It is intriguing that several designed analogues **2**–**6**, which share the epoxy and cyclohexenone structural features of scyphostatin, have been prepared and found to be selective but irreversible inhibitors of N-SMase.[4–6] However, the contribution of these reactive functionalities to the observed biological activity remains unclear. In the course of synthetic studies towards the polar core of scyphostatin, we have prepared three novel analogues **7**–**9** that could help resolve this issue since they lack the epoxy moiety.



Scheme 1. Scyphostatin and synthetic neutral sphingomyelinase inhibitors.



*Scheme 2. Preparation of novel scyphostatin analogues **7**–**9**. Reagents and conditions: a) K_2CO_3, tetrabutylammonium iodide, BnBr, DMF, 78%, b) OsO_4, $K_3Fe(CN)_6$, K_2CO_3, tBuOH/H_2O (1:1), 68%, c) TBSCl, imidazole, DMF, 0°C, 73%, d) PPh_3, DEAD, DPPA, THF, 0–25°C, 87%, e) PPh_3, THF, H_2O, 65°C, 92% (**15**), f) H_2, Pd/C 10%, EtOH, 96% (**18**), g) 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, 4-dimethylaminopyridine, palmitic or sorbic acid, THF, 78% (**16**) or 84% (**19**), h) H_2, Pd/C 10%, MeOH, 97%, i) $Pb(OAc)_4$, AcOH, 5°C, 44% (**7**), 42% (**9**), j) TFA, CH_2Cl_2, 0–25°C, 62%. DMF = N,N-dimethylformamide, THF = tetrahydrofuran, TFA = trifluoroacetic acid.*

Their preparation is depicted in Scheme 2.[7] The benzyl ether of 2-allylphenol **11** was dihydroxylated to provide diol **12**. Selective protection of the primary hydroxyl group, as a *tert*-butyldimethylsilyl (TBS) ether, and subsequent treatment of alcohol **13** with triphenylphosphine, diethyl azodicarboxylate (DEAD) and diphenylphosphoryl azide (DPPA) furnished the corresponding azide **14**.[8] This could be selectively reduced by triphenylphosphine to amine **15**, which was coupled with palmitic acid, as a surrogate for the fatty side chain of scyphostatin, to yield amide **16**. Hydrogenolysis of the benzyl protective group furnished phenol **17**. Alternatively, direct hydrogenolysis of azide **14** and subsequent coupling of aminophenol **18** could accommodate the incorporation of unsaturated fatty acids, such as sorbic acid to yield phenol **19**. Oxidation of phenols **17** or **19** by treatment with lead tetraacetate (Wessely oxidation)[9] afforded *o*-quinol acetates **7** or **9**, respectively, as an

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equimolecular mixture of diastereoisomers. As exemplified by the conversion of **7** to alcohol **8**, selective cleavage of the silyl ether could be accomplished by treatment with trifluoroacetic acid in dichloromethane.

For the evaluation of the newly synthesized compounds **7**–**9** as inhibitors, a raw microsome preparation containing rat brain Mg^{2+}-dependent N-SMase was utilized. The assay was carried out with and without pre-incubation of the compound tested with the enzyme in the absence of substrate. The latter provides an indication of the ability of the compound to compete with the substrate for binding at the active site of the enzyme, whereas the former represents the kinetics of irreversible inhibition. All compounds tested were found to be irreversible N-SMase inhibitors (Figure 1). Compound **7** is a weak inhibitor, while compounds **8** and **9** exhibit comparable activity with the known inhibitor **2** (Table 1).[4]

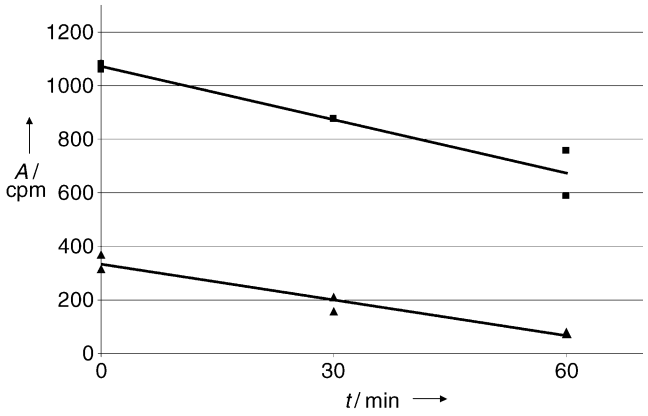

*Figure 1. Time dependence of the inhibition of neutral sphingomyelinase by compound **9** (200 μM in pre-incubation buffer): ▲ = with compound **9**, ■ = control experiment without compound **9**; A* = activity, cpm = counts per minute; *t* = pre-incubation time.

*Table 1. Inhibition of neutral sphingomyelinase by scyphostatin analogues **7**–**9** without and with pre-incubation.[a]*

Compound	Inhibition (without pre-incubation) [%]	Inhibition (with pre-incubation) [%]
7	0	28
8	48	71
9	68	89

[a] The concentration of the compounds in the pre-incubation buffer was 200 μM (final concentration 100 μM). The pre-incubation time was 1 h.

In accordance to previous findings, these results indicate that: i) the presence of a primary hydroxy group increases enzyme inhibition (compare activity of **7** vs. **8**),[5] while ii) an unsaturated side chain contributes to a higher affinity for N-SMase (compare activity of **7** vs. **9**).[6] Furthermore, the results suggest that the epoxy moiety is not a strict requirement for enzyme inhibition.

These findings and insights should contribute to the quest for potent competitive inhibitors of N-SMase and may finally provide a means for the development of novel anti-inflammatory therapeutic strategies. We are concentrating our efforts on the synthesis of the core of scyphostatin as well as the preparation of selective competitive inhibitors of N-SMase.

Experimental Section

Wessely oxidations: A solution of the phenol (0.074 mmol) in acetic acid (4 mL) was cooled to 5 °C and treated with lead tetraacetate (0.081 mmol, 1.1 equiv). The reaction mixture, protected from direct light, was stirred at the above temperature for 2 h. Excess reagent was quenched by addition of ethylene glycol (0.1 mL). A saturated aqueous solution of NaCl (4 mL) was added, and the mixture was extracted with diethyl ether (4 × 5 mL). The combined organic phases were dried over anhydrous Na_2SO_4, the solvent was removed under reduced pressure, and the residue was purified by flash column chromatography (10–50% AcOEt in hexanes gradient) to yield the respective *o*-quinol acetates as an inseparable mixture of diastereoisomers.

7: 44% yield from **17**; ^{1}H NMR (250 MHz, $CDCl_3$) δ = 7.03–6.93 (m, 1H; =CH), 6.50–6.08 (m, 3H; =CH), 5.75, 5.38 (2br d, J = 8.6, 8.9 Hz, 1H (1:1); N*H*CO), 4.23–4.09 (m, 1H; C*H*NHCO), 3.61–3.46 (m, 2H; CH_2OTBS), 2.19–1.91 (m, 4H; CH_2CHNHCO + NHCOCH_2), 2.08, 2.03 (2s, 3H; CH_3CO), 1.69–1.47 (m, 2H; COCH_2CH_2), 1.35–1.21 (m, 24H; $CH_2(CH_2)_{12}CH_3$), 0.92–0.83 (m, 12H; $SiC(CH_3)_3$ + CH_2CH_3), 0.05, 0.03, 0.02, 0.01 (4s, 6H; $Si(CH_3)_2$); IR (thin film, KBr) $\tilde{\nu}_{max}$ = 2959, 2927, 2853, 1753, 1682 cm^{-1}; HR-MS (EI): *m/z*: 577.4173 [M^+], $C_{33}H_{59}NO_5Si$ requires 577.4163.

9: 42% from **19**; ^{1}H NMR (250 MHz, $CDCl_3$) δ = 7.25–6.75 (m, 2H; =CH), 6.60–6.01 (m, 4H; =CH), 5.84–5.30 (m, 3H; =CH), 4.36–4.11 (m, 1H; C*H*NHCO), 3.76–3.46 (m, 2H; CH_2OTBS), 2.17–1.94 (m, 2H; CH_2CHNHCO), 2.09, 2.01 (2s, 3H; CH_3CO), 0.90, 0.87 (2s, 9H; $SiC(CH_3)_3$), 0.04, 0.03, 0.02, 0.01 (4s, 6H; $Si(CH_3)_2$); IR (thin film, KBr) $\tilde{\nu}_{max}$ = 2956, 2924, 2857, 1748, 1676, 1665, 1636, 1616 cm^{-1}; HR-MS (EI): *m/z*: 433.2293 [M^+], $C_{23}H_{35}NO_5Si$ requires 433.2285.

Alcohol **8**: Trifluoroacetic acid (TFA, 0.5 mL) was added to a stirred solution of *o*-quinols **7** (21 mg, 36 μmol) in dichloromethane (1 mL) at 0 °C. The reaction mixture was allowed to warm up gradually to ambient temperature over 45 min. After being stirred at this temperature for an additional 15 min, the solvent was evaporated under reduced pressure. Residual TFA was removed by redissolving the residue in benzene (5 mL) and removal of the solvent under reduced pressure. Further purification by flash column chromatography (50% EtOAc in hexanes) provided free alcohol **8** (11 mg, 62%) as a mixture of diastereoisomers. ^{1}H NMR (250 MHz, $CDCl_3$) δ = 7.06–6.96 (m, 1H; =CH), 6.40–6.08 (m, 3H; =CH), 5.98, 5.66 (2br d, J = 7.4, 8.2 Hz, 1H (1:1); NHCO), 4.18–3.98 (m, 1H; C*H*NHCO), 3.69–3.53 (m, 2H; CH_2OH), 2.24–1.87 (m, 4H; CH_2CHNHCO + NHCOCH_2CH_2), 2.10, 2.09 (2s, 3H; CH_3CO), 1.68–1.46 (m, 2H; NHCOCH_2CH_2), 1.38–1.16 (m, 24H; $CH_2(CH_2)_{12}CH_3$), 0.88 (t, 3H; CH_2CH_3); IR (thin film, KBr) $\tilde{\nu}_{max}$ = 3308, 3078, 2921, 2849, 1749, 1683, 1642, 1551 cm^{-1}; HR-MS (EI): *m/z*: 463.3293 [M^+], $C_{27}H_{45}NO_5$ requires 463.3298.

Purification of N-Smase: Partial purification of neutral sphingomyelinase was carried out as previously described.[6]

Inhibition assay: For the determination of the N-SMase activity, the inhibitors were dissolved in chloroform. A 10 nmol aliquot was dried under a nitrogen stream, redissolved in buffer (40 μL, 75 mM Tris-HCl, pH 7.4, 0.05% (*w/v*) Triton X-100, 5 mM $MgCl_2$) and mixed with the enzyme solution (10 μL). Together with controls, the probes were pre-incubated for 60 and 30 min at 37 °C. After addition of [^{14}C]sphingomyelin (10 nmol, ca. 40000 cpm) in the same buffer (50 μL), the reaction proceeded for another 30 min. The reaction was

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stopped by adding chloroform/methanol (750 µL, 2:1, *v/v*). After addition of water (200 µL), the lipids were extracted, and the radioactivity of the polar upper phase, which contained [^{14}C]phosphorylcholine, was determined by scintillation counting.

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Catalytically Active Tetramodular 6-Deoxyerythonolide B Synthase Fusion Proteins

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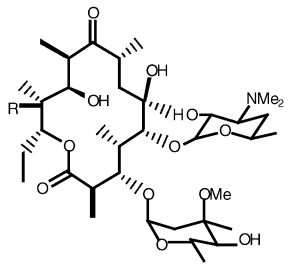
Large numbers of structurally diverse and medicinally important macrocyclic polyketides are produced through the action of the type I modular polyketide synthases (PKS) of actinomycete bacteria. In such systems, the individual catalytic domains required for each successive cycle of polyketide chain extension and subsequent reduction are ordered through covalent links into a multienzyme module. These modules are housed in the order in which they are used on three or more giant protein subunits. An individual giant PKS protein may house a single extension module, as in one subunit of the spiramycin-producing PKS,[1] or as many as six successive extension modules, as in one subunit of the rapamycin-producing PKS.[2] In general, the catalytic advantages of such multifunctional enzymes are thought to include more efficient channelling of enzyme-bound intermediates between successive active sites, and protection of the intermediates from side reactions.[3]

The erythromycin-producing PKS, 6-deoxyerythonolide B synthase (DEBS), which governs the biosynthesis of erythromycin A (**1**) and B (**2**) in *Saccharopolyspora erythraea*, is an



Erythromycin A (**1**): R = OH
Erythromycin B (**2**): R = H

established model system for investigations into the mechanism of polyketide biosynthesis. The three multienzyme polypeptides DEBS 1, DEBS 2 and DEBS 3 are readily separated during their purification from *S. erythraea* extracts, and this has prevented investigation of the overall architecture of the complex.[4–8] In this work we aimed to create tethered versions of DEBS by

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