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Influence of the Scyphostatin Side Chain on the Mode of Inhibition of Neutral Sphingomyelinase

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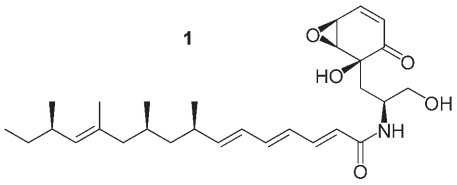
Sphingomyelin is a ubiquitous constituent of cell membranes. Its role is not merely structural; hydrolysis of sphingomyelin by sphingomyelinases (SMases) to generate phosphorylcholine and ceramide is the initial step in the so-called sphingomyelin signal transduction pathway.[1] In this respect, two of the many different forms of SMase identified to date[2] have been the focus of intensive research: the acidic sphingomyelinase (A-SMase), primarily found in the lysosomes,[3] and the Mg^{2+}-dependent, membrane-bound neutral sphingomyelinase (N-SMase).[4] Potent, specific inhibitors of these enzymes might not only prove to be valuable probes in deciphering their biological role but may also lead to the development of novel therapeutics for the treatment of inflammation and immunological and neurological disorders.[5]

Scyphostatin (**1**) was isolated from the culture broth of the cup-shaped (cup = scyphos/σκυφος in ancient Greek) fungus



Dasyscyphus mollissimus, later renamed *Trichopeziza mollissima*.[6] Although it has no activity towards bacterial SMases and inhibits A-SMase at high concentrations (IC_{50} = 49.3 μM), it is a potent (IC_{50} = 1.0 μM) inhibitor of N-SMase.[6a,c] Preliminary kinetic analyses indicated that inhibition of N-SMase was reversible (mixed-type with respect to sphingomyelin).[6b,7] Inhibitory activity was mainly attributed to the polar aminopropanol-substituted, epoxycyclohexenone core of the molecule while the polyunsaturated fatty acid moiety was blamed for its short half-life in the solid state, even when stored at −20 °C.[6b]

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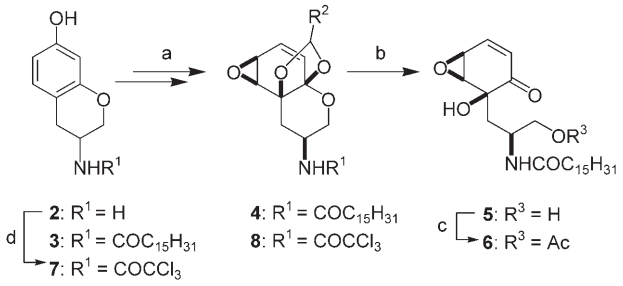
The combination of structural novelty and significant biological activity has made scyphostatin the focus of many synthetic efforts[8,9] as well as the prototype for the design of novel N-SMase inhibitors.[10] The former have led to an elegant total synthesis of (+)-scyphostatin by Katoh and co-workers.[11] It is interesting to note that although the latter efforts have yielded several novel inhibitors of N-SMase, they were all found to be irreversible inhibitors, unlike the natural product.[10a–e,g] These inhibitors shared, to various degrees, the structural motif of scyphostatin. However, both the polar core and the side chain have been modified, making it impossible to attribute this shift in inhibition type.

We recently published a short, diastereoselective route to the fully functionalized polar core of scyphostatin.[12] In this initial implementation of our strategy towards the polar core of scyphostatin, we opted to employ palmitic acid as a surrogate for its fatty acid side chain. Thus, aminobenzopyrane **3** (Scheme 1) was successfully converted, in six steps and 14% overall yield, to the fully protected final intermediate **4**. Treatment with montmorillonite K10 afforded the targeted palmitoyl analogue of scyphostatin **5** in 55% yield, which was converted to the corresponding acetyl derivative **6** in order to provide additional proof of its structure.[12]

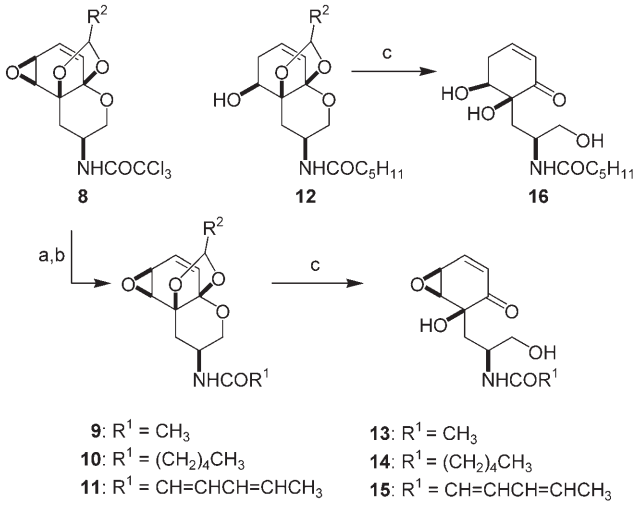


Scheme 1. Synthesis of the palmitoyl analogue of scyphostatin (**5**). a) Ref. [12]; b) montmorillonite K10, CH_2Cl_2, 55%; c) Ac_2O, Py, 78%; d) CCl_3COCCl_3, THF, 60 °C, 96%. R^2 = 4-methoxyphenyl, Py = pyridine.

Herein we present: 1) the evolution of this synthetic strategy to allow a convergent entry to several new analogues of the natural product, and 2) the unexpected inhibitory activity of these analogues towards N-SMase. Their close structural resemblance to scyphostatin has led us to refer to them as kotylostatins (from the ancient Greek vessel kotyle/κοτυλη the shape of which was closely related to that of scyphos/σκυφος).

Protection of the amino function of benzopyran **2** followed by protecting group removal at a latter stage could significantly improve the overall efficacy and versatility of our synthetic sequence. However, the strategic role of the amide carbonyl function in controlling the diastereoselectivity of the approach[12] in combination with the dense and potentially sensitive functionalization of **4**, significantly narrowed the repertoire of suitable protecting groups. Although amino groups are not often protected as trichloroacetamides,[13] the successful use of this protecting group in the total synthesis of sphingofungin E[14] and tetrodotoxin[15] led us to investigate its applicability to our case.

Thus, treatment of aminobenzopyran **2** with hexachloroacetone afforded trichloroacetamide **7** in nearly quantitative yield (Scheme 1). Subsequent diastereoselective conversion to the key intermediate **8** (Scheme 2) was successfully implemented



Scheme 2. Synthesis of scyphostatin analogues **13**–**16**. a) DIBAL-H, toluene, −78 °C; b) Ac_2O, Py, 86% of **9** or hexanoic acid, $(iPr)_2EtN$, PyBop, CH_2Cl_2/DMF (14:1), 73% of **10** or sorbic acid, $(iPr)_2EtN$, PyBop, CH_2Cl_2/DMF (14:1), 76% of **11**; c) montmorillonite K10, CH_2Cl_2, 62% of **13** or 87% of **14** or 56% of **15** or 95% of **16**. R^2 = 4-methoxyphenyl, Py = pyridine, PyBop = (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate.

in six steps and 12% overall yield, in a manner analogous to that for compound **4**.[12] Reaction of **8** with DIBAL-H in toluene at −78 °C reduced the trichloroacetamide moiety to afford an amine, which was isolated as its *N*-Ac derivative **9** in high yield. Alternatively, the crude amine could be coupled with longer chains, saturated or unsaturated, such as caproic or sorbic acid to yield compounds **10** or **11**, respectively. Notably, it was crucial to maintain a low temperature during the deprotection step to avoid concomitant reductive opening of the epoxide ring. This reactivity was exploited for the preparation of compound **12**. Final deprotection of compounds **9**–**12** was accomplished by treatment with montmorillonite K10 to afford scyphostatin analogues **13**–**16** in good yields.

The inhibitory effects of compounds **5** and **6** and the newly synthesized compounds **13**–**16** against N-SMase were evaluated using a microsome preparation containing Mg^{2+}-dependent N-SMase from rat brain (Table 1).[10a–e,g] Compound **16**, which lacks the epoxide group, is inactive, indicating that this functional group is essential for inhibition. Interestingly, compound **13**, which lacks a hydrophobic side chain, showed very weak but measurable inhibitory activity. More notable, however, was the finding that, unlike scyphostatin, compounds **5**, **6**, and **13**–**15** proved to be time-dependent (that is, irreversible) inhibitors, indicating that the *N*-acyl side chain has a remarkable effect on the mode of inhibition.

The palmitoyl analogue of scyphostatin (**5**) proved to be the most potent derivative and was investigated in more detail (Figure 1). Moreover, increasing the concentration of sphingomyelin in the assay attenuates the inhibitory effect of **5** (Figure 2), indicating that inhibitor **5** binds at the active site of N-SMase.

Table 1. Inhibition of neutral sphingomyelinase by scyphostatin analogues **5**–**6** and **13**–**16** without and with preincubation.[a]

Compound	Inhibition (without preincubation) [%]	Inhibition (with preincubation) [%]
5	92	100
6	90	100
13	4	69
14	33	97
15	25	89
16	0	0

[a] The concentration of the compounds was 200 μM in the preincubation buffer (final concentration 100 μM). The preincubation time was 45 min.

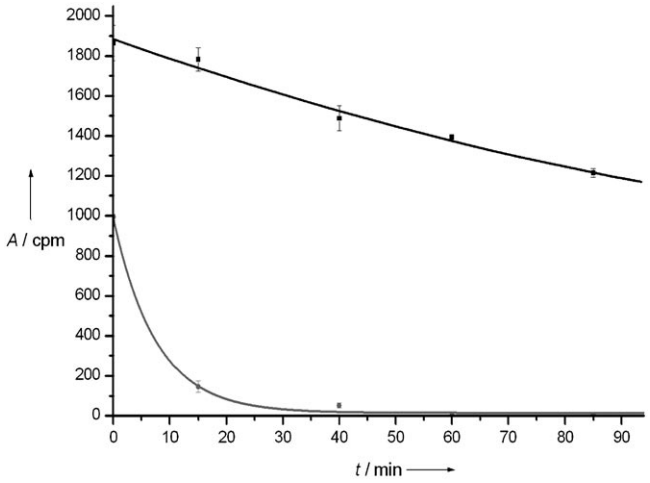


Figure 1. Time dependence of the inhibition of N-SMase by **5** (5 μM final concentration): ● = with compound **5**, ■ = control experiment without compound **5**. *A* = activity in counts per minute; *t* = preincubation time.

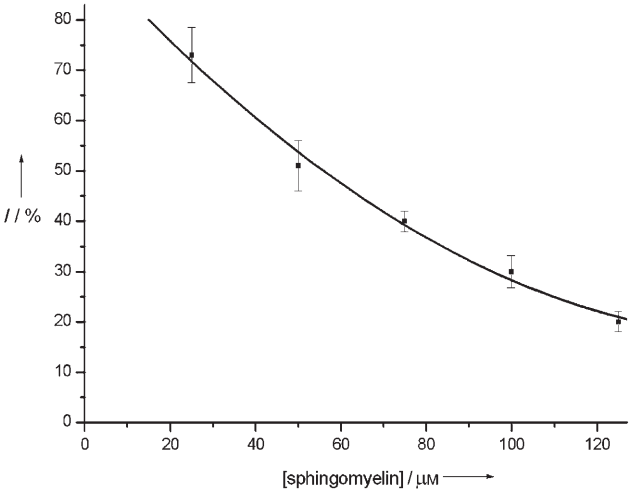


Figure 2. Inhibition of N-SMase by **5** at different concentrations of sphingomyelin (final inhibitor concentration 5 μM, no preincubation).

In conclusion, just as kotyle and scyphos had extremely similar shapes yet different functions (measuring cup and drinking cup, respectively), kotylostatins **5**, **6**, **13**–**15** and scyphostatin (**1**) are closely related structurally, yet exhibit different modes of N-SMase inhibition (irreversible and reversible, respectively). This result indicates that the fatty side chain of scyphostatin has a decisive influence on the mode of inhibition.

Experimental Section

Preparation of compounds 13–16: Montmorillonite K10 (100 mg) was added to a solution of one of the compounds **9–12** (23–26 μmol) in dichloromethane (5 mL). The mixture was stirred at ambient temperature for 10 min and then filtered. The clay was washed on the filter with acetone (4×10 mL), and the combined filtrates were evaporated under reduced pressure. The residue obtained was purified by flash column chromatography (10% methanol in chloroform) to produce compounds **13**–**16**.

13: 62% from **9**; ^{1}H NMR (500 MHz, CD_3OD): $\delta = 7.17$ (dd, $J = 9.9$, 3.9 Hz, 1H, COCH=C*H*), 6.11 (dd, $J = 9.9$, 1.4 Hz, 1H, COC*H*=CH), 4.03–3.98 (m, 1H, C*H*NHCO), 3.66 (d, $J = 3.9$ Hz, 1H, C*H*(O)CHCH), 3.61 (dt, $J = 3.9$, 1.5 Hz, 1H, CH(O)C*H*CH), 3.47 (dd, $J = 10.9$, 5.4 Hz, 1H, C*H*HOH), 3.43 (dd, $J = 10.9$, 5.8 Hz, 1H, CH*H*OH), 2.03 (dd, $J = 14.7$, 3.3 Hz, 1H, C*H*HCHNH), 1.87 (s, 3H, COCH_3), 1.82 (dd, $J = 14.7$, 9.6 Hz, 1H, CH*H*CHNH); ^{13}C NMR (62.5 MHz, $CDCl_3$): $\delta = 199.6$, 173.0, 145.8, 132.0, 77.5, 65.5, 58.2, 49.2, 47.8, 39.6, 22.6; HRMS (ESI): *m/z*: 264.08438 [*M*+Na$^+$], $C_{11}H_{15}NO_5Na$ requires 264.08479.

14: 87% from **10**; ^{1}H NMR (500 MHz, [D_6]acetone): $\delta = 7.20$ (ddd, $J = 9.9$, 3.8, 0.5 Hz, 1H, COCH=C*H*), 6.81 (bd, $J = 7.2$ Hz, 1H, N*H*CO), 6.11 (dd, $J = 9.9$, 1.5 Hz, 1H, COC*H*=CH), 4.03–3.96 (m, 1H, C*H*NHCO), 3.68 (d, $J = 3.9$ Hz, 1H, C*H*(O)CHCH), 3.63 (dt, $J = 3.9$, 1.6 Hz, 1H, CH(O)C*H*CH), 3.57–3.53 (m, 1H, C*H*HOH), 3.48–3.44 (m, 1H, CH*H*OH), 2.14–2.02 (m, 3H, COCH_2 + C*H*HCHNH), 1.84 (dd, $J = 14.6$, 9.4 Hz, 1H, CH*H*CHNH), 1.62–1.51 (m, 2H, COCH$_2$CH_2), 1.34–1.22 (m, 4H, CH_2CH_2), 0.87 (t, $J = 6.7$ Hz, 3H, CH$_2$CH_3); ^{13}C NMR (62.5 MHz, [D_6]acetone): $\delta = 199.9$, 174.4, 146.1, 132.5, 78.3, 66.7, 58.7, 49.5, 48.6, 40.1, 37.5, 33.2, 26.7, 24.1, 15.2; HRMS (ESI): *m/z*: 320.14677 [*M*+Na$^+$], $C_{15}H_{23}NO_5Na$ requires 320.14739.

15: 56% from **11**; ^{1}H NMR (500 MHz, CD_3OD): $\delta = 7.16$ (dd, $J = 9.9$, 3.9 Hz, 1H, COCH=C*H*), 7.09 (dd, $J = 15.2$, 10.6 Hz, 1H, COCH=C*H*CH=), 6.23–6.10 (m, 2H, C*H*=C*H*CH$_3$), 6.07 (dd, $J = 10.0$, 1.5 Hz, 1H, COC*H*=CH), 5.82 (d, $J = 15.1$ Hz, 1H, COC*H*=CHCH=), 4.08–4.01 (m, 1H, C*H*NHCO), 3.66 (d, $J = 3.9$ Hz, 1H, C*H*(O)CHCH), 3.59 (dt, $J = 3.9$, 1.6 Hz, 1H, CH(O)C*H*CH), 3.52 (dd, $J = 11.0$, 5.1 Hz, 1H, C*H*HOH), 3.45 (dd, $J = 11.0$, 5.9 Hz, 1H, CH*H*OH), 2.08 (dd, $J = 14.7$, 3.4 Hz, 1H, C*H*HCHNH), 1.88 (dd, $J = 14.7$, 9.4 Hz, 1H, CH*H*CHNH), 1.84 (d, $J = 6.5$ Hz, 3H, CH=CHCH_3); ^{13}C NMR (62.5 MHz, CD_3OD): $\delta = 199.5$, 168.7, 145.8, 142.5, 138.9, 132.0, 131.1, 122.6, 77.5, 65.5, 58.2, 49.6, 47.9, 39.8, 18.6; HRMS (ESI): *m/z*: 316.11541 [*M*+Na$^+$], $C_{15}H_{19}NO_5Na$ requires 316.11609.

16: 95% from **12**; ^{1}H NMR (500 MHz, CD_3OD): $\delta = 6.77$ (m, 1H, COCH=C*H*), 5.98 (dd, $J = 10.2$, 2.2 Hz, 1H, COC*H*=CH), 4.09–4.02 (m, 2H, C*H*NHCO + C*H*OH), 3.47 (dd, $J = 10.9$, 5.3 Hz, 1H, C*H*HOH), 3.41 (dd, $J = 10.9$, 6.0 Hz, 1H, CH*H*O), 2.82 (ddd, $J = 19.9$, 6.3, 3.0 Hz, 1H, C*H*HCHOH), 2.58 (dm, $J = 19.9$ Hz, 1H, CH*H*CHOH), 2.11–2.07 (m, 2H, COCH_2), 1.94 (d, $J = 6.1$ Hz, 2H, CH_2CHNH), 1.61–1.55 (m, 2H, COCH$_2$CH_2), 1.37–1.25 (m, 4H, CH_2CH_2), 0.92 (t, $J = 7.1$ Hz, 3H, CH$_2$CH_3); ^{13}C NMR (62.5 MHz, CD_3OD): $\delta = 202.6$, 176.1, 146.5, 127.6, 79.7, 75.7, 65.7, 48.1, 37.6, 37.1, 33.7, 32.6, 26.3, 23.5, 14.3; HRMS (ESI): *m/z*: 322.16246 [*M*+Na$^+$], $C_{15}H_{25}NO_5Na$ requires 322.16304.

Purification of N-SMase: Partial purification of neutral sphingomyelinase was carried out as previously described.[10g]

Inhibition assay: For the determination of N-SMase activity the potential inhibitors were dissolved in DMSO. Depending on the desired final concentration (100 μM or 5 μM), an aliquot of 10 nmol or 0.5 nmol, respectively, was mixed with N-SMase buffer (40 μL, 50 mM Tris-HCl, pH 7.4, 0.05% (*v/v*) Triton X-100, 5 mM $MgCl_2$) and the enzyme solution (10 μL). The final concentration of DMSO was below 5%. Together with controls, the probes were preincubated for the indicated times at 37 °C. After addition of 50 μL of N-SMase assay buffer (0.23 nmol [^{14}C]sphingomyelin and 9 nmol bovine sphingomyelin in N-SMase buffer), the reaction proceeded for another 30 min. The reaction was stopped by adding chloroform/methanol (800 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.

Keywords: ceramide · inhibitors · scyphostatin · signal transduction · sphingomyelinases

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