Structural Elucidation of Scyphostatin, an Inhibitor of Membrane-Bound Neutral Sphingomyelinase

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Signal transduction pathways via the sphingomyelin cycle have attracted much attention since ceramide, a product of sphingomyelinase, was found to play an important role in vitamin D_3-induced differentiation in HL-60 cells.[1] Ceramide is an intracellular lipid second messenger and takes part in the regulation of cell proliferation, differentiation, and apoptosis in a wide variety of cell types.[2] In particular, sphingomyelin breakdown by membrane-bound neutral sphingomyelinase (N-SMase) appears downstream of signaling events of inflammatory cytokines including TNFα and IL-1β, and this ceramide generation has been reported to mediate prostaglandin production and cytokine gene expression.[3−7] Although these cytokines are believed to play essential roles in many physiological processes including immune responses, uncontrolled production of the cytokines causes severe tissue damage and leads to a variety of pathological states.[8] Thus, inhibition of N-SMase may lead to regulation of ceramide levels and to therapy for inflammation and autoimmune diseases.

With this aim in mind, N-SMase inhibitors were sought for in fermentation broths of microorganisms, and scyphostatin (**1**) (Figure 1)[9] was discovered in a mycelial extract of *Dasyscyphus mollissimus* SANK-13892. **1** exhibited potent inhibitory activity ($IC_{50} = 1.0\ \mu M$) to N-SMase;[10] on the other hand, acidic SMase was inhibited at high concentrations ($IC_{50} = 49.3\ \mu M$). To our knowledge, this is the first inhibitor of the enzyme from either natural sources or synthetic origin except for ganglioside GM3 ($IC_{50} = 45\ \mu M$).[10] In this report, we describe the structural elucidation of **1**.

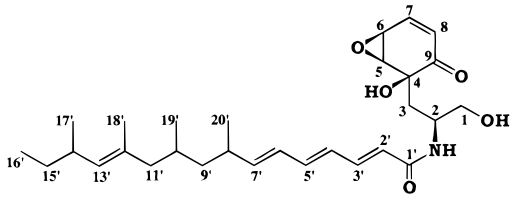

Figure 1. Structure of scyphostatin (**1**).

Table 1. NMR Data of Scyphostatin (**1**) (360 MHz, in CD_3OD)

pos	δ_C	δ_H	pos	δ_C	δ_H
1	65.6	3.45 (1H, dd, $J = 10.9, 5.8$ Hz)	7′	146.4	5.70 (1H, dd, $J = 15.1, 8.7$ Hz)
		3.52 (1H, dd, $J = 10.9, 5.1$ Hz)	8′	36.7	2.35 (1H, m)
2	48.5	4.05 (1H, m)	9′	45.7	1.10 (1H, m)
3	40.3	1.89 (1H, m)			1.33 (1H, m)
		2.08 (1H, dd, $J = 14.7, 3.6$ Hz)	10′	30.0	1.59 (1H, m)
4	78.0		11′	50.1	1.79 (1H, m)
5	58.7	3.67 (1H, d, $J = 3.9$ Hz)			1.89 (1H, m)
6	49.8	3.59 (1H, m)	12′	134.0	
7	146.6	7.15 (1H, m)	13′	134.7	4.84 (1H, m)[a]
8	132.5	6.07 (1H, dd, $J = 9.8, 1.6$ Hz)	14′	35.9	2.27 (1H, m)
9	201.0		15′	32.6	1.19 (1H, m)
1′	169.0				1.33 (1H, m)
2′	124.3	5.89 (1H, d, $J = 14.8$ Hz)	16′	13.0	0.86 (3H, t, $J = 7.4$ Hz)
3′	142.9	7.15 (1H, m)	17′	22.0	0.91 (3H, d, $J = 6.9$ Hz)
4′	129.9	6.25 (1H, dd, $J = 14.9, 11.1$ Hz)	18′	16.9	1.54 (3H,d, $J = 1.3$ Hz)
5′	141.9	6.53 (1H, dd, $J = 14.9, 10.7$ Hz)	19′	20.4	0.83 (3H, d, $J = 6.5$ Hz)
6′	130.4	6.15 (1H, dd, $J = 15.1, 10.7$ Hz)	20′	22.4	1.00 (3H, d, $J = 6.7$ Hz)

[a] Signal was detected with a WEFT experiment.

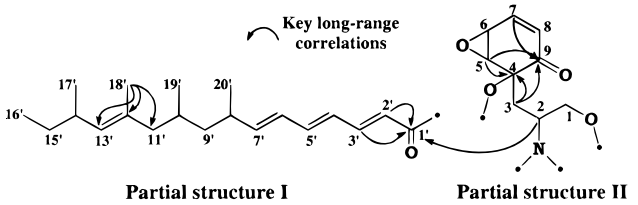

Figure 2. Partial structures of **1**.

Scyphostatin (**1**) [UV λ_{max}(MeOH) (ϵ) 300 nm (41 500), $[\alpha]^{25}_D$ +66.4° (*c* 0.09 in MeOH)] was isolated as a colorless oil and the molecular formula was determined to be $C_{29}H_{43}NO_5$ ($[M + H]^+$, *m/z* 486.3199 Δ −0.5 mmu) on the basis of high-resolution FABMS spectral analyses. The structural study was mainly carried out by interpretation of NMR spectra taken in CD_3OD. ^1H and ^{13}C NMR spectral data are summarized in Table 1. The DQFCOSY spectrum revealed two spin systems, C-2′ through C-11′ and C-13′ through C-16′, which were in turn connected by the C-12′ olefinic quaternary carbon based on long-range correlations of 18′-H with C-11′, C-12′, and C-13′ in HMBC experiments. The geometry of the conjugated triene was determined to be all *E* based on the large coupling constants ($J_{2'-3'} = 14.8$ Hz, $J_{4'-5'} = 14.9$ Hz, and $J_{6'-7'} = 15.1$ Hz) of the corresponding olefinic proton signals, and the C-12′ double bond was determined to be *E* because of the chemical shift of the C-18′ methyl resonance at higher field (16.9 ppm). Long-range correlations of C-1′ with 2′-H and 3′-H were observed revealing that **1** has a conjugated acyl group (partial structure I) as depicted

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(9) The isolation, fermentation, and biological activities of scyphostatin will be reported soon elsewhere.
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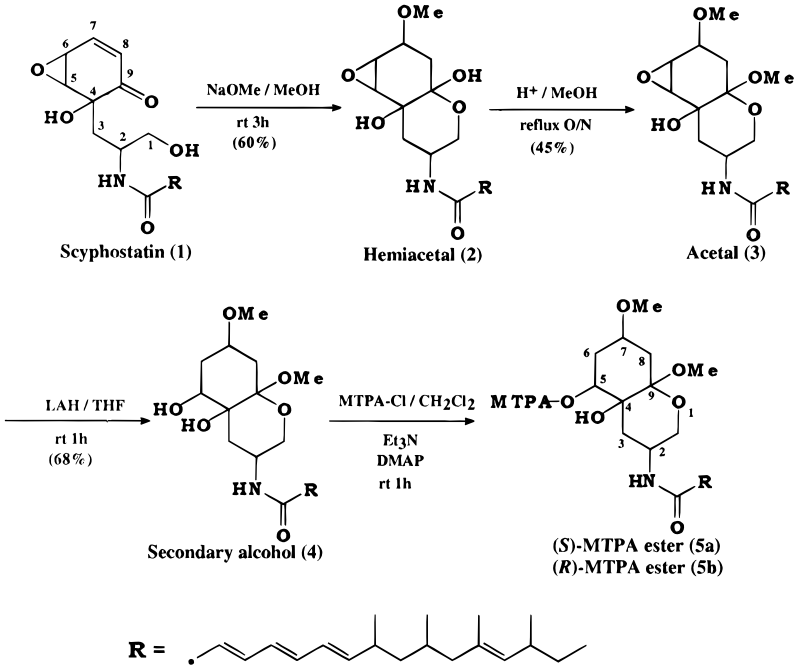
S0002-7863(97)01338-3 CCC: $14.00

Scheme 1



in Figure 2. This partial structure was consistent with the strong UV absorption at 300 nm due to the triene amide chromophore (vide infra).

The existence of the epoxycyclohexenone moiety shown in partial structure II (Figure 2) was determined as follows. Proton−proton connectivities, C-1 through C-3 and C-5 through C-8, were revealed by the DQFCOSY spectrum. The chemical shifts of C-1 (65.6 ppm) and C-2 (48.5 ppm) suggested that they were substituted with oxygen and nitrogen, respectively. The presence of an epoxide ring at C-5 and C-6 was deduced from the chemical shifts and large $^1J_{C-H}$ values of these signals (C-5: 58.7 ppm, 180 Hz; C-6: 49.8 ppm, 181 Hz). The geometry of the C-7 double bond was determined to be *Z*, based on the coupling constant (9.8 Hz) between the two vinyl protons. In HMBC experiments, correlations of a ketone (201 ppm) with 3-H, 5-H, and 7-H and that of an oxygenated quaternary carbon (78.0 ppm) with 3-H and 5-H were observed. Hence, the ketone and the oxygenated quaternary carbon were thought to be connected to form a cyclohexenone ring as shown in Figure 2.

All atoms in the molecular formula of **1** except for three labile hydrogens were included in partial structures I and II. In HMBC experiments, the observation of long-range coupling between 2-H and C-1′ verified that the two partial structures were connected by means of an amide bond between 2-N and C-1′, giving the full structure of **1**.

To elucidate the stereochemistry of the hydrophilic head moiety, **1** was converted to the bicyclic acetal (**4**) and its MTPA esters (**5a**,**b**) as illustrated in Scheme 1. The addition of methanol to **1** under basic conditions produced epoxyhemiacetal (**2**). Heating of **2** in methanol under reflux in the presence of H_2SO_4 gave epoxyacetal (**3**). The latter on reduction with lithium aluminum hydride gave secondary alcohol (**4**) as the sole product. (*S*)- and (*R*)-MTPA esters (**5a,b**) were prepared by reactions of **4** with (*R*)- and (*S*)-MTPA chloride, respectively.

Because the conformation of the hexahydrochroman ring was considered to be fixed, the relative configuration of **5a** was determined from the analysis of a NOESY experiment in which strong NOEs between 2-H and 5-H and between 5-H and 7-H were observed. These results are only consistent with a relative configuration in which two rings are fused in a cis fashion, and the oxygen substituents on C-5 and C-7 are equatorial as shown in Figure 3.

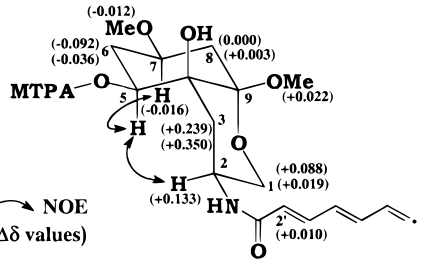


Figure 3. Stereochemistry of the hexahydrochroman ring.

The absolute configuration of **4** was established by the modified Mosher's method.[11] The $\Delta\delta$ ($\delta_{(S)-\text{MTPA ester}} - \delta_{(R)-\text{MTPA ester}}$) values of 1-H, 2-H, 3-H, 2′-H, and the 9-methoxy group were positive, and the $\Delta\delta$ values of 6-H, 7-H, and the 7-methoxy group were negative (Figure 3), indicating the (*S*) configuration at C-5 of **4**. Hence, the stereochemistry of the hydrophilic head moiety of **1** was (2*S*,4*S*,5*S*,6*S*) as shown in Figure 1.

The structure of **1** is composed of a fatty acid and an amino alcohol substituted by a highly oxygenated cyclohexenone moiety. The structural similarity between **1** and ceramide represented by the *N*-acylamino alcohol moiety suggests that **1** may exhibit inhibitory activity as a substrate or product analogue of the enzymatic reaction. Although there are some natural products which have structures analogous to sphingosine or ceramide, for example, fumonisins,[12] sphingofungins,[13] and lipoxamycin,[14] all of which have been reported to inhibit the *de novo* synthesis of sphingolipids, there are as far as we know no previous reports about an SMase inhibitor with significant activity. Thus, **1** may be a useful probe for studying N-SMase-mediated signal transduction.

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