VOL. 52, NO. 6, JUNE 1999 THE JOURNAL OF ANTIBIOTICS pp. 525 — 530

Scyphostatin, a Neutral Sphingomyelinase Inhibitor from a Discomycete, *Trichopeziza mollissima*: Taxonomy of the Producing Organism, Fermentation, Isolation, and Physico-chemical Properties

FUTOSHI NARA, MASAHIRO TANAKA[a], TSUYOSHI HOSOYA[b], KEIKO SUZUKI-KONAGAI[a] and TAKESHI OGITA[a,*]

Biological Research Laboratories, Biomedical Research Laboratories[a] and Tsukuba Research Laboratories[b], Sankyo Co., Ltd., 2-58 Hiromachi 1-chome, Shinagawa-ku, Tokyo 140-8710, Japan Miyukigaoka 33, Tsukuba-shi, Ibaraki 305-0841, Japan

(Received for publication February 10, 1999)

We performed experiments to screen for neutral sphingomyelinase inhibitors using rat brain microsomes as an enzyme source. Among more than 10,000 microbial extracts tested, a mycelial extract of *Trichopeziza mollissima* SANK 13892 exhibited potent inhibitory activity. The active compound, scyphostatin, was purified by a series of chromatographies. Scyphostatin inhibited the enzyme with an IC_{50} value of 1.0 μM.

The breakdown of membrane sphingomyelin (SM) has been shown to play a critical role in intracellular signal transduction. Since OKAZAKI and HANNUN first demonstrated that the differentiation of HL-60 cells to monocytes with vitamin D_3 is accompanied by activation of neutral sphingomyelinase (N-SMase)[1,2], TNFα, IL-1β, INFγ, Fas, NGF and several other protein factors have been shown to use SM turnover in their downstream signaling[3~10]. A growing body of evidence is showing that ceramide, a product of SMase reaction, serves as an intracellular second messenger and transduces the signals down to the nucleus through the activation of specific target molecules. However, the biological outcome of ceramide generation is not uniform and may depend on cell types, topological distribution of ceramide in cells, and/or crosstalk with other signals. To establish a clearer picture of the role of ceramide, specific inhibitors of SMases will be very useful tools. Moreover, these inhibitors can be expected to have clinical value since ceramide generation might be implicated in pathogenic states such as inflammation and immunological and neurological disorders. With these considerations in mind, we searched for low-molecular-weight inhibitors for N-SMase in the extracts of microbial products and found scyphostatin[11,12] in the mycelial extract of *Trichopeziza mollissima* SANK 13892. This paper describes taxonomical studies of the producing strain, screening procedure, fermentation, isolation, and physico-chemical properties of the compound.

Materials and Methods

Materials

All reagents were obtained from Sigma unless otherwise specified. [*N*-methyl-^{14}C] sphingomyelin (bovine) and Wistar-Imamichi rats were purchased from Amersham Co., Ltd. and Japan SLC Co., respectively.

Preparation of an Enzyme Source

We used the rat brain microsomes as a source of N-SMase because it has been reported that the fraction is rich in the activity[13]. The preparation was performed by the following method: Male rats (Wistar-Imamichi, 9~10 weeks old) were sacrificed by decapitation and all of the brain except the cerebellum was removed and washed with cooled phosphate-buffered saline. The brain was suspended in SM buffer (5 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM phenylmethylsul-

fonyl fluoride, 0.1 mM leupeptin) and then homogenized by a Teflon pestle glass homogenizer. The post-nuclear homogenate was centrifuged for 1 hour at 100,000 × *g*, and the pellet was dissolved in SM buffer. The fraction was directly subjected to N-SMase activity. In our basic research, the SMase in rat brain microsomes showed optimal pH activity at 7.5 and was stimulated by divalent cations. Maximum stimulation of SMase activity occurred in the presence of 1 to 3 mM of magnesium or manganese in the assay mixture. Moreover, SMase activity was activated with increasing concentrations of Triton X-100, and the highest activity was obtained at a concentration of 0.3%. These results imply that rat brain microsome generally includes a representative of N-SMase.

Screening procedure

[*N*-methyl-^{14}C] sphingomyelin in a final concentration of 2 mM with a specific activity of 31 μCi/mmol was dried under N_2 and mixed with the assay mixture (100 mM Tris-HCl, pH 7.5, 0.2% Triton X-100, and 5 mM magnesium chloride). After incubation at 48°C for 30 minutes, the mixture repeatedly underwent a 15-second probe-type sonication procedure at 20 watts and was then used as a mixed-micelle. The reaction was started by the addition of an enzyme preparation and a microbial extract. It proceeded for 40 minutes at 37°C and was then terminated and extracted by chloroform : methanol (2 : 1, v/v) followed by repeated pipetting. After phase separation, the portion of the upper phase containing the enzymatically released phosphocholine was removed and the radioactivity was determined by liquid scintillation counting. N-SMase activity was calculated by subtracting the control count run in the condition excluding magnesium. The identity of the product in the upper phase was confirmed by thin layer chromatography as described before[14].

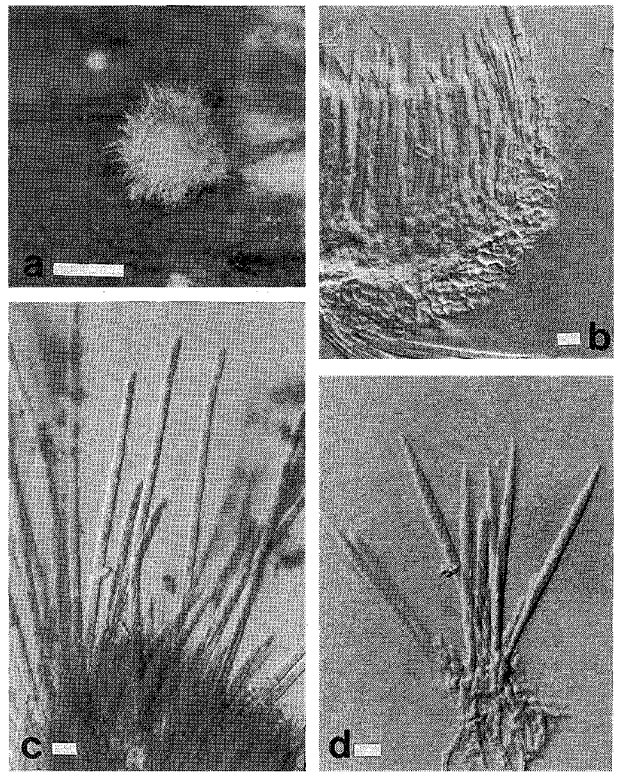
Results and Discussion

Taxonomical Studies of Producing Strain

SANK 13892 was collected in May 1992 in Aomori Prefecture, Japan. The strain was obtained from an ascospore using a single-spore isolation technique. As this discomycete is not identified by cultural features, the dried apothecia were examined for identification. The mycological properties were as follows (Fig. 1).

Apothecia were up to 0.2 mm in diameter, sessile, flat, discoid, greyish orange to yellow, and frilled with hairs.

Fig. 1. Mycological properties of *Trichopeziza mollissima* SANK 12892.

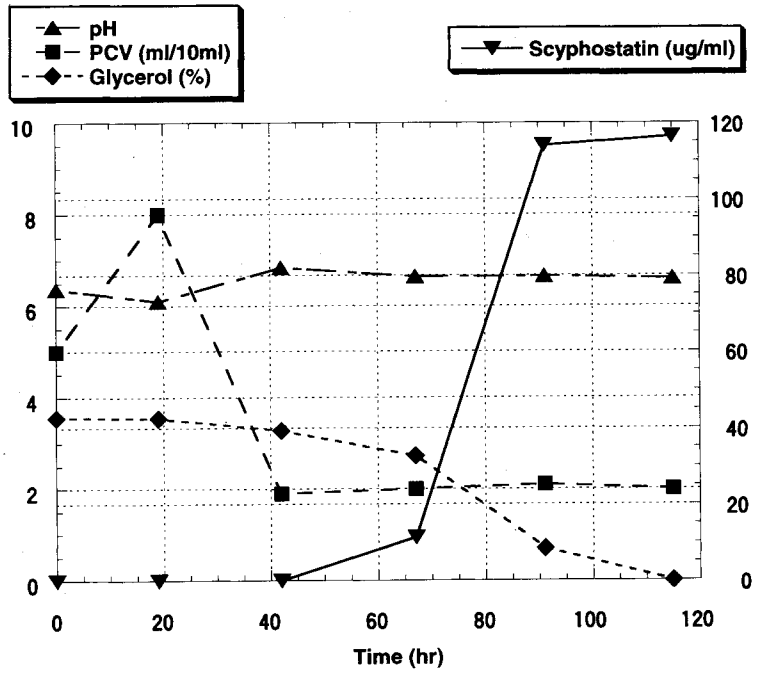


a. Dry apothecium. b. Vertical section of the apothecium showing hymenium, excipulum, and hairs. c. Hairs in squash mount. d. Paraphyses and asci. Scales. a: 1 mm. b~d: 10 μm.

Ectal excipulum were "textura angularis" and composed of pale-colored, thin-walled, polygonal to prismatic cells. Medullary excipulum were "textura intricata" of closely interwoven hyphae running somewhat parallel to the outside. Hairs arose from the outermost layer of the ectal excipulum. They were straight, cylindrical, smooth, thin-walled, multi-septate, partially covered with resinous material, 3.5 μm thick, and up to 200 μm long terminating in an obtuse apex. Asci arose from croziers and were 58~66 × 5.0~6.5 μm, cylindrical, and eight-spored, with apices stained by Melzer's reagent. Paraphyses were lanceolate, c. 20 μm longer than asci, and 74.5~100.0 × 3.5~4.0 μm. Ascospores were bacilliform to fusiform, one-celled, hyaline, and 18.0~26.5 × 2.5~5.0 μm.

The above properties agreed with the description of *Dasyscyphus mollissimus* (Lasch) Dennis[15]. The confusion in the nomenclature for *D. mollissimus* has been discussed and a number of possible synonyms have been

Fig. 2. Fermentation profile of *Trichopeziza mollissima* SANK 13892.

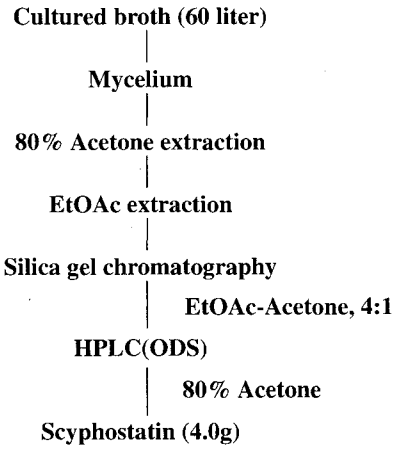


suggested[16]. Since the present taxonomy does not accept the generic name *Dasyscyphus*., it is identified here as *Trichopeziza mollissima* (Lasch) Fuckel.

Fermentation

A mature slant culture of *Trichopeziza mollissima* SANK 13892 was inoculated into 500-ml Erlenmyer flasks containing 100 ml of a medium composed of glycerol 5.0%, potato 5.0%, yeast extract 0.5% and malt extract 0.5%. The pH was not adjusted and the medium was supplemented with 0.02% of anti-foaming agent, CB-442. The flask was shaken under aerobic condition for 5 days at 23°C and 200 r.p.m. Twenty-five ml of each seed culture thus obtained was transferred into 2-liter Erlenmyer flasks containing 500 ml of the same medium and shaken for 6 days. Next, 1500 ml of the second seed culture was inoculated to a 60-liter tank fermentor containing 30-liter of the same medium and cultivated for 7 days at 23°C. The dissolved oxygen level was maintained around 5 p.p.m. by adjusting the agitation rate. As shown in Fig. 2., scyphostatin production emerged after most of the glycerol was consumed and the growth rate had started to decline.

Fig. 3. Isolation procedure for scyphostatin.



Isolation

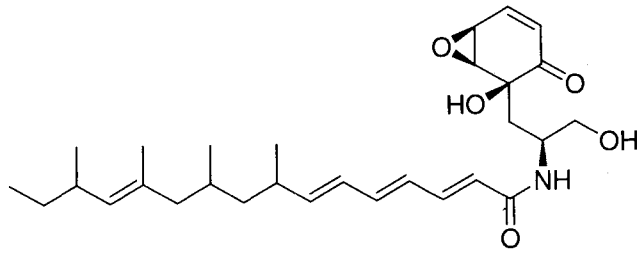
The isolation procedure for scyphostatin is summarized in Fig. 3. Mycelial cake containing predominant amounts of the active principle was extracted with 80% aqueous acetone. The extract was concentrated *in vacuo* to remove acetone and the resulting aqueous solution

Table 1. Physicochemical properties of scyphostatin.

Nature	Pale yellow powder Extremely labile in solid state
Molecular formula	$C_{29}H_{43}NO_5$
HRFABMS	486.3199[M+H]$^+$ 486.3204(calced)
UV λmax(MeOH) nm (ε)	300(41500)
IR vmax(KBr) cm^{-1}	3410, 1655, 1608, 1508
$[\alpha]_D^{25}$	66.4 (*c* 0.09 in MeOH)
Color reaction	Positive to I_2 Negative to ninhydrin

was extracted twice with ethyl acetate. The ethyl acetate extract was concentrated to a reduced volume and applied on a silica gel column equilibrated with ethyl acetate : acetone, 4 : 1. Since scyphostatin is extremely labile in the solid state, great care was taken to keep the solvent from drying up throughout the isolation procedure. The column was eluted with the same solvent and fractions containing scyphostatin were combined. By this stage, the purity of the sample inspected by HPLC detection at U.V. 210 μm was more than 85%, a level sufficient to allow biological evaluation. Further purification was accomplished by HPLC with an ODS column eluted with 80% acetonitrile. Effluent containing scyphostatin was concentrated to remove the organic solvent and the resulting milky aqueous solution was carefully lyophilized. Because of the instability of the compound, lyphilization for prolonged periods should also be avoided. The white powder we obtained from this procedure remained unstable even when stored in a −20°C freezer, and the half-life in such condition was less than 1 month. However, the compound was rather stable in solution and did not decompose when stored in methanol or DMSO solution at −20°C. From the fermentation with the 60-liter tank, 4g of scyphostatin was obtained in pure form.

Fig. 4. Structure of scyphostatin.



Physico-chemical Properties

The physico-chemical properties of scyphostatain are summarized in Table 1. It was obtained as colorless oil which was soluble in organic solvents such as methanol, acetone, chloroform and ethyl acetate, but insoluble in water. It exhibited strong UV absorption at 300 μm, and IR absorption bands at 3,410 cm^{-1} and 1,655 cm^{-1} implied the presence of a hydroxyl group and an amide moiety, respectively. Scyphostatin is not stable in a dry state at room temperature, probably because of the triene moiety. It decomposes to a complex mixture, but it remains stable for months in methanol solution at −20°C. The structure of scyphostatin determined by the spectroscopic method is shown in Fig. 4. The details have been reported in a separate paper[11].

In Vitro Studies on the Inhibitory Modes of Scyphostatin

The preliminary kinetic analyses of scyphostatin were performed using the rat microsome fraction as N-SMase source. The increase in the *Km* value and decrease in the Vmax value shown in the Lineweaver-Burk plots in Fig. 5A indicate that the inhibition was a mixed-type with respect to sphingomyelin. The Dixon plots shown in Fig. 5B did not match a linear fit and the inhibition seemed to synergistically increase as scyphostatin increased. Thus, the inhibition mechanism appeared to be complex. At the next possible opportunity, it will be pertinent to perform kinetic analysis to study the inhibition mechanism using the purified N-SMase.

Antimicrobial Activities

Antimicrobial activities of scyphostatin are shown in Table 2. The compound mainly showed weak activity

Fig. 5. Kinetic analyses of the enzyme inhibition.

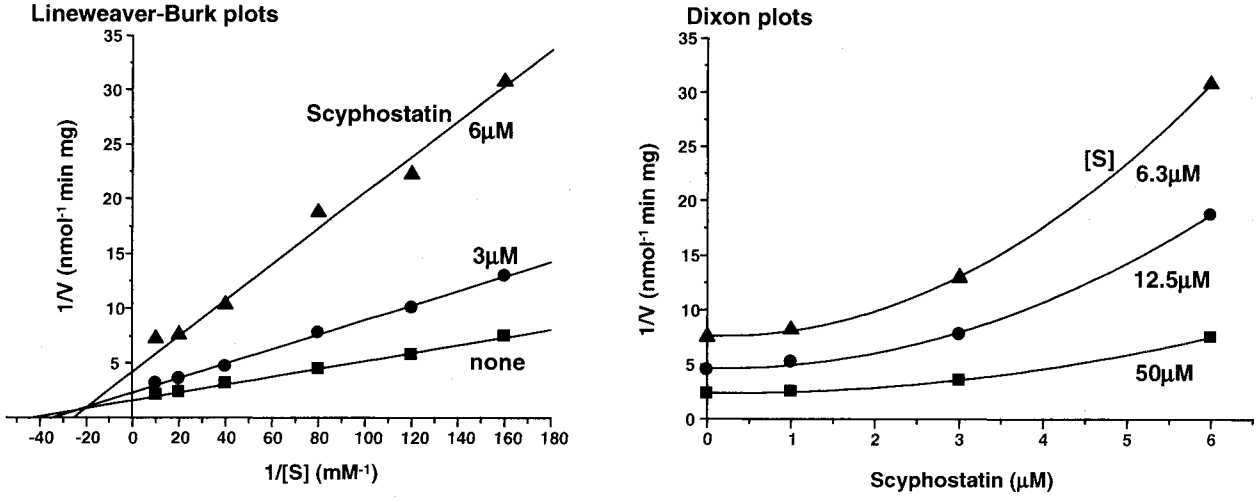


Table 2. Antimicrobial activity of scyphostatin.

Organism	Diameter of Inhibition zone (mm)
Staphylococcus aureus FDA 209P	20
Bacillus subtilis PCI 219	23
Mycobacterium smegmatis ATCC 607	0
Escherichia coli NIHJ	0
Escherichia coli JE 5506	0
Proteus vulgaris OX19	0
Pseudomonas aeruginosa 1046	0
Bacteroides fragilis	10
Candida albicans YU1200	0
Trichophyton mentagrophytes	10
Mycoplasma mycoides subsp.mycoidesPG-1	13

Plate diffusion assay. 40 μg was applied onto 8 μm filter disk.

against Gram-positive bacteria, in contrast to no activity against Gram-negative bacteria. These differences may be due to variable efficiency of scyphostatin penetration into the cell membranes because of the compound's bulky side chain.

References

1) Okazaki, T.; R. M. Bell & Y. A. Hannun: Sphingomyelin turnover induced by vitamin D_3 in HL-60 cells. Role in cell differentiation. J. Biol. Chem. 264: 19076～19080, 1989
2) Okazaki, T.; A. Bielawska, R. M. Bell & Y. A. Hannun: Role of ceramide as a lipid mediator of 1 alpha, 25-dihydroxyvitamin D_3-induced HL-60 cell differentiation. J. Biol. Chem. 265: 15823～15831, 1990
3) Candela, M.; S. C. Barker & L. R. Ballou: Sphingosine synergistically stimulates tumor necrosis factor α-induced prostaglandin E_2 production in human fibroblasts. J. Exp. Med. 174: 1363～1369, 1991
4) Ballou, L. R.; C. P. Chao, M. A. Holness, S. C. Barker & R. Raghow: Interleukin-1-mediated PGE_2 production and sphingomyelin metabolism. Evidence for the regulation of cyclooxygenase gene expression by sphingosine and ceramide. J. Biol. Chem. 267: 20044～20050, 1992
5) Mathias, S.; A. Younes, C. C. Kan, I. Orlow, C. Joseph & R. N. Kolesnick: Activation of the sphingomyelin signaling pathway in intact EL4 cells and in a cell-free system by IL-1β. Science 259: 519～522, 1993
6) Wiegmann, K.; S. Schutze, T. Machleidt, D. Witte & M. Kronke: Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. Cell 78: 1005～1015, 1994
7) Heller, R. A. & M. Kronke: Tumor necrosis factor receptor-mediated signaling pathways. J. Cell. Biol. 126: 5～9, 1994
8) Dobrowsky, R. T.; M. H. Werner, A. M. Castellino, M. V. Chao & Y. A. Hannun: Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. Science 265: 1596～1599, 1994

9) CIFONE, M. G.; R. DE MARIA, P. RONCAIOLI, M. R. RIPPO, M. AZUMA, L. L. LANIER, A. SANTONI & R. TESTI: Apoptotic signaling through CD95 (Fas/Apo-1) activates an acidic sphingomyelinase. J. Exp. Med. 180: 1547~1552, 1994

10) TEPPER, C. G.; S. JAYADEV, B. LIU, A. BIELAWSKA, R. WOLFF, S. YONEHARA, Y. A. HANNUN & M. F. SELDIN: Role for ceramide as an endogenous mediator of Fas-induced cytotoxicity. Proc. Natl. Acad. Sci. USA 92: 8443~8447, 1995

11) TANAKA, M.; F. NARA, K. SUZUKI-KONAGAI, T. HOSOYA & T. OGITA: Structural elucidation of scyphostatin, an inhibitor of membrane-bound neutral sphingomyelinase. J. Am. Chem. Soc. 119: 7871~7872, 1997

12) NARA, F.; M. TANAKA, S. MASUDA-INOUE, Y. YAMASATO, H. DOI-YOSHIOKA, K. SUZUKI-KONAGAI, S. KUMAKURA & T. OGITA: Biological activities of scyphostatin, a neutral sphingomyelinase inhibitor from a discomycete, *Trichopeziza mollissima*. J. Antibiotics 52: 531~535, 1999

13) LISTER, M. D.; C. L. CRAWFORD-REDICK & C. R. LOOMIS: Characterization of the neutral pH-optimum sphingomyelinase from rat brain: inhibition by copper II and ganglioside GM3. Biochim. Biophys. Acta 1165: 314~320, 1993

14) OKAZAKI, T.; A. BIELAWSKA, N. DOMAE, R. M. BELL & Y. A. HANNUN: Characteristics and partial purification of a novel cytosolic, magnesium-independent, neutral sphingomyelinase activated in the early signal transduction of 1 alpha,25-dihydroxyvitamin D_3-induced HL-60 cell differentiation. J. Biol. Chem. 269: 4070~4077, 1994

15) OTANI, Y.: Notes on some cup fungi of the Hyaloscyphaceae collected in Hokkaido, Japan. Trans. Mycol. Soc. Japan 8: 33~42, 1967

16) DENNIS, R. W. G.: A reassessment of *Belonidium* Mont. et Dur. Persoonia 2: 171~191, 1962