Studies on Thermophile Products. IV.[1] Structural Elucidation of Cytotoxic Substance, BS-1, Derived from *Bacillus stearothermophilus*

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A new cytotoxic substance designated as BS-1 was isolated from the autolysate and culture filtrate of *Bacillus stearothermophilus* UK563. On the basis of spectral data, the structure of BS-1 was determined as bis(2-hydroxyethyl) trisulfide and confirmed by direct comparison with the synthetic compound. BS-1 exhibited potent cytotoxicity against leukemia P388-D1, leukemia P388, mastocytoma P815, lymphoma EL4 and lymphoma MOLT4.

Keywords *B. stearothermophilus*; cytotoxicity; structure; bis(2-hydroxyethyl) trisulfide; leukemia; mastocytoma; lymphoma

In connection with our studies on products and constituents of thermophile with potentially useful biological activity, we previously reported the isolation of a new cytotoxic substance designated as BS-1 from the autolysate and culture filtrate of *Bacillus stearothermophilus* UK563.[1b] The present paper describes the structural elucidation and cytotoxicity of BS-1.

Experimental

Materials Hank's balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM) and RPMI1640 medium were obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan) and fetal calf serum (FCS) was obtained from Gibco (Gland Island, NY). Crystal violet, trypan blue and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Co. (St. Louis, MO). All other reagents were of analytical grade. All tumor cell lines listed in Table I were supplied by the Japanese Cancer Research Resources Bank (JCRB).

Analytical Procedures The proton nuclear magnetic resonance (^{1}H-NMR; 600 MHz) and carbon-13 nuclear magnetic resonance (^{13}C-NMR; 150 MHz) spectra were measured with a GE-NMR Omega spectrometer with MeOH-d_4 as a solvent and tetramethylsilane (TMS) as an internal standard. Electron impact mass spectroscopy (EI-MS) and high resolution mass spectroscopy (HR-MS) were obtained with a JEOL JNM-DX300 spectrometer at 30 eV. Ultraviolet (UV) absorption spectra were measured with a MPS-2000 spectrophotometer (Shimadzu Co., Kyoto, Japan).

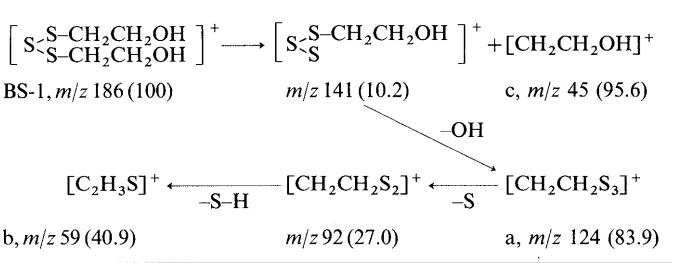
Isolation of BS-1 BS-1 was isolated from the autolysate and culture filtrate of *B. stearothermophilus* UK563 as previously described.[1b]

Synthesis of Bis(2-hydroxyethyl) Trisulfide Bis(2-hydroxyethyl) trisulfide was prepared by the method described by Harpp *et al.*,[2] and its purity was checked with NMR and MS.

Bioassay The cell lines were incubated with the test sample in DMEM or RPMI1640 medium supplemented with 10% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin. After incubation for 48 or 72 h, the cell growth was determined by crystal violet staining,[3] trypan blue dye exclusion[4] or MTT method.[5] Concentration of the sample required for 50% inhibition of cell growth (IC_{50}: μg/ml) was examined by plotting the logarithms of the concentration *versus* the growth rate (percentage of control) of the treated cells. The antimicrobial activity was determined by a serial broth dilution method in bouillon media for gram-positive and gram-negative bacteria and in Sabouraud media for fungus and yeast. Minimum inhibitory concentrations (MIC) were expressed in terms of μg/ml after overnight incubation at 37 °C for bacteria and 48—72 h incubation at 28 °C for fungus and yeast. The antiviral activity was based upon observing the cytopathic effect (CPE) due to viral infection of cultured Vero cells (African green monkey kidney).[6] Vero cells were plated and incubated overnight at 37 °C. Herpes simplex virus (HSV) was added to the host cell medium and was allowed to remain for 1 h at 37 °C. The medium was then removed and replaced with a medium containing the sample or control. After 48 h the plates were examined visually for relative amounts of CPE in the sample and the control.

Results and Discussion

The isolated BS-1 gave a molecular ion peak at *m/z* 185.984 in the HR-MS which indicated the molecular formula $C_4H_{10}O_2^{32}S_3$, as shown in Chart 1. The presence of three sulfur atoms in BS-1 was further substantiated by the two isotopic molecular ion peaks at *m/z* 187.980 ($C_4H_{10}O_2^{32}S_2^{34}S$) and at *m/z* 189.973 ($C_4H_{10}O_2^{32}S^{34}S_2$). The ^{1}H-NMR and the ^{13}C-NMR of BS-1 showed only two signals due to two methylene groups [δ: 3.85, t, J=6.6 Hz and δ_C: 61.03 ppm (–CH_2OH); δ: 3.03, t, J=6.6 Hz and δ_C: 42.00 ppm (–CH_2S–)] to indicate a symmetrical structure containing 1,2,3-trithiane group in BS-1. Further, the UV spectrum of BS-1 (λ_{max} 256 nm) has characteristics of the 1,2,3-trithiane group and the free hydroxy group, respectively.[7] Finally, the structure of BS-1 was determined as bis(2-hydroxyethyl) trisulfide by examination of the EI-MS and the HR-MS as shown in Chart 1. The compound had the molecular formula, $C_4H_{10}O_2S_3$ (*vide supra*) and gave intense and strong fragment peaks at *m/z* 124 (a), 59 (b), and 45 (c). The elemental compositions of each fragment have been reasonably ascertained by HR-MS to show the presence of 1,2,3-trithiane moiety and



			Calcd.	Obsvd.	Relative intensity (%)
$C_4H_{10}O_2^{32}S_3$	(M^+)	(BS-1)	185.984	185.984	100.0
$C_4H_{10}O_2^{32}S_2^{34}S^{a)}$			187.980	187.980	14.6
$C_4H_{10}O_2^{32}S^{34}S_2{}^{a)}$			189.976	189.973	0.7
$C_2H_4^{32}S_3$	(a)		123.948	123.948	83.9
$C_2H_4^{32}S_2^{34}S^{b)}$			125.943	125.943	11.7
$C_2H_3^{32}S$	(b)		58.996	58.996	40.9
$C_2H_3^{34}S^{c)}$			60.991	60.991	25.5
C_2H_5O	(c)		45.034	45.033	95.6

a—c) indicates isotopic peaks in each case.

Chart 1. EI$^-$ and HR-MS Data of BS-1, *m/z* (Rel. Intensity in %)

TABLE I. Cytotoxity of BS-1

	IC_{50} (μg/ml)
P388-D1 mouse leukemia	4
P388 mouse leukemia	3
P815 mouse mastocytoma	0.6
EL4 mouse lymphoma	0.8
BW5147 mouse lymphoma	13
J774-1 mouse leukemia	10
BALB/3T3 mouse embryo	>25
Vero monkey kidney	>25
MOLT4 human lymphoma	3
U937 human lymphoma	12
EJ-1 human bladder carcinoma	13
HLE human hepatoma	13
TYK-nu human ovary carcinoma	10
A549 human lung carcinoma	>25

$-CH_2CH_2OH$ moiety in BS-1. Based on the combined evidence, BS-1 has been assigned as bis(2-hydroxyethyl) trisulfide.

Furthermore, BS-1 isolated from *B. stearothermophilus* was found to be identical with the synthetic compound[2,8] by direct comparison of their ^{1}H-NMR, EI-MS, thin layer chromatographic (TLC) and high performance liquid chromatogaphic (HPLC) data. Synthetic compound BS-1 inhibited the proliferation of P388-D1 at doses ranging from 0.5 to 10 μg/ml as well as the native one, and its 50% inhibitory concentration (IC_{50}) was 4 μg/ml. Further, BS-1 was effective against leukemia P388, mastocytoma P815, lymphoma EL4 and lymphoma MOLT4 at low concentrations. BS-1 has weak cytotoxic activity against lymphoma BW5147, leukemia J774-1 and several human tumor cell lines (Table I). On the other hand, BS-1 did not show antimicrobial activity against *Staphylococcus aureus* 209P, *Bacillus subtilis* ATCC 6633, *Escherichia coli* NIHJ JC-2, *Candida albicans* and *Asperigius fumigatus* (MIC were more than 10 μg/ml), and antiviral activity against HSV (MIC were more than 33 μg/ml).

BS-1 must be a secondary metabolite of the strain UK563, because it was isolated from both autolysate and culture filtrate. Although this compound has already been synthesized as a useful reagent in the manufacture of liquid polymer and in vulcanization technology,[2,8] this report presents the first instance of a natural occurrence and the biological aspect of the compound. Naturally occurring cyclic polysulfide compounds, 1,2,3-trithiane derivatives in tunicate,[9] ascidian,[10] asparagus[11] and alga[7b,12] have been reported to exhibit *in vitro* antimicrobial, antileukemia and cytotoxic properties.[10] Recently, diallyl or alkenyl trisulfide compounds present in garlic and onion oil have been shown to inhibit chemical carcinogenesis.[13] The biological activity of these organosulfur compounds likely originates from unsaturated alkyl groups rather than polysulfide.[13] A novel class of potent antitumor antibiotics, esperamicins[14] and calichemicins[15] containing bicyclo[7.3.1]diynene and methyl trisulfide has been discovered in bacteria. Interestingly, the enediyne system can be readily triggered to aromatize *via* a free radical intermediate by cleavage at the methyl trisulfide moiety. It has been reported that bis(2-hydroxyethyl) trisulfide in aqueous solution can be cleaved to produce (2-hydroxyethyl)perthiyl (RSS·) radicals and 2-hydroxyethanethiol by the reducing radicals; such as e_{aq}^- and $\cdot CO_2^-$.[8a] The cytotoxicity of BS-1 may be due to the radical production, but it requires confirmation.

Since BS-1 consists chemically of a simple structure, it seems to be a degradated metabolite. Several peaks with cytotoxic activity, which were less active than that of BS-1, existed in HPLC on octadecyl silica (ODS). It is possible to consider that the activity originates from BS-1 analogues in strain UK563. Anyway, it is interesting to clarify the physiological significance of trisulfide compound in thermophile.

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