TAN-999 AND TAN-1030A, NEW INDOLOCARBAZOLE ALKALOIDS WITH MACROPHAGE-ACTIVATING PROPERTIES

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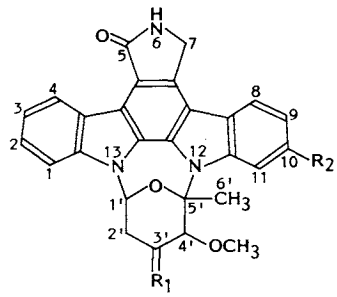
Two new indolocarbazole alkaloids, TAN-999 and TAN-1030A, were isolated from culture broths of *Nocardiopsis dassonvillei* C-71425 and *Streptomyces* sp. C-71799, respectively. Their structures were elucidated on the basis of their reactions, spectroscopic analyses and in particular, comparison of spectral data with that of staurosporine.

These metabolites induced spreading of a murine macrophage cell line, Mm 1. They also augmented the phagocytic activity, Fcγ receptor expression and β-glucuronidase activity of murine macrophage cell lines, Mm 1 and J774A.1. When proteose-peptone elicited peritoneal macrophages from mice were incubated with these metabolites for 2 days, the phagocytosis-dependent respiratory burst of these cells was enhanced. Similar enhancement was also observed when the peritoneal macrophages in mice were modulated by intraperitoneal administration of these metabolites. These results reveal that TAN-999 and TAN-1030A can activate macrophage functions in mice.

Macrophages act as regulatory and effector cells in the host defense system. They are phagocytic and are potentially destructive to invading microorganisms and neoplastic cells.[1,2] Thus, the activation of macrophage function by natural or synthetic substances is expected to be applicable for therapy of microbial infection and cancer.

In our search for immunomodulators of microbial origin, three metabolites having macrophage-activating properties were found in culture broths of two actinomycetes isolated from soil samples. One is a metabolite of *Nocardiopsis dassonvillei* C-71425 and is designated TAN-999. The other two, TAN-1030A and staurosporine,[3,4] are metabolites of *Streptomyces* sp. C-71799. TAN-999 and TAN-1030A are new alkaloids belonging to the staurosporine family (Fig. 1).

Fig. 1. Structures of TAN-999 and TAN-1030A.



TAN-999 (**1**) R_1 = NHCH$_3$, H R_2 = OCH$_3$
TAN-1030A (**2**) R_1 = =NOH R_2 = H
Staurosporine R_1 = NHCH$_3$, H R_2 = H

Materials and Methods

Taxonomic Studies

Culture characterization was carried out following the International Streptomyces Project procedure.[5] The color recorded for the mature culture was described according to the Color Harmony Manual.[6] Cell analysis was performed by the method of HASEGAWA *et al.*[7] Scanning electron microscopy was conducted by the method of TANIDA *et al.*[8]

Fermentation

A loopful of strain C-71425 or C-71799 grown on mature slant culture was inoculated into a 2-liter

Sakaguchi flask containing 500 ml of a sterile seed medium. The flask was shaken on a reciprocal shaker at 28°C for 48 hours. The seed medium consisted of glucose 2%, corn steep liquor 1%, soybean flour 1%, peptone 0.5%, NaCl 0.3% and $CaCO_3$ 0.5%. Five hundred ml of the seed culture were transferred to a 50-liter fermenter containing 30 liters of the same medium, and the fermentation was carried out at 28°C for 48 hours with aeration of 30 liters/minute and agitation of 280 rpm. Six liters of the seed culture were transferred to a 200-liter fermenter containing 120 liters of a production medium consisting of glucose 0.5%, dextrin 5%, soybean flour 3.5% and $CaCO_3$ 0.7%. The fermentation was carried out at 28°C with aeration of 120 liters/minute and agitation of 120 rpm.

Isolation of TAN-999 (**1**)

The culture broth (110 liters) was adjusted to pH 9, EtOAc (110 liters) was added, and the mixture was stirred for 1 hour. After filtration using Hyflo-Super Cel, the aqueous layer was discarded. The extract was washed with water (25 liters) and concentrated. The residue was chromatographed on a column of silica gel (50 g) eluting with EtOAc - MeOH (9 : 1). The active fractions detected by morphological changes in Mm 1 were left standing at 4°C to afford pale yellow crystals of **1** (1.1 g).

Isolation of TAN-1030A (**2**)

The culture broth (35 liters) was filtered using Hyflo-Super Cel and the mycelium was extracted with acetone (15 liters). The extract was concentrated to remove the acetone, and the concentrate (6 liters) was extracted with EtOAc (3 liters × 2) after addition of NaCl (500 g). The extract was washed with water (1.5 liters) and concentrated. The residue was chromatographed on a column of silica gel (125 g) eluting with EtOAc - MeOH (9 : 1). The active fractions were concentrated and again chromatographed on silica gel (50 g), this time eluting with EtOAc. The active fractions were concentrated to give a crude powder of **2** (1.07 g). The crude powder was purified by silica gel column chromatography (50 g) eluting with $CHCl_3$ - MeOH (49 : 1). The bio-active fractions were concentrated and crystallized from $CHCl_3$ - MeOH to give colorless crystals of **2** (110 mg).

Hydrogenation of **2** to **3**

Compound **2** (72 mg) in 60% aqueous acetic acid was hydrogenated over Pt-black (25 mg) at room temperature. The catalyst was filtered off and the filtrate was evaporated. EtOAc (30 ml) was added to the residue, and the mixture was extracted with 0.05 N HCl (25 ml × 4). The aqueous layers were combined, adjusted to pH 9.4 and extracted with EtOAc (50 ml × 3). The extract was washed with a saturated aqueous solution of NaCl, dried over Na_2SO_4 and concentrated. Adding Et_2O to the residue gave a powder of **3** (53 mg).

$[\alpha]_D$ +90.4° (*c* 0.28, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 9.44 (1H, d, J=8.2 Hz), 7.97 (1H, d, J=8.6 Hz), 7.89 (1H, d, J=7.3 Hz), 7.50 (1H, t), 7.44 (1H, t), 7.38 (1H, t), 7.33 (1H, t), 7.29 (1H, d, J=7.9 Hz), 6.58 (1H, d, J=4.0 Hz), 6.33 (1H, s), 5.02 (2H, ABq), 3.75 (2H, m), 3.46 (3H, s), 2.60 (2H, m), 2.33 (3H, s): Secondary ion (SI)-MS m/z 453 $(M+H)^+$

Anal Calcd for $C_{27}H_{24}N_4O_3 \cdot \frac{1}{2}H_2O$: C 70.27, H 5.46, N 12.14.
Found: C 69.95, H 5.26, N 11.93.

Acetylation of **3** to **4**

Ac_2O (0.3 ml) was added to a solution of **3** (30 mg) in pyridine (0.6 ml), and the mixture was left standing at room temperature for 3 hours. The reaction mixture was diluted with EtOAc (20 ml) and washed with 0.05 N HCl (20 ml × 2) and a saturated aqueous solution of NaCl successively. The solution was evaporated to dryness and adding Et_2O to the residue gave a powder of **4** (28 mg).

^1H NMR ($CDCl_3$) δ 9.40 (1H, d, J=7.9 Hz), 7.97 (1H, d, J=9.0 Hz), 7.94 (1H, d, J=8.5 Hz), 7.51 (1H, t), 7.48 (1H, t), 7.39 (2H, t), 7.28 (1H, d, J=9.4 Hz), 6.71 (1H, s), 6.61 (1H, d, J=5.2 Hz), 5.16 (1H, d, J=6.2 Hz), 5.04 (2H, s, like), 4.60 (1H, m), 3.91 (1H, d, J=4.4 Hz), 3.40 (3H, s), 3.09 (1H, dd, J=3.1 and 15.0 Hz), 2.53 (1H, m), 2.39 (3H, s), 0.81 (3H, s): Electron impact (EI)-MS m/z 494 M^+.

Anal Calcd for $C_{29}H_{26}N_4O_4 \cdot \frac{1}{2}H_2O$: C 69.17, H 5.40, N 11.13.
Found: C 68.74, H 5.21, N 10.46.

Murine Macrophage Cell Lines and Media

The cell line, Mm 1[9] was kindly donated by Dr. MAEDA, Chest Disease Research Institute, University of Kyoto. Mm 1 was usually cultured with GIT medium.[10] To determine the expression of Fcγ receptors, Mm 1 was cultured with EAGLE's minimum essential medium (MEM) containing 10% fetal calf serum (FCS). The cell line, J774A.1[11,12] was purchased from the American Type Culture Collection (ATCC). J774A.1 was cultured with RPMI-1640 medium containing 10% FCS. They were incubated at 37°C in an atmosphere of 5% CO_2 in air. Growth of these cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay.[13]

Scanning Electron Microscopy

Mm 1 cells were cultured with each metabolite on coverslips for 2 days. Then, monolayers of the cells on the coverslips were fixed with 2% glutaraldehyde in phosphate-buffered saline (PBS; Ca^{++} and Mg^{++} free) for 1 hour and then in 1% osmium tetroxide in water for 1 hour. The fixed Mm 1 cells were dehydrated in graded ethanol, critical-point dried in liquid CO_2, and coated with gold. The specimen was then viewed with a scanning electron microscope (Hitachi model S-570).

Assay for Phagocytosis

Mm 1 or J774A.1 cells, 2.0×10^5 cells, were cultured in a plastic dish (3.5 cm i.d., Corning Co., U.S.A.) with each metabolite for 2 days. Then, 1.0×10^7 fluorescein isothiocyanate (FITC)-labeled latex particles (1.74 μm i.d., Polyscience Inc., U.S.A.) were added to the culture. After incubating 45 minutes, the cells were washed 3 times with HANKS' balanced salt solution (HBSS). The number of cells which ingested more than 10 particles was counted as the number of phagocytic cells under an inverted fluorescence microscope (Olympus IMT-2; magnification, ×400).

Assay for Fcγ Receptor

Fcγ receptors on the surface of Mm 1 or J774A.1 cells were detected by the EA rosette assay.[14] Sheep red blood cells (SRBC) coated with anti-SRBC IgG (EAγ complexes) were prepared by incubating 4 volumes of SRBC in PBS at 5.0×10^8/ml with one volume of a 1/10,000 dilution of the purified IgG fraction of rabbit anti-SRBC at 37°C for 30 minutes. Mm 1 or J774A.1 was cultured with each metabolite for 2 days and was adjusted to 2.0×10^7 cells/ml, and the suspension was incubated with the EAγ complexes (2.0×10^7/ml) at 0°C for 90 minutes. Cells in the microscopic fields with five or more adherent complexes were counted as Fcγ-receptor mediated rosettes.

Assay for β-Glucuronidase Activity

β-Glucuronidase activity was determined by hydrolysis of phenolphthalein-mono-β-glucuronic acid at pH 4.5.[15] Two-day cultures of Mm 1 (1.0×10^6) in a tissue culture dish (10 cm i.d., Corning Co., U.S.A.) with each metabolite were washed with HBSS, and the adherent cells were lysed with Triton X-100. The cell lysate was centrifuged at $2,500 \times g$ for 10 minutes, and the supernatant was used as the enzyme source. The amount of protein in the supernatant was estimated with the Bio-Rad Protein Assay Kit.

Macrophage Experiments in Mice

Female C57BL/6 CrSlc inbred mice 7 weeks of age were purchased from Shizuoka Laboratory Animal Center, Japan. One ml of 10% Proteose-peptone (PP) in saline was injected ip. The peritoneal exudate cells (PECs) were harvested 96 hours after injection by rinsing the peritoneal cavity with cold EAGLE's MEM containing 10% FCS. The PEC suspension was transferred to a borosilicate glass culture tube (6 × 50 mm, Kimble, U.S.A.) and incubated for 2 hours at 37°C in an atmosphere of 5% CO_2 in air. The PECs were then washed twice with pre-warmed medium to remove non-adherent cells (2.0×10^5 adherent cells/tube). The adherent macrophages were incubated with each metabolite for 2 days under 5% CO_2. The cells were then washed twice with HBSS.

In vivo modulation of peritoneal macrophages with these metabolites was carried out as follows. One ml of 10% PP in saline was administered ip and each metabolite was injected ip 72 hours later. Twenty-four hours after the second injection, PECs were harvested. Three mice were used for each dose of the metabolites, and the PECs from the mice receiving the same dose were mixed together. The PEC suspension

was transferred to a borosilicate glass culture tube and incubated for 1 hour under 5% CO_2. The PECs were washed twice with pre-warmed medium to remove non-adherent cells (1×10^5 adherent cells/tube).

Respiratory Burst of Peritoneal Macrophages

The phagocytosis-dependent respiratory burst in mouse peritoneal macrophages was evaluated by measuring the chemiluminescence response using luminol. The standard reaction mixture contained the peritoneal macrophages described above, 5×10^{-7} M luminol and 1 mg/ml opsonized zymosan (OPZ) in a total volume of 100 μl in a glass culture tube. The reaction was initiated by the addition of the OPZ, and the chemiluminescence was measured for 8 minutes with a luminometer (Picolite model 6106, Packard, U.S.A.)

Chemicals

EAGLE'S MEM, RPMI-1640 medium, HBSS and FCS were obtained from Whittaker M. A. Bioproducts, Inc., U.S.A. GIT medium was purchased from Nippon Seiyaku Co., Japan. The serum was inactivated by heating at 56°C for 30 minutes prior to use. The SRBC and anti-SRBC antibody were purchased from Cooper Biomedical, Inc., U.S.A. Other materials and their sources were as follows: Phenolphthalein-mono-β-glucuronic acid (Sigma Chemical Co., U.S.A.); luminol (Wako Chemical Co., Japan); OPZ (ZAP, Packard Instrument Co. Inc., U.S.A.); PP (Difco Labs., U.S.A.).

Results

Taxonomy of the Producing Organism

Strain C-71425 was isolated from a soil sample collected in Tottori Prefecture, Japan. Cell analysis of the strain showed the presence of *meso*-diaminopimelic acid. Galactose and ribose were present but madurose, xylose, galactose and arabinose were not present in whole-cell hydrolysate. Accordingly, the cell wall type of strain C-71425 is chemotype III/C. Each spore chain of this strain consists of about 20 spores. The spores are cylindrical and have a smooth surface (Plate 1A). The vegetative mycelia of the strain grown in liquid medium divide into rod-shaped, irregular and branching fragments. Sporangia and flagellated spores are not observed. These characteristics of strain C-71425 indicate that it belongs to the genus *Nocardiopsis*. The cultural and physiological characteristics of the strain are shown in Table 1. In

Plate 1. Scanning electron micrographs of the spores of strains C-71425 (A) and C-71799 (B).

The organisms were cultured on ISP-2 agar for 1 week at 28°C (bar, 1 μm).

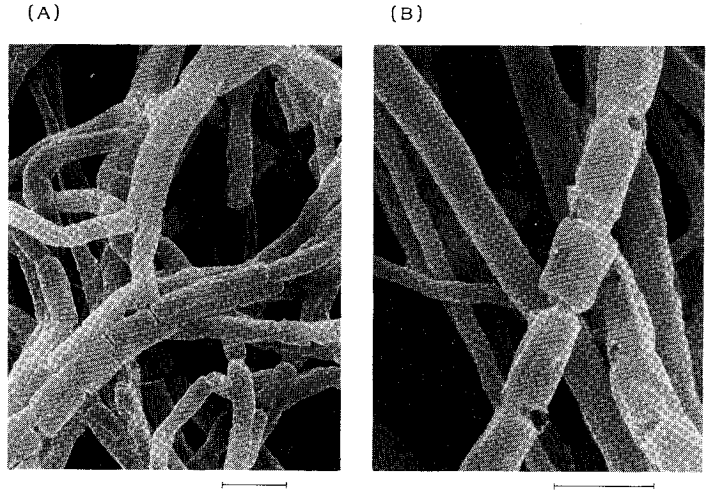


Table 1. Cultural and physiological characteristics of strains C-71425 and C-71799.

	C-71425	C-71799
Cultural characteristics		
Yeast extract-malt extract agar (ISP-2)	G: Good, 2ea (Lt wheat) A: Moderate, white P: None	Good, 3ie (camel) Good, white Light brown
Oatmeal agar (ISP-3)	G: Good, 2ca (Lt ivory) A: Moderate, white P: None	Good, 2ca (Lt ivory) Abundant, white None
Inorganic salts-starch agar (ISP-4)	G: Good, 2ga (maize) A: Moderate, white P: None	Good, 2ca (Lt ivory) Abundant, white None
Glycerol-asparagine agar (ISP-5)	G: Good, 2ga (maize) A: Moderate, white P: None	Good, 2ig (olive gray) Abundant, 13dc (peal gray) None
Peptone-yeast extract-iron agar (ISP-6)	G: Good, 2ic (honey gold) A: Poor P: None	Poor, 2gc (bamboo) None None
Tyrosine agar (ISP-7)	G: Good, 2ea (Lt wheat) A: Moderate, white P: None	Good, 2ie (brown) Abundant, white None
Physiological characteristics		
Temperature range for growth	7~34°C	13~42°C
Nitrate reduction	+	+
Starch hydrolysis	+	+
Milk peptonization	+	+
Milk coagulation	−	−
Gelatin liquefaction	−	−
Utilization of carbon sources		
Positive	Inositol, mannitol, xylose, arabinose, glucose, fructose, rhamnose, sucrose, raffinose	Inositol, mannitol, xylose[a], arabinose[a], glucose[a], rhamnose[a], sucrose, raffinose[a]
Negative	Cellulose	Fructose, cellulose

[a] Weakly positive.
G: Growth, A: aerial mass color, P: soluble pigment.

comparison with known species in the genus *Nocardiopsis*, strain C-71425 has characteristics similar to *N. dassonvillei*[16] and is thought to be the same species. Therefore, the strain is designated *N. dassonvillei* C-71425.

Strain C-71799 was isolated from a soil sample collected in Okinawa Prefecture, Japan. Cell analysis of the strain showed the presence of LL-diaminopimelic acid, and it was classified as chemotype I/NC. The strain forms straight and flexuous mycelia and forms spore chains which consist of about 20 spores each. The spores are cylindrical and have a smooth surface (Plate 1B). The color of the mature sporulated aerial mycelium is in the Gray series. These characteristics of strain C-71799 indicate that it belongs to the genus *Streptomyces*. The cultural and physiological characteristics of strain C-71799 are shown in Table 1. Further studies are needed to determine the taxonomical niche of strain C-71799.

Production and Isolation

TAN-999 was produced by cultivating strain C-71425. The amount of TAN-999 in the culture at 90 hours reached about 50 μg/ml. TAN-1030A was produced by cultivating strain C-71799, and the amount of the metabolite reached a maximum (15 μg/ml) at about 140 hours. Staurosporine was also produced

Table 2. Physico-chemical properties of TAN-999 and TAN-1030A.

Property	TAN-999	TAN-1030A
Appearance	Pale yellow crystals	Colorless crystals
MP (°C, dec)	221	290~295
$[\alpha]_D^{24}$ (*c* 0.5, DMF)	+42°	0°
MS (*m/z*)	496[a] M^+	467[b] $(M+H)^+$
Molecular formula	$C_{29}H_{28}N_4O_4$ ($\frac{1}{2}H_2O$)	$C_{27}H_{22}N_4O_4(H_2O)$
Anal	Calcd Found	Calcd Found
C	68.90, 68.59	66.93, 67.23
H	5.78, 5.64	4.99, 5.07
N	11.08, 10.86	11.56, 11.70
UV λ_{max}^{MeOH} nm (ε)	245 (31,200), 296 (60,300), 341 (19,500), 352 (sh, 16,200), 368 (11,000)	233 (29,400), 244 (sh, 28,000), 263 (sh, 31,300), 275 (sh, 42,000), 289 (71,000), 319 (sh, 13,400), 333 (17,700), 352 (12,100), 369 (13,400)
IR ν_{max} (KBr) cm^{-1}	3430, 2950, 1680, 1590, 1460, 1420, 1360, 1320, 1290, 1260, 1210, 1120, 1040, 840, 800, 760	3430, 3160, 2940, 2860, 1680, 1590, 1460, 1400, 1350, 1320, 1290, 1260, 1230, 1120, 1020, 950, 920, 840, 820, 780, 740
Color reaction		
Positive	Ninhydrin, Barton, Ehrlich, Dragendorff	Barton, Ehrlich
Negative	$FeCl_3$	Ninhydrin, Dragendorff

[a] EI-MS, Jeol TMS-DX-303. [b] SI-MS, Hitachi M-80A.

Table 3. Mobilities of TAN-999, TAN-1030A and staurosporine on TLC and HPLC.

Mobilities	TAN-999	TAN-1030A	Staurosporine
Rf value of TLC[a] (1)	0.11	0.61	0.11
(2)	0.20	0.14	0.20
Retention time of HPLC (minutes)[b]	6.0	4.7	6.3

[a] Adsorbent: Kieselgel 60 F_{254} (E. Merck AG.). Solvent system: (1) EtOAc-MeOH (96:4), (2) $CHCl_3$-MeOH (96:4). Detection: UV lamp at 254 nm.

[b] Equipment: Model 6000A/660/440 (Waters assoc.). Column: ODS, YMC-Pack A-312 (Yamamura Chem. Lab.). Mobile phase: 55% CH_3CN-0.01 M phosphate buffer (pH 6.3). Detection: UV absorbance at 254 nm. Flow rate: 2 ml/minute.

by this strain.

TAN-999 (**1**) and TAN-1030A (**2**) are fat-soluble compounds. Therefore, purification was carried out by extraction with EtOAc, column chromatography using silica gel and crystallization. The active fractions were detected by morphological changes in Mm 1 cells and TLC. The physico-chemical properties and mobilities of **1** and **2** are summarized in Tables 2 and 3. The molecular formulae of **1** and **2** were determined on the basis of the elemental analyses, the molecular ion peaks in the mass spectra and the carbon numbers in the ^{13}C NMR spectra. The UV absorption spectra suggested **1** and **2** have a chromophore similar to the indolocarbazole moiety in staurosporine.

Structural Elucidation

^1H and ^{13}C NMR data are summarized in Tables 4 and 5. The signals were assigned based on 2D NMR experiments, such as ^1H-^1H correlation spectroscopy (COSY), ^1H-^{13}C COSY, long range ^1H-^{13}C COSY (Fig. 2) and nuclear Overhauser and exchange spectroscopy (NOESY). When the ^1H NMR spectrum of **1** was compared with that of staurosporine, an aromatic proton found in staurosporine was lacking and a methoxy signal at 3.95 ppm was observed in **1**. A long range coupling was found between the methoxy protons and C-10 (Fig. 3). Further observation of NOE between the methoxy protons and 9-H

Table 4. ^{1}H NMR spectral data of TAN-999 and TAN-1030A (300 MHz).[a]

Position	TAN-999[b]	TAN-1030A[c]	Staurosporine[c]
1	7.27 (d, J=8.0)	7.70 (d, J=8.2)	7.56 (d, J=8.1)
2	7.46 (t)	7.50 (t)	7.45 (t)
3	7.35 (t)	7.32[d] (t)	7.27[d] (t)
4	9.40 (d, J=7.8)	9.31 (d, J=7.8)	9.30 (d, J=7.9)
6	6.28 (s)	8.58 (s)	8.53 (s)
7	4.96 (ABq, J=17.0)	4.96 (s like)	4.96 (s like)
8	7.75 (d, J=8.5)	7.98 (d, J=8.0)	7.96 (d, J=7.3)
9	6.96 (dd, J=2.0, 8.5)	7.32[d] (t)	7.27[d] (t)
10		7.44 (t)	7.41 (t)
11	7.46 (d, J=2.0)	8.01 (d, J=9.1)	7.98 (d, J=8.4)
1′	6.53 (d, J=5.6)	7.04 (d, J=5.2)	6.68 (s like)
2′	2.38 (m)	3.01 (dd, J=5.2, 14.0)	2.49 (m)
	2.75 (dd, J=4.0, 14.7)	3.63 (d, J=14.0)	
3′	3.35 (m)		3.24 (m)
4′	3.87 (d, J=3.5)	4.73 (s)	4.04 (d, J=3.2)
6′	2.33 (s)	2.47 (s)	2.29 (s)
3′-NCH_3	1.57 (s)		1.44 (s)
4′-OCH_3	3.44 (s)	3.43 (s)	3.32 (s)
10-OCH_3	3.95 (s)		
3′-NOH		10.45 (s)	

[a] The δ values were recorded in ppm downfield from TMS using a Brucker AC-300. Coupling constants in Hz are given in parentheses. [b] In $CDCl_3$. [c] In DMSO-d_6. [d] These signals overlapped.

Table 5. ^{13}C NMR spectral data of TAN-999 and TAN-1030A (75 MHz).[a]

Position	TAN-999[b]	TAN-1030A[c]	Staurosporine[c]	Position	TAN-999[b]	TAN-1030A[c]	Staurosporine[c]
1	106.89 d	108.88 d	108.18 d	11	100.72 d	115.58 d	115.12 d
2	124.88 d	125.21 d	124.76 d	11a	141.04 s	139.82 s	139.34 s
3	119.65 d	119.52 d	118.88 d	12a	130.63 s	128.03 s	129.90 s
4	126.50 d	125.61 d	125.51 d	12b	127.09 s	124.60 s	126.59 s
4a	123.55 s	122.85 s	122.42 s	13a	136.67 s	136.01 s	136.25 s
4b	114.88 s	114.98 s	114.02 s	1′	80.12 d	82.17 d	79.82 d
4c	118.58 s	119.15 s	118.71 s	2′	30.29 t	29.71 t	29.27 t
5	173.81 s	171.77 s	172.18 s	3′	50.36 d	145.12 s	49.98 d
7	45.92 t	45.30 t	45.33 t	4′	84.26 d	83.57 d	82.70 d
7a	131.48 s	132.25 s	131.91 s	5′	91.06 s	96.16 s	91.00 s
7b	114.24 s	114.02 s	113.38 s	6′	29.69 q	28.59 q	29.63 q
7c	118.92 s	123.79 s	123.79 s	3′-NCH_3	33.38 q		33.19 q
8	120.94 d	120.72 d	120.70 d	4′-OCH_3	57.24 q	58.29 q	57.14 q
9	107.94 d	120.13 d	119.63 d	10-OCH_3	55.78 q		
10	157.49 s	124.64 d	124.19 d				

[a] The δ values were recorded in ppm downfield from TMS using a Brucker AC-300. [b] In $CDCl_3$. [c] In DMSO-d_6.

or 11-H (Fig. 3) indicated that the methoxy group is bound to the 10-position. Thus **1** was determined to be 10-methoxy staurosporine.

The difference in **2** and staurosporine was found at the 3′-position. Comparing the ^{13}C NMR spectra, a methine signal at 49.98 ppm in staurosporine collapsed to a quaternary carbon at 145.12 ppm in **2**. In addition the *N*-methyl signal at 33.19 ppm found in staurosporine was absent. In the ^{1}H NMR spectra, instead of the *N*-methyl signal at 1.44 ppm observed in staurosporine a proton signal exchangeable with D_2O was observed at 10.45 ppm in **2**. A long range coupling was found between the D_2O exchangeable proton and C-3′ (Fig. 3). These data suggested **2** has an oxime function at the 3′-position. Upon hydrogenation using Pt-black, compound **2** gave an amino derivative (**3**) which was acetylated with

Fig. 2. Long range ^{1}H-^{13}C COSY spectrum of TAN-999 ($CDCl_3$).

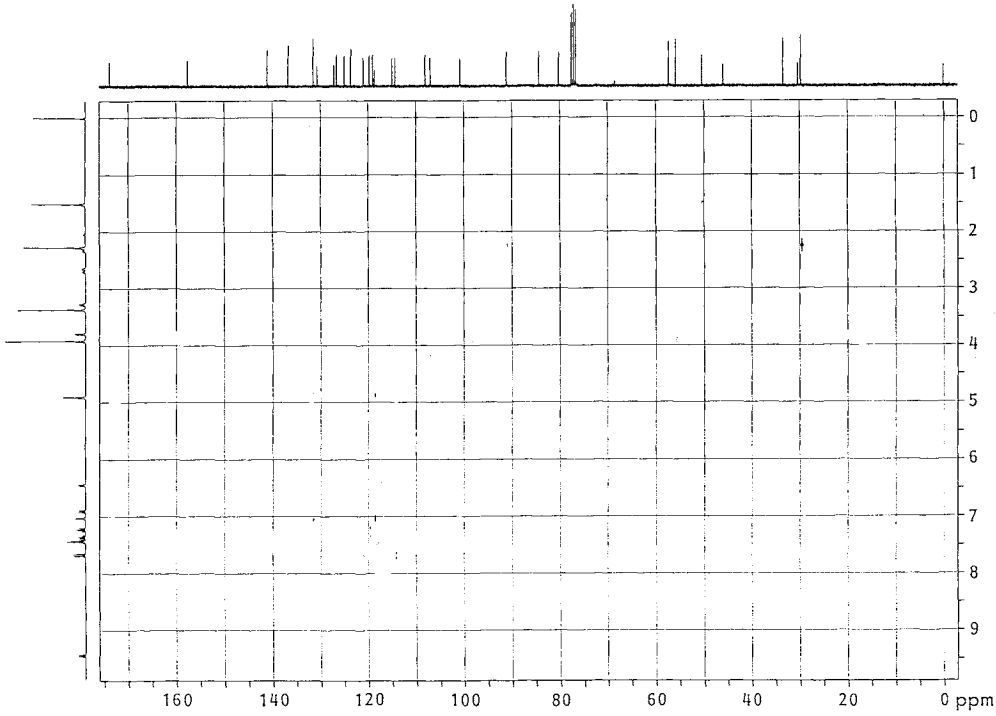
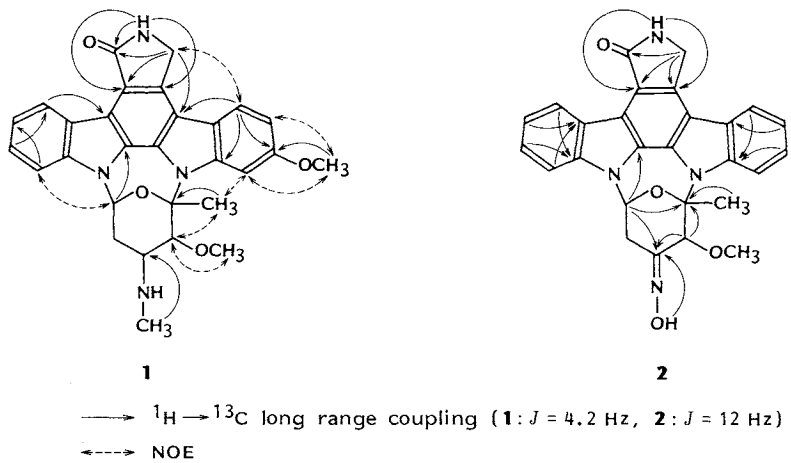


Fig. 3. Long range ^{1}H-^{13}C COSY and NOESY experiments of TAN-999(**1**) and TAN-1030A(**2**).



Ac_2O-pyridine to compound **4** (Fig. 4). These chemical reactions confirmed the presence of an oxime function in **2**. Thus the chemical structure of **2** was determined as shown in Fig. 1.

Biological Activity

TAN-999 induced spreading of Mm 1 cells. When TAN-999 was added to a culture of Mm 1 at the concentrations of 1.3 to 78 ng/ml, more than 50% of the cells drastically elongated. In the control culture without the metabolite, less than 5% of the cells elongated. TAN-1030A also induced spreading of Mm 1 cells at concentrations of 78 to 625 ng/ml (Fig. 5). The elongated cells, like activated macrophages, formed

Plate 2. Scanning electron micrographs of Mm 1 cells.

(A) Drug free, (B) TAN-999 at 2 ng/ml, (C) magnification of (B). Mm 1 cells were cultured with the metabolite for 2 days at 37°C (bar, 2 μm).

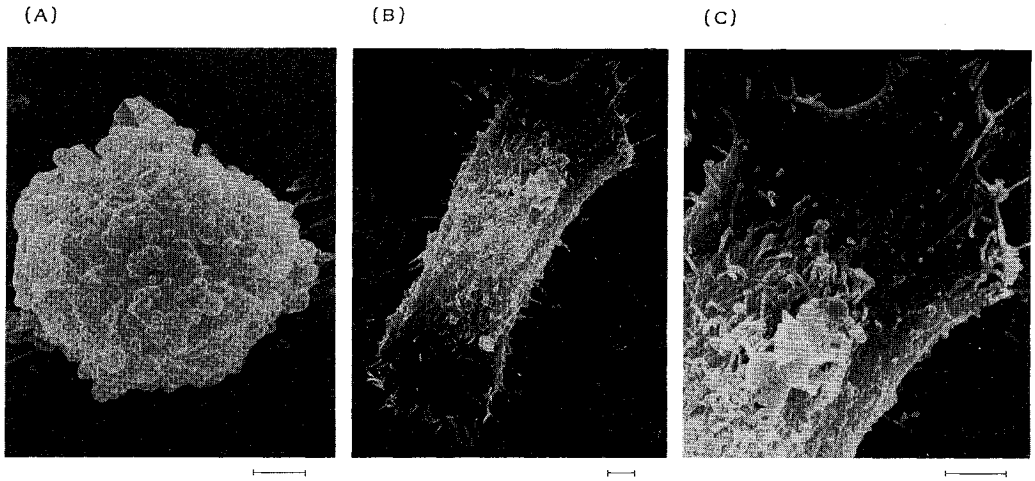
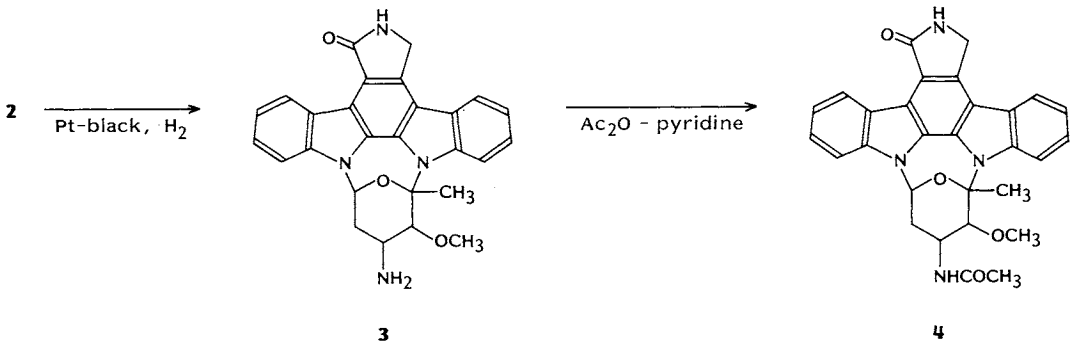


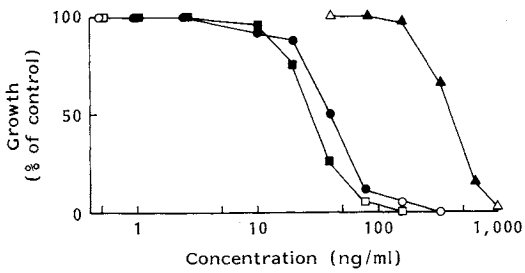
Fig. 4. Chemical reactions of TAN-1030A (**2**).



thin lamellipodia and numerous microvilli and were clearly different from most of the cells in the control culture having a round shape with many blebs (Plate 2). Staurosporine induced spreading of the cells at 1.3 to 39 ng/ml, but it was more toxic to Mm 1 than TAN-999. In addition, compounds **3** and **4** (Fig. 4) induced similar changes in morphology of Mm 1 at 1.3 and 50 ng/ml, respectively (data not shown).

Phagocytosis of exogenous particles is an essential function of macrophages. To clarify the effect of these metabolites on the phagocytic activity of macrophages, further studies using murine macrophage cell lines were carried out. When TAN-999 at 5 ng/ml was added to a culture of Mm 1, the number of phagocytic cells ingesting latex

Fig. 5. Effect of TAN-999 and TAN-1030A on growth and morphology of Mm 1 cells.



The growth of Mm 1 cells was evaluated by MTT assay. Changes in morphology of Mm 1 cells were determined by microscopy with an inverted light microscope. Circles, TAN-999; triangles, TAN-1030A; squares, staurosporine; closed symbols, more than 50% of Mm 1 cells in a microscopic field elongated.

Table 6. Effect of TAN-999 and TAN-1030A on the phagocytic activity, Fcγ receptor expression and β-glucuronidase activity of murine macrophage cell lines, Mm 1 and J774A.1.

Metabolite	Conc (ng/ml)	Mm 1			J774A.1	
		Phago.[a]	FcR[b]	β-Glu.[c]	Phago.[a]	FcR[b]
TAN-999	0	1.0	1.0	1.0	1.0	1.0
	1	2.0	1.6	1.4	10.7	1.1
	2	4.8	1.7	1.3	11.5	1.5
	5	9.6	3.9	1.1	11.7	1.7
TAN-1030A	50	nd	4.7	1.1	2.4	1.3
	100	nd	3.3	1.3	4.8	1.3
	200	nd	1.7	1.2	9.0	1.1
Staurosporine	1	1.8	1.5	1.1	12.0	1.7
	2	9.9	1.6	1.1	11.8	1.3

Mm 1 and J774A.1 cells were cultured with these metabolites for 2 days at 37°C under 5% CO_2.

[a] Ratio of phagocytic positive cells in comparison with the control culture as 1.0. Phagocytic positive cells in the control culture of Mm 1 and J774A.1 were 1.6 and 8.2%, respectively.

[b] Ratio of EA-rosette positive cells in comparison with the control culture as 1.0. EA-rosette positive cells in the control culture of Mm 1 and J774A.1 were 5.4 and 24.0%, respectively.

[c] Ratio of β-glucuronidase activities of modulated cells in comparison with the control cells as 1.0.

nd: Not determined.

Table 7. Phagocytosis-dependent respiratory burst in mouse peritoneal macrophages incubated with TAN-999 and TAN-1030A.

Metabolite	Concentration (ng/ml)	Relative chemiluminescence[a]
TAN-999	0.01	1.27
	0.1	1.93
	1	1.58
TAN-1030A	1	1.74
	10	1.17
	100	1.03
Staurosporine	0.01	1.69
	0.1	1.32
	1	1.09

PECs were harvested 96 hours after administration of PP. The adherent macrophages were obtained by removing non-adherent cells from the PECs and were incubated with these metabolites for 2 days under 5% CO_2. The phagocytosis-dependent respiratory burst was induced by adding opsonized zymosan and was evaluated by measuring the chemiluminescence response with luminol. Each experiment was performed in duplicate and the photon counts were averaged.

[a] Chemiluminescence (photon counts) for 8 minutes of the drug-free control was 14,900.

Table 8. Phagocytosis-dependent respiratory burst in peritoneal macrophages from mice administered TAN-999 and TAN-1030A.

Metabolite	Dose (μg/kg)	Relative chemiluminescence[a]
TAN-999	0.005	1.32
	0.05	1.94
	0.5	1.08
TAN-1030A	0.05	1.12
	0.5	1.59
	5	1.39
Staurosporine	0.005	1.37
	0.05	1.18
	0.5	1.00

Mice received these metabolites 24 hours before PECs were harvested. The respiratory burst was measured by the method described in the footnote of Table 7. Each experiment was performed in duplicate, and the photon counts were averaged.

[a] Chemiluminescence (photon counts) for 8 minutes of the control was 21,900.

particles reached about 9 times that in the control culture (Table 6). A similar effect was obtained when TAN-999 was added to a culture of J774A.1, a cell line which is highly phagocytic. These results indicate that TAN-999 augments phagocytic activity of these murine macrophage cell lines. TAN-1030A and staurosporine also increased the number of phagocytic cells in these cell lines.

Since several functions of macrophages are mediated by Fcγ receptors on their surfaces, the effect of these metabolites on the expression of the Fcγ receptors of Mm 1 and J774A.1 was examined. TAN-999

at a concentration of 5 ng/ml increased the EAγ-rosette formation of Mm 1 cells to 4 times that of the control. The metabolite also significantly enhanced the rosette formation of J774A.1 (Table 6). These results indicate that TAN-999 augments the expression of Fcγ receptors on the surface of the murine macrophage cell lines. TAN-1030A also enhanced the expression of Fcγ receptors at 50 to 100 ng/ml, and similar results were obtained in the presence of staurosporine at 1 to 2 ng/ml.

β-Glucuronidase, a lysosomal enzyme, is an important marker for macrophage functions against bacterial infection. TAN-999 increased the activity of this enzyme. When the metabolite was added to a culture of Mm 1 at 1 ng/ml, the enzyme activity increased 1.4 times. Since the enzyme activity was normal level in the presence of TAN-999 at 5 ng/ml, the metabolite at high concentrations seems to inhibit lysosome function in Mm 1 cells. A significant increase in the enzyme activity was also observed in the presence of TAN-1030A (Table 6). Staurosporine scarcely increased the enzyme activity.

Since a respiratory burst was scarcely detected in Mm 1, the effects of these metabolites on the phagocytosis-dependent respiratory burst in mouse peritoneal macrophages was investigated. When PP-elicited peritoneal macrophages from C57BL/6 mice were incubated with TAN-999 or TAN-1030A, the respiratory bursts in these macrophages were enhanced. The enhancement by TAN-999 occurred at concentrations of 0.01 to 1 ng/ml and reached a maximum at 0.1 ng/ml (2 times higher than the drug-free control). An increase in the respiratory burst in the macrophages also occurred in the presence of TAN-1030A at concentrations of 1 to 10 ng/ml (Table 7). Similar results were obtained by adding staurosporine at 0.01 to 0.1 ng/ml.

When less than 1/10,000 of the lethal doses of the metabolites were injected ip 24 hours before harvesting peritoneal cells, the peritoneal macrophages showed a greatly enhanced phagocytosis-dependent respiratory burst. The increase in respiratory burst occurred with 0.005 to 0.05 μg/kg of TAN-999 and with 0.5 to 5 μg/kg of TAN-1030A (Table 8). These results indicate that these metabolites modulate macrophages to an activated state in mice. Peritoneal macrophages from staurosporine treated mice showed an increase in respiratory burst only at 0.05 μg/kg.

Discussion

Due to their phagocytic function and their ability to secrete certain effector molecules, macrophages contribute to the host's ability to protect itself against invasion by microorganisms and against development of tumors. Our present results show that TAN-999 and TAN-1030A enhance non-specific phagocytic activity and the expression of Fcγ receptors in murine macrophage cell lines. These metabolites also increase lysosomal enzyme activity in these cells. Moreover, phagocytosis-dependent respiratory burst in mouse peritoneal macrophages was stimulated by TAN-999 and TAN-1030A *in vitro* and *in vivo*. These findings imply that these microbial metabolites may augment macrophage functions in animal and human bodies, resulting in the potentiation of defense mechanisms against infection and tumor development in these hosts. In preliminary experiments, the growth of B16 melanoma in syngeneic C57BL/6 mice was inhibited by ip pretreatment with TAN-999 (data not shown).

Staurosporine is a potent inhibitor of protein kinases,[17,18] especially protein kinase C. TAN-999 also inhibits protein kinase C (Dr. KOZAI of our Central Research Division; unpublished data). Moreover, we found that genistein, an inhibitor of tyrosine kinase,[19,20] induced spreading of Mm 1 cells and enhanced the respiratory burst in mouse peritoneal macrophages. These facts suggest that the activation of macrophages is regulated by protein kinases. Specific inhibitors of protein kinases are expected to act as macrophage activators which could protect against tumor development and microbial infection.

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References

1) WALKER, W. S. & R. B. HESTER: The functional heterogeneity of macrophages. *In* The Reticuloendothelial System. A Comprehensive Treaties. Vol. 6. *Ed.*, J. A. BELLANTI *et al.*, pp. 27~42, Plenum Press, 1984
2) FIDLER, I. J. & A. J. SCHROIT: Recognition and destruction of neoplastic cells by activated macrophages: discrimination of altered self. Biochim. Biophys. Acta 948: 151~173, 1988
3) ŌMURA, S.; Y. IWAI, A. HIRANO, A. NAKAGAWA, J. AWAYA, H. TSUCHIYA, Y. TAKAHASHI & R. MASUMA: A new alkaloid AM-2282 of *Streptomyces* origin. Taxonomy, fermentation, isolation and preliminary characterization. J. Antibiotics 30: 275~282, 1977
4) FURUSAKI, A.; N. HASHIBA & T. MATSUMOTO: X-Ray crystal structure of staurosporine: A new alkaloid from *Streptomyces* strain. J. Chem. Soc. Chem. Commun. 1978: 800~801, 1978
5) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
6) JACOBSON, E.; W. C. GRAUVILLE & C. E. FOGS (*Ed.*): Color Harmony Manual. 4th Ed. Container Corporation of America, 1958
7) HASEGAWA, T.; M. TAKIZAWA & S. TANIDA: A rapid analysis for chemical grouping of aerobic actinomycetes. J. Gen. Appl. Microbiol. 29: 319~322, 1983
8) TANIDA, S.; T. HASEGAWA, M. MUROI & E. HIGASHIDE: Dnacins, new antibiotics. I. Producing organism, fermentation, and antimicrobial activities. J. Antibiotics 33: 1443~1448, 1980
9) KYOIZUMI, S.; K. TESHIGAWARA, S. SAKAGUCHI & T. MASUDA: A cloned cell line, Mk 1, possessing Ia antigens and accessory cell activity. J. Immunol. 128: 2586~2594, 1982
10) KITANO, K.; Y. SHINTANI, Y. ICHIMORI, K. TSUKAMOTO, S. SASAI & M. KIDA: Production of human monoclonal antibodies by heterohybridomas. Appl. Microbiol. Biotechnol. 24: 282~286, 1986
11) RALPH, P. & I. NAKOINZ: Direct toxic effects of immunopotentiators on monocytic, myelomonocytic, and histocytic or macrophage tumor cells in culture. Cancer Res. 37: 546~550, 1977
12) RALPH, P. & I. NAKOINZ: Antibody-dependent killing of erythrocyte and tumor targets by macrophage-related cell lines: Enhancement by PPD and LPS. J. Immunol. 119: 950~954, 1977
13) TADA, H.; O. SHIHO, K. KUROSHIMA, M. KOYAMA & K. TSUKAMOTO: An improved colorimetric assay for interleukin 2. J. Immunol. Methods 93: 157~165, 1985
14) KERBEL, R. S. & B. E. ELLIOTT: Detection of Fc receptors. Methods in Enzymol. 93: 113~147, 1983
15) WREGHT, D. C. & S. E. MALAIVESTA: The mobilization and extracellular release of granular enzymes from human leukocytes during phagocytosis. J. Cell Biol. 53: 788~797, 1972
16) MEYER, J.: *Nocardiopsis*, a new genus of the order *Actinomycetales*. Int. J. Syst. Bacteriol. 26: 487~493, 1976
17) TAMAOKI, T.; H. NOMOTO, I. TAKAHASHI, Y. KATO, M. MORIMOTO & F. TOMITA: Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. Biochem. Biophys. Res. Commun. 135: 397~402, 1986
18) NAKANO, H.; E. KOBAYASHI, I. TAKAHASHI, T. TAMAOKI, Y. KUZUU & H. IBA: Staurosporine inhibits tyrosine-specific protein kinase activity of Rous sarcoma virus transforming protein p60. J. Antibiotics 40: 706~708, 1987
19) OGAWARA, H.; T. AKIYAMA, J. ISHIDA, S. WATANABE & K. SUZUKI: A specific inhibitor for tyrosine protein kinase from *Pseudomonas*. J. Antibiotics 39: 606~608, 1986
20) AKIYAMA, T.; J. ISHIDA, S. NAKAGAWA, H. OGAWARA, S. WATANABE, N. ITOH, M. SHIBUYA & Y. FUKAMI: Genistein, a specific inhibitor of tyrosine-specific protein kinases. J. Biol. Chem. 262: 5592~5595, 1987