TAN-1496 A, C AND E, DIKETOPIPERAZINE ANTIBIOTICS WITH INHIBITORY ACTIVITY AGAINST MAMMALIAN DNA TOPOISOMERASE I

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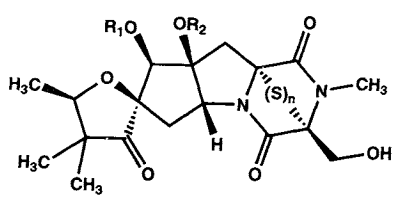
Fungal metabolites with an *epi*-oligothiadiketopiperazine structure, TAN-1496 A, C and E, were isolated from the culture broth of *Microsphaeropsis* sp. FL-16144. Their molecular formulas were determined to be $C_{22}H_{28}N_2O_9S_2$, $C_{22}H_{28}N_2O_9S_3$ and $C_{22}H_{28}N_2O_9S_4$, respectively. Structures were determined by comparing the NMR data with those of known diketopiperazine antibiotics, sirodesmins. These metabolites inhibited the relaxation of supercoiled pBR322 DNA by calf thymus topoisomerase I but did not affect the decatenation of kinetoplast DNA by calf thymus topoisomerase II at concentration up to 500 μM. They strongly suppressed the growth of various murine and human tumor cells and induced apoptosis. Moreover, various derivatives were synthesized to investigate the relationship of their functional groups and biological activities.

The death rate due to malignant tumors has been steadily increasing, and in the area of cancer chemotherapy, we are in need of potent antitumor agents to inhibit the proliferation of such cancers. DNA topoisomerase I (Topo I), a nuclear enzyme responsible for DNA metabolism[1], has been proposed as an intracellular target for cancer therapeutics[2]. In fact, the alkaloid camptothecin (CPT) and several of its analogs specifically inhibit Topo I and show considerable efficacy against a broad spectrum of human tumor xenografts.[3~5] In our search for Topo I inhibitors of microbial origin, five metabolites were found in the culture broth of a fungus strain isolated from a soil sample. Three of them, TAN-1496 A (**1**), C (**3**) and E (**5**), were proved to be new compounds having specific inhibitory activity against Topo I (Fig. 1). The other two, TAN-1496 B (**2**) and D (**4**), were found to be identical to sirodesmin A and its deacetyl derivative, respectively. This paper describes taxonomic studies of the producing organism and fermentation, isolation, structure determination, biological activities and modification of the antibiotics.

Fig. 1. Structures of TAN-1496 A~E.



	n	R_1	R_2
A	2	Ac	Ac
B	2	Ac	H
C	3	Ac	Ac
D	2	H	H
E	4	Ac	Ac

Results

Fermentation

A spore suspension of *Microsphaeropsis* sp. FL-16144 was inoculated into a 2-liter Sakaguchi flask containing 500 ml of a sterile seed medium consisting of glucose 2%, soluble starch 3%, soybean flour 1.5%, corn steep liquor 1%, Polypepton 0.5%, yeast extract 0.3%, NaCl 0.3% and $CaCO_3$ 0.5%. The flask was shaken on a reciprocal shaker at 24°C for 48 hours. One and a half liters of the seed culture was transferred to a 200-liter fermentor containing 120 liters of a production medium consisting of glucose 1%, dextrin 4%, soybean flour 0.5%, Polypepton 0.5%, meat extract 0.5%, $FeSO_4$ 0.05%, $MnSO_4$ 0.05%, $MgSO_4$ 0.05%, KH_2PO_4 0.1% and $CaCO_3$ 0.5%. Fermentation was carried out at 24°C with aeration at 120 liters/minute and agitation at 180 rpm. The production of the major metabolite, **1**, reached a maximum (81 μg/ml) 114 hours after inoculation.

Taxonomy of the Producing Organism

Culture characterization was carried out following the methods described by MALLOCH[6] and SUTTON[7]. Scanning electron microscopy was conducted by the method of TANIDA *et al.*[8].

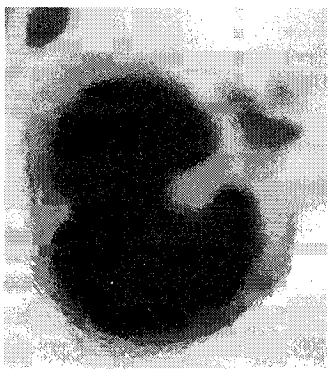
Strain FL-16144 was isolated from a soil sample collected in Ibaragi prefecture, Japan. Macroscopic characteristics of strain FL-16144 on malt extract agar were as follows: the colony is ventinous with a regular periphery; the aerial hyphae are grayish white and develop concentrically; the reverse side of the colony is yellowish brown at the center and pale yellow to pale yellowish brown at the periphery; no pigment production is observed.

Microscopic characteristics of the strain were as follows: the hyphae are $1 \sim 3$ μm in diameter, hyaline to pale brown, immersed, branched and septate; the pycnidia are globose to irregular and immersed; peridia are thin-walled, hyaline to pale brown, somewhat gelatinous; the conidiogenous cell ampulliform is monophialidic; conidia are $4.0 \sim 4.5 \times 2.2 \sim 2.8$ μm, subglobose to ellipsoid, aseptate, smooth and brown (plate 1). These characteristics indicate that strain FL-16144 belongs to the genus *Microsphaeropsis*. Therefore, the strain is designated *Microsphaeropsis* sp. FL-16144.

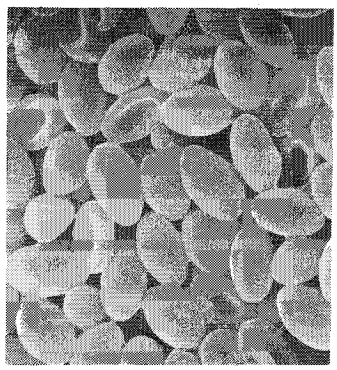
Plate 1. Light and scanning electron micrographs of strain FL-16144.

The organism was cultured on malt extract agar for 2 weeks at 28°C. A, a pycnidium (×400); B, spores (×5,000).

A



B



Isolation

The general procedure for the isolation of **1**~**5** is summarized in Fig. 2. The antibiotics are neutral, fat-soluble compounds; therefore solvent extraction, silica gel chromatography and preparative reversed-phase HPLC are used for purification. The amount of bioactive components was estimated by the inhibitory activity against Topo I or analyzed by HPLC: column, octadecylsilane (ODS), mobile phase, 45% acetonitrile/0.01 M phosphate buffer (pH 6.3).

Chemical Characterization

Compounds **1**~**5** showed positive color reactions with iodine and potassium permanganate and

Fig. 2. Isolation procedure for TAN-1496 A, B, C, D and E.

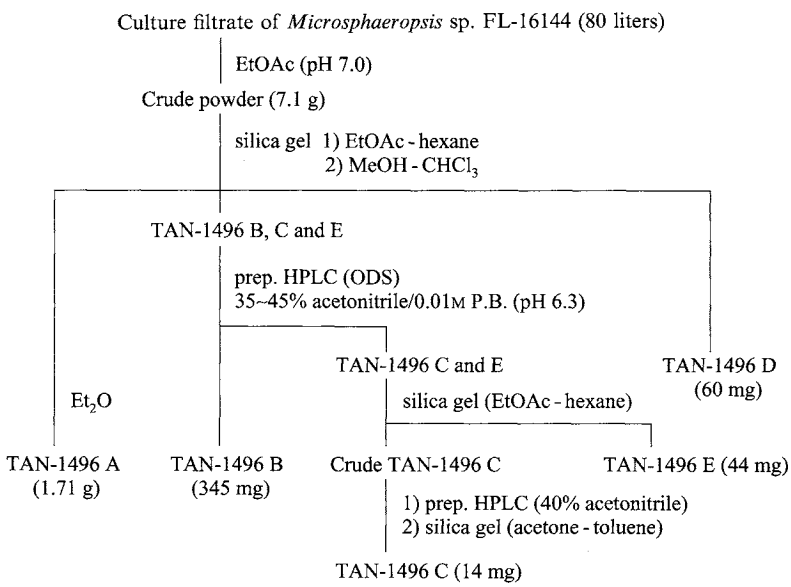
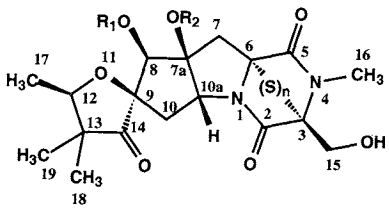


Table 1. Physico-chemical properties of TAN-1496.

Property		1	2	3	4	5
EI-MS		528 (M^+)	486 (M^+)		444 (M^+)	
		464 (M^+-2S)	422 (M^+-2S)	464 (M^+-3S)	380 (M^+-2S)	464 (M^+-4S)
Analysis	C,	49.99/49.99	49.37/49.74	47.13/46.80	48.64/49.23	44.58/44.91
Calcd/Found	H,	5.34/ 5.47	5.39/ 5.68	5.03/ 5.17	5.44/ 5.56	4.76/ 4.68
	N,	5.30/ 5.25	5.76/ 5.33	5.00/ 4.82	6.30/ 6.48	4.73/ 4.75
	S,	12.13/12.20	13.18/11.85	17.15/16.53	14.42/14.31	21.64/21.55
Formula		$C_{22}H_{28}N_2O_9S_2$	$C_{20}H_{26}N_2O_8S_2$	$C_{22}H_{28}N_2O_9S_3$	$C_{18}H_{24}N_2O_7S_2$	$C_{22}H_{28}N_2O_9S_4$
UV λ_{max} (ε)		230 (sh, 4,200)	230 (sh, 4,900)	250 (3,700)	230 (sh, 4,600)	211 (12,400)
						290 (1,400)
IR (KBr, cm^{-1})		1760, 1690	1755, 1690	1750, 1680	1745, 1680	1745, 1680
CD (MeOH)		231 (−105100)	231 (−99600)	212 (+71400)	232 (−83300)	213 (+105100)
[θ] nm		261 (+27700)	261 (+26700)	264 (−68100)	261 (+22200)	241 (−56800)
		313 (−4200)	315 (−2400)			320 (−5900)
HPLC* Rt (minutes)		6.5	3.5	10.1	2.4	10.8
TLC** Rf		0.38	0.26	0.34	0.24	0.18

* ODS (YMC-Pack A-312), 45% acetonitrile - 0.01 M phosphate buffer (pH 6.3).
** Silica gel 60 (Merck, Art. 5715), EtOAc - hexane = 2 : 1.

Table 2. ^{13}C NMR data of TAN-1496 (75 MHz, $CDCl_3$, δ ppm).



No.		1	2	3	4	5	No.		1	2	3	4	5
2	Q	162.6	162.8	164.6	162.8	166.3	13	Q	46.1	46.2	46.2	46.1	46.1
3	Q	76.7	76.5	79.0	76.6	77.7	14	Q	218.2	216.5	218.6	218.5	217.8
5	Q	165.6	165.5	168.2	165.7	168.7	15	CH_2	60.9	60.8	62.6	60.7	63.3
6	Q	75.1	75.0	75.0	74.9	76.3	16	CH_3	27.4	27.4	27.9	27.6	29.0
7	CH_2	41.1	40.7	46.6	40.8	48.0	17	CH_3	14.9	15.0	14.8	15.0	14.9
7a	Q	88.6	84.9	87.9	85.1	88.7	18	CH_3	18.2	18.2	18.2	18.1	18.2
8	CH	79.5	79.5	79.2	79.6	82.1	19	CH_3	19.3	19.2	19.3	19.1	19.3
9	Q	89.4	89.9	86.7	90.3	86.7	R_1	$COCH_3$	20.4	20.5	20.3	—	20.3
10	CH_2	38.6	37.9	39.0	38.0	37.3		$COCH_3$	169.4	169.5	169.3	—	170.0
10a	CH	67.4	68.5	67.7	67.2	69.2	R_2	$COCH_3$	21.2	—	21.2	—	21.5
12	CH	82.0	82.7	81.9	82.6	82.1		$COCH_3$	169.5	—	169.4	—	169.5

Table 3. ^1H NMR data of TAN-1496 (300 MHz, $CDCl_3$, δ ppm).

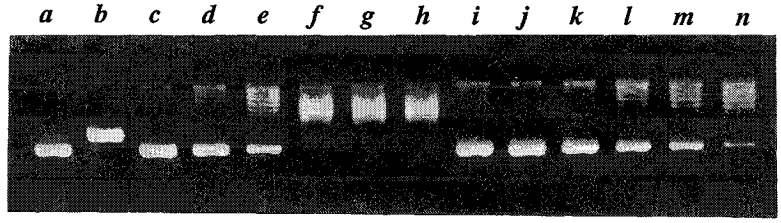
No.	1	2	3	4	5
7	3.39 (d)	3.07 (d)	3.27 (d)	2.65 (d)	3.13 (d)
	3.62 (d)	3.14 (d)	3.64 (d)	3.20 (d)	3.47 (d)
8	5.61 (s)	5.74 (s)	5.25 (s)	4.66 (d)	5.31 (s)
10	2.04 (dd)	1.96 (dd)	2.17 (dd)	1.96 (dd)	2.02 (dd)
	2.55 (dd)	2.57 (dd)	2.51 (dd)	2.51 (dd)	2.44 (dd)
10a	4.88 (t)	4.48 (t)	4.94 (d)	4.44 (dd)	5.33 (dd)
12	4.09 (q)	4.22 (q)	4.05 (q)	4.27 (q)	4.16 (q)
15	4.25 (dd)	4.25 (dd)	4.06 (dd)	4.25 (dd)	4.05 (dd)
	4.33 (dd)	4.32 (dd)	4.22 (dd)	4.32 (dd)	4.37 (dd)
15-OH	3.48 (dd)	3.49 (dd)	ND	3.46 (dd)	3.08 (t)
16	3.16 (s)	3.15 (s)	3.26 (s)	3.16 (s)	3.11 (s)
17	1.27 (d)	1.31 (d)	1.27 (d)	1.28 (d)	1.27 (d)
18	0.94 (s)	0.96 (s)	0.97 (s)	0.94 (s)	0.95 (s)
19	1.02 (s)	1.03 (s)	1.00 (s)	1.07 (s)	1.00 (s)
R_1	2.06 (s, Ac)	2.13 (s, Ac)	2.03 (s, Ac)	3.00 (d, H)	2.06 (s, Ac)
R_2	2.09 (s, Ac)	3.51 (s, H)	2.04 (s, Ac)	3.56 (s, H)	2.06 (s, Ac)

ND: not detected.

negative color reactions with ninhydrin and Ehrlich, Dragendorff and Sakaguchi reagents. These antibiotics are easily soluble in methanol, acetone, ethyl acetate, chloroform, *etc.* The IR spectra showed characteristic absorption in the carbonyl region. The molecular formula of **1** was determined from the elemental analysis, EI-MS and ^{13}C NMR spectrum. Those of other components were determined in the same manner as listed in Table 1. The ^{13}C and ^1H NMR spectra of **1**~**5** are listed in Tables 2 and 3. Compounds **3** and **5** had additional one and two sulfur atoms in the molecule, respectively. In the EI-MS spectra the major peaks were detected as $M^+ - nS$ ($n=2\sim4$), while the molecular ion peaks (E^+) were detected as the minor ones (**1**, **2** and **4**) or not detected (**3** and **5**). On treatment of **1** with triphenylphosphine in dichloromethane

Fig. 3. Effect of TAN-1496 A on the relaxation activity of Topo I.

Electrophoresis was carried out using 0.7% agarose and 0.1% SDS at 100 V for 35 minutes. *Lane a*, ccc DNA control; *lane b*, linear DNA control; *lanes c* to *h*, TAN-1496 A; *lanes i* to *n*, camptothecin. Drug concentrations were as follows: *lanes c* and *i*, 250 μM; *lanes d* and *j*, 125 μM; *lanes e* and *k*, 62.5 μM; *lanes f* and *l*, 31.3 μM; *lanes g* and *m*, 15.7 μM; *lanes h* and *n*, 7.9 μM.



or tetrahydrofuran (THF), the monosulfide derivative (**6**, see Fig. 6) was obtained without cleavage of the sulfide bond. These phenomena[9] are characteristic features of *epi*-oligothiadiketopiperazine compounds such as gliotoxin[10] and sporidesmins[11]. Therefore, **1**~**5** are regarded as belonging to this family.

Table 4. Inhibitory activity of TAN-1496 A, C and E against calf thymus Topo I.

Conc. (μM)	**1**	**3**	**5**
500	++	++	++
250	+	+	+
125	+	+	+
62.5	−	w	w

++: strong inhibition; +: evident inhibition; w: slight inhibition; −: no inhibition.

Structure Determination

The results of 1D- and 2D-NMR analyses of **1** suggested the presence of five partial structures, (a) N–CH_3, (b) –CH_2–C(OAc)–CH(OAc)–, (c) –CH_2–OH, (d) –CH–CH_2– and (e) O–CH–CH_3, in the molecule. The NMR data revealed that **2** and **4** had one or no acetyl groups in the molecules, respectively. Among the diketopiperazine antibiotics, sirodesmin A[12] has almost the same partial structures, except for one acetyl group. Comparison of the physico-chemical data of **1** and sirodesmin A indicated that the two were analogous. In addition, **2** and sirodesmin A had identical ^{1}H NMR, ^{13}C NMR and CD spectra. Compound **4** was previously reported to be obtained by deacetylation of sirodesmin A. Differences between **1** and **2** were derived from the presence of one acetyl group. This indicated that **1** was an acetate of **2** (=sirodesmin A). Although an acetate of sirodesmin A has been reported[12], it is the acetate of the primary hydroxy group. Considering structural components (b) and (c), the absolute structures of **1** as well as **3** and **5** were determined to be as shown in Fig. 1. Thus **1**, **3** and **5** are concluded to be new *epi*-oligothiadiketopiperazine antibiotics.

Biological Activity

The inhibitory effect of **1** on the relaxation of plasmid DNA by Topo I is shown in Fig. 3. Compound **1** inhibited the Topo I activity in a dose-dependent manner. The inhibitory effect of **3** and **5** were comparable to that of **1** (Table 4). Sirodesmin A (**2**) also inhibited this enzyme, but the inhibitory effect was weaker than that of compounds **1**, **3** and **5** (data not shown).

To determine whether these metabolites are specific inhibitors of Topo I, the effect of **1** on the decatenation of kDNA by topoisomerase II (Topo II) was tested. Compound **1** did not inhibit the enzyme activity even at a concentration of 500 μM, although m-AMSA, a specific inhibitor of Topo II, still inhibited Topo II at a concentration of 4 μM (Fig. 4). This indicates that the inhibitory activity of **1** is specific for Topo I. Neither **3** nor **5** affected the decatenation activity of Topo II at concentrations up to 500 μM.

Fig. 4. Effect of TAN-1496 A on the decatenation activity of Topo II.

Electrophoresis was carried out using 1.0% agarose and 0.1% SDS at 100 V for 20 minutes. *Lane a*, catenated kinetoplast DNA control; *lane b*, decatenated minicircle control; *lane c* to *j*, m-AMSA; *lanes k* to *o*, TAN-1496 A. Drug concentrations were as follows: *lanes c* and *l*, 250 μM; *lanes d* and *m*, 125 μM; *lanes e* and *n*, 62.5 μM; *lanes f* and *o*, 31.3 μM; *lane g*, 15.7 μM; *lane h*, 7.9 μM; *lane i*, 4 μM; *lane j*, 2 μM; *lane k*, 500 μM.

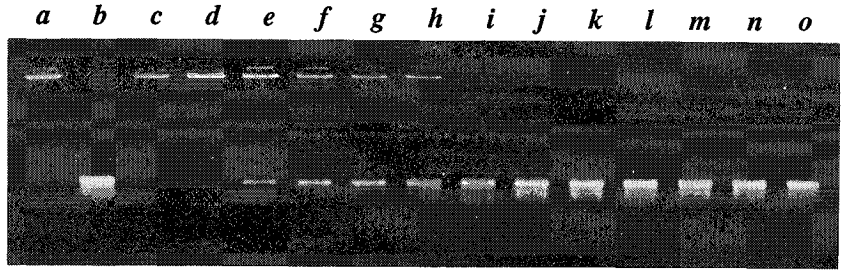


Table 5. Growth inhibitory activity of TAN-1496 A, C and E in murine and human tumor cell lines.

Cell line	IC_{50} (ng/ml)		
	1	**3**	**5**
P815 murine mastcytoma	8.5	18.7	21.2
EL4 murine lymphoma	3.9	9.5	7.5
B16 murine melanoma	57.4	113.0	115.3
HeLa S3 human epitheloid carcinoma	16.2	21.3	28.7
WiDr human colon adenocarcinoma	14.7	20.5	21.8
A549 human lung carcinoma	18.4	37.4	40.8
G361 human melanoma	16.0	25.6	28.3

Each cell line was cultured with each metabolite in a 96-well plate in the presence of drug for 72 hours. The proliferation was evaluated by MTT assay. Initial cell concentrations were as follows: EL4 and P815, 2×10^4/ml; HeLa S3, WiDr, A549 and G361, 4×10^4/ml; B16, 5×10^4/ml.

The growth inhibitory activities of these metabolites against murine and human tumor cells are shown in Table 5. They strongly suppressed the growth of all tumor cell lines tested.

The analysis of chromosomal DNA extracted from tumor cells treated with **1** is shown in Fig. 5. DNA fragmentations corresponding to the nucleosomal ladders were detected in SW48 and HeLa S3 cell lines. Similar results were also obtained in other cell lines at higher concentrations or with longer exposure. These results indicate that **1** induces apoptosis of tumor cells.

Antimicrobial activities of **1**, **3** and **5** are shown in Table 6. They were active against Gram-positive bacteria but showed no antimicrobial activity against Gram-negative bacteria, yeasts or fungi.

Fig. 5. Effect of TAN-1496 A on the induction of apoptosis in various human tumor cell lines.

Electrophoresis was carried using 0.7% agarose and 0.1% SDS at 100 V for 40 minutes. *Lane a*, øx-174/HincII marker; *lane b*, WiDr human colon adenocarcinoma; *lane c*, G361 human melanoma; *lane d*, A549 human lung carcinoma; *lane e*, SW48 human colon adenocarcinoma; *lane f*, HeLaS3 human epitheloid carcinoma.

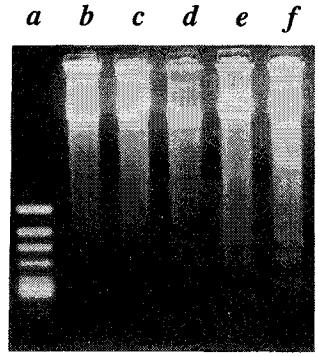


Table 6. Antimicrobial spectra of TAN-1496 A, C and E.

Test organism	MIC (μg/ml)		
	1	**3**	**5**
Escherichia coli K2	>100	>100	>100
Escherichia coli NIHJ JC-2	>100	>100	>100
Proteus mirabilis ATCC 21100	>100	>100	>100
Proteus vulgaris IFO3045	>100	>100	>100
Proteus morganii IFO 3168	>100	>100	>100
Klebsiella pneumoniae IFO 3317	>100	>100	>100
Serratia marcescens IFO 3046	>100	>100	>100
Salmonella typhimurium IFO 12529	>100	>100	>100
Salmonella enteritidis IFO 3313	>100	>100	>100
Citrobacter freundii IFO 12681	>100	>100	>100
Pseudomonas aeruginosa IFO 3080	>100	>100	>100
Alcaligenes faecalis IFO 13111	>100	>100	>100
Bacillus subtilis PCI 219	12.5	12.5	12.5
Bacillus cereus IFO 3514	3.13	1.57	3.13
Bacillus pumilus IFO 3813	6.25	3.13	6.25
Bacillus megaterium IFO 12108	1.57	0.78	1.57
Staphylococcus aureus FDA 209P	6.25	12.5	6.25
Micrococcus luteus IFO 12708	3.13	6.25	3.13
Micrococcus flavus IFO 3242	<0.78	<0.78	<0.78
*Mycobacterium avium** IFO 3154	12.5	12.5	12.5
*Mycobacterium phlei** IFO 3158	>100	>100	>100
*Mycobacterium smegmatis** ATCC 607	>100	>100	>100
Candida albicans† IFO 0583	>100	>100	>100
Cryptococcus neoformans† IFO 0410	>100	>100	>100
Saccharomyces cerevisiae† IFO 0209	>100	>100	>100
Candida parakrusei† IFO 0640	>100	>100	>100
Penicillium chrysogenum† IFO 4626	>100	>100	>100
Aspergillus niger† IFO 4066	>100	>100	>100

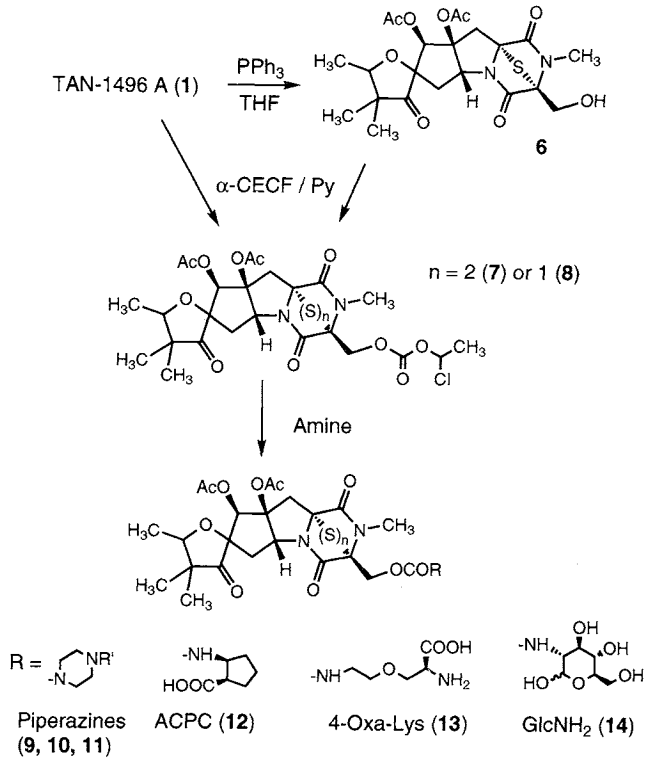
Minimum inhibitory concentration (MIC) was determined by the agar dilution method with antibiotic medium 3+0.5% yeast extract, *TSA+3% glycerin or †TSA+1% glucose.

Chemical Modification

TAN-1496 components are potent inhibitors of Topo I as well as proliferation of tumor cells *in vitro*; however, the *epi*-oligothiadiketopiperazine antibiotics are known to be highly toxic substances[9]. Optimal chemotherapeutic coefficient is required for improved antitumor activity. In addition, TAN-1496 is very lipophilic that it is hardly dissolved in an aqueous solution, and it has very low bioavailability *in vivo*. Therefore, we searched for more bioavailable and less toxic derivatives by means of chemical modifications. For these purposes, (I) introduction of a hydrophilic group and (II) reductive cleavage of the disulfide bond were examined.

Compound **1** has a hydroxymethyl group, which seemed to be a key function for the first purpose. The general method for preparation of carbamate derivatives is as follows. Compound **1** was treated with α-chloroethyl chloroformate (α-CECF) and pyridine in dichloromethane to afford the carbonate (**7**). This compound gave the monosulfide derivative (**8**) on treatment with triphenylphosphine. Carbonate **7** or **8** was mixed with amines bearing protected (unprotected) hydrophilic functions such as amines, carboxylic acids, alcohols to give the corresponding carbamates. In this manner were prepared piperazine carbamates **9** (R′=H, n=2), **10** (R′=Me, n=2) and **11** (R′=H, n=1), 2-aminocyclopentane-1-carboxylic acid[13]

Fig. 6. Preparation of carbamate derivatives.



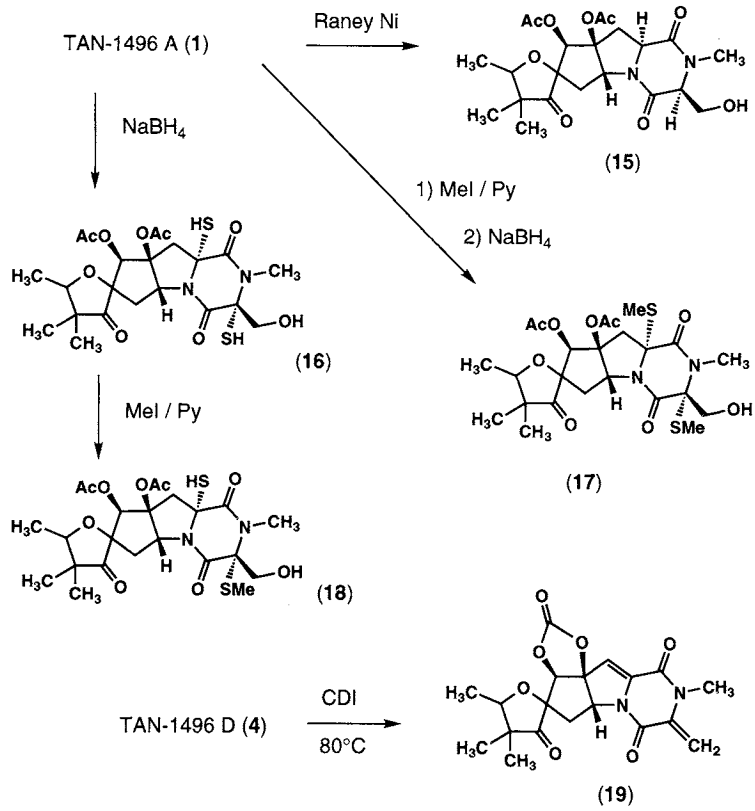
(ACPC) carbamate **12** (n=2), 4-oxalysine[14] carbamate **13** (n=2) and D-glucosamine carbamate **14** (n=2) as shown in Fig. 6. These derivatives are water-soluble, and the solubilities of **9**, **10** and **11** (hydrochlorides) in water are more than 10 mg/ml.

Compound **1** has a disulfide bridge on the diketopiperazine ring. Reduction with Raney nickel (W-2) in ethanol gave completely desulfurized compound **15**. Treatment with sodium borohydride ($NaBH_4$) in methanol cleaved the disulfide bond to give dithiol derivative **16**. It was assumed that this product would be oxidized back to the disulfide in the presence of oxygen; therefore, the free thiol groups should be protected. Compound **1** was *S*-methylated with iodomethane in pyridine and then reduced with $NaBH_4$ in methanol to give bis(methylthio) derivative **17** in good yield. Compound **16** was monomethylated with iodomethane and pyridine in dichloromethane to afford monomethylthio derivative **18**. In the ^1H-^1H COSY experiment on **18**, the SH proton (δ 4.43 ppm, s) showed cross peaks with the C7-methylene protons (δ 3.04 and 3.42 ppm). Therefore, **18** was proved to have the methylthio group (δ 2.18 ppm) on C3 position. When **4** was heated with 1,1'-carbonyldiimidazole (CDI) in toluene, it gave cyclic carbonate **19**. These results are summarized in Fig. 7.

Biological Activity of TAN-1496 Analogs

Inhibitory effects on topoisomerases and growth of HeLa cells with **1** and the obtained derivatives (**6**~**19**) are listed in Table 7. The monosulfides **6** and **11** caused weak or no growth inhibition in spite of their inhibitory activity against Topo I. This apparent disagreement between these two activities seemed to be derived from the difference in assay methods. The incubation time needed for the enzymatic reaction

Fig. 7. Derivatives afforded by disulfide bond cleavage.



of Topo I was about 40 minutes at 37°C, while that for growth of HeLa cell was 72 hours at the same temperature. The half-life period ($T_{1/2}$) of **6** was 2 hours at 37°C in a phosphate buffer (pH 7.0); therefore, **6** did not exist throughout the growth inhibition assay. This lability may be caused by strain of the bicyclo-[2,2,1]-ring system.

As for the Topo I inhibitory activity of carbamate derivatives as well as the intermediate carbonate derivatives, **7**~**14** exhibited comparable activity to **1**. On the other hand, **9**, **13** and **14** inhibited Topo II as well as Topo I. Most of these water-soluble derivatives showed much weaker growth inhibitory activity. These facts indicated that introduction of a hydrophilic group was not favorable for specificity against Topo I or growth inhibition of tumor cells. Among these water-soluble derivatives, **10** showed specificity for Topo I and strong growth inhibitory activity.

Table 7. Biological activity of derivatives of TAN-1496.

Compound	MIC (μg/ml)		Ratio Topo II/I	IC_{50} (μg/ml) HeLa
	Topo I	Topo II		
1	18.8	>600	>32	0.016
6	18.8	300	16	2.1
7	37.5	37.5	1	0.066
8	75	300	4	5.1
9	37.5	37.5	1	0.56
10	18.8	75	4	0.034
11	18.8	300	16	3.2
12	37.5	150	4	0.83
13	37.5	37.5	1	>10
14	37.5	37.5	1	0.46
15	>600	>600	—	2.86
16	37.5	300	8	0.009
17	>600	>600	—	>10
18	75	150	2	>10
19	37.5	37.5	1	0.072

While cleavage of the disulfide bond resulted in the loss of the both activities in most cases, dithiol **16** had satisfactory activities. This compound has two thiol groups, and it is soluble in weak alkaline aqueous solutions, for example 2% sodium bicarbonate. Because of these properties, **16** may make a good antitumor agent.

It is of much interest that **19** inhibited topoisomerase activity and proliferation of tumor cells without any sulfur atoms. Comparing the biological properties of **1** and **19** could be useful to investigate the significance of the disulfide bond in this family of antibiotics.

Discussion

We described here new Topo I inhibitors belonging to the *epi*-oligothiadiketopiperazine class of fungal metabolites. These metabolites suppressed the growth of various tumor cells and induced apoptosis. This implies that the inhibitory effect of these metabolites on Topo I may play an important role in apoptotic death of tumor cells[15,16].

TAN-1496 A does not stabilize the Topo I-DNA reaction intermediate "cleavable complex" (data not shown), whereas CPT is known to inhibit Topo I by stabilizing it[17]. Moreover, this metabolite does not intercalate into DNA (data not shown). These data indicate that TAN-1496 A may inhibit Topo I itself before it binds to DNA[18]. These findings suggest that these metabolites would be a novel type of antitumor agent with a mode of action different from that of CPT. They are also expected to be potent tools to shed light on the function of Topo I such as transcription and recombination in eukaryotic cells, because stabilization of cleavable complex causes the fragmentation of replicating chromosomal DNA and subsequent S-phase-specific cytotoxicity[17,19].

In another study, we confirmed that gliotoxin weakly inhibited Topo I, like sirodesmin A (data not shown). This may indicate that *epi*-polythiadiketopiperazines commonly have property of Topo I inhibition[20].

Experimental

Materials

Supercoiled pBR322 DNA and proteinase K were purchased from WAKO Pure Chemical Co., Osaka, Japan. Catenated kDNA was purchased from TopoGEN Inc., Ohio, USA. Calf thymus Topo I and RNase were purchased from Takara Shuzo Co., Kyoto, Japan. Topo II was purified to homogeneity from calf thymus gland as described by SCHOMBURG *et al.*[21]. EAGLE's MEM, RPMI1640 medium and FBS were purchased from Whittaker M. A. Bioproducts Inc., Maryland, USA. FBS was inactivated by heating at 56°C for 30 minutes prior to use. CPT and 4'-(9-acridinylamino)-methanesulfon-m-anisidine (m-AMSA) were purchased from Sigma Chemical Co., Missouri, USA. Culture plates were purchased from Corning Laboratory Sciences Co., New York, USA. Antibiotic medium 3 and trypticase soy agar were purchased from Difco Laboratories Co., Michigan, USA and Becton Dickinson and Co., Maryland, USA, respectively.

Relaxation Activity of Topo I

Topo I activity was determined by detecting the conversion of supercoiled pBR322 DNA to its relaxed form. The reaction mixture contained 50 mM Tris-HCl buffer (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 30 μg/ml of bovine serum albumin (BSA), pBR322 DNA (0.25 μg), 2 μl of sample solution and 1 unit of Topo I in a total volume of 20 μl. The mixture was incubated at 37°C for 40 minutes The reaction was terminated by adding 4 μl of loading buffer containing 0.25% bromophenol blue, 40% glycerol and 2.5% SDS. The reaction mixture was subjected to agarose gel electrophoresis at 100 V for 35 minutes in the presence of 0.1% SDS in 89 mM Tris-borate buffer (pH 8.9) containing 2 mM EDTA. Gels were stained with ethidium bromide and washed thoroughly with distilled water. The DNA band was visualized over UV light and photographed with Polaroid type 667 positive/negative film. One unit of Topo I activity was defined as the amount of the enzyme that converted 0.25 μg of supercoiled pBR322 DNA to its relaxed form within 40 minutes at 37°C.

Decatenation Activity of Topo II

Topo II activity was determined by detecting the conversion of catenated kinetoplast DNA (kDNA) to minicircle monomers. The reaction mixture contained 50 mM Tris-HCl buffer (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP, 0.5 mM DTT, 30 μg/ml of BSA, kDNA (0.2 μg), 2 μl of sample solution and 1 unit of Topo II in a total volume of 20 μl. The mixture was incubated at 37°C for 20 minutes. DNA was analyzed by agarose gel electrophoresis as described above. One unit of the Topo II activity was defined as the amount of the enzyme that decatenated 0.2 μg of kDNA within 20 minutes at 37°C.

Cytotoxicity

EL4 murine lymphoma, P815 murine mastcytoma and SW48 human colon adenocarcinoma cell lines were purchased from the American Type Culture Collection (ATCC). B16 murine melanoma cells were a gift from Japanese Research Resources Bank. HeLa S3 human epitheloid carcinoma, WiDr human colon adenocarcinoma, A549 human lung carcinoma and G361 human melanoma cells were obtained from the Institute for Fermentation, Osaka. All cell lines except EL4 and P815 were cultured with EAGLE's minimum essential medium (EAGLE's MEM) containing 10% fetal bovine serum (FBS), 5 mM glutamine and 20 μg/ml of gentamicin. EL4 and P815 cell lines were cultured with RPMI1640 containing FBS, 5 mM glutamine, 20 μg/ml of gentamicin and 0.05 mM 2-mercaptoethanol. In these conditions, all the cells became confluent within 72 hours incubation. The cells at the indicated concentration in 0.1 ml of the culture medium were incubated with each metabolite at 37°C for 72 hours in an atmosphere of 5% CO_2 in air. Growth of these cell lines was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay[22].

Detection of DNA Fragmentation

One million cells in 3 ml of the culture medium were plated in a 6 well plate. After 18 hours incubation, **1** was added followed by incubation for another 18 hours. The cells were then harvested, and genomic DNA was isolated essentially following the method of MILLER *et al.*[23]. Harvested cells were resuspended in 300 μl of nuclei lysis buffer consisting of 10 mM Tris-HCl (pH 8.2), 400 mM NaCl and 2 mM EDTA. The cell lysates were digested by incubation with 50 μl of 10 mg/ml proteinase K and 25 μl of 10% SDS at 37°C overnight. The reaction mixture was shaken vigorously with 100 μl of saturated NaCl. After centrifugation, 350 μl of supernatant was mixed with 700 μl of ethanol. Precipitated DNA was dried, dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and incubated at 37°C for 1 hour in the presence of 10 units of RNase A. The purified chromosomal DNA was analysed by agarose gel electrophoresis as described above.

Antimicrobial Activity

Antimicrobial activities of **1**, **3** and **5** were determined by the agar dilution method. Antibiotic medium 3 supplemented with 0.5% yeast extract was used for common bacteria. Trypticase soy agar (TSA) supplemented with 3% glycerol was used for acid-fast bacteria and TSA supplemented with 1% glucose was used for yeasts and fungi. Bacteria were grown at 37°C for 18 hours, whereas yeasts and fungi were grown at 28°C for 48 hours.

General

The UV and CD (JASCO J-20 with DP-501N) spectra were measured at 23~28°C in methanol. The IR spectra were measured in KBr pellets. The EI-MS spectra were measured on a Jeol JMS-DX303 instrument. The δ-values in the NMR spectra were recorded in ppm downfield from tetramethylsilane (TMS) using a Bruker AC-300 spectrometer.

Isolation of **1**, **2**, **3**, **4** and **5**

The culture filtrate (80 liters) of *Microsphaeropsis* sp. FL-16144 was adjusted to pH 7.0 and extracted with EtOAc (27 liters) twice. The organic layer was washed with 2% sodium bicarbonate (25 liters), 0.02 N HCl (25 liters) and water (25 liters, twice) and concentrated. The remainder was triturated with hexane to give a powder (7.1 g). This powder was dissolved with a minimum volume of MeOH, adsorbed on silica gel (*ca.* 10 g) and dried in air. The crude material on silica gel was chromatographed on a column of silica

gel (500 ml). Fraction-I (Fr.-I), eluted with EtOAc - hexane (1 : 2), was concentrated to give a crude powder of **1** (4.4 g). Fr.-II, eluted with EtOAc - hexane (2 : 1), gave a mixture of **2**, **3**, **4** and **5** (767 mg) as a yellowish powder. Fr.-III, eluted with EtOAc - hexane (3 : 1), afforded a mixture of **1**, **2**, **3** and **5** (214 mg) as a pale yellow powder. The powders from Fr.-I and Fr.-II were independently re-chromatographed on silica gel (300 and 80 ml) eluting with MeOH - $CHCl_3$. Fr.-I gave powders of pure **1** and a mixture of **1**, **2** and **3** (561 mg). The former was crystallized from Et_2O to afford **1** (1.71 g) as fine crystals. Fr.-II gave powders of crude **3** (286 mg), pure **4** (60 mg) and a mixture of **2**, **3** and **5** (379 mg). The crude powder of **3** was re-chromatographed on silica gel (30 ml) eluting with EtOAc - hexane to give a crude powder of **3** (221 mg). The mixtures from Fr.-I and Fr.-II and the powder from Fr.-III were mixed and subjected to preparative reversed-phase HPLC (ODS, YMC-pack, S-363, I-15) with a mobile phase of 35% and 45% acetonitrile - 0.01 M phosphate buffer (pH 6.3) to afford **1** (251 mg) and **2** (345 mg) and a mixture of **3** and **5** (129 mg) as pale yellow powders. This mixture was chromatographed on silica gel (20 ml) eluting with EtOAc - hexane to give powders of **5** (44 mg) and crude **3** (79 mg). The latter and the crude powder (201 mg) of **3** from Fr.-II were mixed and subjected to prep. HPLC with a mobile phase of 40% acetonitrile - 0.01 M phosphate buffer (pH 6.3) to give a crude powder of **3** (49 mg). This was again chromatographed on silica gel (10 ml) eluting with EtOAc - toluene to give **3** (14 mg) as a white powder.

Mono-desulfurization of **1** (**6**)

A solution of **1** (195 mg, 0.369 mmol) in THF (6.5 ml) was stirred with triphenylphosphine (100 mg, 1.0 eq) at ambient temperature for 15 hours. The reaction mixture was diluted with EtOAc (7 ml) and hexane (3 ml) and washed with water (5 ml). The aqueous layer was mixed with a mixture of EtOAc and hexane (2 : 1, 6 ml). The organic layers were combined and washed with water and brine (10 ml), dried over anhydrous sodium sulfate and concentrated to give an oil (309 mg). This oil was applied to a silica gel column (Merck, Silica gel 60, Art. 7734, 10 g) and eluted with a mixed solvent of EtOAc and hexane to give **6** (148 mg, 0.299 mmol, 81%) as a white powder.

Anal Calcd for $C_{22}H_{28}N_2O_9S$: C 53.22, H 5.68, N 5.64, S 6.46.
Found: C 53.46, H 6.00, N 5.29, S 6.41.

UV λ_{max}^{MeOH} 211 nm (ε 5,600), 261 (1,800) IR: 3445, 2975, 2930, 1755, 1725, 1645, 1460, 1430, 1370, 1240, 1150, 1075, 1010 (cm^{-1}) ^{13}C NMR ($CDCl_3$): 219.5 (Q), 173.1 (Q), 172.4 (Q), 169.6 (Q), 169.5 (Q), 94.5 (Q) 88.0 (Q), 81.9 (CH), 80.0 (Q), 79.4 (Q), 76.3 (CH), 63.2 (CH), 59.3 (CH_2), 46.3 (Q), 38.4 (CH_2), 31.4 (CH_2), 28.4 (CH_3), 21.2 (CH_3), 20.4 (CH_3), 19.4 (CH_3), 18.3 (CH_3), 14.8 (CH_3).

α-Chloroethylcarbonation of **1**(**7**)

A solution of **1** (990 mg, 1.87 mmol) in dichloromethane (33 ml) was stirred with pyridine (0.23 ml, 1.5 eq) and α-CECF (43% in dichloromethane solution, 0.56 ml, 1.2 eq) at 0°C for 30 minutes. Additional pyridine (0.23 ml) and α-CECF (0.17 ml, 0.3 eq) were added to the mixture, and 2.5 hours later, the reaction mixture was mixed with 5% ammonium chloride (50 ml). The aqueous layer was extracted with a mixture of EtOAc and hexane (1 : 2, 60 ml) twice. The organic layers were combined, washed with 0.05 N HCl twice, 2% sodium bicarbonate, water and brine (50 ml, each), dried over anhydrous sodium sulfate and concentrated to give a powder (1.31 g). This was applied to a silica gel column (Merck, Silica gel 60, Art. 7734, 40 g) and eluted with a mixed solvent of EtOAc and hexane (1 : 2 ~ 2 : 3) to give **7** (1.15 g, 1.81 mmol, 97%) as a white powder.

Anal Calcd for $C_{25}H_{31}N_2O_{11}S_2Cl$: C 47.28, H 4.92, N 4.41, S 10.10, Cl 5.58.
Found: C 47.19, H 5.14, N 4.18, S 10.23, Cl 5.39.

IR: 3500, 2980, 2940, 1760, 1700, 1430, 1380, 1240, 1110, 1070, 1010 (cm^{-1}) ^{13}C NMR ($CDCl_3$): 218.2/218.3 (Q), 169.5 (Q), 169.4 (Q), 164.9 (Q), 160.1 (Q), 151.9 (Q) 89.3 (Q), 88.6 (Q), 85.2/85.2 (CH), 81.9 (CH), 79.4 (CH), 75.8/75.6 (Q), 75.2/75.1(Q), 67.7 (CH), 63.4/64.0 (CH_2), 46.1 (Q), 41.2 (CH_2), 38.4/38.5 (CH_2), 28.3 (CH_3), 25.1/25.2 (CH_3), 21.3 (CH_3), 20.4 (CH_3), 19.3 (CH_3), 18.1 (CH_3), 14.9 (CH_3).

α-Chloroethylcarbonation of **6** (**8**)

A solution of **6** (381 mg, 0.768 mmol) in dichloromethane (12 ml) was stirred with pyridine (0.12 ml, 2.0 eq) and α-CECF (43% in dichloromethane solution, 0.23 ml, 1.2 eq) at 0°C for 90 minutes. Additional pyridine (0.06 ml) and α-CECF (0.04 ml, 0.2 eq) were added to the mixture. One hour later, the reaction

mixture was diluted with a mixture of EtOAc and hexane (1 : 1, 30 ml). The mixture was washed with 0.05 N HCl twice, 2% sodium bicarbonate, water and brine (15 ml, each), dried over anhydrous sodium sulfate and concentrated to give a powder (467 mg). This was applied to a silica gel column (Merck, Silica gel 60, Art. 7734, 20 g) and eluted with a mixed solvent of EtOAc and hexane (1 : 2 ~ 2 : 3) to give **8** (458 mg, 0.759 mmol, 99%) as a white powder.

Anal Calcd for $C_{25}H_{31}N_2O_{11}SCl$: C 49.79, H 5.18, N 4.65, S 5.32, Cl 5.88.
Found: C 49.71, H 5.32, N 4.50, S 5.26, Cl 5.78.

IR: 3440, 2980, 2940, 1760, 1730, 1430, 1370, 1240, 1150, 1070, 1010 (cm^{-1}) ^{13}C NMR ($CDCl_3$): 219.4 (Q), 172.6/172.6 (Q), 169.7 (Q), 169.6 (Q), 169.4 (Q), 152.3 (Q) 94.5/94.6 (Q), 88.0 (Q), 85.2/85.1 (CH), 81.6 (CH), 79.0 (Q), 78.4 (Q), 76.2/75.1 (CH), 63.4 (CH), 62.5 (CH_2), 46.3 (Q), 38.5 (CH_2), 31.2 (CH_2), 28.3 (CH_3), 25.1 (CH_3), 21.1 (CH_3), 20.4 (CH_3), 19.4 (CH_3), 18.3 (CH_3), 14.7 (CH_3).

Piperazine Carbamate of **1** (**9**)

A solution of **7** (176 mg, 0.277 mmol) in dichloromethane (6.0 ml) was stirred with triethylamine (0.15 ml, 4 eq) and piperazine (49 mg, 2 eq) at 0°C for 60 minutes. The reaction mixture was mixed with EtOAc (8 ml), hexane (8 ml) and 0.16 N HCl (10 ml). The aqueous layer was washed with a mixture of EtOAc and hexane (1 : 1, 10 ml), adjusted to pH 8.4 and extracted with EtOAc (10 ml) three times. The EtOAc layers were combined, washed with 2% sodium bicarbonate, water and brine (15 ml, each), dried over anhydrous sodium sulfate and concentrated to give a powder of **9** (171 mg). This powder was dissolved in 0.1 N HCl (2.6 ml) and freeze-dried to give **9** hydrochloride (159 mg, 0.235 mmol, 85%) as a white powder.

Anal Calcd for $C_{27}H_{37}N_4O_{10}S_2Cl \cdot H_2O$: C 46.65, H 5.65, N 8.06, S 9.22, Cl 5.10.
Found: C 46.54, H 5.84, N 7.85, S 8.17, Cl 4.90.

IR: 3440, 2970, 2940, 1750, 1700, 1430, 1370, 1240, 1170, 1080, 1050 (cm^{-1}) ^{13}C NMR (free amine, $CDCl_3$): 218.2 (Q), 169.6 (Q), 169.4 (Q), 165.2 (Q), 160.5 (Q), 153.7 (Q), 89.3 (Q), 88.7 (Q), 81.9 (CH), 79.8 (CH), 75.9 (Q), 75.5 (Q), 67.7 (CH), 61.6 (CH_2), 46.1 (Q), 41.2 (CH_2), 38.5 (CH_2), 28.3 (CH_3), 21.3 (CH_3), 20.4 (CH_3), 19.3 (CH_3), 18.1 (CH_3), 14.9 (CH_3).

N-Methylpiperazine Carbamate of **1** (**10**)

A solution of **7** (156 mg, 0.246 mmol) in dichloromethane (5.0 ml) was stirred with triethylamine (0.17 ml, 5 eq) and *N*-methylpiperazine (0.05 ml, 2 eq) at 0°C for 90 minutes. The reaction mixture was mixed with EtOAc (5 ml), hexane (5 ml) and 0.18 N HCl (10 ml). The organic layer was extracted with 10% ammonium chloride (6 ml). The aqueous layers were combined, washed with a mixture of EtOAc and hexane (1 : 1, 6 ml), adjusted to pH 8.4 and extracted with EtOAc (10 ml) three times. The EtOAc layers were combined, washed with 2% sodium bicarbonate, water and brine (10 ml, each), dried over anhydrous sodium sulfate and concentrated to give a powder of **10** (161 mg, quant.). This powder (60 mg) was dissolved in 0.1 N HCl (0.8 ml) and freeze-dried to give **10** hydrochloride (57 mg) as a white powder.

Anal Calcd for $C_{28}H_{39}N_4O_{10}S_2Cl \cdot 1.5H_2O$: C 46.82, H 5.89, N 7.80, S 8.93, Cl 4.94.
Found: C 46.94, H 5.79, N 7.79, S 8.70, Cl 4.79.

IR: 3440, 2970, 2940, 1750, 1700, 1460, 1430, 1370, 1240, 1180, 1110, 1080, 1050 (cm^{-1}) ^{13}C NMR (free amine, $CDCl_3$): 218.2 (Q), 169.5 (Q), 169.4 (Q), 165.2 (Q), 160.5 (Q), 153.6 (Q) 89.3 (Q), 88.7 (Q), 81.9 (CH), 79.7 (CH), 75.9 (Q), 75.5 (Q), 67.7 (CH), 61.6 (CH_2), 54.6 (CH_2), 46.1 (CH_3), 46.0 (Q), 44.0 (CH_2), 41.5 (CH_2), 38.5 (CH_2), 28.3 (CH_3), 21.2 (CH_3), 20.4 (CH_3), 19.3 (CH_3), 18.1 (CH_3), 14.9 (CH_3).

Piperazine Carbamate of **8** (**11**)

A solution of **8** (676 mg, 1.12 mmol) in dichloromethane (22 ml) was stirred with triethylamine (0.31 ml, 2 eq) and piperazine (197 mg, 2 eq) at 0°C for 90 minutes. The reaction mixture was mixed with hexane (40 ml) and 0.2 N HCl (17 ml). The organic layer was extracted with water (15 ml). The aqueous layers were combined, washed with a mixture of EtOAc and hexane (1 : 2, 30 ml), adjusted to pH 8.4 and extracted with EtOAc (30 ml) three times. The EtOAc layers were combined, washed with water and brine (30 ml, each), dried over anhydrous sodium sulfate and concentrated to give a powder of **11** (561 mg). This powder was dissolved in 0.1 N HCl (9.0 ml) and freeze-dried to give **11** hydrochloride (551 mg, 0.854 mmol, 76%) as a white powder.

Anal Calcd for $C_{27}H_{37}N_4O_{10}SCl \cdot 2H_2O$: C 47.61, H 6.07, N 8.23, S 4.71, Cl 5.20.
Found: C 47.46, H 5.97, N 8.06, S 4.37, Cl 6.18.

IR: 3440, 2970, 2940, 1720, 1630, 1440, 1370, 1250, 1160, 1110, 1080, 1040 (cm^{-1}) ^{13}C NMR (free amine, $CDCl_3$): 219.3 (Q), 173.0 (Q), 170.2 (Q), 169.6 (Q), 169.5 (Q), 153.9 (Q) 94.6 (Q), 88.1 (Q), 81.6 (CH), 79.6 (Q), 78.8 (Q), 76.5 (CH), 63.4 (CH), 59.3 (CH_2), 46.2 (Q), 45.7 (CH_2), 45.1 (CH_2), 38.3 (CH_2), 31.4 (CH_2), 28.2 (CH_3), 21.2 (CH_3), 20.5 (CH_3), 19.4 (CH_3), 18.3 (CH_3), 14.8 (CH_3).

2-Aminocyclopentanecarboxylic Acid Carbamate of **1** (**12**)

A solution of **7** (336 mg, 0.529 mmol) and (1*R*,2*S*)-2-aminocyclopentanecarboxylic acid *tert*-butyl ester (98 mg, 1.0 eq) in dichloromethane (11 ml) was stirred with triethylamine (0.22 ml, 3 eq) and *N*,*N*-dimethylaminopyridine (DMAP, 8 mg) at 0°C for 2 hours. The reaction mixture was mixed with EtOAc (10 ml), hexane (10 ml) and 0.2 N HCl (10 ml). The aqueous layer was extracted with EtOAc (10 ml) twice. The organic layers were combined, washed with 0.1 N HCl, 2% sodium bicarbonate, water and brine (15 ml, each), dried over anhydrous sodium sulfate and concentrated to give a powder (0.38 g). This was applied to a silica gel column (Merck, Silica gel 60, Art. 7734, 10 g) and eluted with a mixed solvent of EtOAc and hexane (2 : 3 ~ 1 : 1) to give the *tert.*-butyl ester of **12** (147 mg, 0.199 mmol, 38%) as a white powder. This powder (75 mg) was mixed with TFA (0.2 ml) for 30 minutes and dried *in vacuo* to give **12** (75 mg) as a white powder. Compound **12** (19 mg) was dissolved in 2% sodium bicarbonate (5 ml), desalted with Microacilizer G1 (Asahi Kasei Corp.), concentrated and freeze-dried to give the sodium salt of **12** (17 mg) as a white powder.

Anal Calcd for $C_{29}H_{36}N_3O_{12}S_2Na \cdot 11H_2O$: C 38.53, H 6.47, N 4.65, S 7.09.
Found: C 38.37, H 4.56, N 4.37, S 7.24.

IR: 3430, 2970, 1750, 1690, 1560, 1380, 1240, 1110, 1080, 1050 (cm^{-1}) ^{13}C NMR (free acid, $CDCl_3$): 218.2 (Q), 170.8 (Q), 169.7 (Q), 169.7 (Q), 165.2 (Q), 160.6 (Q), 154.7 (Q) 89.2 (Q), 88.7 (Q), 82.0 (CH), 79.8 (CH), 75.7 (Q), 75.6 (Q), 67.7 (CH), 60.8 (CH_2), 54.6 (CH), 46.6 (CH), 46.1 (Q), 41.5 (CH_2), 38.3 (CH_2), 31.8 (CH_2), 28.4 (CH_3), 27.6 (CH_2), 22.3 (CH_2), 21.3 (CH_3), 20.4 (CH_3), 19.3 (CH_3), 18.1 (CH_3), 14.9 (CH_3).

4-Oxalysine Carbamate of **1** (**13**)

A solution of **7** (130 mg, 0.20 mmol) and 4-oxalysine *tert*-butyl ester di-acetic acid salt (69 mg, 1.05 eq) in DMF (4.0 ml) was stirred with triethylamine (0.09 ml, 3 eq) at room temperature for 3.5 hours. The reaction mixture was evaporated, mixed with 0.02 N HCl (15 ml) and washed with a mixture of EtOAc and hexane (1 : 2, 15 ml) twice. The organic layers were combined and extracted with 0.02 N HCl (10 ml) twice. The aqueous layers were combined, adjusted to pH 8.5 and extracted with EtOAc (12 ml) three times. The EtOAc layers were combined, washed with 2% sodium bicarbonate, water and brine (15 ml, each), dried over anhydrous sodium sulfate and concentrated to give a powder (128 mg). This (114 mg) was mixed with TFA (0.5 ml) at ambient temperature for 30 minutes. The reaction mixture was evaporated and diluted with Et_2O to give a powder. This powder (75 mg) was subjected to preparative HPLC (column, ODS; YMC-pack D-ODS-5, mobile phase, 30% acetonitrile/0.01 M P. B.; pH 6.3). The fractions eluted with 310 ~ 350 ml of the solvent were concentrated, desalted with Microacilizer G1 and lyophilized to give a white powder (22 mg). This powder (11 mg) was desalted with Diaion HP-20 (Mitsubishi Chemical Industries Ltd.) chromatography eluting with aqueous MeOH. The fraction eluted with 80% MeOH was concentrated and lyophilized to give **13** (6 mg, 8.5 μmol, 5.7%) as a white powder.

Anal Calcd for $C_{28}H_{38}N_4O_{13}S_2 \cdot 3.0H_2O$: C 44.44, H 5.86, N 7.40, S 8.47.
Found: C 44.20, H 5.40, N 7.48, S 8.07.

IR: 3440, 2970, 1750, 1700, 1630, 1380, 1240, 1100, 1050 (cm^{-1}) ^{13}C NMR (D_2O): 224.2 (Q), 175.6 (Q), 175.5 (Q), 169.3 (Q), 164.3 (Q), 159.8 (Q), 92.5 (Q), 92.2 (Q), 86.2 (CH), 82.9 (CH), 78.4 (Q), 78.2 (Q), 72.7 (CH_2), 70.1 (CH_2), 63.2 (CH_2), 57.6 (CH), 49.2 (Q), 43.5 (CH_2), 43.3 (CH_2), 40.9 (CH_2), 31.4 (CH_3), 23.8 (CH_3), 22.7 (CH_3), 21.2 (CH_3), 20.4 (CH_3), 16.9 (CH_3).

D-Glucosamine Carbamate of **1** (**14**)

A solution of **7** (389 mg, 0.613 mmol) and D-glucosamine acetic acid salt (203 mg, 1.5 eq) in DMF (8.0 ml) was stirred with triethylamine (0.15 ml, 2 eq) at 0°C for 5 hours and then with DMAP (8 mg) for

4 hours. The reaction mixture was evaporated, mixed with water (20 ml) and washed with a mixture of EtOAc and hexane (1 : 1, 15 ml) twice. The organic layers were combined and extracted with water (10 ml). The aqueous layers were combined, washed with a mixture of EtOAc and hexane (1 : 1, 10 ml), mixed with NaCl (3.0 g) and extracted with EtOAc (15 ml) three times. The EtOAc layers were combined, washed with 10% NaCl and brine (15 ml, each), dried over anhydrous sodium sulfate and concentrated to give a powder (122 mg), which was subjected to prep. HPLC (same as above). The fractions eluted with 320 ~ 420 ml of the solvent were concentrated, extracted with EtOAc (10 ml) three times. The EtOAc layers were combined, washed with brine (10 ml), dried over anhydrous sodium sulfate and evaporated to give a crude powder (45 mg). This powder was triturated with Et_2O to afford **14** (42 mg, 0.057 mmol, 9%) as a white powder.

Anal Calcd for $C_{29}H_{39}N_3O_{15}S_2 \cdot 5H_2O$: C 42.28, H 5.99, N 5.10, S 7.78.
Found: C 42.29, H 5.03, N 4.79, S 7.84.

IR: 3410, 2970, 2940, 1750, 1700, 1540, 1430, 1370, 1240, 1080, 1050 (cm^{-1}) ^{13}C NMR ($CDCl_3$): 219.8 (Q), 171.4 (Q), 171.3/171.4 (Q), 166.7 (Q), 162.1 (Q), 157.1/157.4 (Q), 97.0/92.7 (CH), 90.5 (Q), 90.0 (Q), 83.4 (CH), 80.9 (CH), 77.9/75.8 (CH), 77.3/77.2 (Q), 76.8/76.7 (Q), 72.7/73.0 (CH), 72.0/72.3 (CH), 68.8 (CH), 62.8 (CH_2), 61.8/61.7 (CH_2), 57.8/60.5 (CH), 47.1 (Q), 42.2 (CH_2), 39.4 (CH_2), 28.9/28.8 (CH_3), 21.3 (CH_3), 20.4 (CH_3), 19.6 (CH_3), 18.5 (CH_3), 15.1 (CH_3).

Complete Desulfurization of **1** (**15**)

A solution of **1** (160 mg, 0.303 mmol) in ethanol (16 ml) was stirred with Raney Ni (W2, *ca.* 1.0 g) at room temperature for 2.5 hours. The reaction mixture was filtered with Millex GV (0.22 μm). The filtrate was evaporated and applied to a silica gel column (Merck, Silica gel 60, Art. 7734, 5 g) and eluted with 10% methanol-EtOAc to give **15** (37 mg, 0.079 mmol, 26%) as a white powder.

Anal Calcd for $C_{22}H_{30}N_2O_9$: C 56.64, H 6.48, N 6.01.
Found: C 56.39, H 6.62, N 5.98.

IR: 3480, 3400, 2970, 2940, 1750, 1650, 1470, 1370, 1310, 1230, 1120, 1080, 1050 (cm^{-1}) ^{13}C NMR ($CDCl_3$): 219.5 (Q), 169.4 (Q), 169.2 (Q), 167.8 (Q), 165.0 (Q), 90.0 (Q), 88.6 (Q), 81.4 (CH), 76.2 (CH), 67.1 (CH), 61.5 (CH), 59.9 (CH_2), 46.7 (Q), 39.6 (CH_2), 37.1 (CH_2), 30.2 (CH_3), 21.3 (CH_3), 20.4 (CH_3), 19.6 (CH_3), 18.7 (CH_3), 14.7 (CH_3).

Reductive Cleavage of the Disulfide Bond of **1** (**16**)

A solution of **1** (1.11 g, 2.10 mmol) in methanol (37 ml) was stirred with $NaBH_4$ (44 mg, 2 eq) at room temperature for 4.5 hours. Additional $NaBH_4$ (10 mg, 0.5 eq) was added every 30 minutes during the first 90 minutes. The reaction mixture was evaporated and mixed with 0.3 M citric acid (30 ml) and EtOAc (50 ml). The aqueous layer was extracted with EtOAc (15 ml) twice. The EtOAc layers were combined, washed with 10% ammonium chloride, water and brine (30 ml, each), dried over anhydrous sodium sulfate and concentrated to give a powder (1.14 g), which was triturated with Et_2O to afford **16** (932 mg, 1.76 mmol, 84%) as a white powder.

Anal Calcd for $C_{22}H_{30}N_2O_9S_2$: C 49.80, H 5.70, N 5.28, S 12.09.
Found: C 49.80, H 5.89, N 5.23, S 12.16.

IR: 3500, 2970, 2940, 1750, 1670, 1430, 1380, 1240, 1090, 1050 (cm^{-1}) ^{13}C NMR ($CDCl_3$): 218.1 (Q), 169.7 (Q), 169.5 (Q), 167.8 (Q), 163.8 (Q), 89.0 (Q), 87.5 (Q), 82.0 (CH), 81.3 (CH), 68.7 (Q), 68.6 (Q), 68.6 (CH), 65.9 (CH_2), 50.8 (CH_2), 46.1 (Q), 39.7 (CH_2), 28.5 (CH_3), 21.4 (CH_3), 20.4 (CH_3), 19.3 (CH_3), 18.2 (CH_3), 15.0 (CH_3).

S,S'-Dimethylation and Disulfide Bond Cleavage of **1** (**17**)

A solution of **1** (246 mg, 0.465 mmol) in pyridine (1.0 ml) and iodomethane (5.0 ml) was stirred at room temperature for 2 hours. The reaction mixture was diluted with methanol (4 ml) to dissolve the precipitates. $NaBH_4$ (78 mg, 4 eq) was added to the reaction mixture, and after 1 hour, additional $NaBH_4$ (78 mg) was added. The reaction mixture was then stirred for 2 hours, evaporated and partitioned between EtOAc (12 ml) and water (16 ml). The aqueous layer was extracted with EtOAc (12 ml) twice. The EtOAc layers was combined, washed with 0.02 N HCl, 2% sodium bicarbonate, water and brine (10 ml, each), dried over anhydrous sodium sulfate and concentrated to give a powder (306 mg), which was triturated

with Et_2O and hexane to afford **17** (101 mg, 0.181 mmol, 39%) as a white powder.

Anal Calcd for $C_{24}H_{34}N_2O_9S_2 \cdot 0.5\,Et_2O$: C 52.42, H 6.60, N 4.70, S 10.76.
Found: C 52.34, H 6.60, N 4.78, S 10.85.

IR: 3460, 2970, 2930, 2880, 1750, 1670, 1650, 1430, 1380, 1240, 1110, 1090, 1050 (cm^{-1}) ^{13}C NMR ($CDCl_3$): 218.4 (Q), 170.1 (Q), 169.6 (Q), 165.7 (Q), 163.0 (Q), 89.4 (Q), 88.0 (Q), 81.9 (CH), 81.4 (CH), 72.6 (Q), 70.4 (Q), 68.3 (CH), 64.4 (CH_2), 46.1 (Q), 43.4 (CH_2), 40.5 (CH_2), 28.7 (CH_3), 21.6 (CH_3), 20.5 (CH_3), 19.3 (CH_3), 18.2 (CH_3), 15.5 (CH_3), 15.0 (CH_3), 13.4 (CH_3).

S-Methylation of **16** (**18**)

A solution of **16** (94 mg, 0.177 mmol) in dichloromethane (3 ml) was stirred with pyridine (0.14 ml, 10 eq) and iodomethane (0.05 ml, 4 eq) at room temperature for 5 hours. The reaction mixture was diluted with EtOAc (20 ml) and Et_2O (4 ml), washed with 0.02 N HCl, 2% sodium bicarbonate, water and brine (10 ml, each), dried over anhydrous sodium sulfate and concentrated to give a powder (58 mg). This powder was subjected to silica gel thin layer chromatography (Merck, Silica gel 60, Art. 5717, 200 × 150 mm) developing with EtOAc. The band of Rf 0.4~0.6 was separated and eluted with 10% methanol - EtOAc. The eluate was evaporated to give **18** (11 mg, 0.02 mmol, 11%) as a white powder.

Anal Calcd for $C_{23}H_{32}N_2O_9S_2$: C 50.72, H 5.92, N 5.14, S 11.77.
Found: C 50.47, H 5.98, N 4.88, S 11.38.

IR: 3450, 2970, 2930, 1750, 1670, 1430, 1380, 1240, 1090, 1050 (cm^{-1}) ^1H NMR ($CDCl_3$): 0.96 (3H, s), 1.02 (3H, s), 1.29 (3H, d, J=6.4 Hz), 2.04 (6H, s), 2.18 (3H, s), 2.39 (1H, dd, J= 13.7, 9.5 Hz), 2.55 (1H, dd, J=13.7, 9.2 Hz), 2.78 (1H, br, OH), 3.04 (1H, dd, J=15.7, 1.2 Hz), 3.16 (3H, s), 3.42 (1H, d, J=15.7 Hz), 3.92 (1H, dd, J=12.0, 7.6 Hz), 4.18 (1H, q, J=6.4 Hz), 4.43 (1H, s, SH), 4.48 (1H, br d, J=12.0 Hz), 5.15 (1H, t, J=9.3 Hz), 5.85 (1H, s). ^{13}C NMR ($CDCl_3$): 218.3 (Q), 169.8 (Q), 169.4 (Q), 168.2 (Q), 161.5 (Q), 89.1 (Q), 87.5 (Q), 82.0 (CH), 81.4 (CH), 70.8 (Q), 69.0 (Q), 68.5 (CH), 63.4 (CH_2), 50.2 (CH_2), 46.1 (Q), 40.0 (CH_2), 29.2 (CH_3), 21.5 (CH_3), 20.4 (CH_3), 19.3 (CH_3), 18.2 (CH_3), 15.0 (CH_3), 13.5 (CH_3).

Cyclic Carbonation of **4** (**19**)

A solution of **4** (110 mg, purity 83%, 0.205 mmol) in toluene (5.5 ml) was stirred with 1,1'-carbonyldiimidazole (170 mg, 5 eq.) at 80°C for 4 hours. The reaction mixture was filtered. The filtrate was diluted with EtOAc (10 ml) and hexane (5 ml), washed with 10% ammonium chloride, water and brine (10 ml, each), dried over anhydrous sodium sulfate and concentrated to give a powder (147 mg). This powder was subjected to silica gel thin layer chromatography (Merck, Silica gel 60, Art. 5717, 200 × 200 mm) developing with a mixture of EtOAc - hexane (2 : 1). The band of Rf 0.53~0.66 was separated and eluted with EtOAc. The eluate was evaporated to give **19** (45 mg, 0.116 mmol, 57%) as a white powder.

Anal Calcd for $C_{19}H_{20}N_2O_7 \cdot 1.5\,H_2O$: C 54.94, H 5.58, N 6.74.
Found: C 55.02, H 5.21, N 6.54.

IR: 3450, 2980, 1820, 1760, 1690, 1640, 1610, 1430, 1350, 1230, 1170, 1100, 1070 (cm^{-1}) ^{13}C NMR ($CDCl_3$): 217.6 (Q), 153.6 (Q), 152.9 (Q), 152.2 (Q), 137.3 (Q), 136.8 (Q), 110.4 (CH), 105.3 (CH_2), 95.2 (Q), 86.3 (Q), 85.1 (CH), 82.9 (CH), 66.2 (CH), 46.5 (Q), 39.4 (CH_2), 29.9 (CH_3), 18.9 (CH_3), 18.2 (CH_3), 14.3 (CH_3).

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