Marine Natural Products. XXXI.[1] Structure–Activity Correlation of a Potent Cytotoxic Dimeric Macrolide Swinholide A, from the Okinawan Marine Sponge *Theonella swinhoei*, and Its Isomers

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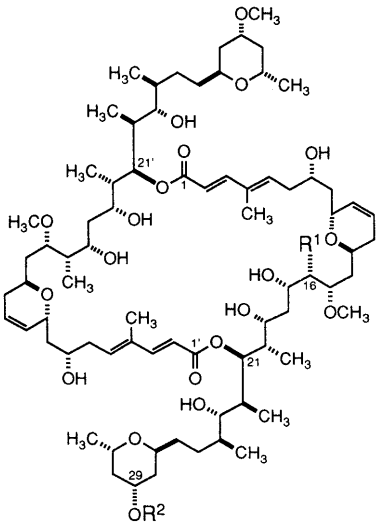
Acidic treatment of swinholide A (1), which was characterized as the major cytotoxic macrolide from the Okinawan marine sponge *Theonella swinhoei*, provided several isomeric macrolides having different size of the dilactone ring structure. From the structure–activity correlation viewpoint, the *in vitro* cytotoxicities and *in vivo* antitumor activities of these dimeric macrolides, together with two monomeric macrolides which were synthesized from 1, have been examined.

Keywords marine sponge; *Theonella swinhoei*; swinholide A; cytotoxicity; antitumor activity; macrolide dimeric

In our continuing search for new bioactive substances from marine organisms, we have investigated the constituents of the Okinawan marine sponge *Theonella swinhoei* and have isolated five new bioactive tridecapeptide lactones, named theonellapeptolides Ia—e,[2,3] two new 3-keto-4-methylene steroids, theonellasterone and conicasterone, and a Diels–Alder type dimeric steroid, named bistheonellasterone,[1] as well as four potent cytotoxic dimeric macrolides, named swinholides A (**1**), B (**2**), and C (**3**) and isoswinholide A (**4**).[4–7]

Among those swinholide analogs, swinholides A (**1**), B (**2**), and C (**3**) were shown to exhibit potent cytotoxicities against L1210 and KB cell lines with IC_{50} values of 0.03, 0.30, and 0.14 μg/ml (for L1210) and 0.04, 0.04, and 0.05 μg/ml (for KB), respectively. However, isoswinholide A (**4**), which differs from **1** only in the size of the dilactone ring, was found to show a weaker cytotoxicity [IC_{50} 1.35 μg/ml (L1210) and 1.1 μg/ml (KB)] than **1**, **2**, and **3**.

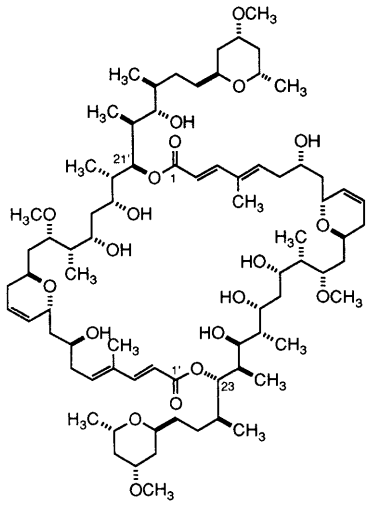
We have also examined the cytotoxicities of several derivatives (**5**—**9**) of swinholide A (**1**), which were prepared during the course of the structure elucidation study of **1**.[6] Interestingly, the monomeric free acid **6** was shown to exhibit considerably weakened cytotoxicity (IC_{50} 14 μg/ml) against KB cells while the monomeric methyl ester **5** was found to lack cytotoxicity. On the other hand, the monomeric alcohol **7**, which was obtained by diisobutyl aluminum hydride (DIBAL) reduction of **1**, was found to show little growth inhibition for KB cells (13.7% inhibition at 10 μg/ml). Furthermore, the octaformate **8** and the diacetonide **9**, in which hydroxyl groups in the macrolide ring of swinholide A (**1**) were protected, were also found to show very little growth inhibition for KB cells (51.1%



swinholide A (**1**): $R^1 = R^2 = CH_3$

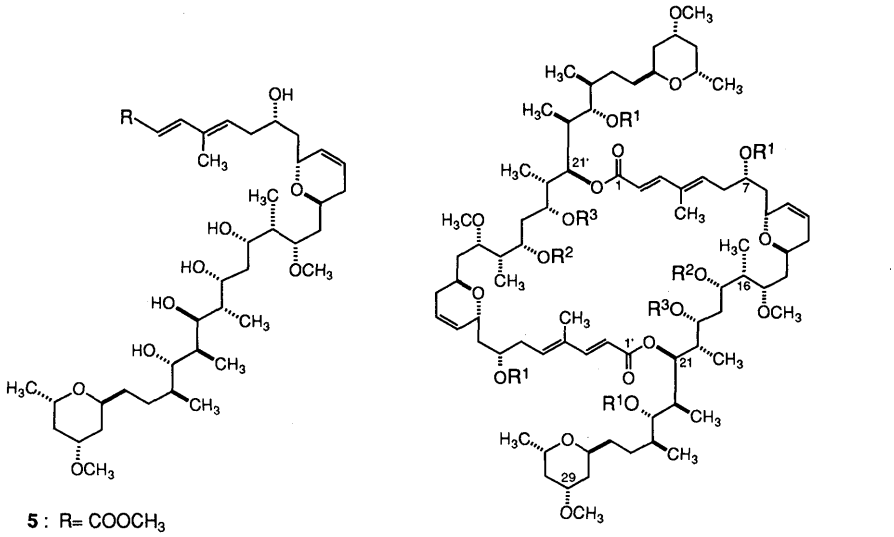
swinholide B (**2**): $R^1 = H$, $R^2 = CH_3$

swinholide C (**3**): $R^1 = CH_3$, $R^2 = H$



isoswinholide A (**4**)

Chart 1



5: R= $COOCH_3$
6: R= COOH
7: R= CH_2OH

8: $R^1 = R^2 = R^3 = CHO$
9: R^1= H, R^2, R^3= $-C(CH_3)_2-$

Chart 2

TABLE I. Cytotoxic Activities of Swinholide A (**1**) towards Human Carcinoma Cell Lines

Tumor cell line and origin		IC_{50} (μg/ml)
KB	Oral, epidermoid cell carcinoma	0.04
SW-480	Colon, adenocarcinoma	0.070
KATO-III	Gastric, adenocarcinoma	0.050
HT-1080	Fibrosarcoma	0.017
T-24	Bladder, transitional cell carcinoma	0.046
PC-3	Lung, adenocarcinoma	6.0
PC-8	Lung, adenocarcinoma	0.12
PC-9	Lung, adenocarcinoma	0.13
PC-10	Lung, squamous cell carcinoma	0.11
PC-13	Lung, large cell carcinoma	0.10
QG-56	Lung, squamous cell carcinoma	0.043
Daudi	Burkitt lymphoma	0.036

inhibition at 50 μg/ml and 19.3% inhibition at 10 μg/ml, respectively). These results have led us to presume that the presence and the size of the macrocyclic dilactone-ring as well as the three-dimensional conformation of the ring may be essential for exhibiting the cytotoxic activity.

In our parallel study, the cytotoxicities of the major dimeric macrolide swinholide A (**1**) against various tumor cell lines have been examined. As shown in Table I, **1** was found to exhibit potent cytotoxicities with different selectivities against those tumor cell lines, which had originated from various human carcinomas. Among twelve cell lines examined, the most significant difference of 350 times was observed between cytotoxicity against HT-1080 fibrosarcoma cells (IC_{50} 0.017 μg/ml) and cytotoxicity against PC-3 lung adenocarcinoma cells (IC_{50} 6.0 μg/ml). These findings as well as the above-mentioned results have led us to investigate in more detail the structure–activity correlation of these dimeric macrolides.[8,9]

As reported in our previous paper,[7] treatment of swinholide A (**1**) with *p*-toluenesulfonic acid monohydrate (*p*-TsOH) in chloroform at room temperature for 7 h

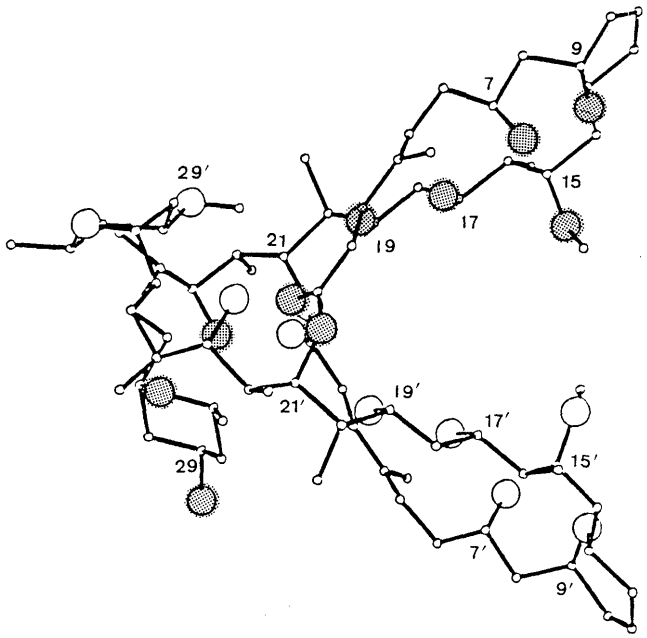


Fig. 1. A Possible Molecular Conformation of Swinholide A (**1**) Based on the Energy Calculation Study

provided isoswinholide A (**4**, 12%) and some isomers together with recovered **1** (60%). On the other hand, we have obtained a possible molecular conformation of swinholide A (**1**) as shown in Fig. 1 by energy calculation based on the X-ray crystallographic analysis data for a swinholide A derivative.[10] It is interesting to note that the oxygen atoms in this model conformation are all directed to the interior of the dilactone ring structure. So, it has been presumed that the lactonic linkages of swinholide A (**1**) may migrate readily upon acidic treatment to provide several isomeric dilactones of different sizes, and the cytotoxicities of those dilactones are of interest.

To shed light on the correlation between chemical

structures (the dilactone structures in particular) and cytotoxic activities, we have prepared several dilactone isomers from swinholide A (**1**). Thus, *p*-TsOH treatment of **1** in methylene chloride was carried out at room temperature for 3 d to convert **1** to afford the 23,17′-diolide (**10**), the 23,23′-diolide (**11**, obtained as the major isomer), isoswinholide A (23,21′-diolide, **4**), the 23,19′-diolide (**12**), and the 19,19′-diolide (**13**) together with recovered **1** (21,21′-diolide) in 9 : 20 : 11 : 13 : 10 : 8 ratio, respectively. Furthermore, it was found that similar *p*-TsOH treatment of isoswinholide A (**4**) also furnished the same isomeric mixture as demonstrated by reversed-phase high-performance liquid chromatography (HPLC) analysis of the reaction product. It is noteworthy that swinholide A (**1**), obtained most abundantly from the sponge, may not be the most thermodynamically favored isomer.

Each of **10**, **11**, **12**, or **13** gave its quasi-molecular ion peak at *m/z* 1411 $(M+Na)^+$ in the fast atom bombardment mass spectrum (FAB-MS), indicating that these products have the same molecular formula $C_{78}H_{132}O_{20}$ as that of swinholide A (**1**) and are lactone linkage isomers. The proton nuclear magnetic resonance (^{1}H-NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra of the 23,17′-diolide (**10**) and 23,19′-diolide (**12**) were very complicated due to their asymmetric dimeric natures. The 23,23′-diolide (**11**) and 19,19′-diolide (**13**) both gave 39 carbon signals in the ^{13}C-NMR spectra due to their C_2 symmetrical structures.

^{1}H–^{1}H correlation spectroscopy (COSY) and homonuclear Hartmann Hahn spectroscopy (HOHAHA) studies of the major 23,23′-diolide (**11**) allowed us to assign all the ^{1}H signals. Thus, a low-field-shifted signal of H_{23} [observed at δ 4.97 (br d)] was observed to couple with the signals of H_{22} [δ 1.88 (m)] and H_{24} [δ 1.90 (m)], both of which were observed to couple further with methyl doublets of C_{22} [δ 0.90 (d)] and C_{24} [δ 0.91 (d)], respectively. The HOHAHA spectrum of **11** further showed the connectivity of methyl protons at C-24 (δ 0.91) to H_{23} and geminal methylene protons at C-25 (δ 1.52 and 1.30), which were further shown to be coupled with other methylene protons at C-26 (δ 1.25 and 1.90). Consequently, the 23,23′-diolide **11** has been elucidated to have a 48-membered macrocyclic dilactone structure as shown.

The location of the lactone linkages in another C_2 symmetrical dimeric macrolide, the 19,19′-diolide (**13**), has been determined by means of HOHAHA experiments.

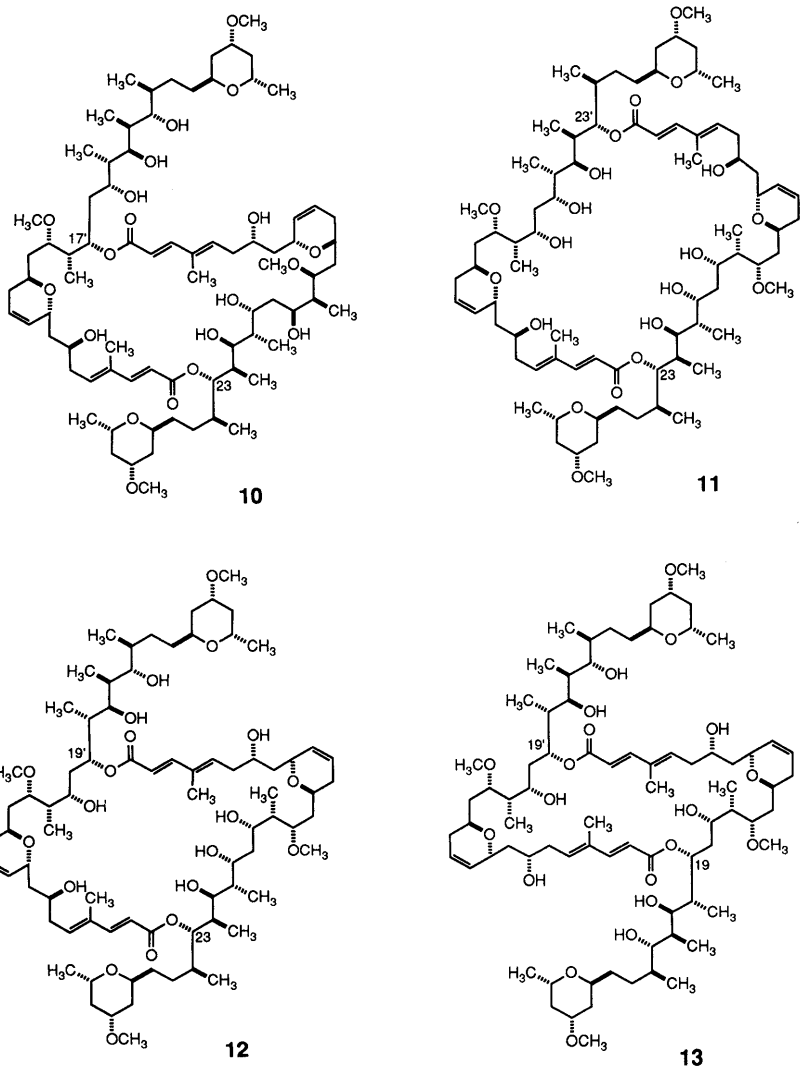


Chart 3

TABLE II. ^{1}H-NMR Data for **11**, **13**, **15**, and **16** in $CDCl_3$

	11	**13**	**15**	**16**
2	5.82 (d, J=15.5)	5.78 (d, J=15.5)	5.80 (d, J=15.5)	5.87 (d, J=15.5)
3	7.42 (d, J=15.5)	7.49 (d, J=15.5)	7.38 (d, J=15.5)	7.39 (d, J=15.5)
4-Me	1.82 (s)	1.79 (s)	1.81 (s)	1.85 (s)
5	6.11 (br dd, J=*ca.* 8, 8)	6.05 (br dd, J=*ca.* 8, 8)	5.89 (br dd, J=*ca.* 8.5, 8.5)	6.02 (br dd, J=*ca.* 8, 8)
6	2.34, 2.43 (m)[a]	2.24, 2.43 (m)[a]	*ca.* 2.5 (m)[b]	2.46 (m)[b]
7	4.09 (m)	4.06 (m)	4.01 (m)	4.03 (m)
8	1.46, 1.75 (m)[a]	1.50, 1.77 (m)[a]	*ca.* 1.3, 1.75 (m)[b]	1.50, 1.78 (m)[b]
9	4.52 (br d, J=*ca.* 10)	4.50 (m)	4.47 (br d, J=10)	4.44 (br d, J=9.5)
10	5.66 (br d, J=*ca.* 10)	5.67 (br d, J=*ca.* 8.5)	5.67 (br d, J=*ca.* 10)	5.70 (br d, J=*ca.* 9.5)
11	5.79 (m)	5.82 (m)	5.82 (m)	5.80 (dddd, J=2, 2, 5, 9.5)
12a	2.10 (br d, J=*ca.* 16)	2.08 (m)	2.02 (m)	2.08 (m)
12b	1.95 (m)	1.95 (m)	1.95 (m)	2.00 (m)
13	3.77 (m)	3.73 (m)	3.40 (m)	3.65 (m)
14	1.52, 2.00 (m)[a]	1.53, 2.05 (m)[a]	*ca.* 1.6, 1.82 (m)[b]	1.68, 1.95 (m)[b]
15	3.82 (m)	3.83 (m)	3.50 (m)	3.72 (m)
15-OMe	3.38 (s)	3.36 (s)	3.37 (s)	3.35 (s)
16	1.65 (m)	1.70 (m)	1.70 (m)	1.70 (m)
16-Me	0.79 (d, J=7)	0.86 (d, J=6)	0.81 (d, J=7)	0.76 (d, J=7.5)
17	3.52 (m)	3.67 (m)	3.70 (m)	3.81 (m)
18	1.65, 1.87 (m)[a]	1.70, 1.97 (m)[a]	*ca.* 1.6, 1.70 (m)[b]	1.45, 1.57 (m)[b]
19	4.00 (m)	5.57 (m)	3.89 (m)	3.91 (m)
20	1.90 (m)	1.97 (m)	2.02 (m)	2.06 (m)
20-Me	0.79 (d, J=7)	0.86 (d, J=7)	0.93 (d, J=7)	0.92 (d, J=6.5)
21	3.52 (m)	3.70 (m)	5.24 (dd, J=1.5, 10.5)	3.54 (m)
22	1.88 (m)	1.75 (m)	1.95 (m)	1.82 (m)
22-Me	0.90 (d, J=7)	0.87 (d, J=6)	0.87 (d, J=7)	0.91 (d, J=7)
23	4.97 (br d, J=*ca.* 11.5)	3.33 (m)	3.36 (dd, J=2, 9.5)	4.97 (dd, J=2, 10)
24	1.90 (m)	1.80 (m)	1.70 (m)	1.90 (m)
24-Me	0.91 (d, J=7)	0.99 (d, J=6.5)	1.02 (d, J=7)	0.96 (d, J=6.5)
25	1.30, 1.52 (m)[a]	1.23, 1.92 (m)[a]	*ca.* 1.3, 1.44 (m)[b]	1.32, 1.54 (m)[b]
26	1.25, 1.90 (m)[a]	1.25, 1.84 (m)[a]	1.25, 2.02 (m)[b]	1.27, 1.90 (m)[b]
27	4.03 (m)	3.97 (m)	3.99 (m)	4.00 (m)
28	1.65, 1.83 (m)[a]	1.62, 1.83 (m)[a]	*ca.* 1.6, 1.82 (m)[b]	1.65, 1.82 (m)[b]
29	3.55 (m)	3.55 (m)	3.55 (dddd, J=4.5, 4.5, 10, 12)	3.55 (br d, J=*ca.* 9.5)
29-OMe	3.34 (s)	3.34 (s)	3.35 (s)	3.34 (s)
30	1.23, 2.00 (m)[a]	1.20, 1.97 (m)[a]	1.17, 2.02 (m)[b]	1.24, 2.00 (m)[b]
31	3.71 (m)	3.70 (m)	3.72 (m)	3.72 (m)
31-Me	1.23 (d, J=6)	1.21 (d, J=6)	1.20 (d, J=6.5)	1.23 (d, J=6.5)

a) Four-proton intensity in total. *b*) Two-proton intensity in total.

Thus, a low-field-shifted signal of H_{19} [observed at δ 5.57 (m)] was shown to be connected to an oxymethine signal of H_{17} at δ 3.67, which was shown to be coupled with a methine signal of H_{16} (δ 1.70). Furthermore, the methine signal of H_{16} was shown to be coupled with an oxymethine signal of H_{15} (δ 3.83), which was further shown to be connected to an oxymethine signal of H_{13}. In consequence, the structure of the 19,19′-diolide has been elucidated as **13**, having a 40-membered macrocyclic dilactone structure.

The ^{1}H-NMR spectrum of an asymmetrical dilactone, the 23,19′-diolide (**12**), showed proton signals ascribable to both the 23,23′-diolide (**11**) and the 19,19′-diolide (**13**). One low-field-shifted signal at δ 4.97 (due to H_{23}) was shown to be coupled with a methine proton signal of H_{24} (δ 1.90), which was further observed as coupled with methylene proton signals due to H_{25} (δ 1.27 and 1.49). Another low-field-shifted signal at δ 5.63 (due to $H_{19'}$) was shown to have the same connectivity as in the 19,19′-diolide (**13**) by COSY and HOHAHA experiments. Consequently, the structure of the 23,19′-diolide has been elucidated as **12**, having a 44-membered macrocyclic dilactone structure.

Finally, the 23,17′-diolide (**10**) showed two low-field-shifted methine signals at δ 4.94 ($H_{17'}$) and 4.90 (H_{23}) in its ^{1}H-NMR spectrum. The signal of H_{23} (δ 4.90) was shown to possess the same connectivity as in the 23,23′-diolide (**11**) by means of COSY and HOHAHA experiments. The signal of $H_{17'}$ (δ 4.94) was shown to be coupled with a methine signal of $H_{16'}$ at δ 2.15, which was further observed as coupled with an oxymethine signal of $H_{15'}$ at δ 3.78. The HOHAHA spectrum of **10** further led to a connection of the oxymethine signal of $H_{15'}$ to an oxymethine signal of $H_{13'}$ at δ 3.70. Consequently, the structure of the 23,17′-diolide has been elucidated as **10**, having a 42-membered macrocyclic dilactone structure. As mentioned above, swinholide A (**1**) with a 44-membered dilactone ring was obtained as the major macrocyclic diolide from the fresh marine sponge, whereas the 23,23′-diolide (**11**) having a 48-membered dilactone structure has been shown to be the most thermodynamically favored isomer among these six isomeric macrocyclic diolides (**1**, **4**, **10**, **11**, **12**, and **13**).

Next, in order to distinguish the conformational features of these dimeric macrolides, their circular dichroism (CD) spectra were measured. As shown in Table IV, these dimeric macrolides gave characteristic CD spectra, in some

TABLE III. ^{1}H-NMR Data for **10** and **12** in $CDCl_3$

	10		**12**	
	H_n	$H_{n'}$	H_n	$H_{n'}$
2	5.75, 5.85 (d, J=15.5)		5.78, 5.83 (d, J=15.5)	
3	7.32, 7.40 (d, J=15.5)		7.34, 7.51 (d, J=15.5)	
4-Me	1.80 (s)		1.79 (s)	
5	6.13, 6.17 (br dd, J=*ca.* 7, 8.5)		6.03, 6.22 (br dd, J=*ca.* 6.5, 6.5)	
6a	2.45, 2.50 (ddd, J=8.5, 8.5, 16)		2.40, 2.44 (m)	
6b	2.35 (m), 2.37 (br d, J=*ca.* 8, 15.5)		2.20, 2.30 (m)	
7	4.03, 4.08 (m)		4.06, 4.10 (m)	
8	1.49, 1.58, 1.74, 1.86 (m)[a]		1.52, 1.68, 1.76 (m)[a]	
9	4.50 (br d, J=*ca.* 7.5)		4.51, 4.53 (m)	
10	5.65 (br d, J=*ca.* 9.5)		5.66 (m)	
11	5.77 (m)		5.79 (m)	
12a	2.05 (m)		2.20 (m)	
12b	1.95 (m)		1.84 (m)	
13	3.70 (m)		3.87 (m)	
14	1.54, 1.96 (m)	2.05 (m)	1.44, 2.15 (m)	1.95 (m)
15	3.87 (m)	3.78 (m)	3.72 (m)	3.75 (m)
15-OMe	3.39 (s)	3.28 (s)	3.32, 3.36 (s)	
16	1.70 (m)	2.15 (m)	1.72 (m)	1.66 (m)
16-Me	0.78 (d, J=7)	0.92 (d, J=7)	0.78 (d, J=7)	0.82 (d, J=7)
17	4.25 (br d, J=*ca.* 10.5)	4.94 (dd, J=5.5, 11.5)	3.84 (m)	3.71 (m)
18	1.46, 1.58, 1.90 (m)[a]		1.50, 1.60 (m)	1.65, 2.00 (m)
19	3.83 (m)	4.08 (m)	4.22 (br d, J=*ca.* 10)	5.63 (m)
20	1.65 (m)	1.75 (m)	1.72 (m)	1.95 (m)
20-Me	0.80 (d, J=7)	0.92 (d, J=7)	0.77 (d, J=7)	0.88 (d, J=7)
21	4.01 (m)	3.95 (br d, J=*ca.* 9.5)	4.02 (m)	3.56 (m)
22	1.88 (m)	1.80 (m)	1.92 (m)	1.72 (m)
22-Me	0.87 (d, J=7)	0.97 (d, J=7)	0.89 (d, J=7.5)	0.92 (d, J=7.5)
23	4.90 (dd, J=2.5, 12.5)	3.40 (m)	4.97 (dd, J=3.5, 9)	3.44 (m)
24	1.90 (m)	1.65 (m)	1.90 (m)	1.78 (m)
24-Me	0.92 (d, J=7)	0.72 (d, J=7)	0.83 (d, J=7.5)	0.89 (d, J=7.5)
25	1.26, 1.52 (m)[a]		1.27, 1.49 (m)	1.33, 1.52 (m)
26	1.20, 1.25 (m)[a]		1.26, 1.90 (m)	1.52 (m)
27	4.04 (m)		4.05 (m)	
28	1.65, 1.85 (m)[a]		1.60, 1.80 (m)[a]	
29	3.55 (m)		3.54 (m)	
29-OMe	3.35 (s)		3.34 (s)	
30	1.20, 1.96 (m)[a]		1.22, 1.90 (m)[a]	
31	3.70 (m)		3.71 (m)	
31-Me	1.21, 1.22 (d, J=6)		1.19, 1.20 (d, J=6.5)	

a) Four-proton intensity in total.

of which the exciton couplings presumably arose from two 2,4-dienoate chromophores. In other words, these isomers take distinct three-dimensional ring conformations in solution. The cytotoxic activities against L1210 and KB cell lines and toxicity against brine shrimp *Artemia salina* of these dimeric macrolides were then examined and the results are summarized in Table IV. Swinholide A (**1**) showed cytotoxic activity and toxicity over fifty times as strong as other macrocyclic diolides. It is interesting to note that swinholide A (**1**) was isolated from the marine sponge *Theonella swinhoei* as the major macrolide constituent and was shown to exhibit significant cytotoxicity and strong toxicity even though it is thermodynamically less favored as compared with allied macrocyclic diolides (*i.e.* swinholide family).

In 1986, Moore and his group characterized a cytotoxic monomeric macrolide scytophycin C (**14**), which was isolated from the culture of the terrestrial blue-green alga *Scytonema pseudohofmanni*.[11] As was pointed out previously,[5,6] the atomic array in the structure of the monomeric unit in swinholide A (**1**) is mostly very similar to that in scytophycin C (**14**) and, in addition, the configurations at the asymmetric carbons in **1** are mostly

TABLE IV. CD Data and Cytotoxic Activities of Swinholide A (**1**) and Its Isomers

Macrolide	CD max. (MeOH) nm ($\Delta\varepsilon$)	IC_{50} (μg/ml) L1210	IC_{50} (μg/ml) KB	Brine shrimp LD_{100} (μg/ml)
Swinholide A (**1**) (21,21′-diolide)	280 (−5)	0.004	0.011	0.3
10 (23,17′-diolide)	280 (−10) 258 (+3)	0.11	0.84	>15
11 (23,23′-diolide)	284 (−37) 255 (+35)	0.58	1.8	14
Isoswinholide A (**4**) (23,21′-diolide)	283 (−4)	0.12	1.4	15
12 (23,19′-diolide)	282 (+25) 256 (−19)	1.3	2.1	>15
13 (19,19′-diolide)	283 (+34) 256 (−36)	1.1	1.2	>15

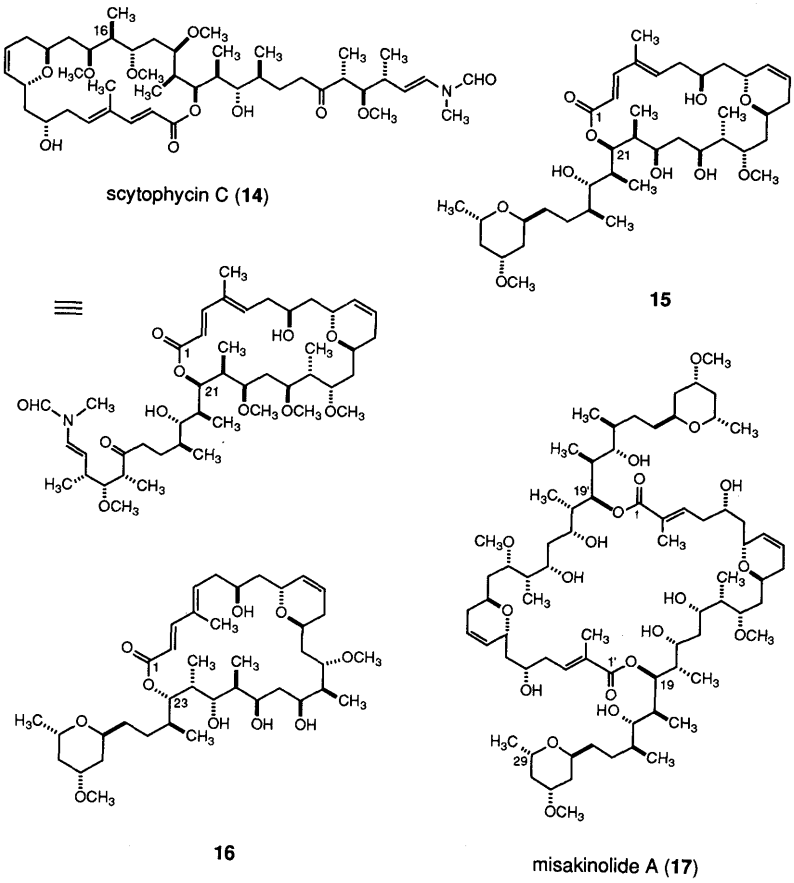


Chart 4

the same as those in **14**. Therefore, we wondered whether "a lactone" derivable from the monomeric unit of **1** might exhibit cytotoxicity. To examine this question, we have prepared monomeric lactones from **1**.

As described above, alkaline hydrolysis of swinholide A (**1**) afforded the monomeric acid **6** in quantitative yield. After various attempts, it was found that treatment of **6** with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) and 4-dimethylaminopyridine provided the 21-olide (**15**) and the 23-olide (**16**) in 41% and 24% yields, respectively. Both **15** and **16** gave the quasi-molecular ion peak at m/z 695 $(M+H)^+$ in their FAB-MS, which indicated that these products are isomers with the same molecular formula, $C_{39}H_{66}O_{10}$, corresponding to one half of swinholide A (**1**) and are lactone isomers in regard to the monomeric unit in **1**. The chemical structures of the 21-olide and the 23-olide have been elucidated as **15** and **16**, respectively, on the basis of COSY experiments, which have allowed us to assign all ^1H signals (Table II).

The 21-olide (**15**) thus obtained was shown to exhibit moderate [less than one-tenth of the cytotoxicity of parent swinholide A (**1**)] cytotoxicity (IC_{50} values of 0.76 and 0.13 μg/ml against L1210 and KB cell lines), while the 23-olide (**16**) was found to exhibit very weak cytotoxicity [IC_{50} 6.5 (L1210) and 7.5 (KB) μg/ml].

Another dimeric macrolide, misakinolide A (**17**), which possesses a 40-membered dilactone structure, was isolated from another Okinawan marine sponge of *Theonella* sp. The stereostructure of **17** is the same as that of swinholide A (**1**) except that **17** lacks one conjugated double bond in each monomeric unit of **1**.[12-14] In regard to the *in vitro* cytotoxicity and *in vivo* antitumor activity, misakinolide

TABLE V. Effects of Swinholide A (**1**) and Its Isomers on P388 Leukemia in CDF_1 Mice

	Dose (mg/kg)[a]	Day	T/C (%)
Swinholide A (**1**)	0.003	1, 5	110
(21,21'-diolide)	0.01	1, 5	110
	0.03	1, 5	115
	0.05	1, 5	Toxic
Isoswinholide A (**4**)	0.01	1, 5	110
(23,21'-diolide)	0.05	1, 5	105
23,23'-Diolide (**11**)	0.01	1, 5	125
	0.05	1, 5	120
	0.1	1, 5	Toxic
21-Olide (**15**)	1	5, 10	110
	3	5, 10	110
	10	5	Toxic

a) The compounds were dissolved in DMSO and injected i.p.

TABLE VI. Inhibition of Macromolecule Synthesis in L1210 Cells by Swinholide A (**1**)

Swinholide A (μg/ml)	% inhibition of ^3H uptake		
	Thymidine	Uridine	Leucine
0.004	13.6	15.7	12.6
0.010	47.1	49.0	41.0

A (**17**) is an interesting compound. Thus, **17** was reported to exhibit not only potent cytotoxicity [IC_{50} 0.035 μg/ml (L1210)] but also antitumor activity [T/C 140% at the dosage of 0.31 mg/kg (mice) against P388 leukemia].

We have examined the antitumor effects of swinholide A (**1**) and its isomers on P388 leukemia in CDF_1 mice. As shown in Table V, we found unexpectedly that the macrocyclic diolides, swinholide A (**1**), isoswinholide A (**4**), and 23,23′-diolide (**11**), were toxic and did not show promising antitumor activity. The 21-olide (**15**), the monomeric lactone, was also examined but showed no antitumor activity at 3 mg/kg dose.

Finally, to clarify the mechanism of the cytotoxicity of swinholide A (**1**), the inhibition of macromolecule synthesis in L1210 cells caused by swinholide A (**1**) was examined and the results are shown in Table VI. Swinholide A (**1**) was found to inhibit the uptake of tritium-labeled thymidine, uridine, or leucine and these inhibitions were dose-dependent. These results suggest that some mechanism(s) other than the inhibition of DNA, RNA or protein synthesis may be involved in its antitumor properties. Further studies are in progress on the mode of antitumor action of swinholide A (**1**).

Experimental

The instruments used to obtain physical data and the experimental conditions for chromatography were the same as those described in our previous paper.[3]

Alkaline Hydrolysis of Swinholide A (1) Giving 6 A solution of **1** (150 mg) in MeOH (1 ml) was treated with 1 N aqueous KOH (0.5 ml) and the whole was stirred at 25 °C for 4 h. After addition of 1 N aqueous HCl (0.6 ml), the reaction mixture was partitioned into an AcOEt–H_2O mixture. The AcOEt-soluble portion was taken and washed with brine, then dried over $MgSO_4$. Removal of the solvent from the AcOEt solution under reduced pressure furnished **6** (144 mg). **6**: A white powder, $[\alpha]_D$ −32.7° (c=1.1, $CHCl_3$, 21 °C). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3390, 2930, 1680, 1614, 1378, 1075. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.37 (d, J=15.5 Hz, H_3), 6.04 (br dd, J=*ca.* 7, 7 Hz, H_5), 5.81 (m, H_{11}), 5.78 (d, J=15.5 Hz, H_2), 5.65 (dd, J=2, 10 Hz, H_{10}), 4.51 (br d, J=*ca.* 8 Hz, H_9), 4.14 (br d, J=*ca.* 8 Hz, H_7), 4.04 (m), 3.86 (m), 3.80 (m), 3.73 (m), 3.55 (ddd, J=4.5, 10, 14.5 Hz), 3.37, 3.33 (both s, $H_{15\text{-}OMe,29\text{-}OMe}$), 3.30 (m, H_{23}), 2.44 (ddd, J=7, 7.5, 15 Hz, $H_{6a(6b)}$), 2.35 (ddd, J=7, 7, 15 Hz, $H_{6b(6a)}$), 2.14 (d, J=15 Hz), 1.78 (s, $H_{4\text{-}Me}$), 1.00 (d, J=7 Hz), 0.87 (d, J=6.5 Hz), 0.84 (d, J=7 Hz), 0.76 (d, J=7 Hz). HR-FAB-MS: Obsd: m/z 751.435. Calcd for $C_{39}H_{68}KO_{11}$: 751.440 $(M+K)^+$.

Reductive Degradation of Swinholide A (1) Giving 7 A solution of **1** (100 mg) in dry toluene (2 ml) was treated dropwise with 1.5 M DIBAL solution (0.7 ml) at 0 °C and the whole was stirred at 0 °C under an N_2 atmosphere for a further 1 h. The reaction was quenched by adding ether saturated with water and 4 N aqueous NaOH, and the precipitates were removed by filtration. The filtrate was evaporated under reduced pressure to give a crude product (92 mg), which was purified by HPLC [Cosmosil $5C_{18}$, MeOH–H_2O (5 : 1)] to furnish **7** (49 mg). **7**: A white powder, $[\alpha]_D$ −27.3° (c=0.2, $CHCl_3$, 21 °C). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3400, 2930, 1465, 1378, 1074, 967. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.29 (d, J=15.5 Hz, H_3), 5.82 (ddd, J=10.5, 4.5, 2 Hz), 5.76 (dt, J=15.5, 6 Hz, H_2), 5.67 (br dd, J=*ca.* 2, 10.5 Hz), 5.57 (br dd, J=*ca.* 7, 7 Hz), 4.57 (br dd, J=*ca.* 7, 7 Hz), 4.53 (br d, J=*ca.* 10 Hz), 4.20 (d, J=6 Hz, H_1), 3.74 (dddd, J=3, 3, 6.5, 6.5 Hz), 3.69 (ddd, J=2.5, 6.5, 6.5 Hz), 3.41, 3.35 (both s, $H_{15\text{-}OMe,29\text{-}OMe}$), 2.17 (br d, J=*ca.* 17 Hz), 1.78 (s, $H_{4\text{-}Me}$), 1.22 (d, J=6.5 Hz), 1.04 (d, J=7 Hz), 0.89 (d, J=5.5 Hz), 0.87 (d, J=6 Hz), 0.77 (d, J=7 Hz). HR-FAB-MS: Obsd: m/z 721.480. Calcd for $C_{39}H_{70}NaO_{10}$: 721.476 $(M+Na)^+$.

Acidic Treatment of Swinholide A (1) Giving 10, 11, 12, and 13 A solution of **1** (80 mg) in CH_2Cl_2 (2 ml) was treated with p-TsOH (3 mg). The mixture was stirred at room temperature for 3 d and then partitioned into a mixture of EtOAc–water saturated with $NaHCO_3$. The EtOAc layer was taken and washed with brine, then dried over $MgSO_4$. The solvent was removed from the EtOAc solution under reduced pressure, and the crude product was subjected to HPLC [Cosmosil $5C_{18}$, MeOH–H_2O (7 : 1)] to furnish swinholide A (**1**, 8 mg), the 23,17′-diolide (**10**, 9 mg), the 23,23′-diolide (**11**, 20 mg), isoswinholide A (**4**, 11 mg), the 23,19′-diolide (**12**, 13 mg), and the 19,19′-diolide (**13**, 10 mg) in order of elution.

10: A white powder, $[\alpha]_D$ −21.6° (c=1.7, $CHCl_3$, 25 °C). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3450, 3020, 2970, 1685, 1615, 1175, 980. ^{1}H-NMR: as given in Table III. ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C: 196.9, 167.0, 151.2, 150.3, 140.3, 139.8, 134.4, 134.3, 130.0, 129.7, 123.9, 123.8, 115.7, 114.8, 80.1, 79.9, 78.1, 77.6, 77.0, 74.6, 73.5, 73.3, 72.0, 71.8, 71.3, 70.0, 68.7, 68.4, 67.7, 67.3, 64.9, 64.8, 64.6, 57.4, 56.8, 55.4, 55.3, 40.8, 40.7, 40.6, 40.4, 38.7, 38.6, 38.5, 38.0, 37.5, 37.2, 36.5, 35.8, 35.3, 35.1, 35.0, 34.9, 34.6, 33.0, 31.0, 30.8, 29.8, 29.3, 29.0, 27.8, 25.2, 21.8 (2C), 17.1, 16.9, 12.6, 12.4, 11.0, 10.9, 10.4, 10.2, 8.8, 7.7. HR-FAB-MS: Obsd: m/z 1389.936. Calcd for $C_{78}H_{133}O_{20}$: 1389.939 $(M+H)^+$. **11**: A white powder, $[\alpha]_D$ −42.5° (c=4.2, $CHCl_3$, 25 °C). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3430, 3030, 2940, 1685, 1615, 1265, 1180, 990. ^{1}H-NMR: as given in Table II. ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C: 169.6, 151.6, 139.9, 134.3, 129.9, 123.8, 114.7, 79.5, 77.1, 76.7, 75.1, 73.4, 71.8, 71.2, 68.1, 66.9, 65.3, 64.9, 57.6, 55.4, 42.1, 40.6, 39.8, 38.5, 37.7, 36.7, 36.1, 35.8, 35.2, 33.0, 30.6, 29.4, 25.1, 21.8, 17.1, 12.9, 12.6, 9.6, 8.7. HR-FAB-MS: Obsd: m/z 1389.939. Calcd for $C_{78}H_{133}O_{20}$: 1389.939 $(M+H)^+$. **12**: A white powder, $[\alpha]_D$ +12.2° (c=3.4, $CHCl_3$, 25 °C). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3430, 3040, 2940, 1680, 1615, 1180, 980. ^{1}H-NMR: as given in Table III. ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C: 169.8, 168.9, 152.1, 151.0, 140.5 (2C), 134.5, 134.4, 130.0, 129.8, 123.8, 123.4, 115.1, 114.7, 80.0, 78.4, 76.6, 74.8, 73.4, 71.7, 71.5, 71.0, 70.9, 70.6, 68.9, 67.3, 66.6, 66.3, 65.6, 64.8, 64.4, 57.6, 57.2, 55.3, 41.2, 40.6, 40.5, 40.3, 39.4, 38.7, 38.6, 38.1, 37.6 (2C), 37.0, 36.2, 35.8, 35.1, 34.9, 34.7, 33.9, 33.2, 31.1, 30.0, 29.8, 29.2, 29.1, 26.3, 25.5, 21.8, 17.2, 17.0, 12.5, 12.4, 11.0, 9.9, 9.5 (2C), 8.8. HR-FAB-MS: Obsd: m/z 1389.942. Calcd for $C_{78}H_{133}O_{20}$: 1389.939 $(M+H)^+$. **13**: A white powder, $[\alpha]_D$ +20.9° (c=0.5, $CHCl_3$, 25 °C). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3420, 3020, 2940, 1685, 1610, 1170, 1080, 970. ^{1}H-NMR: as given in Table II. ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C: 169.2, 151.4, 139.9, 134.8, 130.0, 123.8, 115.0, 79.7, 73.4, 72.2, 71.7, 70.9, 68.3, 66.9, 64.8, 57.1, 55.3, 40.8, 40.3, 40.2, 38.7, 38.6, 37.8, 37.2, 35.6, 35.2, 34.8, 34.6, 30.9, 29.8, 29.4, 29.2, 27.9, 21.9, 16.9, 12.6, 10.4, 9.6. HR-FAB-MS: Obsd: m/z 1389.941. Calcd for $C_{78}H_{133}O_{20}$. 1389.939 $(M+H)^+$.

Lactonization of 6 Giving 15 and 16 A solution of **6** (41 mg) in CH_2Cl_2 was treated with EDCI (24 mg) and 4-dimethylaminopyridine (14 mg). The reaction mixture was stirred at 25 °C for 3 h. The solvent was evaporated under reduced pressure, then the crude product was subjected to HPLC (Cosmosil $5C_{18}$AR, MeOH–H_2O (3 : 1)] to furnish the 21-olide (**15**, 17 mg) and the 23-olide (**16**, 10 mg).

15: A white powder. ^{1}H-NMR: as given in Table II. HR-FAB-MS: Obsd: m/z 695.471. Calcd for $C_{39}H_{67}O_{10}$: 695.473 $(M+H)^+$. **16**: A white powder. ^{1}H-NMR: as given in Table II. HR-FAB-MS: Obsd: m/z 695.474. Calcd for $C_{39}H_{67}O_{10}$: 695.473 $(M+H)^+$.

Assay of *in Vitro* Antitumor Activity The tetrazolium-based semi-automated colorimetric assay (MTT assay) developed by Carmichael *et al.*[15] was modified and used for the *in vitro* assay. Briefly, 2000 cells in 180 μl of Rosewell Park Memorial Institute Medium 1640 (RPMI-1640 medium) (Nissui Pharmaceutical Co., Osaka) supplemented with 10% heat-inactivated fetal bovine serum (Nipro Co., Osaka), penicillin (100 units/ml) and streptomycin (0.1 μg/ml) were seeded in a 96-well flat-bottomed microtest plate (InterMed, Roskilde, Denmark) and 20 μl aliquots of drug solutions of graded concentrations were simultaneously added to triplicate wells. The plate was incubated for 3 d at 37 °C in a humidified atmosphere of 5% CO_2. MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide](Sigma Chemical Co., St. Louis, MO, U.S.A.) was prepared at a concentration of 2 mg/ml in Dulbecco's phosphate-buffered saline without calcium and magnesium and stored in a refrigerator. On day 3, MTT reagent (25 μl) was added to each well. After another 4-h incubation at 37 °C, the microplate was centrifuged at 3000 rpm for 10 min and the medium was removed by aspiration. To solubilize the resulting MTT-formazan, 0.2 ml of dimethyl sulfoxide (DMSO) was added to each well followed by thorough mixing with a mechanical plate mixer. Absorbance at 540 nm (OD_{540}) was measured with an ImmunoReader NJ-2000 (InterMed Japan, Tokyo). The percentage cell growth inhibition was calculated by means of the following formula: % cell growth inhibition = $(1-T/C)\times 100$, where C is the mean OD_{540} of the control group and T is that of the treated group. The 50%-inhibitory drug concentration (IC_{50} value) was determined graphically from the dose-response curve with at least 3 drug concentration points.

Assay of *in Vivo* Antitumor Activity Swinholide A (**1**) and its isomers were tested for antitumor activity against intraperitoneally implanted murine leukemia P388 in CDF_1 mice. One million cells were intraperitoneally transplanted on day 0. The mice were randomly assigned to several experimental groups, each consisting of 6 mice. The compounds were dissolved in DMSO and injected intraperitoneally on days 1 and 5 or 5 and 10 after tumor inoculation at the stated doses. The median survival time of the control group was 10 d. The ratio of median survival time of the treated mice (*T*) *vs.* the control mice (*C*) was calculated.

Inhibition of Macromolecular Synthesis in L1210 Cells RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Nipro Co., Osaka) and antibiotics was used as the cell culture medium (RPMI–FCS). Murine L1210 leukemia cells (10^5 cells/ml in RPMI–FCS) were prepared as a target. Swinholide A (**1**) was dissolved in DMSO at graded concentrations from 1 to 0.0001 μg/ml. Cell suspension (198 μl) and sample solution (2 μl) were mixed in a 96-well U-bottomed micro test plate (NUNC, Roskilde, Denmark). In this case, the final drug concentrations were from 10 to 0.001 ng/ml. The plate was incubated in a humidified atmosphere of 5% CO_2 for 24 h. Four hours before the cell harvest, [6-^{3}H]thymidine (0.75 μCi in 15 μl of saline: specific activity 28 Ci/mmol), [5-^{3}H]uridine (0.75 μCi in 15 μl of saline: specific activity 28 Ci/mmol), and [3,4,5-^{3}H]-L-leucine (1.5 μCi in 15 μl of saline: specific activity 5 Ci/mmol) purchased from New England Nuclear (Boston, Mass) were added to the culture as precursors of DNA, RNA, and protein synthesis, respectively. Cells were harvested on a glass-fiber disk using a PHD™ cell harvester (Cambridge Technology, Watertown, Mass). The disk was successively washed with water and dried. Radioactivity was determined with an LSC-1000 liquid scintillation counter (Aloka Co., Tokyo) using Scintisol EX-H counting solution (Wako Pure Chemical Industries, Osaka). Each experiment was performed in quadruplicate. Inhibition of macromolecular synthesis was calculated from the incorporation of tritiated precursors into the macromolecular fraction of cells on the disk using the following formula: percentage inhibition $(\%) = (A - B) \times 100$, where A is the mean tritium count (DPM) of the control group and B is that of the treated group.

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