CALICHEAMICINS, A NOVEL FAMILY OF ANTITUMOR ANTIBIOTICS†

3. ISOLATION, PURIFICATION AND CHARACTERIZATION OF CALICHEAMICINS β_1^{Br}, γ_1^{Br}, α_2^{I}, α_3^{I}, β_1^{I}, γ_1^{I} AND δ_1^{I}

May D. Lee, Joann K. Manning, David R. Williams, Nydia A. Kuck, Raymond T. Testa and Donald B. Borders

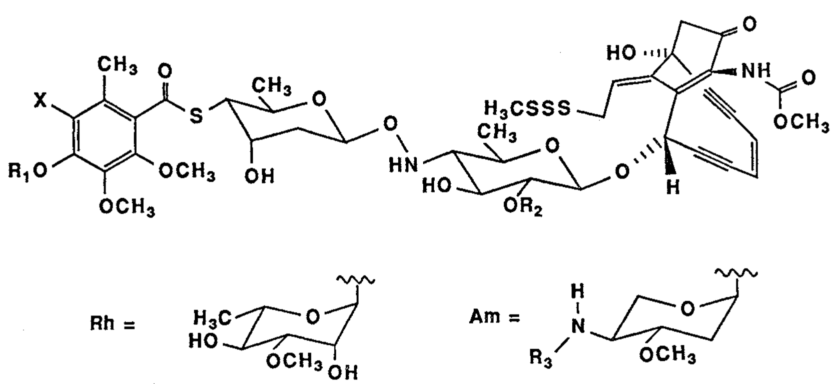
American Cyanamid Company, Medical Research Division, Lederle Laboratories, Pearl River, New York 10965, U.S.A.

(Received for publication December 16, 1988)

Novel antitumor antibiotics, calicheamicins β_1^{Br}, γ_1^{Br}, α_2^{I}, α_3^{I}, β_1^{I}, γ_1^{I} and δ_1^{I} were recovered from the fermentation broth of *Micromonospora echinospora* ssp. *calichensis* by solvent extraction, selective precipitation, normal phase, reversed phase and partition chromatography. The individual components were characterized by their UV, IR, ^1H and ^{13}C NMR spectral data.

During the course of our search for new fermentation-derived antitumor agents guided by the biochemical prophage induction assay (BIA),[1,2] a partially purified broth extract of *Micromonospora echinospora* ssp. *calichensis* (NRRL 15839) demonstrated potent activity *in vivo* against murine tumors P388 and B16. Two components, calicheamicins, β_1^{Br} (**1**) and γ_1^{Br} (**2**), were isolated and characterized. Subsequent strain improvement and media studies led to the production of calicheamicins α_2^{I} (**3**), α_3^{I} (**4**), β_1^{I} (**5**), γ_1^{I} (**6**) and δ_1^{I} (**7**) by new strains of *M. echinospora* ssp. *calichensis*, NRRL 15975 and

Table 1. Chemical structures of calicheamicins β_1^{Br}, γ_1^{Br}, α_2^{I}, α_3^{I}, β_1^{I}, γ_1^{I} and δ_1^{I}.



Calicheamicin	X	R_1	R_2	R_3
β_1^{Br} (**1**)	Br	Rh	Am	$CH(CH_3)_2$
γ_1^{Br} (**2**)	Br	Rh	Am	CH_2CH_3
α_2^{I} (**3**)	I	H	Am	CH_2CH_3
α_3^{I} (**4**)	I	Rh	H	
β_1^{I} (**5**)	I	Rh	Am	$CH(CH_3)_2$
γ_1^{I} (**6**)	I	Rh	Am	CH_2CH_3
δ_1^{I} (**7**)	I	Rh	Am	CH_3

† This paper is dedicated to Professor Kenneth L. Rinehart in honor of his 60th birthday.

NRRL 18149.[3,4] The chemical structures of the calicheamicins are shown in Table 1. The stereochemistry for the glycosidic linkage to the aglycone has been changed since our preliminary reports on the structure elucidation of calicheamicin γ_1^{I}, the major component of the complex.[5,6] A full paper on the details of the structure elucidation of the calicheamicins is in preparation. The isolation, characterization and antimicrobial activities of the calicheamicins are described in this report. The taxonomy of the producing organism and the production of the calicheamicins will be reported

Fig. 1. Process for the isolation of calicheamicins β_1^{Br} and γ_1^{Br} from the fermentation of NRRL 15839.

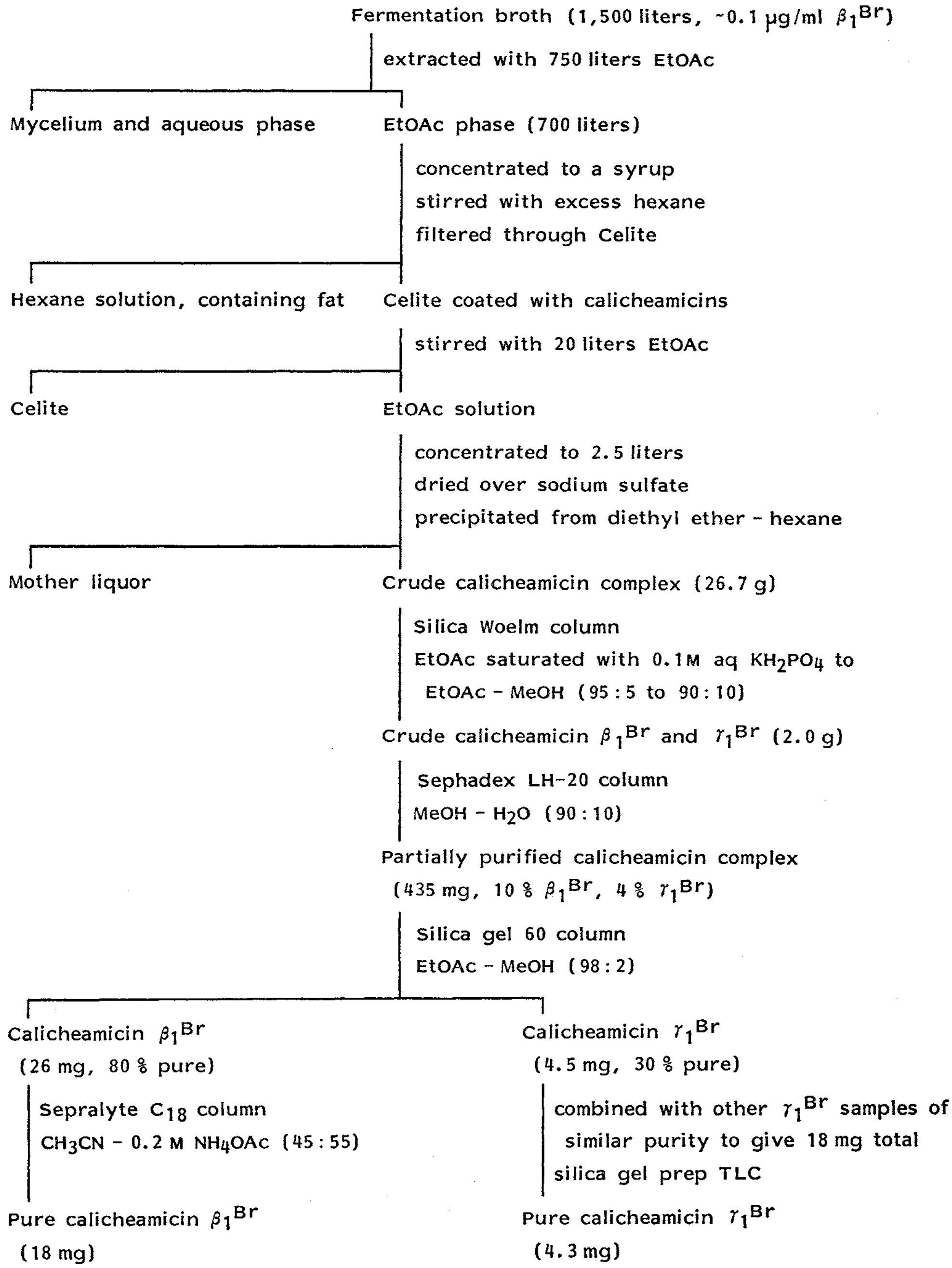


Table 2. Physico-chemical properties of calicheamicins β_1^{Br}, γ_1^{Br}, α_2^{I}, α_3^{I}, β_1^{I}, γ_1^{I} and δ_1^{I}.

	Calicheamicin						
	β_1^{Br} (1)	γ_1^{Br} (2)	α_2^{I} (3)	α_3^{I} (4)	β_1^{I} (5)	γ_1^{I} (6)	δ_1^{I} (7)
$[\alpha]_D^{26}$ (EtOH)	−49° (*c* 0.1)	−93° (*c* 0.11)				−103° (*c* 0.45)	
FAB-MS[a]	1,334/1,336 (M+H)		1,208 (M+H)	1,211 (M+H) 1,233 (M+Na)		1,368 (M+H)	1,354 (M+H)
HRFAB-MS	1,258.3699 (^{79}Br)[b] M+H−CS_2, Δ 2.3 mmu 1,260.3726 (^{81}Br) M+H−CS_2, Δ 7.0 mmu					1,368.2878[a] M+H, Δ 5.7 mmu	
MW	1,333/1,335	1,319/1,321	1,207	1,210	1,381	1,367	1,353
Molecular formula	$C_{56}H_{78}N_3O_{21}S_4Br$	$C_{55}H_{74}N_3O_{21}S_4Br$	$C_{48}H_{62}N_3O_{17}S_4I$	$C_{47}H_{59}N_2O_{19}S_4I$	$C_{56}H_{78}N_3O_{21}S_4I$	$C_{55}H_{74}N_3O_{21}S_4I$	$C_{54}H_{72}N_3O_{21}S_4I$
Anal	Found / Calcd					Found / Calcd	
C	48.61 / 50.37					48.81 / 48.28	
H	5.64 / 5.70					5.41 / 5.41	
N	2.93 / 3.15					2.75 / 3.07	
O	— / 25.19					— / 24.58	
S	9.10 / 9.60					9.03 / 9.36	
Br	5.51 / 6.00						
I						9.21 / 9.29	
IR (cm^{-1})	3440, 2970, 2930, 1720, 1680, 1460, 1390, 1330, 1245, 1160~980	3440, 2980, 2940, 1720, 1680, 1460, 1420, 1395, 1385, 1335, 1245, 1160~980	3400, 2980, 2940, 1720, 1680, 1560, 1510, 1460, 1410, 1385, 1340, 1310, 1245, 1150~980	3440, 2980, 2940, 1720, 1680, 1455, 1415, 1390, 1320, 1240, 1150~980	3450, 2970, 2930, 1720, 1680, 1460, 1415, 1390, 1325, 1240, 1160~980	3440, 2980, 2940, 1720, 1680, 1455, 1415, 1390, 1325, 1240, 1200, 1150~980 (Fig. 6)	3440, 2980, 2940, 1720, 1680, 1455, 1415, 1390, 1320, 1240, 1140~950

Appearance: Off-white amorphous powder, solubility: practically insoluble in water, soluble in CH_2Cl_2, EtOAc and the lower alcohols, stability: fairly stable between pH 4.5 and pH 7.0, very sensitive to acids and bases, poor stability in alcohols and aqueous alcohols, quite stable in CH_2Cl_2 and EtOAc, UV: identical for all components, no apparent absorption maximum, the UV spectrum of calicheamicin γ_1^{I} is shown in Fig. 7.

[a] Sulfalane matrix.

[b] Dithiothreitol/dithioerythritol (magic bullet) matrix.

separately.

Isolation of Calicheamicins β_1^{Br} and γ_1^{Br}

Most of the calicheamicins found in the fermentation broth of *M. echinospora* ssp. *calichensis* were associated with the mycelium and were recovered by extracting the whole fermentation mash with ethyl acetate. The organic extract, containing the antibiotic complex and large amounts of fat, was concentrated to remove the ethyl acetate. The residue was stirred with excess hexane. The antibiotic complex and other hexane insoluble material forming a sticky and oily suspension was collected by filtration through a pad of Celite. The calicheamicins, recovered from the Celite by ethyl acetate extraction, were precipitated into a mixture of diethyl ether and hexane. Purification and separation of components β_1^{Br} and γ_1^{Br} was accomplished by repeated chromatography on silica gel, Sephadex LH-20, and C_{18} bonded silica as depicted in Fig. 1 and in the Experimental. The entire chromatographic purification procedure was guided by the BIA.

Characterization of Calicheamicins β_1^{Br} and γ_1^{Br}

The physico-chemical properties of calicheamicins β_1^{Br} and γ_1^{Br} were summarized in Table 2 and their chromatographic properties were summarized in Tables 3 and 4. They were isolated as off-white amorphous powders, practically insoluble in water, soluble in dichloromethane, ethyl acetate and the lower alcohols. Preliminary fast atom bombardment mass spectral (FAB-MS, thioglycerol matrix) and field desorption mass spectral (FD-MS) analysis, although failed to determine the molecular weight of calicheamicin β_1^{Br}, suggested that it contained bromine. High-resolution electron impact mass spectral (HREI-MS) analysis identified the presence of sulfur in the low mass fragments. The presence of bromine and sulfur as well as nitrogen, oxygen and carbon in calicheamicin β_1^{Br} was confirmed by electron spectroscopy for chemical analysis (ESCA). The ^{13}C NMR (Table 5) and

Table 3. Mobility of calicheamicins β_1^{Br} (1), γ_1^{Br} (2), α_2^{I} (3), α_3^{I} (4), β_1^{I} (5), γ_1^{I} (6) and δ_1^{I} (7) on TLC.

Solvent system	Rf value						
	1	2	3	4	5	6	7
EtOAc saturated with 0.1 M KH_2PO_4 (aq)	0.24	0.18	0.61	0.55	0.24	0.18	0.11
3% 2-propanol in EtOAc saturated with 0.1 M KH_2PO_4 (aq)	0.35	0.28	0.75	0.69	0.35	0.28	0.19
EtOAc - MeOH (95 : 5)	0.36	0.27	0.73	0.61	0.36	0.27	0.20

Adsorbent: Silica gel 60 F_{254} pre-coated aluminum sheets, 0.2 mm layer thickness, EM reagents.
Detection: $UV_{254\,nm}$ quenching and bioautography using the biochemical induction assay.

Table 4. Mobility of calicheamicins β_1^{Br} (1), γ_1^{Br} (2), α_2^{I} (3), α_3^{I} (4), β_1^{I} (5), γ_1^{I} (6) and δ_1^{I} (7) in HPLC.

HPLC system	Retention time (minutes)						
	1	2	3	4	5	6	7
System I	7.8	6.5	15.7	3.5	8.2	6.8	5.3
System II	3.6	3.0	8.5	1.5	3.9	3.2	2.4

System I: Column, Ultrasphere-ODS, 5 μm, 4.6 mm×25 cm, Altex-Beckman; mobile phase, CH_3CN - 0.2 M NH_4OAc, pH 6.0 (55 : 45), 1.0 ml/minute; detection, UV absorbance at 254 and 280 nm; instrument, Hewlett-Packard, HP1090M.

System II: Column, Microsorb, 3 μm, 4.6 mm×5 cm, Rainin; mobile phase, CH_3CN - 0.2 M NH_4OAc, pH 6.0 (50 : 50), 1.0 ml/minute; detection, UV absorbance at 254 and 280 nm; instrument, Hewlett-Packard, HP1090M.

Table 5. ^{13}C NMR spectral data of calicheamicins β_1^{Br}, γ_1^{Br}, α_2^{I}, α_3^{I}, β_1^{I}, γ_1^{I} and δ_1^{I}.

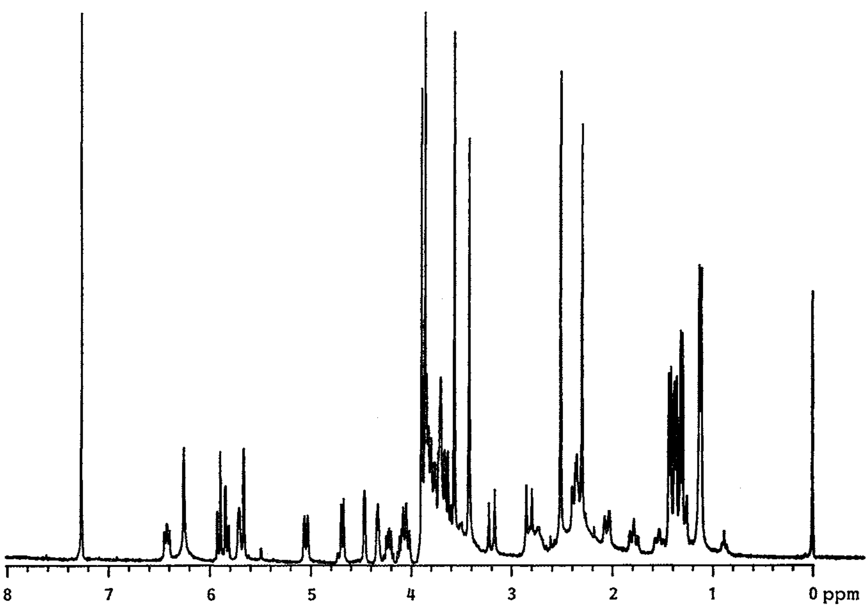
β_1^{Br} (1)[a]	β_1^{I} (5)[a]	γ_1^{Br} (2)[b]	γ_1^{I} (6)[a,c]	α_3^{I} (4)[b]	α_2^{I} (3)[b]	δ_1^{I} (7)[b]
192.4	192.6	192.9	192.4 s	192.6 s	193.2	192.8
191.7	192.3	192.5	191.9 s	192.4 s	192.8	192.7
			175.1 s			
154.8	*154.2	155.6	154.5 s	154.7 s	155.5	155.5
149.5 s	151.8	149.6	151.6 s	151.6 s	151.6	151.5
149.3 s	150.9	149.5	150.7 s	150.5 s	149.2	150.3
145.7		146.5	145.1 s	*142.3	*145.8	146.6
144.6 s	*143.2	144.7	143.1 s	143.0 s	137.1	142.8
138.8 s	*139.2	138.0	139.1 s	136.8 s	137.0	137.7
130.8 s	133.4	131.8	133.4 s	133.5 s	133.1	133.2
131.2 s	131.2	131.1	131.0 s	130.7 s	131.3	131.4
130.2 s	130.1	130.4	130.3 s	130.4 s	127.1	130.1
126.5 d	126.4	126.9	126.3 d	127.3 d	126.3	126.6
124.4 d	124.4	124.9	124.4 d	124.7 d	125.1	124.7
123.4 d	123.4	123.0	123.4 d	123.4 d	122.7	122.7
102.5 d	102.6	103.0	102.7 d	103.1 d		103.0
100.8 s	100.8	101.3	101.0 s	101.0 s	100.8	100.9
100.2	100.1	98.8	100.3 s	98.6 s	100.4	98.9
99.7 d	99.6	100.2	99.7 d	100.1 d	100.1	99.8
99.7 d	99.6	100.0	99.6 d	103.4 d	98.2	99.6
97.4 d	97.4	97.8	97.4 d		98.0	97.5
115.1 s	93.6	115.3	93.5 s	93.7 s	85.4	93.4
88.2 s	88.1	88.4	88.1 s	87.6 s	87.4	88.1
83.3 s	83.3	82.9	83.0 s	83.1 s	82.6	82.5
81.0 d	80.8	81.0	81.0 d	80.8 d		80.4
77.2		77.4	76.0 d	74.5 d	79.0	77.2
76.1 d	76.2	76.1	75.8 d		71.8	76.0
72.1 s	72.2	71.7	72.2 s	72.1 s	71.8	71.3
71.9 d	71.8	71.8	72.0 d	71.3 d		71.1
71.1 d	71.1	71.1	71.2 d	70.9 d	71.0	70.7
70.2 d	70.4	70.8	70.5 d	70.8 d		70.6
69.7 d	69.6	70.1	69.8 d	70.1 d	70.3	69.8
69.1 d	69.1	69.7	69.2 d	69.5 d	69.7	69.5
68.4 d	68.4	68.23	68.5 d	70.0 d	69.6	67.7
68.4 d	68.4	68.18	68.4 d	68.0 d	67.9	67.7
66.9 d	68.4	67.2	67.2 d	67.1 d	67.7	66.8
68.0 d	67.0	69.4	67.2 d	67.3 d		66.8
61.7 q	62.2	61.9	61.7 q	61.8 q	61.4	61.4
62.4 t	61.6	61.8	61.3 t		61.2	61.0
61.0 q	60.9	61.1	60.9 q	61.0 q	60.7	60.6
57.8 d	57.9	59.3	60.2 d		60.7	60.4
57.2 q	57.2	57.3	57.3 q	57.3 q		57.0
56.3 q	56.3	56.3	56.3 q		56.3	56.1
54.6 t	54.8	54.1	54.7 t	53.7 t	53.8	53.6
52.7 q	52.8	52.7	52.7 q	53.3 q	53.3	52.4
51.7 d	51.6	51.6	51.8 d	51.5 d	51.3	51.0
		42.1	42.2 t		41.4	
39.2 t	39.2	39.5	39.4 t	39.2 t	39.6	39.0
36.9 t	36.9	37.6	37.0 t	37.4 t	37.4	37.3
34.3 t	34.3	34.0	34.1 t		33.1	33.5
19.7 q	25.4	19.7	25.4 q	25.3 q	24.7	25.0
22.8 q	22.8	22.8	22.8 q	22.7 q	22.7	22.4
			20.8 q			

Table 5. (Continued)

β_1^{Br} (1)[a]	β_1^{I} (5)[a]	γ_1^{Br} (2)[b]	γ_1^{I} (6)[a,c]	α_3^{I} (4)[b]	α_2^{I} (3)[b]	δ_1^{I} (7)[b]
18.9 q	18.9	19.0	19.0 q	19.0 q	18.8	18.5
17.64 q	17.6	17.9	17.7 q	18.0 q	17.7	17.6
17.60 q	17.6	17.6	17.6 q	17.5 q		17.2
		14.4	14.4 q		*12.0	
47.8 d	47.9					
22.4 q	22.4					
23.5 q	23.4					
						33.3

[a] Recorded at 75 MHz in $CDCl_3$ on a Nicolet NT-300.
[b] Recorded at 75 MHz in $CDCl_3$ with a few drops of CD_3OD on a Nicolet NT-300.
[c] The spectral data of its NH_4OAc salt is reported since all the signals are sharp.
* Broad diffuse signals.

Fig. 2. ^1H NMR spectrum of calicheamicin β_1^{Br} in $CDCl_3$ (300 MHz).

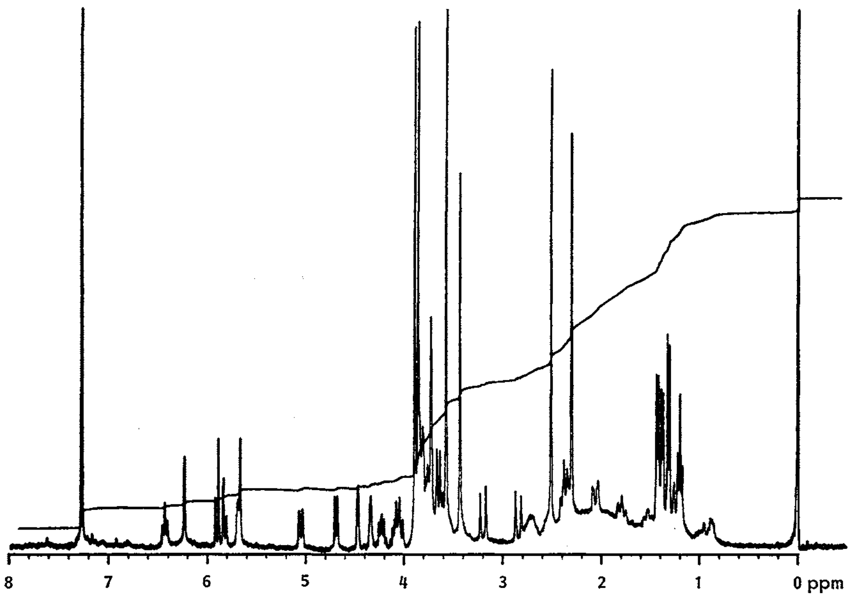


^1H NMR (Figs. 2 and 3) data suggested that both calicheamicins β_1^{Br} and γ_1^{Br} contained 50~60 carbons and 3~4 glycosidic units, and that the difference between the two components was an isopropyl substitution in β_1^{Br} *versus* an ethyl substitution in γ_1^{Br}. Both calicheamicins β_1^{Br} and γ_1^{Br} are active in the BIA at <1 pg/ml concentration and are extremely potent as antibacterials (Table 6). The physico-chemical properties described above in conjunction with their extremely high potency defined calicheamicins β_1^{Br} and γ_1^{Br} as novel antitumor antibiotics.

Discovery of the Iodine Containing Calicheamicins

In an attempt to increase the fermentation yields of calicheamicins β_1^{Br} and γ_1^{Br}, various bromides were supplemented to the fermentation medium. No obvious effects were observed. However, when the fermentation medium was supplemented with sodium iodide, a dramatic increase in the fermentation yields, based on the BIA, was observed. Analysis of the broth extracts by TLC-bioautography

Fig. 3. ^{1}H NMR spectrum of calicheamicin γ_1^{Br} in $CDCl_3$ (300 MHz).



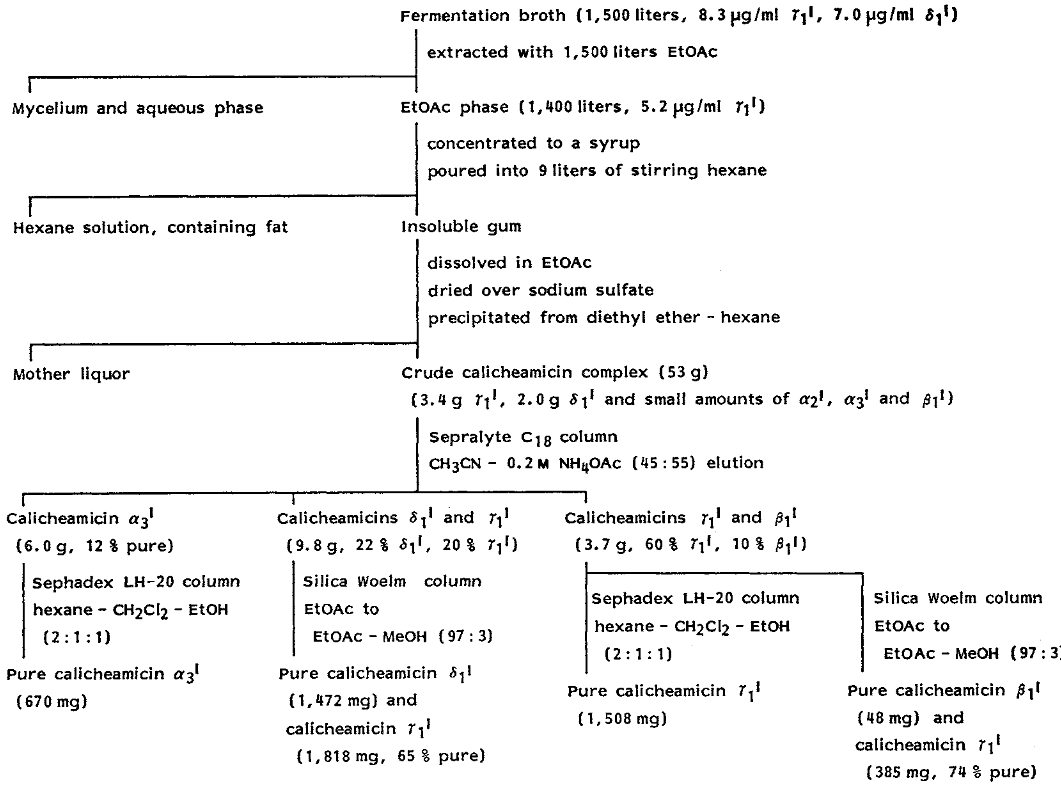
against the BIA showed that the titer of both the β_1^{Br} and γ_1^{Br} components increased considerably. HPLC analysis of the same extracts, however, did not show improved yields of either components, instead, two new peaks chromatographing a little slower than β_1^{Br} and γ_1^{Br} were found.

During a bioassay development study it was discovered accidentally that the antibacterial activity of calicheamicin β_1^{Br} was inhibited completely by the addition of dithiothreitol to the assay medium. Subsequent HPLC studies showed that the addition of approximately 50-fold excess of dithiothreitol to an acetonitrile solution (0.1 mg/ml) of calicheamicin β_1^{Br} caused the degradation of $>95\%$ of the antibiotic within 30 minutes. The broth extracts of the sodium iodide supplemented fermentations were analyzed by HPLC, with and without the addition of excess dithiothreitol to the HPLC samples. The two peaks associated with components β_1^{Br} and γ_1^{Br}, the two peaks chromatographing just after them, as well as two other peaks were degraded by dithiothreitol. This interesting observation suggested that the increased titer, based on the BIA, in these fermentations was due to components related to calicheamicins β_1^{Br} and γ_1^{Br}, possibly iodinated analogues.

Isolation of Calicheamicins α_2^I, α_3^I, β_1^I, γ_1^I and δ_1^I

The antibiotic complex was recovered by extracting the whole fermentation mash with one equal volume of EtOAc. The EtOAc solution containing the calicheamicins was concentrated, defatted and selectively precipitated, as shown in Fig. 4, to give crude calicheamicin complex containing 6.4% γ_1^I component, 3.7% δ_1^I component, and small amounts of components α_2^I, α_3^I and β_1^I. The individual components were conveniently separated by reverse phase column chromatography using Sepralyte C_{18} and further purified by silica gel normal phase or Sephadex LH-20 partition column chromatography. In general, due to higher fermentation yields, the isolation and purification of the iodine containing calicheamicins were much more straight forward than that of the bromine containing components. Due to the generally poor stability of the calicheamicins, in-process samples were best kept as precipitated solids rather than in solution.

Fig. 4. Process for the isolation of calicheamicins α_3^I, β_1^I, γ_1^I and δ_1^I from the fermentation of strain NRRL 18149.

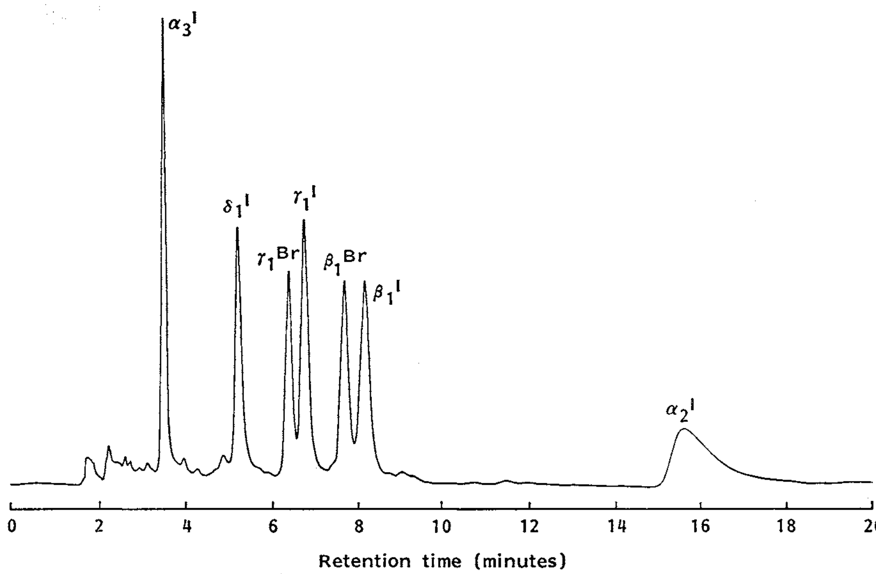


The amount of calicheamicin α_2^I present in the fermentation broth and in the crude calicheamicin complex was approximately the same as that of calicheamicin α_3^I. However, due to the much slower chromatography of α_2^I than the other components in the reverse phase system used for separating the individual components, it was not recovered after the Sepralyte C_{18} column chromatography. A normal phase system would have been the choice if the goal were to isolate and purify quantities of calicheamicin α_2^I from the crude calicheamicin complex. In the course of degradation studies, however, it was found that calicheamicin α_2^I can be conveniently prepared from the major component of the complex, γ_1^I, by mild acid hydrolysis. The chromatographic properties of this material was identical to that present in the crude calicheamicin complex.

Characterization of Calicheamicins α_2^I, α_3^I, β_1^I, γ_1^I and δ_1^I

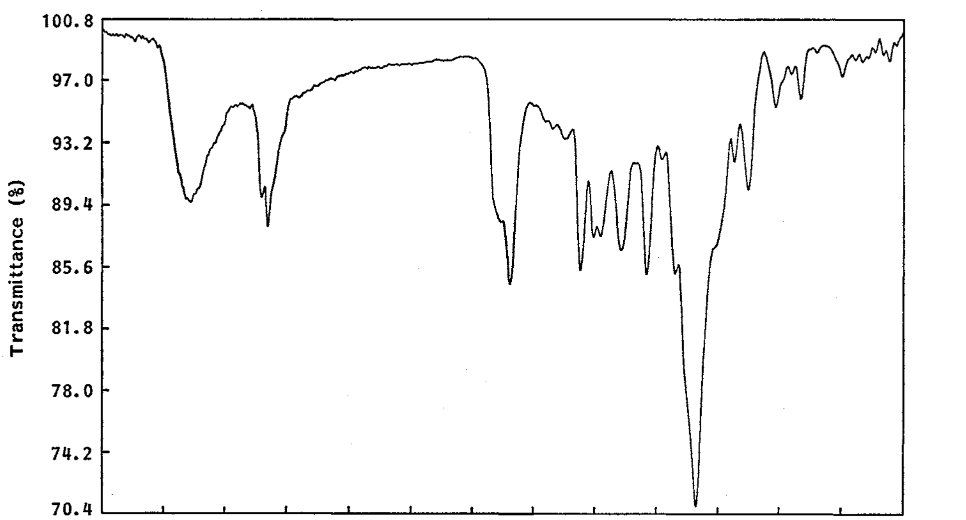
The physico-chemical properties of calicheamicins α_2^I, α_3^I, β_1^I, γ_1^I and δ_1^I were summarized in Table 2 and their chromatographic properties were summarized in Tables 3 and 4. An analytical separation of the individual components of both the iodinated and the brominated calicheamicins is shown in Fig. 5. The brominated components could not be separated from the corresponding iodinated analogues by normal phase chromatography on silica gel. In general it was not practical to fractionate the brominated from the iodinated analogues in a preparative scale. Fortunately, the fermentation condition which gave the best yield of the iodinated calicheamicins produced negligible amounts of the corresponding brominated components, and unless iodides were supplemented in the fermentation media no iodinated components were produced.

Fig. 5. Analytical HPLC separation of a mixture of purified calicheamicins using System I described in Table 4.



The UV trace at 280 nm resulting from a 5-μl injection of a solution containing approximately 0.1 mg/ml of calicheamicin $\alpha_2{}^{\mathrm{I}}$ and 0.05 mg/ml of each of the other components is shown.

Fig. 6. IR spectrum of calicheamicin $\gamma_1{}^{\mathrm{I}}$ (KBr disc).



The IR absorption spectrum of calicheamicin $\gamma_1{}^{\mathrm{I}}$, the major component of the complex, is shown in Fig. 6. The IR spectra of components $\beta_1{}^{\mathrm{Br}}$, $\gamma_1{}^{\mathrm{Br}}$, $\beta_1{}^{\mathrm{I}}$ and $\delta_1{}^{\mathrm{I}}$ are practically superimposable with it, all showing strong absorption between 3600 and 3200 cm^{-1} due to large amounts of hydrogen bonded hydroxyl groups, carbonyl absorptions at 1720 and 1680 cm^{-1} for aryl or α,β-unsaturated esters and ketones, and strong C-O stretches between 1140 and 1040 cm^{-1}. The IR spectra of components $\alpha_2{}^{\mathrm{I}}$ and $\alpha_3{}^{\mathrm{I}}$ showed a little more variation between 1500 and 1200 cm^{-1}, nevertheless, the major bands, even in this region, remain unchanged. The UV absorption spectrum of calicheamicin γ_1^I is shown in Fig. 7, no acid or base shifts were observed. The UV spectra of the other components were identical to it.

The ^{13}C NMR (Table 5) and ^1H NMR (Figs. 2, 3, 8 and 9) data confirmed that the only difference between calicheamicins β_1^{Br} and β_1^I, and between calicheamicins γ_1^{Br} and γ_1^I, was a bromine *versus* an iodine substitution on a sp^2 carbon (carbon chemical shifts at 115.0 vs. 93.6 ppm and 115.3 vs. 93.5 ppm). It was also quite clear that the structural differences between calicheamicins β_1^I, γ_1^I and δ_1^I (Fig. 10) reside in an alkyl substitution, which is isopropyl for β_1^I, ethyl for γ_1^I and methyl for δ_1^I, on a hetero-atom. The NMR data (Table 5, Figs. 11 and 12) also pointed out that calicheamicins α_2^I and α_3^I differ from γ_1^I by having one less sugar moiety, although not the same glycoside was missing in the two components.

The calicheamicins are structurally related to the esperamicin produced by *Actinomadura verrucosospora*, strain H964-62 (BBM-1675, ATCC 39334).[7~10] Veractamycins (PD 114,759, PD 115,028, CL-1577 antibiotics) produced by *A. verrucosospora* subsp. *veractimyces* (ATCC 39363),[11~17] FR-900405 (WS 6049-A), FR-900406 (WS 6049-B)[18~20] produced by *Actinomadura pulveracea* sp. nov. No. 6049 and CL-1724 antibiotics produced by *Actinomadura* sp. (NRRL 15758)[21] have also been described. These antibiotics are very closely related to, if not identical

Fig. 7. UV spectrum of calicheamicin γ_1^I (0.02 mM in MeOH).

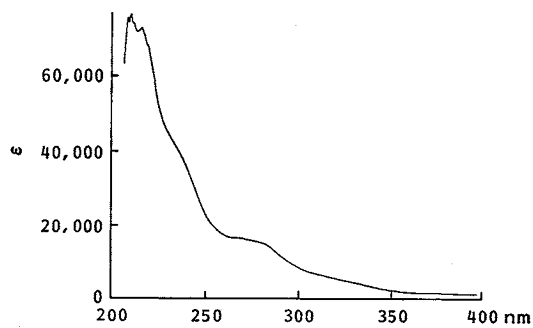


Fig. 8. ^1H NMR spectrum of calicheamicin β_1^I in $CDCl_3$ (300 MHz).

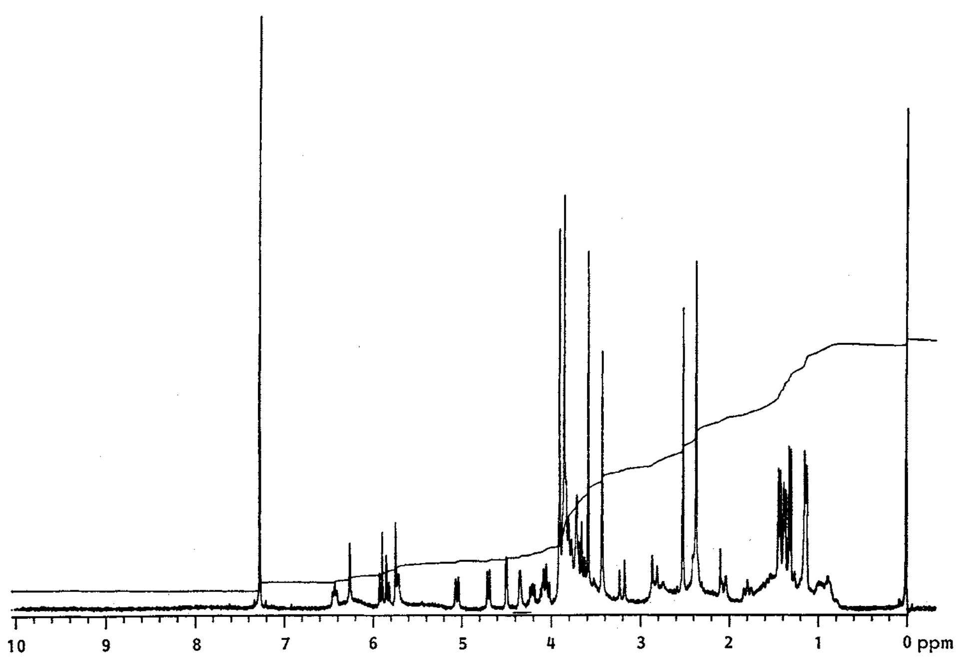


Fig. 9. ^{1}H NMR spectrum of calicheamicin $\gamma_1{}^{I}$ (NH_4OAc salt) in $CDCl_3$ (300 MHz).

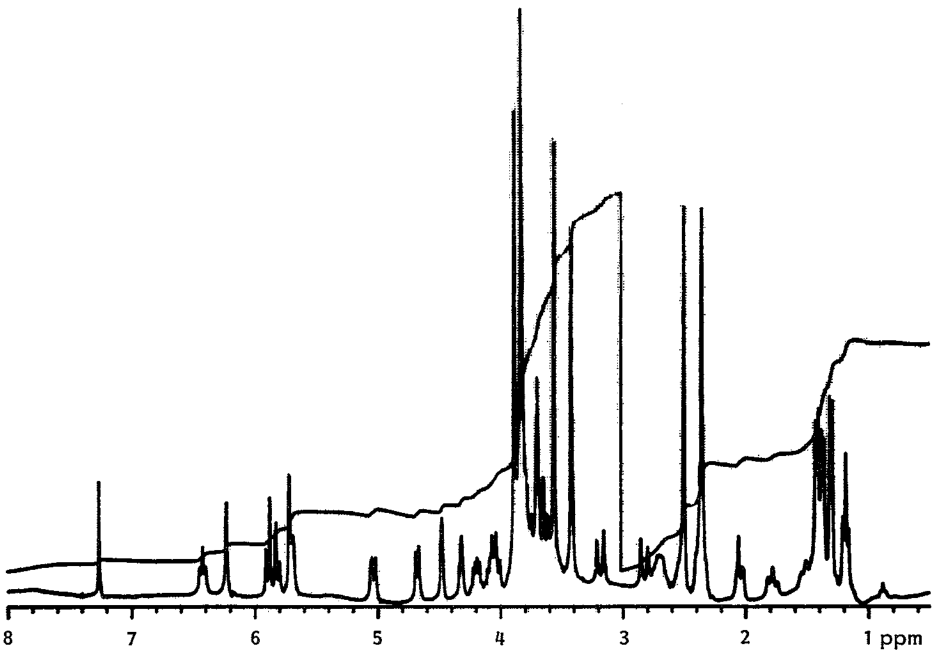


Fig. 10. ^{1}H NMR spectrum of calicheamicin $\delta_1{}^{I}$ in $CDCl_3$ (300 MHz).

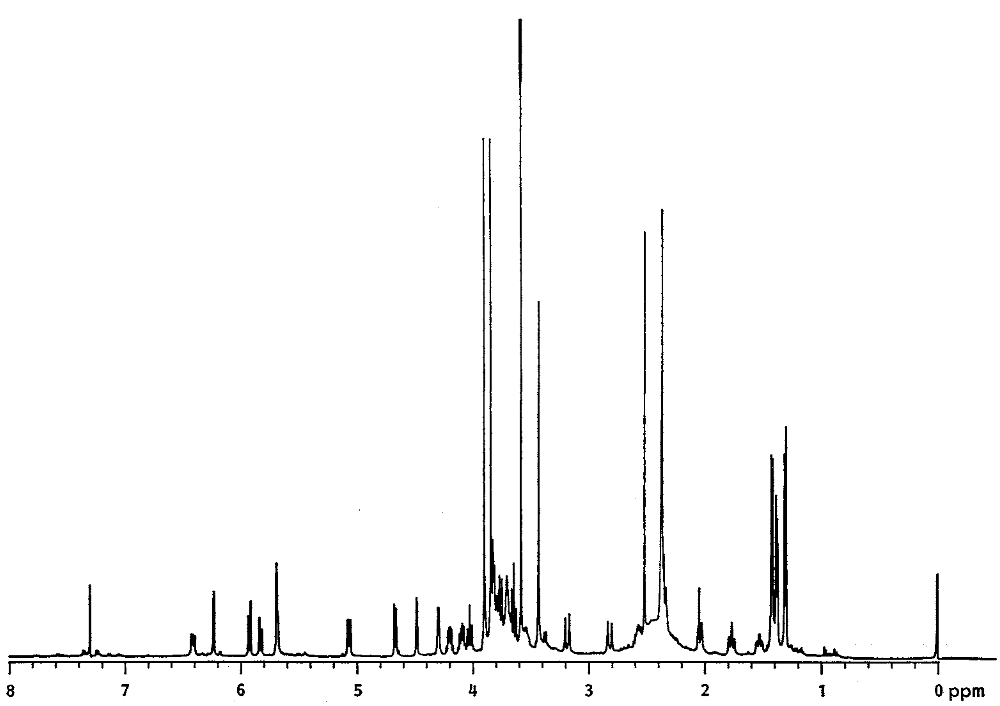


Fig. 11. [1]H NMR spectrum of calicheamicin α_2^I in $CDCl_3$ - CD_3OD (300 MHz).

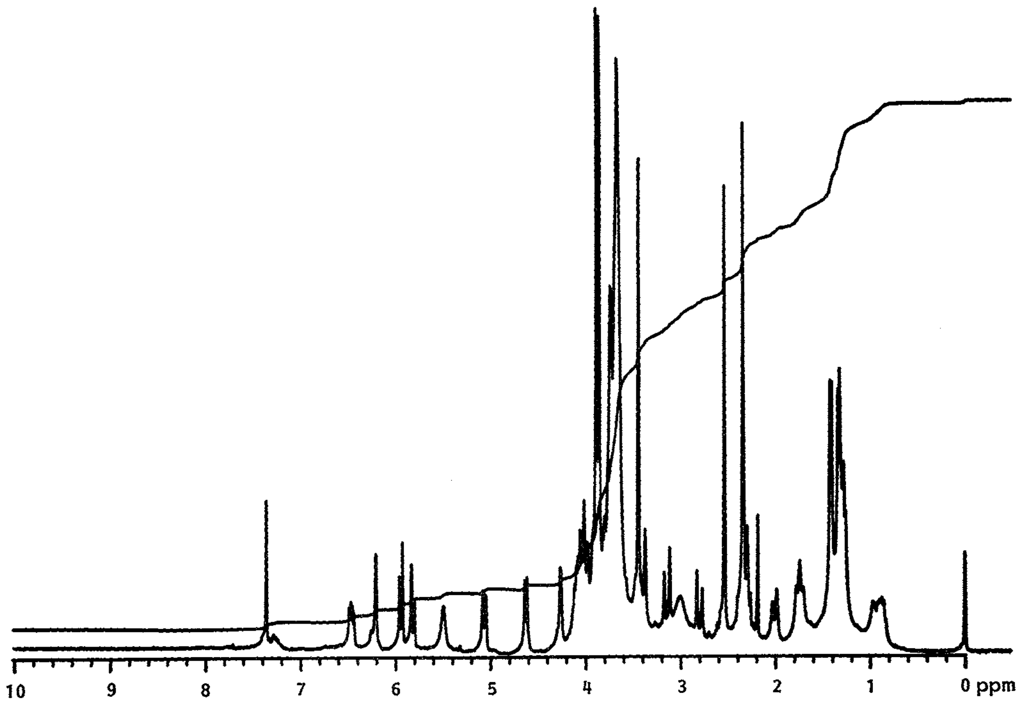
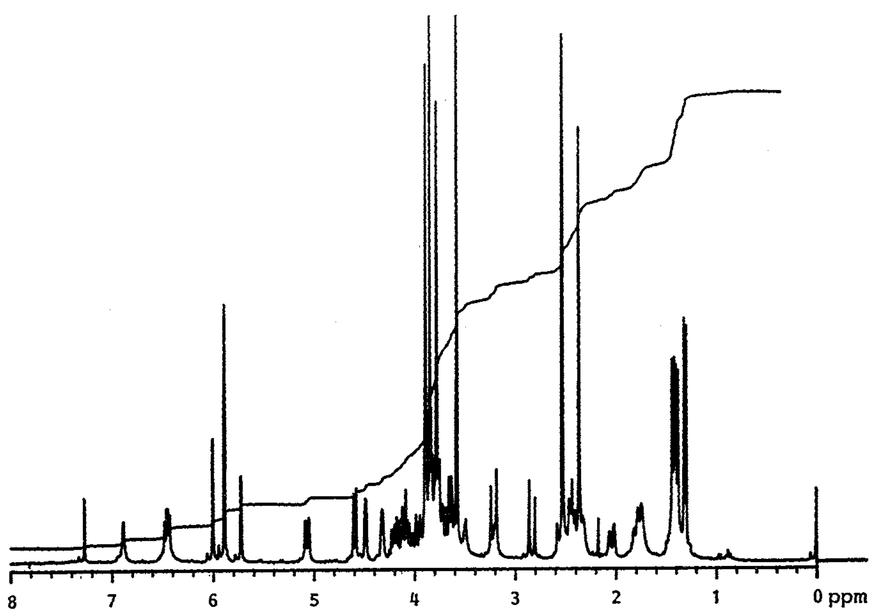


Fig. 12. [1]H NMR spectrum of calicheamicin α_3^I in $CDCl_3$ (300 MHz).



to, the esperamicins.

Biological Activities

The individual components of the calicheamicins are active in the biochemical prophage induc-

Table 6. Antimicrobial activity of the calicheamicins.

Organism (strains tested)	MIC (μg/ml) range[a]						
	β_1^{Br}	γ_1^{Br}	α_2^{I}	α_3^{I}	β_1^{I}	γ_1^{I}	δ_1^{I}
Escherichia coli (3)	0.12~0.25	0.25~0.5	0.06~0.12	1	0.25~0.5	0.25	0.06~0.12
Klebsiella pneumoniae (2)	0.12~0.25	0.5	0.01~0.25	0.25~2	0.5	0.25	0.12
Enterobacter sp. (2)	0.25~0.5	0.5	0.06~0.5	2	0.25~0.5	0.5	0.12~0.25
Serratia sp. (2)	0.12	0.25~0.5	0.25~0.5	2	0.25~0.5	0.12~0.25	0.03~0.12
Morganella morganii (1)	0.5	0.5	0.12	1	1	0.25	0.12
Providencia stuartii (1)	0.25	1	0.25	2	0.5	0.25	0.25
Citrobacter sp. (2)	0.12	0.25~0.5	0.03~0.12	0.25	0.25~0.5	0.12~0.25	0.12
Acinetobacter sp. (2)	0.06~0.12	0.25	0.25	1~2	0.25	0.06~0.12	0.06~0.12
Pseudomonas aeruginosa (2)	0.25~0.5	0.5~1	0.06~0.5	1~2	0.25~0.5	0.12~0.25	0.12
Staphylococcus aureus (5)	≤0.0005	≤0.0005	≤0.0005	≤0.0005~0.02	≤0.0005	≤0.0005	≤0.0005
S. epidermidis sp. (2)	≤0.0005	≤0.0005	≤0.0005	≤0.0005	≤0.0005	≤0.0005	≤0.0005
Streptococcus faecalis (2)	≤0.0005~0.004	≤0.0005~0.06	≤0.0005	≤0.0005~0.02	≤0.0005~0.03	≤0.0005~0.008	≤0.0005~0.004
Micrococcus luteus (1)	≤0.0005	≤0.0005			≤0.0005	≤0.0005	
Bacillus subtilis (1)	≤0.0005	≤0.0005			≤0.0005	≤0.0005	

[a] MICs were determined by the standard agar-dilution method in Mueller-Hinton medium.

tion assay at concentrations less than 1 pg/ml, extremely active against Gram-positive bacteria, and highly active against Gram-negative bacteria (Table 6). The calicheamicins are potent DNA damaging agents giving rise to sequence specific double stranded DNA cleavages.[22] The individual components show significant activity and extraordinary potency against experimental murine tumors such as P388 and L1210 leukemias and solid neoplasms Colon 26 and B-16 melanoma with optimal dose at 0.15~5 μg/kg, depending on the component.[23] Details on the antitumor activities of the calicheamicins will be published in a separate report.

Experimental

General

UV absorption spectra were recorded with a Hewlett-Packard 8450A UV/VIS spectrophotometer. IR spectra were determined as KBr discs using a Nicolet Fourier transformation (FT)-IR spectrometer. Mass spectrometry was attempted in EI and FAB (glycerol, sulfalane or dithiothreitol/dithioerythritol matrix) ionization modes, determined either using a VG Analytical Instruments Model ZAB-SE mass spectrometer or at the University of Illinois. The ^{1}H and ^{13}C NMR spectra were recorded using a Nicolet NT-300 spectrometer at 300 and 75 MHz respectively. The samples were prepared in $CDCl_3$. A drop of CD_3OD was added to some samples where the $CDCl_3$ solutions were not clear.

TLC and HPLC

TLC was done on E. Merck Silica gel 60 F_{254} pre-coated aluminum sheets (0.2 mm layer thickness) or Whatman High Performance TLC (HPTLC) plates, Type HP-KF or Type LHP-KF both with fluorescent indicator, using solvent systems shown in Table 3. The calicheamicins could be detected by $UV_{254\,nm}$ quenching when the samples are relatively clean, otherwise detection was done by bioautography using the biochemical prophage induction assay.[2] HPLC analysis was carried out with a Waters ALC/GPC 200 Series Liquid Chromatograph equipped with Waters WISP 710B sample processor or with a Hewlett-Packard HP 1090 Series M Liquid Chromatograph. There different reversed phase analytical columns were used during the study: Altex-Beckman Ultrasphere-ODS (5 μm, 4.6 mm×25 cm) with Waters Guard-PAK Precolumn Module and μBondapak C_{18} Precolumn Inserts, Waters NOVA-PAK C_{18} Radial-PAK Cartridge (4 μm, 5 mm×10 cm) with RCM-100 Radial Compression Module and Waters Guard-PAK Precolumn Module and μBondapak C_{18} Precolumn Inserts, and Rainin Microsorb (3 μm, 4.6 mm×5 cm) with Microsorb guard (3 μm, 4.6 mm×1.5 cm) and Waters In-Line Precolumn Filter. Solvent systems and flow rates were as shown in Table 4. The conditions used for the NOVA-PAK Radial Compression column were similar to those used for the Microsorb column. The resolution of the calicheamicins by the NOVA-PAK Radial Compression column was inferior to the other two columns used, however, it was much more economical to use the Radial Compression system since it was possible to recover the resolution of a cartridge by decompression and recompression during the rinsing cycle.

Preparative Column Chromatography

Closed glass columns manufactured by Altex Scientific or ACE Glass fitted with Altex type 1/4-28 thread low pressure Teflon tube end fittings with stainless steel washer and flanged Teflon tube end were used. Model 396 Milton-Roy Instrument miniPumps equipped with pulse dampers and NUPRO 8CPA Series Adjustable In-line relief valves or Rainin Rabbit HPLC pumps equipped with Rainin Electronic Pressure Monitors were used to deliver the eluent onto the columns.

The following adsorbents were used for preparative column chromatography: Silica Woelm (32~63 μm, silica gels for chromatography with elevated pressure, Woelm Pharma GmbH & Co.), Silica gel 60 (Kieselgel 60, 40~63 μm, E. Merck), Bio-Sil A (20~40 μm, Bio-Rad Laboratories), Sephadex LH-20 (Pharmacia Fine Chemicals) and Sepralyte C_{18} (35~63 μm, 60 Å porosity, Analytichem International). Silica gel columns were slurry packed in EtOAc under pressure and were equilibrated with the first eluent. Sepralyte C_{18} columns were slurry packed in MeOH under pressure and were equilibrated with the eluent. Sephadex LH-20 were pre-equilibrated with the eluent and slurry packed into columns.

Column fractions were first assayed by the BIA and those positive were analyzed by TLC (E. Merck Silica gel 60 F_{254} pre-coated aluminum sheets, eluted with 3% 2-propanol in EtOAc saturated with 0.1 M KH_2PO_4) and detected by bioautography using the BIA. Pooled column fractions from eluent containing only organic solvents were concentrated to dryness, and the residue was redissolved in EtOAc, dried over anhydrous Na_2SO_4, concentrated to a small volume and precipitated by addition of hexane. Pooled column fractions from eluents containing KH_2PO_4 were first extracted once with water and the organic phase was concentrated, dried, and precipitated. Pooled column fractions from Sephadex LH-20 columns eluted with MeOH - H_2O (90:10) were first concentrated to remove MeOH and the calicheamicins were extracted into EtOAc, dried, concentrated, and precipitated. Pooled column fractions from reversed phase Sepralyte C_{18} columns were first concentrated to remove most of the CH_3CN and the calicheamicins were extracted into EtOAc, the EtOAc solution was washed once with water and was then dried, concentrated and the calicheamicins precipitated by addition of hexane.

Isolation of Calicheamicins β_1^{Br} and γ_1^{Br} from the Fermentation of NRRL 15839

The whole harvest mash (1,500 liters) was adjusted to pH 6 and was extracted with 750 liters of EtOAc. The EtOAc phase (700 liters) was concentrated to a syrup which was stirred with excess hexane and the mixture was filtered through diatomaceous earth. The diatomaceous earth cake was thoroughly mixed with 20 liters of EtOAc and filtered. The filtrate was concentrated to 2.5 liters, dried over excess anhydrous Na_2SO_4 and precipitated by addition of diethyl ether and hexane to give 26.7 g of crude calicheamicin complex containing approximately 0.3% calicheamicin β_1^{Br} and a very small amount of calicheamicin γ_1^{Br}.

The crude calicheamicin complex was divided evenly into three portions and chromatographed on three separate Silica Woelm columns (2.5×100 cm) packed and equilibrated with EtOAc saturated with 0.1 M aqueous KH_2PO_4. The columns were first eluted with the same solvent at a flow rate of 3 ml/minute for 18 hours, collecting 18 ml fractions. The eluent was changed to EtOAc - MeOH (95:5) and elution continued for 8 hours. Finally the columns were eluted with EtOAc - MeOH (90:10) for 10 hours. Fractions containing calicheamicins β_1^{Br} and γ_1^{Br} from the three columns were pooled and worked up to give 2.0 g of crude calicheamicin β_1^{Br} complex containing calicheamicin γ_1^{Br}.

A 1.9-g sample of the above was chromatographed on a Sephadex LH-20 column (2.5×100 cm) equilibrated with MeOH - H_2O (90:10) at a flow rate of 1.2 ml/minute, collecting 15 ml fractions. Fractions 21~26, containing most of the calicheamicins β_1^{Br} and γ_1^{Br} were pooled and worked up to give 435 mg of partially purified calicheamicin complex containing approximately 10% calicheamicin β_1^{Br} and 4% calicheamicin γ_1^{Br}.

The partially purified calicheamicin complex above was divided evenly and chromatographed on two Silica gel 60 column (1.5×100 cm), packed and equilibrated with EtOAc - MeOH (98:2), at a flow rate of 1 ml/minute, collecting 12 ml fractions. Fractions containing primarily calicheamicin β_1^{Br} were pooled and worked up to give 26 mg of 80% pure calicheamicin β_1^{Br}. Fractions containing calicheamicin γ_1^{Br}, chromatographing just after calicheamicin β_1^{Br}, were also pooled and worked up to yield 4.5 mg of 30% pure calicheamicin γ_1^{Br}.

The 80% pure calicheamicin β_1^{Br} obtained above was chromatographed on a reverse phase (Sepralyte C_{18}) column (1.5×45 cm) eluting with CH_3CN - 0.2 M NH_4OAc (45:55) at 1.5 ml/minute collecting 7 ml fractions. Fractions containing pure calicheamicin β_1^{Br} were pooled and worked up to give 18 mg of analytically pure calicheamicin β_1^{Br}.

The 30% pure calicheamicin γ_1^{Br} sample was combined with other samples of similar quality derived from similar fermentations and processed to give a total of 18 mg and was further purified by preparative TLC (Silica gel GF pre-coated tapered plate, Analtech) developing with 2% 2-propanol in EtOAc saturated with 0.1 M aqueous KH_2PO_4. The major quenching band under short wavelength UV lamp (254 nm), chromatographing at Rf 0.5, was excised and the antibiotic was washed off the adsorbent with 10% 2-propanol in EtOAc saturated with 0.1 M aqueous KH_2PO_4. The solution was concentrated and the residue was redissolved in EtOAc, washed with a small amount of water, dried over anhydrous Na_2SO_4, reconcentrated and precipitated by addition of hexane to give 4.3 mg of pure calicheamicin γ_1^{Br}.

Isolation of Calicheamicins α_3^I, β_1^I, γ_1^I and δ_1^I from the Fermentation of NRRL 18149

The whole harvest mash (1,500 liters, containing 12.5 g of calicheamicin γ_1^I and 10.5 g of calicheamicin δ_1^I) was mixed thoroughly with 1,500 liters of EtOAc for 3 hours. Filter aid was added and the mash-solvent mixture was filtered. The EtOAc phase (1,400 liters, containing 7.3 g of calicheamicin γ_1^I) was concentrated to 100 liters and was adjusted to pH 6~7 with 2 N NaOH; any aqueous phase present was removed. The EtOAc phase was further concentrated to 20 liters and any aqueous phase and interfacial fats were removed. The EtOAc phase was finally concentrated to a golden yellow syrup and was poured slowly into 7~8 times its volume of rapidly stirring hexane. The hexane solution was decanted and the insoluble gum, containing the calicheamicins, was redissolved in 3 liters of EtOAc, dried over anhydrous Na_2SO_4, concentrated to a small volume and precipitated by addition of diethyl ether and hexane to give 53 g of crude calicheamicin complex, containing 3.4 g of calicheamicin γ_1^I, 2.0 g of calicheamicin δ_1^I and samll amounts of calicheamicins α_2,^{I} α_3^I and β_1^I.

A 7.2-g sample of the crude calicheamicin complex obtained above was divided evenly and chromatographed on two Sepralyte C_{18} columns (2.5×23 cm) eluting with CH_3CN - 0.2 M aqueous NH_4OAc (45:55) at 12 ml/minute, collecting sixty 24-ml fractions from each column. Fraction containing calicheamicins γ_1^I and β_1^I were pooled and worked up to yield 504 mg of partially purified calicheamicin γ_1^I (60% pure) containing β_1^I. Fractions containing calicheamicins α_3^I and δ_1^I (co-eluting with a large portion of the γ_1^I present), eluting off the column ahead of calicheamicin γ_1^I were pooled separately and worked up to yield 812 mg of partially purified calicheamicin α_3^I (12% pure) and 1,336 mg of partially purified mixture of calicheamicins δ_1^I and γ_1^I (22% δ_1^I, 20% γ_1^I).

A 309-mg sample of partially purified calicheamicin γ_1^I (66% pure) was chromatographed on a Sephadex LH-20 column (1.5×90 cm) equilibrated with hexane - CH_2Cl_2 - EtOH (2:1:1). The column was eluted with the same solvent system at 1.5 ml/minute and twenty-five 20-ml fractions were collected. Fractions containing pure calicheamicin γ_1^I were pooled and worked up to yield 194 mg of analytically pure calicheamicin γ_1^I.

A 1.05-g sample of partially purified calicheamicin γ_1^I containing β_1^I (61% γ_1^I, 10% β_1^I) was chromatographed on a Silica Woelm column (1.5×45 cm) packed and equilibrated with EtOAc. The column was eluted with EtOAc at 3.6 ml/minute for 1 hour, the eluent was changed to EtOAc - MeOH (97:3), and the elution was continued for 2 hours; 18-ml fractions were collected during the entire elution. Fractions containing pure calicheamicin β_1^I were pooled and worked up to yield 48 mg of pure calicheamicin β_1^I. Fractions containing calicheamicin γ_1^I were also worked up to yield 385 mg of 74% pure calicheamicin γ_1^I which could be rechromatographed on a Sephadex LH-20 column as described above to give pure calicheamicin γ_1^I.

A partially purified mixture of calicheamicins δ_1^I and γ_1^I (1.8 g, containing 648 mg of γ_1^I and 540 mg of δ_1^I) was chromatographed on a Silica Woelm column (1.5×45 cm) packed and equilibrated with EtOAc. The column was eluted with EtOAc at 3 ml/minute for 1 hour, the eluent was changed to EtOAc - MeOH (97:3), and the elution was continued for 2 hours; 15-ml fractions were collected during the entire elution. Fractions containing pure calicheamicin δ_1^I were pooled and worked up to yield 367 mg of analytically pure calicheamicin δ_1^I. Fractions containing calicheamicin γ_1^I were also worked up to yield 574 mg of 65% pure calicheamicin γ_1^I.

A partially purified calicheamicin α_3^I sample (1.8 g, containing 310 mg of α_3^I) was chromatographed on a Sephadex LH-20 column (1.5×90 cm) equilibrated with hexane - CH_2Cl_2 - EtOH (2:1:1). The column was eluted with the same solvent system at 4 ml/minute and forty-five 20-ml fractions were collected and analyzed as before. Fractions containing pure calicheamicin α_3^I were pooled and worked up as before to yield 289 mg of analytically pure calicheamicin α_3^I.

Preparation of Calicheamicin α_2^I from Calicheamicin γ_1^I

A 300-mg sample of partially purified calicheamicin γ_1^I (60% pure) was dissolved in 60 ml of 2% (by weight) HCl in MeOH and the solution was allowed to remain at room temperature for 6 hours.

The reaction mixture was then neutralized by addition of saturated methanolic solution of K_2CO_3. The precipitated potassium chloride was filtered off and the solution was concentrated to dryness. The EtOAc soluble portion of the residue was concentrated and precipitated from hexane to yield 135 mg of crude calicheamicin α_2^I.

The crude calicheamicin α_2^I obtained above was purified by chromatography on a Bio-Sil A column (1.5×20 cm) eluting with CH_2Cl_2 - MeOH (96:4) to give 34 mg of analytically pure calicheamicin α_2^I identical to the calicheamicin α_2^I present in the fermentation of NRRL 18149 by TLC and HPLC analyses.

Acknowledgment

The authors gratefully acknowledge the support and contributions of many co-workers in the Microbial Physiology, Microbial Chemistry, Antimicrobial Chemotherapy, Chemotherapy Research, and Analytical Service Departments at the Medical Research Division of the American Cyanamid Company. In particular, we wish to thank Mrs. L. Barbieri and Ms. L. Camp for technical assistance.

References

1) Elespuru, R. K. & M. B. Yarmolinsky: A colorimetric assay of lysogenic induction designed for screening potential carcinogenic and carcinostatic agents. Environ. Mutagen. 1: 55～78, 1979
2) Elespuru, R. K. & R. J. White: Biochemical prophage induction assay: A rapid test for antitumor agents that interact with DNA. Cancer Res. 43: 2819～2830, 1983
3) Fantini, A. A.; J. D. Korshalla, F. Pinho, N. A. Kuck, M. J. Mroczenski-Wildey, M. Greenstein, W. M. Maiese & R. T. Testa: LL-E33288 antibiotics, a complex of novel potent antitumor agentis: Taxonomy, fermentation and biological activity. Program and Abstracts of the 26th Intersci. Conf. on Antimicrob. Agents Chemother., No. 227, p. 137, New Orleans, Sept. 28～Oct. 1, 1986
4) Lee, M. D.; G. O. Morton, T. S. Dunne, D. R. Williams, J. K. Manning, M. Siegel, C. C. Chang & D. B. Borders: LL-E33288 antibiotics. A complex of novel potent antitumor agents. Isolation and chemical characterization. Program and Abstracts of the 26th Intersci. Conf. on Antimicrob. Agents Chemother., No. 228, p. 137, New Orleans, Sept. 28～Oct. 1, 1986
5) Lee, M. D.; T. S. Dunne, M. M. Siegel, C. C. Chang, G. O. Morton & D. B. Borders: Calichemicins, a novel family of antitumor antibiotics. 1. Chemistry and partial structure of calichemicin γ_1^I. J. Am. Chem. Soc. 109: 3464～3466, 1987
6) Lee, M. D.; T. S. Dunne, C. C. Chang, G. A. Ellestad, M. M. Siegel, G. O. Morton, W. J. McGahren & D. B. Borders: Calichemicins, a novel family of antitumor antibiotics. 2. Chemistry and structure of calichemicin γ_1^I. J. Am. Chem. Soc. 109: 3466～3468, 1987
7) Golik, J.; J. Clardy, G. Dubay, G. Groenewold, H. Kawaguchi, M. Konishi, B. Krishnan, H. Ohkuma, K. Saitoh & T. W. Doyle: Esperamicins, a novel class of potent antitumor antibiotics. 2. Structure of esperamicin X. J. Am. Chem. Soc. 109: 3461～3462, 1987
8) Golik, J.; G. Dubay, G. Groenewold, H. Kawaguchi, M. Konishi, B. Krishnan, H. Ohkuma, K. Saitoh & T. W. Doyle: Esperamicins, a novel class of potent antitumor antibiotics. 3. Structures of esperamicins A_1, A_2, and A_{1b}. J. Am. Chem. Soc. 109: 3462～3464, 1987
9) Konishi, M.; H. Ohkuma, K. Saitoh, H. Kawaguchi, J. Golik, G. Dubay, G. Groenewold, B. Krishnan & T. W. Doyle: Esperamicins, a novel class of potent antitumor antibiotics. 1. Physico-chemical data and partial structure. J. Antibiotics 38: 1605～1609, 1985
10) Konishi, M.; K. Saitoh, H. Ohkuma & H. Kawaguchi (Bristol-Myers): BBM-1675, a new antitumor antibiotic complex. Brit. UK Patent Appl. 2,141,425 A, Dec. 19, 1984
11) Bunge, R. H.; T. R. Hurley, T. A. Smitka, N. E. Willmer, A. J. Brankiewicz, C. E. Steinman & J. C. French: PD 114,759 and PD 115,028, novel antitumor antibiotics with phenomenal potency. I. Isolation and characterization. J. Antibiotics 37: 1566～1571, 1984
12) Bunge, R. H.; T. R. Hurley, J. B. Tunac, J. C. French & T. A. Smitka (Warner-Lambert): Antibiotic/antitumor compounds and their production. Eur. Pat. Appl. 0,132,082 A2, Jan. 23, 1985
13) Brankiewicz, A. J.; R. H. Bunge, J. C. French, C. Hokanson, R. Hurley & J. P. Schaumberg (Warner-Lambert): CL-1577D and CL-1577E antibiotic/antitumor compounds. Their production and use. U.S. 4,539,203, Sept. 3, 1985
14) Wilton, J. H.; G. C. Hokanson & J. C. French: The structures of the U.V. chromophoric fragments

of the antitumor antibiotics, PD 114,759 and PD 115,028. J. Chem. Soc. Chem. Commun. 1985: 919~920, 1985
15) WILTON, J. H.; C. D. RITHNER, G. C. HOKANSON & J. C. FRENCH: A novel sulfur-containing hexose from the antitumor antibiotics, PD 114,759 and PD 115,028. J. Antibiotics 39: 1349~1350, 1986
16) FRY, D. W.; J. L. SHILLIS & W. R. LEOPOLD: Biological and biochemical activities of the novel antibiotic PD 114,759 and related derivatives. Invest. New Drugs 4: 3~10, 1986
17) MAMBER, S. W.; W. G. OKASINSKI, C. D. PINTER & J. B. TUNAC: Bacterial DNA reactivity of the novel antitumor antibiotics PD 114,759 and PD 115,028 (veractamycins A and B). J. Antibiotics 40: 1044~1049, 1987
18) IWAMI, M.; S. KIYOTO, M. NISHIKAWA, H. TERANO, M. KOHSAKA, H. AOKI & H. IMANAKA: New antitumor antibiotics, FR-900405 and FR-900406. I. Taxonomy of the producing strain. J. Antibiotics 38: 835~839, 1985
19) KIYOTO, S.; M. NISHIKAWA, H. TERANO, M. KOHSAKA, H. AOKI, H. IMANAKA, Y. KAWAI, I. UCHIDA & M. HASHIMOTO: New antitumor antibiotics, FR-900405 and FR-900406. II. Production, isolation, characterization and antitumor activity. J. Antibiotics 38: 840~848, 1985
20) KIYOTO, S.; T. SHIBATA, Y. KAWAI, Y. HORI, O. NAKAYAMA, H. TERANO, M. KOHSAKA, H. AOKI & H. IMANAKA: New antitumor antibiotic, FR-900405. III. Mechanism of action of FR-900405. J. Antibiotics 38: 955~956, 1985
21) YOUNG, C. A.; J. P. SCHAUMBERG, G. C. HOKANSON, J. C. FRENCH & J. B. TUNAC (Warner-Lambert): CL-1724 antibiotic compounds, their production and use. U.S. 4,554,162, Nov. 19, 1985
22) ZEIN, N.; A. M. SINHA, W. J. MCGAHREN & G. A. ELLESTAD: Calicheamicin γ_1^{I}: An antitumor antibiotic that cleaves double-stranded DNA site specifically. Science 240: 1198~1201, 1988
23) THOMAS, J. P.; S. G. CARVAJAL, H. L. LINDSAY, R. V. CITARELLA, R. E. WALLACE, M. D. LEE & F. E. DURR: LL-E33288 antibiotics. A complex of novel potent antitumor agents: Antitumor activity and tolerance in mice. Program and Abstracts of the 26th Intersci. Conf. on Antimicrob. Agents Chemother., No. 229, p. 138, New Orleans, Sept. 28~Oct. 1, 1986