New Betaclamycin and Aclarubicin Analogs Obtained by Prolonged Microbial Conversion with an Aclarubicin-negative Mutant

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Microbial conversion of β-rhodomycinone and aklavinone using an aclarubicin-negative *Streptomyces galilaeus* mutant afforded new anthracycline antibiotics CG21-C and CG1-C which had a rednosyl-2-deoxyfucosyl-rhodosaminyl trisaccharide residue at C-7 of each added aglycone. They were produced only when a prolonged conversion culture took place. Because the usual conversion products containing a cinerulosyl-2-deoxyfucosyl-rhodosaminyl residue were at first accumulated and then decreased during further cultivation, it was evident that they occurred by the modification of terminal cinerulose. The isolation, purification, and structural determination are described, and cytotoxicity *in vitro* against cultured L1210 cells and the formation mechanism are discussed.

We have shown that a microbial conversion of various anthracyclines or anthracyclinones using antibiotic-negative mutants of certain anthracycline producers is a useful way of obtaining targeted derivatives with improved antitumor activity.[1~8] In one case this was done using an antibiotic-negative mutant KE303, which was artificially derived from anthracycline antibiotic aclarubicin-producing *Streptomyces galilaeus* MA144-M1.[1~5,8] Strain KE303 is damaged at a step of aklavinone formation in aclarubicin biosynthesis, but kept glycosidation ability to produce aclarubicin from exogenously added aklavinone. The glycosidic part of aclarubicin is a cinerulosyl-2-deoxyfucosyl-rhodosaminyl trisaccharide attached to aklavinone at C-7. Potent anthracyclines with the same trisaccharide residue, such as betaclamycin A (CG7),[1,2] 2-hydroxyaclacinomycin A,[3] and trisarubicinol,[4] were thus obtained when β-rhodomycinone, 2-hydroxyaklavinone, and carminomycinone, respectively, were bioconverted with the strain KE303. We found that prolonged conversion culture with this strain produced new anthracyclinone trisaccharides in which the terminal sugar cinerulose was replaced by a different sugar, rednose.

In this paper, we describe the production of the new anthracycline antibiotics, CG21-C and CG1-C (Fig. 1) by a prolonged microbial conversion of β-rhodomycinone and aklavinone by strain KE303, their structural determination, and biological activity against the cultured L1210 cells.

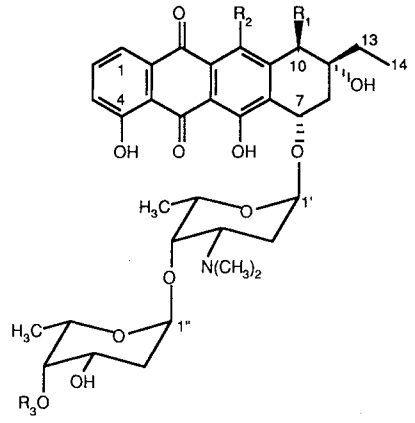
Results

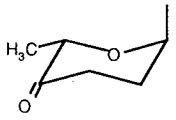
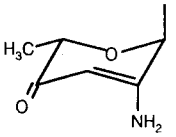
Conditions for Microbial Conversion

The aclarubicin (ACR)-negative mutant KE303 used is a non-anthracycline pigmented strain capable of producing the parental ACR (7-*O*-(cinerulosyl-2-deoxyfucosyl-rhodosaminyl)-aklavinone (AKN)) from exogenous AKN. Betaclamycin A (BCM-A) (7-*O*-(cinerulosyl-2-deoxyfucosyl-rhodosaminyl)-β-rhodomycinone (β-RMN)) was also obtained from β-RMN by conversion with strain KE303. The usual conditions for their formation was that the substrate aglycones were added to a 24-hour culture followed by a further 48-hour cultivation. In the course of optimizing the conditions, we found that a prolonged conversion culture gave an additional spot when the conversion products were analyzed by TLC.

Conversion culture was carried out as previously

Fig. 1. Structures of CG21-C (**1**), CG1-C (**2**), and related antibiotics.



Compound	R_1	R_2	R_3
Betaclamycin A	OH	OH	
Aclarubicin	$COOCH_3$	H	〃
CG21-C (**1**)	OH	OH	
CG1-C (**2**)	$COOCH_3$	H	〃
CG21-B	OH	OH	H

described,[1] except that cultivation was extended by about 96 hours. The products were examined by TLC and HPLC analyses after solvent extraction. Table 1 shows the results of bioconversion of β-RMN with KE303 culture during prolonged cultivation. After 2-days conversion, a single major product (Rf value: 0.51) corresponding to BCM-A was obtained at a maximum level with a 27.4% conversion rate. No other conversion products were detected at this point. However, it decreased gradually with appearance of new spots (Rf values: 0.17 and 0.67) during further cultivation. They

Table 1. Time course of microbial conversion of β-rhodomycinone.

Conversion time (days)	1	2	3	4
β-Rhodomycinone (13.0)*	19.6	3.9	N.D.	N.D.
Betaclamycin A (8.8)	4.2	27.4	23.1	8.4
Betaclamycin B (13.5)	N.D.	N.D.	5.5	4.5
CG21-B (4.1)	N.D.	N.D.	2.5	8.0
CG21-C (5.2)	N.D.	N.D.	4.0	22.8
pH	6.0	6.5	8.3	8.7

Values indicate conversion rate of products or residual rate of a substrate (%).
* Retention time (minutes).
HPLC conditions
Column: YMC A312 (ODS)
Mobile phase: MeCN - 0.05 M ammonium formate (pH 4.0), 38 : 62.
Flow rate: 1.0 ml/minute
Detection: 495 nm.

Fig. 2. Isolation procedure for the products.

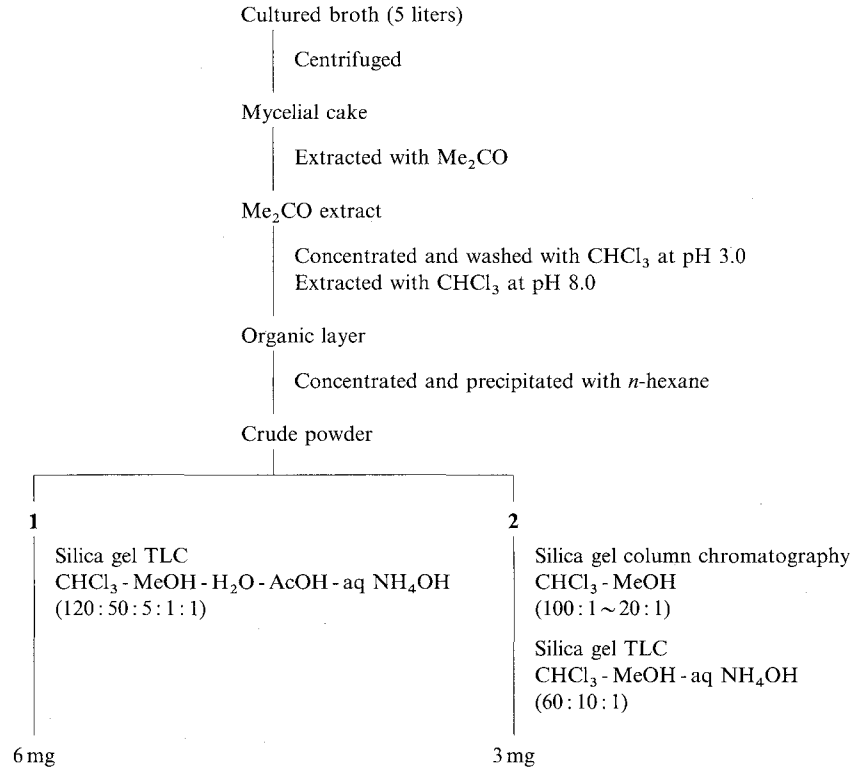


Table 2. Physico-chemical properties of **1** and **2**.

	1	**2**
Appearance	Orange powder	Yellow powder
Molecular formula	$C_{40}H_{50}N_2O_{15}$	$C_{42}H_{52}N_2O_{15}$
HRFAB-MS (m/z)	799.3278 ($(M+H)^+$, $\Delta -1.2$ mmu)	825.3431 ($(M+H)^+$, $\Delta -1.5$ mmu)
MP (°C, dec)	187~190	142~145
$[\alpha]_D^{20}$ ($CHCl_3$)	+43° (c 0.02)	+36° (c 0.01)
UV $\lambda_{max}^{90\%\ MeOH}$ nm ($E_{1cm}^{1\%}$)	203 (284), 235 (528), 255 (339), 285 (360), 495 (188), 529 (124)	229 (411), 262 (294), 282 (294), 431 (115)
IR ν_{max} (KBr) cm^{-1}	1600	1672, 1624

Table 3. ^1H NMR data for **1** and **2**.

Proton	**1**	**2**
1-H	7.89 d (7.3)	7.84 d (7.3)
2-H	7.73 t (7.3)	7.70 t (7.3)
3-H	7.33 d (7.3)	7.32 d (7.3)
7-H	5.16 br s	5.27 d (3.7)
8-Ha	2.1~2.3	2.52 dd (15.4, 3.7)
8-Hb		2.29 d (15.4)
10-H	4.88	4.11 s
11-H	—	7.70 s
13-Ha	1.7~1.9	1.65~1.8
13-Hb		1.45~1.55
14-CH_3	1.11 t (7.3)	1.09 t (7.3)
16-CH_3	—	3.70 s
4-OH	—	12.05 br s, 12.69 br s
6-OH	—	
1′-H	5.51 br s	5.52
2′-CH_2	1.8~1.9	1.55~1.75
3′-H	2.1	2.3
4′-H	3.78 br s	3.74 br s
5′-H	4.03 q (6.6)	4.00 q (6.6)
6′-CH_3	1.30 d (6.6)	1.28 d (6.6)
3′-$N(CH_3)_2$	2.18 s	2.20 s
1″-H	5.06 br d (4)	5.04 br s
2″-Ha	2.1	2.12 dd (12.5, 4.4)
2″-Hb	1.9	1.85 br t
3″-H	4.13 m	4.16 br m
4″-H	3.81 br s	3.78 br s
5″-H	4.55 q (6.6)	4.62 br q
6″-CH_3	1.22 d (6.6)	1.23 d (6.6)
1‴-H	5.29 s	5.29 s
3‴-H	5.18 s	5.26 s
5‴-H	4.57 q (6.6)	4.54 q (6.6)
6‴-CH_3	1.42 d (6.6)	1.43 d (6.6)
2‴-NH_2	6.16 br s	4.74 br s
Solvent	$CDCl_3$-CD_3OD (10:1)	$CDCl_3$

The coupling constants (Hz) are in parentheses.

Table 4. ^{13}C NMR data for **1** and **2**.

Carbon	**1**	**2**
1	119.77	120.17
2	137.23	137.37
3	124.89	124.82
4	162.54	162.60
4a	116.20	115.85
5	191.02	192.74
5a	112.28	114.69
6	156.59	162.17
6a	135.02	132.94
7	70.59	70.72
8	32.67	33.77
9	72.19	71.71
10	66.17	57.13
10a	138.68	142.64
11	157.16	120.95
11a	111.67	131.36
12	186.46	181.39
12a	133.58	133.53
13	30.33	32.16
14	6.53	6.69
15	—	171.31
16	—	52.52
1′	101.51	101.49
2′	29.24	29.66
3′	61.65	61.57
4′	74.20	74.03
5′	68.48	68.26
6′	17.91	17.87
3′-NMe^2	43.26	43.18
1″	99.38	99.36
2″	34.04	34.20
3″	65.45	65.39
4″	82.36	83.29
5″	66.98	66.68
6″	17.04	17.43
1‴	95.03	94.90
2‴	161.33	158.42
3‴	95.09	97.50
4‴	195.06	194.13
5‴	72.19	72.92
6‴	15.91	15.63

Solvents used were the same as shown in Table 3.

were initially identified as CG21-B and betaclamycin B (BCM-B) by Rf values in comparison with the authentic samples which have been previously isolated by microbial bioconversion.[5,8] The lower spot was separated into 2 peaks (RT: 4.1 and 5.2 minutes) by HPLC analysis. The later peak was unknown and designated as CG21-C (**1**). **1** was accumulated at a peak level after 4-days cultivation. The maximum conversion yield of **1** was about 23%. Similarly, it was found that prolonged microbial conversion of AKN with KE303 culture also gave a new product, named CG1-C (**2**), with decrease of initially accumulated ACR (data not shown).

Isolation and Purification

A large scale conversion was carried out using 500-ml Erlenmeyer flasks each containing 50 ml medium. After a 1-day cultivation, the substrate β-RMN or AKN was added at a final concentration of 30 μg/ml, and cultivation was continued for a further 4 days. The products were isolated and purified from a total 5 liters of conversion broth according to the procedures summarized in Fig. 2. The conversion broth (5 liters) was centrifuged and the product extracted with acetone from the mycelial cake. The acetone extract was concentrated and washed with $CHCl_3$, after the pH was adjusted to 3.0 with phosphoric acid. The products were then extracted with $CHCl_3$ at pH 8.0. The organic layer was concentrated and an excess of *n*-hexane was added to it to precipitate the crude products. Purification was performed by silica gel column chromatography and then by preparative silica gel TLC.

Structural Elucidation

The physico-chemical properties of new products, **1** and **2**, are shown in Table 2. Their molecular formulas were determined to be $C_{40}H_{50}N_2O_{15}$ and $C_{42}H_{52}N_2O_{15}$, respectively, by high-resolution FAB-MS analysis, and their molecular masses were 13 mass units higher than those of BCM-A and ACR, respectively. Both compounds also showed a characteristic absorption peak at around 285 nm. By qualitative TLC analysis of component sugars and aglycone after complete acid hydrolysis, it was shown that the compounds contained the same aglycone as the added substrate and at least two sugars, rhodosamine (Rf value: 0.14, skyblue) and 2-deoxyfucose (Rf value: 0.54, grayish blue).

^1H and ^{13}C NMR data for **1** and **2** are shown in Tables 3 and 4. The chemical shift assignments and sugar linkage sites were determined by ^1H-^1H COSY, DEPT, and HMBC experiments. There were three anomeric protons and one olephinic proton at δ 5.0~5.5 observed in ^1H NMR analysis for each compound. In ^{13}C NMR spectra, the chemical shifts of both compounds were superimposable on those of BCM-A[2] and ACR,[9] respectively, except for the terminal sugar moiety. It was shown that the chemical shifts of the terminal sugar moiety of **1** and **2** were identical to those assigned for rednose residue which was contained in anthracycline antibiotic rudolphomycin.[10] A broad singlet signal of amino proton at C-2‴ for **1** and **2** was observed at δ 6.16 and 4.74, respectively, and a long range coupling between 3‴-H and C-1‴ was observed for both compounds. The small coupling constants (~4 Hz) of the anomeric protons indicated the glycosidic bonds are all α.

From these findings, the structures of **1** and **2** were determined as shown in Fig. 1.

Biological Activity

The inhibitory activity of **1**, **2**, and related antibiotics on the growth of cultured murine leukemic L1210 cells is shown in Table 5. The products exhibited a diminished cytotoxic activity as compared with parental BCM-A and ACR. This is in contrast to analogs with a variety of neutral terminal sugars which exhibit a similar cytotoxic activity among themselves, and indicates that exchange with a basic terminal sugar markedly affected the cytotoxicity.

Discussion

We reported previously that microbial conversion of various anthracyclinones using a non-ACR producing mutant KE303 produced hybrid anthracyclines which have the constituent glycosidic part of ACR, a cinerulosyl-2-deoxyfucosyl-rhodosaminyl (RN-F-CA) trisaccharide, on the aglycone at C-7. In this study, we found that prolonged microbial conversion gave a new anthracycline metabolite in which the glycosidic part was changed to a rednosyl-2-deoxyfucosyl-rhodosaminyl

Table 5. Inhibitory activities of the products and related compounds on growth of cultured L1210 leukemia cells.

Compound	Structure		IC_{50} (μg/ml)
	Aglycone	Sugar*	
CG21-C (**1**)	β-Rhodomycinone	RN-dF-Red	0.15
Betaclamycin A	β-Rhodomycinone	RN-dF-CA	0.01
CG1-C (**2**)	Aklavinone	RN-dF-Red	0.15
Aclarubicin	Aklavinone	RN-dF-CA	0.02

Cultured L1210 leukemia cells (5×10^4 cells/ml) were exposed for 48 hours to the drugs and viable cells were counted by coulter counter. IC_{50} is expressed as a drug concentration required to inhibit by 50% control growth of cultured L1210 cells.

* RN: Rhodosamine, dF: 2-deoxyfucose, Red: Rednose, CA: Cinerulose A.

(RN-F-Red) residue. This trisaccharide residue was the same as that of rudolphomycin which was isolated by DOYLE *et al.*[10] as one component of anthracycline bohemic acid complex produced by an *Actinosporangium* species strain. We have not detected this sugar type in anthracycline analogs from any fermentation beer of ACR,[9] rhodirubins,[11] and obelmycins.[12] A time course analysis of the conversion yield for **1** indicated that the origin of **1** was BCM-A because the decrease of the initially accumulated BCM-A was followed by the increase in **1** yield during further cultivation.

We have found earlier that a specific oxidoreductase plays a role in the production of ACR analogs by ACR fermentations. This enzyme catalyzed the conversion of ACR to its sugar analog aclacinomycin Y by dehydrogenation of the terminal sugar cinerulose. The sugar residue thus obtained was aculose[9] with a highly reactive α, β-unsaturated ketone structure. It was clear that a major ACR analog, aclacinomycin B (ACM-B), was produced by the intermolecular formation of an ether bond between terminal aculose and the second 2-deoxyfucose. The microbial conversion of AKN and β-RMN also gave rise to such ether-bond analogs, ACM-B and BCM-B, respectively, at a low level. For these reason, we deduced that **1** and **2** were formed by the addition of ammonia to an α, β-unsaturated ketone on the terminal aculose to give rednose. It seemed that the prolonged cultivation needed for yield of **1** or **2** was related to the accumulation of ammonia in the culture because the pH of broth rapidly elevated to above 8 after 3-days conversion.

The cytotoxic activity of **1** and **2** were 7~15 fold weaker than those of BCM-A and ACR, respectively. MATSUZAWA *et al.* reported that IC_{50} values for rudolphomycin (ε-pyrromycinone-RN-F-Red) and MA144 A2 (ε-pyrromycinone-RN-F-CA) were 36 nM and 7 nM, respectively.[13] Our data is in good accordance with their results, indicating that a replacement of cinerulose in the trisaccharide by rednose caused a significant decrease in cytotoxicity. β-RMN glycosides such as BCM-A and oxaunomycin[14] have good antitumor activity against leukemia L1210 *in vivo*, but they induce a weight loss due to their side effects near at a maximum effective dose. The cytotoxicity of **1** is moderate as well as that of doxorubicin. Therefore, we expect that **1** would have an more improved antitumor activity with less side effect.

Experimental

General

MP's were determined on a Kofler hot stage microscope and are uncorrected. UV spectra were recorded on a Hitachi EPS 3T and IR spectra (KBr pellet) on a Hitachi EPI-GS Spectrophotometer. ^1H and ^{13}C NMR were recorded with a JEOL JNM-GSX400 spectrometer at 400 MHz and 100 MHz, respectively. Chemical shifts were expressed in δ values (ppm) with TMS as an internal standard and coupling constants were given in J (Hz). Mass spectra were recorded with a JEOL JMS-SX102A spectrometer. Specific rotations were recorded on a Jasco DIP-181 digital polarimeter. TLC analysis was performed on Silicagel 60 F_{254} (E. Merck).

Microorganism

The aclarubicin-negative mutant strain KE303[1] of *S. galilaeus* MA144-M1 was used for the microbial conversion, grown at 28°C on YS agar slant (yeast extract 0.3%, soluble starch 1.0%, and agar 1.5%, pH 7.2) and then stored at 5°C before use.

Microbial Conversion

A seed culture was grown aerobically at 28°C for 48 hours in a 500-ml Erlenmeyer flask containing 100 ml of the following medium: soluble starch 0.5%, glucose 0.5%, soybean meal 1.0%, yeast extract 0.1%, NaCl 0.1%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, pH 7.4. This culture was employed as an inoculum (2%, v/v) for a 500-ml Erlenmeyer flask containing 50 ml of conversion medium. Conversion medium consisted of soluble starch 20 g, glucose 10 g, soybean meal 30 g, NaCl 3 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 1 g, yeast extract 1 g, $CuSO_4 \cdot 5H_2O$ 0.005 g, $FeSO_4 \cdot 7H_2O$ 0.0008 g, $MnCl_2 \cdot 4H_2O$ 0.0064 g, $ZnSO_4 \cdot 2H_2O$ 0.0016 g in 1000 ml of tap water, pH 7.4. Cultivation was carried out at 28°C on a rotary shaker (200 rpm). After 24 hours, a methanol solution of a substrate (3 mg/ml) was added to the growing culture to give a final concentration of 30 μg/ml and the cultivation was continued for a further 96 hours.

Analysis of Products

For the detection of the products of microbial conversion, to 2 ml of the culture broth an equal volume of acetone and 0.1 ml of 1 M Tris-HCl buffer (pH 8.0) were added. After agitation, products were extracted with 2 ml of $CHCl_3$ and the organic layer was evaporated *in vacuo* to dryness. The residual anthracycline pigments were analyzed by TLC and HPLC. TLC was performed using a developing solvent of $CHCl_3$ - MeOH - HCOOH - H_2O (70 : 10 : 0.2 : 0.1). HPLC was performed on a Shimadzu HPLC system consisting of LC-6A pump, SPD-6AV detector, and Chromatopak C-R3A integrator with a reverse-phase column, YMC-A312 (ODS) (150 × 6 mm i.d., Yamamura Chemical Laboratories Co. Ltd.). Acetonitrile - 0.05 M ammonium formate buffer (pH 4.0) (38 : 62) was used as a mobile phase and run at a flow rate of 1.0 ml/minute. Samples were dissolved in 100 μl of the mobile phase and 10 μl of the sample solution was injected. Absorbance was monitored at 495 nm.

CG21-C (1)

β-Rhodomycinone (150 mg) was converted using 5 liters of the culture of strain KE303. The cultured broth was centrifuged and the product was extracted with 5 liters of acetone from the mycelial cake. The acetone extract was evaporated *in vacuo* to about 200 ml and

adjusted to pH 3.0 with phosphoric acid. This solution was washed with a half volume of $CHCl_3$ and extracted with $CHCl_3$ at pH 8.0. The organic layer was evaporated *in vacuo* to a small volume and an excess of *n*-hexane was added to precipitate a crude powder of the product. The crude powder was purified twice by silica gel TLC (Silicagel 60 PF_{254}, E. Merck) using $CHCl_3$ - MeOH - H_2O - AcOH - concd NH_4OH (120 : 50 : 5 : 1 : 1) as a developing solvent. The relevant band (Rf: 0.6) was scraped from the TLC plate and extracted with $CHCl_3$ - MeOH (5 : 1). The product was extracted with 0.1 M acetate buffer (pH 3.0) and re-extracted with $CHCl_3$ at pH 8.0. The $CHCl_3$ layer was washed with H_2O, dried over anhydrous sodium sulfate, and evaporated *in vacuo* to a small volume. To the concentrate, an excess of *n*-hexane was added to precipitate a reddish orange powder (6 mg) of **1**.

CG1-C (**2**)

Conversion of aklavinone (150 mg) and preparation of the crude powder of product was carried out in the same way as described above. Crude **2** was dissolved in $CHCl_3$, applied to a column of silica gel (Wakogel C-200), and eluted with $CHCl_3$ - MeOH (100 : 1 ~ 20 : 1). The active fractions were pooled, washed with H_2O, and evaporated *in vacuo* to dryness. The concentrate was further purified by silica gel TLC (Silicagel 60 PF_{254}, E. Merck) using $CHCl_3$ - MeOH - concd NH_4OH (60 : 10 : 1) as a developing solvent. The relevant band (Rf: 0.6) was scraped from the TLC plate and extracted with $CHCl_3$ - MeOH (7 : 1). The extract was washed with H_2O and evaporated *in vacuo* to dryness. The residual pigment was dissolved in 5 ml of 0.1 M acetate buffer (pH 3.0), washed with 2 ml of toluene and extracted with $CHCl_3$ at pH 8.0. The $CHCl_3$ layer was washed with H_2O, dried over anhydrous sodium sulfate, and evaporated *in vacuo* to a small volume. To the concentrate, an excess of *n*-hexane was added to precipitate a yellow powder (3 mg) of **2**.

Qualitative Determination of Aglycone and Sugars by TLC

The product (0.5 mg) was dissolved in 1 ml of 0.1 N HCl and heated at 85°C for 30 minutes in a water bath. The aglycone, thus obtained, was extracted with $CHCl_3$. The $CHCl_3$ layer was evaporated *in vacuo* to dryness and the pigment residue was then subjected to TLC using a developing solvent of $CHCl_3$ - MeOH (30 : 1).

Alternatively, the aqueous layer containing sugar components was neutralized by addition of silver carbonate with a small amount of charcoal and centrifuged. The supernatant fluid was concentrated *in vacuo* and subjected to TLC using a developing solvent of BuOH - AcOH - H_2O (4 : 1 : 1). Sugars were detected by spraying with *p*-anisaldehyde - H_2SO_4 (5% each) in 90% EtOH and heating at 90°C. Aclarubicin was also hydrolyzed under the same condition and the aqueous layer was used as a source of authentic sugars including L-rhodosamine, 2-deoxy-L-fucose, and L-cinerulose; Rf values of them were 0.14, 0.54, and 0.80, respectively.

Biological Activity

In vitro cytotoxicity in cells of murine L1210 leukemia was assayed according to the method previously described.[15]

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