BIOSYNTHESIS OF ANTHRACYCLINE ANTIBIOTICS BY *STREPTOMYCES GALILAEUS*

II. STRUCTURE OF NEW ANTHRACYCLINE ANTIBIOTICS OBTAINED BY MICROBIAL GLYCOSIDATION AND BIOLOGICAL ACTIVITY

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New anthracycline antibiotics derived from ε-, γ- and β-rhodomycinones and ε-isorhodomycinone by the microbial glycosidation using an aclacinomycin-negative mutant, the strain KE303, of *Streptomyces galilaeus* MA144-M1 were studied to elucidate their structures and biological activities. These antibiotics were the products in which the anthracyclinones added as precursors were linked at C-7 or C-10 position with the same trisaccharide moiety (cinerulosyl-2-deoxyfucosyl-rhodosaminyl group) as in the parental antibiotic aclacinomycin A. In addition to antimicrobial activity, they exhibited the growth inhibition of cultured L1210 leukemia cells and the marked inhibition against DNA and RNA synthesis.

In the preceding paper[1] we examined the microbial conversion of anthracyclinones by their addition to cultures using an aclacinomycin-negative mutant, the strain KE303, of *Streptomyces galilaeus* MA144-M1, and found that some of anthracyclinones tested were converted to biologically active anthracycline glycosides. Thus, several new anthracyclines were obtained. As described in the preceding paper[1], anthracycline glycosides produced biosynthetically from aklavinone, 10-decarbomethoxyaklavinone[2], 4-O-methylaklavinone[2] and ε-pyrromycinone were known compounds[1], and identified to be aclacinomycin A (CG1), 10-decarbomethoxyaclacinomycin A (CG2), 4-O-methylaclacinomycin A (CG3) and cinerubin A (CG4), respectively. On the other hand the other products CG5 to 8 derived from ε-, γ- and β-rhodomycinone and ε-isorhodomycinone were shown to be new anthracyclines in view of the fact that their sugar moieties contain cinerulose A, which has not been found in the other anthracyclines than aclacinomycin A[3] and cinerubin A[4].

This paper deals with the structural elucidation of new anthracyclines CG5, 6, 7 and 8; named 11-hydroxyaclacinomycin A, 11-hydroxycinerubin A, 10-decarbomethoxy-10,11-dihydroxyaclacinomycin A and 4‴-dehydrorhodomycin Y, respectively.

Results and Discussion

New anthracycline glycosides produced from anthracyclinones by their addition to cultures of the mutant strain KE303 of *S. galilaeus* MA144-M1 were named as CG compounds as follows: CG5 (11-hydroxyaclacinomycin A) derived from ε-rhodomycinone, CG6 (11-hydroxycinerubin A) from ε-isorhodomycinone, CG7 (10-decarbomethoxy-10,11-dihydroxyaclacinomycin A) from β-rhodomycinone and CG8 (4‴-dehydrorhodomycin Y) from γ-rhodomycinone.

Structures of CG5, CG6, CG7 and CG8

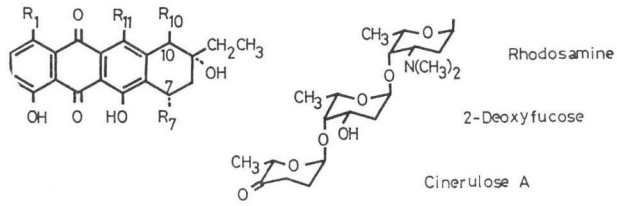
Treatments of CG5, CG6, CG7 and CG8 in 0.1 N hydrochloric acid at 85°C for 30 minutes gave aglycones and sugar moieties. The aglycone moieties of CG5, CG6, CG7 and CG8 were identified to be ε-rhodomycinone, ε-isorhodomycinone, β-rhodomycinone and γ-rhodomycinone, which were added to the fermentation medium, respectively, by direct comparison of their melting points, Rf values, IR and mass spectra. Sugar moieties were determined on silica gel TLC, and all compounds were shown to be composed of rhodosamine, 2-deoxyfucose and cinerulose A as detected in acid hydrolysates of aclacinomycin A.

The partial methanolysis of these compounds in 0.01 N methanolic hydrogen chloride-acetone at room temperature for 45 minutes resulted in a methyl glycoside and the corresponding rhodosaminyl-aglycone which yielded rhodosamine and aglycones by further acid hydrolysis. Methyl glycoside was identical with the authentic methyl L-cinerulosyl-2-deoxy-L-fucoside[4] obtained from the methanolysis of aclacinomycin A by the direct comparison of their Rf values and color developments.

ε-Rhodomycinone, ε-isorhodomycinone and γ-rhodomycinone aglycones which were found in CG5, CG6 and CG8, respectively, have only one hydroxyl group at C-7 or C-10 which should be the possible site for glycosidic linkage. On the other hand, CG7 has two hydroxyl groups for glycosidic linkage at the C-7 and C-10 positions, as found in β-rhodomycinone glycosides isolated from *S. purpurascens*[5].

In order to determine the site of glycosidic linkage, CG7 was reductively cleaved by hydrogenolysis on palladium catalyst, and the product gave two red spots of aglycones on TLC using benzene - acetone - formic acid (100: 15: 1 v/v/v) as developing solvent. The starting material, CG7, stuck to the origin on the TLC and the faster developing spot was suggested to be 10-deoxy-γ-rhodomycinone (=7,10-dideoxy-β-rhodomycinone) which was reduced at the C-7 and 10 positions of β-rhodomycinone, because the amount of this spot increased with the time of hydrogenolysis. On the other hand, the major

Table 1. Structures of CG compounds.



Compound	R_1	R_{11}	R_{10}	R_7
ACM (AKN-Sugar)	H	H	CO_2CH_3	O-Sugar
CG 5 (ε-RMN-Sugar)	H	OH	CO_2CH_3	O-Sugar
CG 6 (ε-IsoRMN-Sugar)	OH	OH	CO_2CH_3	O-Sugar
CG 7 (β-RMN-Sugar)	H	OH	OH	O-Sugar
CG 8 (γ-RMN-Sugar)	H	OH	O-Sugar	H

Abbreviation: ACM = Aclacinomycin A, AKN = Aklavinone, ε-RMN = ε-Rhodomycinone, ε-IsoRMN = ε-Isorhodomycinone, β-RMN = β-Rhodomycinone and γ-RMN = γ-Rhodomycinone.

Table 2. Chemical shift-assignments of ^{1}H-NMR spectra of CG compounds.

H	ACM ppm (*J* Hz)	CG5 ppm (*J* Hz)	CG6 ppm (*J* Hz)	CG7 ppm (*J* Hz)	CG8 ppm (*J* Hz)
1-H	7.79 dd (7, 1.6)	7.85 dd (7, 1.6)	—	7.84 dd (7, 1.6)	7.87 dd
2-H	7.65 t (7, 7)	7.68 t (7, 7)	7.27 s	7.70 t (7, 7)	7.67 t
3-H	7.25 dd (7, 1.6)	7.28 dd (7, 1.6)		7.28 dd (7, 1.6)	7.26 dd
4-OH	11.95 b		12.26		12.19
6-OH	12.64 b		12.30		12.70
11-OH	—		12.80		13.80
1-OH	—	—	12.90	—	—
7-H	5.28 b (W1/2, 6)	5.24 b	5.24 b	5.13 b	2.9~m (7-CH_2)
8-CH_2	2.4~m	2.4~m	2.4~m	2.4~m	2.4~m
9-OH	4.57 bs	4.60 bs	4.59 bs	4.13 bs	
10-H	4.11 bs (W1/2, 2.6)	4.28 bs	4.28 bs	4.90 bs	4.97 bs
11-H	7.65 s	—	—	—	—
13-CH_2	1.7~m	1.7~m	1.7~m	1.7~m	1.7~m
14-Me	1.09 t	1.12 t	1.13 t	1.11 t	1.08 t
16-OMe	3.70 s	3.71 s	3.72 s	—	—
1′-H	5.52 b (W1/2, 5.5)	5.52 b	5.53 b	5.50 b	5.40 b
2′-CH_2	1.9~m	1.9~m	1.9~m	1.9~m	1.9~m
3′-H	2.4~m				
3′-NMe_2	2.17 s	2.18 s	2.20 s	2.17 s	2.19 s
4′-H	3.78 bs (1~)			3.75 bs	3.70 bs
5′-H	4.56 q (6.5, 1~)	4.56 q (6.5, 1~)	4.56 q (6.5, 1~)	4.56 q (6.5, 1~)	4.52 q (6.5, 1~)
6′-Me	1.16 d (6.5)	1.16 d (6.5)	1.16 d (6.5)	1.16 d (6.5)	1.15 d (6.5)
1″-H	5.03 b (W1/2, 5~)	5.03 b	5.04 b	5.04 b	5.00 b
2″-CH_2	1.9~m	1.9~m	1.9~m	1.9~m	1.9~m
3″-H	4.1~m	3.5~m	3.5~m	3.5~m	4~ m
4″-H	3.7~bs		3.68 bs	3.67 bs	3.64 bs
5″-H	4.02 q (6.5, 1~)	4.02 q (6.5, 1~)		4.03 q (6.5, 1~)	3.90 q (6.5, 1~)
6″-Me	1.29 d (6.5)	1.28 d (6.5)	1.28 d (6.5)	1.29 d (6.5)	1.26 d (6.5)
1‴-H	5.07 t (6, 6)	5.07 t (6, 6)	5.08 t (6, 6)	5.07 t (6, 6)	5.05 t (6, 6)
2‴-CH_2	2~ m	2~ m	2~ m	2~ m	2~ m
3‴-CH_2	2.4~m	2.4~m	2.4~m	2.4~m	2.4~m
5‴-H	4.50 q (6.5, 1~)	4.50 q (6.5, 1~)	4.50 q (6.5)	4.50 q (6.5)	4.48 q (6.5)
6‴-Me	1.33 d (6.5)	1.33 d (6.5)	1.33 d (6.5)	1.33 d (6.5)	1.31 d (6.5)

Spectra were measured in $CDCl_3$ using TMS as the internal reference.

spot migrating with Rf value of 0.38 was deduced to be 7-deoxy-β-rhodomycinone (=γ-rhodomycinone) when the aglycone was linked at C-7 position to the sugar moiety, or 10-deoxy-β-rhodomycinone in the case of having the sugar moiety linked at the C-10 position. In the PMR spectrum of the major aglycone recrystallized from acetone, two methylene groups at δ 1.8~ and δ 2.8~ were shown to be

coupled with each other and assigned as C-8 and C-7 methylene protons, respectively. Moreover, the doublet at δ 4.63 (J=5.0) was assigned to C-10 proton, coupling with the doublet at δ 4.28 (J=5.0) which was assigned to C-10 hydroxyl group. The results indicated that the major aglycone obtained by hydrogenolysis is identical with γ-rhodomycinone, and thus, the sugar moieties linked to the C-7 position of β-rhodomycinone in CG7.

The structures of the compounds CG5, CG6, CG7 and CG8 are deduced to be cinerulosyl-2-deoxyfucosyl-rhodosaminyl-ε-rhodomycinone, -ε-isorhodomycinone, -β-rhodomycinone and -γ-rhodomycinone, respectively, as illustrated in Table 1. The chemical shifts and assignments of their PMR and CMR spectra are shown in Tables 2 and 3, respectively. In both spectra, the peaks corresponding to the three sugars are superimposable in all of these CG compounds, indicating that they have the same trisaccharide moiety.

Biological Activity

The antimicrobial activities of the CG compounds tested by the broth dilution method are shown in Table 4. These anthracyclines are active against Gram-positive bacteria.

The effects of CG compounds on the cytotoxicity and synthesis of DNA and RNA of cultured L1210 leukemia cells were examined by the comparative experiment with aclacinomycin A, and the results are summarized in Table 5. For the determination of cytotoxicity, the log. of the cell survivals on day 2 was plotted *versus* drug concentration, and IC_{50} values were estimated. CG5, CG6 and CG7 inhibited strongly the growth of L1210 cells and their IC_{50} values were about 0.01 μg/ml similar to that of aclacinomycin A, while CG8 was approximately 10-times less cytotoxic when compared with aclacinomycin A.

The previous studies concerning the inhibi-

Table 3. ^{13}C-Chemical shift-assignments of CG compounds.

C	ACM*	CG 5	CG 6	CG 7	CG 8
1	120.1	119.6	157.9	119.7	119.4
2	137.3	137.0	129.6	137.1	137.1
3	124.8	124.8	124.8	124.9	124.3
4	162.6	162.6	158.0	162.7	162.5
6	162.2	156.9	157.1	156.9	156.4
11	120.9	157.0	156.4	157.3	158.3
5	192.7	190.7	189.2	190.7	190.9
12	181.3	186.1	189.2	186.2	185.6
4a	115.9	116.1	111.7	116.0	116.1
5a	114.7	111.5	112.7	112.1	110.7
11a	131.5	111.2	111.4	111.5	110.3
12a	133.5	133.4	111.6	133.3	133.7
6a	132.9	135.5	135.0	135.2	136.8
10a	142.7	136.2	136.1	138.7	141.0
7	70.6	70.7	70.7	70.4	27.0
8	33.8	33.4	33.4	30.5	21.0
9	71.7	71.2	71.2	71.8	71.8
10	57.2	52.4	52.4	66.7	70.5
13	32.2	32.4	32.4	32.9	30.8
14	6.7	6.8	6.8	6.6	6.7
15	171.3	171.4	171.4	—	—
16	52.5	52.2	52.2	—	—
1′	101.6	101.8	101.8	101.7	97.1
2′	29.3	29.3	29.2	29.3	29.6
3′	61.6	61.6	61.6	61.6	61.5
4′	74.1	74.1	74.1	74.1	74.5
5′	66.8	66.8	66.8	66.8	66.7
6′	17.0	17.0	17.0	17.0	17.0
NMe_2	43.3	43.3	43.3	43.3	43.3
1″	100.2	100.2	100.1	100.2	100.1
2″	34.4	34.4	34.3	34.3	34.3
3″	65.4	65.4	65.4	65.4	65.3
4″	83.0	83.0	83.0	83.0	82.9
5″	68.4	68.5	68.5	68.5	68.5
6″	17.9	17.9	17.9	17.9	18.2
1‴	99.4	99.4	99.5	99.4	99.4
2‴	27.7	27.7	27.7	27.7	27.7
3‴	33.5	33.5	33.5	33.5	33.5
4‴	210.0	210.0	210.0	210.0	210.1
5‴	71.8	71.8	71.8	71.8	71.8
6‴	14.8	14.8	14.8	14.8	14.8

In ppm (δ), obtained from $CDCl_3$ solution containing TMS as internal reference.

* ref. 3).

Table 4. Antimicrobial activity.

Organisms	Minimum inhibitory concentration (μg/ml)				
	ACM	CG5	CG6	CG7	CG8
Staphylococcus aureus FDA 209P	3.1	0.8	0.4	6.2	12.5
Bacillus subtilis ATCC 6633	0.8	0.4	0.2	1.6	3.1
Bacillus cereus ATCC 9634	0.2	0.2	0.2	1.6	6.2
Bacillus megaterium NRRL B-938	0.8	0.1	0.4	3.1	6.2
Sarcina lutea ATCC 9341	0.8	0.2	0.2	0.8	6.2
Micrococcus flavus	0.2	0.2	0.2	0.8	6.2
Corynebacterium bovis 1810	1.6	0.4	0.4	1.6	3.1
Pseudomonas fluorescens NIHJ B-254	>50	>50	>50	>50	>50
Mycobacterium smegmatis ATCC 607	3.1	0.8	0.8	6.2	25
Candida albicans IAM 4905	50	50	50	50	>50

Broth dilution method.

Table 5. Antitumor activity of new anthracycline antibiotics against L1210 leukemia *in vitro*.

Compound	IC_{50} (μg/ml)			DNA/RNA ratio
	Growth (On day 2)	DNA synthesis	RNA synthesis	
ACM	0.01	0.65	0.09	7.2
CG 5	0.01	0.70	0.08	8.8
CG 6	0.01	1.25	0.11	11.4
CG 7	0.01	0.57	0.15	3.8
CG 8	0.11	0.85	0.28	3.0

tory effects on the macromolecular synthesis of L1210 cells have revealed that aclacinomycin A is a potential inhibitor of RNA synthesis[6,7]. CG5 and CG6 also inhibited preferably RNA synthesis of L1210 cells while the same degree of inhibition of DNA synthesis as in the case of aclacinomycin A was caused at 8~10-times the drug concentration than needed for inhibition of RNA synthesis. CG7 was a less potential inhibitor of RNA synthesis, but somewhat stronger inhibitor for DNA synthesis than aclacinomycin A. In spite of one-order less cytotoxicity, CG8 exhibited the inhibitory effects similar to those of aclacinomycin A for the DNA and RNA syntheses.

Experimental

General

Melting points were determined on a Kofler hot-stage microscope and were uncorrected. PMR and CMR spectra were recorded on a Varian XL-100 spectrometer using tetramethylsilane as reference. The mass spectrum was taken by a Hitachi RMU-6 mass spectrometer with direct inlet system.

Thin-layer chromatography (TLC) was carried out on a silica gel 60 F_{254} plate (E. Merck & Co.) using the solvent systems of chloroform - methanol (10: 1, and/or 30: 1, v/v), benzene - acetone - formic acid (100: 15: 1, v/v/v) and *n*-butanol - acetic acid - water (4: 1: 1, v/v/v). Detection of sugars was done by spraying TLC plate with the mixture of 5% *p*-anisaldehyde and 5% sulfuric acid in ethanol and heating at 90°C for color development.

Mild hydrolysis of CG compounds

Isolation, purification and preliminary characterization of the compounds were described in the preceding paper[1].

A solution of a CG compound (20 mg) in 4 ml of 0.1 N hydrochloric acid was heated in a water bath at 85°C for 30 minutes. The precipitable pigment (aglycone, about 6 mg) was removed by centrifugation and purified by crystallization from acetone. The aqueous acidic layer was neutralized by addition of silver carbonate, and the precipitate was centrifuged off. After extraction of partially hydrolyzed products with chloroform, the aqueous layer was concentrated *in vacuo* and analyzed for sugar components by TLC using the solvent system of *n*-butanol - acetic acid - water (4: 1: 1, v/v/v) following by the visualization. Three sugars corresponding to rhodosamine, 2-deoxyfucose and cinerulose A located at Rf values of 0.12, 0.56 and 0.82, respectively, on TLC were detected in the hydrolyzates of all CG compounds. The aglycones were identified by co-chromatography with authentic aglycones on TLC with several solvent systems described in General Section and by spectral analyses. The aglycones of CG5, CG6, CG7 and CG8 were ε-rhodomycinone (mp. 191~194°C, MS: *m/z* 428 (M^+)), ε-isorhodomycinone (mp. 227°C, MS: *m/z* 444 (M^+)), β-rhodomycinone (mp. 230°C, MS: *m/z* 386 (M^+)) and γ-rhodomycinone (mp. 240°C, MS: *m/z* 370 (M^+)), respectively.

Partial methanolysis of CG compounds

To a solution of a CG compound (2 mg) in dry acetone (0.2 ml) were added dry methanol (0.02 ml) and 0.1 N hydrochloric acid in absolute methanol (0.02 ml). The mixture was allowed to stand at room temperature for 45 minutes in the dark, neutralized by addition of silver carbonate and filtered. The filtrate was evaporated to dryness followed by two 2 ml extractions with ether. After concentration, the ether extract was spotted on a silica gel plate, and developed with ethyl acetate followed by visualization with *p*-anisaldehyde reagent. Methyl glycoside giving a greyish-blue spot (Rf 0.59) co-migrated with the authentic methyl glycoside (methyl cinerulosyl-2-deoxyfucoside) prepared from aclacinomycin A. The ether insoluble pigment (rhodosaminyl aglycone) was hydrolyzed with 0.1 N hydrochloric acid at 85°C for 30 minutes, and rhodosamine and respective aglycone were determined by TLC according to the same manner described above.

Hydrogenolysis of CG7

A solution of CG7 (20 mg) in dry methanol (10 ml) was hydrogenated over 5% $Pd/BaSO_4$ (27 mg) at room temperature under atmospheric pressure for over night. The reaction mixture was filtered and evaporated to about 0.5 ml. The residue was separated into aglycones, sugar moieties and starting material by Sephadex LH-20 column (ϕ 1.2×18 cm) chromatography using the solvent system of chloroform - methanol (1: 2, v/v). The initial fraction containing γ-rhodomycinone and minor 10-deoxy-γ-rhodomycinone was evaporated and purified by preparative TLC with benzene - acetone - formic acid (100: 15: 1, v/v/v). γ-Rhodomycinone was recrystallized from acetone to yield 3.5 mg: mp. 239~241°C, MS: *m/z* 370 (M^+), PMR (dioxane-d_8) δ in ppm: 1.04 (3H, t, J=7.5, H-14), 1.5~2.1 (4H, m, H-8 and -13), 2.5~3.0 (2H, m, H-7), 4.28 (1H, d, J=5.0, OH-10, exchange with D_2O), 4.63 (1H, d, J=5.0, H-10), 7.30 (1H, d.d, J=1.5, and 8.0, H-3), 7.71 (1H, t, J=8.0, H-2), 7.86 (1H, d.d, J=1.5 and 8.0, H-1), 12.15, 12.70 and 13.74 (OH, exchange with D_2O).

Cell culture

L1210 leukemia cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 20% calf serum at 37°C under a 95% air-5% CO_2 atmosphere. Nine tenth ml of cell suspension was placed into each culture tube, and 0.1 ml of the diluted compound solution, which was dissolved in acetate buffer (2×10^{-2} M, pH 3.5) at the concentration of 1 mg/ml and then diluted with phosphate-buffered saline, was added to give the desired concentration of 0.02~0.5 μg/ml on day 0. The cell growth was periodically determined using a hemocytometer over the course of 3 days by counting viable cells stained with trypane blue (0.17%). Cytotoxicity was expressed as IC_{50} of the control growth on day 2.

Incorporation of labelled precursors into nucleic acids

L1210 cells grown in RPMI 1640 medium containing 20% calf serum were collected in early log phase and resuspended in RPMI 1640 medium containing 10% calf serum to 7×10^5 cells per ml. To the suspensions were added one tenth volume of diluted test compound solutions and the pre-incubated for 15 minutes at 37°C. Thymidine-2-^{14}C and uridine-2-^{14}C purchased from New England Nuclear Co. Ltd. were added with 0.05 μCi/ml, respectively. Immediately after the incubation for

60 minutes at 37°C, the reaction was terminated by rapid chilling onto the ice-water bath followed by adding 1 ml of cold 10% trichloroacetic acid (TCA) to 1 ml of the reaction mixture. The precipitate was collected by centrifugation and washed twice with 2 ml of cold 5% TCA, and then dissolved in 0.25 ml of 99% formic acid. The radioactivities were counted in 10 ml of BRAY's scintillator with a Aloka LSC-653 liquid scintillation spectrometer.

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