QUARTROMICIN†, A COMPLEX OF NOVEL ANTIVIRAL ANTIBIOTICS

I. PRODUCTION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND ANTIVIRAL ACTIVITY

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A strain of *Amycolatopsis orientalis* No. Q427-8 (ATCC 53884) was found to produce a complex of new antiviral antibiotics, quartromicin which consisted of at least six components A_1, A_2, A_3, D_1, D_2 and D_3. Structural studies suggested that they are a novel type of molecules unrelated to any known antibiotics. Each component of quartromicin exhibited antiviral activity against herpes simplex virus type 1, influenza virus type A and human immunodeficiency virus.

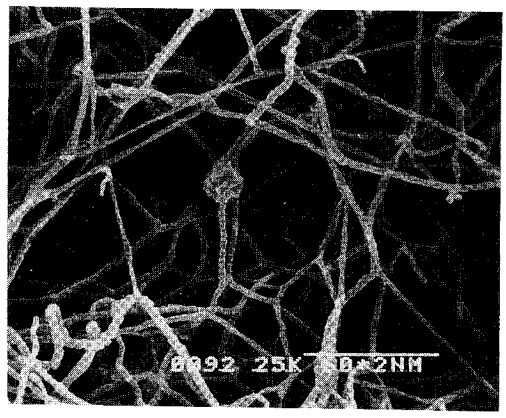
In our continuing search for novel bioactivities in microbial metabolites, a new actinomycete strain isolated from a soil sample collected in Maharastra state, India, was found to produce a complex of new antiviral antibiotics, the quartromicins. The antibiotic complex was extracted from the broth filtrate using non-ionic porous polymer resin and purified by column chromatography to isolate six components A_1, A_2, A_3, D_1, D_2 and D_3. They showed inhibitory activity against herpes simplex virus type 1, influenza virus type A and human immunodeficiency virus with different degrees of potency. This paper reports the taxonomy of the producing organism, fermentation, isolation, physico-chemical properties and anti-HSV and anti-influenza virus activity of the quartromicin components. The structure determination and anti-HIV activity of the quartromicin complex will be reported elsewhere.

Taxonomical Studies

Morphology

Substrate and aerial hyphae are long, monopodially branched, and partially zig-zag-shaped. Partial fragmentation of substrate hyphae occurs at the periphery of intact colony after incubation for 3 weeks. The aerial hyphae bear long straight chains of oblong spores ($0.6 \times 0.6 \sim 3.0\,\mu m$) with smooth surface (Fig. 1). Motile spores, sporangia or synnemata are not formed.

Fig. 1. Scanning electron micrograph of aerial spore chains of strain Q427-8.



Medium: ISP-5. Cultivation: 28°C for 2 weeks.

Cultural and Physiological Characteristics[1,2]

Colorless or yellowish colony is covered with white aerial mycelium. Carotenoid yellow pigment is formed, but melanoid and other distinct pigments

† Quartromicin was originally called as BU-3889V.

are not formed (Table 1). The temperature range for growth is 19°C to 40°C. Among 25 sugars examined, acid production is observed in 22 sugars except for D-melezitose, cellulose and dulcitol (Table 2).

Table 1. Cultural characteristics of strain Q427-8.

Medium	Growth	Aerial mycelium	Reverse color	Soluble pigment
Sucrose-nitrate agar (CZAPEK-Dox agar)	Moderate	Poor; white (263)	Light yellow (86)	None
Tryptone-yeast extract broth (ISP No. 1)	Moderate and not turbid	—	—	Pale yellow (89)
Yeast extract-malt extract agar (ISP No. 2)	Good	Abundant; white (263)	Brilliant orange yellow (67)	Light orange yellow (70)
Oatmeal agar (ISP No. 3)	Moderate	Moderate; white (263)	Colorless	None
Inorganic salts-starch agar (ISP No. 4)	Moderate	Moderate; white (263)	Vivid orange yellow (66)	Pale yellow (89)
Glycerol-asparagine agar (ISP No. 5)	Moderate	Moderate; white (263)	Pale yellow (89)	None
Peptone-yeast extract-iron agar (ISP No. 6)	Poor	No or scant; whitish	Colorless	None
Tyrosine agar (ISP No. 7)	Moderate	Moderate; white (263)	Pale yellow (89)	Pale yellow (89)

Observation after incubation at 28°C for 3 weeks.
Color name: ISCC-NBS color-name charts.

Table 2. Physiological characteristics of strain Q427-8.

Decomposition of:		Erythritol	+
Adenine	−	D-Galactose	+
Casein	+	D-Glucose	+
Hypoxanthine	+	Inositol	+
Tyrosine	+	Lactose	+
Xanthine	+	D-Mannitol	+
Decarboxylation of:		D-Melezitose	−
Benzoate	−	Melibiose	+
Citrate	−	α-Methyl-D-glucoside	+
Mucate	−	Raffinose	+
Succinate	+	Rhamnose	+
Tartrate	−	D-Sorbitol	+
Production of:		Trehalose	+
Nitrate reductase	−	D-Xylose	+
Amylase	+	Utilization of:	
Urease	+	Cellulose	−
Esculinase	+	Dulcitol	−
Gelatinase	+	D-Fructose	+
Tyrosinase	−	D-Mannose	+
Growth on or in:		Salicin	+
Lysozyme broth	−	Soluble starch	+
5% NaCl	+	Sucrose	+
Growth at:		Temperature:	
10°C	−	Growth range (°C)	19～40
45°C	−	Optimal growth (°C)	25～34
Acid produced from:		No growth (°C)	16 and 43
Adonitol	+	Tolerance to:	
D-Arabinose	+	NaCl, 1%～8%	+
L-Arabinose	+	9%	−
Cellobiose	+	pH, 5.0～10.5	+

Chemotaxonomy

Whole cell hydrolysate contains *meso*-diaminopimelic acid, galactose, arabinose and rhamnose. Hence, the cell wall belongs to Type IV_A[3]. Phospholipids contain phosphatidylethanolamine, hydroxylated phosphatidylethanolamine, phosphatidylglycerol and phosphatidylinositol, therefore the strain belongs to Type P-II[4]. Glycolate test is negative (*N*-acyl type of peptidoglycan: acetyl)[5]. Mycolic acid is absent and menaquinone MK-9 (H_4) is observed as a major component[6].

These taxonomical studies indicated that strain Q427-8 is closely related to *Amycolatopsis orientalis*[7] and differentiated from *Amycolatopsis mediterranei*, *Amycolatopsis rugosa*, *Amycolatopsis sulphurea*, *Amycolatopsis fastidiosa* and *Amycolatopsis methanolica*. Thus, strain Q427-8 was identified as *A. orientalis*, and has been deposited with the American Type Culture Collection, Rockville, Maryland under the accession No. ATCC 53884.

Production

A loopful slant culture of *A. orientalis* Q427-8 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the seed medium consisting of soluble starch 0.5%, glucose 0.5%, fish meat extract (Mikuni) 0.1%, NZ-case (Sheffield) 0.2%, NaCl 0.2% and $CaCO_3$ 0.1% (adjusted to pH 7.0 before sterilization). The flask was shaken on a rotary shaker (200 rpm) at 32°C for 4 days. Five ml of the above seed culture was transferred into 500-ml Erlenmeyer flasks each containing 100 ml of the production medium consisted of soluble starch 2%, beet molasses (Nihon Tensai Seito) 1% and $CaCO_3$ 0.5% (adjusted to pH 7.0 before sterilization). The fermentation was carried out at 28°C for 7 days on a rotary shaker (200 rpm). The antiviral activity in the fermentation broth was determined by the cytopathic effect reduction assay method using herpes simplex virus type 1. The production reached a maximum of 50~100 μg/ml after 6 to 7 days fermentation.

A large scale fermentation was carried out in a 200-liter fermenter. Two liters of the seed culture prepared by the above method was transferred into the fermenter containing 120 liters of the production medium described above. The fermentation was run at 32°C for 7 days with stirring at 250 rpm and aeration at 120 liters/minute.

Extraction

The fermentation broth (210 liters, 50~100 μg/ml) was filtered using a Sharples-type centrifuge. The supernatant was adjusted to pH 7.0 with 6N HCl and stirred vigorously with Diaion HP-20 resin (20 liters) for 1 hour and filtered. The resin was washed with water (30 liters) and 20% aqueous methanol (30 liters) and then eluted with 80% aqueous acetone (pH 8.0, 20 liters) two times. Active effluents were combined and concentrated *in vacuo* to an aqueous solution which was applied to a column of Diaion HP-20 (2.4 liters). The column was developed with water and 80% aqueous methanol (9 liters), successively. The bioactive methanolic eluate was evaporated to an aqueous solution which was washed with ethyl acetate. The aqueous layer was taken up and concentrated to afford a crude solid of quartromicin (31.6 g).

Isolation

All operations described hereafter were conducted in a dark room. The crude solid (23.8 g) obtained above was chromatographed on a silica gel column (Wakogel C-200, 350 ml) developed with BuOH - PrOH - conc NH_4OH - H_2O (5 : 5 : 1 : 1, 2 liters and 3 : 3 : 1 : 1, 4 liters, stepwise). First active eluates were combined and concentrated to yield a crude solid of quartromicin D mixture (1.47 g) and the second gave a mixture solid of quartromicin A (6.3 g).

The D mixture solid (1.4 g) was subjected to reversed-phase C18 column chromatography (YMC-ODS, AM type, Yamamura Chem. Lab. Co., Ltd., 800 ml). The column was developed with 0.022 M phosphate buffer solution containing an increasing amount of MeOH (30%, 40% and 50%) and the eluate was examined by TLC (RP-18, Merck; MeOH - 0.022 M phosphate buffer, pH 7 = 50 : 50). Concentration of the first active eluate (Rf 0.18) followed by desalting using a Diaion HP-20 column gave a semi-pure solid of component D_3 (260 mg). The second (Rf 0.14) and third (Rf 0.10) active eluates afforded respectively components D_2 (154 mg) and D_1 (97 mg) as solids. These materials were further purified by preparative HPLC (column: Capcell pak C18, Shiseido, 30 × 250 mm, mobile phase: MeOH - 0.05 M Sørensen buffer, pH 8, 30 ~ 55% linear gradient, detection: UV 254 nm). The peaks corresponding to D_1, D_2 and D_3 were collected and desalted to afford reasonably pure solids of quartromicins D_1 (15 mg, 96% purity by HPLC), D_2 (32 mg, 92% purity) and D_3 (77 mg, 91% purity), respectively.

The A mixture solid (390 mg) was applied to column of YMC-ODS (800 ml) developed with 0.022 M phosphate buffer solution containing an increasing amount of MeOH (25%, 28%, 30% and 35%) and eluates were checked by TLC used above. The three active eluates, showing Rf 0.55, 0.43 and 0.22, were worked up to afford homogenous solids of quartromicins A_3 (70 mg, 97% purity), A_2 (87 mg, 97% purity) and A_1 (54 mg, 91% purity), respectively. Further purification of A_1 (52 mg) was carried out by preparative HPLC mentioned above to yield a pure sample of quartromicin A_1 (39 mg, 98% purity).

Physico-chemical Properties

Quartromicin components were isolated as pale-yellow amorphous powders of weakly acidic nature. Quartromicin D_1 was crystallized from MeOH - EtOAc to obtain pale-yellow needles. Quartromicins A_1, A_2 and A_3 were soluble in water and dimethyl sulfoxide, slightly soluble in methanol and acetone but practically insoluble in other organic solvents. Quartromicins D_1, D_2 and D_3 were soluble in dimethyl sulfoxide but only slightly soluble in water and methanol. Both components A_1 and D_1 showed positive reactions to iodine, ferric chloride and 2,4-dinitrophenylhydrazine but were negative to ninhydrin and Sakaguchi tests. Quartromicin A_1 was positive to anthrone reagent while D_1 was negative to the test. All components of quartromicin were labile to light. Upon exposure to fluorescence light at room temperature, they gradually decomposed. The TLC and HPLC chromatograms of quartromicin components are shown in Table 3.

The physico-chemical properties of quartromicins A_1, A_2, A_3, D_1, D_2 and D_3 are summarized in Tables 4 and 5. The UV spectra of the six components were quite similar, exhibiting the maxima at around 238 and 302 nm in water or methanol and no shift was observed in acidic or alkaline media. The IR spectrum of quartromicin A_1 (Fig. 2) is similar to that of other components, showing characteristic bands at 1710, 1620, 1550 and 1450 cm^{-1} (Tables 4 and 5). The ^1H NMR spectra of quartromicins A_1 and D_1 are shown in Figs. 3 and 4, respectively. The ^{13}C NMR spectra (Table 6) indicated the presence of 39 carbons in quartromicins A_1 and A_3 but 33 carbons in quartromicin D_1. The carbon resonances of quar-

Table 3. TLC and HPLC of quartromicin components.

System[a]	Quartromicin					
	A_1	A_2	A_3	D_1	D_2	D_3
S-1	0.05	0.05	0.05	0.34	0.30	0.28
S-2	0.22	0.43	0.55	0.10	0.14	0.18
S-3	11.6	6.8	4.2	17.9	12.5	9.5

[a] S-1: TLC (Rf), SiO_2 plate, BuOH - PrOH - conc NH_4OH - H_2O (3 : 3 : 1 : 1). S-2: TLC (Rf), RP-18 plate, MeOH - 0.022 M phosphate buffer, pH 7 (50 : 50). S-3: HPLC (Rt, minutes) Capcell pak C18, MeOH - 0.05 M Sørensen buffer, pH 8.0, 30 ~ 55% gradient.

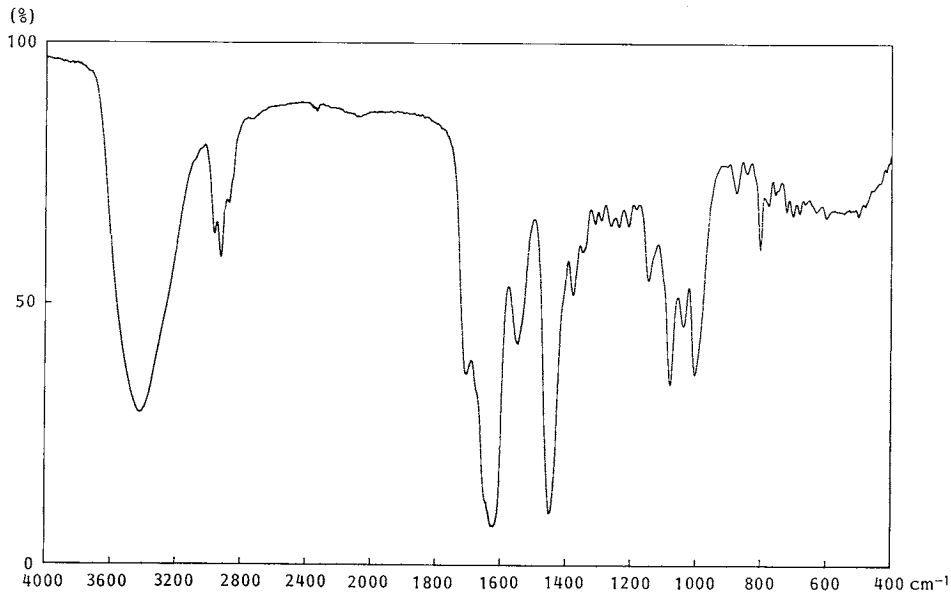
Table 4. Physico-chemical properties of quartromicins A_1, A_2 and A_3.

	Quartromicin A_1	Quartromicin A_2	Quartromicin A_3
MP (°C)	>250	>250	>250
$[\alpha]_D^{25}$ (*c* 0.5, H_2O)	+180°	+108°	+36°
Molecular formula	$C_{78}H_{88}O_{30}$	$C_{78}H_{90}O_{30}$	$C_{78}H_{92}O_{30}$
MW	1,504	1,506	1,508
Negative	1,525 $(M-2H+Na)^-$		
FAB-MS	1,541	1,543	1,545
$((M-2H+K)^-, m/z)$			
UV $\lambda_{max}^{H_2O}$ (ε) nm	238 (84,000), 302 (56,300)	238 (74,100), 302 (59,600)	237 (74,200), 301 (64,900)
IR (KBr) cm^{-1}	3420, 1710, 1620, 1550, 1450, 1150, 1100~1000	3410, 1710, 1620, 1550, 1450, 1140, 1100~1000	3410, 1710, 1620, 1550, 1440, 1150, 1100~1000

Table 5. Physico-chemical properties of quartromicins D_1, D_2 and D_3.

	Quartromicin D_1	Quartromicin D_2	Quartromicin D_3
MP (°C)	>250	>250	>250
$[\alpha]_D^{25}$ (*c* 0.4, pyridine)	+34°	−13°	−17°
Molecular formula	$C_{66}H_{68}O_{20}$	$C_{66}H_{70}O_{20}$	$C_{66}H_{72}O_{20}$
MW	1,180	1,182	1,184
Negative	1,201 $(M-2H+Na)^-$		1,205 $(M-2H+Na)^-$
FAB-MS	1,217	1,219	1,221
$((M-2H-K)^-, m/z)$			
UV $\lambda_{max}^{H_2O}$ (ε) nm	235 (66,000), 302 (52,500)	237 (62,200), 301 (50,400)	237 (54,200), 300 (47,200)
IR (KBr) cm^{-1}	3430, 1710, 1620, 1550, 1455, 1380, 1080, 1000, 800	3420, 1710, 1630, 1550, 1450, 1080, 1000	3420, 1720, 1620, 1540, 1455, 1080, 1000

Fig. 2. IR spectrum of quartromicin A_1 (KBr).



tromicins A_1 and D_1 corresponded well to each other except that the resonances at δ 99.1, 72.0, 70.4, 70.3, 69.3 and 61.9 of A_1 were absent in D_1. Based on the ^{13}C-^1H COSY spectral analysis, these signals were assigned to those of a galactopyranoside moiety. This finding suggested that quartromicin A_1

Fig. 3. ^{1}H NMR spectrum of quartromicin A_1 (400 MHz, D_2O).

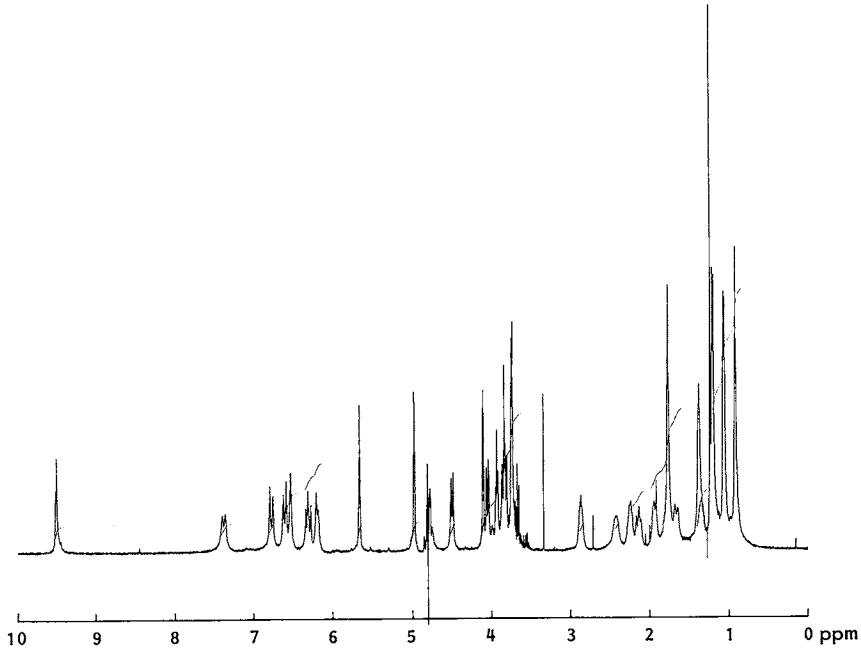
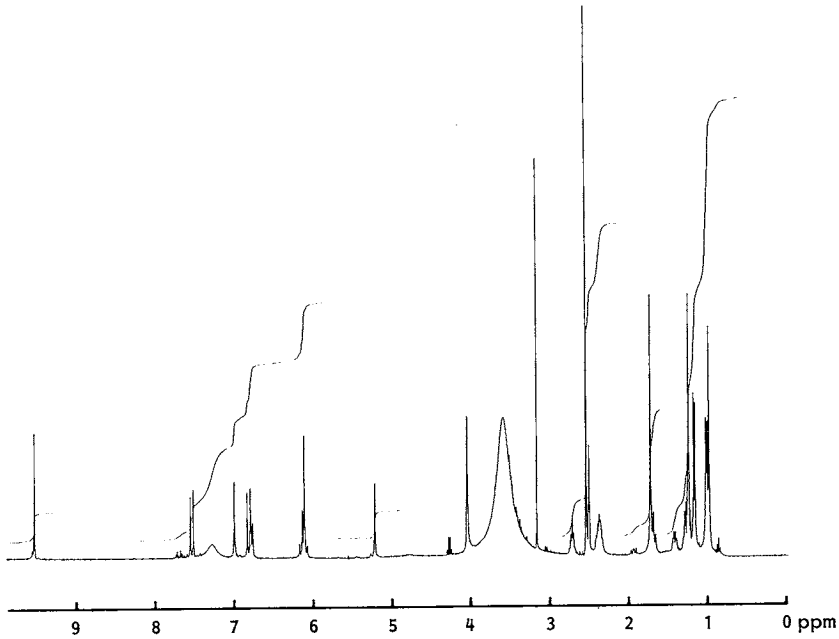


Fig. 4. ^{1}H NMR spectrum of quartromicin D_1 (400 MHz, DMSO-d_6).

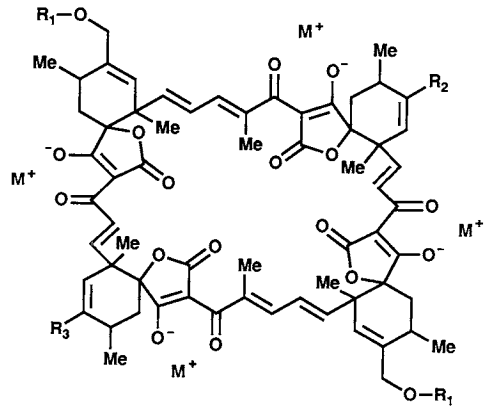


was a galactoside derivative of D_1. In fact, quartromicin A_1 afforded D_1 and methyl D-galactoside upon mild acid methanolysis. Similarly, quartromicin A_2 was converted to D_2 and A_3 to D_3. The molecular formulae of quartromicins A_1 and D_1 were determined to be $C_{78}H_{88}O_{30}$ and $C_{66}H_{68}O_{20}$, respectively, by negative FAB-MS (Jeol JMS-SX 102, matrix: glycerol-thioglycerol) and ^{13}C NMR spectra. Their microanalytical

Table 6. ^{13}C NMR data of quartromicins A_1, A_3 and D_1 (100 MHz).

Carbon	Quartromicin A_1 (D_2O)	Quartromicin A_3 (D_2O)	Quartromicin D_1 (DMSO-d_6)	Carbon	Quartromicin A_1 (D_2O)	Quartromicin A_3 (D_2O)	Quartromicin D_1 (DMSO-d_6)
1	199.3 s	199.5 s	197.3 s	21	87.9 s	87.5 s	85.0 s
2	198.4 d	199.3 s	194.6 d	22	87.5 s	72.2 d	83.3 s
3	198.2 s	196.8 s	193.5 s	23	72.0 d	70.6 t	—
4	196.7 s	184.8 s	192.7 s	24	70.8 t	70.6 d	62.4 t
5	183.7 s	177.8 s	181.7 s	25	70.4 d	70.1 d	—
6	177.1 s	176.7 s	174.0 s	26	70.3 d	69.1 d	—
7	176.8 s	146.6 d	173.9 s	27	69.3 d	65.1 t	—
8	157.5 d	145.1 d	154.5 d	28	61.9 t	61.8 t	—
9	145.6 d	139.7 s	146.4 d	29	45.7 s	45.7 s	45.5 s
10	143.4 d	137.9 s	143.1 d	30	45.4 s	44.0 s	43.5 s
11	143.2 s	137.2 d	142.4 s	31	36.6 t	36.4 t	36.0 t
12	137.9 s	135.8 s	141.4 s	32	32.8 t	33.4 t	33.6 t
13	137.8 d	132.9 d	137.9 d	33	28.7 d	28.6 t	27.5 d
14	135.3 s	130.7 d	133.3 s	34	25.5 d	27.4 d	25.0 d
15	132.7 d	129.6 d	126.6 d	35	22.8 q	23.8 q	25.3 q
16	130.3 d	127.7 d	125.7 d	36	22.5 q	22.6 q	21.7 q
17	127.6 d	99.9 s	124.2 d	37	20.9 q	20.6 q	20.3 q
18	99.9 s	98.8 d	96.8 s	38	18.5 q	18.5 q	19.1 q
19	99.1 d	98.2 s	—	39	12.6 q	12.8 q	12.1 q
20	98.0 s	88.6 s	93.7 s				

Fig. 5. Structures of quartromicin components.



	R_1	R_2	R_3
Quartromicin A_1	α-D-Galactopyranosyl	CHO	CHO
Quartromicin A_2	α-D-Galactopyranosyl	CHO	CH_2OH
Quartromicin A_3	α-D-Galactopyranosyl	CH_2OH	CH_2OH
Quartromicin D_1	H	CHO	CHO
Quartromicin D_2	H	CHO	CH_2OH
Quartromicin D_3	H	CH_2OH	CH_2OH

M^+: Metal ion.

data revealed the absence of nitrogen, sulfur and halogen atoms but did not agree with the calculated values due to the persence of salt (data not shown). As detailed in the separated paper on the structural studies[8], quartromicins contain four tetronic acid moieties which have strong affinity for metal ions (Fig. 5)[9,10], and are difficult to be freed from the metals by conventional methods. The 324 mass unit difference

between quartromicins A_1 and D_1 corresponded to 2 mol of galactose, suggesting that A_1 is a dimeric structure of two 39 carbon compounds. The ^1H and ^{13}C NMR spectra of quartromicin A_3 were very similar to those of A_1 except that the spectra of A_3 lacked the aldehyde signal (δ_H 9.50, δ_C 198.4) observed in those of A_1, but showed a hydroxymethyl signal (δ_H 4.24 and 4.14, δ_C 65.1) instead. While the spectra of A_2 exhibited both the aldehyde and hydroxymethyl signals with about half the intensity compared with that of other common signals. These findings suggested that components A_2 and A_3 are reduced analogs of A_1 with one or both of the two aldehydes of the latter being converted to hydroxymethyl. In fact, upon treatment with $NaBH_4$, both A_1 and A_2 were converted rapidly to A_3.

The structures of quartromicins A_1, A_2 and A_3 have been determined by spectrometric methods and reported in a separate paper[8]. They are unusual symmetrical macrocyclic ring compounds containing four tetronic acids and two D-galactoses (Fig. 5). Quartromicins D_1, D_2 and D_3 are des-D-galactosyl analogues, respectively of quartromicins A_1, A_2 and A_3.

Antiviral Activity

The cytopathic effect (CPE) reduction assay was used to evaluate the antiviral activity of quartromicins A_1, A_2, A_3, D_1, D_2 and D_3 against herpes simplex virus type 1 (KOS strain) infection in Vero cells and influenza virus A (Victoria strain) infection in MDCK cells *in vitro*. Vero and MDCK cells were grown in EAGLE's MEM (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 50 μg/ml amikacin. The cell suspension (200 μl) containing 2×10^4 cells was inoculated to each well of 96- well microplates and cultured at 37°C for 72 hours under a humidified 5% CO_2 −95% air environment. The growth medium in each well was replaced with 250 μl of fresh medium (EAGLE MEM without serum) containing a quartromicin antibiotic with various concentrations, and 50 μl of medium containing approximately $30 \times TCID_{50}$ (50% tissue culture infection dose) of the virus was added to each well. For cytotoxicity test, the same set of wells without virus were prepared. After 72 hours of incubation, the degrees of inhibition of the virus-induced CPE and the drug-induced cytotoxicity were determined by using a neutral red-uptake method. CPE reduction activity is expressed as ID_{50} which is defined as the concentration of compound required to reduce CPE by 50% as compared to the control (no virus and no compound). The concentration of the compound which showed 50% inhibition of the host cells growth is defined as TD_{50} (50% toxic dose).

Acyclovir and ribavirin were used as the reference compounds of anti-HSV activity and anti-influenza virus activity, respectively. The results are shown in Table 7. Quartromicins A_1, A_2 and A_3 exhibited potent antiviral activity against HSV infection (ID_{50}: 11 μg/ml) but showed little or no antiviral activity against influenza virus infection. By contrast, quartromicins D_1, D_2 and D_3 demonstrated antiviral activity against influenza virus infection with ID_{50} values of 6.8~24 μg/ml, but showed less anti-HSV activity.

Table 7. Antiviral activity against herpes simplex virus type 1 and influenza virus A.

	HSV-Vero cell		Influenza virus-MDCK cell	
	ID_{50} (μg/ml)	TD_{50} (μg/ml)	ID_{50} (μg/ml)	TD_{50} (μg/ml)
Quartromicin A_1	11	>100	>100	>100
Quartromicin A_2	11	>100	>100	>100
Quartromicin A_3	11	>100	88	>100
Quartromicin D_1	92	>100	6.8	>100
Quartromicin D_2	35	>100	9.9	68
Quartromicin D_3	20	>100	24	>100
Acyclovir	0.09	>100		
Ribavirin			15	>100

Data represent mean values of two separate experiments.

Discussion

Quartromicin is a complex of novel antiviral antibiotics produced by a strain of *A. orientalis*. Six components of the complex, quartromicins A_1, A_2, A_3, D_1, D_2 and D_3 exhibited inhibitory activity against herpes simplex virus type 1, and influenza virus type A. The structural studies indicated that quartromicin had a novel macrocyclic symmetrical structure composed of four tetronic acid unit. There have been reported several macrocyclic antibiotics containing a tetronic acid unit, *e.g.* kijanimicin[10], tetrocarcin[11], MM 46115[12], chlorothricin[13] and PA-46101 A and B[14]. They differ from quartromicin in having only one tetronic acid in the molecules and in being asymmetrical molecular structures.

The tetronic acid is known to form chelates with various metal cations, and strong chelation of kijanimicin and tetrocarcin with metals has been reported. Quartromicin also produces strong chelation with metal ions and has so far been obtained only as the metal chelate after various attempts to remove the metals. Qualitative and quantitative determination of the metals in quartromicin will be reported separately.

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