ISOLATION, CHARACTERIZATION AND BIOLOGICAL ACTIVITIES OF CONCANAMYCINS AS INHIBITORS OF LYSOSOMAL ACIDIFICATION

JE-TAE WOO, CHIKARA SHINOHARA, KAORU SAKAI,
KEIJI HASUMI and AKIRA ENDO

Department of Applied Biological Science, Tokyo Noko University,
Fuchu-shi, Tokyo 183, Japan

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Four new analogues of concanamycin family, designated concanamycins D, E, F and G, were isolated from the mycelium of *Streptomyces* sp. A1509 by solvent extraction, silica gel column chromatography and HPLC. Structures of these compounds were identified by the combination of spectroscopic analyses. All of these compounds were structurally related to concanamycins A, B and C, which had been isolated previously, and inhibited the acidification of rat liver lysosomes at $10^{-11} \sim 10^{-9}$ M concentration. The structure-activity study showed that the 18-membered macrolide ring and the 6-membered hemiketal ring portions of the molecules of concanamycin family are responsible for potent inhibitory activity.

Concanamycins A (**1**), B (**2**) and C (**3**) were first isolated from *Streptomyces diastatochromogenes* S-45 by KINASHI *et al.*[1]. Absolute configuration of concanamycin A (antibiotic X-4357B) was established by WESTLEY *et al.*[2]. Recently we have demonstrated that concanamycin B is a potent inhibitor of the formation of cholesteryl ester induced by oxidized low density lipoprotein (LDL) in macrophage J774[3]. This inhibition was found to be due to the inhibition of ATP-dependent acidification of endosomes and lysosomes. The present studies deal with isolation, structures and biological activities of new concanamycins **4** (D), **5** (E), **6** (F) and **7** (G).

Materials and Methods

Materials

ATP, nigericin, oleic acid and FITC-dextran were purchased from Sigma. [^{14}C]Oleic acid was obtained from ICN Radiochemicals. Bafilomycin B_1 was isolated from *Streptomyces* sp. A23[4]. Human LDL ($d = 1.019 \sim 1.063$ g/ml) and fetal calf lipoprotein-deficient serum (LPDS) were prepared by ultracentrifugation[5]. LDL was oxidized by exposure to 5 μM $CuSO_4$ in phosphate-buffered saline (PBS) at 37°C[6].

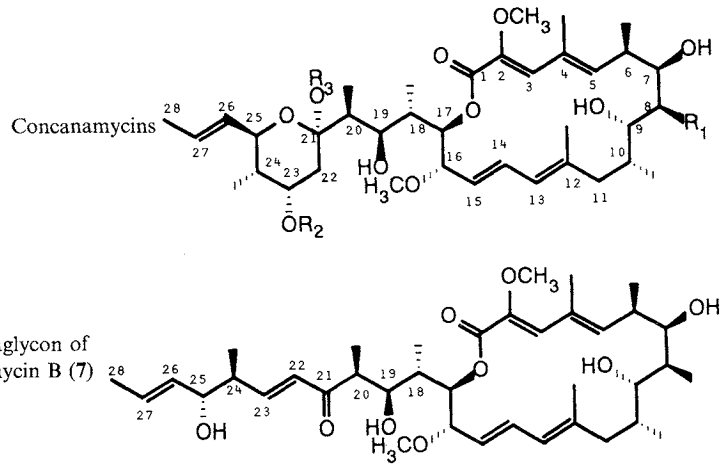
Taxonomic Studies

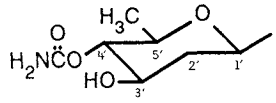
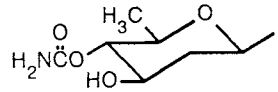
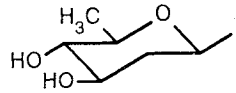
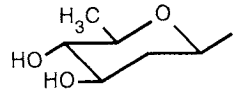
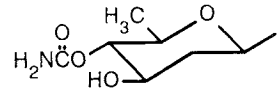
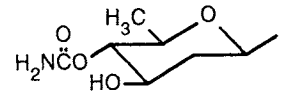
The media and procedures employed in cultural and physiological characterization were described by SHIRLING and GOTTLIEB[7]. Each culture was incubated at 27°C for 2 weeks before observation. Color names were based on the Color Standard of Nihon Shikisai Co., Japan. Cell wall analysis was performed by the methods of BECKER *et al.*[8] and YAMAGUCHI[9]. Utilization of carbon sources was examined according to the method of PRIDHAM and GOTTLIEB[10].

Cells

Mouse macrophage cell line J774 A.1 was obtained from Japanese Cancer Resources Bank. The cells were grown in monolayer in medium A (DULBECCO's modified EAGLE's medium containing 100 units/ml benzylpenicillin and 100 μg/ml streptomycin) supplemented with 10% fetal calf serum. For assays, 1.5×10^5 cells were seeded into 24-well dishes (2 cm^2/well) in 0.4 ml of medium A supplemented with 10% fetal

Fig. 1. Structures of concanamycins.



Compound	R_1	R_2	R_3
Concanamycin A (**1**)	CH_2CH_3		H
Concanamycin B (**2**)	CH_3		H
Concanamycin C (**3**)	CH_2CH_3		H
Concanamycin D (**4**)	CH_3		H
Concanamycin E (**5**)	H		H
Concanamycin F (**6**)	CH_2CH_3	H	H
O-Methyl concanamycin B (**8**)	CH_3		CH_3

calf serum (day 0). On day 1, cells were switched to medium B (medium A supplemented with 10% fetal calf LPDS) and used for experiments on day 2.

[^{14}C]Oleate Incorporation into Cholesteryl Ester

Cells were incubated in 0.3 ml of medium B containing 100 μg protein/ml oxidized LDL and 100 μM [^{14}C]oleate (1×10^4 dpm/nmol) in complex with albumin[5]. In experiments where indicated, oxidized LDL was omitted. After incubation at 37°C for 3 hours, cells were washed with PBS and extracted twice

with 0.5 ml of *n*-hexane-isopropanol (3:2). The extract was evaporated to dryness, dissolved in 40 μl *n*-hexane, and then submitted to TLC.[5] Cholesteryl ester was detected by autoradiography, followed by scraping and counting for radioactivity.

Preparation of Lysosomes and Determination of ATP-dependent Acidification

Macrophage J774 grown in 90-mm dishes received 2 mg/ml FITC-dextran in 5 ml of medium A containing 10% serum. After incubation at 37°C for 5 minutes, cells were chased in a medium lacking FITC-dextran for 30 minutes to label lysosomes. Cells were washed three times with PBS and then collected by scraping in a buffer (pH 7.4) containing 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA and 250 mM sucrose. Particulate fraction of the cells was prepared by ultracentrifugation as described by MARSH *et al.*[11]. Aliquots (200 μg protein) were diluted to 1 ml in a buffer containing 20 mM Tris-trimethylamine (pH 7.4), 100 mM NaCl, 50 mM KCl and 5 mM $MgCl_2$. After incubation at room temperature for 10 minutes, the mixture received 2 mM ATP (potassium salt). ATP-dependent acidification of FITC-dextran-labeled lysosomes were determined by the method of SCHMID *et al.*[12]. Lysosomes from rat liver was prepared by sucrose density gradient centrifugation as described by SCHNEIDER and CHIN[13]. Acidification of rat liver lysosomes was determined by fluorescence quenching of acridine orange[14]. Initial rate of acidification of lysosomes was obtained from fluorescence-quenching curves.

Results

Identification of the Strain A1509

The producing strain A1509 was isolated from a soil sample collected in the suburbs of Tokyo. The cultural characteristics of this strain are shown in Table 1. The aerial mycelium was light brownish gray and branched. It bore more than 20 spores in a spiral chain. Spores were cylindrical-shaped and smooth-surfaced ($0.5 \sim 0.7 \times 1.0 \sim 1.2\,\mu$m). Whole cell hydrolysate contained LL-diaminopimelic acid. The strain utilized L-arabinose, D-xylose, D-glucose, D-fructose, sucrose, inositol, L-rhamnose, raffinose and D-mannitol. The strain showed following physiological characteristics: Optimal growth 24~37°C; positive in nitrate reduction, gelatin liquefaction, starch hydrolysis, milk peptonization and melanin production; negative in milk coagulation. Based on these observations, it was concluded that the strain A1509 belongs to the genus *Streptomyces*.

Table 1. Cultural characteristics of the strain A1509.

Medium	Growth	Aerial mycelium	Soluble pigment
Yeast extract-malt extract agar	Good	Abundant; light brownish gray	Faint, brownish
Oatmeal agar	Moderate	Moderate; light brownish gray	None
Inorganic salts-starch agar	Good	Abundant; light brownish gray	Faint, brownish
Glycerol-asparagine agar	Moderate	Abundant; light brownish gray	None
Peptone-yeast extract-iron agar	Moderate	None	Dark brown
Tyrosine agar	Good	Abundant; light brownish gray	Brown
Sucrose-nitrate agar	Moderate	Abundant; light brownish gray	Faint, brownish
Glucose-asparagine agar	Good	Moderate; light brownish gray	Faint, brownish
Nutrient agar	Moderate	None	Faint, brownish

Fermentation

Streptomyces sp. A1509 was grown in 500 ml Sakaguchi flasks containing 80 ml of the medium consisting of 1% glucose, 3% soluble starch, 0.5% peptone, 1% soybean meal, 0.5% yeast extract, 0.2% $CaCO_3$ and 0.02% CB442. The flasks were shaken on a reciprocal shaker (200 rpm) at 28°C for 4 days and the cultures (240 ml) transferred into 15 liters of the same medium in a 30-liter jar fermenter. The fermentation was carried out at 28°C for 4 days under aeration at 10 liters/minute and agitation at 250 rpm.

Isolation

The mycelium was obtained by filtration from 2 batches of the above cultures (36.5 liters) and washed with water. This mycelium was extracted with 7.5 liters of acetone. The extract was concentrated to 4.1 liters, extracted with 10.3 liters of dichloromethane at pH 8.0. The solvent layer was dried over sodium sulfate, and evaporated *in vacuo* to dryness, giving 21.7 g of an oily residue. HPLC analysis of this residue using silica ODS showed the presence of 7 compounds that inhibited cholesteryl ester formation in macrophage J774 (Fig. 2). The residue was dissolved in dichloromethane and absorbed on a silica gel column (56 × 360 mm). After washing with hexane and hexane-acetone (90:10) successively, the column was developed successively with hexane-acetone (80:20 and 60:40). The active fractions eluted with hexane-acetone (80:20) were combined and subjected to preparative HPLC in Inertsil Prep-ODS column (30 × 250 mm) with acetonitrile-water (55:45) to give 1.4 mg of compound **6**. The active fractions of the hexane-acetone (60:40) eluate were submitted to HPLC as described above, yielding **1** (13 mg), **2** (98 mg), **3** (12.7 mg), **4** (3.6 mg), **5** (1.6 mg) and **7** (4.1 mg). By comparison of spectral data with those described previously[1], **1**, **2** and **3** were identified as concanamycins A, B and C, respectively. The identification of concanamycin B was further established by direct comparison with an authentic sample, kindly provided by Dr. MIZOUE of Taisho Pharmaceutical Company. The hemiketal hydroxyl group in **1** and **2** was readily methylated to give *O*-methyl derivatives of **1** and **2** (**8**) by treating with methanol.

Physico-chemical Properties and Structural Determinations

The physico-chemical properties of compounds **1**, **4**, **5**, **6** and **7** are summarized in Table 2. Compounds **4**, **5** and **7** were obtained as colorless thin plates and compound **6** as a pale yellow plate. They were all soluble in dichloromethane, ethyl acetate, acetonitrile, acetone and lower alcohols but not in water. UV and IR spectra of these compounds were quite similar to those of concanamycins A, B and C. The UV spectra (measured in EtOH) of these compounds indicated in common the presence of an $\alpha,\beta,\gamma,\delta$-unsaturated ester (285 nm) and a conjugated diene (245 nm). Based on the data of FAB-MS, NMR, UV and IR analyses in comparison with those of concanamycins A, B and C, the molecular formulae of these compounds were assigned as shown in Table 2.

Fig. 2. HPLC pattern of crude preparation of concanamycins.

Column: Inertsil Prep-ODS (6 × 250 mm); mobile phase: acetonitrile-water (55:45); flow rate: 2 ml/minute; detection: at 245 nm.

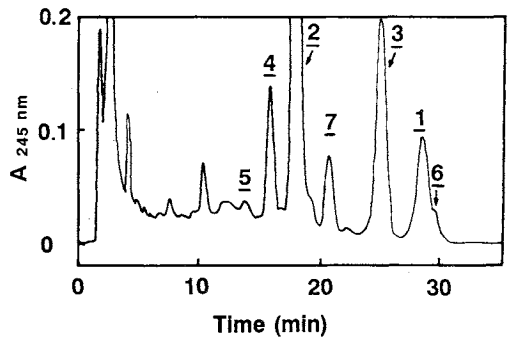


Table 2. Physico-chemical properties of concanamycins.

Compound	Nature	Molecular formula	Molecular weight	FAB-MS (m/z)	UV λ_{max}^{EtOH} nm (ε)	IR ν_{max} ($CHCl_3$) cm^{-1}
1	Colorless thin plate	$C_{46}H_{75}NO_{14}$	865	888 $(M+Na)^+$, 904 $(M+K)^+$	245 (31,500), 285 (15,000)	3448, 2966, 2933, 2878, 1718, 1686, 1618, 1458, 1438, 1389, 1363, 1279, 1251, 1167, 1106, 1067, 1029, 967, 918
4	Colorless thin plate	$C_{44}H_{72}O_{13}$	808	831 $(M+Na)^+$, 847 $(M+K)^+$	245 (30,800), 285 (16,000)	3448, 2969, 2933, 2881, 1686, 1618, 1458, 1375, 1363, 1251, 1196, 1164, 1107, 1070, 1021, 967
5	Colorless thin plate	$C_{44}H_{71}NO_{14}$	837	860 $(M+Na)^+$, 876 $(M+K)^+$	245 (21,300), 285 (14,100)	3448, 2967, 2935, 2879, 1718, 1688, 1618, 1458, 1376, 1364, 1251, 1163, 1108, 1068, 1029, 989
6	Pale yellow thin plate	$C_{39}H_{64}O_{10}$	692	715 $(M+Na)^+$, 731 $(M+K)^+$	245 (24,700), 285 (14,000)	3448, 2958, 2927, 2856, 1686, 1618, 1459, 1375, 1364, 1249, 1106, 967
7	Colorless thin plate	$C_{38}H_{60}O_9$	660	683 $(M+Na)^+$, 699 $(M+K)^+$	245 (32,800), 285 (14,500)	3448, 2970, 2877, 1936, 1686, 1618, 1458, 1376, 1356, 1248, 1105, 969

In the ^{13}C NMR spectra, some carbon signals of the macrolide ring were very broad and some others were not observed, perhaps because of conformational flexibility of the 18-membered macrolide ring (data not shown). Therefore, the structural elucidation was established on the basis of UV, IR, FAB-MS and ^1H NMR spectral analyses by comparison with concanamycin A. 270 MHz ^1H NMR spectra were measured at $-20°C$ in $CDCl_3$. The chemical shift assignments of the proton signals were carried out by either decoupling or ^1H-^1H COSY, and long-range ^1H-^1H COSY. The results for compounds **1**, **4**, **5**, **6** and **7** are listed in Table 3.

In ^1H NMR data for **4**, two olefinic methine signals at 3-H (δ_H 6.39) and 5-H (δ_H 5.63) and three olefinic methine signals at 13-H (δ_H 5.82), 14-H (δ_H 6.54) and 15-H (δ_H 5.16) were assigned to an $\alpha,\beta,\gamma,\delta$-unsaturated ester and to a conjugated diene of the 18-membered macrolide ring, respectively. Allylic couplings between 5-H and 4-CH_3 and between 13-H and 12-CH_3 were observed in the ^1H-^1H COSY spectrum. Two other olefinic methine signals at 26-H (δ_H 5.22) and 27-H (δ_H 5.51) were assigned to an isolated double bond attached to the 6-membered hemiketal ring. The configuration of the disubstituted double bonds was deduced as *E* from the large coupling constants ($J=15.0$ Hz) between 14-H and 15-H and between 26-H and 27-H. The chemical shifts of the *geminal* coupled methylene protons at 22-H_2 (δ_H 1.10 and 2.33, $J=11.0$ Hz) were attributed to an axial and equatorial proton in the 6-membered hemiketal ring, respectively. The methine signal at 1′-H (δ_H 4.55, $J=9.0$ and 1.5 Hz) was assigned to an anomeric proton of a β-pyranoside moiety. These diagnostic NMR data suggested that **4** is similar to concanamycin A. The downfield-shifted methine signal of 8-H (δ_H 2.87) coupled with methyl protons (δ_H 1.10 d, $J=7.0$ Hz) revealed that an ethyl group attached to C-8 of concanamycin A was replaced by a methyl group in **4**. Furthermore, the upfield-shifted signal of 4′-H (δ_H 3.08 br t, $J=9.0$ Hz) and no carbamide proton signal were observed in comparison with those of concanamycin A, indicating decarbamylation of the hydroxyl group attached to C-4′. From these observations and physico-chemical properties, it was suggested that **4** is a decarbamyl derivative of concanamycin B.

Table 3. ^{1}H NMR (270 MHz) data[a] for compounds **1**, **4**, **5**, **6** and **7**.

Proton No.	**1**	**4**	**5**	**6**	**7**
2-OCH_3	3.53 s	3.52 s	3.55 s	3.54 s	3.53 s
3-H	6.37 s	6.39 s	6.37 s	6.38 s	6.41 s
4-CH_3	1.92 br s	2.00 br s	2.05 br s	1.96 br s	1.98 br s
5-H	5.65 br d (10.0)	5.63 br d (10.0)	5.89 br d (10.0)	5.64 br d (10.0)	5.80 br d (9.0)
6-H	2.71 m	2.58 m	2.66 m	2.70 m	3.60 m
6-CH_3	1.01 d (7.2)	1.01 d (7.0)	1.05 d (6.2)	1.06 d (6.2)	1.00 d (6.8)
7-H	3.81 br d	4.01 br d	4.02 m	3.80 br d (8.0)	3.70 br d
8-H	1.48 m	2.87 m	—	1.48 m	2.89 m
8-H_2	—	—	1.50 m,	—	—
	—	—	2.97 m	—	—
8-CH_3	—	1.10 d (7.0)	—	—	1.16 d (6.6)
8-CH_2CH_3	1.12 m	—	—	0.94 m	—
8-CH_2CH_3	0.82 t (7.0)	—	—	0.80 d (7.0)	—
9-H	3.20 br d (11.0)	3.06 br d	3.51 m	3.19 br d (11.0)	3.08 br d (10.0)
10-H	2.25 m	2.34 m	2.19 m	2.31 m	2.36 m
10-CH_3	1.07 d (6.0)	1.04 d (7.0)	0.80 d (7.0)	1.00 d (6.5)	1.00 d (6.7)
11-H_2	1.94 m	1.77 m	1.96 m	1.94 m	1.49 m
12-CH_3	1.83 br s	1.85 br s	1.70 d	1.85 br s	1.80 br s
13-H	5.74 br d (10.5)	5.82 br d (10.5)	5.94 br d (10.5)	5.75 br d (10.5)	5.84 br d (10.8)
14-H	6.54 dd (15.0, 10.5)	6.54 dd (15.0, 10.5)	6.36 dd (15.0, 10.5)	6.54 dd (15.0, 10.5)	6.49 br q (15.8, 10.8)
15-H	5.16 dd (15.0, 9.0)	5.16 dd (15.0, 9.5)	5.18 dd (15.0, 9.2)	5.18 dd (15.0, 9.0)	5.19 dd (15.8, 9.6)
16-H	3.81 t (9.0)	3.82 br t (9.5)	3.76 t (9.2)	3.82 t (9.0)	3.73 t (9.6)
16-OCH_3	3.23 s	3.24 s	3.22 s	3.24 s	3.22 s
17-H	4.98 br d (9.0, 1.0)	5.03 br d (9.5)	4.95 br d (9.2)	4.99 br d (9.0)	5.09 br d (9.6)
18-H	2.14 m	2.14 m	2.19 m	2.14 m	2.09 m
18-CH_3	0.81 d (6.7)	0.80 d (6.6)	0.77 d (6.9)	0.82 d (7.0)	0.92 d (6.0)
19-H	4.00 m	3.98 br d (10.5)	3.95 m	4.00 dd	3.72 dd
20-H	1.75 m	1.72 m	1.75 m	1.73 m	2.86 m
20-CH_3	1.05 d (6.9)	1.04 d (7.0)	1.03 d (7.0)	1.06 d (6.8)	1.10 d (6.7)
22-H	—	—	—	—	6.26 br d (15.8)
22-H_2	1.16 m,	1.10 m,	1.13 m,	1.22 m, 2.36 dd	—
	2.30 dd (11.0, 4.0)	2.33 dd (11.0, 4.0)	2.30 dd (11.0, 4.0)		—
23-H	3.73 m	3.73 m	3.74 m	3.70 m	6.85 dd (15.8, 8.0)
24-H	1.25 m	1.22 m	1.24 m	1.22 m	2.36 m (8.0, 6.7)
24-CH_3	0.89 d (6.5)	0.86 d (6.2)	0.85 d (6.5)	0.92 d (6.4)	0.99 d (6.7)
25-H	3.96 dd (10.0, 7.8)	3.94 br t	3.93 dd (10.8, 8.0)	3.94 dd (10.0, 7.8)	3.87 t (7.2)
26-H	5.24 m (15.0, 7.8, 1.5)	5.22 m (15.0, 7.8)	5.24 m (15.0, 8.0)	5.23 m (15.0, 7.8)	5.41 br d (15.5, 7.2)
27-H	5.52 dq (15.0, 6.4)	5.51 dq (15.0, 6.6)	5.52 dq (15.0, 6.6)	5.53 m (15.0, 6.8)	5.66 dq (15.5, 6.2)
28-H_3	1.55 d (6.4)	1.57 d (6.6)	1.55 d (6.6)	1.57 d	1.69 br d (6.2)
1′-H	4.52 br d (9.0)	4.55 dd (9.0, 1.5)	4.53 br d (9.0)	—	—
2′-H (ax)	1.66 dd	1.60 dd	1.63 dd	—	—
2′-H (eq)	2.18 dd	2.13 dd	2.18 dd	—	—
3′-H	3.74 m (9.0, 6.0)	3.60 m	3.72 m	—	—
4′-H	4.26 t (9.0)	3.08 br t (9.0)	4.26 t (9.0)	—	—
5′-H	3.34 dq (9.0, 6.0)	3.20 dq (9.0, 6.0)	3.34 m (9.0, 6.0)	—	—
5′-CH_3	1.23 d (6.0)	1.27 d (6.0)	1.22 d (6.0)	—	—
OCO-NH_2	4.90 s	—	4.78 s	—	—

[a] ^{1}H NMR spectra were measured at −20°C in $CDCl_3$. Chemical shifts are in ppm downfield of internal TMS (J=Hz).

Abbreviations: br s, broad singlet; br d, broad doublet; br t, broad triplet; br q, broad quartet.

FAB-MS spectrum (*m/z* 860, $(M+Na)^+$ and *m/z* 876, $(M+K)^+$) indicated that **5** is different from concanamycin A only by 28 mass units. In the ^1H NMR spectrum of **5**, the signals of an ethyl group attached to C-8 of concanamycin A were absent and *geminal* coupled signals of 8-H_2 (δ_H 1.50 and 2.97) were appeared. Other spectral data of **5** corresponded well with those of concanamycin A. Thus **5** differs from concanamycin A only in the lack of an ethyl substituent at C-8.

No signal of a carbamide and a sugar moiety including the anomeric proton was observed in ^1H NMR spectrum of **6**. Other ^1H NMR data shown in Table 2 were quite similar to those of concanamycin A. FAB-MS spectrum showed that the molecular weight of **6** (692) was smaller than that of concanamycin A (**1**) by 173, which corresponds to 4'-carbamyl-2'-deoxyrhamnosyl moiety present in concanamycin A (**1**). These observations indicated that **6** is the aglycone of concanamycin A.

The UV, IR and ^1H NMR spectral analyses revealed that the structure of the 18-membered macrolide ring of **7** was identical with that of **4**. The ^1H NMR spectrum of **7** indicated that **7** lacked both the 6-membered hemiketal ring and the β-pyranoside in Fig. 1. The existence of an α,β-unsaturated ketone was deduced from the carbonyl absorption at 1690 cm^{-1} in the IR spectrum of **7** and the downfield-shifted olefinic methine signals at 22-H (δ_H 6.26 br d, $J=15.8$ Hz) and 23-H (δ_H 6.85 dd, $J=15.8$ and 8.0 Hz) in the ^1H NMR spectrum. Two olefinic methine signals (δ_H 5.41 br d, $J=15.5$ and 7.2 Hz and δ_H 5.66 dq, $J=15.5$ and 6.2 Hz) were assigned to 26-H and 27-H of an isolated double bond, respectively. The coupling constants between 14-H and 15-H, 22-H and 23-H, and 26-H and 27-H indicated that the disubstituted double bonds had the *E* configurations. Thus the structure of **7** was determined as the anhydroaglycone of concanamycin B.

From all these findings the structures of **4**, **5**, **6** and **7** (designated concanamycins D, E, F and G, respectively) were determined as illustrated in Fig. 1.

Biological Activity

All concanamycins studied inhibited cholesteryl ester synthesis from [^{14}C]oleate by 50% at a concentration of 8~720 nM (Table 4) in J774 macrophages incubated with oxidized LDL, while no inhibition of cholesteryl ester synthesis was observed when cells were incubated in the absence of oxidized LDL (Fig. 3A). The results are consistent with those previously obtained with **2**[3]. **2** inhibited the acidification of lysosomes in J774 cells by 50% and 99% at 0.09 nM, and 2.00 nM, respectively (Fig. 3B). Similar inhibition was obtained with rat liver lysosomes; the inhibition by the 8 analogues studied was 50% at a concentration of 0.038~1.500 nM (Table 4). The inhibitory potency of the compounds against lysosomal acidification was in parallel with that against [^{14}C]oleate incorporation into cholesteryl ester. The IC_{50}values for **1**, **2**, **3**, **4** and **5** in the inhibition of lysosomal acidification ranged from 0.038 to 0.086 nM. **6**, which lacks 4-carbamyl-2-deoxy-β-D-rhamnose moiety of **1**, was comparable to **1**, **2**, **3**, **4** and **5** in inhibitory potency. However, **8** was 5 times less active than that of **2**,

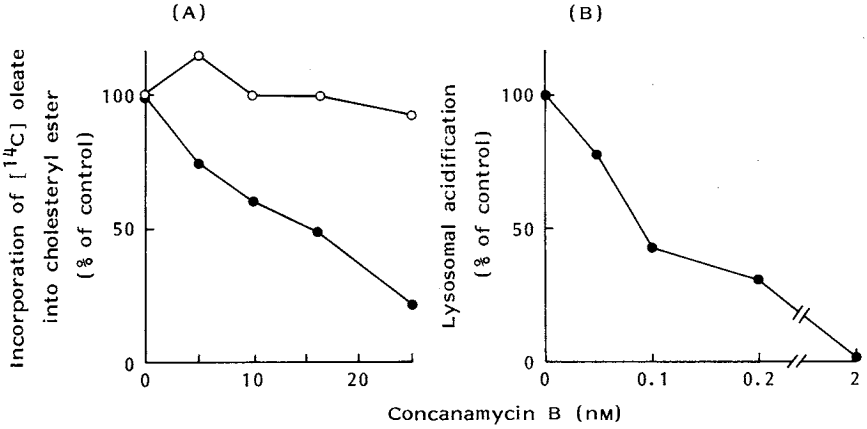
Table 4. Inhibition of cholesterol esterification and lysosomal acidification by concanamycins and bafilomycin B_1.

Compound	IC_{50} (nM)	
	[^{14}C]Oleate incorporation into cholesteryl ester	Lysosomal acidification
1	14	0.061
2	14	0.060
3	8	0.048
4	8	0.085
5	17	0.038
6	12	0.050
7	720	1.500
8	68	0.340
Bafilomycin B_1	8	0.100

Fig. 3. Effects of **2** on the incorporation of [^{14}C]oleate into cholesteryl ester and acidification of lysosomes in J774 macrophages.

A: On day 2, each monolayer of macrophages received 0.1 mM [^{14}C]oleate in complex with albumin and the indicated concentrations of **2** in the presence (●) or absence (○) of 100 μg protein/ml of oxidized LDL. After incubation at 37°C for 3 hours, [^{14}C]oleate incorporated into cholesteryl ester was determined. Each value represents the average of duplicate determinations.

B: J774 cells grown in medium A supplemented with 10% fetal calf serum were incubated with 2 mg/ml FITC dextran to label lysosomes. Particulate fractions were isolated and ATP-dependent acidification was measured as described under Materials and Methods.



and **7** (anhydroaglycone of **2**) was 25 times less active than **2**.

Bafilomycin B_1, known as an inhibitor of proton-ATPase[15], inhibited [^{14}C]oleate incorporation into cholesteryl ester in J774 cells and lysosomal acidification in rat liver, giving IC_{50} values comparable to those for concanamycins (Table 4).

Discussion

1, **2** and **3** have been isolated from *Streptomyces diastatochromogenes* as antibiotics by KINASHI *et al.*[1]. In the present studies we isolated four new compounds of concanamycin family, **4**, **5**, **6** and **7**, from *Streptomyces* sp. A1509. This strain produced concanamycins A, B and C as well as the four new concanamycins. **2** was the major product of the strain. Thus, 98 mg of the compound was obtained from 36.5 liters of culture broth, as compared to 1~13 mg for other concanamycins.

1, **2** and **5** have a 6-membered hemiketal ring bound to the 4-carbamyl-2-deoxy-β-D-rhamnosyl moiety. Neither 2-deoxy-β-D-rhamnosyl moiety nor 4-carbamyl-2-deoxy-β-D-rhamnosyl moiety seemed to be involved in the expression of biological activities since **3**, **4** and **6**, which lack these groups, have an inhibitory activity comparable to that of **1** and **2**. The hydroxyl group on the hemiketal ring as well as the hemiketal ring itself seems to play an important role in biological activity since *O*-methylconcanamycin B (**8**) and anhydroaglycon of **2** (**7**) were 5 and 25 times less active than that of **2**, respectively. However, **7** inhibited lysosomal acidification at a concentration of 10^{-9} M, suggesting that the macrolide ring plays a key role in biological activities. These hypotheses are supported by the data that bafilomycins, macrolide antibiotics having 6-membered hemiketal and 16-membered lactone rings, are potent inhibitors of lysosomal acidification of rat liver. Recently, BOWMAN *et al.* reported that bafilomycins inhibited vacuolar H^+-ATPase activity of *Neurospora crassa*[15]. Effects of concanamycins on the H^+-ATPase activity are to be studied.

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