CLOSTOMICINS, NEW ANTIBIOTICS PRODUCED BY *MICROMONOSPORA ECHINOSPORA* SUBSP. *ARMENIACA* SUBSP. NOV.

I. PRODUCTION, ISOLATION, AND PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

SATOSHI ŌMURA, NOBUTAKA IMAMURA, RUIKO ŌIWA, HIROSHI KUGA, RIMIKO IWATA, ROKURO MASUMA and YUZURU IWAI

The Kitasato Institute and School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo 108, Japan

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A soil isolate named as *Micromonospora echinospora* subsp. *armeniaca* subsp. nov. KMR-593 was found to produce at least five related antibiotics, clostomicins, active against Gram-positive bacteria including anaerobes. From the physico-chemical properties, one of these components was identified with lipiarmycin and others were found to be new antibiotics. Each component includes two chlorine atoms and the molecular weights of A and B_2, C, and D are 1,058, 1,042 and 1,056, respectively. The structural differences were characterized by NMR analyses.

In the course of our screening for new antibiotics from actinomycetes, a strain KMR-593 was found to produce new antibiotics exhibiting a potent activity especially against *Clostridium* sp. The antibiotics, designated clostomicins, were isolated from the cultured broth of strain KMR-593, which was classified as a new subspecies of *Micromonospora echinospora* from taxonomic studies described in the next paper[1].

This paper deals with the production, isolation, physico-chemical and biological properties and the structural relationships among the components of clostomicin.

Production and Isolation

The stock culture of strain KMR-593 was inoculated into 100 ml of a seed medium consisting of glucose 0.1%, starch 2.4%, peptone 0.3%, meat extract 0.3%, yeast extract 0.5%, $CaCO_3$ 0.4% in a 500-ml Erlenmeyer flask and incubated at 27°C for 120 hours. Nine hundred milliliters of the seed culture was transferred into a 50-liter jar fermentor containing 25 liters of a production medium (pH 7.0) consisting of soluble starch 2.0%, dry yeast 1.0%, $CaCO_3$ 0.3% and the aerobic fermentation was carried out at 27°C. The antibiotic production started at 20 hours after the inoculation, then gradually increased and reached a maximum at 155 hours. The cultured broth (50 liters) was centrifuged to obtain about 45 liters of a supernatant fluid and 5.0 kg of wet mycelial cake. The cake was extracted with 70% aqueous acetone (10 liters). After the removal of acetone by evaporation under reduced pressure, the aqueous residual solution (500 ml) was extracted three times with benzene (250 ml). The combined organic extract was concentrated to a small volume (50 ml), and then poured into *n*-hexane (500 ml) to yield an active precipitate (dry weight 720 mg). The supernatant fluid of the cultured broth was applied to a Diaion HP-20 column (1 liter). After washing with water and then 30% aqueous acetone, the active adsorbed principles were eluted with 70% acetone. The eluate was concentrated to 100 ml and extracted three times with benzene (100 ml). The concentrated (50 ml) extract was mixed with *n*-hexane (500 ml) to yield an active precipitate (dry weight 480 mg). Both precipitates

Table 1. Rf values on silica gel TLC and retention time on HPLC (column YMC: AM-324, CH_3CN - H_2O - HCOOH, 63: 37: 1; flow rate 2.0 ml/minute).

	A	B_1	B_2	C	D
Rf: silica gel TLC					
Benzene - Me_2CO (1: 1)	0.57	0.47	0.35	0.48	0.48
$CHCl_3$ - MeOH (9: 1)	0.53	0.50	0.35	0.54	0.50
Rt: HPLC (ODS)					
CH_3CN - H_2O - HCOOH (63: 37: 1)	11.8	13.4	13.4	16.8	20.0

obtained from the mycelial cake and the supernatant fluid were combined and applied to a preparative reversed-phase HPLC column (ODS) using acetonitrile - water - formic acid (63: 37: 1) as a developing solvent system. Four active fractions eluted at different retention times were evaporated to dryness yielding crude materials; a (175.5 mg), b (251.0 mg), c (102.6 mg) and d (116.7 mg). Subsequently fraction was purified by preparative silica gel HPLC column with chloroform - methanol (22: 1). Fraction b was further separated into two active components, B_1 (27.1 mg) and B_2 (9.5 mg) as pure form. The other fractions gave each single active compound; A (15.4 mg), C (18.0 mg) and D (39.4 mg).

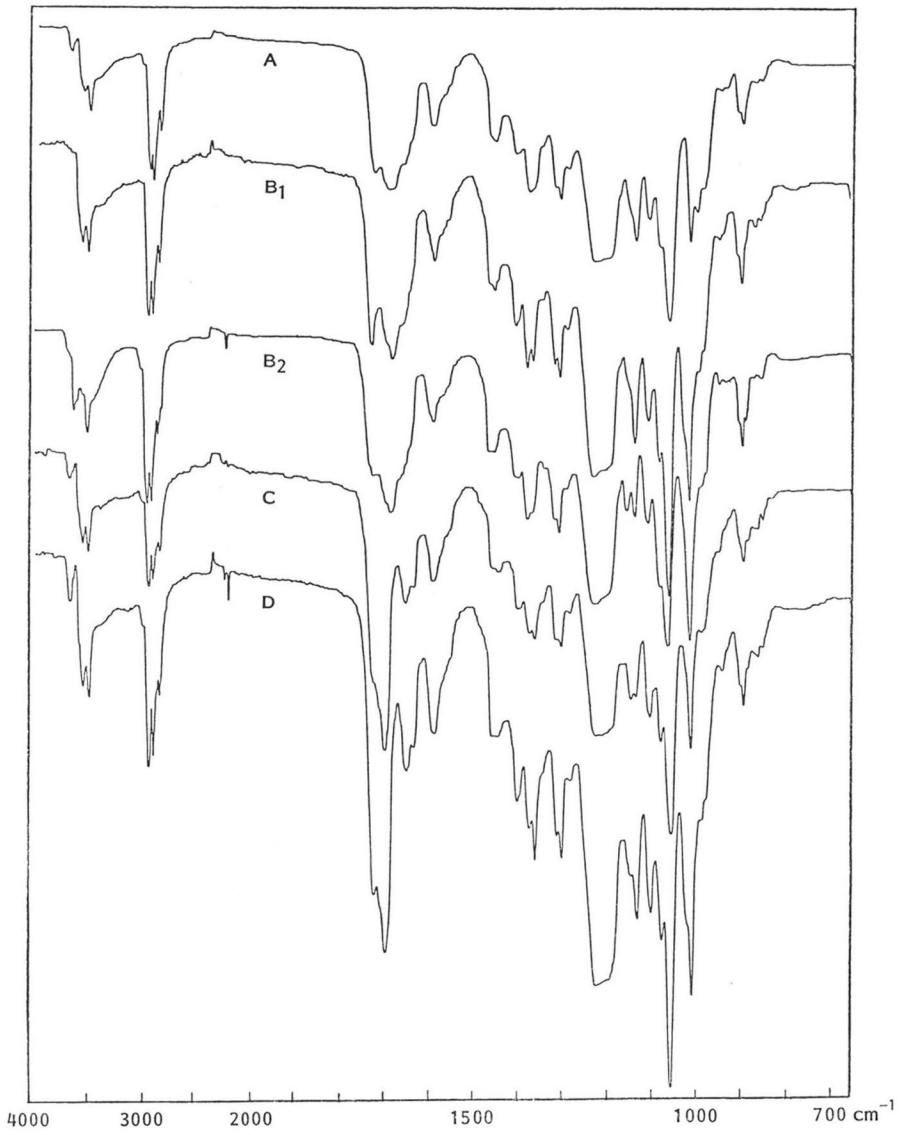
The antimicrobial activity during isolation was assayed by the paper-disk method using *Clostridium perfringens* as test organism. Table 1 shows the Rf values of each components on silica gel TLC and retention time on the ODS column [acetonitrile - water - formic acid (63: 37: 1); flow rate 2.0 ml/minute].

Physico-chemical Properties and Structural Relationship

The UV spectra of the antibiotics showed similar characteristics, *i.e.*, λ_{max}^{MeOH} nm 230, 245 (sh), 270 (sh), 315~320 (sh). The values of $[\alpha]_D^{20}$ (*c* 1.0, methanol) of A, B_1, B_2, C and D were +9.8°, −3.6°, −2.8°, +53.9° and +55.0°, respectively. The IR spectra are demonstrated in Fig. 1. Although the above physico-chemical properties suggest homologous structures, the clostomicins seem to be devided into two groups, indicated by the differences in the carbonyl group region in the IR spectra; one of them includes A, B_1 and B_2 and the other C and D. The EI-MS spectra showed no molecular ion peaks, but the peaks at *m/z* 206 and 208 suggest that all components include halogen atoms. The presence of two chlorine atoms was confirmed by the elemental analysis of main component D (Found (%) C 58.00, H 7.22, Cl 6.60) and by ^{13}C NMR analysis of D. The ^{13}C NMR spectra of the antibiotics revealed 52 carbons for each component except component C which showed 51 carbons. Aforementioned data suggested that the component B_1 might be identical with lipiarmycin[2], the partial structures of which were recently reported[3]. All the published partial structures of lipiarmycin were recognized by the decoupling experiments of ^1H NMR of B_1, and the ^{13}C NMR data[2] of lipiarmycin in $CDCl_3$ were coincident with those of B_1. Therefore, component B_1 is identical with lipiarmycin.

On the basis of the partial structures of B_1 (lipiarmycin), the structural relationships of the components were studied by ^1H and ^{13}C NMR and FD-MS spectroscopic analyses. At first, A and B_2 were compared with B_1. The ^1H NMR spectra of A and B_2 (Fig. 2) showed some obvious differences from that of B_1 especially at the region of 3.5~5.5 ppm. The ^1H NMR spectrum of A exhibited a proton signal of a doublet at 3.89 ppm, which is coupled with a proton signal at 4.72 ppm. The latter also is coupled with a doublet proton at 4.12 ppm which was attributable as Hb (Fig. 4) by the comparison with the spectrum of B_1. Thus, protons at 3.89 and 4.72 ppm were assigned as Hd and Hc,

Fig. 1. IR spectra of clostomicins.



respectively. The downfield-shift of Hc and the upfield-shift of Hd indicate that an acyl group is attached at the 3-position of the partial structure **1** in A instead of the 4-position as in B_1. Since the ^1H NMR spectrum of B_2 displayed a proton signal at 5.32 ppm which is coupled with two protons attributed as Ha at 4.68 ppm with a small coupling constant and as Hc at *ca.* 3.7 ppm, the acyl group could be substituted at the 2-position of the partial structure **1** in place of the 4-position as in B_1. The position of the substituent acyl group seems to be the only difference between substances A, B_1 and B_2.

According to the ^{13}C NMR spectrum (Fig. 3) the other component D possesses an additional ketone carbonyl (205.1 ppm), and lacks the oxygenated methine carbon at 62.2 ppm in B_1. The ^1H NMR spectrum of D showed a methyl signal of an acetyl group at 2.19 ppm. Since the oxygenated methine carbon signal at 62.2 ppm was assigned as C_A of the partial structure **2** in lipiarmycin[3] and

Fig. 2. ^{1}H NMR spectra (90 MHz) of clostomicins.

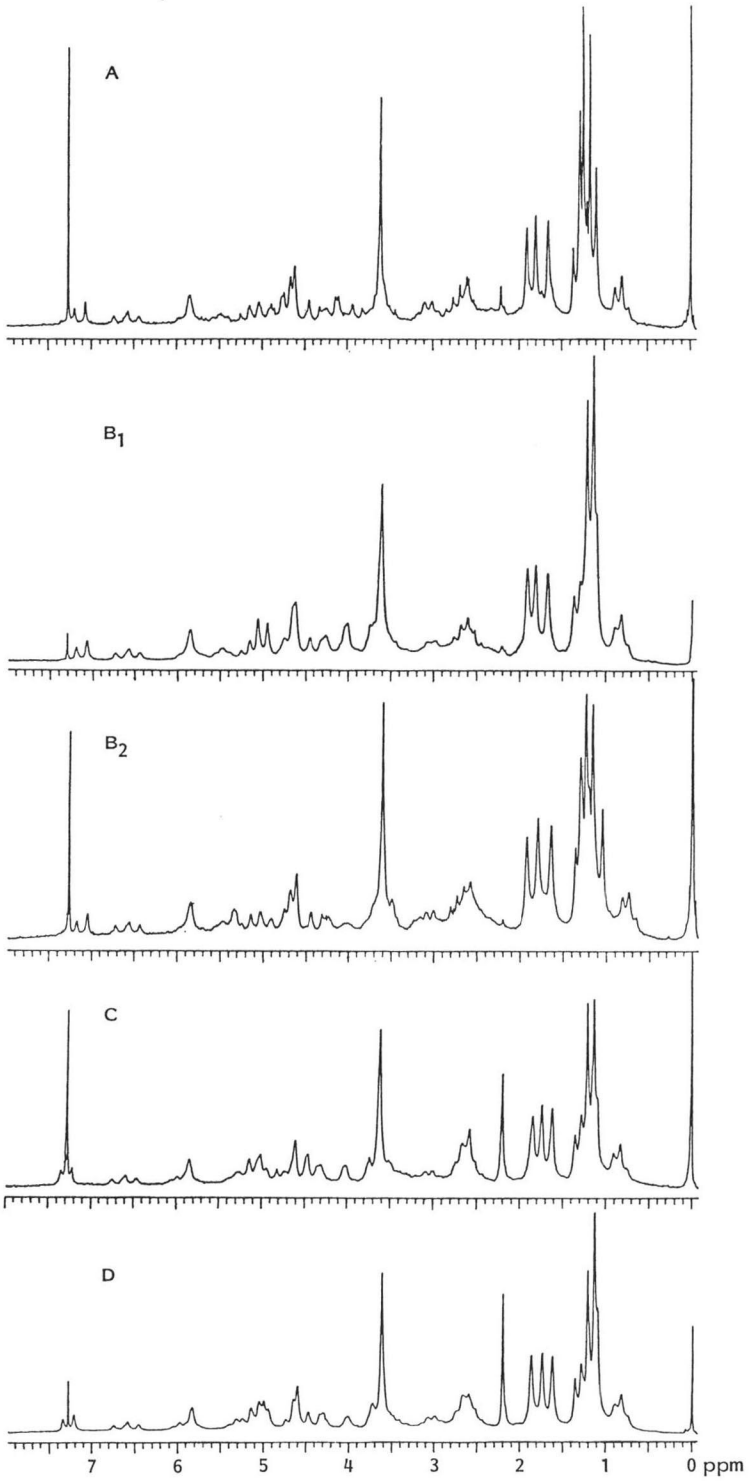


Fig. 3. ^{13}C NMR spectra (25.5 MHz) of clostomicins.

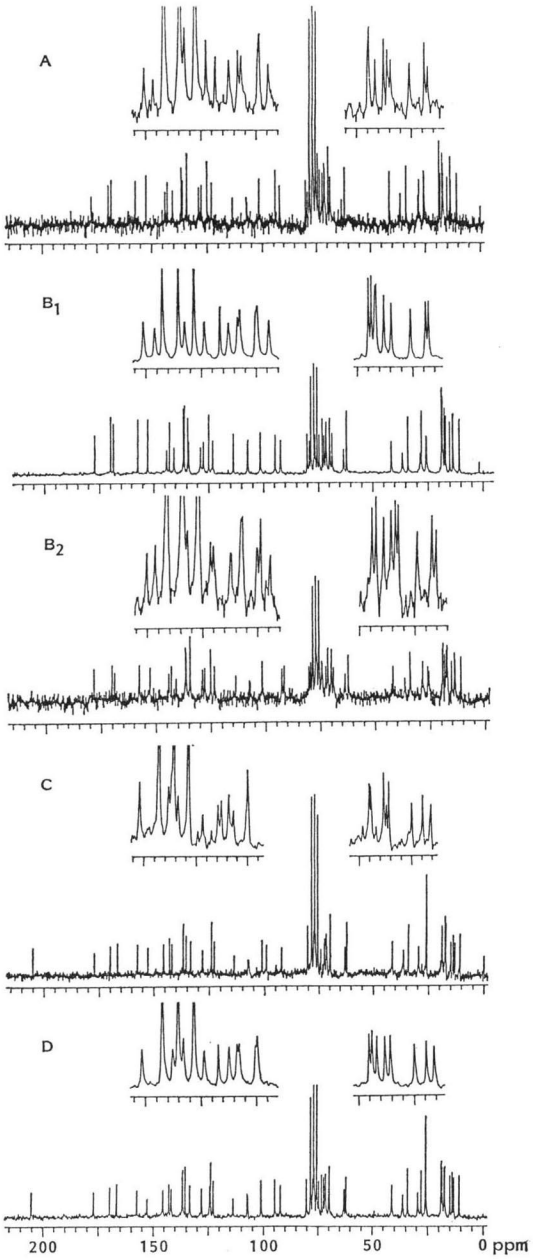
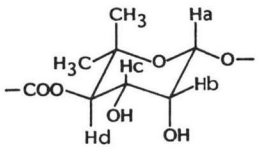
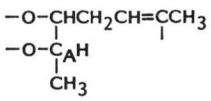


Fig. 4. Two partial structures among eight ones of lipiarmycin (clostomicin B_1).



1



2

the decoupling experiments of ^1H NMR of D indicated the absence of the methine group, the carbon of C_A would be converted to ketone carbonyl carbon in the component D. The same structural conversion must occur in C because of a similar observation in the spectra of component C. Furthermore, the ^{13}C NMR spectrum of C lacks one more carbon at 28.6 ppm in B_1, which had been assigned as an equatorial methyl group at the 5-position of the partial structure **1**[3]. Since a 7-ppm downfield-shift of the anomeric carbon of **1** was observed and it was attributable to the removal of γ-steric effect on the anomeric proton and γ-axial methyl group[4], the reported assignment of lipiarmycin would be reversed in our compound C and the carbon signal at 28.6 ppm should be assigned as an axial methyl group at the 5-position of the partial structure **1**. The ^1H NMR spectrum of C also indicated the lack of one methyl group at 1.10 ppm in D. Thus, the component C would lack the axial methyl group at the 5-position of the partial structure **1** as compared to the component D. The FD-MS spectra of C and D showed $(M+Na)^+$ ion at m/z 1,063 and 1,077, respectively, confirming the structural relationship between C and D.

Because B_1 seems to be a dihydro-derivative of D, the molecular mass on MS of B_1 should be m/z 1,056. Although the molecular formula of lipiarmycin has been reported to be $C_{52}H_{72}Cl_2O_{18\sim19}$[3], it should be revised to be $C_{52}H_{74}Cl_2O_{18}$ (MW 1,057.97) from aforementioned data.

Biological Properties

The antimicrobial activities of clostomicins were measured by the conventional paper-disk method at a concentration of 1.0 mg/ml, and the diameters of inhibition zones were demonstrated in Table 2.

Table 2. Antimicrobial activities of clostomicins.

Test microorganism	Diameter of inhibition zone (mm)*				
	A	B_1	B_2	C	D
Staphylococcus aureus FDA 209P	19.4	18.0	14.7	10.2	±
Bacillus subtilis PCI 219	19.9	19.4	15.4	13.9	11.4
B. subtilis PCI 219**	22.5	22.5	18.2	16.7	13.2
Micrococcus luteus ATCC 9341	20.4	18.0	17.4	19.0	16.5
Mycobacterium smegmatis ATCC 604	22.0	17.6	17.4	15.0	13.3
Xanthomonas oryzae KB88	13.0	15.0	12.2	11.7	±
Acholeplasma laidlawii PG8	19.4	19.0	17.1	17.2	14.6
Clostridium perfringens ATCC 32624	36.7	36.8	33.2	33.0	32.4
C. difficile ATCC 9689	36.3	33.4	30.0	31.7	24.4

* 8 mmϕ paper-disk method, 1 mg/ml solution.
** On synthetic medium.

Clostomicins exhibited strong antibacterial activities against Gram-positive anaerobic bacteria such as *Clostridium perfringens* and *C. difficile*, but showed less activities against Gram-negative bacteria. The antibiotics did not show any toxic effect when they were administered intraperitoneally at 100 mg/kg to mice.

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