A NEW ANTIBIOTIC, DIENOMYCIN. I

SCREENING METHOD, ISOLATION AND CHEMICAL STUDIES

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New antibiotics, dienomycins A, B and C were isolated from the culture filtrate of the strain MC67-C1 by a chemical screening method using WOOD reagent. Dienomycins are derivatives of (4-phenylbutadienyl) pipecolin. Dienomycins A and B contain an alkoxyl group, and instead, C contains a hydroxyl group. Dienomycins are the first examples of microbial metabolites having piperidine and phenylbutadiene structures. Dienomycin A is active only against mycobacteria.

Screening of culture filtrates of microorganims for their antimicrobial activities has led to the discovery of various types of valuable compounds. If, however, culture filtrates are screened with chemical reactions, one would expect to find new compounds which might elucidate biosynthetic sequences. The authors have screened culture filtrates for their color reactions to the WOOD reagent[1] which is positive for purines, pyrimidines or imidazole derivatives. A compound found in this way also showed antimicrobial activities and was named dienomycin. The organism producing this compound belongs to actinomycetes. As shown by the chemical studies described in the next paper, dienomycin is not a purine, pyrimidine or imidazole derivative but a piperidine compound. Two analogues of this compound were also found in the same culture filtrates. In this paper, the isolation, physical and chemical properties and biological activities of dienomycins are reported.

Screening Method

Approximately 4 μl each of culture filtrates of microorganisms taken from soil samples is spotted on a thin-layer plate (microcrystalline cellulose powder* or silica gel, 20×20 cm) at 3-cm distance from one edge of the plate to make a row of 17~19 spots at 1-cm intervals and the plate is developed with a solvent system, usually butanol - acetic acid - water (120 : 30 : 50). The detection method with WOOD reagent[1] is as follows: first, developing solvents (especially acetic acid) must be completely removed from the plate by allowing to stand in the air for a long time or by heating, if necessary. The plate is then heated to 100°C, sprayed with a mixture (1 : 1) of 0.4 %

* "Avicel" from Funakoshi Yakuhin, Japan was used.

bromophenol blue in acetone and 2 % silver nitrate solution while hot, and allowed to cool. The blue-colored plate is immersed carefully into water in a large bowl without disturbing the surface of the plate. The water is changed, if necessary, until clear-stained blue spots appear on a light background. The visualized blue spots are then checked carefully and spots having the same Rf values as components of nutrient are omitted. If a clear, unique spot is found, the isolation of the substance corresponding to the spot is undertaken. This reagent was selected for the detection of the above-mentioned compounds because the sensitivity and selectivity for purines, pyrimidines and imidazoles is superior to other reagents tested.

The dienomycins are the first substances to be isolated by this procedure. They have characteristic antibacterial activity against some strains of *Mycobacterium tuberculosis*.

Production and Isolation

The strain producing dienomycin was isolated from a soil sample collected at Nepal and the laboratory number MC67-C1 was given to this strain, the characteristics of which will be reported in a separate paper.

For laboratory production of dienomycin, 125 ml of a medium consisting of glycerol 2.5 %, meat extract 0.5 %, peptone 0.5 %, yeast extract 1.0 %, NaCl 0.2 %, $MgSO_4 \cdot 7H_2O$ 0.05 %, K_2HPO_4 0.05 % and $CaCO_3$ 0.3 % (w/v) (pH 7.0) was inoculated with a slant on KRAINSKY's glucose asparagine agar slant and cultured for 48 hours at 27°C. Each inoculum was transferred to 125 ml of the same medium in a 500-ml Sakaguchi-flask and cultured for 60~70 hours at 27°C on a reciprocating shaker (8 cm amplitude, 130 strokes per minute). When tested by thin-layer chromatography (TLC) with "Avicel" and a solvent system of butanol - acetic acid - water (120 : 30 : 50) and three coloration reagents (WOOD, EHRLICH and diacetyl, which is a sensitive reagent for guanidine group), the fermented broth showed a broad zone of Rf 0.65~0.85. The broth (10 liters) was filtered at harvested pH 6.2~6.4 and the filtrate was extracted with *n*-butanol (4 liters × 2). The combined extracts were evaporated *in vacuo* at 45°C to give a solid which, by TLC on silica gel with a solvent system of ethyl acetate - methanol (5 : 1) (Solvent A) showed, by sulfuric acid coloration, five spots of Rf 0.87, 0.48, 0.40, 0.26 and 0.10. The substance of Rf 0.87 was positive for EHRLICH reagent, the substances of Rf 0.48, 0.40 and 0.27 were positive both for WOOD and EHRLICH reagents and the substance of Rf 0.10 was positive only for diacetyl reagent. In these color reactions, the colored spots caused by WOOD and diacetyl reagents appeared immediately, but the spots by EHRLICH reagent needed 1 hour to 2 days to emerge. The material (9.2 g) obtained above was chromatographed on a column of alumina (Woelm acid-form, 280 g, 2.6 × 55 cm) with Solvent A and the eluate positive for WOOD reagent was collected and evaporated to dryness. The resultant residue (4.8 g) was again chromatographed on a silica gel column (Mallinckrodt 500 g, 5.0 × 58 cm) with Solvent A and the eluates were tested by TLC with silica gel and WOOD reagent. The substance having an Rf 0.48 was eluted between 900~1,450 ml. This portion when evaporated gave a crystalline substance, which was recrystallized from ethyl acetate-

methanol to afford colorless needles; yield 870 mg. This substance was named dienomycin A. The substance having Rf 0.40 was eluted between 1,450~2,200 ml, and was treated similarly and the solid obtained was recrystallized from ethyl acetate-methanol affording colorless needles; yield 700 mg. This substance was named dienomycin B. The substance having an Rf 0.26 was eluted in a fraction of 2,500~3,700 ml, which was treated as described above and the resultant crystals were recrystallized from methanol by adding acetone to give colorless needles: yield 200 mg. This substance was named dienomycin C.

Physical and Chemical Properties and Partial Structures

Dienomycins A, B and C isolated were found to be a mixture of hydrochlorides (major) and sulfates (minor). The salts were recrystallized from a mixture of ethyl acetate - methanol - water containing hydrochloric acid (pH of the solution was about 2) to give sulfate-free hydrochlorides. The physical properties of these dienomycin hydrochlorides are shown in Table 1.

Table 1. Physical properties of dienomycins

	Dienomycin A hydrochloride		Dienomycin B hydrochloride		Dienomycin C hydrochloride	
Melting point (°C)	212~214°		280~281° (sealed tube) Sublimed at 210°		252~253°	
Rotation $[\alpha]_{589}^{20}$ (*c* 1, methanol)	+85°		+80°		+65°	
Molecular weight of free base (mass spectrum, m/e)	313		285		243	
Elemental analysis	Found	Calcd. for $C_{20}H_{27}NO_2 \cdot HCl$	Found	Calcd. for $C_{18}H_{23}NO_2 \cdot HCl$	Found	Calcd. for $C_{16}H_{21}NO \cdot HCl$
C	68.94	68.65	67.49	67.17	68.73	68.68
H	7.66	8.07	7.83	7.52	7.89	7.93
N	4.14	4.00	4.39	4.35	5.03	5.01
O	9.54	9.15	10.28	9.94		5.72
Cl	9.98	10.13	10.55	11.02	12.39	12.67

Preparation of the free base of dienomycin C: An aqueous suspension of dienomycin C hydrochloride was adjusted to pH 10 with a dilute sodium hydroxide solution and the resultant free base was extracted into benzene and evaporated to give dienomycin C free base; mp 130~131°C, $[\alpha]_{589}^{20}$ +85° (*c* 1.0, methanol). Found: C 78.96, H 8.84, N 5.48. Calcd. for $C_{16}H_{21}NO$: C 78.97, H 8.70, N 5.76%.

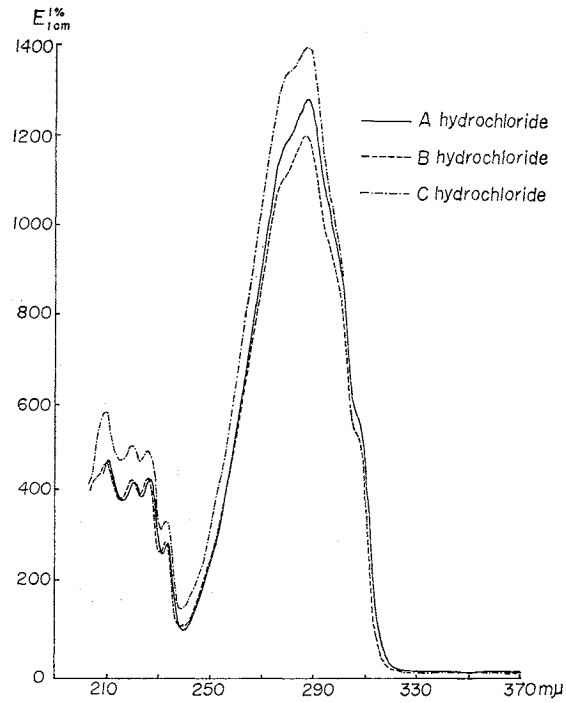
Dienomycin A and B hydrochlorides are soluble in methanol, ethanol, chloroform and dimethyl sulfoxide, sparingly soluble in water and insoluble in benzene, ethyl acetate and ethers; dienomycin C hydrochloride also shows similar solubility in the above-mentioned solvents except chloroform, in which the hydrochloride is insoluble. Dienomycin C free base is soluble in methanol, ethanol, benzene, chloroform, dimethyl sulfoxide and acetone and insoluble in ether and water.

An aqueous solution of dienomycin A or B hydrochloride at 60°C is stable at pH 2 for one day and at pH 7.0 for 1 hour, but slightly unstable at pH 9; dienomycin C hydrochloride is stable under such conditions.

The ultraviolet spectra of dienomycin A, B and C hydrochlorides closely resemble

each other and have identical patterns throughout the whole pH range as shown in Fig. 1; for instance, dienomycin A hydrochloride shows maxima at the following wave length (ε): 211 (14,800), 220 (14,700), 227 (14,700), 234 (10,500), 280 (sh., 40,900), 287 (44,100) 297 (sh., 33,600) and 307 mμ (sh., 19,600) either in methanol, 0.01 N methanolic hydrochloric acid or 0.01 N methanolic sodium hydroxide. Their ultraviolet spectra are quite similar to that of 1-phenylbutadiene[2] having a *trans-trans* configuration (maxima at 225, 227, 234, 275, 282, 292 and 302 mμ) suggesting the presence of this chromophore in the dienomycins.

Fig. 1. Ultraviolet absorption spectra of dienomycins.



The infrared spectra of dienomycin A and B hydrochlorides are presented in Fig. 2 and that of C hydrochloride and its free base in Fig. 3.

Comparison of these spectra furnishes the following information: (1) The presence of ester groups: as for dienomycin A, the peaks (1740, 1185, 1150 and 1120 cm^{-1}) are ascribable to an alkyl ester group (RCOOR). As for B, the peaks (1735, 1243 and 1228 cm^{-1}) are ascribable to an acetoxyl

Fig. 2. Infrared spectra of dienomycin A hydrochloride and B hydrochloride in KBr disk.

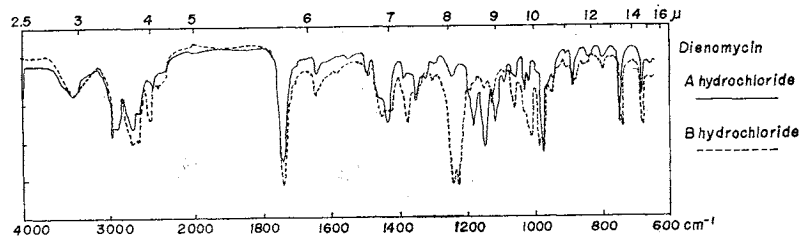
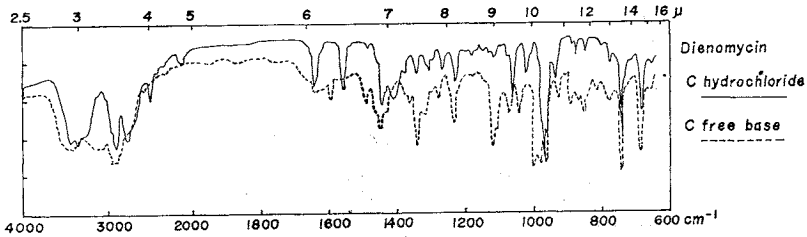


Fig. 3. Infrared spectra of dienomycin C hydrochloride and C free base in KBr disk.



group (CH_3COOR). C has no such groups, but, instead, has free hydroxyl group ($\sim$ 3400 cm^{-1}). (2) The basic structures of dienomycins may be similar and commonly have a hydroxyl group, which is free in C and esterified in A and B with a fatty acid.

In the mass spectrum of dienomycin A hydrochloride, only two prominent peaks (m/e 313 and 226) are discerned. The peak at m/e 313, which is the highest numbered peak of all peaks other than the accompanying small natural isotopic peaks, corresponds to $C_{20}H_{27}NO_2^+$. On the other hand, elemental analysis of dienomycin A hydrochloride (see Table 1) indicates that the most probable empirical formula will be $C_{20}H_{27}NO_2 \cdot HCl$, and this is supported also by the integrated proton counts of the nmr spectrum of dienomycin A (see later). These data suggest that m/e 313 is the molecular ion of A free base and the ion is formed by splitting of the hydrochloric acid from dienomycin A hydrochloride. In the case of dienomycin B hydrochloride, also two prominent peaks (m/e 285 and 226) appear; and from the elemental analysis and the nmr data of B hydrochloride, the peak m/e 285 is assigned to the molecular ion ($C_{18}H_{23}NO_2^+$) of B free base. In dienomycin C hydrochloride, also two prominent peak (m/e 243 and 226) are recognized; and likewise the former peak is assigned to the molecular

Fig. 4. The nmr spectrum of dienomycin A hydrochloride in $CDCl_3$.

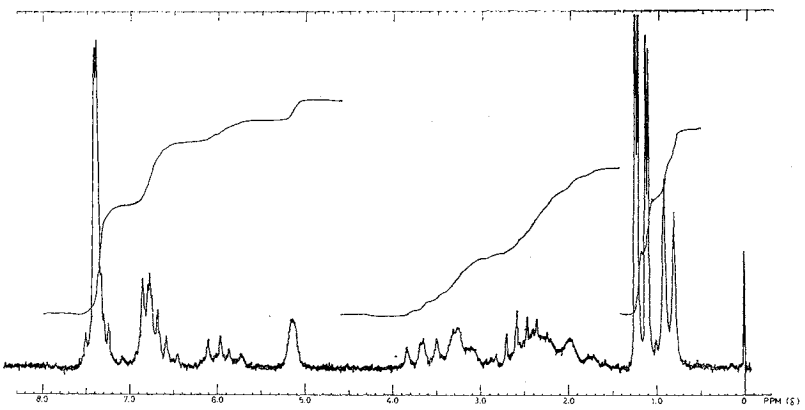


Fig. 5. The nmr spectrum of dienomycin B hydrochloride in $CDCl_3$.

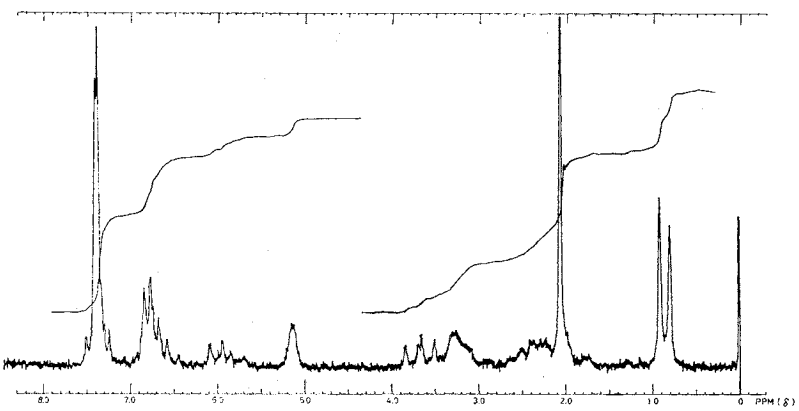
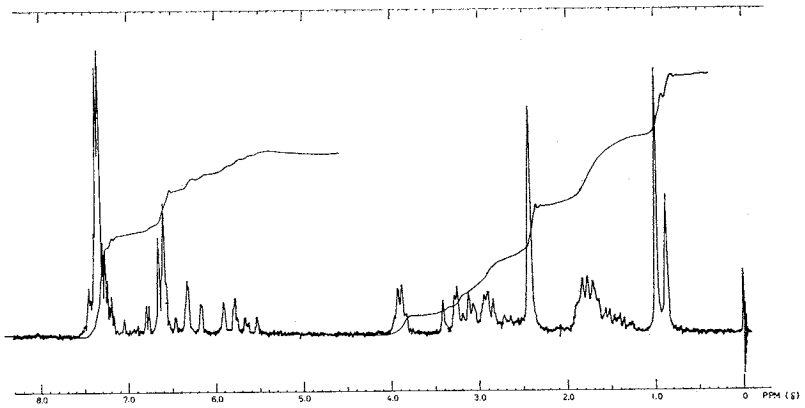


Fig. 6. The nmr spectrum of dienomycin C base in $CDCl_3$.



ion ($C_{16}H_{21}NO^+$) of C free base. Another characteristic feature of the mass spectra is that the peak of m/e 226 is observed in all of dienomycins. Since the values of $M^+ - 226$ in dienomycins A, B and C are 87, 59 and 17 which correspond to C_3H_7COO, CH_3COO and OH, respectively, the fragment (m/e 226) is ascribed reasonably to the ions produced by cleavage of C-O bond between the basic structure and a hydroxyl or acyloxyl group.

The nmr spectra of dienomycin A and B hydrochlorides and C free base were measured with a Varian A-60D spectrometer (60 MHz) in deuteriochloroform using tetramethylsilane as an internal reference (Figs. 4, 5 and 6).

Each of the three compounds shows the signals due to a $>CH-CH_3$ (δ 0.8~0.9, 3-proton doublet, J 7 Hz), a phenyl (δ 7.1~7.5, 5-proton multiplet) and olefinic protons (δ 5.6~7.0, 4-protons, many sharp peaks). Additionally, dienomycin A shows the signals due to an isopropyl group (δ 1.19 and 1.21, each 3-proton doublet, J 7 Hz, $(C\underline{H}_3)_2CH$; δ 2.5 (or 2.6), one proton septet, J 7 Hz, $(CH_3)_2C\underline{H}$). Dienomycin B shows the signals due to an acetyl group (δ 2.08, 3-proton singlet). Dienomycin C shows, neither the signals of higher alkyl nor an acyl group, but shows a signal (δ 2.42, 2-proton singlet, disappeared on deuteration) due to a hydroxyl and an imino(?) hydrogen.

On hydrogenation over palladium black, dienomycin A, B and C hydrochlorides in 90 % ethanol solution absorbed 2.03, 2.19 and 1.97 moles of hydrogen per mole, respectively.

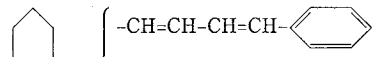
From the above spectral data, dienomycins A, B and C must resemble each other closely except for their acyloxyl (or hydroxyl) side chains. In A, for instance, the presence of 4-phenylbutadienyl, isobutyroyloxyl ($(CH_3)_2CHCO_2$) and methyl side-chain groups is indicated. If the sum ($C_{15}H_{19}O_2$) of these is subtracted from the parent molecule ($C_{20}H_{27}NO_2$), C_5H_8N is left; addition of three hydrogens corresponding to the three side chains gives $C_5H_{11}N$ and this formula requires a mono-cyclic structure. Therefore, the remaining problem necessary to determine the gross structure of the antibiotics is to decide the functional nature of the nitrogen and the size of the ring.

On electrometric titration of dienomycin A and B hydrochlorides in 50 % methanol,

the values of pKa' 8.8 and 9.1 were obtained, respectively. On high voltage paper electrophoresis (3,500 V/42 cm, 65 mA/20 cm, 15 min.; Toyo Roshi paper No. 51 using a buffer of formic acid - acetic acid - water (25 : 75 : 900 by volume), dienomycin A, B and C hydrochlorides showed a single spot with $Rf_{alanine}$ 0.75, 0.92 and 1.05, respectively.

To clarify the nature of the nitrogen atom, dienomycin B was acetylated to give a mono-acetylated derivative; the detailed preparation of which will be described in the following paper. The infrared spectrum of the acetyl derivative shows the band (1645 cm^{-1}) assignable to amide I but no bands near 1550 cm^{-1} (amide II). Furthermore, on treatment with RYDON-SMITH reagent, the derivative shows no color reaction indicating the absence of an amide-type hydrogen. These results show that the nitrogen is of the type of imino group and, consequently, the nitrogen is in a ring system. A piperidine moiety satisfies all the properties of the N-containing skeleton.

Thus, the partial structures for dienomycins A, B and C are presented as follows:



Biological Properties

The minimal inhibitory concentrations of dienomycins A, B and C are listed in Table 2.

Dienomycins are weakly antibacterial, and dienomycin A is the most active. Mycobacteria are sensitive to the compound; the growths of *M. phlei* and *M. tuberculosis* $H_{37}Rv$ are completely inhibited at a concentration of 25 mcg/ml of culture medium. It is of interest to find that the substitution at the hydroxyl group by esterification produces a powerful influence on antibacterial activity.

The acute toxicities LD_{50} of dienomycin A, B and C hydrochlorides to mice by

Table 2. Antibacterial spectra of dienomycins

Test organisms	M. I. C.* (mcg/ml)		
	A	B	C
Staphylococcus aureus FDA 209P	>100	>100	>100
Staphylococcus aureus Terajima	>100	>100	>100
Staphylococcus aureus Smith	100	>100	>100
Staphylococcus aureus 193	>100	>100	>100
Micrococcus flavus FDA 16	50	>100	>100
Bacillus subtilis NRRL B-558	100	>100	>100
Bacillus subtilis PCI 219	100	>100	>100
Bacillus cereus ATCC 10702	>100	>100	>100
Bacillus anthracis	50	>100	>100
Corynebacterium bovis 1810	100	>100	>100
Escherichia coli NIHJ	>100	>100	>100
Escherichia coli K-12 CS-2	>100	>100	>100
Escherichia coli K-12 ML1629	>100	>100	>100
Shigella flexneri 1a(Ew 8)	100	>100	>100
Salmonella typhosa	>100	>100	>100
Proteus vulgaris OX 19	>100	>100	>100
Proteus rettgeri GN 311	>100	>100	>100
Proteus rettgeri GN 466	>100	>100	>100
Pseudomonas aeruginosa A3	>100	>100	>100
Serratia marcescens	>100	>100	>100
Klebsiella pneumoniae PCI 602	100	>100	>100
****Mycobacterium smegmatis*** ATCC 607	50	>100	>100
****Mycobacterium phlei***	25	100	>100
*****Mycobacterium tuberculosis*** $H_{37}Rv$	25	62.5	

* Minimal inhibitory concentration of dienomycins by agar streak method using nutrient agar
** 1.0 % glycerol nutrient agar
*** KIRCHNER's medium

intraperitoneal injection were 90, 360 and 45 mg/kg, respectively.

Discussion

We designed a screening procedure using WOOD reagent to search for compounds related to purine- and pyrimidine-bases, however, the first substance obtained by this screening turned out to be an antibiotic complex having another kind of heterocyclic ring. It is noteworthy that the screening procedure disclosed the presence of piperidine compounds among microbial metabolites. Another unusual characteristic feature of the molecules of dienomycin is the phenylbutadienyl group, which, we believe, has never been found even in the alkaloids of higher plants.

Acknowledgement

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