NEW BASIC WATER-SOLUBLE ANTIBIOTICS BD-12 AND BY-81. II*

ISOLATION, PURIFICATION AND PROPERTIES

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Isolation, purification and properties of two new basic water-soluble antibiotics, BD-12 and BY-81, are reported. These antibiotics are considered to be new members of the streptothricin-like group of antibiotics.

In the course of antibiotic screening, a new basic water-soluble antibiotic tentatively named BD-12 was isolated from the culture filtrate of *Streptomyces luteocolor* nov. sp. MCRL-0357. Later, another new antibiotic designated as BY-81 has been obtained from the fermentation broth of *S. olivoreticuli* nov. var. MCRL-0358.

Because of the similarity in chemical and biological nature of BD-12 and BY-81, the authors wish to report the isolation, purification, physico-chemical properties and biological characteristics of both antibiotics together in the present paper. The taxonomic studies of the antibiotic-producing strains and antibiotic productions have been reported in the foregoing paper[1].

Isolation and Purification[(a)]

Similarly to other basic, water-soluble antibiotics, BD-12 or BY-81 in the filtered broth was concentrated by adsorption on Amberlite IRC-50 (Na^+ type), followed by elution with dilute hydrochloric acid. After neutralization with Amberlite IR-45 (OH^- type), the eluate was concentrated *in vacuo* and finally freeze-dried. The crude hydrochloride of BD-12 or BY-81 thus obtained was then converted to the corresponding water-insoluble picrate, from which the hydrochloride was recovered by treating with pyridine hydrochloride in methanol. As ninhydrin-positive contaminants were detected by thin-layer chromatography on MN 300 cellulose powder (solvent 2),

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(a) Activity was assayed by a cup or pulp-disc method using *B. subtilis* PCI 219 as a test organism. The purified hydrochloride of each antibiotic was used as a standard material. Compositions of the solvents used for chromatography during isolation and purification procedures are, unless otherwise noticed, as follows :

Solvent 1 : *n*-PrOH - pyridine - AcOH - H_2O (15 : 10 : 3 : 12)

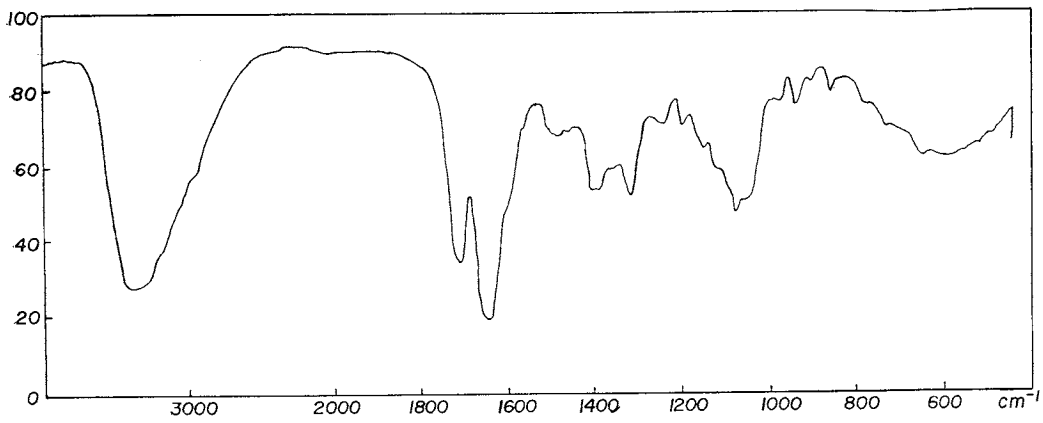
Solvent 2 : *n*-BuOH - pyridine - AcOH - H_2O (15 : 10 : 3 : 12)

the hydrochloride was further purified by chromatography on cellulose powder columns, using solvent 2 for BD-12 or solvent 1 for BY-81. Each fraction of the eluate collected on the fraction collector was assayed for antibiotic activity and also examined for the presence of contaminant(s) by thin-layer chromatography. The fractions containing only the antibiotic were gathered and freed from solvent by extraction with large amounts of petroleum ether. The resulting aqueous layer was freeze-dried to give the amorphous powder of the desired antibiotic.

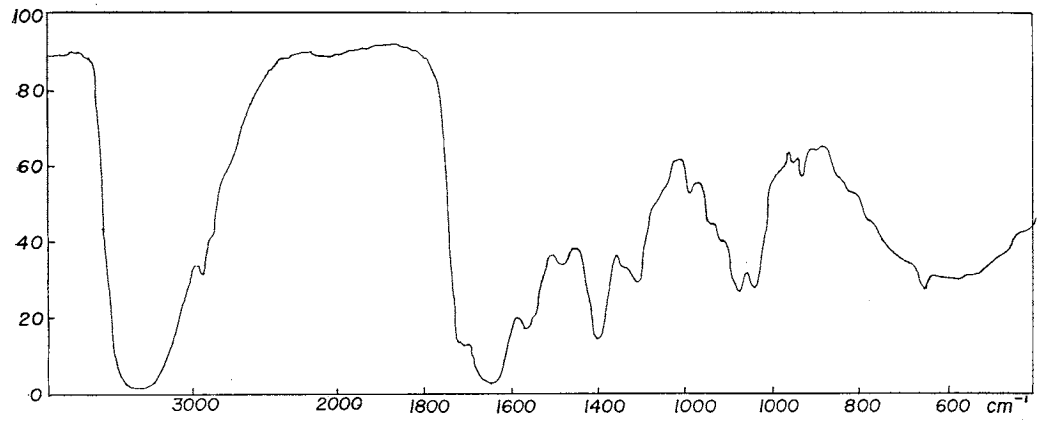
Further purification procedures are different for the two antibiotics. BD-12 hydrochloride obtained as above was again purified through its picrate[b]. Purified hydrochloride regenerated from the picrate was recrystallized from MeOH-EtOH (1 : 1) mixture. Thus, BD-12 hydrochloride was obtained as white rhombic crystals.

Unlike BD-12, BY-81 hydrochloride could not be converted to the picrate again without decomposition, so BY-81 was further purified by repeating the cellulose powder column chromatography using solvent 2. BY-81 recovered as hydrochloride was recrystallized from MeOH-EtOH (1 : 3) mixture to yield the pure BY-81 hydro-

Fig. 1. Infrared absorption spectra (KBr)
(a) BD-12·hydrochloride



(b) BY-81·hydrochloride



(b) BD-12 picrate (recryst. from EtOH) : yellow plates, m. p. 160~165.5°C (decomp.). Anal. Found : C 37.41, H 4.11, N 18.84 %.

Fig. 2. Summarized papergram of BD-12 and BY-81 (Bioautography with *B. subtilis*)

Solvent : A : wet BuOH
B : 1.5 % ammonium chloride aq. solution
C : phenol - H_2O (3 : 1)
D : 50 % aq. acetone
E : *n*-BuOH(40 ml)-MeOH(10 ml)-H_2O (20 ml)+methylorange (1.5 g)
F : *n*-BuOH(40 ml)-MeOH(10 ml)-H_2O (20 ml)
G : benzene - MeOH (4 : 1)
H : H_2O

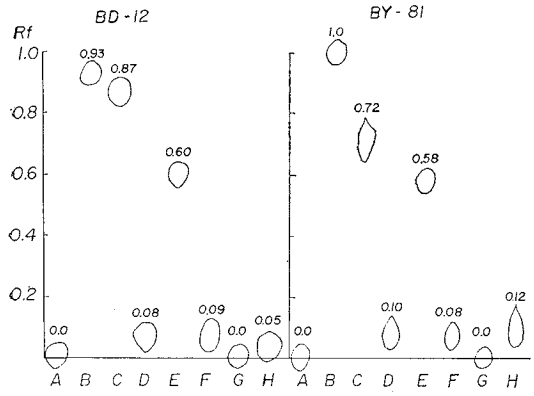


Fig. 3. Salting-out paper chromatography of BD-12 and BY-81 with ammonium chloride solution (Bioautography with *B. subtilis*)

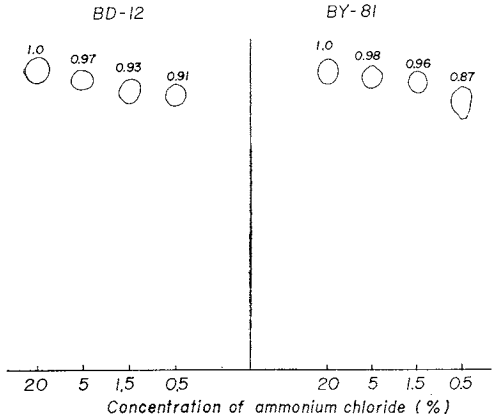


Table 2. Paper chromatography of BD-12 and BY-81 (Biautography with *B. subtilis*)

Solvent system	Rf values	
	BD-12	BY-81
n-PrOH - pyridine - AcOH - H_2O (60 : 40 : 10 : 30) plus 1.2 g Na *p*-hydroxybenzene sulfonate/ 140 ml	0.36	0.24
wet BuOH plus 2 % *p*-toluene sulfonic acid	0.29	0.17
n-BuOH - pyridine - AcOH - H_2O (15 : 10 : 3 : 12)	0.54	0.45
BuOH - AcOH - H_2O (4 : 1 : 5)	0.21	0.07

Table 1. Physico-chemical properties of BD-12 and BY-91 hydrochlorides.

	BD-12·HCl	BY-81·HCl
Appearance	colorless hygroscopic crystal (rhombic)	colorless crystalline powder
m. p.	200.5~201.5°C (decomp.)	142~143°C (decomp.)
Formula (tentative) and elementary analysis	$C_{19}H_{35}N_7O_{12}\cdot 2HCl$ Anal. Calcd. : C 36.43, H 5.95, N 15.65, Cl 11.32 mol. wt. 626.48 Found : C 36.37, H 6.31, N 15.65, Cl 11.75 mol. wt. 614 (titration)	$C_{23}H_{45}N_9O_{14}\cdot HCl$ Anal. Calcd. : C 39.01, H 6.55, N 17.80, Cl 5.01 mol. wt. 708.15 Found : C 39.30, H 6.41, N 17.99, Cl 4.53 mol. wt. 648 (titration)
pKa'[a]	7.1 and (9.5)	4.9, 7.4 and (10.0)
Electrophoresis[b]	moved toward cathode 4.1 cm (pH 5.0) 0.95 cm (pH 8.0)	moved toward cathode 5.5 cm (pH 5.0) 1.0 cm (pH 8.0)
Optical rotation	$[\alpha]_D^{25}$ −75° (c 1, H_2O)	$[\alpha]_D^{20}$ −60° (c 1, H_2O)
Ultraviolet absorption	end absorption in water and acidic water alkaline water : λ_{max} 260 mμ ($E^{1\%}_{1cm}$ 6.5)	end absorption in water, acidic water or in alkaline water
N.M.R.[c]	δ 2.83 ppm (s, 3H) 3.03 ppm (s, 3H)	δ 3.04 ppm (s, 3H)
Stability	stable at neutral pH	stable at neutral pH
Solubility	soluble in H_2O, MeOH slightly soluble in EtOH insoluble in most organic solvents	readily soluble in H_2O soluble in MeOH slightly soluble in EtOH insoluble in most organic solvents

a) The pKa' values were obtained by titration in H_2O. Taking account of decomposition during titration on alkaline side, pKa' values in parentheses are of slight accuracy.

b) Ten volt/cm, 2.5 hours in 1/15 M phosphate buffer.

c) The N.M.R. spectra were determined in D_2O with the Japan Electron Co. J.N.M.-60 spectrometer at 60 MHz. DSS was used as an internal standard.
s : singlet.

chloride as a white crystalline powder.

Starting from 105 liters (374 mcg/ml) of filtered broth of the strain MCRL-0357, 4.1 g of BD-12 hydrochloride was obtained, and from 247 liters (69.5 mcg/ml) of that of the strain MCRL-0358, 0.73 g of BY-81 hydrochloride was recovered.

Physico-chemical Properties

Some of the physico-chemical properties of hydrochlorides of BD-12 and BY-81 are summarized in Table 1. Infrared absorption spectra in KBr tablet are given in Fig. 1a and 1b. Titration curves from which pKa values were obtained suggested that BD-12 is a dibasic compound, while BY-81 is a monoacidic-dibasic compound. BD-12 was positive to ninhydrin, TOLLENS, ELSON-MORGAN and ferric hydroxamic acid reactions, and decolorized aqueous permanganate solution, while it was negative to ferric chloride, HOPKINS-COLE, MILLON, maltol, SAKAGUCHI, MOLISCH, iodoform, nitroprusside and sulfuric acid reactions. It gave doubtful reactions to BENEDICT, FEHLING and biuret reagents. Color reactions of BY-81 were identical with those of BD-12 except biuret reaction to which BY-81 was negative.

Table 3. Antimicrobial spectrum of BD-12 and BY-81 hydrochlorides. (Serial dilution method)

Test organisms	Medium	M.I.C. (mcg/ml)	
		BD-12	BY-81
Staphylococcus aureus FDA 209 P	I	3.7	8.7
Staphylococcus aureus SMITH	I	7.5	—
Staphylococcus aureus TERASHIMA	I	7.5	17.5
Staphylococcus aureus (PC, SM, TC, CP-R)	I	7.5	17.5
Streptococcus hemolyticus	II	30	>70
Diplococcus pneumoniae	III	30	>70
Neisseria meningitidis 13077 group A	III	15	17.5
Bordetella pertussis TOHAMA	III	30	35
Bacillus subtilis PCI 219	I	1.8	4.35
Corynebacterium diphtheriae PARK-WILLIAMS No. 8	II	7.5	70
Clostridium welchii PB 6 K K-1	VI	60	>70
Clostridium tetani	VI	>60	>70
Mycobacterium tuberculosis $H_{37}Rv$	IV	50~100	35
Escherichia coli K-12	I	3.7	8.7
Escherichia coli NIHJ	I	3.7	8.7
Salmonella typhi T-58	I	3.7	8.7
Salmonella typhimurium	I	15	35
Shigella dysenteriae	I	1.8	8.7
Shigella flexneri 2a	I	0.9	4.35
Klebsiella pneumoniae	I	3.7	8.7
Pseudomonas aeruginosa	I	1.8	—
Pseudomonas fluorescens	I	30	4.35
Proteus vulgaris	I	3.7	4.35
Willia anomala	V	>60	70
Hansenula anomala	V	15	8.7
Torula utilis	V	>60	70
Saccharomyces cerevisiae	V	7.5	4.35
Candida albicans	V	>60	70
Aspergillus niger	V	>60	>70
Aspergillus oryzae	V	>60	>70
Aspergillus fumigatus	V	>60	>70
Penicillium notatum	V	>60	—
Penicillium chrysogenum	V	>60	—

Abbreviation : -R : resistant, CP : chloramphenicol, PC : penicillin G, SM : streptomycin, TC : tetracycline
Medium : I : nutrient broth (Difco), II : brain heart infusion broth (Difco), III : brain heart infusion broth (Difco) supplemented with serum, IV : modified DUBOS medium, V : SABOURAUD's liquid medium, VI : ZEISLER's blood agar

The summarized and salting-out paper chromatograms are shown in Figs. 2 and 3 respectively. In Table 2 are listed some additional Rf values with the solvents which effectively differentiated both antibiotics.

Biological Properties

Antimicrobial activities of BD-12 and BY-81 hydrochlorides are summarized in Table 3. As evident from the table, both antibiotics are mainly active against Gram-positive and Gram-negative bacteria. The cross resistance with streptothricin is observed

with both antibiotics.

Mice survived more than 20 days after intravenous administration of doses less than 50 mg/kg. However, similarly to streptothricin, necrotoxic symptoms were observed at the injection site in the tails of the mice on sixth to eighth days after injection of doses over 12.5 mg/kg.

BD-12 and BY-81 showed a protective effect in mice against the infection of *Staphylococcus aureus* SMITH strain (diffuse type), effective dose being 12.5~25 mg/kg with BD-12 and 25~50 mg/kg with BY-81.

Table 4. Comparison of BD-12 and BY-81 with other basic water-soluble antibiotics by ascending paper chromatography (Bioautography with *B. subtilis*)

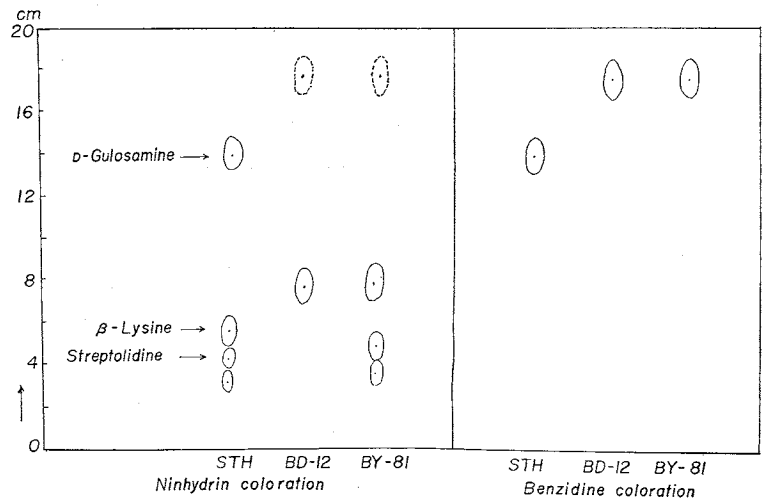
Antibiotics	Rf values	
	Solvent A	Solvent B
BD-12	0.36	0.54
BY-81	0.24	0.45
Actinospectacin	0.52	0.60
Dihydrostreptomycin	0.21	0.42
Gentamicin	0.20	0.40
Glebomycin	0.25	0.45
Hydroxystreptomycin	0.18	0.38
Kanamycin	0.12	0.23
Kikumycin A	0.35	0.48
Lincomycin	0.75	0.74
Neomycin	0.07	0.14
Netropsin	0.31	0.50
Paromomycin	0.11	0.22
Racemomycin A	0.18	0.38
Racemomycin B	0.07	0.20
Sporaviridin	0.51	0.78
Streptomycin	0.23	0.42
Streptothricin	0.18	0.38
Viomycin	0.11	0.28

Solvent A : *n*-PrOH (60 ml), pyridine (40 ml), AcOH (10 ml), H_2O (30 ml), Na *p*-hydroxybenzene sulfonate (1.2 g)
Solvent B : *n*-PrOH - pyridine - AcOH - H_2O (15 : 10 : 3 : 12)

Discussion

Physico-chemical properties and paper chromatographic behavior of BD-12 and BY-81 suggested that these antibiotics were members of the so-called basic, water-soluble antibiotic group. Thus, direct comparison of both antibiotics with streptothricin and other typical antibiotics of the same group was attempted by paper chromatography. As shown in Table 4, no antibiotics identical with BD-12 were found, while glebomycin[2] gave nearly the same Rf values as BY-81. Glebomycin, albovertiicillin[3] and capreomycin[4] were reported to give similar patterns of summarized and salting-out paper chromatograms as those of BY-81. However, these three antibiotics were readily differentiated from BY-81 by their physico-chemical properties. Since no other antibiotics were found in the literature[5] which have similar properties with BD-12 and BY-81, both antibiotics are considered to be new antibiotics.

Fig. 4. Paper chromatograms of hydrolysates of BD-12, BY-81 and streptothricin (STH) (Descending method with a solvent : pyridine - AcOEt - AcOH - H_2O (5 : 5 : 1 : 3))



As mentioned above, BD-12 and BY-81 could be obviously distinguished from streptothricin, but the cross-resistance of BD-12 and BY-81 with streptothricin and the necrotoxic phenomena observed with both antibiotics suggested that these three antibiotics are closely related to each other in their structures. Thus, the preliminary examination of the acid hydrolysates of these antibiotics was attempted by paper chromatographic procedure. Hydrolysis was done at 100°C for 8 hours in 6 N hydrochloric acid in sealed ampules. Paper chromatograms obtained by the descending method with a solvent consisting of pyridine - AcOEt - AcOH - H_2O (5 : 5 : 1 : 3) were visualized by spraying independently with ninhydrin and benzidine reagents. As shown in Fig. 4, spots detected on paper chromatograms of BD-12 and BY-81 hydrolysates did not correspond to any of streptolidine (roseonine), β-lysine or D-gulosamine, three main constituents of streptothricin. Chromatograms of hydrolysates of BD-12 and BY-81 suggested the presence of two common components. The one which ran slightly faster than β-lysine was positive to ninhydrin reagent, while the other which moved faster than D-gulosamine was essentially negative (later weakly positive) to ninhydrin reagent and positive to benzidine reagent.

After the completion of the present work, two new streptothricin-like antibiotics, LL-AC 541[6] and LL-AB 664[7], were reported by the Lederle group. On acid hydrolysis, the former is reported to give glycine, streptolidine, N-methyl-α-D-gulocosamine and others, while the latter is suggested to give glycine, N-methylstreptolidine and others. Finding of glycine, N-methylstreptolidine and N-methyl-α-D-gulosamine as components of streptothricin-like antibiotics is of significant value in classifying streptothricin group antibiotics. It will be interesting to determine if the above mentioned common components of BD-12 and BY-81 are identical with any of the above substances.

Literature

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Addendum

After the submission of the present paper, Lederle antibiotics were kindly sent to authors from Dr. E. L. Patterson of Lederle Laboratories. On direct chromatographic comparison of Lederle antibiotics with author's, no solvent systems were found which differentiated LL-AB 664 from BD-12, and LL-AC 541 from BY-81. SF-701 substance, a member of streptothricin-like antibiotics, recently reported by T. Tsuruoka *et al.* (J. Antibiotics 21 : 237~238, 1968) is also supposed to be identical with or closely related to any of the above antibiotics. On acid hydrolysis, SF-701 substance gave N-methylglycine in place of β-lysine.