ISOLATION OF A NEW WATER-SOLUBLE BASIC ANTIBIOTIC A-396-I

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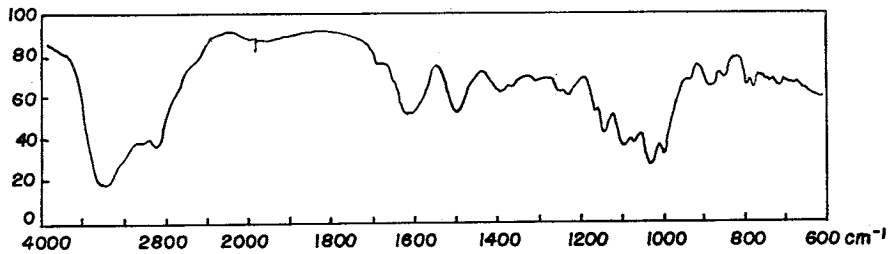
Two water-soluble basic antibiotics A-396-I and II were isolated from a culture broth of *Streptoverticillium eurocidicus* A-396. Both antibiotics gave similar analytical data indicative of a molecular formula $C_{19\pm1}H_{35\pm2}O_{13}N_3$, but different optical activities: A-396-I, $[\alpha]_D$ +11.6° (in water), and A-396-II, $[\alpha]_D$ +21.8° (in water). A comparison with known antibiotics using thin-layer chromatography suggested that A-396-I is a new antibiotic and A-396-II is identical with hygromycin B.

A streptomyces strain A-396 was isolated from a soil sample collected at Shimane Prefecture. Morphological, cultural and physiological characteristics of the strain were studied and the results indicated it to be *Streptoverticillium eurocidicus*.

When this strain was grown in suitable media produced in nearly equal amounts of two water-soluble basic antibiotics designated as A-396-I and II. Both antibiotics were isolated by adsorption procedure on ion-exchange resin and activated carbon. They were separated from each other by preparative layer chromatography on silica gel, further purified through crystalline reineckates. Finally hydrochlorides and free bases of both antibiotics were obtained.

The antibiotic A-396-I is freely soluble in water, slightly soluble in lower alcohols, and insoluble in other common organic solvents. Basicity is demonstrated by paper electrophoresis carried out in buffer solutions of various pHs. No ultraviolet absorption was noted in studies using aqueous solutions. The infrared absorption spectrum is shown in Fig. 1.

Fig. 1. Infrared absorption spectrum of A-396-I hydrochloride (KBr).



The antibiotic A-396-I gave a positive ninhydrin reaction, and negative SAKAGUCHI, PAULY and FEHLING reactions.

Elemental analysis and molecular weight determination correspond to a molecular formula $C_{19\pm1}H_{35\pm2}O_{13}N_3$.

The above described properties of A-396-I were also noted with A-396-II. An apparent difference between the both antibiotics is shown in their optical rotatory activities: A-396-I hydrochloride, $[\alpha]_D^{28°}$ +6.9°±0.5° (*c* 1.037, H_2O), A-396-I free base, $[\alpha]_D^{28°}$ +11.6°±0.5° (*c* 0.936, H_2O); A-396-II hydrochloride, $[\alpha]_D^{23°}$ +9.2°±0.5° (*c* 1.012, H_2O); and A-396-II free base, $[\alpha]_D^{23°}$ +21.8°± 0.5° (*c* 1.020, H_2O).

Thin-layer chromatography (TLC) on silica gel with a solvent system of chloroform-methanol-4% aqueous ammonia (2:1:1, upper layer) was carried out, comparing with related known antibiotics. A-396-I migrated faster, and A-396-II exhibited the same mobility as hygromycin B[1,2,3] and destomycin A,[4,5,6,7] as shown in Fig. 2.

Fig. 2. Thin-layer chromatogram of A-396-I, II and related antibiotics.

Solvent system: Chloroform-methanol-4% aqueous ammonia (2:1:1).

1. A-396-I
2. A-396-II
3. Hygromycin B
4. Destomycin A
5. Destomycin B

The antibiotic A-396-I is active against gram-positive bacteria, gram-negative bacteria, mycobacteria and some of fungi and protozoa (Table 1). It exhibits moderately acute toxicity to mice by intravenous injection: LD_{50} 12.50 mg/kg (9.51~15.50 mg/kg).

The biological activities of A-396-II are shown to be almost similar to those of A-396-I so far as tested.

Table 1. Antimicrobial spectrum of A-396-I

Test organism	MIC (mcg/ml)
Bacillus subtilis PCI 219	12.5
Bacillus anthracis	50
Staphylococcus aureus 209 PJC-I	6.25
Corynebacterium diphtheriae Tront	1.25
Escherichia coli NIHJ JC-2	50
Klebsiella pneumoniae	12.5
Salmonella typhimurium	50
Salmonella typhosa	25
Shigella sonnei OHARA	25
Pseudomonas aeruginosa	50
Mycobacterium tuberculosis $H_{37}R_V$	1.25
Candida albicans	>50
Trichophyton rubrum	>50
Epidermophyton floccosum	50
Piricularia oryzae	>50
Colletotrichum sp.	25
Gloeosporium kaki	25
Fusarium oxysporum	12.5
Trichomonas vaginalis 4F	20

Many antibiotics with water-soluble and basic character have been isolated from actinomycetes. However, from elemental analytical data, the antibiotics A-396-I and II are differentiated from most of them, and only a few antibiotics remain to be compared. Actinospectacin[8] ($C_{14}H_{24}O_7N_2$) is differentiated by its negative ninhydrin reaction and low toxicity. Hygromycin B ($C_{20}H_{37}O_{13}N_3$ [9], $[\alpha]_D$ +19.2° in water), Destomycin A ($C_{20}H_{37}O_{13}N_3$, $[\alpha]_D$ +7° in water) and destomycin B (C 45.34, H 7.37, N 7.69, O 39.40, N-CH_3 3.98%, $[\alpha]_D$ +6° in water) are quite similar to the antibiotics A-396-I and II.

On TLC, A-396-I is distinguished from them. Therefore, it is considered to be a new antibiotic. A-396-II is identical with hygromycin B as judged from its TLC behavior and optical activity. Studies on the structural feature of A-396-I are now in progress.

Experimental

Production:

The streptomyces strain A-396 was inoculated on 800 ml medium consisting of starch 1.0 %, meat extract 0.5 %, peptone 0.5 %, yeast extract 0.25 % and NaCl 0.5 %, pH 7.0, in a 2-liter Erlenmeyer flask. It was cultured for 48 hours at 27°C on a rotary shaking machine. The culture was then transferred to 15 liters medium consisting of starch 1.0 %, glycerin 0.5 %, soybean meal 1.0 %, corn steep liquor 0.5 %, NaCl 0.3 %, K_2HPO_4 0.1 % and KH_2PO_4 0.1 %, pH 7.0, in a 30-liter jar fermentor. Fermentation was carried out for 3 days at 28°C under agitation of 250 r.p.m. and aeration of 20 liters per minute.

Isolation:

About 30 liters of the cultured broth obtained were adjusted to pH 3.0 with diluted hydrochloric acid and filtered with filter-aid. The filtrate was then adjusted to pH 7.0 with sodium hydroxide and passed through a 3-liter column of IRC-50 (Na type). The column was eluted with 1 N hydrochloric acid. The eluate having antimicrobial activity against *Bacillus subtilis* on an assay plate was treated with 60 g of Darco G-60 on adjusting to pH 7.0. The carbon was extracted with 300 ml of acid aqueous methanol three times. The extract was adjusted to pH 5.0 with IR-4B (OH type) and lyophilized. Trituration of the residue with acetone resulted in approx. 2 g of colorless powder which was a crude mixture of A-296-I and 1I in nearly equal amounts.

Preparative thin-layer chromatography:

The crude mixture of A-396-I and II (*ca.* 250 mg) was applied to a silica gel plate (Silica gel GF, thickness 750 μ, 20×100 cm). The plate was developed with chloroform - methanol - 4 % ammonia (2 : 1 : 1, upper layer), and development with the same solvent system was repeated after air-dried. Separated zones of A-396-I and II were visualized by iodine and extracted with acid aqueous methanol. After adjusting pH 5.0 with IR-4B (OH type) each extratct was freeze-dried to obtain crude powders of A-396-I hydrochloride (approx. 80 mg) and A-396-II hydrochloride (approx. 90 mg).

Purification of A-396-I and II:

The crude preparation (500 mg) of A-396-I hydrochloride obtained as above was converted to crystalline reineckate by the usual metohd. A purified reineckate (700 mg) was prepared by recrystallization from hot water. One half portion (350 mg) of the reineckate was regenerated to hydrochloride (135 mg) by treatment with pyridine hydrochloride, and the other half (350 mg) was recovered as free base (107 mg) by passing through a Dowex 1×8 (OH type) column followed by lyophilization.

A-396-I reineckate: red crystal, no definite melting point.

Anal. Found: C 24.95, H 3.87, N 19.16, Cr 10.40, MW 1147 (osmometry in tetrahydrofuran).

Calcd. for $C_{19}H_{35}O_{13}N_3 \cdot 3[Cr(NH_3)_2(SCN)_4]$:
C 25.37, H 3.61, N 20.04, Cr 10.63 %, MW 1467.28.

A-396-I hydrochloride: colorless hygroscopic powder, decomposes at above *ca.* 190°C.

Anal. Found: C 35.45, H 6.52, N 6.56, Cl 15.93.
Calcd. for $C_{19}H_{35}O_{13}N_3 \cdot 3HCl$: C 36.65, H 6.10, N 6.75, Cl 17.09 %.

A-396-I free base: colorless amorphous powder, m. p. 185~195°C (decomp.).

Anal. Found: C 45.42, H 7.07, N 7.93.
Calcd. for $C_{19}H_{35}O_{13}N_3$: C 44.46, H 6.82, N 8.18 %.

Similarly, A-396-II was purified, and the hydrochloride and free base were prepared.

A-396-II hydrochloride: colorless hygroscopic powder, decomposes at above *ca.* 190°C.

Anal. Found; C 36.91, H 6.67, N 6.30, Cl 16.87 %.

A-396-II free base: colorless amorphous powder, m. p. 170~180°C (decomp.).

Anal. Found: C 44.32, H 6.86, N 7.59 %.

Thin-layer chromatographic examination:

Silica gel GF (Merck) and Eastman Chromatogram Sheet 6060 were used with chloroform - methanol - 4 % aq. ammonia (2 : 2 : 1, upper layer) and (2 : 1 : 1, upper layer). Chromatography was carried out with multiple development, continuous development and circular development techniques, respectively, as well as with their combination, because differences of the mobilities were considered to be very small. All results indicated that A-396-I migrated faster than others, A-396-II, hygromycin B and destomycin A exhibited similar mobilities, and destomycin B gave slower mobility. One example is illustrated in Fig. 2.

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References

1) MANN, R. L. & W. W. BROMER: The isolation of a second atibiotic from *Streptomyces hygroscopicus*. J. Am. Chem. Soc. 80 : 2714~2716, 1958
2) WILEY, P. F.; M. V. SIGAL, Jr. & O. WEAVER: Degradation products of hygromycin B. J. Org. Chem. 27 : 2793~2796, 1962
3) MCGUIRE, J. M. & R. L. MANN: Hygromycin B, its production and treatment of intestinal parasites. U. S. Pat. 3,018,220, Jan. 23, 1962; Japan Pat. 18,441, Dec. 20, 1960
4) KONDO, S.; M. SEZAKI, M. KOIKE, M. SHIMURA, E. AKITA, K. SATOH & T. HARA: Destomycin A and B, two new antibiotics produced by a *Streptomyces*. J. Antibiotics, Ser. A 18 : 38~42, 1965
5) KONDO, S.; M. SEZAKI, M. KOIKE & E. AKITA: Destomycin A. The acid hydrolysis and the partial structure. J. Antibiotics, Ser. A 18 : 192~194, 1965
6) KONDO, S.; E. AKITA & M. SEZAKI: A new polyhydroxy amino acid, destomic acid, the hydrolysis product of destomycin A. J. Antibiotics, Ser. A 19 : 137~138, 1966
7) KONDO, S.; E. AKITA & M. KOIKE: The structure of destomycin A. J. Antibiotics, Ser. A 19 : 139~140, 1966
8) BERGY, M. E.; T. E. EBLE & R. R. HERR: Actinospectacin, a new antibiotic. IV. Isolation, purification, and chemical properties. Antibiot. & Chemoth. 11 : 661~664, 1961
9) Private communication from Dr. N. NEUSS.