NEW ANTICOCCIDIAL ANTIBIOTICS, WS-5995 A AND B

I. ISOLATION AND CHARACTERIZATION

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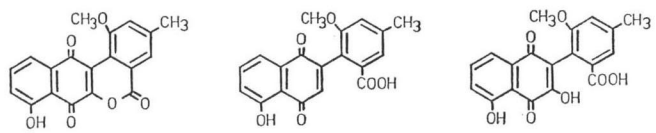
WS-5995 A, B and C are produced by a new strain of *Streptomyces* designated *Streptomyces auranticolor*. These antibiotics were purified by solvent extraction followed by chromatography on silica gel and then crystallized. WS-5995 A ($C_{19}H_{12}O_6$, m.p., 289~291°C) and WS-5995 B ($C_{19}H_{14}O_6$, sublimation at 300°C) protect chickens from infection with *Eimeria tenella*, a species of coccidia, which produces morbidity or mortality in chickens. WS-5995 C ($C_{19}H_{14}O_7$, m.p., 288~290°C), a biologically inactive component, was found to be converted to WS-5995 A on treatment with trifluoroacetic anhydride.

The control of coccidiosis is a serious problem for the poultry industry. Therefore, we undertook a screening program directed toward the isolation and evaluation of new anticoccidial substances. As a result, we isolated from a soil sample collected at Mt. Takao, Tokyo Prefecture, a strain of *Streptomyces* designated strain No. 5995, which was found to produce two new antibiotics, WS-5995 A and B, and a related inactive component, WS-5995 C. The producing microorganism has been identified as *Streptomyces auranticolor* sp. nov. by IKUSHIMA *et al*[1].

Studies in our laboratories[2,3] have shown that these antibiotics have the chemical structures shown in Fig. 1. This paper describes the fermentation, and isolation procedures along with biological and chemical properties of WS-5995 compounds.

Fig. 1. Structures of WS-5995 A, B and C.



WS-5995 A WS-5995 B WS-5995 C

Methods

Fermentation

The growth of *Streptomyces auranticolor* sp. nov. on mature (10 days) slant cultures was used to inoculate four 500-ml flasks containing 100 ml each of sterile seed medium containing 1% soluble starch, 1% glycerin, 1% cotton seed meal, and 1% dried yeast. The flasks were shaken on a rotary shaker (220 rpm, 2-inch throw) for 2 days at 30°C. The content of the flasks was used to inoculate 20 liters of fermentation medium in a stainless-steel fermentor. The composition of the fermentation medium is as follows: 3% glycerin, 0.5% oatmeal, 0.25% dried yeast, 0.05% KH_2PO_4 and 0.05% $Na_2HPO_4 \cdot 12\,H_2O$. The fermentation was carried out for 7 days at 30°C, an air flow rate of 20 liters per minute and an agitation rate of 300 rpm. A typical fermentation profile is shown in Fig. 2.

Fig. 2. Time course of fermentation.

The medium contained 3% glycerin, 0.5% oat meal, 0.25% dried yeast, 0.05% KH_2PO_4 and 0.05% $Na_2HPO_4 \cdot 12H_2O$.

Jar fermentor (20 liters) was operated with agitation of 300 rpm at 30°C. Mycelia were weighed after heating at 80°C for 10 hours.

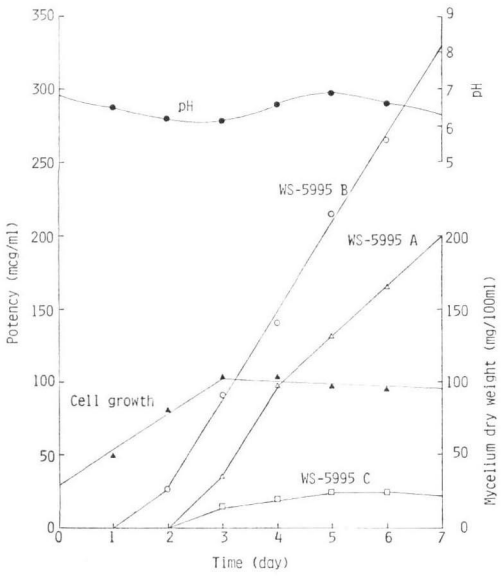
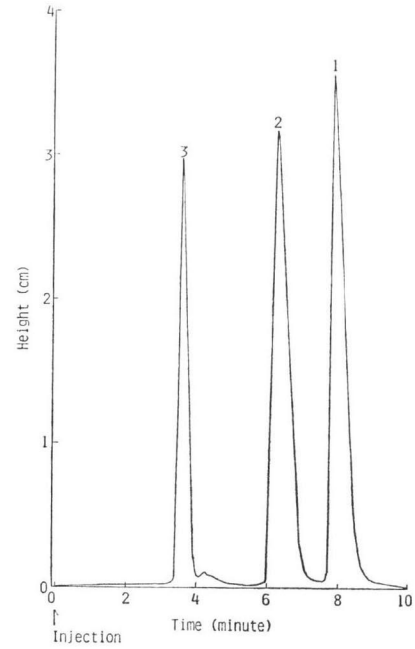


Fig. 3. High performance liquid chromatography of standard WS-5995 A, B and C.

Model separation of WS-5995 A, B and C was carried out using mixtures of equal amounts of the three components (100 mcg/ml) in tetrahydrofuran. 5 μl of the solution were injected. Peak: 1=WS-5995 A; 2=WS-5995 B; 3=WS-5995 C.



Detection of the antibiotics

The antibiotics present in the fermentation broth or in preparations obtained during the purification process were detected by their anticoccidial activity or by high performance liquid chromatography (HPLC).

(1) Test of anticoccidial activity: Two week-old White Leghorn chickens were used. Samples were given by oral administration three times per day from day 0 to day 2 of the experiment. After the first medication on day 0, chickens were infected by oral intubation with 30,000 sporulated oocysts of *Eimeria tenella*. Mortality rate and average weight gain of the treated, infected chickens were compared with those of nontreated, infected and nontreated, uninfected controls at day 5. The criteria for determination of efficacy of the drugs on coccidiosis were those described by JOHNSON *et al.*[4]. Gross cecal lesions produced by *E. tenella* were scored as follows: 0, no lesion; 1, a few discrete pinpoint lesions; 2, moderate cecal involvement; 3, marked cecal involvement, moderate hemorrhage; 4, maximal cecal involvement, massive hemorrhage or death due to the infection.

(2) High performance liquid chromatography: HPLC was carried out using a Waters Model 6000 A pump with a Waters Model U6K injector. Chromatography was monitored by a UV detector, Waters Model 440 at 254 nm. For analytical purpose, a steel column (4 mm inside diameter, 250 mm length) packed with a LiChrosorb RP-18 (Merck, Darmstadt) was used at a flow rate of 1.3 ml/min. The mobile phase was a mixture of tetrahydrofuran, ethyl alcohol and MCILVAINE's citrate-phosphate buffer (pH 3.6) (6: 3: 1). Good resolution of this group of antibiotics was obtained with this mobile phase. A typical chromatogram of a standard solution is illustrated in Fig. 3. Calibration curves were constructed using mixtures of equal amounts of the three components of WS-5995 in tetrahydrofuran. Five μl of the solutions containing 100, 200 or 300 μg/ml of the antibiotics were injected. The extinctions corresponding to the peak areas were plotted against the amount of the antibiotics added.

Assay for the antimicrobial activity

Antimicrobial activity was determined by the serial agar dilution streak method. One loopful of an overnight culture of each test organism in an appropriate medium was streaked on plates containing graded a concentration of the drugs and the minimal inhibitory concentration (MIC) was expressed in terms of μg/ml after incubation at 37°C for 18 hours for bacteria, and 48~72 hours at 30°C for *Mycobacterium*, yeast and fungi.

Conversion of WS-5995 C to WS-5995 A

To a solution of WS-5995 C (1 g) in anhydrous tetrahydrofuran (5 ml) was added trifluoroacetic anhydride (3 ml). WS-5995 A (800 mg) was recrystallized from tetrahydrofuran.

Results

Isolation of WS-5995 A, B and C

Fermentation broth (20 liters) was filtered with the aid of filteraid (Radiolite). The filtrate (15 liters) was concentrated *in vacuo* to 2 liters, and was extracted twice with 3 liters of ethyl acetate. The extract was concentrated *in vacuo* and the crude oily material obtained was dissolved in 10 ml of benzene and applied to a 1 liter silica gel column. The column was washed with 1 liter of *n*-hexane, and WS-5995 A was eluted with benzene. WS-5995 B was eluted with a mixture of benzene - ethyl acetate (3: 1). The fractions containing WS-5995 A were combined and concentrated to dryness. The crude solid thus obtained was crystallized from hot tetrahydrofuran yielding 140 mg of the antibiotic as orange needles.

The fractions containing WS-5995 B were combined and concentrated to oily material, which was crystallized from ethanol yielding 600 mg of the antibiotic as yellow needles.

To obtain WS-5995 C component, the residual aqueous layer of the culture filtrate was extracted with 2 liters of ethyl acetate at pH 2.0. The extract was concentrated to dryness. The dry residue was crystallized from hot tetrahydrofuran yielding 48 mg of WS-5995 C as orange needles.

Physicochemical Properties of WS-5995 A, B and C

The physicochemical properties of the three components are shown in Table 1. WS-5995 A, obtained as orange needles, is sparingly soluble in most organic solvents. Elemental analysis gave the formula, $C_{19}H_{12}O_6$, and the molecular weight (336) required by this formula was confirmed by its mass

Table 1. Physicochemical properties of WS-5995 A, B and C.

	WS-5995 A	WS-5995 B	WS-5995 C
Appearance	orange needles	yellow needles	orange needles
m.p. (°C)	289~291	300 (sublimation)	288~290
Molecular form.	$C_{19}H_{12}O_6$	$C_{19}H_{14}O_6$	$C_{19}H_{14}O_7$
Molecular weight	336	338	354
Elemental analysis; Calcd.	C : 67.85 H : 3.60	C : 67.45 H : 4.17	C : 64.40 H : 3.98
Found	C : 68.00 H : 3.53	C : 67.32 H : 4.34	C : 64.13 H : 3.91
Color reaction	$FeCl_3$	$FeCl_3$	$FeCl_3$
Solubility; soluble	hot THF	MeOH, $CHCl_3$, EtOAc	MeOH, EtOH, THF
insoluble	H_2O, $CHCl_3$, EtOAc	H_2O, Benzene	H_2O, Ether, Hexane
Rf value*			
Benzene - MeOH (4:1)	0.88	0.31	0.21
Benzene - EtOAc (5:1)	0.60	0.10	0.00

Rf value*: Thin-layer chromatography on silica gel sheet (Merck)

Fig. 4. UV Absorption spectra of WS-5995 A, B and C.

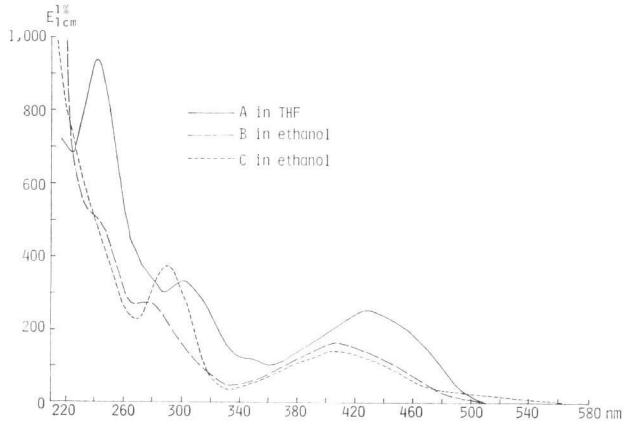
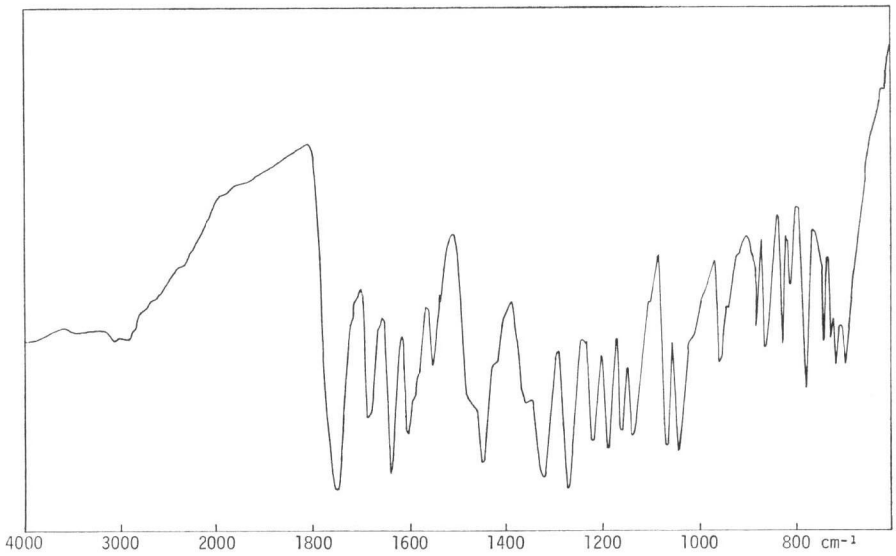


Fig. 5. IR Spectrum of WS-5995 A (KBr).



spectrum. The IR spectrum shown in Fig. 5 shows bands at 1640 cm^{-1} and 1750 cm^{-1} characteristic of a hydrogen bonded quinone group and lactone group, respectively. Its UV absorption spectrum shown in Fig. 4 [λ_{max}^{THF}: 242 nm ($E_{1cm}^{1\%}$, 946), 303 nm ($E_{1cm}^{1\%}$, 343), and 434 nm ($E_{1cm}^{1\%}$, 260)] resembles that of a perihydroxy-1,4-naphthoquinone.

WS-5995 B is a yellow crystalline material which is soluble in methanol, ethyl acetate, and chloroform. Elemental analysis and mass spectrometry established the molecular formula presented in Table 1. The presence of a benzoic acid moiety and 5-hydroxy-1,4-naphthoquinone was suggested by absorption bands in its IR spectrum (Fig. 6) at 1720 cm^{-1}, and 1680 and 1640 cm^{-1}, respectively. The UV spectrum of the antibiotic is shown in Fig. 4.

WS-5995 C is an orange crystalline material and soluble in methanol and tetrahydrofuran. The IR spectrum of this compound is shown in Fig. 7, with the following significant absorption maxima in KBr: 3300 ~ 2400 cm^{-1} (carboxy group), 1680 cm^{-1} (quinone carbonyl group) and 1640 cm^{-1} (chelated

Fig. 6. IR spectrum of WS-5995 B (KBr).

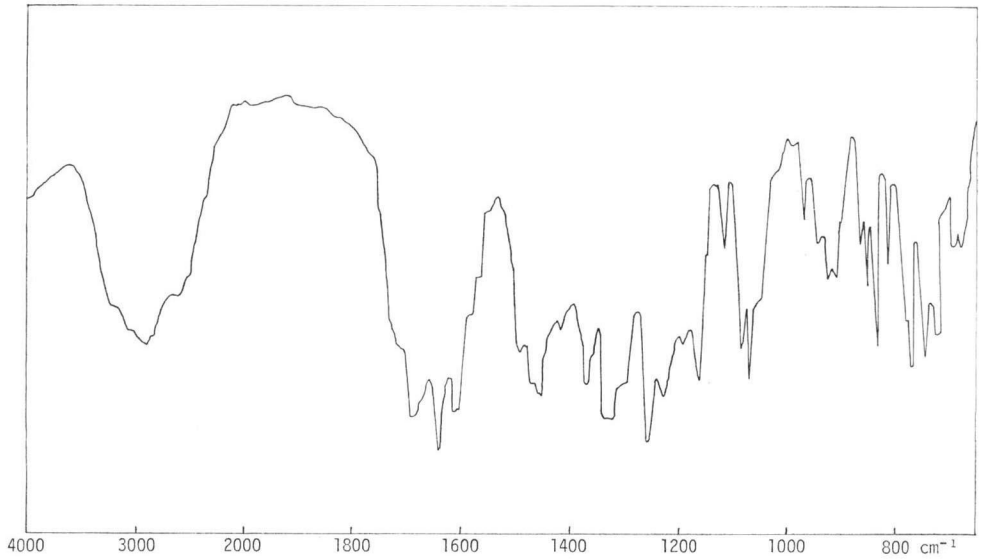
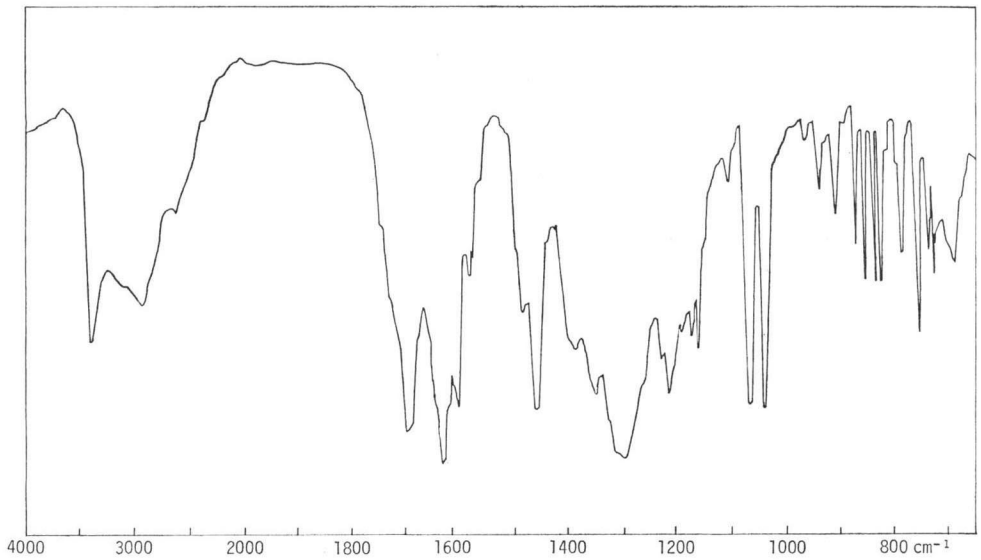


Fig. 7. IR Spectrum of WS-5995 C (KBr).



quinone carbonyl group). The UV spectrum shown in Fig. 4 indicates the presence of a naphthoquinone moiety.

The chemical structures of WS-5995 A, B and C are shown in Fig. 1. WS-5995 C possesses a hydroxy carboxylic acid group which can be converted to a lactone. Since WS-5995 A is the most effective of the three components on coccidiosis caused by *E. tenella* in chickens, WS-5995 C was converted to WS-5995 A by treatment with trifluoroacetic anhydride.

Biological Activities

The antimicrobial spectrum of WS-5995 A, B and C is shown in Table 2. Only WS-5995 B showed

Table 2. Antimicrobial spectra of WS-5995 A, B and C. (Agar dilution method).

Test organisms	M.I.C. (mcg/ml)		
	A	B	C
* *Staphylococcus aureus* 209 P JC-1	>100	>100	>100
* *Bacillus subtilis* ATCC-6633	>100	>100	>100
* *Escherichia coli* NIHJ JC-2	>100	>100	>100
* *Klebsiella* 417	>100	>100	>100
* *Proteus vulgaris* IAM-1025	>100	100	>100
* *Proteus mirabilis* 501	>100	>100	>100
* *Salmonella entritidis*	>100	>100	>100
* *Pseudomonas aeruginosa* NCTC-10490	>100	>100	>100
** *Candida albicans* YU-1200	>100	>100	>100
** *Trichophyton interdigitale*	>100	>100	>100
*** *Trichomonas vaginalis* 4 FM	>100	50	>100
**** *Mycobacterium* SP-607	>100	>100	>100

* H.I-Agar ** SABOUROUD *** V-bouillon **** DUBOS

Table 3. Effect of WS-5995 A, B and monensin against *Eimeria tenella* infection.

Dose (mg/bird/3 days)	WS-5995 A		WS-5995 B		Monensin	
	Score[a]	W.G.I[b]	Score	W.G.I	Score	W.G.I
Inf. med. 40	0	95.2	0	101.8	0	−41.0
20	0	108.3	0	89.5	0	51.4
10	0	106.3	0	94.7	0	68.1
5	0.3	97.2	0	89.5	2.3	97.9
2.5	2.0	91.7	3.0	68.4	2.7	91.7
1.2	2.0	108.3	-	-	4.0	98.6
Inf. unmed.	4.0	60.0				
Uninf. unmed.	0	100.0				

Score[a] : 0. 1. 2. positive effect
3. 4. no effect
W.G.I[b]: Weight gain index

weak activity against *Proteus vulgaris* and *Trichomonas vaginalis* and the two other components are devoid of any significant antimicrobial activity.

The anticoccidial activity of the antibiotics is shown in Table 3. WS-5995 A and B showed excellent protective activity against *E. tenella* infections. Birds treated with WS-5995 A at a dose larger than 10 mg/bird/day and B at 2.5 mg/bird/day showed no lesions at all. Average weight gain index of birds treated with WS-5995 A compared favorably with the weight gain per bird in the uninfected, nontreated control group. The percent relative weight gain of the WS-5995 B-treated birds was about 90% or more. No anticoccidial activity was observed with WS-5995 C.

The acute oral toxicity of the antibiotics is very low. The LD_{50} values of WS-5995 A and B in mice by the oral route are >200 mg/kg and >1,000 mg/kg, respectively. WS-5995 A and B were administered by the oral route to chickens at a dose of 1,000 mg/kg and 200 mg/kg, respectively. No toxic symptoms were observed during a 2-week period of observation following administration.

Discussion

It is well documented that polyether antibiotics produced by *Streptomyces* are effective in the treatment of coccidial infection of poultry.[5,6] In addition, several chemically synthesized compounds containing the naphthoquinone skeleton are also known to be anticoccidial substances.[7,8] Although the physicochemical properties of WS-5995 components described in this paper suggested the presence of a naphthoquinone moiety in their structure, the antibiotics can clearly be differentiated from these synthetic compounds on the basis of the chemical and biological properties. This is the first report of anticoccidial naphthoquinone antibiotics from fermentation products. Furthermore, the structure elucidation of WS-5995 A[2] has shown that the antibiotic is the first example of a 5H-benzo[d]naphtho-[2,3-b]pyran ring system found in nature.

WS-5995 A and B showed excellent protective efficacy against *E. tenella* infection as shown in Table 3. Thus, the antibiotics may be promising anticoccidial agents, however, further examination of the protective activity against various pathogenic coccidia in an expanded battery and floor pen testings are needed.

References

1) Ikushima, H.; E. Iguchi, M. Kohsaka, H. Aoki & H. Imanaka: *Streptomyces auranticolor* sp. nov., a new anticoccidial antibiotics producer. J. Antibiotics 33: 1103~1106, 1980
2) Tanaka, H.; Y. Ito, H. Ikushima, Y. Kawai, M. Okamoto, M. Kohsaka, H. Aoki & H. Imanaka: The structure and synthesis of WS-5995 A from *Streptomyces auranticolor* sp. nov. I. Tetrahedron Lett., in press
3) Tanaka, H.; Y. Ito, H. Ikushima, Y. Kawai, M. Okamoto, M. Kohsaka, H. Aoki & H. Imanaka: The structure and synthesis of WS-5995 A from *Streptomyces auranticolor* sp. nov. II. Agric. Biol. Chem., to be submitted
4) Johnson, J. & W. M. Reid: Anticoccidial drugs: Lesion scoring technique in battery and floor-pen experiments with chickens. Exp. Parasitol., 28: 30~36, 1977
5) Shumard, R. F. & M. E. Callender: Monensin, a new biologically active compound: Anticoccidial activity. Antimicr. Agents & Chemoth. -1967: 369~377, 1968
6) Reid, W. M.: Progress in the control of coccidiosis with anticoccidials planned immunization. Am. J. Vet. Res. 36: 593~596, 1975
7) Merck: Naphthoquinone derivatives. U. K. Patent 1,091,810. September 5, 1966
8) Merck: 2-(4-Cyclohexylcyclohexyl)-3-hydroxy-1,4-naphthoquinone in controlling coccidiosis in poultry. U. S. Patent 3,347,742. Oct. 17, 1967