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Structure and Synthesis of New Anticoccidial Antibiotics Isolated from Streptomyces Auranticolor

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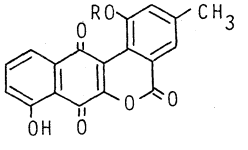
The structures of two new anticoccidial antibiotics, WS-5995 A and B, produced by *Streptomyces auranticolor*, were determined as **I** and **II**, respectively, on the basis of spectral and chemical evidence.

WS-5995 A (**I**), having 5*H*-benzo[*d*]naphtho[2,3-*b*]pyran as its mother skeleton, was synthesized by coupling the diazonium salt prepared from the anthranilic acid (**IX**) to 3-hydroxy juglone (**VIII**).

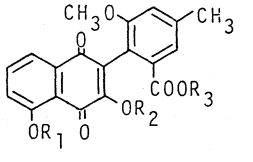
It is well-documented that such polyether antibiotics[1] as monensin and lasalocid are effective against coccidiosis, which is a serious problem for the poultry industry. In the course of screening new antibiotics, we isolated three pigments from a new strain of *Streptomyces*, identified as *Streptomyces auranticolor* sp. nov., [2,3] two of which showed excellent protective activity against *Eimeria tenella* infection.[3] We report[4] here the structural elucidation of these pigments, tentatively named WS-5995 A and WS-5995 B, and the synthesis of the former.

The first pigment, WS-5995 A (**I**), has the molecular formula $C_{19}H_{12}O_6$ which was confirmed by elemental analysis and mass spectroscopy ($M^+=336$). Its IR (ν_{max} 1750, 1670 and 1640 cm^{-1}) and UV (λ_{max} 242, 303 and 426 nm) spectra suggested the presence of a juglone[5] moiety in addition to a lactone group in this molecule. The ^{1}H-NMR spectrum of **I** exhibited signals at δ2.38 (3H, s, aromatic methyl), 3.72 (3H, s, methoxy) and 6.80～7.45 (5H, m, aromatic protons).



(I) R=Me

(VI) R=H



(III) $R_1=R_2=R_3=H$

(IV) $R_1=H$, $R_2=R_3=Me$

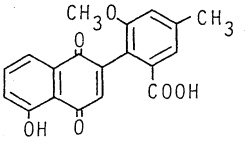
(V) $R_1=Ac$, $R_2=R_3=Me$

Hydrolysis of **I** with 5% sodium hydroxide gave the hydroxy-acid (**III**) which coincided with the third pigment, WS-5995 C, having no protective acitivity against the same coccidial infection. Treatment of **III** with trifluoroacetic anhydride regenerated the parent pigment (**I**)

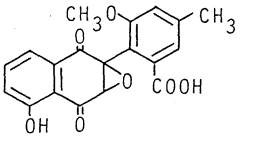
in a good yield, indicating the presence of a lactone ring in the pigment (**I**).

On the other hand, methylation of **III** with ethereal diazomethane gave the trimethoxy-compound (**IV**), (mp 164～166°C), which was smoothly acetylated in the usual manner to yield the monoacetate (**V**). The NMR spectrum of **V** showed clearly differentiate signals corresponding to five aromatic protons indicating two groups. The first group consisting of three protons exhibited a type of ABC-pattern ($\delta_{H9}=7.36$, $J_{9,10}=7.8$ Hz, $J_{9,11}=2.0$ Hz, $\delta_{H10}=7.72$, $J_{10,9}=7.8$ Hz, $J_{10,11}=7.8$ Hz, $\delta_{H11}=8.08$, $J_{11,10}=7.8$ Hz, $J_{11,9}=2.0$ Hz) similar to 3-acetoxy juglone.[6] The second group consisting of two protons showed signals which were indicated that these protons were arranged in *meta*-position to each other.

Treatment of **I** with boron tribromide in methylene chloride at room temperature resulted in the cleavage of a methoxy group to give the di-phenolic compound (**VI**), (mp 197～199°C). The phenol (**VI**) did not show the carbonyl band at 1670 cm^{-1} observed in the parent pigment (**I**) and the strength of the carbonyl band at 1640 cm^{-1} increased instead. This result indicated that the newly-created phenolic hydroxyl group formed a strong hydrogen bond with the carbonyl observed at 1670 cm^{-1} in **I**. All the evidence allowed us to propose the structure (**I**) for WS-5995 A.



(II)



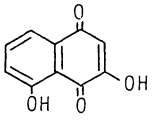
(VII)

The second pigment, WS-5995 B(**II**), has the molecular formula $C_{19}H_{14}O_6$ and spectroscopic properties (ν_{max} 3300～2400, 1680 and 1640 cm^{-1} and λ_{max} 276 and 410 nm), suggesting that the pigment is the deoxy-*seco*-acid (**II**) of **I**. The NMR spectrum of **II** showed a signal at δ 6.80 attributable to a proton at the 3-position of the juglone moiety. Confirmation of the proposed WS-5995 B structure (**II**) was provided by conversion of **II** to **I** as follows.

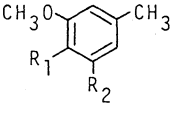
Oxidation of **II** with 30% hydrogen peroxide in the presence of sodium bicarbonate gave the epoxide (**VII**)[7] which was, without further purification, treated with boron trifluoride etherate to give **I** as the sole product, confirming the structure (**II**).

Next, we focused our efforts on the synthesis of **I**.

Our strategy to construct the skeleton of the pigment rested on the diazo coupling of the anthranilic acid (**IX**) to 3-hydroxy juglone (**VIII**). The segment (**IX**) was prepared from 2,3-dimethoxy-5-methyl-benzoic acid (**X**)[8], the carboxylic acid group of **X** being protected as an oxazoline ring in the conventional manner.[9]



(VIII)



(IX) $R_1=NH_2$, $R_2=COOH$

(X) $R_1=OCH_3$, $R_2=COOH$

(XI) $R_1=OCH_3$, $R_2=$ 

(XII) $R_1=NH_2$, $R_2=$ 

This was achieved by treating the oxazoline (**XI**) with lithium amide in tetrahydrofuran to give the amine (**XII**) in a 45% yield.[10] Hydrolysis of **XII** with 10% hydrochloric acid furnished **IX**.

Coupling [11] **VIII** to **IX** was accomplished by

adding a solution of the diazonium salt (prepared from **IX** with sodium nitrite and dilute hydrochloric acid) to a solution of **VIII** in 5% aqueous potassium hydroxide. This gave **III**, which was, without purification, converted to **I** in the same manner as mentioned above.

All the spectral data of the synthetic WS-5995 A were identical to those of WS-5995 A from natural sources.

To our knowledge, WS-5995 A (**I**) is the first naturally occurring compound possessing 5*H*-benzo[*d*]naphtho[2,3-*b*]pyran as the mother skeleton.

EXPERIMENTAL

All melting points were determined with a Yanagimoto microscopic hot-stage apparatus and were uncorrected. IR spectra were recorded by a Jasco IRA-2 grating spectrophotometer. ^{1}H-NMR spectra were obtained with a JEOL-JNM-PMX 60 NMR spectrometer. Mass spectra were determined on a Hitachi RMU-6M spectrometer with a direct, heated inlet system.

Isolation. Fermentation broth (20 liters)[3] was filtered with filteraid (Radiolite). The filtrate (15 liters) was concentrated to a volume of *ca.* 2 liters under reduced pressure and extracted with ethyl acetate (3 liters). The extract was evaporated under reduced pressure to afford an oily residue which was chromatographed on silica gel in benzene. Elution with benzene gave WS-5995 A (140 mg) which was crystallized from tetrahydrofuran, mp 289~291°C; MS *m/z*: 336 (M^+); UV λ_{max}^{THF} nm(ε): 242 (31,800), 303 (8,700) and 426 (8,700); IR ν_{max}^{KBr} cm^{-1}: 1750, 1670 and 1640; NMR $\delta_{TMSP}^{NaOD\text{-}D_2O}$: 2.38 (3H, s), 3.72 (3H, s) and 6.80~7.45 (5H, m). *Anal.* Found: C, 68.00, H, 3.53. Calcd for $C_{19}H_{12}O_6$: C, 67.85, H, 3.60%.

Elution with benzene–ethyl acetate (3 : 1) gave WS-5995 B (600 mg) which was crystallized from ethanol, sublimation at 300°C; MS *m/z*: 338 (M^+); UV λ_{max}^{EtOH} nm (ε): 240 (sh) (17,200), 276 (8,800) and 410 (5,700); IR ν_{max}^{KBr} cm^{-1}: 3300~2400, 1720, 1680 and 1640; NMR $\delta_{TMS}^{CDCl_3}$: 2.40 (3H, s), 3.76 (3H, s), 6.80 (1H, s), 7.0~7.6 (5H, m), 10.1 (1H, br. disappeared with D_2O)and 12.00 (1H, br.s. disappeared with D_2O). *Anal.* Found: C, 67.32, H, 4.34. Calcd for $C_{19}H_{14}O_6$: C, 67.45, H, 4.17%.

The residual aqueous layer of the culture filtrate was adjusted to pH 2 and extracted with ethyl acetate (2 liters). The extract was evaporated under reduced pressure to afford WS-5995 C (48 mg), which was crystallized from tetrahydrofuran, mp 288~290°C; MS *m/z*: 354 (M^+); UV λ_{max}^{EtOH} nm: 288 (13,500) and 412 (5,300); IR ν_{max}^{KBr} cm^{-1}: 3300~2400, 1680 and 1640; NMR $\delta_{TMS}^{DMSO\text{-}d_6}$: 2.45 (3H, s), 3.75 (3H, s), 3.5~4.5 (1H, br. disappeared with D_2O), 7.13~7.88 (5H, m) and 11.58 (1H, s disappeared with D_2O). *Anal.* Found: C, 64.13, H, 3.91. Calcd for $C_{19}H_{14}O_7$: C, 64.40, H, 3.98%.

*Conversion of WS-5995 A (**I**) to WS-5995 C (**III**).* A solution of **I** (100 mg) in 1 N sodium hydroxide (5 ml) was stirred at room temperature for 5 min, acidified with dilute hydrochloric acid and extracted with ethyl acetate. The extract was washed with water, dried and evaporated under reduced pressure to afford **III** (80 mg).

Conversion of **III** *to* **I**. A solution of **III** (1 g) in dry tetrahydrofuran (5 ml) and trifluoroacetic anhydride (3 ml) was stirred at room temperature for 5 min. The usual work-up gave **I** (800 mg).

Methylation of the hydroxy-acid (**III**). A solution of **III** (500 mg) in methanol (10 ml) was treated with ethereal diazomethane (prepared from *p*-toluenesulfonyl-*N*-methyl-*N*-nitrosoamide) and the mixture was allowed to stand at room temperature for 1 hr. Removal of the solvent gave a residue which was chromatographed on silica gel in chloroform. Elution with chloroform gave **IV** (300 mg) which was crystallized from ethanol, mp 164~166°C; MS *m/z*: 382 (M^+); IR ν_{max}^{KBr} cm^{-1}: 1725, 1655, 1640 and 1612; NMR $\delta_{TMS}^{CDCl_3}$: 2.43 (3H, s), 3.75 (9H, s), 6.94 (1H, br. s), 7.0~7.4 (1H, m), 7.46 (1H, br. s) and 7.45~7.6 (2H, m). *Anal.* Found: C, 66.12, H, 4.53. Calcd for $C_{21}H_{18}O_7$: C, 65.96, H, 4.75%.

Acetylation of the trimethoxy-compound (**IV**) A solution of **IV** (500 mg) and acetic anhydride (5 ml) in pyridine (5 ml) was allowed to stand at room temperature overnight and concentrated under reduced pressure to dryness to leave a residue which was taken up in methylene chloride. The methylene chloride solution was washed with dilute hydrochloric acid, water, aqueous sodium bicarbonate and water and then dried. Removal of the solvent gave a residue which was chromatographed on silica gel in chloroform. Elution with chloroform gave **V** (450 mg) which was crystallized from ethanol, mp 127~128°C; MS *m/z*: 424 (M^+); IR ν_{max}^{KBr} cm^{-1}: 1700, 1725, 1675 and 1620; NMR $\delta_{TMS}^{CDCl_3}$: 2.41 (3H, s), 2.44 (3H, s), 3.70 (9H, s), 7.00 (1H, d, J=1.8 Hz), 7.36 (1H, dd, J=7.8 and 2.0 Hz), 7.52 (1H, d, J=1.8 Hz), 7.72 (1H, t, J=7.8 Hz) and 8.08 (1H, dd, J=7.8 and 2.0 Hz). *Anal.* Found: C, 65.21, H, 4.60. Calcd for $C_{23}H_{20}O_8$: C, 65.09, H, 4.75%.

Cleavage of the methyl ether of **I**. Boron tribromide (1 ml) was added to a solution of **I** (100 mg) in dry methylene chloride (100 ml). The mixture was stirred at room temperature for 2 hr, washed with water, dried and evaporated under reduced pressure to afford **VI** (70 mg), which was crystallized from tetrahydrofuran, mp 190~192°C; MS *m/z*: 322 (M^+); IR ν_{max}^{nujol} cm^{-1}: 1760, 1640, 1605 and 1575; NMR $\delta_{TMSP}^{NaOD\text{-}D_2O}$: 2.27 (3H, s), 6.50 (2H, s) and 6.8~7.8 (3H, m). *Anal.* Found: C, 67.11, H,

3.21. Calcd for $C_{18}H_{10}O_6$: C, 67.08, H, 3.13%.

Transformation of WS-5995 B (**II**) *to WS-5995 A* (**I**). Aqueous hydrogen peroxide (30%, 1.5 ml) was added to a solution of WS-5995 B (100 mg) and sodium bicarbonate (800 mg) in methanol (15 ml) at 0°C and the mixture was stirred at room temperature for 5 hr.

Removal of the solvent under reduced pressure gave a residue which was taken up in ethyl acetate. The solution was washed with water, dried and concentrated to dryness under reduced pressure to leave a residue which was dissolved in methylene chloride (30 ml).

Boron trifluoride etherate (2 ml) was added to the solution and the whole was allowed to stand at room temperature overnight. The solution was then poured into ice-cooled water and extracted with ethyl acetate. The extract was washed with water, dried and evaporated under reduced pressure to leave a residue which was chromatographed on silica gel in chloroform. Elution with chloroform gave **I** (50 mg) (identical with WS-5995 A isolated from natural sources in all respects).

4,4-Dimethyl-2-(2,3-dimethoxy-5-methylphenyl)-oxazoline (**XI**). Ethyl chloroformate (8.6 g) was added to a solution of 2,3-di-methoxy-5-methylbenzoic acid (**X**) (15.7 g) and triethylamine (8.9 g) in dry methylene chloride (120 ml) with stirring at −20°C and stirring was continued for 1 hr. 2-Amino-2-methyl propanol (9.6 g) was added to the solution at the same temperature. The whole was warmed to room temperature, stirred for 7 hr, washed with dilute hydrochloric acid, water, aqueous sodium bicarbonate and water, and finally dried. Removal of the solvent gave *N*-[2-(1-hydroxy-2-methylpropyl)]-2,3-dimethoxy-5-methylbenzamide (19.2 g) which was crystallized from ether–*n*-hexane, mp 73~75°C; IR ν_{max}^{KBr} cm^{-1}: 3440, 3345 and 1642. *Anal.* Found: C, 66.62, H, 8.01, N, 5.24. Calcd for $C_{14}H_{21}NO_4$: C, 62.90, H, 7.92, N, 5.24%.

To a solution of the benzamide (19 g) in dry ether (150 ml) was added dropwise thionyl chloride (21 ml) and the mixture was stirred at room temperature for 15 min. Removal of the ether by decantation gave an oily residue, which was washed with ether (30 ml) three times.

After adding 20% aqueous sodium hydroxide to the residue, the solution was extracted with ether. The extract was washed with water, dried and evaporated under reduced pressure to afford **XI** (14.3 g), bp 124~125°C/2 mmHg; IR ν_{max}^{film} cm^{-1}: 1650 and 1588. *Anal.* Found: C, 67.10, H, 7.76, N, 5.64. Calcd for $C_{14}H_{19}NO_3$: C, 67.44, H, 7.68, N, 5.62%.

4,4-Dimethyl-2-(2-amino-3-methoxy-5-methylphenyl)-oxazoline (**XII**). A suspended solution of **XI** (5 g) and lithium amide (60 g) in dry tetrahydrofuran (80 ml) was stirred under nitrogen at room temperature for three days, poured into ice-cooled water, and extracted with ether. The extract was washed with water, dried and evaporated under reduced pressure to leave a residue which was chromatographed on silica gel in chloroform. Elution with chloroform gave **XII** (1.2 g) which was crystallized from ether, mp 135~137.5°C; IR ν_{max}^{KBr} cm^{-1}: 3510, 3340, 1635 and 1610; NMR $\delta_{TMS}^{CDCl_3}$: 1.35 (6H, s), 2.25 (3H, s), 3.83 (3H, s), 3.97 (2H, s), 6.1 (2H, br.), 6.67 (1H, d, J=2Hz) and 7.17 (1H, m). *Anal.* Found: C, 66.37, H, 7.75, N, 11.80. Calcd for $C_{13}H_{18}N_2O_2$: C, 66.64, H, 7.74, N, 11.96%.

3-Methoxy-5-methyl-anthranilic acid (**IX**). A solution of **XII** (500 mg) in 10% hydrochloric acid (42 ml) was refluxed for 14 hr and concentrated to a volume of *ca.* 10 ml under reduced pressure. The resulting solution was adjusted at pH 4.6~4.8 with dilute ammonium hydroxide and extracted with ethyl acetate. The extract was dried and evaporated under reduced pressure to afford **IX** (170 mg) which was crystallized from ethanol-water, mp 170~172°C; IR ν_{max}^{KBr} cm^{-1}· 1655; NMR $\delta_{TMS}^{CD_3COCD_3}$: 2.28 (3H, s), 3.88 (3H, s), 6.87 (1H, d, J=1.8 Hz), 6.95 (3H, br. s) and 7.17 (1H, m). *Anal.* Found: C, 59.56, H, 6.02, N, 7.75. Calcd for $C_9H_{11}NO_3$: C, 59.66, H, 6.12, N, 7.73%.

WS-5995A (**I**). A solution of sodium nitrite (80 mg) in water (4 ml) was added to a solution of **IX** (170 mg) in dilute hydrochloric acid (conc. HCl–H_2O/0.15 ml~4 ml) on an ice-salt bath, and the solution was added to a solution of 3-hydroxy juglone (**VIII**) (180 mg) in 5% aqueous potassium hydroxide (10 ml) at 40~45°C which was stirred at the same temperature for 1 hr. After cooling to room temperature, the reaction mixture was acidified with dilute hydrochloric acid and extracted with ethyl acetate. The extract was washed with water, dried and evaporated under reduced presssure to leave a residue which was taken up in ethyl acetate (5 ml). To the solution was added trifluoroacetic anhydride (1 ml) at room temperature and the whole was stirred for 1 hr. The reaction mixture was washed with aqueous sodium bicarbonate and water, dried and evaporated under reduced pressure to leave a residue which was chromatographed on silica gel in chloroform. Elution with chloroform gave WS-5995 A (20 mg) which was identical in all respects to WS-5995 A from natural sources.

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