Isolation, Structure, and Coccidiostat Activity of Coccidiostatin A

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Coccidiosis is one of the more common and costly diseases in poultry that is caused by various *Eimeria* species. In our quest to discover coccidiostats from natural products, we discovered a microbial fermentation extract that exhibited *in vivo* anticoccidial activity. Fractionation of this extract led to the discovery of two potent antiprotozoals, emecorrugatin A (**1**) and coccidiostatin A (**2**). The former compound exhibited only *in vitro* activity, whereas the latter new compound exhibited *in vivo* activity against *Eimeria* species in chickens at 150 ppm dosed in chicken feed. The isolation, structure elucidation, relative configuration, and activity of coccidiostatin A (**2**) are described.

Coccidiosis is a self-limiting disease of the intestinal lining produced by the invading protozoan parasites of the genus *Eimeria.* It is a major disease of the poultry industry, leading to high mortality and severe loss of productivity.[1] The disease is characterized by droopiness, paleness of the comb, diarrhea, and occasionally blood in the droppings. The death rate may be quite high, both in chicks and in adults. The majority of anticoccidials control the disease by inhibiting the development of the parasite at an early stage before the host-damaging stage is reached. The leading anticoccidials are ionophores represented by salinomycin, monensin, narasin, diclazuril, toltrazuril, nicarbazin, decoquinate, maduramycin, and lasalocid. In 2003 the estimated market for anticoccidials was $455 million, an increase of 0.2% over the previous year.[2] The widespread use of anticoccidials has revolutionized the poultry industry by reducing mortality and production losses caused by coccidiosis. However resistance to the in-feed coccidiostats traditionally used to control coccidiosis has also risen. Hence, there is an urgent need for chemically diverse coccidiostats to enter into the market. There is also an increased acceptance by the poultry industry worldwide of live vaccines to control coccidiosis. This increase is measurable by sales and by market penetration of products such as Immucox.[1]

We have taken a variety of natural product screening approaches to discover coccidiostats including a mechanism of action (MOA) based approach such as inhibition of cGMP-dependent kinase (PKG) and an empirical approach. From the MOA-based screening approaches, we recently reported discovery of tenellones as PKG inhibitors.[3] In the empirical approaches, we utilized *Besnoitia jellisoni* as surrogate organism in a whole-cell *in vitro* assay (Ex-Bes assay: extended *Besnoitia* assay) as well as *Eimeria*-infected chickens (MAC assay: minibird assay for coccidiosis) for an *in vivo* test. An extract of *Penicillium rugulosum* displayed activity in both of these assays. Bioassay-guided fractionation of the extract using both assays simultaneously led to the isolation of two compounds with disparate activities. Emecorrugatin A (**1**) showed potent activity only in the Ex-Bes assay, whereas the new compound, coccidiostatin A (**2**) (Figure 1), exhibited activity only in the *in vivo* MAC assay. The isolation, structure elucidation, and biological activities of emecorrugatin A (**1**) and coccidiostatin A (**2**) are herein described.

Producing Organism and Fermentation. The producing organism was isolated from pitch pine needles collected in Monmouth County, NJ, and was identified as *P. rugulosum*. The culture was fermented on solid vermiculite-based production media.

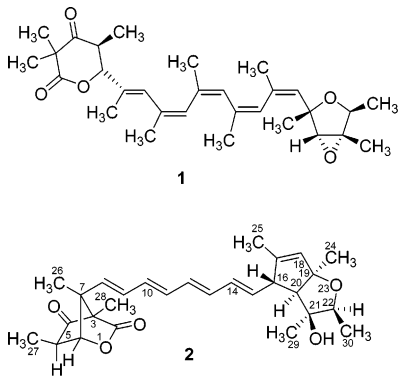

Figure 1. Structures of emecorrugatin A (**1**) and coccidiostatin A (**2**).

Isolation of Emecorrugatin A (1) and Coccidiostatin A (2). A 9 L fermented solid-state growth was extracted with 9 L of methyl ethyl ketone (MEK) to afford an extract that exhibited activities in the Ex-Bes and MAC assays. The MEK extract was concentrated and suspended in aqueous MeOH and extracted with EtOAc. The EtOAc extract was purified by silica gel chromatography to yield two distinct active fractions, an Ex-Bes- and a MAC-active fraction. Subsequent purification of these fractions by reversed-phase HPLC afforded emecorrugatin A (**1**, 125 mg, 138 mg/L) from the Ex-Bes-active fraction and coccidiostatin A (**2**, 87 mg, 10 mg/L) from the MAC-active fraction, both as yellow powders. Emecorrugatin A (**1**) possessed a tetrahydrofuran-epoxide moiety connected to a six-membered keto-lactone through a pentaene chain. Soon after its isolation and structure elucidation by us, it was published by Fujimoto et al. as a toxic metabolite from the Ascomycete *Emericella corrugate*, and the structure was confirmed by comparison of its NMR and UV data.[4]

Coccidiostatin A (2). High-resolution ESIMS analysis of coccidiostatin A (**2**) revealed a molecular formula of $C_{28}H_{36}O_5$ indicating 11 degrees of unsaturation. The ^{13}C NMR spectrum of **2** in CD_3CN (Table 1) corroborated the assigned molecular formula. The NMR spectra were also acquired in other solvents (e.g., C_5D_5N and CD_2Cl_2), but CD_3CN provided the best overall resonance dispersion, and assignments in CD_3CN are summarized in Table 1. The IR spectrum of **2** showed absorption bands assignable for keto (ν_{max} 1712 cm^{-1}) and hydroxy groups (ν_{max} 3414 cm^{-1}). The UV spectrum showed absorption bands at λ_{max} 279, 290, 306, 321, and 357 nm, indicating the presence of a polyene chain. The DEPT spectrum of coccidiostatin A indicated the presence of seven methyls and five sp^3 and nine olefinic methines. Two of the methines appearing as a doublet at δ_H 5.00 (J = 2.0 Hz) and a

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Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Assignments of Coccidiostatin A (**2**) in CD_3CN

position	δ_C	mult	δ_H (mult, *J* in Hz)	HMBC (C → H)
2	173.1	C°		H-6, 28
3	70.9	C°		H-6, 8, 28
4	209.9	C°		H-5, 6, 27, 28
5	44.5	CH	2.87, dq, 2.0, 7.0	H-6, 27
6	86.2	CH	5.00, d, 2.0	H-8, 26, 27
7	59.6	C°		H-8, 9, 28, 26
8	131.5	CH	5.77, d, 15	H-27
9	134.7	CH	6.43, dd, 15, 10	
10	133.1	CH	6.31, dd, 15, 10	
11	135.5	CH	6.30, dd, 15, 10	
12	132.5	CH	6.28, dd, 15, 10	
13	135.1	CH	6.36, dd, 15, 10	
14	132.05	CH	6.20, dd, 15, 10	
15	139.4	CH	5.62, dd, 15, 10	H-16, 20, 25
16	52.4	CH	3.27, d, 10	H-14, 15, 18, 20, 25
17	145.7	C°		H-15, 16, 18, 20
18	132.12	CH	5.36, t, 1	H-16, 20, 24, 25
19	93.3	C°		H-16, 18, 20, 24
20	66.5	CH	2.11, brs	H-15, 18, 16, 24, 29
21	80.4	C°		H-16, 22, 29, 30
22	81.4	CH	3.68, q, 6	H-29, 30
24	26.8	CH_3	1.31, s	H-20
25	15.5	CH_3	1.62, brs	H-18
26	17.5	CH_3	1.13, s	H-8
27	12.2	CH_3	1.16, d, 7	H-5
28	5.5	CH_3	1.06, s	
29	21.8	CH_3	1.09, s	H-20, 22
30	13.3	CH_3	1.03, d, 6	H-22

quartet at δ_H 3.68 (J = 6.0 Hz) were oxygenated. The remaining seven carbons were quaternary in nature, as indicated by the ^{13}C NMR spectrum. Of these, two were assigned to carbonyls [an alicyclic ketone (δ_C 209.9) and an ester (δ_C 173.1)], one to an sp^2 carbon (δ_C 145.7), and four to sp^3 carbons (δ_C 59.6, 70.9, 80.4, and 93.3), of which the latter two were oxygenated. Among the seven methyl groups, two were secondary and the rest were tertiary. The methyl groups and the other proton-bearing carbons were correlated to the respective protons by an HMQC experiment and are listed in Table 1. The all-*E* tetraene functionality was assembled on the basis of the UV spectrum and the ^{1}H NMR, COSY, HMQC, and ^{13}C NMR spectra. Measurement of the magnitude of *J* values (15 and 10 Hz) established the all-*E* geometry of the olefinic moieties. Determination of *J* values was possible only after resolution enhancement using the Varian's resolv command (with line broadening of −1.5 Hz) for processing the data. The two bicyclic systems were assembled at each end of the tetraene chain by analysis of HMBC correlations and the ^{1}H−^{1}H spin systems deduced by a 2D-COSY experiment. H-15 exhibited a strong COSY correlation with H-16 and provided evidence for the linkage of the right-hand-side bicyclic structure through C-16. The HMBC correlations of methyl groups H_3-25 to C-16, C-17, and C-18; H_3-24 to C-18, C-19, and C-20; H_3-29 to C-20, C-21, and C-22; and H_3-30 to C-21 and C-22, along with two- and three-bond HMBC correlations of H-16 and H-20 to C-15 (Table 1), established the assembly of the fused tetrahydrofuranyl-cyclopentene bicyclic structure. Similar two- and three-bond HMBC correlations from the methyl groups H_3-26 to C-3, C-6, C-7, and C-8; H_3-27 to C-4, C-5, and C-6; and H_3-28 to C-2, C-3, C-4, and C-7, together with correlations of H-6 to C-2, C-3, C-4, and C-5, established the bicyclic ketolactone and its linkage at the left-hand side of the tetraene. ESIMS analysis of coccidiostatin A (**2**) yielded a major fragment at *m*/*z* 365 due to loss of the C4 unit from the cleavage of the tetrahydofuran ring (Figure 3).

The substitution of the bridgehead carbon C-7 at the 1,4-position (C-3 and C-6) of the six-membered ketolactone ring forces the ring into a boat conformation placing H-6 and CH_3-28 in pseudoequatorial dispositions. H-6 exhibited a small coupling (J = 1 Hz) with H-5 consistent with a dihedral angle of ∼90° observed in the Dreiding model when H-5 was in pseudoaxial orientation, as shown in **2**. H-9 exhibited strong NOESY correlations (Figure 2) to H_3-28 and H-6 and did not show a NOESY correlation with H_3-27. Consideration of the NOESY correlations together with potential steric crowding imposed by the CH_3-27 and the tetraene unit if they were placed on the same side led to the assignment of the tetraene unit on the right side of the molecule and the CH_3-26 on the left side. The relative configuration of the bicyclic tetrahydrofuranyl-cyclopentene unit was determined by application of *J* values, NOESY correlations, and examination of the Dreiding model. H-20 exhibited strong NOESY correlations with H_3-24, indicating a C19−C20 *cis* ring fusion between the tetrahydrofuran and cyclopentene rings. H-20 also showed a strong NOESY correlation with H-15, whereas H-14 of the *trans* olefin exhibited a NOESY correlation with H-16, indicating that H-16 and H-20 should be *trans* disposed. H-16 and H-20 did not show any coupling, consistent with the ∼90° dihedral angle observed in the Dreiding model when H-16 and H-20 were placed in *trans* arrangements. H-20 showed strong NOESY correlation with H_3-29 and H-22, suggesting their through-space proximity and their *syn* facial relationship, which was further supported by strong NOESY correlations of H-22 with H_3-29 and H_3-24. On the basis of these data structure **2** was proposed for coccidiostatin A. The structure as drawn represents the independent relative configuration of the individual bicyclic units. They could not be correlated to each other.

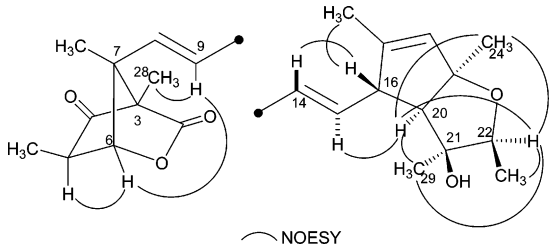

Figure 2. NOESY (mixing time = 300 ms) correlations of coccidiostatin A (**2**).

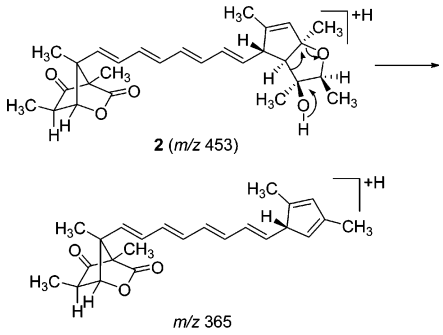

Figure 3. ESIMS fragmentation of coccidiostatin A (**2**).

Biological Activity. Purified compounds were tested in Ex-Bes and MAC assays. In the *in vitro* Ex-Bes assay *B. jellisoni* trachyzoites were treated with the test compounds and the potency was expressed as ED_{50} (concentration of compound that caused 50% reduction of trachyzoites compared to untreated control represented by an average of two experiments performed in duplicate by visual read). Apicidin[5] was used as a positive control, exhibiting an ED_{50} value of <0.28 uM. In the *in vivo* MAC assay the *Eimeria*-infected chickens were fed their regular diet mixed with the test compound, and the percentage of viable oocysts were counted to ascertain the potency compared to untreated control. Robenidine (2, 4, and 8 ppm) and monensin (121 and 64 ppm) were used as controls.

Emecorrugatin A (**1**) was a potent inhibitor of the growth of *B. jellisoni* and exhibited ED_{50}'s of 1.39 ± 0.4 μM in the Ex-Bes assay. However this compound was inactive in the *in vivo* MAC assay at 416 ppm when administered with the feed. It was also

inactive when administered ip at 28 mg/kg. However, coccidiostatin A exhibited no activity in the Ex-Bes assay ($ED_{50} \gg 2$ mg/mL), but was efficacious in the MAC assay at 75−150 ppm against several species of *Eimeria*. Coccidiostatin A (**2**) was partially active at both 150 and 75 ppm versus *E. acervulina* in the MAC assay with 74 ± 15% and 64 ± 12% reduction of oocyst compared to control, respectively. At the 150 ppm level the compound was also partially active against *E. tenella* and *E. mitis*, exhibiting 59 ± 10% and 44 ± 10% oocyst reduction compared to the control, respectively. No activity was observed against *E. maxima* and *E. praecox* at both concentrations. There was no indication of toxicity at 500 ppm, the highest dose tested.

In summary, we have described herein the isolation, structure elucidation, and relative configuration of coccidiostatin A, a new bis-bicyclic tetraene natural product that showed relatively broad spectrum coccidiostat activity. However its low potency prevents it from being developed as a commercially useful cocidiostat that could be used to treat chickens.

Experimental Section

General Experimental Procedures. For general experimental procedures see ref 5.

Producing Organism. The producing fungus (MF6599 = GB5133) was isolated from decayed pitch pine (*Pinus rigida*) needles collected in Monmouth County, New Jersey, and was identified as *Penicillium rugulosum* by morphological comparisons.[6]

Fermentation of *P. rugulosum*: Seed Medium. The composition of the seed medium (in g/L, unless otherwise noted) was as follows: corn steep powder (2.5), tomato paste (40.0), oat flour (10.0), glucose (10.0), and a trace elements solution (10.0 mL/L). The trace elements solution was prepared in 0.6 N HCl and had the following composition (in g/L): $FeSO_4 \cdot 7H_20$ (1.0), $MnSO_4 \cdot H_20$ (1.0), $CuCl_2 \cdot 2H_2O$ (0.025), $CaCl_2$ (0.1), H_3BO_4 (0.056), $(NH_4)_6Mo_7O_{24} \cdot 4H_20$ (0.019), and $ZnSO_4 \cdot 7H_2O$ (0.2). The seed medium was prepared with distilled water, and the pH was adjusted to 6.8 prior to sterilization. The medium was dispensed at 50 mL per 250 mL in plain Erlenmeyer flasks. Cotton closures were used. Sterilization was conducted at 121 °C for 20 min.

Frozen vials of vegetative growth of cultured *P. rugulosum* were stored in 10−20% glycerol at −75 °C. The vials were thawed to room temperature and used to inoculate seed cultures at 1.0 mL per 50 mL of seed medium. The cultures were grown on a gyratory shaker (220 rpm) for 3 days at 25 °C and 85% relative humidity, until a sufficient amount of biomass was obtained.

Production Medium. The composition of the solid substrate fermentation medium consisted of a solid portion and a liquid portion. The solid portion was 675 mL of vermiculite, which was added to a 2 L roller bottle, which was plugged with a latex closure, autoclaved for 60 min, and dried for 30 min. The liquid portion of the medium composition (in g/L) included glucose (150.0), glycerol (20.0), yeast extract (4.0), $NaNO_3$ (1.0), monosodium glutamate (3.0), Na_3HPO_4 (0.5), $MgSO_4 \cdot 7H_2O$ (1.0), K-elements (1.0 mL/L), and $CaCO_3$ (8.0). The K-elements solution employed in the liquid portion of the medium was prepared with distilled H_2O with a composition (in g/L) of $FeCl_3 \cdot 6H_2O$ (5.8), $MnSO_4 \cdot H_2O$ (0.1), $CoCl_2 \cdot 6H_2O$ (0.02), $CuSO_4 \cdot 5H_2O$ (0.015), $Na_2MoO_4 \cdot 2H_2O$ (0.012), $ZnCl_2$ (0.02), $SnCl_2 \cdot 2H_2O$ (0.005), H_3BO_3 (0.01), KCl (0.02), and concentrated HCl (2.0 mL/L). The liquid portion of the medium was prepared in distilled H_2O, wherein the pH was adjusted to 7.0 before addition of $CaCO_3$, and then 220 mL was dispensed in 500 mL bottles and sterilized at 121 °C for 15 min. A 12 mL aliquot of seed growth was placed into 220 mL of the liquid portion of the production medium. This was swirled vigorously to disperse the biomass. The contents were dispensed by pouring into a 2 L roller bottle vessel, which contained 675 mL of steam-sterilized large-particle vermiculite. The contents of the roller bottle were shaken/mixed to ensure homogeneous inoculation and coverage. The roller bottles were incubated horizontally, revolving at approximately 4 rpm on a Wheaton roller apparatus, at 22 °C and 75% relative humidity for 18 days.

Isolation of Emecorrugatin A (1) and Coccidiostatin (2). A 9 L solid fermentation was extracted with 9 L of MEK by shaking for 60 min and was concentrated to remove most of the MEK, leaving an aqueous residue, which was resuspended in 50% aqueous MeOH (2 L) and extracted sequentially with CH_2Cl_2 and EtOAc. All the organic extracts showed Ex-Bes and MAC activity. The organic extracts were pooled, concentrated under reduced pressure to a small volume, adsorbed onto silica gel, and loaded on a sintered funnel containing 500 g of silica gel. The sample in the funnel was eluted with 2 L each of 10, 20, 50, and 75% EtOAc in hexane under vacuum. The fractions eluted with 10−20% EtOAc were active in the Ex-Bes assay, but were inactive in the MAC assay. The 50−75% EtOAc fractions were active in the MAC assay, but were inactive in the Ex-Bes assay.

The 10−20% EtOAc fractions were combined together and concentrated to give a syrup, which was dissolved in MeOH and extracted with hexane to remove the fatty material. The MeOH extract was concentrated to 10 mL. One milliliter of this MeOH extract was purified by reversed-phase HPLC (Zorbax RX C-8, 21.6 × 250 mm, starting with 50% aqueous CH_3CN for 10 min and then to 80% CH_3CN in 20 min at a flow rate of 8 mL/min). The fractions eluting at 44 min were lyophilized to give 125 mg of emecorrugatin A (**1**) (titer 138 mg/L). The MAC-active EtOAc extracts (50−75% EtOAc from silica gel) were combined and evaporated to dryness. The red residue was dissolved in 2 mL of MeOH and purified by reversed-phase HPLC (Zorbax RX C-8, 21.6 × 250 mm, eluting with 55% aqueous CH_3CN at a flow rate of 10 mL/min). Four repetitive preparative HPLC runs of this extract yielded 87 mg of coccidiostatin A (**2**) (titer ~10 mg/L) as a yellow powder. Accurate optical rotation could not be measured (Perkin-Elmer 241 polarimeter) due to the instability of the absorption at Na wavelength. UV (MeOH) λ_{max} (log ϵ) 279 (4.55), 290 (4.54), 306 (4.53), 321 (4.46), 357 (3.84); CD (MeOH) λ (θ) 234 (−1547), 278 (1734), 312 (−3195), 324 (−4212); IR (ZnSe) ν_{max} 3414, 2930, 1712, 1452, 1379, 1087 cm^{-1}; ^1H and ^{13}C NMR (see Table 1); ESIMS (*m/z*) 453 (M + H), HRESIMS (*m/z*) 365.2106 (calcd for $C_{24}H_{28}O_3$+H, 365.2111).

Ex-Bes Assay. Microtiter plates were seeded to a total well concentration of 1.0×10^4 cells/well with human foreskin fibroblast (HFF) cells in minimum essential medium (MEM) containing 0.5% gentamicin, 1% antibiotic-antimycotic solution, and 1% fetal bovine serum. The cells were incubated for 48 h at 37 °C. *B. jellisoni* trachyzoites were harvested from the peritoneum of mice and diluted to 2.0 × 104 parasites/mL with MEM containing 0.5% gentamicin, 1% antibiotic-antimycotic solution, and 1% fetal bovine serum. Half the plates were infected with *B. jellisoni* trachyzoites to a final concentration of 4×10^3 parasites per well. The infected and uninfected plates were incubated for 2−4 h at 37 °C. A positive control (described below) and the test compound in DMSO (DMSO test concentration of 0.05%) were introduced (2-fold serial dilutions tested in duplicate) to the infected and uninfected plates, and the plates were incubated for 4 days at 37 °C. The standard test concentrations for the positive control and test compounds of unknown activity were from 2000 to 0.0078 μg/mL. After incubation, crystal violet stain was added to the plates and observed visually. Violet wells were scored 1−3 (1 = least intense, 3 = most intense) according to their color intensity, which correlated to the Ex-Bes potency of the test compound.

On activity plates, a no drug control lane or well revealed only slight or no crystal violet color. This is because the parasite, *B. jellisoni*, destroyed the HFF cell monolayer to the point of disappearance. Since the cell monolayer had been destroyed, and free parasites do not stain crystal violet, the well was not stained. However, if the test compound or drug was active against *B. jellisoni*, then the cell monolayer was partially or fully intact, depending upon the activity of the test compound at the given concentration. In this case, the undestroyed cell monolayer was stained with the crystal violet stain and the well color was violet. Degrees of this drug activity were observed and scaled in a scoring system for each well. The mean score was calculated for duplicate test well serial dilutions, and the ED_{50} of the compound was determined as the concentration of the test compound with a mean score of 2 in the assay; no confidence intervals could be statistically calculated due to the subjective nature of the visual scoring. Confirmation of activity was shown after two repetitions of identical activity from two different assays.

The uninfected plates that contained the drug should be colorless if the compound tested was toxic to the cells, thereby providing an indication about the toxicity of the test compounds to the HFF cell monolayer. The wells of uninfected toxicity plates when containing nontoxic test drug or compound were intensely violet, indicating that the crystal violet stain has stained the HFF cell monolayer that has remained intact.

The natural product apicidin, a fungal metabolite isolated at Merck & Co., Inc. from endophytic fungi (*Fusarium pallidoroseum*) on twigs

collected in Costa Rica, was used as a positive control for both activity and toxicity in this assay.[5] Apicidin was solubilized in 100% DMSO, diluted 1:2000, allowing for a final DMSO test concentration of 0.05, then serially diluted 2-fold (tested in duplicate) from 1 to 0.0078 μg/mL. At the higher concentrations (0.25 to 1 μg/mL), apicidin was known to be toxic (partial to full, respectively) to the HFF cells. At concentrations ≤ 0.125 μg/mL, apicidin showed full efficacy, as measured by the assay readout, for antiparasitic activity with no toxicity.

Spectrum, Efficacy Study, and MAC Assay. In the spectrum study, 1-day-old male and female leghorn chickens were obtained from a commercial hatchery and housed in a holding room at 35 °C. At one week of age they were placed on medicated or basal diet, using two chicks per treatment group. All but the normal control chicks received ~25 000 sporulated oocysts, following 48 h of premedication. The inoculum was a mixture of *Eimeria mitis*, *E. maxima*, *E. acervulina*, *E. tenella*, and *E. praecox.* At the termination of the experiment the chicks were euthanized, the weight gains were determined, the intestinal tract was examined for coccidial lesions, and the oocysts from a 48 h accumulation of the excreta were counted. The MAC assay was a simplified version of the spectrum study in which the chickens were infected with only two species of coccidian, namely, *E. acervulina* and *E. tenella.*

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