CP-78,545, A NEW MONOCARBOXYLIC ACID IONOPHORE ANTIBIOTIC RELATED TO ZINCOPHORIN AND PRODUCED BY A *STREPTOMYCES*

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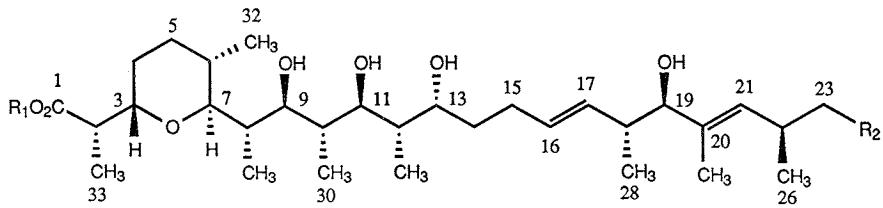
A new monocarboxylic acid ionophore antibiotic related to zincophorin, CP-78,545 (**1**), was found in the culture broth of *Streptomyces* sp. N731-45. CP-78,545 was extracted with organic solvents and purified by column chromatography. The metabolite, which is active *in vitro* against certain Gram-positive bacteria, as well as the anaerobe *Treponema hyodysenteriae*, and a coccidium *Eimeria tenella*, was isolated as a water insoluble magnesium salt (**2**) in 2 : 1 (ligand/metal) stoichiometry. The structure of CP-78,545 was elucidated by spectroscopic (NMR and MS) methods, and the relative stereochemistry was determined by single-crystal X-ray analysis of the cadmium salt (**3**). CP-78,545, *i.e.*, 24-dehydrozincophorin, is unique since its molecular backbone contains a terminal double bond previously not found in other polyether ionophores.

Many polyether antibiotics are capable of complexing and transporting monovalent cations. A number of polyethers are commercially important as feed additives, acting as anticoccidial agents and enhancing feed efficiency in ruminants. An interesting monocarboxylic acid ionophore with a high



CP-78,545 free acid (**1**)	R_1=H	R_2=CH=CH_2
CP-78,545 Mg-salt (**2**)	R_1=½Mg	R_2=CH=CH_2
CP-78,545 Cd-salt (**3**)	R_1=½Cd	R_2=CH=CH_2
Zincophorin Zn/Mg-salt (**4**)	R_1=½Zn(Mg)	R_2=CH_2CH_3
Griseochelin Mg-salt (**5**)[a]	R_1=½Mg	R_2=CH_2CH_3

[a] The stereochemistry is unknown: See ref 2.

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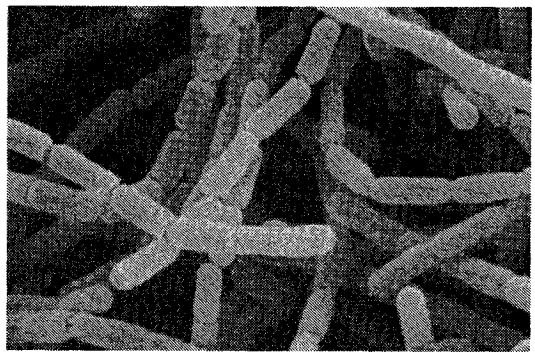
affinity for divalent cations, particularly zinc, was isolated from a strain of *Streptomyces griseus* and given the trivial name zincophorin (**4**)[1] (or griseochelin (**5**)[2]). Recently, a systematic degradation of zincophorin and the total synthesis of this antibiotic have been published by DANISHEFSKY *et al.*[3,4]. In the present paper, we report the discovery of antibiotic CP-78,545 (**1**), the second member in the zincophorin family. The structure of CP-78,545 is particularly noteworthy in that, unlike the parent antibiotic, it is the first monocarboxylic acid ionophore of the polyether type that contains a terminal olefin.

Fig. 1. Scanning electron micrograph of spore chains of *Streptomyces* sp. N731-45 on oatmeal agar at 15 days, ×12,000.



Strain

The CP-78,545 producing strain, *Streptomyces* sp. N731-45, was isolated from a soil sample collected in Awaji Island, Hyogo Prefecture, Japan. It was found to produce narrow dimensions of the hyphae, an aerial mycelium, an unfragmented substrate mycelium, and spores borne in chains on the aerial mycelium. The culture N731-45 is characterized by its melanin production, gray aerial mycelium, straight to flexuous spore chains, and spores with a smooth surface (Fig. 1). It utilizes glucose, arabinose, fructose, inositol, mannitol, raffinose, rhamnose, sucrose and xylose. The whole-cell hydrolysates contained LL-diaminopimelic acid, glucose, mannose and rhamnose. The results of whole-cell analyses established its belonging in the genus *Streptomyces*. Compared with related species of *Streptomyces*, it resembles *Streptomyces actuosus* ATCC 25431 in its biochemical properties, but differs from the latter in that it shows poor rather than moderate growth at 45°C and in its cultural characteristics on a variety of media (*e.g.*, yeast extract - malt extract agar, glucose - asparagine agar, BENNETT's agar, casein agar, CZAPEK - sucrose agar, gelatin agar and starch agar).

Fermentation

The vegetative inoculum of the CP-78,545 producing culture was developed by transferring spores of *Streptomyces* sp. N731-45 to a 300-ml Erlenmeyer flask containing 30 ml of an inoculum medium with the following composition (in g/liter): Cerelose 10, corn starch 10, corn steep liquor 5.0, NZ-Amine YTT 5.0, calcium carbonate 3.0 and cobalt chloride 0.002. The pH of the medium was adjusted to 7.0 before autoclaving. The inoculated flask was incubated for 72 to 96 hours at 28°C on a rotary shaker operating at 150 rpm. Two ml of the resulting vegetative growth was used to inoculate a 300-ml Erlenmeyer flask containing 80 ml of the same medium. The inoculated flask was incubated for 72 hours then used in its entirety to inoculate 3,000 ml of the following medium (in g/liter): Cerelose 10, soy flour 10, corn starch 10, sodium chloride 5.0, corn fermentable solids 5.0, calcium carbonate 1.0 and cobalt chloride 0.002. The pH of the medium was adjusted to 7.1 with an NaOH solution before autoclaving. The fermentation was carried out for 5 days at 28°C in a 5-liter jar fermenter. The fermenter was agitated at 1,700 rpm and sterile air was sparged at 1 v/v/m. P-2000 (Union Carbide) was used as an antifoam agent. For tank fermentation, 1.6 liters of inoculum prepared as described above was used to inoculate a 200-liter fermenter containing 105 liters of the same medium

as used in the jar fermenters. Antibiotic potency of the broth was carried out by using a disc assay against *Bacillus subtilis* ATCC 6633 or *Staphylococcus aureus* ATCC 6538.

Isolation

Sixty liters of the culture broth from twenty 5-liter jar fermenters was extracted with methyl isobutyl ketone (20 liters). The crude extract was concentrated under reduced pressure to afford a dark oil (4.0 g) that was chromatographed on 500 g of Silica gel 60 (70-230M ASTM; E. Merck) in hexane - EtOAc (1 : 1). The activity was followed by TLC on silica gel plates using EtOAc as the eluent. The ionophore was visualized as a bright purple coloration using vanillin - EtOH - H_3PO_4 spray reagent (3 g vanillin in 85 ml of EtOH and 15 ml of 85% H_3PO_4), followed by heating to 80°C. The appropriate fractions were combined and concentrated under reduced pressure to give 0.5 g of an oily product. The oil was dissolved in acetone and treated with Darco G60, filtered, and evaporated. The resulting oil was dissolved in EtOAc and washed with pH 10 Na_2HPO_4 solution and dried over anhydrous Na_2SO_4. This adjustment of the pH, followed by treatment with Na_2SO_4, would normally convert a monovalent polyether to the corresponding sodium salt. However, CP-78,545, with a high affinity for divalent cations such as magnesium, was inert toward this procedure (see below). Thus, this step is probably unnecessary in the present case. CP-78,545 was isolated analytically pure by crystallization from EtOAc to afford 110 mg as the magnesium salt **2**. Material from the mother liquor (300 mg after concentration) was chromatographed on a flash-chromatography column on Silica gel 32-63 (Universal Adsorbents) using hexane - EtOAc with increasing amounts of EtOAc as eluting solvent to give 150 mg of slightly impure CP-78,545 Mg-salt. Further purification of the latter material could be accomplished by preparative HPLC using a Dynamax-60A silica gel column with hexane - EtOAc - AcOH (49 : 50 : 1) as an eluting solvent.

Structural Determination

CP-78,545 free acid (**1**) was obtained by treatment of a chloroform solution of the magnesium salt **2** with an aqueous solution of EDTA. CP-78,545 Cd-salt (**3**) was prepared from the free acid **1** using cadmium oxide, and the resulting crystals were suitable for X-ray crystallographic studies (see below). The physico-chemical properties of the free acid **1** and its magnesium salt **2** are summarized in Table 1.

Table 1. Physico-chemical properties of CP-78,545 free acid (**1**) and Mg-salt (**2**).

Property	1	2
MP (°C)	94~98	221~222
$[\alpha]_D^{25}$ (*c* 1.0, $CHCl_3$)	+6.7°	+18.2°
Empirical Formula	$C_{33}H_{58}O_7$	$(C_{33}H_{57}O_7)_2Mg$
MW	566.8	1,156
Elemental analysis		
Calcd:	C 69.93, H 10.31	C 68.58, H 9.94
Found:	C 69.64, H 10.69	C 68.82, H 9.89
IR $\nu_{max}^{CHCl_3}$ cm^{-1}	3540, 3400, 3260, 1725	3540, 3350, 3220, 2920, 2860, 1560
UV (MeOH)	End absorption	End absorption
EI-MS *m/z* (%)	566 (0.1) $C_{33}H_{58}O_7$, 549 (13) $C_{33}H_{57}O_6$, 428 (75) $C_{24}H_{44}O_6$, 287 (10) $C_{15}H_{27}O_5$, 229 (41) $C_{12}H_{21}O_4$, 171 (100) $C_9H_{15}O_3$, 153 (39) $C_9H_{13}O_2$	589 (8) $C_{33}H_{57}O_7Mg$, 451 (0.3) $C_{24}H_{43}O_6Mg$, 428 (14) $C_{24}H_{44}O_6$, 229 (12) $C_{12}H_{21}O_4$, 211 (11) $C_{12}H_{19}O_3$, 171 (49) $C_9H_{15}O_3$, 153 (36) $C_9H_{13}O_2$, 97 (100) C_6H_9O

Spectroscopic data and elemental analyses were consistent with $C_{33}H_{58}O_7$ for the free acid **1**, and $(C_{33}H_{57}O_7)_2Mg$ for the magnesium salt **2**. The electron impact mass spectrum (EI-MS) of the free acid **1** indicated an ion at *m/z* 549 (M−OH, 13%; calcd for $C_{33}H_{57}O_6$: 549.4154, found: 549.4024). An extremely low intensity parent ion was observed at *m/z* 566. The positive fast atom bombardment mass spectrum (FAB-MS), using a dithioerythritol - dithiothreitol (1 : 3) matrix, gave cationized molecules *m/z* 704 (M−16+$C_4H_{10}O_2S_2$ (from the matrix)) and 549 (M−OH). In contrast, the EI-MS of the magnesium salt **2** gave no parent ion. However, an ion at *m/z* 589 (M−$C_{33}H_{57}O_7$, 8%; calcd for $C_{33}H_{57}O_7Mg$: 589.3953, found: 589.3975) was observed that was consistent with the postulated empirical formula. Furthermore, the positive FAB-MS of **2** using the same matrix as above gave characteristic peaks that were in agreement with a 2:1 (organic ligand/magnesium) stoichiometry,

Table 2. ^{13}C and ^1H NMR chemical shift data for CP-78,545 Mg-salt (**2**) and griseochelin Mg-salt (**5**)[3].

Carbon	2		5	
	^{13}C shift[a]	^1H shift[b]	^{13}C shift[a]	^1H shift[b]
C-1	182.84 (0)	—	182.85 (0)	—
C-2	41.00 (1)	3.06	41.04 (1)	3.07
C-3	76.71 (1)	4.04	76.47 (1)	4.08
C-4	24.94 (2)	1.62	25.01 (2)	1.46, 1.65
C-5	27.14 (2)	1.24, 1.46	27.23 (2)	1.24, 1.45
C-6	32.24 (1)	1.51	32.28 (1)	1.50
C-7	75.65 (1)	3.80	75.69 (1)	3.85
C-8	32.64 (1)	2.00	32.74 (1)	2.01
C-9	86.32 (1)	3.32	86.33 (1)	3.37
C-10	35.99 (1)	2.63	36.02 (1)	2.64
C-11	83.41 (1)	3.64	83.52 (1)	3.69
C-12	35.99 (1)	1.73	36.29 (1)	1.75
C-13	68.25 (1)	4.24	68.23 (1)	4.27
C-14	34.99 (2)	1.48, 1.78	35.13 (2)	1.46, 1.78
C-15	28.39 (2)	2.03, 2.14	28.34 (2)	2.08, 2.18
C-16	131.57 (1)	5.42	131.33 (1)	5.49
C-17	134.27 (1)	5.32	134.58 (1)	5.37
C-18	41.67 (1)	2.14	41.72 (1)	2.19
C-19	81.64 (1)	3.48	81.80 (1)	3.16
C-20	134.12 (0)	—	133.88 (0)	—
C-21	134.38 (1)	5.08	135.09 (1)	5.08
C-22	32.24 (1)	2.48	31.82 (1)	2.41
C-23	41.89 (2)	1.98	40.03 (2)	1.20, 1.28
C-24	137.26 (1)	5.70	20.59 (2)	1.26
C-25	115.65 (2)	4.94	14.18 (3)	0.86
C-26	20.47 (3)	0.94	20.89 (3)	0.94
C-27	11.01 (3)	1.56	10.99 (3)	1.59
C-28	17.47 (3)	0.76	17.49 (3)	0.81
C-29	10.45 (3)	1.11	10.50 (3)	1.14
C-30	12.60 (3)	0.54	12.57 (3)	0.59
C-31	11.01 (3)	1.04	10.99 (3)	1.08
C-32	17.57 (3)	0.74	17.44 (3)	0.78
C-33	15.61 (3)	0.96	15.50 (3)	1.01

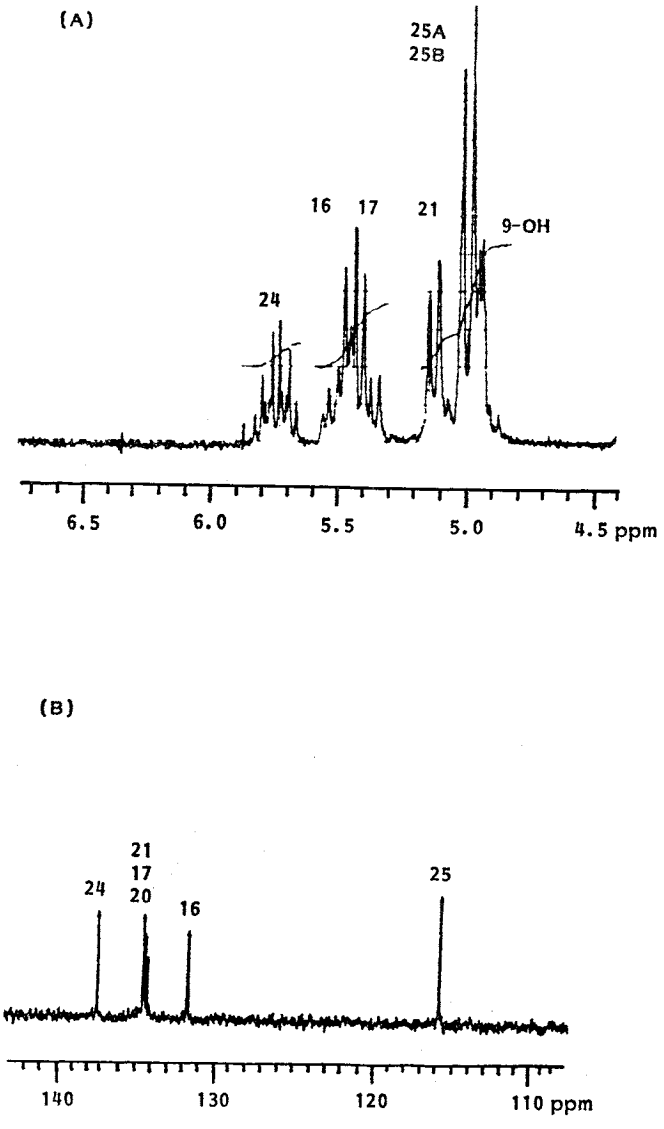
[a] In ppm from TMS in $CDCl_3$ solution; number of attached protons in parentheses.

[b] In ppm from TMS in $CDCl_3$ solution.

i.e., $(C_{33}H_{57}O_7)_2Mg$: *m/z* 1,334 ((M+H)+Mg+$C_4H_{10}O_2S_2$ (from the matrix)), 1,333 (M+Mg+$C_4H_{10}O_2S_2$), 1,156 (M+H), 897 ($C_{33}H_{57}O_7Mg$+2($C_4H_{10}O_2S_2$)), 743 ($C_{33}H_{57}O_7Mg$+$C_4H_{10}O_2S_2$) and 589 ($C_{33}H_{57}O_7Mg$, base peak).

The ^{13}C and ^1H NMR spectra, including ^{13}C distortionless enhancement by polarization transfer (DEPT), ^1H homonuclear correlation spectroscopy (COSY) and ^1H-^{13}C heteronuclear correlation spectroscopy (HETCOR) experiments, of CP-78,545 Mg-salt were most informative, and Table 2 gives the complete chemical shift assignments. Since the Mg-salt of zincophorin has not been reported, our spectra were instead compared with those for the Mg-salt of griseochelin (**5**), which was known to be closely related to or identical with zincophorin. Griseochelin has the same gross structure as zincophorin, however, an X-ray structure has not been determined for griseochelin and the

Fig. 2. ^1H (250 MHz) (A) and ^{13}C NMR (62.5 MHz) (B) spectra for CP-78,545 Mg-salt (**2**) in $CDCl_3$. Only the olefinic region is shown.



exact stereochemistry is unknown[2]. Indeed, a remarkable similarity between CP-78,545 Mg-salt (**2**) and griseochelin Mg-salt (**5**) was noted as regards to ^{1}H and ^{13}C NMR data, with the exception of the chemical shifts for C-23, C-24 and C-25, and the protons that are attached to these carbons (Table 2). Some assignments for **2** were made by analogy with the chemical shifts and assignments for griseochelin Mg-salt (**5**), since a detailed NMR analysis had already been performed for this compound[2]. The following ^{13}C connectivities for **2** were assumed: C(4)-C(5), C(5)-C(6), C(7)-C(8), C(8)-C(9), C(11)-C(12), C(12)-C(13), C(14)-C(15), C(15)-C(16) and C(19)-C(20).

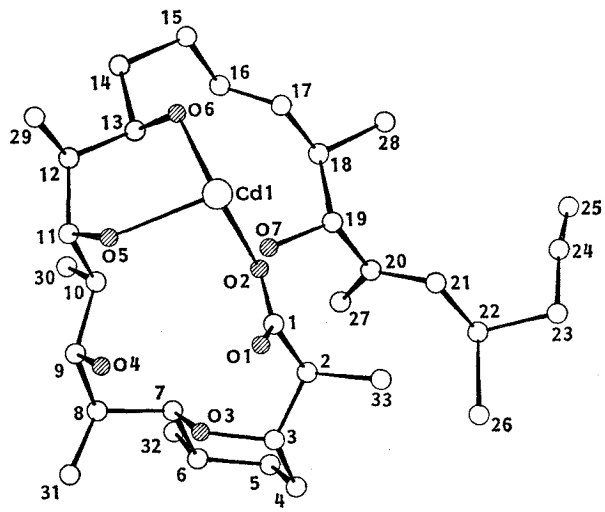
The difference in empirical formulas between zincophorin (griseochelin) and CP-78,545 was two hydrogens, and suggested that CP-78,545 was possibly a dehydro-analog of the former. Inspection of the ^{1}H and ^{13}C NMR spectra of CP-78,545 Mg-salt (**2**) did in fact show the presence of an extra double bond (Fig. 2) relative to zincophorin (griseochelin). Furthermore, the ^{13}C chemical shifts of the olefinic carbons for this additional double bond, and the number of hydrogens attached to each carbon (*i.e.*, C-24, δ_c 137.26 (1H) and C-25, δ_c 115.65 (2H)), were consistent with an unsubstituted double bond at the side-chain terminus.

X-Ray Analysis of CP-78,545 Cd-Salt (3)

The structure and relative stereochemistry of **3** was determined by X-ray crystallography using a crystal which measured $0.32\times0.10\times0.08$ mm. Diffraction measurements were made on an Enraf-Nonius CAD-4 fully automated diffractometer using graphite monochromated CuKα radiation (λ= 1.54184 Å). Preliminary indications of the unit cell based on 25 randomly selected reflections revealed monoclinic symmetry with the following lattice parameters: a=12.801(2) Å, b=12.210(4) Å and c= 22.944(3) Å with β=90.7°. The space group, based on the observed systematic extinctions was assigned as $P2_1$ (No. 4), Z=2 with one molecule of composition $(C_{38}H_{57}O_7)_2Cd$ forming the asymmetric unit. The volume was 3,585 Å^3 and the calculated density was 1.15 g/cm^3. There were 5364 reflections collected with $2\theta\leq112°$; of those reflections 4433 (83%) with $|\geq3\sigma|$ were adjudged observed.

Due to the isomorphous nature of the unit cell with zincophorin Zn/Mg-salt (**4**), the coordinates of zincophorin[1] were input into the WFO option in MULTAN 80. The phasing of 434 E_S ($E_S\geq$

Fig. 3. Crystal structure of CP-78,545 Cd-salt (**3**).
Only one-half of the complex is shown.



1.512) resulted in an electron density map which revealed 67 out of the 81 non-hydrogen atoms. Multiple iterations of refinement and the weighted Fourier option in MULTAN 80 resulted in solution of the entire structure. Hydrogen atoms were not included in the structure factor calculations. The side chains of the two ligands exhibit extreme disorder and have associated with them large thermal factors. However, full matrix refinement of the non-hydrogen atoms has resulted in convergence of the crystallographic reliability factors to the following values; unweighted of 0.064 and a weighted residual of 0.081.

Table 3. *In vitro* antimicrobial activity of CP-78,545 Mg-salt (**2**).

Organism	MIC (μg/ml)
Staphylococcus aureus 01A106	25
Streptococcus pyogenes 02C203	1.56
Escherichia coli 51A538	>100
Salmonella choleraesuis 58B015	>100
Pasteurella multocida 59A006	>100
P. haemolytica 59B018	>100
Mycoplasma bovis 93A001	0.78
Treponema hyodysenteriae 94A007	0.39

The crystal structure of the Cd-salt of CP-78,545 (**3**) is shown in Fig. 3. Only one-half of the complex is shown. Chelation to the cation is octahedrally through the carboxylate and the hydroxyls on C-11 and C-13 of the two molecules of CP-78,545, which was also the case for zincophorin[1]. The entire complex is C_2-symmetric about an axis passing through the Cd ion. Bond distances for C(16)-C(17), C(20)-C(21) and C(24)-C(25) were 1.359, 1.433 and 1.396 Å, respectively, which are consistent with the three assigned double bonds. Although the absolute stereochemistry of CP-78,545 remains to be clarified, it seems most reasonable to assume that it is the same as zincophorin because of their similar optical rotation values[1].

Biological Properties

CP-78,545 Mg-salt (**2**) exhibited *in vitro* antibiotic activity against certain Gram-positive bacteria such as *Bacillus*, *Staphylococcus* and *Streptococcus* sp., as well as *Mycoplasma bovis*, and the anaerobe *Treponema hyodysenteriae* (the causative agent of swine dysentery), but was devoid of Gram-negative activity. The MIC values are shown in Table 3. The antibiotic **2** is active *in vitro* against a coccidium *Eimeria tenella* in a tissue culture assay, but was inactive *in vivo* at levels of 100 and 200 mg/kg in feed versus *E. tenella* coccidial infections in chickens. A similar spectrum and potency were observed previously with zincophorin[1] and griseochelin[2].

Experimental

General Methods

MP's were determined on a Thomas-Hoover capillary apparatus and are uncorrected. Spectral data were recorded on the following instruments: NMR, Bruker WM-250 (250 MHz; equipped with an Aspect-3000 data system, using $CDCl_3$ solutions in a 5-mm dual $^{13}C/^1H$ probe); IR, Perkin-Elmer 1420; EI-MS, A.E.I. MS-30 (at an ionizing potential of 70 eV); FAB-MS, VG-70/250-S; and optical rotations, Perkin-Elmer 141.

CP-78,545 Free Acid (1)

To a solution of CP-78,545 Mg-salt (200 mg) in $CHCl_3$ (60 ml) was added 60 ml of an aqueous suspension of EDTA (30 mg). The mixture was allowed to stir for 20 minutes and the $CHCl_3$ layer was separated, washed with water (2×20 ml water) and evaporated. Based on IR analysis, some Mg-salt was still present, so the above procedure was repeated to afford 168 mg of **1** as a white solid. Physico-chemical properties are given in Table 1.

CP-78,545 Cd-Salt (3)

To a solution of CP-78,545 free acid (80 mg) in acetone (20 ml) was added an aqueous suspension

of CdO (162 mg) in 10 ml deionized water. The mixture was allowed to stir for 1 hour and filtered, and the filtrate was concentrated *in vacuo*. The filtrate was extracted twice with $CHCl_3$ and the combined $CHCl_3$ extracts were evaporated to afford 40 mg of **3** as a white solid. A representative crystal of **3** was obtained by slow evaporation from ether upon standing at ambient temperature for 4 weeks.

Single Crystal X-Ray Analysis of **3**

All data processing was done on a NMRVAX 11/750 (Digital Equipment Corp.) using the Enraf Nonius SDP-PLUS programs and MULTAN 80, a system of computer programs for the automatic solution of crystal structures from X-ray diffraction data: P. MAIN *et al.* The programs URANUS and SKK-PUB, programs to generate plot and tables, respectively, were written by S. K. KEARSLEY, Yale University, 1985.

Antimicrobial and Anticoccidial Assays

MICs were determined as described by DIRLAM *et al.*[5] except that all anaerobes were tested on Tryptose agar (Difco) supplemented with 5% bovine blood (TBA) and incubated 48 hours at 39°C in a Coy (Ann Arbor, Mich.) anaerobe chamber containing an N_2 - CO_2 - H_2 (80 : 10 : 10) atmosphere. MICs for aerobes were determined in an identical manner except that brain heart infusion agar (Difco) was used and plates were incubated aerobically at 37°C for 18~20 hours.

The evaluation of drug activity against *E. tenella* coccidial infections in chickens was conducted as described by CHAPPEL *et al.*[6]

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