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CP-60,993, a new dianemycin-like ionophore produced by *Streptomyces hygroscopicus* ATCC 39305: fermentation, isolation and characterization

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SUMMARY

CP-60,993, 19-epi-dianemycin, is a novel polycyclic ether antibiotic produced by *Streptomyces hygroscopicus* ATCC 39305. Fermentation recovery, purification and crystallization were achieved using standard procedures. CP-60,993 was characterized as a monocarboxylic acid. Elemental analysis suggested a molecular formula of $C_{47}H_{78}O_{14}$ for the free acid and $C_{47}H_{77}O_{14}$ Na for the sodium salt. Crystalline form CP-60,993 sodium salt shows the following properties: m.p. 193~205°C, $E_{1cm}^{1\%} = 157$ at 232 nm, $[\alpha]_D^{25°C} + 11.0$ (c 1, methanol). The structure, determined by MS, PMR and CMR, differs from dianemycin only in the stereochemistry at position 19. This was confirmed by X-ray crystallography carried out on the rubidium salt of CP-60,993. It exhibited activity in vitro against Gram-positive and anaerobic bacteria, efficacy against *Eimeria coccidia* in vivo in poultry, and stimulation in vitro of rumen propionic acid production.

INTRODUCTION

CP-60,993, shown in Fig. 1 [3], was isolated from the fermentation broth of a culture identified as *Streptomyces hygroscopicus* ATCC 39305. It was

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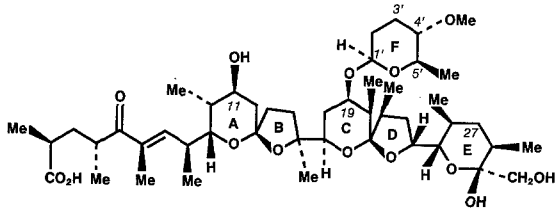


Fig. 1. The structure of CP-60,993, 19-epi-dianemycin.

purified by a series of chromatography columns using silica gel. The migration pattern on TLC and its vanillin color response, when compared with other members of the ionophore structural class, was used as an initial determination of novelty. CP-60,993 had a UV spectrum typical of the dianemycin [7] group. Spectral analysis by NMR indicated that it was a monoglycosylated ionophore similar to, but different from dianemycin. Comparisons of CP-60,993 with related members of the dianemycin group of antibiotics indicated that it was novel when it showed a different R_f on TLC and color response with vanillin reagent. The MS data from CP-60,993 revealed that its structure was isomeric with dianemycin and its novelty was assured when the NMR data proved that it differed from dianemycin structurally at position 19.

MATERIALS AND METHODS

The culture was isolated from a soil sample collected in Washington, DC. It was characterized as a strain of *Streptomyces hygroscopicus* (Jensen) Waksman and Henrici [3]. It has been deposited at the American Type Culture Collection under the accession umber ATCC 39305.

Table 1

Fermentation parameters for CP-60,993

Stage	Media		Conditions
1. Lyophile	Pridham's yeast extract agar		Maintained under refrigeration
2. Slant culture	PYEA	g/l	pH 7.3
	Yeast extract (Difco)	4	7 to 10 days old slant maintained
	Malt extract (Difco)	10	at room temperature
	Dextrose	4	
	Deionized water	905 ml	
	Agar	20	
	Coconut milk	50 ml	
3. Inoculum flasks	JDYTT		
	Cerelose	10	pH 7.1-7.2
	Corn starch	5	Fermentation run for 3 to 5 days
	NZ-amine YTT	5	28 ± 1°C, 150 cpm
	Corn steep liquor	5 ml	
	Cobalt choride	0.005	
	Calcium carbonate	3	
4. Jar fermentors	C′		
and	Cerelose	10	pH 6.9 to 7.1
inoculum tanks	Corn starch	10	1 to 5% inoculum
	Soy bean meal	10	Fermentation run for 4 to 6 days
	Distillers solubles	10	30 ± 1°C
	Calcium carbonate	1	1700 rpm (JAR)
	Sodium chloride	5	600 rpm (Tanks)
	Cobalt chloride	0.002	
	P-2000 antifoam	1 ml	
5. Production tanks	Same as above		Same as above

The culture was maintained (Table 1) on Pridham's yeast extract agar plants consisting of 10 g malt extract (Difco), 4 g yeast extract (Difco), 4 g dextrose, 50 ml of fresh coconut milk and made up to 1 liter with deionized water (pH 7.3). The inoculum was grown in a medium consisting of cerelose 0.1%, casein 0.5%, starch 0.5%, corn steep liquor 0.5%, calcium carbonate 0.3% and cobalt chloride 0.0002%. A 5% inoculum was used to seed the production run in the same medium or in the following one: cerelose 1%, corn starch 1%, soybean meal 1%, distillers solubles 1%, calcium carbonate 0.1%, sodium chloride 0.5%, cobalt chloride 0.0002% and P2000 antifoam (1 ml/l). The

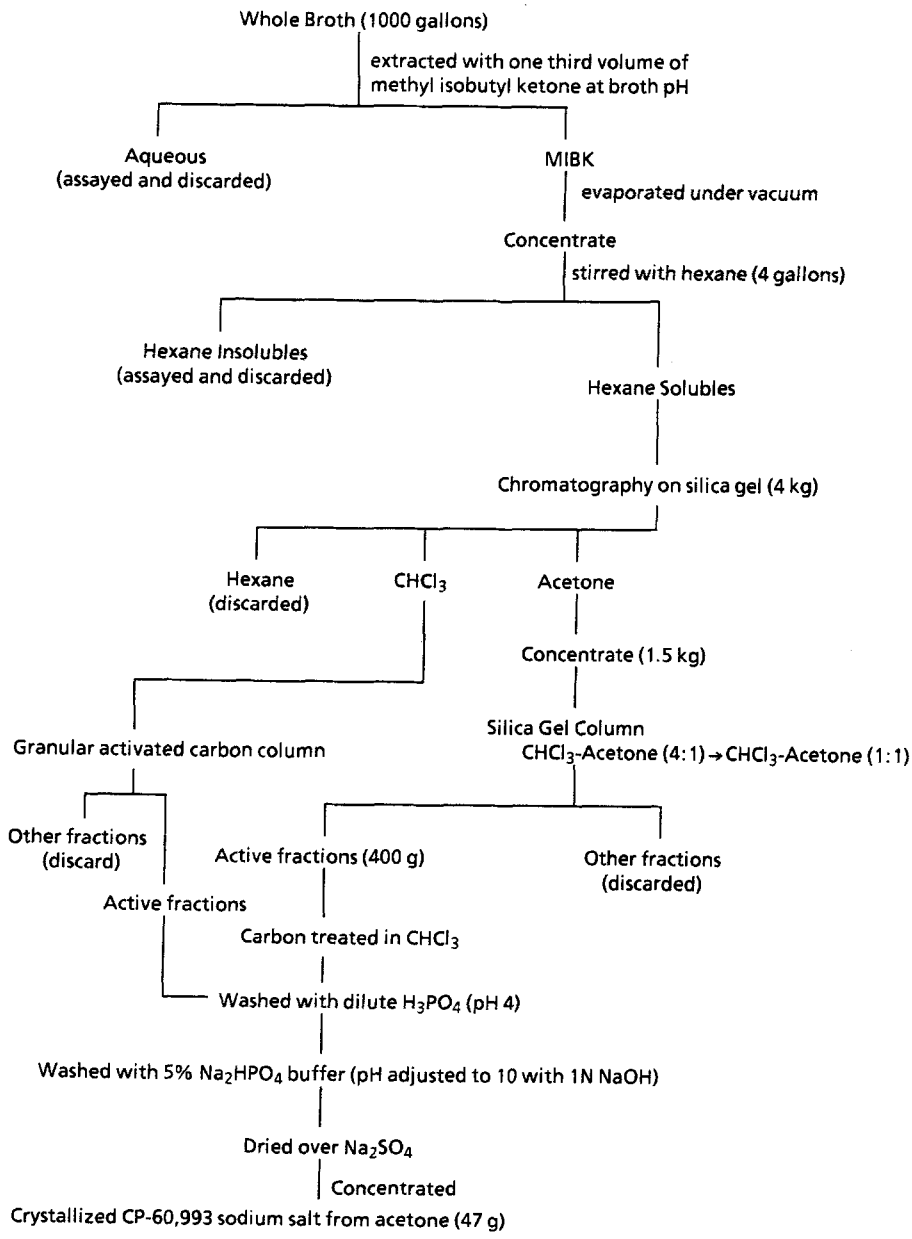
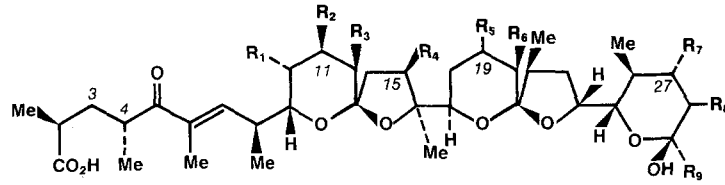
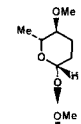
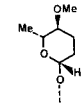
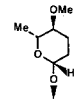
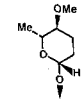
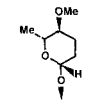


Fig. 2. Isolation and purification of CP-60,993.

fermentation was run at 30°C for 4 to 6 days. The antibiotic titers were followed by using a disc assay on a sensitive strain of *Bacillus subtilis* ATCC 6633. Antibiotic titers could also be determined by extracting the whole broth with an immiscible solvent spotting the solvent extract on a TLC plate and run in a system such as neat ethyl acetate. CP-60,993 could be visualized by UV at 254 nm, or by spraying the developed plate with vanillin (3 g dissolved in 75 ml of ethanol to which 25 ml of 85% phosphoric acid is added) and heating at 80°C. CP-60,993 appears as a purple spot.

The isolation (Fig. 2) of the antibiotic was accomplished as follows: 1000 gallons of the whole broth was extracted with 1/3 volume of methyl isobutyl ketone, the solvent concentrated and the residue triturated with hexane. The hexane concentrate was chromatographed on silica gel in hexane followed by elution with chloroform and acetone. The active cuts in the chloroform fractions were treated with carbon. The acetone cuts were concentrated and rechromatographed on a second silica gel column. The column was developed with a chloroform/acetone gradient. Eluates were collected and assayed, the active cuts were combined, treated with carbon, filtered and mixed with the chloroform filtrate from this first column. After washing with dilute phosphoric acid, the chloroform was washed with 5% sodium phosphate at pH 10, separated and dried over anhydrous sodium sulfate. The solvent



Compound	R_f TLC System A	B	C	Vanillin Reagent Color	Dianemycin-related Ionophores R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	Ref.
CP-60,993	.35	.46	.58	Purple	CH_3	OH	H	H		CH_3	H	◄CH_3	CH_2OH	2
Dianemycin	.25	.36	.56	Purple	CH_3	OH	H	H		CH_3	H	◄CH_3	CH_2OH	6
CP-53,607	.35	.40	.61	Red	CH_3	OH	H	H	H	CH_3	H	◄CH_3	CH_3OH	3
Lenoremycin	.35	.50	.56	Orange	H		CH_3	H	H	CH_3	H	◄CH_3	CH_2OH	1
Endusamycin	.40	.44	.63	Green	CH_3	OH	H		H	CH_3	H	◄CH_3	CH_2OH	9
CP-47,224	.30	.38	.61	Green	CH_3	OH	H		H	CH_3	OCH_3	◄CH_3	CH_2OH	4

TLC Systems A: EtOAc
B: $CHCl_3$-CH_3COCH_3 1:1
C: $CHCl_3$-MeOH 9:1

Fig. 3. Comparative R_fs and vanillin color response of selected dianemycin related ionophores.

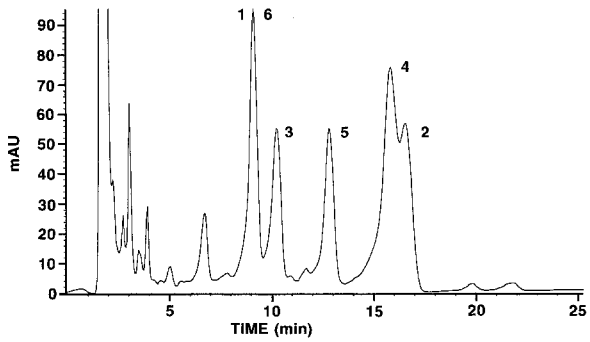


Fig. 4. HPLC comparison of selected dianemycin-related ionophores. Chemosorb 5-ODS-H (4.5 × 150 mm); System: MeOH:0.1 M HCO_2NH_4 = 90:10; Flow rate: 1 ml/min; Oven temp: 40°C; Diode-array Detector: UV 232 mm.

was concentrated, and the CP-60,993 crystallized from acetone as the sodium salt (47 g).

Once the ionophore CP-60,993 had been isolated and purified, its structure and novelty were assessed. Because of the similarity in UV spectra, the antibiotic was run on TLC plates against a number of dianemycin-related standards to determine its relative Rf and color formation with vanillin reagent. This can be done with crude or purified extracts. When crystalline material became available, an HPLC system was developed that separated CP-60,993 from most members of its class, as shown in Fig. 3. TLC and HPLC were complementary in that separations in one system were enhanced or confirmed by the other. The HPLC was run on a Chemcosorb 5 ODS-H column (4.5 × 150 mm) using a MeOH/0. 1 M HCO_2NH_4 (90:10) system (Fig. 4). Detection was done by UV at 232 nm. Following the TLC and HPLC work, the novelty of CP-60,993 suggested by TLC and HPLC was confirmed by NMR spectral analysis and an X-ray structure determination on the rubidium salt. CP-60,993 was thus characterized as a monocarboxylic acid. Elemental analysis established a molecular formula of $C_{47}H_{78}O_{14}$ for the free acid and $C_{47}H_{77}O_{14}Na$ for the sodium salt. Some physicochemical properties are listed in Table 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

Table 2

Physico-chemical data for CP-60,993 sodium salt and free acid

Property		Na salt				Free acid		
MP (°C)		193–205				85–95		
$[a]_D^{25\approx}$ (c 1.0, MeOH)		+11.0°				+15.6°		
UV λ_{MAX}^{MEOH} nm (log E)		232 (4.15)				232 (4.17)		
MW		889.12				867.13		
Empirical formula		$C_{47}H_{77}O_{14}Na$				$C_{47}H_{78}O_{14}$		
		C	H	O	Na	C	H	O
Elemental analysis	Calc:	63.49	8.73	25.20	2.58	65.10	9.07	25.83
	Found:	63.10	8.86	25.46	2.58	65.45	8.94	26.61
Color reaction[a]		Purple				Purple		
Solubility	Soluble:	hexane, $CHCl_3$ methanol acetone,				hexane, $CHCl_3$, acetone, methanol		
	Insoluble:	H_2O				H_2O		

[a] 3% vanillin in 3:1 EtOH 85% H_3PO_4, heat at 80°C.

Bruker WM-500 (500 MHz; equipped with an Aspect 3000 data system, using $CDCl_3$ solutions in 5 mm dual $^{13}C^1H$ probe); EI-MS, A.E.I. MS-30 (at an ionizing potential of 70 eV); optical rotations, Perkin-Elmer 141 and an HP-1090 liquid chromatograph.

Table 3

^{13}C chemical shifts of CP-60,993 and dianemycin in $CDCl_3$

Carbon Function-	CP-60,993 ality	Dianemycin Na salt[a]	Na salt[b]
1	COO	184.00	183.8
2	CH	40.07(2.50)	40.2
3	CH_2	41.43(1.03, 1.77)	41.5
4	CH	37.52(3.47)	37.5
5	C = O	206.41	206.2
6	C =	133.77	133.6
7	CH =	144.85(6.72)	144.9
8	CH	36.05(2.67)[c]	37.8
9	CHO	69.71(4.65)	69.6
10	CH	36.16(1.86)	35.9
11	CHO	70.28(3.88)	70.4
12	CH_2	34.01(1.68,1.93)	34.0
13	OCO	106.94	106.9
14	CH_2	39.59(1.81, 2.06)	39.7
15	CH_2	32.37(1.84, 2.02)	32.2
16	CO	86.08	86.6
17	CHO	76.78(3.25)	75.7
18	CH_2	25.24(1.55)	25.4
19	CHO	73.98(3.79)	79.2
20	CH	37.46(2.39)	34.6
21	OCO	110.90	109.8
22	CH	35.59(2.47)	35.9
23	CH_2	29.69(1.44, 2.33)	29.9
24	CHO	78.44(4.40)	77.9
25	CHO	72.86(3.90)	73.2
26	CH	32.96(1.32)	32.9
27	CH_2	36.46(1.37, 1.51)	36.5
28	CH	35.94(1.50)	35.9
29	OCOH	98.63	98.5
30	CH_2OH	65.25(3.29, 4.65)	65.3
2-Me		19.53(1.04)	19.5
4-Me		14.50(1.10)	16.9[d]
6-Me		11.22(1.79)	11.2
8-Me		17.00(1.09)	14.4[d]
10-Me		10.05(0.77)	10.0
16-Me		25.03(1.48)	26.6
20-Me		7.04(1.01)[e]	13.1
22-Me		14.65(1.03)	16.1
26-Me		17.55(0.87)	17.7
28-Me		16.68(0.92)	16.7
	Deoxy sugar at C-19:		
1′	OCHO	100.92(4.52)	102.4
2′	CH_2	30.46(1.56, 1.89)	30.6
3′	CH_2	26.93(1.33, 2.24)	27.0
4′	CHO	79.95(2.86)	79.9
5′	CHO	74.66(3.32)	74.5
4′	OMe−	56.92(3.38)	56.7
5′-Mw	−	18.30(1.26)	18.5

[a] Values in parenthesis are ^{13}C (attached 'H) shifts in ppm from TMS in CDC_3 solution, measured at 126 MHz (500 MHz).

[b] K. Mizoue et al., [9].

[c] These values are in good agreement with those found for a related structure, endusamycin Na salt: δC-13 35.9 and δH-1 2.64 [10].

[d] These values were likely permuted in the assignments for dianemycin [9].

[e] Differences observed (i.e. 6 ppm) is due to the relationship of the 20-Me and the oxygen of the sugar at C-19.

Preparation of free acid of CP-60,993

The CP-60,993 sodium salt (150 mg) was dissolved in 100 ml of diethyl ether. The dissolved sample was combined with 100 ml of pH 2 HCl solution. The mixture was shaken vigorously for approximatley 5 min. The organic phase was separated and extracted once with deionized distilled H_2O and then evaporated to a white foam. IR indicated that the free acid had been formed.

Preparation of Rb salt of CP-60,993

The free acid was dissolved in 100 ml of $CHCl_3$. $RbCO_3$ (150 mg in 100 ml distilled, deionized H_2O) was added to the $CHCl_3$ and the mixture was shaken vigorously for 5 min. The organic phase was separated and extracted once with deionized, distilled H_2O and then evaporated to a white solid. The Rb salt was recrystallized using $CHCl_3$, EtOAc and hexane, heating the solution slightly. The solution was left to evaporate slowly producing suitable crystals for X-ray.

Table 4

Mass fragmentation pattern of CP-60,993

Fragment	Mass	Lenoremycin	Dianemycin	CP-53,607	CP-60,993
RAB	379	377	377	377	377
RABCD	547	545	543	–	543
ABCDE	495	493	491	493	491
ABCDE-H_2O	477	475	–	475	473
CDE	327	–	325	–	325
CDE-H_2O	309	309	307	309	307

Single crystal X-ray analysis of CP-60,993 Rb salt

A representative crystal was surveyed and a 1 Å data set (maximum sin $\beta/\lambda = 0.5$) was collected on a Nicolet R3m/μ diffractometer. Atomic scattering factors were taken from the International Tables for X-ray Crystallography. All crystallographic calculations were facilitated by the SHELXTL system. The diffractometer data were collected at room temperature.

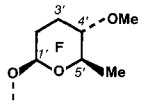
DISCUSSION AND RESULTS

FAB mass spectral data imply that dianemycin and CP-60,993 are isomers. The ^{13}C and ^{1}HMR data show that both have the same structural units (Table 3). Characteristic mass fragments (Table 4), show the same distribution of mass over AB, DC and E ring systems (excluding the $-OR$ substituents on rings A and C, which are cleaved as

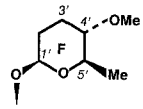
Table 5

Antimicrobial spectrum of CP-60,993 sodium salt

Test organism	Code #	MIC μg/ml
Staphylococcus aureus	01A005	3.125
S. aureus	01A110	3.125
S. aureus	01A539	3.125
S. aureus	01A543	3.125
S. epidermidis	01B111	3.12
Streptococcus pyogenes	02C203	$\leq$0.025
Erysipelothrix rhusiopathiae	04A005	0.39
Lactobacillus casei	09B001	$\leq$0.10
L. catenaforme	09C001	$\leq$0.10
Corynebacterium pyogenes	11D001	1.56
Haemophilus parahaemolyticus	54B002	25
Pasteurella multocida	59A013	$\geq$100.0
P. haemolytica	59B018	$\geq$100.0
Bordetella bronchiseptica	73A006	$\geq$100.0
Bacteroides vulgatus	78E032	25
Fusobacterium plauti	84G001	$\leq$0.10
F. necrophorum	84C004	25
Moraxella bovis	93A001	0.78
Treponema hyodysenteriae	94A001	0.10
T. hyodysenteriae	94A002	0.38



Dianemycin
I



CP-60,993
II

Fig. 5. 'F' ring of CP-60,993 and dianemycin.

ROH), sugar and side chain. The most principal (70 e.v.) EI mass fragments in the $300 < m/z < 440$ range are accountable as simple cleavages of a hypothetical system:

R	AB	CD	E
211	168	168	159

with the premise that if either the AB or CD ring systems contain an OR substituent, it is lost as ROH with a resulting mass two units lower. These data provide a linearly independent set of conditions from which the separate masses of R, AB, CD and E can be determined, if the AB and CD rings both contain nonidentical $-OR$ substituents, their position is undetermined.

Two permutations occur in known members of the dianemycin class. One involves the position of the methyl group in the A-ring, which was determined to be at the 10-position by tracking (COSY) the sequence of coupled protons form H7 to H11. The second more difficult question is the position of the sugar, which gives a m/z 129 ion (absent in CP-53,607, Table 5). Since there is only one secondary alcohol function, this was accomplished indirectly by establishing (heteronuclear correlation) that the methine carbon showing a geminal $-O^2H$ isotope shift is bonded to H11, assigned in the sequence above.

Our construction thus converges on the dianemycin structure, and the differences in the NMR spectra all focus on the C-ring. The sequence of structure units in this ring is unaltered (COSY), but the

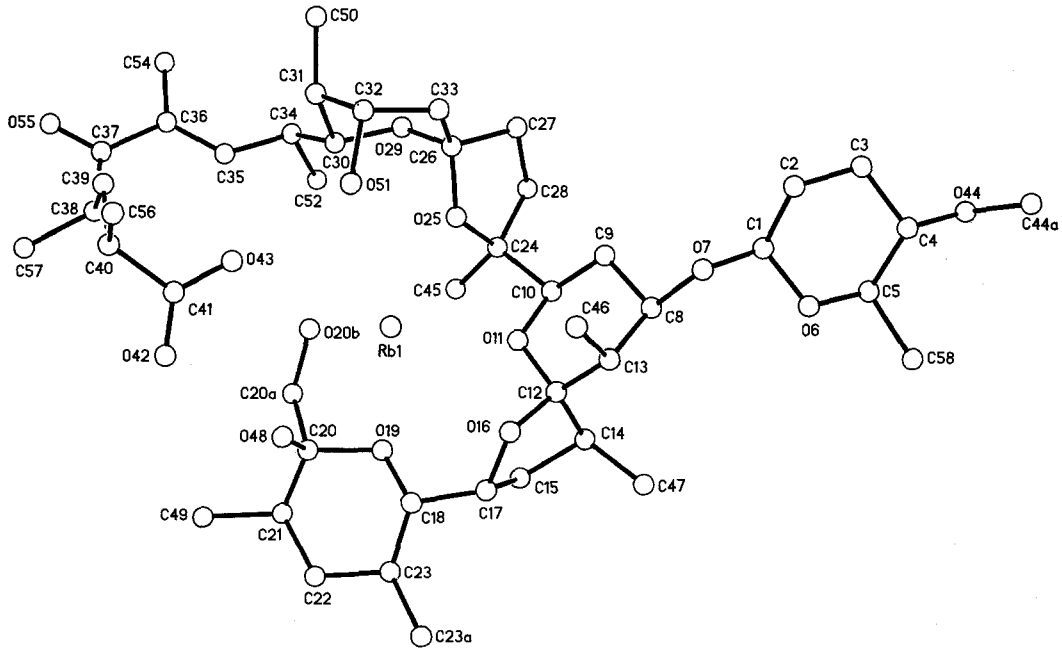


Fig. 6. Stereoscopic drawing of the absolute configuration of the CP-60,993 Rb salt.

Table 6

The in vitro stimulation of rumen propionic acid production by the sodium salt of CP-60,993

Compound	Dosage (μg/g)	% Rumen propionic acid (untreated control = 100%)	% Volatile fatty acids
CP-60,993	8	146	99
	4	144	98
	2	141	99
	1	136	103
Monensin	10	150	95

Criteria for evaluation, Kellogg [8].

coupling constants differ, indicating the presence of stereoisomers. The C-ring in dianemycin has the arrangement shown in Fig. 1. In the Na salt of CP-60,993 in benzene-d_6, both H19 and H17 are coupled to the same H18 proton by an amount ($^3J_{H,H}$–11H_z) indicative of a trans-diaxial array. The necessary condition for this is a configurational inversion at either the 17 or 19 position; in the former event, there would also have to be a conformational change to the opposite chair. Since the H19-H20 coupling remains small ($<5H_z$), a conformation change would also necessitate inversion at C20; hence, the alternatives are either inversion at C19 with no conformational change, or inversions at C17 and C20 with a conformational change. Since the coupling constants tell us nothing about the configuration at C21, there are a total of four acceptable permutations of configuration in the C-ring. Structure II in Fig. 5 involves the minimum change, indicating the compound to be the 19-epimer of dianemycin. X-ray crystallographic analysis of the corresponding rubidium salt revealed the complete structure and the absolute configuration of 19-epi-dianemycin as indicated in Fig. 6.

BIOLOGICAL DATA

Like dianemycin, CP-60,993 is a moderately active Gram-positive antibiotic with little or no Gram-negative activity. It showed good in vitro activity against *Treponema hyodysenteriae*, the causative agent of swine dysentery (Table 5). It also induced a change in the proportions of volatile fatty

Table 7

Efficiency data for CP-60,993 sodium salt against *Eimeria* infections in chickens[a]

Drug	Species	Dose (μg/g of feed)	Lesion control[a] (%)	Weight gain (%)
CP-60,993	*Eimeria tenella*[b]	100	95	9.5
		75	56.5	46.5
		50	73.3	42.3
		25	58.7	50.7
	Eimeria acervulina[c]	100	80	33
		75	90	35
		50	90.5	55.5
		25	76.5	48.5
Monensin	*Eimeria tenella*	100	77	94
		50	53	40
	Eimeria cervulina	100	87	81
		50	40	45

[a] The criteria for evaluation, see Chappel et al. [6].
[b] Average of three assays.
[c] Average of two assays.

acids (acetate, butyrate and propionate) produced in rumen fluid by increasing the molar proportion of propionate (Table 6). This change in RPA can be used as a predictor of an ionophores potential as an enhancer of swine and cattle growth promotion.

The CP-60,993 was quite active against *Eimeria coccidia* (Table 7) when administered from 25 to 100 μg/g of feed, but the weight gains were poor. It had an LD_{50} ca 15 mg/kg when administered to mice by oral gavage.

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