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CP-61,405, a novel polycyclic pyrrolether antibiotic produced by *Streptomyces routienii* Huang sp. nov.

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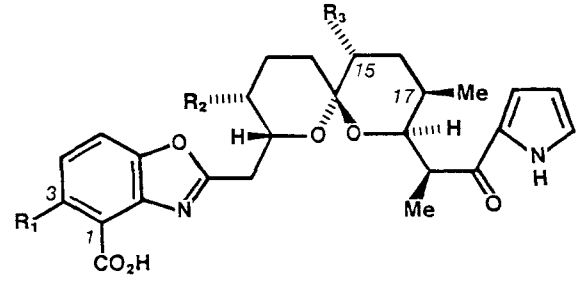
SUMMARY

CP-61,405 ($C_{26}H_{29}N_2O_7Na$) is a novel polycyclic pyrrolether antibiotic produced by a new species, *Streptomyces routienii* Huang sp. nov. (ATCC 39446). Recovery, fractionation and purification were achieved using standard procedures. The crystalline form includes the CP-61,405 sodium salt, m.p. 334–335°C, $[\alpha]_D^{25°C}$ + 315° (c = 1, chloroform). The structure is shown below. CP-61,405 was co-produced with the polycyclic ether antibiotics salinomycin and epi-17-deoxy-(0–8)-salinomycin. It exhibited activity in vitro against gram-positive and anaerobic bacteria, efficacy against poultry coccidia and stimulation in vitro of propionic acid production.

INTRODUCTION

In our search for novel anticoccidial agents, a lyophilized culture broth was found to be very active versus *Eimeria* coccidia in vivo and a stimulator of rumen propionic acid in vitro. Fractionations of the broth revealed the presence of at least three antibiotics, salinomycin [6], epi-17-deoxy-(0–8)-salinomycin (SY-2) [10] and a novel pyrrolether antibiotic CP-61,405 [6]. The former antibiotics are clearly related structurally, while the elaboration of the latter ionophore in the fermentation was un-



		R_1	R_2	R_3
1	CP-61,405	OH	H	H
2	X-14885A	OH	Me	H
3	AC7230	OH	Me	Me
4	A-23187	NHMe	Me	Me
5	Cezomycin	H	Me	Me

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Table 1

Cultural characteristics of *Streptomyces routienii* ATCC 39446

Agar medium	Amount of growth; Texture of colony	Color of colony surface; Color of aerial mycelium[a]	Color of colony reverse; Soluble pigment[a]
Yeast extract-malt extract (ISP-2)[b]	Good; raised, wrinkled	White, pale yellow, tan to brown (2 ba, 3 gc, 3 ie); white to pale yellow (2 ba)	Brown (3 gc, 4 ie); yellowish brown (3 lc)
Oatmeal (ISP-3)	Moderate to good; slightly raised, smooth, or as isolated colonies	White, cream to pale yellow (1.5 ca, 2 ba); white to pale yellow (2 ba)	Cream (1.5 ca); pale yellowish (1.5 ca)
Inorganic salts-starch (ISP-4)	Moderate; raised, roughened to wrinkled	White to cream (2 ca); white to pale yellow (2 ba)	Brown (4 ie); grayish yellow (2 gc)
Glycerol-asparagine (ISP-5)	Poor to moderate; slightly raised, smooth, or as isolated colonies	Pale yellowish (2 ca, 2 ea); white to pale yellow (2 ba)	Same as surface; pale yellowish (1.5 ca)
Gordon-Smith tyrosine	Moderate to good; slightly raised, smooth to slightly granular, or as isolated colonies	White to pale yellow (2 ba); white to pale yellow	Pale yellowish (2 ea); dark yellow (2 lc)
Czapek-sucrose	Good; raised, wrinkled	Yellowish to tan (2 ea, 3 gc, 3 ie); none	Yellowish brown (3 ic); yellowish brown (3 lc)
Glucose-asparagine	Moderate to good; raised, wrinkled to smooth, or as isolated colonies	White, cream to dark yellowish (2 ca, 2 lc); white	Dark yellowish (2 lc); greenish yellow (1 ga)
Calcium-malate	Moderate; slightly to moderately raised, smooth to granular, or as isolated colonies	White, cream to pale yellow (2 ba); white to pale yellow	Pale yellowish (2 ca, 2 ea); cream (1.5 ca)
Casein	Moderate to good; slightly raised to raised, smooth to wrinkled, or as isolated colonies	Pale yellow to tan (2 ea, 3 gc); none	Yellowish green (1.5 ia, 2 ia); yellowish brown (3 lc)
Bennett	Good; raised, wrinkled	White, pale yellow, tan to brown (2 ba, 4 gc, 3 ie); white to pale yellow	Brown (3 ic, 3 lc); yellowish (2 ia)

[a] The color scheme used was Color Harmony Manual, 4th Ed., 1958, Container Corporation of America, Chicago, IL, U.S.A.

[b] Cultural characteristic studies on various media according to Waksman [9] and Shirling and Gottlieb [8].

anticipated. This find is an example of one of the many reasons that the isolation of metabolites from *Actinomycete* fermentation is challenging and often rewarding.

Salinomycin was readily identified by its migration pattern in TLC and its color response with vanillin reagent. The epi-17-deoxy-(0–8)-salinomycin was identified based on its NMR, TLC pattern and a comparison with an authentic sample kindly supplied to us by Kaken Pharmaceutical Co. of Japan. The identity of CP-61,405 proved more elusive because of the low broth titers and the need for repetitive chromatography to separate it from other metabolites. Once crystalline material became available, spectral analysis proved helpful in categorizing the antibiotic and placing it in the pyrrolether group of antibiotics. It has been compared in house with authentic samples of X-14885A (2) [11] and A-23187 (4) [1] and with the literature reference data supplied for AC7230 (3) [12], the newest member of the series, and cezomycin (5) [3]. Though all are structurally related, CP-61,405 is different and is a novel member of the pyrrolether family of antibiotics.

MATERIALS AND METHODS

The microorganism used to produce CP-61,405 was isolated from a soil sample found in Dazaifu, Fukuoka Prefecture, Japan. It is characterized (Tables 1 and 2) by a pale yellow aerial mycelium, a negative melanin reaction, smooth to warty spores that are arranged in a straight to flexuous chain, and the presence of LL-diaminopimelic acid, galactose and mannose in the whole cell hydrolysate [8,9]. The spores were globose, oval to elliptical, 0.8–1.0 μm in diameter or 1.0–1.4 $\times$ 0.7–1.0 μm, smooth or warty, as revealed by scanning electron microscopy (Fig. 1).

The culture does not utilize arabinose, inositol rhamnose, or xylose as a carbon source. Glucose, fructose, mannitol, raffinose and sucrose support growth. These characteristics distinguish it from all known species of *Streptomyces* of the yellow series. Based on a combination of the physiological, biochemical, morphological and cultural properties, the culture is considered a new species of *Streptomyces* and is named *Streptomyces routienii* Huang sp. nov. The culture has been deposited with the

Table 2

Biochemical properties of *Streptomyces routienii*[a] ATCC 39446

Melanin production	–	Utilization of:	
H_2S production	–	glucose	+
Gelatin liquefaction	+	arabinose	–
Starch hydrolysis	+	fructose	+
Nitrate reduction			
organic nitrate	–	inositol	–
dextrose nitrate	+	mannitol	+
Decomposition of cellulose	–		
Milk			
coagulation	+	raffinose	+
peptonization	–		
Casein digestion	+	rhamnose	–
Tyrosine digestion	±	sucrose	+
Digestion of calcium malate	+	xylose	–

[a] For methodology of the biochemical tests, see Huang [4].

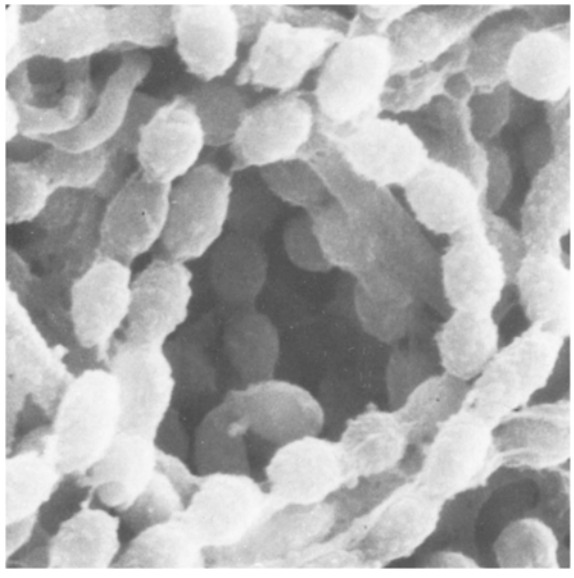

Fig. 1. Scanning electron microscopy of spore chains of 14-day-old *Streptomyces routienii* ATCC 39446; ×15000.

American Type Culture Collection under the accession number ATCC 39446.

The culture was maintained on PYEA agar 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, then adjusted to pH 7.3 with 1 N NaOH. 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 a production run in the following medium: soy flour 0.75%, corn starch 1%, distillers solubles 0.5%, calcium chloride 0.025%, ferrous sulfate 0.0005%, magnesium sulfate crystals 0.005%, sodium citrate 0.2% and cobalt chloride 0.0005%. The fermentation was run at 30°C for 120–168 h (Fig. 2). The antibiotic titers were followed by using a disc assay on a sensitive strain of *Staphylococcus aureus* ATCC 6538, or of *Bacillus subtilis* ATCC 6633. The composition and relative proportions of the ionophores produced during the fermentation could be gauged by extracting the whole broth into chloroform, separating the solvent and concentrating it. The residue was reconstituted in 200 μl of fresh solvent, spotted on a silica gel TLC plate and developed in ethyl acetate. CP-61,405 could be visualized by observing at 366 μm (black light), which showed the antibiotic as a deep blue fluorescent spot against a grey background, or by spraying with 3% vanillin in 75 ml EtOH and 25 ml of 85% phosphoric acid and heating to 80°C. Under these conditions the CP-61,405 turned light pink, the SY-2 deep yellow and salinomycin a dark green color. The plate can also be overlaid with *Staphylococcus aureus* in agar containing tetrazolium. The antibiotics, after incubating at 37°C overnight, are visualized as white spots against a red background.

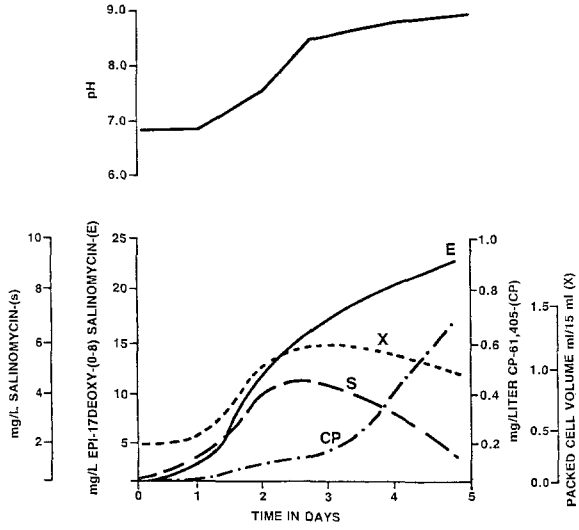

Fig. 2. Growth curve of CP-61,405 in jar fermenters.

The antibiotics were isolated (Fig. 3) from 200 gallons of whole broth by extraction at natural pH into methylisobutyl ketone. After concentrating, the residue was chromatographed on silica gel using a heptane to chloroform to chloroform/acetone (5:1) gradient. The active cuts were concentrated, treated with Darco G-60 carbon and rechromatographed on silica gel using a heptane to neat ethyl acetate gradient. Activity was followed by TLC and the cuts combined accordingly. After concentration, the SY-2 was obtained by precipitation from the residue with heptane. The filtrate was concentrated and rechromatographed repeatedly in chloroform to chloroform/acetone 3:1 to separate the salinomycin from the CP-61,405. Rechromatographing the CP-61,405 cuts in a heptane/ethyl

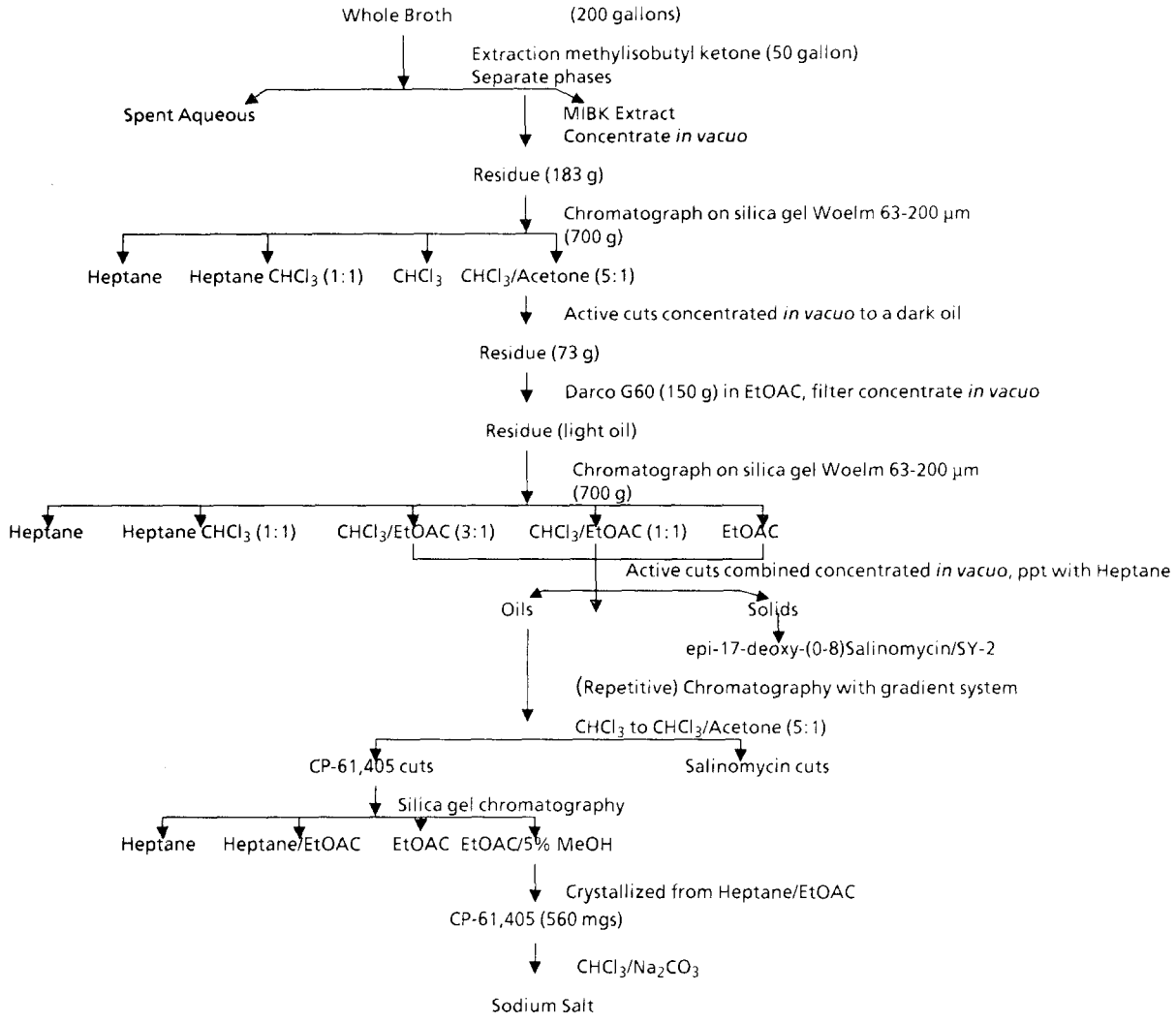

Fig. 3. Isolation and purification of CP-61,405.

acetate to 5% methanol gradient in ethyl acetate gave a fraction that yielded crystalline CP-61,405.

DISCUSSION

The spectroscopic properties of CP-61,405 such as UV (Fig. 4), IR (Fig. 5) and ^{1}H-NMR spectra (Fig. 6) were very similar to those of A-23187 and X-14885A. The mass spectrum (Fig. 7) (free acid: MH^+ 482) was different from that for A-23187 (free acid: MH^+ 523) or for X-14885A (free acid: MH^+ 496).

The analytical data plus 26 carbon-13 lines led to the estimated composition:

	A_i	P_i	N_i
C	12.011	62.25%	26
H	1.008	5.80	28.87
N	14.007	5.69	2.04
O	15.999	(26.26)	(8.23)

We observed 27 protons and three exchangeable ones. A 2-carboxypyrrole fragment and a 1,2,3,4-tetra-substituted benzene ring was also apparent.

Table 3

Properties of CP-61,405, sodium salt

Property	CP-61,405 Na^+				
Melting point (°C)	334–335				
$[\alpha]_D^{25°C}$ (c = 1, chloroform)	+315°				
Ultraviolet (MeOH)	$A_{1cm}^{1\%}$ 287 (258 μm); 400 (308 μm)				
Infra-red (KBr)	2.9, 3.2, 3.5, 6.1, 6.2, 6.4, 6.65, 7.3, 7.45, 8.15, 9.15 and 12.5 μm				
Molecular weight	504.5				
Empirical formula	$C_{26}H_{29}N_2O_7Na$				
Elemental analysis		C	H	N	O	Na
	calcd.	61.77	5.98	5.54	21.65	4.77
	found	62.25	5.80	5.69	21.66	4.60
Color reaction	vanillin/H_3PO_4/heat to 80°C: pink					
Solubility						
soluble	hexane, chloroform, ethyl acetate, methanol, acetone					
insoluble	H_2O					

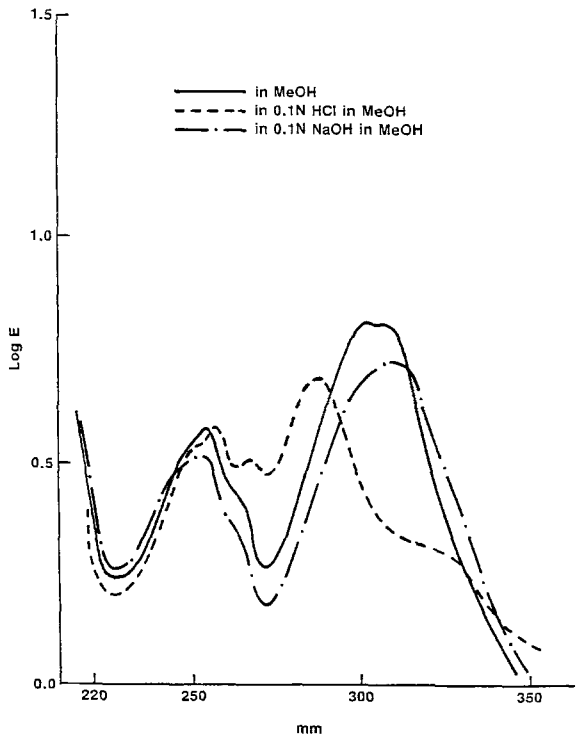

Fig. 4. UV spectra of CP-61,405.

With this information in hand, the A-23187 was used as a model to see how the data fitted.

Electron impact mass spectrometry of CP-61,405 (Fig. 8) gave a number of peaks, including an M^+ at m/z 482 ($C_{26}H_{30}N_2O_7$) and m/z 44 (CO_2), not shown, due to cleavage at (a). Subsequent cleavage at (b) results in a peak at m/z 149 ($C_8H_7NO_2$). Fragmentation at (c) gives rise to a peak at m/z 123 (C_7H_9NO) (see peak at 124). Evidence for the presence of the pyrrole moiety is provided by the base peak at m/z 94 (C_5H_4NO) due to cleavage at (d) and a peak at m/z 66 (C_4H_4N) from fragmentation at (e).

Based on ^{1}H-NMR, the exchangeable (e_2) proton at 14.43 ppm can be established as a phenolic hydroxyl at position 3 (X = OH), and the lone methyl group tracked to position 17 by spin decoupling, completing the structure.

CP-61,405 as indicated in Table 4, is a potent, gram-positive antibiotic. It had no gram-negative or antifungal activity at the levels tested and did not protect mice against *Staphylococcus aureus* when administered by the oral or subcutaneous route.

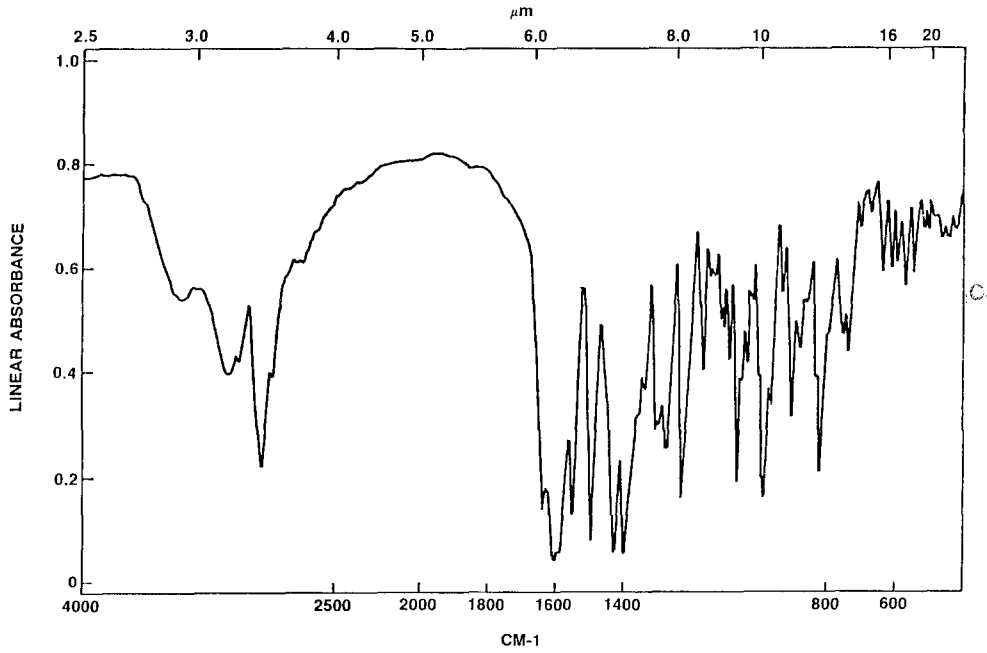

Fig. 5. IR spectra (KBr) of CP-61,405.

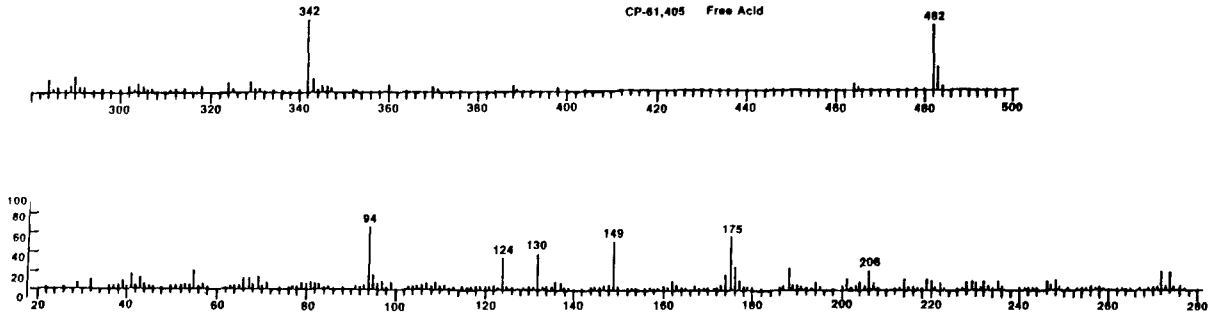

Fig. 6. MS spectra of CP-61,405.

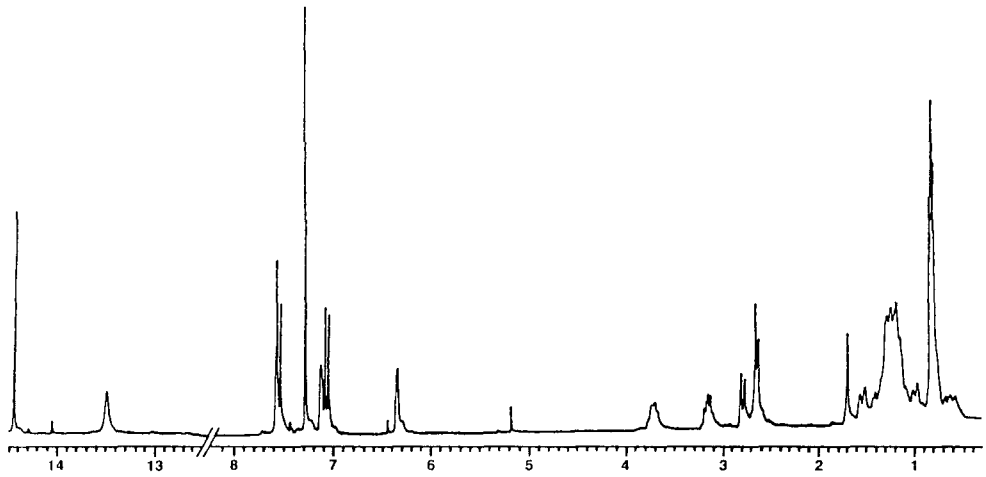

Fig. 7. ^{1}H-NMR spectrum of CP-61,405 at 250 MHz in $CDCl_3$.

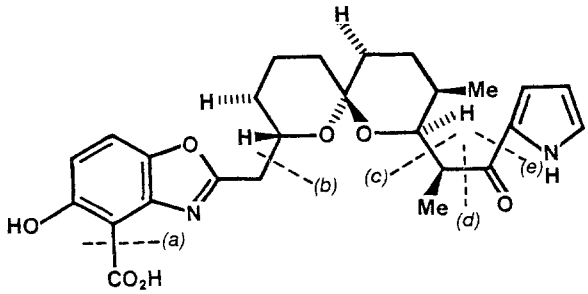

Fig. 8. Mass spectral fragmentation of CP-61,405 free acid.

The pyrrolether antibiotic was weakly active against *Eimeria tenella* coccidia when administered in feed above 150 ppm (Table 5). It also induced a change in the proportion of volatile fatty acids (acetate, propionate and butyrate) produced in the rumen by increasing the molar proportion of propionate in the rumen fluids (Table 6).

A-23187 has found wide use in many pharmacological assays [7], and as potential pharmacological agent in its own right. CP-61,405 has been shown to have similar properties and can be substituted for A-23187 in many assay systems (T.J. Carty, Pfizer Inc., personal communication).

Table 4

Antimicrobial spectrum of CP-61,405

Test organisms		CP-61,405 MIC (μg/ml)
Staphylococcus aureus	01A005	0.39
S. aureus	01A052	0.78
S. aureus	01A160	0.78
S. epidermidis	01B087	0.39
S. epidermidis	01B111	0.39
Streptococcus faecalis	02A006	1.56
S. pyogenes	02C040	0.78
Micrococcus luteus	07A001	0.78
Bacillus subtilis	06A001	0.20
Escherichia coli	51A125	>50
E. coli	51A266	>50
Proteus morganii	57G001	>50
Klebsiella pneumoniae	53A009	>50
Pasteurella multocida	59A001	0.78
Bacillus fragilis	78C004	0.78
B. vulgatus	78E025	200
Fusobacterium necrophorum	84C004	200
F. necrogenes	84B001	1.56
Tetrahymena hyodysenteriae	94A001	3.12
T. hyodysenteriae	94A002	6.25

Table 5

Efficacy data for CP-61,405, Sy-2 and salinomycin against coccidial infections in chickens

Species	Drug	(μg/g)	% weight gain	% control lesions[a]
Eimeria tenella	CP-61,405	150	42	69
		100	51	10
		75	4	1
	Sy-2	100	63	5
		50	26	5
		25	9	−18
	Salinomycin	60	85	100
		30	95	65
	infected controls	none	0	0

[a] For the evaluation criteria see Chappel et al. [2].

Table 6

In vitro rumen propionic acid activity (RPA) of Sy-2, CP-61,405 and salinomycin

Compound	Dosage (μg/ml)	RPA (%)[a]
Sy-2	20	215
	10	194
	5	184
	2.5	162
CP-61,405	20	158
	10	145
	5	128
	2.5	123
Salinomycin	10.0	188

[a] Scoring based on the method of D.W. Kellog (5).

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