CC-1065 (NSC 298223), A POTENT NEW ANTITUMOR AGENT

IMPROVED PRODUCTION AND ISOLATION, CHARACTERIZATION AND ANTITUMOR ACTIVITY

DAVID G. MARTIN,* CAROLYN BILES, SHIRLEY A. GERPHEIDE, LADISLAV J. HANKA, WILLIAM C. KRUEGER, J. PATRICK MCGOVREN, STEPHEN A. MIZSAK, GARY L. NEIL, JULIANNA C. STEWART and JERONIMO VISSER

Research Laboratories, The Upjohn Company, Kalamazoo, Michigan 49001, U.S.A.

(Received for publication June 15, 1981)

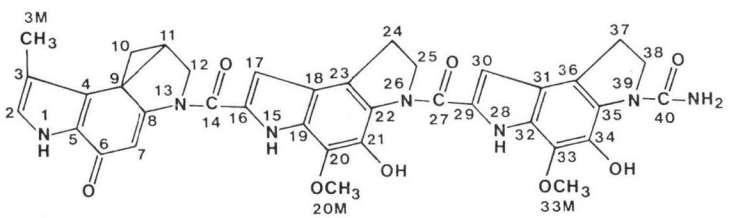
CC-1065 (NSC 298223) is a potent new antitumor antibiotic with a unique structure produced by *Streptomyces zelensis*. Improved production, isolation, and assay methods are described along with physico-chemical properties and antitumor activity.

Screening for soil cultures producing agents displaying both cytotoxic activity against L1210 cells in culture and *in vivo* activity against P388 leukemia in mice afforded a culture, *Streptomyces zelensis*, producing a new, potent antitumor antibiotic, CC-1065. Its production, *in vitro* biological activity, microbiological assays, and taxonomy have already been described[1]; a preliminary communication announced the structure[2] shown in Fig. 1. Details of the structure determination have recently been described[3]. A molecular model of CC-1065 displayed a remarkable curvature, shape, and charge density peculiarly efficient for nestling in the grooves of the DNA double helix. Such features, combined with the presence of an efficient alkylating function in the form of a cyclopropane-conjugated-dienone suggested that CC-1065 may be a selective alkylating agent for DNA. Preliminary studies have established an extremely efficient inhibition of DNA synthesis[4] and an extraordinarily strong interaction with double stranded DNA[3,5]. This report describes improved production and isolation methodology, characterization data, and *in vivo* activity against experimental mouse tumors.

Production

Previously described shake flask fermentation titers of 2~3 μg/ml[1] were increased to approximately 6~9 μg/ml by systematically modifying the production media. Peak titers were usually realized after 96 hours of incubation. Production of CC-1065 by *Streptomyces zelensis* proved highly

Fig. 1. Structure of CC-1065.



* Person to whom correspondence should be sent.

dependent upon the quantity of cobalt, iron, and manganese in the seed or production media and was adversely affected by the presence of ammonium ions and the substitution of chloride ions for sulfate ions.

Stock cultures of *Streptomyces zelensis* were frozen plugs prepared from heavy surface growth on agar and preserved in a liquid nitrogen storage tank. The improved inoculation medium contained 5 g of Bacto-Tryptone (Difco Laboratories, Detroit, Mich., U.S.A.), 3 g of yeast extract (Difco), 1 g of dextrin (A. E. Staley Mfg., Decatur, Ill., U.S.A.), 1 g of $MgSO_4 \cdot 7H_2O$, 20 mg of $FeSO_4 \cdot 7H_2O$, 10 mg of $ZnSO_4 \cdot 7H_2O$, 2 mg of $MnCl_2 \cdot 4H_2O$, and 20 mg of $CoCl_2 \cdot 6H_2O$ per liter of distilled water. It was inoculated with the stock culture and incubated on a 250 rpm rotary shaker for 48 hours at 28°C. The seed was used to inoculate the production media at a rate of 5% (v/v).

The improved production medium contained 30 g of Hi Starch (Kraus Milling Co., Milwaukee, Wisc., U.S.A.), 30 g of black strap molasses (Knappen-Milling Co., Augusta, Mich., U.S.A.), 15 g of fish meal (Zapata-haynie, Houston, Texas, U.S.A.), 15 g of cotton seed meal (Traders Oil Meal Co., Fort Worth, Texas, U.S.A.), 2.5 g of sodium citrate, 1.0 g of $MgSO_4 \cdot 7H_2O$, 5 g of $CaCO_3$, 0.5 g of KCl, and 2 g of Na_2HPO_4 per liter of tap water (pH 7.2 prior to autoclaving). The fermentation was carried out in 500 ml non-stippled flasks (each containing 100 ml) at 28°C on a rotary shaker (250 rpm) for 96 to 120 hours.

Assay

In addition to tube dilution assays based on inhibition of growth of L1210 cells in culture or the antimicrobial activity of CC-1065 already described[1], investigations toward improving its titer in fermentations or its isolation were greatly facilitated by thin-layer chromatographic (TLC) methods developed after the initial isolation of a small sample of the homogeneous antitumor agent. TLC methods were especially beneficial since the presence of contaminating *in vitro* activities in fermentations and crude fractions complicated the interpretation of tube dilution assays. Moreover, CC-1065 appeared to lack conventional antimicrobial activity against organisms growing on agar trays since it binds strongly to paper and doesn't diffuse significantly in agar. The significant observation that the homogeneous antitumor agent could be visualized on silica gel plates by bioautographic methods led to the development of a useful TLC bioautography assay. In this assay, samples containing 0.2 to 1 μg of CC-1065 were chromatographed on Baker flex silica gel 1 B-F (J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.) with solvents consisting of chloroform - methanol - ammonia (90: 10: 0.5) or methylethylketone - acetone - water (80: 17: 3) and the air-dried sheet bioautographed on *Bacillus subtilis* or *Sarcina lutea*. Analysis of samples (such as fermentation broth) containing <10 μg/ml was facilitated by enrichment through extraction and concentration. Although TLC bioautography greatly increased the visibility of minute amounts of CC-1065 in fermentations and crude fractions, a more quantitative assay was desired for fine tuning fermentation titers and purification procedures. A TLC densitometric assay was developed to aid these improvement efforts. Samples were chromatographed on channeled silica gel plates (Whatman LK6DF) with the solvent systems used in TLC bioautography. The plate was then scanned in a recording variable wavelength densitometer (Schoeffel Model SD 3000) set at 364 nm, the UV maximum of CC-1065. Comparison of the appropriate peak height from a sample with that from known amounts of pure CC-1065 provided a reasonable estimate of the amount of agent present in the sample. Samples containing as little as 0.02 μg of CC-1065 were estimated. This quantitation permitted a much more reliable evaluation of differing fermentation and purification procedures.

Isolation and Purification

Two major obstacles complicated initial isolation efforts. Low early fermentation titers were retrospectively estimated at approximately 200 μg/liter by comparing the dose of broth solids required for an equivalent response against P388 leukemia in mice to that obtained with a known dose of pure CC-1065. In addition, as discussed in the previous section, no convenient correlating *in vitro* assay for monitoring the antitumor activity was available. Therefore, initial isolation efforts were monitored by both L1210 *in vitro* and P388 leukemia in mice. The solvent extractability, extreme potency, and availability of in-house *in vivo* assay capability allowed the successful use of an *in vivo* monitor.

Initital Isolation

Fermentation broth was filtered at harvest pH (7.2) through a celite pad. The pad was extracted with acetone - methanol (1: 1), and the extract was evaporated under reduced pressure to an aqueous concentrate, combined with the clarified broth, and extracted with three portions of *n*-butanol. After evaporation of the solvent, the residue was triturated with cyclohexane and then toluene and chromatographed on silca gel with methylethylketone - acetone - ammonium hydroxide (80: 20: 2). Fractions displaying substantial *in vitro* and *in vivo* enrichment were then fractionated by silica gel HPLC with chloroform - methanol - ammonium hydroxide (80: 20: 4). Strongly enriched fractions were then further resolved by silica gel HPLC with chloroform - methanol - ammonium hydroxide (96: 6: 0.5) yielding a few mg of chromatographically homogeneous CC-1065. Improved TLC assay systems, based on the properties of this sample, were then developed which allowed substantial improvements in fermentation and purification.

Modified Isolation

Approximately 17 liters of 4-day fermentation broth (pH 8.6) were adjusted to pH 10 with ammonium hydroxide, diluted with an equal volume of water, and extracted with two portions (30 liters and then 16 liters) of methylene chloride. The combined extracts were concentrated to dryness under reduced pressure; the residue was triturated with 1 liter of cyclohexane and the insoluble solid extracted with 1.4 liters of acetone. The extract, containing 132 mg of CC-1065 by TLC densitometry, was evaporated to dryness under reduced pressure, and the resulting residue was triturated with 50 ml of methanol and chilled overnight affording 165 mg of crude CC-1065 (72% pure by TLC densitometry). Chromatography on silica gel with chloroform - methanol - ammonium hydroxide (85: 15: 1) afforded fractions which were monitored by TLC densitometry; evaporation of appropriate fractions and trituration of the residue with methanol afforded pure CC-1065.

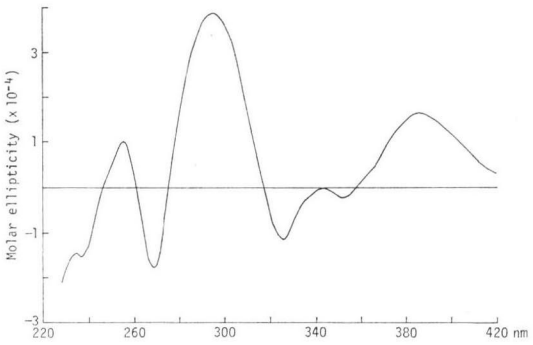
Physico-chemical Properties

CC-1065 displayed good solubility (>10 mg/ml) in dimethylacetamide, dimethylformamide, dimethylsulfoxide, and pyridine; moderate solubility (<100 μg/ml) in dioxane, tetrahydrofuran, and acetone; less solubility in chlorinated hydrocarbons; and very slight solubility in hydrocarbons, alcohols, and water. The amorphous amber solid obtained as described appeared spectrally and biologically equivalent to crystalline forms. The agent crystallized readily as clustered needles, but a highly solvated granular form obtained from dimethylformamide - acetone - methanol was used for X-ray crystallography[3].

In the ultraviolet, a dioxane solution of CC-1065 exhibited strong end absorption with shoulders at 236 (ε 36,100) and 258 (ε 31,200) and a maximum at 364 nm (ε 49,100). The asymmetry present in CC-1065 was clearly reflected in its very characteristic CD spectrum (Fig. 2) carried out on a Cary 60

spectropolarimeter with a Model 6003 CD attachment calibrated with D-10-camphorsulfonic acid[6]. Large molar ellipticities were observed at 388 (+16,000), 327 (−11,000), 297 (+38,000), and 269 nm (−16,000). The IR spectrum (Fig. 3) was consistent with the presence of NH and OH groups, amide carbonyls, and unsaturation.

Fig. 2. CD Spectrum of CC-1065 in dioxane.



NMR spectroscopy contributed vital information to the structure determination[8]. The ^{13}C spectrum (Fig. 4) was consistent with a pure compound containing 11 aliphatic and 26 unsaturated carbons. Table 1 lists the tentative assignments of the signals to the carbons numbered

Fig. 3. IR Spectrum of CC-1065 as Nujol mull recorded on a Digilab model 14D Fourier transform spectrophotometer.

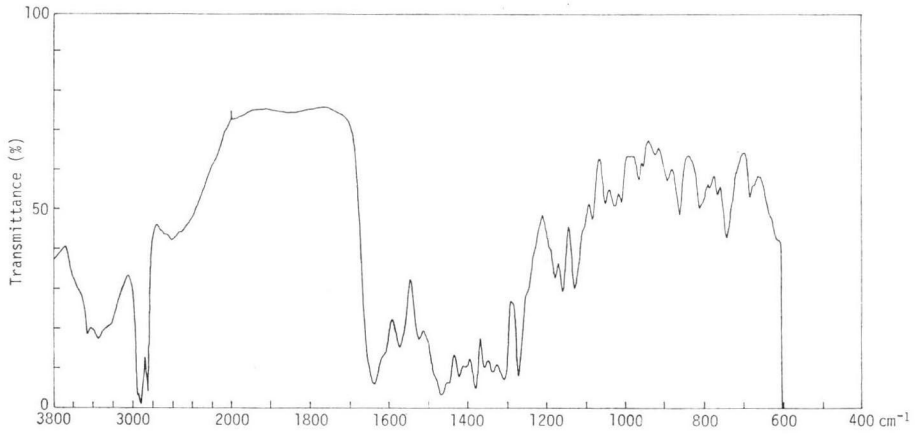


Fig. 4. ^{13}C NMR spectrum of CC-1065 in DMSO-d_6 recorded on a Varian CFT 20 spectrometer.

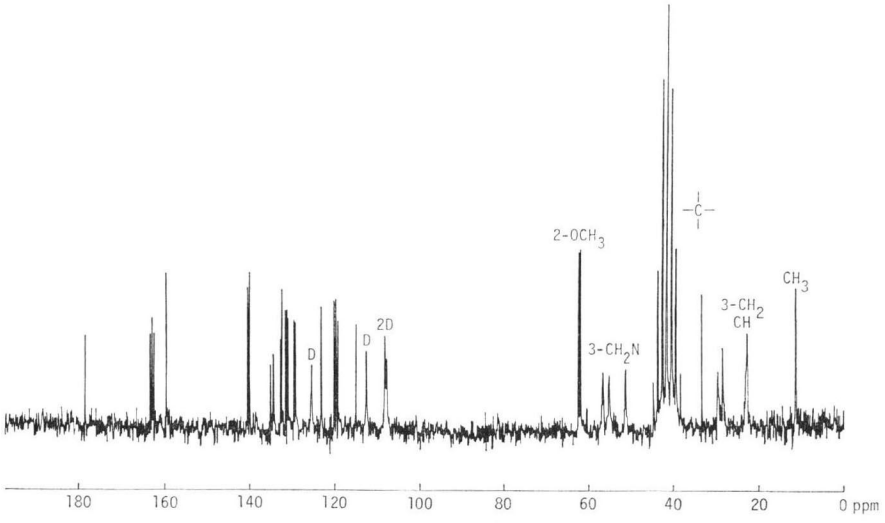
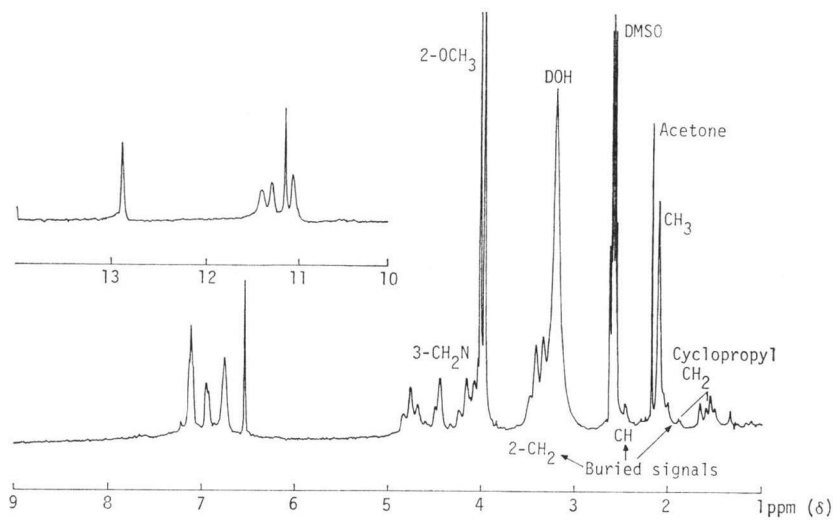


Table 1. ^{13}C NMR shifts for CC-1065.

Chemical shift*	Multiplicity**	Tentative assignment	Chemical shift	Multiplicity	Tentative assignment
9.5	Q	C3M	121.3	S	C23
20.9	D	C11	123.5	D	C2
21.6	T	C10	127.2	S	C16
26.6	T	C24	127.5	S	C29
27.6	T	C37	128.9	S	C22
31.5	S	C9	129.1	S	C35
49.4	T	C12	129.5	S	C4
53.3	T	C25	130.4	S	C19
54.8	T	C38	130.7	S	C32
60.0	Q	C20M	132.4	S	C21
60.3	Q	C33M	133.1	S	C34
105.9	D	C17	138.0	S	C20
106.3	D	C30	138.4	S	C33
110.6	D	C7	157.5	S	C40
113.0	S	C3	157.5	S	C5
117.3	S	C18	160.2	S	C27
117.7	S	C31	160.7	S	C8
118.2	S	C36	161.2	S	C14
			176.4	S	C6

* Chemical shifts in ppm relative to internal TMS.
** S=singlet, D=doublet, T=triplet, Q=quartet.

Fig. 5. ^1H NMR spectrum of CC-1065 in DMSO-d_6 recorded on a Varian XL-100-15-spectrometer.



in Fig. 1. Appropriate ^{13}C shift assignments were facilitated by using PDE I and II[7] as models.

The proton NMR spectrum (Fig. 5) displayed seven exchangeable protons (δ 12.88, 11.44, 11.41, 11.20, 11.06, and 6.84 (2H)] and confirmed the presence of four protons on unsaturated carbons [all singlets, δ 7.06 (2H), 6.86, and 6.45]. The chemical shifts of the two *O*-methyl groups (δ 3.89 and

Table 2. Mouse tumor systems against which CC-1065 meets the NCI initial activity criterion.[a,b]

Tumor		Drug		Best		Reproducible	
Site	System	Route	Schedule	% T/C (cures)	O.D.[d]	% T/C	O.D.
i.p.	ADJ-PC6	i.p.	Days 1, 5, 9	173	50		
i.p.	B16	i.p.	Days 1~9	181	10	159	200
s.c.	CD8F1	i.p.	Days 1, 8, 15, 22, 29	38[c]	33	42	12.5
i.p.	Colon 26	i.p.	Days 1~9	150 (2/10)	50		
〃	〃	〃	Days 1, 5	142	30		
i.p.	L1210	i.p.	Day 1	148	300		
〃	〃	〃	Days 1, 5, 9	144	10	125	15
〃	〃	〃	Days 1~9	138	3	137	37.5
i.p.	P388	i.p.	Day 1	195	200	162	200
〃	〃	〃	Days 1, 5, 9	211	30	170	180
〃	〃	〃	Days 1~9	163	25	136	13.5
i.p.	P388/AMSA	i.p.	Day 1	133	110		
i.p.	P388/DHAD	i.p.	Days 1, 5, 9	141	64.8	136	250

[a] Testing conducted by NCI screeners: ADL, SRI, IIT, Upjohn.
[b] CC-1065 was inactive upon initial or confirmation testing in s.c. colon xenograft, s.c. colon 38, s.c. lung xenograft, i.v. Lewis lung, s.c. breast xenograft, i.p. P388/Adria.
[c] Tumor growth inhibition model; lowest T/C is best.
[d] Optimum dose, μg/kg/day.

3.85) and the methyl (δ 2.00), indicated they were on unsaturated carbons. Two of the methylenes on nitrogen appeared as triplets at δ 4.66 and 4.04. Irradiation of a four-proton multiplet at δ 3.26 collapsed them to singlets. The third methylene on nitrogen appeared as the AB of an ABX pattern centered at δ 4.38. The methine, X of the ABX pattern, was found by spin decoupling to be buried under the DMSO signals. This methine was also coupled to the remaining cyclopropyl methylene which appeared as broad singlets at δ 1.98 and 1.48. The aliphatic methylene groups were hidden under the DOH peak and only partially visible in higher temperature spectra.

Field desorption mass spectrometry indicated a molecular weight of 703 consistent with $C_{37}H_{33}N_7O_8$, the composition of the determined structure.

Antitumor Activity

The activity of CC-1065 (NSC 298223) against mouse tumor systems is summarized in Table 2. In the human tumor cloning assay, one-hour exposure to CC-1065 at 1 ng/ml killed 70% of the clonogenic cells from the following tumor types: breast, colorectal, ovarian, and pancreatic carcinomas, small cell and adeno-carcinoma of the lung, neuroblastoma, and melanoma[4].

Acknowledgment

The authors gratefully acknowledge the support and contributions of many coworkers in the Cancer Research, Fermentation Research and Development, and Physical and Analytical Chemistry units at The Upjohn Company and thank Dr. CARTER COOK at the University of Illinois for the field desorption mass spectrum and Mrs. KAY ZINS for the preparation of this manuscript. This work was supported in part by Contracts NO1-CM-43753 and NO1-CM-77100 with the Division of Cancer Treatment, NIH, DHEW.

References

1) HANKA, L. J.; A. DIETZ, S. A. GERPHEIDE, S. L. KUENTZEL & D. G. MARTIN: CC-1065 (NSC 298223), a new antitumor antibiotic. Production, *in vitro* biological activity, microbiological assays and taxonomy of the producing microorganism. J. Antibiotics 31: 1211~1217, 1978
2) MARTIN, D. G.; C. G. CHIDESTER, D. J. DUCHAMP & S. A. MIZASK: Structure of CC-1065 (NSC 298223), a new antitumor antibiotic. J. Antibiotics 33: 902~903, 1980
3) CHIDESTER, C. G.; W. C. KRUEGER, S. A. MIZSAK, D. J. DUCHAMP & D. G. MARTIN: The structure of CC-1065, a potent antitumor agent and its binding to DNA. J. Amer. Chem. Soc., submitted to
4) BHUYAN, B. K.; K. A. NEWELL, E. G. ADAMS, S. L. CRAMPTON & D. D. VON HOFF: CC-1065 (NSC 298223), a most potent antitumor agent: biological activity, cell-kill and DNA synthesis inhibition kinetics. Proc. Am. Assoc, Cancer Res. 22: 224, 1981
5) SWENSON, D. H.; W. C. KRUEGER, A. H. LIN, S. L. SCHPOK & L. H. LI: CC-1065, a novel antitumor agent that interacts strongly with double stranded DNA. Proc. Am. Assoc. Cancer Res. 22: 216, 1981
6) KRUEGER, W. C. & L. M. PSCHIGODA: Circular dichroism calibration by Kramers-Kronig transform methods. Anal. Chem. 43: 675~677, 1971
7) ENOMOTO, Y.; Y. FURUTANI, H. NAGANAWA, M. HAMADA, T. TAKEUCHI & H. UMEZAWA: Isolation and characterization of PDE-I and II, the inhibitors of cyclic adenosine-3′,5′-monophosphate phosphodiesterase. Agric. Biol. Chem. 42: 1331~1336, 1978