NOVEL ANTITUMOR AGENTS CI-920, PD 113,270 AND PD 113,271

II. ISOLATION AND CHARACTERIZATION

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A complex of structurally related compounds that exhibit *in vivo* antileukemic activity was isolated from fermentation broths of a new streptomycete. The components of this complex are water soluble phosphate esters containing a conjugated triene system. The isolation and characterization of three of these components are described.

In the course of our screening program for new antitumor agents, a *Streptomyces* isolate was found that produces a complex of novel compounds possessing good antileukemic activity in mice. A description of the producing organism and the fermentation conditions used are reported in the preceding paper[1]. The antitumor activity of CI-920 and its analogs will be reported separately[2]. The present paper describes the isolation and characterization of three components of the complex: CI-920 (PD 110,161), PD 113,270 and PD 113,271.

Isolation Procedure

Analytical Methods

CI-920 and its congeners are essentially devoid of antimicrobial activity and their concentrations in fermentation beers and various fractions are best determined by high pressure liquid chromatography (HPLC). These assays were performed using a Waters Associates Model 201/401 HPLC instrument and μBondapak C_{18}-silica gel columns with UV detection at 254 nm. Two useful solvent systems are 0.1 M sodium phosphate buffer (pH 6.8) - acetonitrile (88: 12, System A) and (82: 18, System B). Approximate retention times, at a flow rate of 2.0 ml/minute, for PD 113,271, CI-920 and PD 113,270 are 2.7, 5.0 and >12 minutes, respectively, in System A and <1.0, 2.0, and 5.0 minutes, respectively, in System B. Titers of the major component, CI-920, in beers produced in 30-liter stir-jars and in 760-liter tanks varied between $350\sim450$ μg/ml.

Fractionation Procedure

Because CI-920 and its analogs are unstable above pH 8 and very labile in dilute acid, they can only be obtained in salt form and isolation steps must be performed within the pH range of $5.5\sim7.5$. The fermentation beer, prepared in a 760-liter tank, was treated with 22.5 kg of Celite 545 and filtered at pH 6.5. The filtered beer (644 liters) was passed through a 30-cm (ID) column containing 34 liters of Dowex-1X2 (Cl^-). HPLC analysis showed that the effluent and subsequent water wash (117 liters) did not contain detectable amounts of CI-920 or its congeners. The Dowex resin was eluted with several bed volumes of 1 M NaCl - methanol (1: 1). The eluates were assayed by HPLC and the fractions containing the majority of the CI-920 analogs were pooled (64 liters) and treated with 3.8 volumes (242 liters) of acetone. The resulting mixture was chilled overnight at 5°C. The clear supernatant was re-

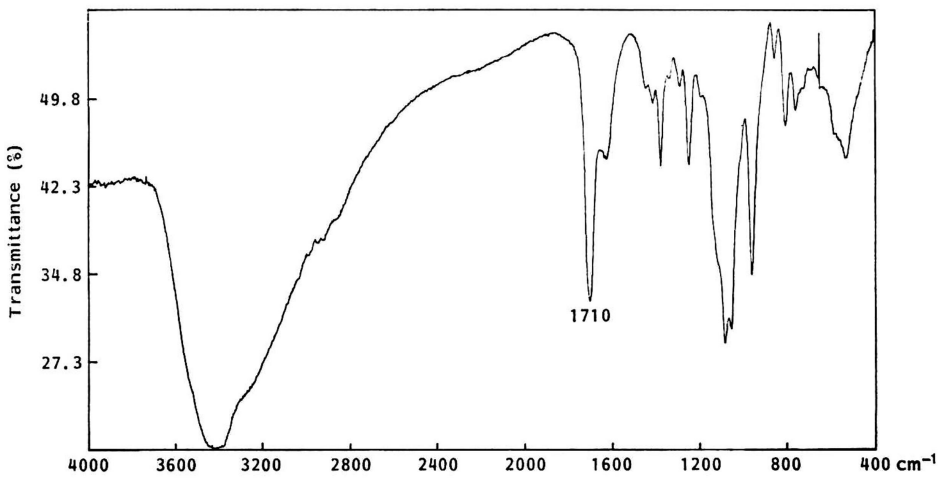
moved and concentrated *in vacuo* to 26.5 liters.

The concentrate was applied to the top of 50 liters of Diaion HP-20 resin packed in water in a 15-cm (ID) column. The resin was then washed with water (100 liters) until appreciable quantities of compounds absorbing at 254 nm were detected. At this point the following fractions were collected: Fraction A; 36 liters H_2O, Fraction B; 96 liters H_2O - MeOH (80: 20), Fraction C; 30 liters H_2O - MeOH (50: 50), Fraction D; 30 liters H_2O - MeOH (70: 30), Fraction E, 30 liters MeOH.

Isolation of CI-920

Fractions B and C were pooled and concentrated *in vacuo* to 17.5 liters. This concentrate was added to a 15-cm (ID) column containing 50 liters (25 kg) of 100 μm C_{18}-reverse phase silica gel (Analytichem Inc., USA) packed in 0.05 M sodium phosphate buffer (pH 6.8). The column was developed at 1.05 kg/cm^2 pressure with 0.05 M phosphate buffer (pH 6.8) - acetonitrile (95: 5). The eluates were monitored by a UV recorder and each fraction was analyzed by HPLC. After 186 liters of this eluent had removed the relatively small amount of PD 113,271 and several UV-absorbing impurities that were present in the charge, most of the CI-920 was eluted with 0.05 M phosphate buffer (pH 6.8) - acetonitrile (93: 7). The fractions that contained CI-920 as the only UV-absorbing component were combined (186 liters) and concentrated to 48 liters *in vacuo* and stored at 5°C. This concentrate was filtered and, in two portions, was desalted over 25 liters of HP-20 resin packed in water in a 15-cm (ID) column. After a water wash (90 liters), the adsorbed CI-920 was eluted with 30% MeOH. The eluates containing the majority of the CI-920 were combined (108 liters), concentrated, and lyophilized at pH 6.5 to afford 40 g of CI-920 as a nearly white solid. Siliceous material originating from the reverse phase silica gel was removed by dissolving the product in methanol (1 g/10 ml) followed by the addition of one volume of ethanol. After the mixture was filtered, the filtrate was converted to an aqueous solution *in vacuo* and lypohilized to yield CI-920. The purity of this product was about 90% as determined by HPLC and UV analysis. A sample of more than 96% pure CI-920 can be prepared by reverse phase chromatography on 40 μm C_8 or C_{18}-silica gel using approximately 2% or 10%, respectively, acetonitrile in 0.1 M phosphate buffer (pH 6.8). CI-920 is eluted more sharply and in optimal purity when a gradient elution is used. For example, chromatography of 2.0 g of 90% pure CI-920 on 1.8 liters (900 g) 40 μm C_{18}-silica gel in a 5-cm (ID) stainless steel column using a 0~10% acetonitrile gradient in 0.05 M sodium phosphate buffer (pH 6.8)

Fig. 1. IR spectrum of CI-920 in KBr.



afforded 1.0 g of 98% pure CI-920 after desalting on HP-20: λ max (methanol) 268 nm (A 80.5) with inflections at 259 and 278 nm; $[\alpha]_D$ +33° (c 1, 0.1 M phosphate buffer (pH 7)). The infrared, ultraviolet, and ^{1}H NMR spectra of CI-920 are shown in Figs. 1, 2, and 3. CI-920 isolated in the above manner is a hydrated, partial sodium salt that is very hygroscopic and difficult to analyze. Elemental analysis of a carefully purified and dried sample showed:

Anal. Calcd. for $C_{19}H_{25.25}O_9P \cdot Na_{1.75} \cdot 1.5H_2O$ (495.9): C 46.02, H 5.74, P 6.25, Na 8.11, H_2O 5.45.
Found: C 45.91, H 5.38, P 6.40, Na 8.38, H_2O 4.48.

Isolation of PD 113,271

Fraction A, described above, contained most of the PD 113,271 and several closely related polar components. It was concentrated and lyophilized to afford 68 g of a crude solid. A similar product obtained from previous fermentations was chromatographed on 25 kg of 100 μm C_{18}-silica gel packed in 0.05 M phosphate buffer (pH 6.8) in a 15-cm (ID) column. After the column was washed with 0.05 M phosphate buffer (pH 6.8) (58 liters) and 100 liters of 0.05 M phosphate buffer (pH 6.8) - acetonitrile (96: 4), most of the PD 113,271 was eluted with 184 liters of 0.05 M phosphate buffer - acetonitrile (94: 6). The fractions that contained PD 113,271 as the only UV-absorbing component, as determined by HPLC, were combined and concentrated to 20 liters. This concentrate was filtered and desalted, in two portions, on 27 liters of HP-20 resin. Lyophilization of the PD 113,271 fractions at pH 6.5 yielded 15.4 g of 90% pure PD 113,271 as a pale yellow solid. A pure

Fig. 2. UV spectrum of CI-920 in methanol.

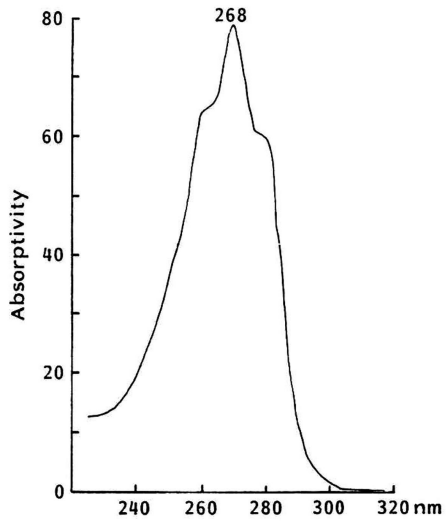
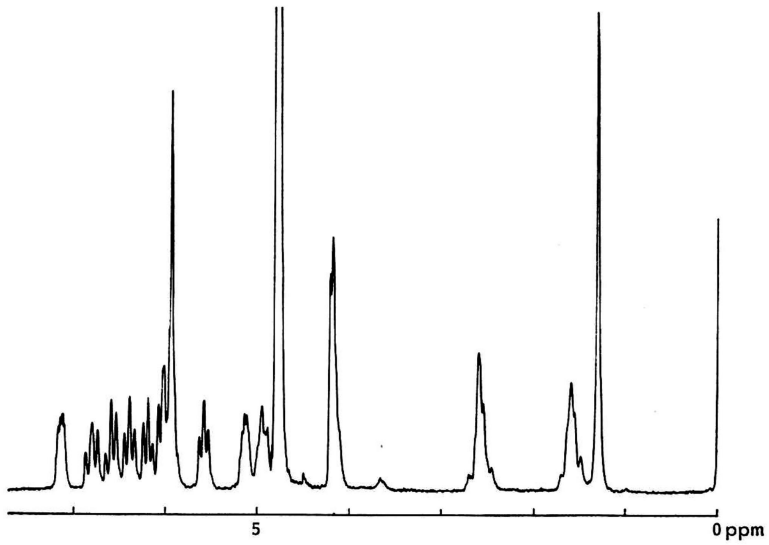


Fig. 3. 200 MHz ^{1}H NMR spectrum of CI-920 in D_2O.



sample of this compound was obtained by rechromatography over 40 μm C_8-silica gel as described for CI-920: λ max (methanol) 268 nm (A 77.4) with inflections at 260 and 278 nm.

Isolation of PD 113,270

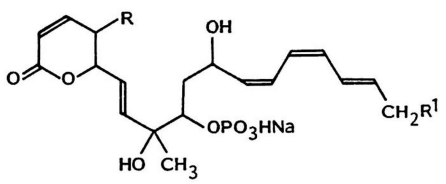
Fraction E, described above, was concentrated and lyophilized to afford 13 g of a brown solid. This material was dissolved in 500 ml of 0.05 M phosphate buffer (pH 7.1) and chromatographed over 1.9 kg of 40 μm C_{18}-silica gel using a gradient elution of 8~16% acetonitrile in 0.05 M buffer (pH 7.1). The fractions collected were analyzed by HPLC using 0.05 M phosphate buffer (pH 7.1) - acetonitrile (77: 23) as the eluent. The fractions containing PD 113,270 were combined, desalted on 1 liter of HP-20 resin, and lyophilized to yield 2.6 g of a brown solid. This product, in 15 ml of buffer, was rechromatographed on 1.9 kg of C_{18}-silica gel using 0.05 M sodium acetate buffer (pH 5.1) - methanol - acetonitrile (70: 20: 10) as the eluent. The fractions containing PD 113,270 as the only UV-absorbing component were combined, desalted on HP-20 resin, and lyophilized to yield 1.1 g of PD 113,270 with a purity >98% by HPLC: λ max 267 nm (A 86.2) with inflections at 259 and 277 nm; $[\alpha]_D$ +31° (c 1, 0.1 M phosphate buffer (pH 7)).

Discussion

The infrared and ultraviolet spectra of CI-920, PD 113,270 and PD 113,271 are nearly identical. However, these compounds can be readily distinguished by differences in their ^1H and ^{13}C NMR spectra. A FAB-mass spectrum of CI-920 shows m/z peaks at 453 (monosodium salt+H) and 475 (disodium salt +H) which correspond to a molecular weight of 430 for the free acid form of CI-920.

Elemental analysis reveals that CI-920 contains 6.4% phosphorus. The ^{31}P NMR spectrum shows a doublet at 0.50 ppm (J=9.9 Hz) downfield from 85% phosphoric acid which identifies CI-920 as a phosphate ester. Facile dephosphorylation of CI-920 by alkaline phosphatase* further shows that this compound is a monosubstituted phosphate ester. Although many antibiotics are phosphonate derivatives only a relatively few, such as moenomycin[4], diumycin, prasinomycin, and macarbomycin, are phosphate esters[5]. CI-920 is readily distinguished from most of these latter antibiotics by its prominent ultraviolet absorption spectrum (Fig. 2) which is characteristic of conjugated trienes. A search of the literature for antibiotics that are both conjugated trienes and phosphate esters shows that the compound most closely related to CI-920 is the antibiotic, proticin[6].

Proticin, $C_{31}H_{44}O_7PNa$ (MW 582), is a lipophilic antibiotic produced by a bacterium and is readily extracted from fermentation beers with 1-butanol. It is very active against several bacteria, particularly *Proteus* sp., contains about 4.4% phosphorus and, in addition to a conjugated triene chromophore, exhibits an intense ultraviolet absorption maximum at 235 nm (A 101). Proticin is soluble in chloroform and acetone and only slightly soluble in acidic water. CI-920 and its congeners are devoid of antibacterial activity, are poorly extracted from fermentation beers, and do not exhibit an ultraviolet absorption maximum between 225~250 nm. CI-920 is insoluble in chloroform and acetone and is very soluble in water at all pHs. These properties show that CI-920, PD 113,270 and PD 113,271 are novel antitumor agents. Structures **1**, **2** and **3** for the monosodium salts of these compounds have been established on the basis of chemical degradations and nuclear magnetic resonance spectral data. Details of these structure determinations will be presented in a forthcoming communication[3].



1	CI-920	R=H, R^1=OH
2	PD 113,270	R=R^1=H
3	PD 113,271	R=R^1=OH

* This reaction and the properties of the C_{19}-alcohol product will be described in a future publication.[3]

Acknowledgments

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