CYTOSTATIN, A NOVEL INHIBITOR OF CELL ADHESION TO COMPONENTS OF EXTRACELLULAR MATRIX PRODUCED BY *Streptomyces* sp. MJ654-NF4

II. PHYSICO-CHEMICAL PROPERTIES AND STRUCTURE DETERMINATION

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The structure of cytostatin was determined to be 5,6-dihydro-5-methyl-6-(6-hydroxy-1,5-dimethyl-4-phosphonooxy-7,9,11-tridecatrienyl)-2*H*-pyran-2-one sodium salt on the basis of physico-chemical properties and NMR studies.

Cytostatin is a novel inhibitor of cell adhesion to components of the extracellular matrix, laminin and collagen type IV, produced by *Streptomyces* sp. MJ654-NF4. The taxonomy of the producing strain, fermentation, isolation and biological properties of cytostatin have been reported in the preceding paper[1].

In this paper, we report the physico-chemical properties and structure determination of cytostatin (**1** in Fig. 1).

Results and Discussion

Physico-chemical Properties of **1**

Physico-chemical properties of **1** are summarized in Table 1. The sodium salt of **1** was isolated as yellowish powders from the fermentation broth of *Streptomyces* sp. MJ654-NF4. The molecular formula of **1** was determined to be $C_{21}H_{33}O_7P$ as a free acid by analyses of HRFAB-MS (calcd: *m/z* 427.1886; found: *m/z* 427.1898 $(M-H)^-$), ^{13}C NMR and ^{31}P NMR spectral data. The IR spectrum of **1** showed a strong absorption at 1715 cm^{-1} suggesting

Fig. 1. Structure of cytostatin.

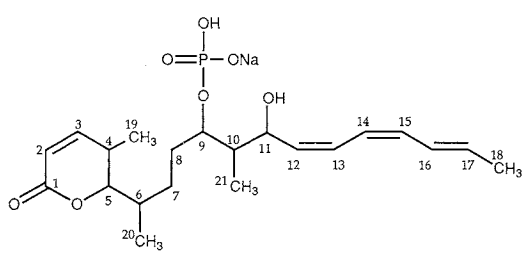


Table 1. Physico-chemical properties of cytostatin.

Appearance	Yellowish powder
Molecular formula	$C_{21}H_{33}O_7PNa$ (sodium salt)
HRFAB-MS (*m/z*)	427.1898 $(M-H)^-$ (Calcd for $C_{21}H_{33}O_7P$ *m/z* 427.1886)
UV λ_{max} nm (log ε)	208 (3.94), 260 (sh, 4.24), 267 (4.33), 277 (sh, 4.22)
IR (KBr) cm^{-1}	3400, 2975, 2925, 1715, 1450, 1380, 1260, 980, 830
Solubility	
Soluble in:	MeOH, DMSO, $CHCl_3$, EtOAc, H_2O
Insoluble in:	*n*-Hexane
Color reaction	$FeCl_3$, anisaldehyde-H_2SO_4, I_2, ammonium molybdate-perchloric acid
Rf value[a]	0.29

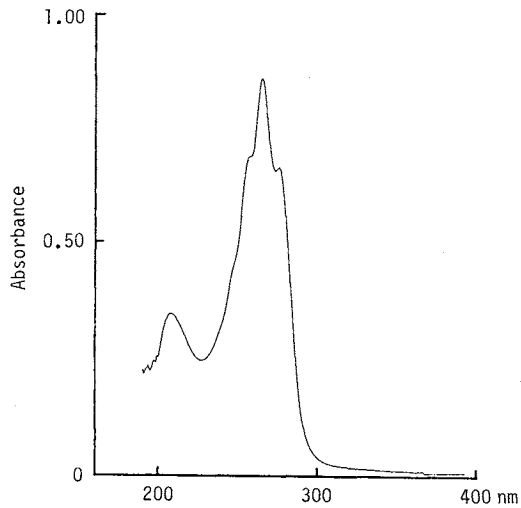
[a] Silica gel TLC (Merck Art No. 5554). *n*-BuOH-AcOH-H_2O, 4:1:1.

the presence of α,β-unsaturated ester or lactone. The UV spectrum of **1** in MeOH is shown in Fig. 2. The characteristic UV spectrum with λ_{max} (log ε) at 260 (4.24), 267 (4.33) and 277 (4.22) was typical of compounds possessing a conjugated triene moiety[2]. Compound **1** showed positive responses to ferric chloride, anisaldehyde-H_2SO_4 and ammonium molybdate-perchloric acid (for phosphoric acid) reagents on TLC plates.

Structure Determination of 1

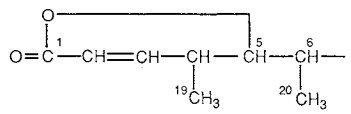
The molecular formula of **1** was determined as $C_{21}H_{33}O_7P$. The presence of phosphoryl ester was suggested by the ^{31}P NMR spectrum, in which a signal was observed at δ 2.59 ($^3J_{P-O-C-H}=8.9$ Hz) and positive color reaction to ammonium molybdate-perchloric acid reagent. This was further confirmed by the result in which the treatment of **1** with alkaline phosphatase gave a dephosphorylated compound of **1** as described below. The ^{13}C NMR spectrum of **1** in CD_3OD showed 21 carbon signals which were assigned to four methyls, two methylenes, six methines, eight olefinic carbons and one ester carbon by the distortionless enhancement by polarization transfer (DEPT) experiments. In the ^1H NMR spectrum of **1**, a total of 30 unexchangeable protons including 12 methyl protons, 4 methylene protons, 6 methine protons and 8 olefinic protons were clarified by combined use of heteronuclear single quantum coherence spectrum (HSQC)[3]. Therefore, one hydroxyl group and phosphoryl group as mentioned above were predicted as the remaining functions. Thus, all oxygen atoms consisting of hydroxyl, phosphoryl and carboxyl function were identified.

Fig. 2. UV spectrum of cytostatin (in MeOH).

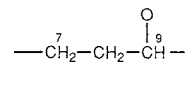


^1H-^1H COSY and homonuclear Hartmann-Hahn (HOHAHA)[4] experiments indicated the three partial structures (Unit A, B and C) as shown in Fig. 3. Among the partial structures, Unit B and C were deduced by ^1H-^1H COSY experiments. The presence of an α,β-unsaturated δ-lactone moiety (see Unit A), which was suggested by the IR spectrum, was confirmed as follows. A methine carbon at δ_C 85.6 (tentatively identified as C-5) was shown to bear an unexchangeable oxygen atom based on

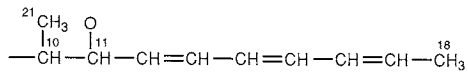
Fig. 3. Partial structures of cytostatin.



Unit A



Unit B



Unit C

Fig. 4. ^{1}H-^{13}C long range coupling by HMBC experiment.

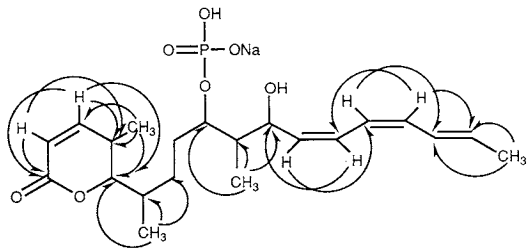


Table 2. ^{1}H and ^{13}C NMR data of cytostatin in CD_3OD.

Position	δ_C (100 MHz)	δ_H (400 MHz)	
1	167.5	—	
2	120.1	5.93	(d, 9.6)[a]
3	155.0	7.14	(dd, 6.4, 9.6)
4	31.7	2.58	(m)
5	85.6	4.11	(dd, 10.4, 10.4)
6	35.6	1.81	(m)
7	29.3	1.22, 1.80	(m)
8	31.6	1.50, 2.06	(m)
9	74.0	4.47	(m)
10	43.8	1.52	(m)
11	68.9	4.61	(dd, 9.4, 9.4)
12	134.5	5.41	(dd, 9.4, 10.0)
13	126.1	6.57	(dd, 10.0, 11.4)
14	123.9	6.28	(dd, 10.8, 11.4)
15	131.4	5.97	(dd, 10.8, 10.8)
16	128.2	6.56	(dd, 10.8, 14.8)
17	131.9	5.75	(m)
18	18.5	1.80	(d, 6.8)
19	10.9	1.00	(d, 6.9)
20	14.9	0.98	(d, 6.9)
21	9.1	0.78	(d, 6.8)

[a] Proton signal multiplicity and coupling constant (J=Hz).

the chemical shift and its behavior in deuterated solvent. The methine proton (δ_H 4.11, 5-H) showed couplings to a methine proton (δ_H 1.81, 6-H) and another methine proton (δ_H 2.58, 4-H) in the ^{1}H-^{1}H COSY spectrum. An olefinic proton at δ_H 7.14 (3-H) showed couplings to the 4-H proton and another olefinic proton at δ_H 5.93 (2-H). These two olefinic protons showed couplings to an ester carbon (C-1) at δ_C 167.5 in heteronuclear multiple bond correlation (HMBC)[5] spectrum. These results indicated that C-1 was connected to an olefinic carbon (C-2) resulting in formation of δ-lactone ring. This was further confirmed by a long range selective proton decoupling (LSPD) experiment between the methine proton (δ_H 4.11, 5-H) and the ester carbon (δ_C 167.5, C-1). Thus, the olefinic protons at δ_H 5.93 and δ_H 7.14 were assignable to the α-proton and β-proton, respectively, of the α,β-unsaturated δ-lactone.

The connectivities of the Unit A, Unit B and Unit C were determined as follows. The HMBC experiment of **1** showed long range couplings between a methyl group (δ_H 0.98, 20-H) of Unit A and a methylene carbon (δ_C 29.3, C-7) of Unit B. The methyl group (δ_H 0.78, 21-H) of Unit C showed a long range coupling to an oxygen-bearing methine carbon (δ_C 74.0, C-9) of Unit B. These correlations confirmed the connectivity between C-6 (Unit A) and C-7 (Unit B) and between C-9 (Unit B) and C-10 (Unit C). Results from HMBC experiments of **1** are shown in Fig. 4.

A final problem to be elucidated was the position of phosphoryl group. It was considered to be located on an oxygen bearing methine carbon at C-9 or C-11. Treatment of **1** with alkaline phosphatase of calf intestine in an aqueous solution at 37°C for 2 hours gave a yellowish oil of **2**. The molecular formula of **2** was determined to be $C_{21}H_{32}O_4$ by HRFAB-MS indicating the dephosphorylated compound of **1**. In the ^{13}C NMR spectra of **1** and **2**, an upfield shift of the C-9 carbon from δ_C 74.0 in **1** to δ_C 72.0 in **2** was observed. The upfield shift was ascribed to the dephosphorylation at the C-9 position. Furthermore, a phosphorus-carbon coupling (δ_C 74.0, $^{2}J_{P-O-C}$=5.0 Hz) detected in ^{13}C NMR spectrum of **1** verified the bond type of phosphoryl ester at C-9 position. Consequently, the C-11 methine carbon was concluded to bear a hydroxyl group. The geometries of the conjugated triene system were established as Z, Z, E on the basis of their coupling constants ($J_{12,13}$=10.0, $J_{14,15}$=10.8 and $J_{16,17}$=14.8 Hz), respectively.

Thus, the total structure of **1** excluding its absolute configuration was determined as shown in Fig.

1. Assignments of ^{1}H NMR and ^{13}C NMR of **1** are summarized in Table 2.

Among the known antibiotics, **1** structurally resembles CI-920[6,7] and sultriecin[8] which have a triene and α,β-unsaturated δ-lactone in the molecules. However, **1** is clearly different from them in terms of the absence of a double bond between C-6 and C-7. The structure-activity relationships of **1** are now under study.

Experimental

General

IR and UV spectra were recorded on a Hitachi 260-10 spectrometer and a Hitachi 228A spectrometer, respectively. ^{1}H (400 MHz), ^{13}C (100 MHz) and ^{31}P (162 MHz) NMR spectra in CD_3OD were obtained on a JEOL JNM-A400 spectrometer. Chemical shifts values are given in ppm downfield of internal TMS, the solvent lines of CD_3OD and H_3PO_4. FAB-MS spectra were measured on a JEOL JMS-SX102 mass spectrometer. TLC was performed on a silica gel plate (Kieselgel 60 F_{254}, Merck) using a solvent mixture of *n*-BuOH - AcOH - H_2O (4 : 1 : 1).

Dephosphorylation of Cytostatin (Preparation of 2)

A solution of alkaline phosphatase (10 mg, calf intestine, Biozyme Laboratories Ltd.) and **1** (20 mg) in 0.3% sodium hydrogen carbonate (5.3 ml) was incubated at 37°C for 2 hours. The reaction mixture was applied on a column of Diaion HP-20 (10 ml, Mitsubishi Chemical Industries Ltd.) equilibrated with water. After washing the column with water, adsorbed materials were eluted with MeOH. Each fraction containing **2** was collected and concentrated under reduced pressure. The residue was applied on a Sephadex LH-20 column (150 ml) and eluted with MeOH. The eluate was concentrated *in vacuo* to yield 7.0 mg of **2** as a yellowish oil. FAB-MS *m/z* 371 $(M+Na)^+$; UV (MeOH) λ_{max} 205, 260 (sh), 267, 277 (sh) nm; ^{13}C NMR (100 MHz, CD_3OD) δ 167.4, 155.1, 134.0, 132.5, 132.1, 128.0, 126.0, 122.7, 120.0, 85.6, 72.0, 69.6, 44.9, 35.0, 32.5, 31.6, 30.2, 18.5, 14.9, 10.9 and 10.0.

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