NOVEL ANTITUMOR ANTIBIOTIC PHOSPHOLINE

2. STRUCTURE DETERMINATION

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Phospholine, an antitumor antibiotic, has the molecular formula of $C_{25}H_{40}NO_8P$ and posesses a δ-lactone and a phospholic acid ester as functional groups. Its structure was determined based on interpretation of fast atom bombardment MS, ^1H NMR, ^{13}C NMR, ^1H-^1H correlation spectroscopy (COSY), ^{13}C-^1H COSY, heteronuclear multiple bond correlation spectroscopy, elemental analysis and chemical modifications.

Phospholine, an antitumor antibiotic, was isolated from the culture broth of *Streptomyces hygroscopicus*. The fermentation, isolation, characterization and biological properties have been reported in the preceding paper. In this paper, the structure determination of phospholine is described.

Results and Discussion

Fast atom bombardment (FAB)-MS of phospholine shows peaks at *m/z* 514 (M+1), 536 (M+Na) and 496 (M+1−H_2O). The molecular weight of phospholine is supposed to be 513. The fragmentation of 496 *m/z* suggests that the parent molecule is readily dehydrated in the process of measuring MS spectrum. A molecular formula of the dehydrated compound was determined to be $C_{25}H_{38}NO_7P$ from the high-resolution (HR)FAB-MS (496.2457). So the molecular formula of phospholine was determined to be $C_{25}H_{40}NO_8P$ (MW 513). The ^{13}C NMR spectrum of phospholine shows 23 carbon signals. This result does not agree with the molecular formula obtained from the FAB-MS spectrum. The ^{13}C-^1H correlation spectroscopy (COSY) spectrum of phospholine shows that methylene carbon resonances at 34.4 and 27.1 ppm indicate each two overlapping methylene carbons, respectively. So the number of carbon atoms constructing phospholine molecule is identified to be 25 carbons.

Phospholine is amphoteric from the characterization on various ion-exchange resins. This is furthermore confirmed by the color reactions; phospholine shows the positive reaction to ninhydrin and ammonium molybdate - perchloric acid. These results suggest the presence of a primary amino group and a phosphoric group. The IR spectrum shows prominent absorptions at 1720 and 1100 cm^{-1}, which suggest the presence of an α,β-unsaturated lactone and a phosphoric group[1,2]. Phospholine was hydrolyzed to dephosphoric phospholine (MW 433) on treatment with alkaline phosphatase of calf intestine (Fig. 3). The

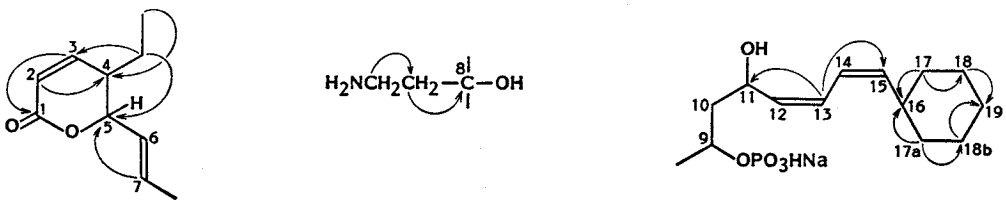
Table 1. Summary of the functional groups of phospholine.

Functional groups	
Carbonyl carbon	1
sp^2 carbon	8
sp^3 tertiary carbon	1
sp^3 methine carbon	5
sp^3 methylene carbon	9
sp^3 methyl carbon	1

UV spectrum of phospholine in methanol shows an absorption at 234 nm, which indicates the presence of α,β-unsaturated δ-lactone and/or conjugated diene. The ^{13}C NMR spectrum and ^{13}C-^{1}H COSY spectrum exhibits 25 carbon signals which are divided into the classes as shown in Table 1.

The ^{1}H NMR signals at 6.05 and 7.10 ppm are assigned to the α-proton and β-proton, respectively, of the α,β-unsaturated lactone. The ^{1}H-^{1}H COSY spectrum shows that the signal at 2.55 ppm is coupled to the above olefinic protons, a methine proton exhibiting a signal at 5.1 ppm and a pair

Fig. 1. Partial structures **A**, **B** and **C** of phospholine.



A B C

Arrows indicate a part of HMBC correlations from protons to carbons.

Fig. 2. ^{1}H-^{1}H COSY spectrum of phospholine.

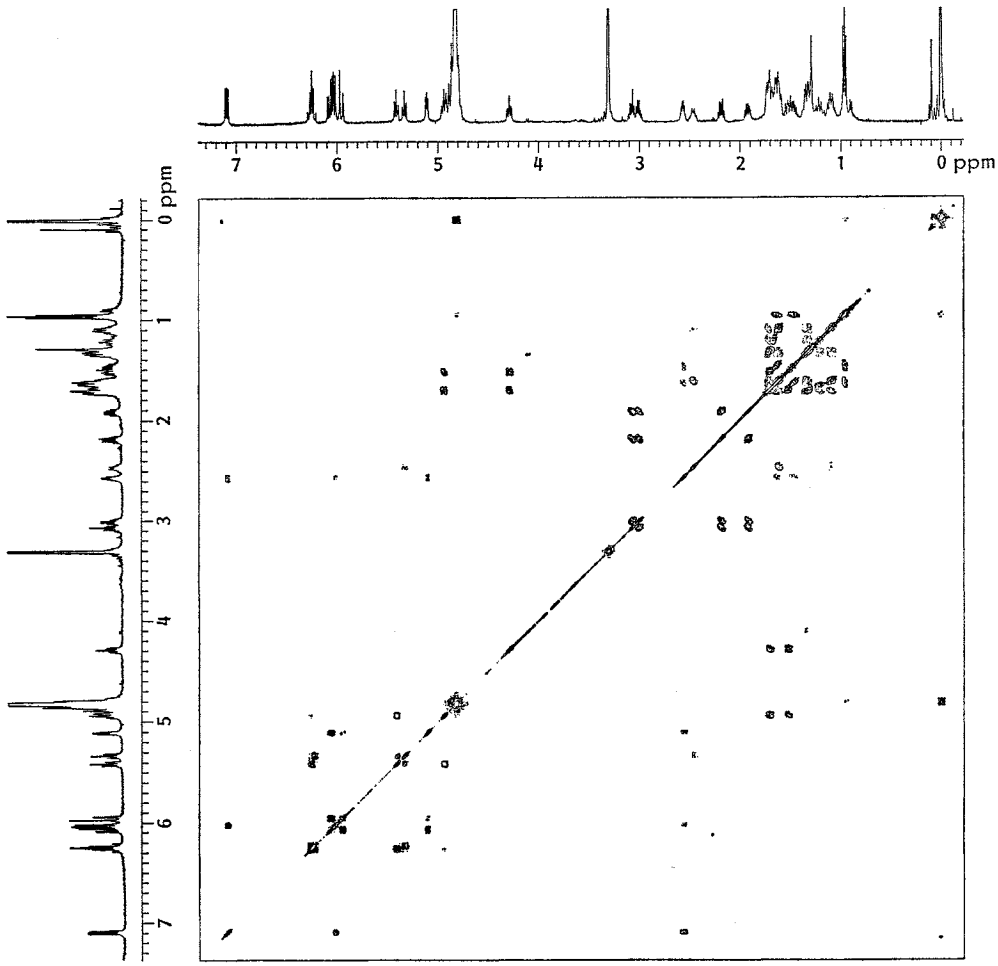
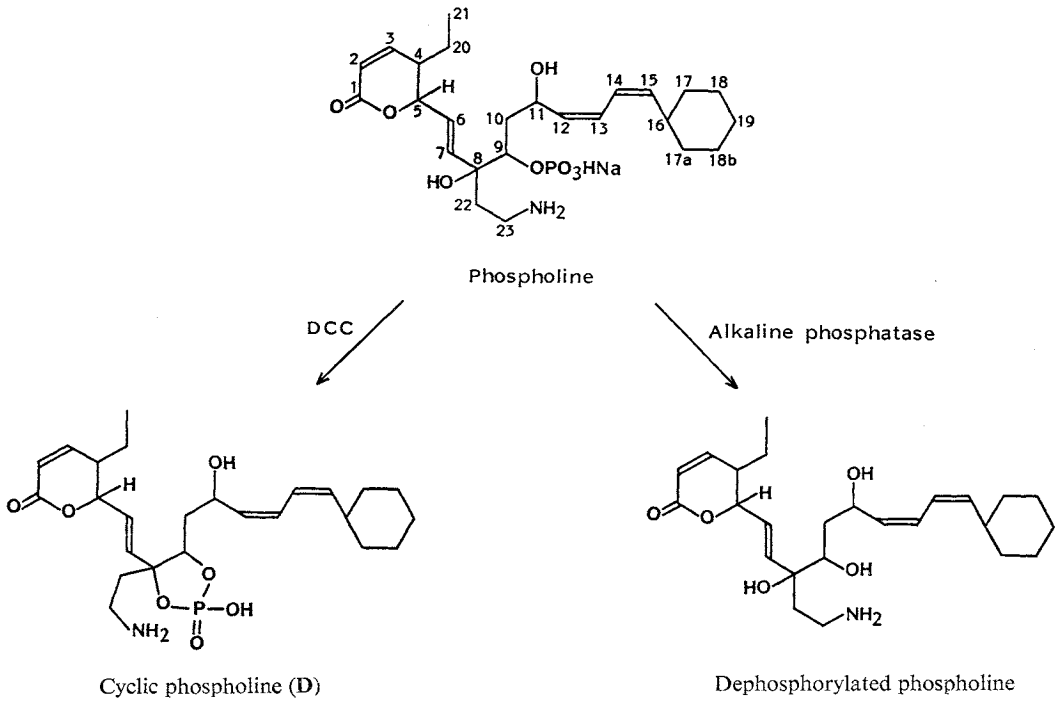


Fig. 3. Structure of phospholine and cyclic phospholine (**D**).



protons of unequivalent metylene at 1.65 and 1.48 ppm, respectively, which are coupled to a methyl proton at 0.95 ppm. A signal at 5.1 ppm is coupled to an other olefinic protons at 5.95~6.10 ppm. From above ^{1}H NMR data, a partial structure **A** can be constructed (Fig. 1). The chemical assignment for above protons are closely agreed with the NMR data reported by HOKANSON and FRENCH[1], with the exception of an ethyl group substituted at 2.55 ppm signal position. Phospholine has 8 degrees of unsaturation based on a calculation of the molecular formula. The partial structure **A** has 4 degrees of unsaturation. The degrees of unsaturation in the residual partial structure of phospholine is calculated to be 4, in which two double bonds and one phosphoric acid ester are contained. The partial structure **B** is constructed from the ^{1}H NMR spectrum. The residual partial structure **C** in phospholine has one cyclic structure to satisfy the degree of unsaturation. From ^{1}H-^{1}H COSY spectrum (Fig. 2), the partial structure **C** is able to be constructed (Fig. 1). The presence of a monosubstituted cyclohexane ring was determined from the ^{13}C-^{1}H COSY spectrum; four carbon signals at 37.7 (methine carbon), 26.9 (methylene carbon), 27.1 (methylene carbon) and 34.4 ppm (methylene carbon) are observed, in which two signals of the latter show the pair carbons. These chemical signals agreed with the calculated data of an equatorial monosubstituted cyclohexane[3,4]. To satisfy the analytical data of phospholine the quarternary carbon C-8 of the partial structure **B** connects the vinyl carbon C-7 of the partial structure **A** to the methine carbon C-9 of the partial structure **C**. The connection of these partial structures was further confirmed by the heteronuclear multiple-bond correlation (HMBC) spectrum; there are HMBC couplings of the four protons at C-6, C-7, C-9 and C-10 to the quaternary carbon C-8 of the partial structure **B**. The NMR spectrum data of structure constructed from each partial structures are similar to that of CI-920[1].

The position of a phosphoric acid ester is attributed to the methine carbon C-9 which splits to

doulet ($^2J_{P-C}$=3.7 Hz) at 78.5 ppm. To ascertain the position of a phosphoric acid ester, phospholine was treated with dicyclohexylcarbodiimide (DCC) to give a cyclic phospholine (**D**) (Fig. 3) *via* a phosphoric group. The ^{13}C NMR data of a cyclic phospholine (**D**) is shown in Table 2. The carbon signal at 8-position shows a downfield shift by 8.7 ppm compared with that of phospholine. This low field shift[5] at 8-position suggests that the hydroxy group of 8-position was newly phosphorylated by a neighboring phosphoric acid group with the treatment with DCC. So the phosphoric acid ester in parent phospholine is positioned at the 9-carbon.

The structure of phospholine is shown in Fig. 3. From a search of the literatures for antibiotics that are both phosphoric acid ester and conjugated dienes, we could find the related antibiotics, MA-5000[6] and AF-273[7], respectively.

Table 2. ^{13}C NMR spectrum (ppm) of phospholine and cyclic phospholine.

Carbon No.	Phospholine	Cyclic phospholine
1	166.5	166.1
2	121.1	121.0
3	152.8	152.8
4	40.6	40.1
5	82.4	81.3
6	127.5	129.3
7	138.2	132.5
8	77.1	85.3
9	78.5	81.9
10	40.6	38.3
11	64.5	64.7
12	134.7	134.7
13	124.5[a]	125.0[a]
14	123.1[a]	122.3[a]
15	140.0	140.9
16	37.7	37.7
17	34.4	34.3
17[a]	34.4	34.3
18	27.1	27.0
18[a]	27.1	27.0
19	26.9	26.9
20	22.8	22.6
21	11.4	11.2
22	34.0	31.3
23	37.4	36.4

[a] Interchangeable.

Experimental

IR spectra were recorded for KBr discs on a Hitachi 260-50 spectrometer and UV spectra were obtained on a Shimadzu UV-240 spectrometer. ^1H and ^{13}C NMR spectra were obtained on Jeol GSX-500 spectrometer. FAB-MS were recorded on a Jeol JMS-DX 300 (HF) spectrometer and the matrix was quoated.

Dephosphoric Phospholine

An solution of alkaline phosphatase (600 mg, calf intestine, Sigma Chemical Company) and phospholine (50 mg) in H_2O (26 ml) and 5% sodium hydrogen carbonate (0.6 ml) was incubated at 37°C for 15 hours. The reaction mixture was extracted with ethyl acetate (50 ml×3). The extract was chromatographed on Sephadex LH-20 (Pharmacia) column (1.0 i.d.×34 cm) using ethyl acetate as a solvent. Fractions containing dephosphoric phospholine were collected, concentrated to yield 2.8 mg of dephosphoric phospholine as a hygroscopic mass: Chemical ionization (CI)-MS *m/z* 434 (M+1), 416 (M−H_2O); IR (KBr) cm^{-1} 1710; $[\alpha]_D^{20}$ +58° (*c* 0.5, MeOH); ^{13}C NMR (125.65 MHz, CD_3OD) δ 166.0, 153.1, 140.9, 137.5, 135.7 127.3, 124.9, 123.0, 121.5, 82.8, 77.1, 75.0, 65.7, 41.0, 40.8, 38.1, 37.7, 35.2, 34.7, 34.0, 27.5, 27.4, 27.3, 23.1, 11.8.

Cyclic Phospholine

A solution of phospholine (51.7 mg) and DCC (2.1 g) in pyridine (20 ml) was stirred at room temperature for 115 hours. After concentration *in vacuo*, the residue was extracted with ethyl acetate, followed by butanol. After the butanol extract was concentrated, the residue was chromatographed on preparative TLC (Kieselgel 60) using CH_3CN - H_2O (5:1) as a solvent. Fractions containing cyclic phospholine was collected and concentrated. The residue was supplied to Sephadex LH-20 (1.0 i.d.×21 cm) column chromatograph using MeOH as a solvent. Fractions containing cyclic phospholine was collected and concentrated to dryness to give a light yellow amorphous powder (15 mg):

MP 182°C (dec); IR (KBr) cm^{-1} 1710, 1210, 1095, 1040; FAB-MS *m/z* 496 (M+1); ^{13}C NMR data are listed in Table 2.

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