J. Antibiot. 58(9): 573–582, 2005

ORIGINAL ARTICLE

THE JOURNAL OF ANTIBIOTICS

A pH-Stability Study of Phoslactomycin B and Analysis of the Acid and Base Degradation Products

Suparna Das Choudhuri, Sloan Ayers, William H. Soine, Kevin A. Reynolds

Received: June 8, 2005 / Accepted: September 1, 2005

Abstract Phoslactomycin B (PLM-B), a potent and selective inhibitor of serine threonine phosphatase is of interest for its antitumor, antifungal and antiviral activity. Described herein is an evaluation of the solution stability of phoslactomycin B at various pH and temperature conditions. Phoslactomycin B was produced from a NP1 mutant strain of *Streptomyces* sp. HK-803 and purified by semi-preparative HPLC. A study of PLM-B degradation was carried out in the pH range of 2~10 at 30°C and 50°C using an HPLC assay. The PLM-B decomposition was observed to exhibit a U-shaped pH profile and demonstrated both acid and base-catalyzed decomposition. The decomposition could be described by the equation $k_{OBS}=k_H\times10^{-pH}+k_{OH}\times10^{pH-14}$ ($k_H=45\pm7\,M^{-1}h^{-1}$; $k_{OH}=448\pm73\,M^{-1}h^{-1}$). PLM-B was found to be most stable at pH 6.63. The major acid and base products were separated and purified. Mass spectroscopic and NMR analysis revealed hydrolysis of the α,β-unsaturated lactone provided the major degradation product under base conditions. Two other products in which hydration of the α,β-unsaturated double bond preceded hydrolysis or methanolysis of the lactone were obtained. Under acidic condition MS and NMR analysis revealed that a dehydration step provided a C_9-C_{11} phosphorinane derivative of PLM-B as one of the major products. The remaining acid degradation products were shown to be mixture of various dehydration products containing an additional double bond in central core of the PLM-B carbon skeleton. The major acid and base degradation products had dramatically reduced antifungal activity despite retaining the same structural core.

Keywords phoslactomycin, stability, phosphatase inhibitor

Introduction

Streptomyces produce a series of structurally novel antifungal and antitumor agents named phoslactomycins (PLMs) [1, 2], phospholine [3, 4], phosphazomycin A [5, 6], leustroducsins [7, 8] and fostriecin [9]. These agents are structurally related and contain an unusual phosphate ester, an α,β-unsaturated δ-lactone and either a conjugated diene or triene chain. PLMs (Figure 1) were isolated from several *Streptomyces* strains including *Streptomyces* sp. HK 803 [10].

This class of compounds are highly potent and selective inhibitors of protein phosphatases including type 1 (PP1) and type 2 (PP2A), which is proposed to be the principle mechanism for their antitumor activity [11~14]. It has been reported that a phase I clinical trial of fostriecin at the National Cancer Institute when concerns with drug purity and drug storage stability proved problematic [9, 15]. Multiple different approaches have been taken to overcome these problems, notably on the issue of supply of the pure natural product and the preparation of analogs. In the case of fostriecin numerous total syntheses have been reported [16~22]. This synthetic approach has also provided

K. A. Reynolds (Correspondence author), ISBDD Suite 212B, Biotechnology Park, 800 East Leigh Street, Richmond, VA 23219, E-mail: reynoldsk@pdx.edu
S. D. Choudhuri, S. Ayers, W. H. Soine: Institute for Structural Biology and Drug Discovery and Department of Medicinal Chemistry, Virginia Commonwealth University, Richmond VA-23219

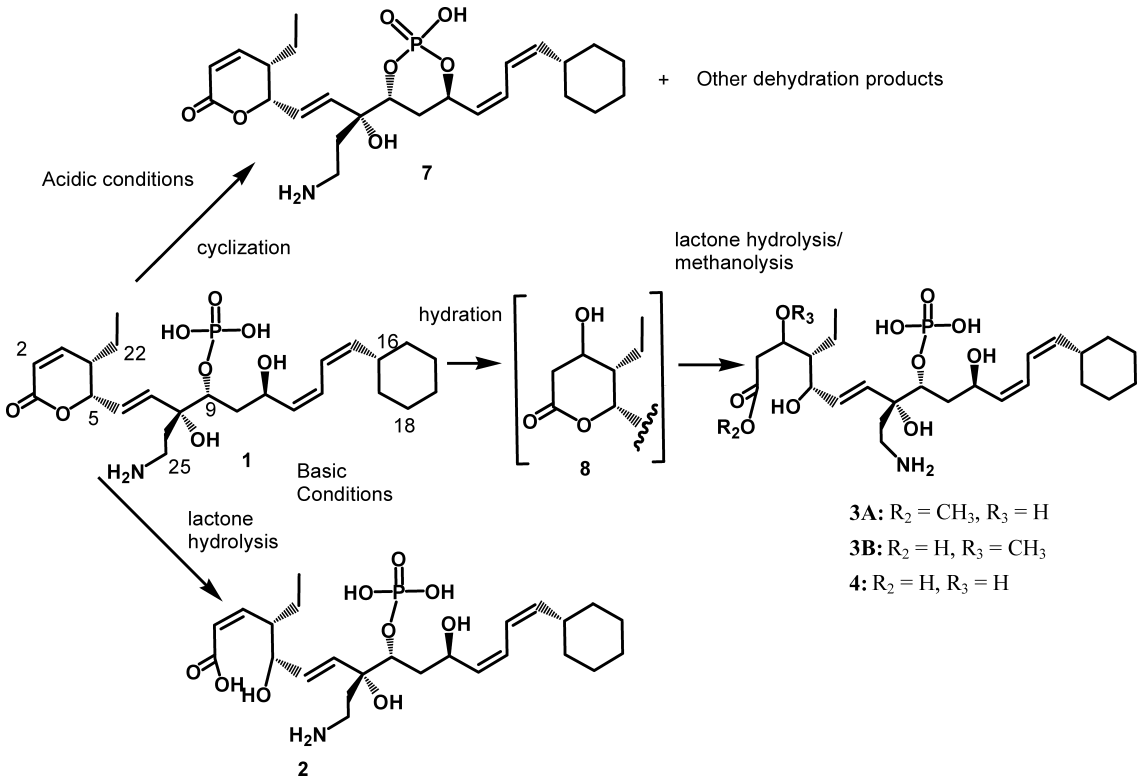

Fig. 1 A proposed degradation mechanism for PLM B (**1**) in acidic and basic aqueous medium.

numerous fostriecin analogs which have been used to demonstrate that the unsaturated lactone plays a fundamental role in selective protein phosphatase inhibition [23]. In the case of phoslactomycins, a different approach of genetic engineering has been reported [10].

Phoslactomycins (PLMs) are produced in low yield by *Streptomyces* sp. HK803 as a mixture of structurally related compounds namely A to F [2]. Biosynthetic studies have indicated that PLM-B (Figure 1) may be a common intermediate in the biosynthetic pathway and that subsequent hydroxylation at C18 and esterification gives rise to the remaining PLM analogs [24]. The PLM biosynthetic gene cluster was cloned and sequenced and an ORF ($\Delta plmS_2$) encoding the putative cytochrome p450 monooxygenase putatively responsible for this hydroxylation was identified [10]. An NP1 mutant ($\Delta plmS_2$) was generated and shown to produce only PLM-B at titers six fold higher than that observed for the wild type strain [10].

Issues pertaining to the stability of either fostriecin, phoslactomycin or any other member of this class of compounds have not been addressed. This report details an analysis of PLM-B degradation under a range of pH and temperature conditions. The major decomposition products were isolated and characterized by mass spectroscopy and NMR analysis. Overall, PLM-B was observed undergo both acid and base-catalyzed degradation but to have reasonable solution stability at pH 6.6 ($t_{1/2}$=72 days, 50°C).

Materials and Methods

Strain and Culture Conditions

The mutant species NP1 of *Streptomyces* HK 803 yielding only PLM-B has been described previously [10]. The NP1 mutant strain was grown at 28°C on SY agar medium (1.0% soluble starch, 0.1% yeast extract, 0.1% N-amine, type A at pH 7.0). A loopful of inoculum (from SY agar plate) was inoculated in a baffled 500 ml flask containing 75 ml of the production medium (2.0 % glucose, 0.1% beef extract, 1.0 % soybean flour, 0.2 % NaCl, 0.005 % K_2HPO_4 and 0.2% L-phenylalanine at pH 7.0) and was incubated in the dark for 96 hours on a rotary shaker (New Brunswick Model G-25D), at 200 rpm and 28°C.

Equipment

HPLC was performed on a system equipped with Waters 600 pump connected to Waters 2487 Dual Absorbance Detector. The semi-preparative and the analytical columns used were Phenomenex C18, 5 μm (250×10.0 mm) and

Waters Symmetry C18 5 μm. (4.6×250 mm), respectively. The mobile phase was solvent A; acetonitrile/0.05% formic acid (20 : 80), solvent B; acetonitrile/0.05% formic acid (80 : 20), Gradient conditions; 0%B～60%B from 0～60 minutes. The flow rate for semi-preparative HPLC was 3 ml/minute and 1 ml/minute for analytical HPLC. Detection of PLM-B and PLM-B degradation products was carried out by UV analysis (235 nm). Mass spectroscopic analysis was carried out using a Waters 2990 Separation Module, Waters 2996 Photodiode Array Detector, and Micromass ZMD 4000 mass spectrometer. The electrospray probe was operated at 3.5 kV with a cone voltage of 50 V. The source and desolvation temperatures were 150°C and 400°C respectively. Spectra were processed using Masslynx^{TM} 3.4 V (Micromass, Manchester, UK). NMR spectra were acquired on a Varian Inova 400 MHz spectrometer with a Sun Microsystems Ultra 1 processor and VNMR version 5.1c software. All constant temperature incubations were done using an incubator (±0.5°C, Precision Scientific, Chicago, IL.)

Isolation, Purification and Quantification of PLM-B

After completion of fermentation, a typical 24×75 ml fermentation broth was centrifuged. The precipitant were treated with 70% acetone then centrifuged, filtered and the acetone was evaporated. This filtrate was combined with the fermentation broth and loaded on a 500 ml XAD-4 (Sigma-Aldrich) column. The column was washed with deionized water (five column volumes) and eluted with 100 % methanol. The methanol eluate containing the active component was concentrated to dryness to give a brown residue. The residue was dissolved in methanol, filtered (0.2 μl PTFE filter, Millipore Corp, MA), and purified by reverse-phase semi-preparative HPLC using the semi-preparative column and the conditions described above. Fractions containing PLM-B were pooled and concentrated under reduced pressure to remove acetonitrile. The remaining aqueous solution was lyophilized to obtain PLM-B. The purity of the PLM-B was verified using an analytical column (retention time 37 minutes). In methanol PLM-B exhibited a λ_{max} at 235 nm [10] (ε=0.3180×10^4). A 2 mg/ml solution of PLM-B was used to construct a standard curve for quantification of PLM-B by HPLC analysis. These analyses revealed that typical fermentation titers of 10 mg/liter (before purification) and 2 mg/liter (after purification).

Preparation of Buffers

For kinetic analysis, chemicals used for the preparation of buffers were of analytical grade (Fisher Scientific). The following buffers were used KCl - HCl (pH 2.0), glycine - HCl (pH 3.0), sodium citrate - citric acid (pH 4.0), potassium phosphate (pH 5.0), potassium phosphate (pH 6.0), potassium phosphate (pH 7.0), Tris - HCl (pH 8.0), boric acid (pH 9.0), carbonate bicarbonate (pH 10.0) and sodium phosphate - NaOH (pH 11.0). The ionic strength of the buffers was maintained at 0.1 M.

Decomposition of PLM-B

The decomposition reactions were initiated by addition of 100 μl of 19～23 μmol PLM-B (dissolved in methanol) in 900 μl of buffer previously equilibrated to either 30°C or 50°C. All incubations contained 10% methanol to maximize solubility of PLM-B and were carried out in Teflon-lined screw-cap vials. Aliquots (100 μl) were withdrawn at short time intervals for pH of 2.0, 3.0, 9.0 and 10.0 (fast degradation), analyzed until the concentration of PLM-B was negligible and at longer time intervals for pH 4.0, 5.0, 6.0, 7.0 and 8.0 (slow degradation) until the concentration had decreased by more than 75%. Analysis of the decomposition was carried out by HPLC using the same the conditions used to analyze for PLM-B purity.

Isolation and Characterization of PLM-B Degradation Products

Isolation of the degradation products was achieved using the same HPLC conditions and semi-preparative column used to purify PLM-B. Fractions corresponding to each decomposition product were collected and then evaporated to dryness under reduced pressure at 30°C. The purity of the isolated decomposition products were then confirmed by HPLC. NMR, mass spectroscopy and UV analyses were obtained for each of the pure major degraded products as well as PLM-B.

PLM-B (**1**): (Phosphoric acid mono-{1-[1-(2-amino-ethyl)-3-(3-ethyl-6-oxo-3,6-dihydro-2*H*-pyran-2-yl)-1-allyl]-7-cyclohexyl-3-hydroxy-hepta-4,6-dienyl}ester). MS *m/z* 514 $[M+H]^+$, *m/z* 512 $[M-H]^-$; UV λ_{max} 235 nm (in methanol), ε=0.3180×10^4; ^{1}H NMR (400 MHz, CD_3OD): 7.10 (1H, dd, *J*=4.4, 9.2 Hz, 3-H), 6.30～6.20 (2H, m, 13-H, 14-H), 6.09～5.93 (3H, m, 2-H, 6-H, 7-H), 5.43 (1H, dd, *J*=8.0, 8.0 Hz, 12-H), 5.33 (1H, dd, *J*=8.0, 8.0 Hz, 15-H), 5.11 (1H, dd, *J*=4.4, 4.4 Hz, 5-H), 4.95 (1H, m, 11-H), 4.28 (1H, dd, *J*=9.2, 9.2 Hz, 9-H), 3.13～2.99 (2H, m, 25-H), 3.48 (1H, m, 16-H), 2.55 (1H, m, 4-H), 2.02～1.93 (2H, 2 m, 24-Ha, 24-Hb) 1.74～0.95 (8H, m, 17-H, 18-H, 19-H, 20-H, 21-H, 22-H, 23-H).

^{13}C NMR (400 MHz, CD_3OD): 136.20 (C-7), 133.47 (C-12), 139.41 (C-15), 123.68 (C-13), 121.88 (C-14), 120.08 (C-2), 126.71 (C-6), 115.32 (C-3), 81.34 (C-5), 77.60 (C-9), 63.69 (C-11), 39.54 (C-4), 36.70 (C-16), 36.44 (C-25),

39.60 (C-10), 33.31 (C-21), 26.0 (C-18), 26.05 (C-20), 25.99 (C-19), 25.97 (C-17), 10.42 (C-23).

Degradation product **2**: (8-(2-Amino-ethyl)-15-cyclohexyl-4-ethyl-5,8,11-trihydroxy-9-phosphonooxy-pentadeca-2,6,12,14-tetraenoic acid). MS *m/z* 532 $[M+H]^+$, *m/z* 530 $[M-H]^-$; UV 235 nm; ^{1}H NMR (400 MHz, CD_3OD): 6.29~6.20 (2H, m, 13-H, 14-H), 6.03 (1H, d, *J*=11.6 Hz, 2-H), 5.91~5.85 (1H, dd, *J*=7.2, 15.6 Hz, 6-H), 5.62 (1H, d, *J*=15.6 Hz, 7-H), 5.51 (1H, dd, *J*=10.2, 10.2 Hz, 3-H), 5.42 (1H, dd, *J*=8.4, 8.4 Hz, 12-H), 5.32 (1H, dd, *J*=8.0, 8.0 Hz, 15-H), 5.10 (1H, m, 11-H), 4.25 (1H, dd, *J*=8.0, 8.0 Hz, 9-H), 4.14 (1H, dd, *J*=5.6, 5.6 Hz, 5-H), 3.3 (1H, m, 4-H), 3.06 (1H, m, 25-H), 2.48~2.45 (1H, m, 16-H), 2.16~2.13 (1H, m, H-24b), 1.93~1.86 (1H, m, H-24a), 2.01~0.95 (8H, m, 10-H, 17-H, 18-H, 19-H, 20-H, 21-H, 22-H, 23-H).

Degradation product **3**: (8-(2-Amino-ethyl)-15-cyclohexyl-4-ethyl-3,5,8,11-tetrahydroxy-9-phosphonooxy-pentadeca-6,12,14-trienoic acid methyl ester). MS *m/z* 564 $[M+H]^+$, *m/z* 562 $[M-H]^-$; UV 235 nm, ^{1}H NMR (400 MHz, CD_3OD): 6.29~6.19 (2H, m, 13-H, 14-H), 6.02~5.96 (1H, dd, *J*=8.0, 15.6 Hz, 6-H), 5.63 (1H, d, *J*=16.0 Hz, 7-H), 5.41 (1H, dd, *J*=4.8, 4.8 Hz, 12-H), 5.28 (1H, dd, *J*=9.6, 9.6 Hz, 15-H), 5.10 (1H, m, 11-H), 4.29 (1H, dd, *J*=12.8, 12.8 Hz, 9-H), 3.81 (1H, dd, *J*=0.4, 4.0 Hz, 5-H), 3.13 (1H, m, 3-H), 3.08 (1H, m, 25-H), 2.59~2.37 (2H, m, 4-H, 16-H), 2.28~0.95 (10H, m, 2-H, 10-H, 17-H, 18-H, 19-H, 20-H, 21-H, 22-H, 23-H, 24-H).

Degradation product **7**: (6-{5-Amino-3-[6-(4-cyclohexyl-buta-1,3-dienyl)-2-hydroxy-2-oxo-2λ^5-[1,3,2]dioxaphosphinan-4-yl]-3-hydroxy-pent-1-enyl}-5-ethyl-5,6-dihydri-pyran-2-one). MS *m/z* 496 $[M+H]^+$, *m/z* 494 $[M-H]^-$; UV 235~237 nm, ^{1}H NMR (400 MHz, CD_3OD): 7.10 (1H, dd, *J*=4.8, 10.0 Hz, 3-H), 6.22~6.15 (1H, dd, *J*=12.0, 15.2 Hz, 13-H), 6.07~5.96 (4H, m, 2-H, 6-H, 7-H, 14-H), 5.69~5.65 (2H, m, 12-H, 15-H), 5.12~5.09 (1H, dd, *J*=4.8, 6.8 Hz, 5-H), 4.61 (1H, dd, *J*=9.2, 15.6 Hz, 9-H), 4.54~4.48 (1H, m, 11-H), 2.99 (2H, t, 25-H), 2.62~2.58 (1H, m, 4-H), 2.37 (1H, m, 16-H), 2.01~0.95 (8H, m, 10-H, 17-H, 18-H, 19-H, 20-H, 21-H, 22-H, 23-H). ^{13}C NMR: 151.68 (C-3), 141.84 (C-15), 136.43 (C-7), 132.86 (C-13), 130.97 (C-12), 127.22 (C-6), 126.67 (C-14), 120.08 (C-2), 81.68 (C-5), 79.30 (C-9), 77.0 (C-11), 48.34 (CD_3OD), 35.93 (C-25), 39.65 (C-4), 41.20 (C-16), 37.57 (C-10), 35.43 (C-24), 10.35 (C-23), 21.85 (C-22), 26.13~33.07 (C-17, C-18, C-19, C-20, C-21).

Antifungal Assay

Antifungal activity was determined using agar diffusion tests against *Rhodotorula glutinitis* (ATCC 2527). The strain was rehydrated with a few drops of Butterfields buffer (International Bioproducts) and 100 μl of this suspension was transferred to a standard potato dextrose agar (PDA) plate (Culture Media and Supplies, Oswego, IL). The plate was incubated at 25°C for 3 days. the pink colonies were harvested on the tip of a micro loop and smeared onto PDA slants in 50 ml centrifuge tubes and incubated under the same conditions. These were stocks for the entire assay and stored at room temperature for several weeks. In each slant about 10 ml of Butterfields buffer was added and vortexed. The resulting suspension was transferred in a sterile 50 ml tube and diluted to 50 ml. About 100 μl of this cell suspension was overlaid on a PDA plate. Sterile assay discs (~7mm diameter, from Whatman No.1 filter paper) were loaded with 20 μl of pure PLM-B as well as both the acidic (pH 2.0) and basic (pH 10.0) degradation products and placed on the inoculated plates. Two different concentrations (0.05 mg/ml and 0.08 mg/ml) of PLM-B and the degradation products were used. The plates were incubated at 25°C for 3 days, and an inhibition zone surrounding the discs was measured.

Results and Discussion

Kinetic Analysis of PLM-B Degradation

The aqueous stability of PLM-B was determined at 50°C and 30°C by measuring the disappearance of the PLM-B peak in the HPLC analysis. The decomposition of PLM-B at 30°C was slowest in the pH range of 5~8. The slow decomposition rate at 30°C, and the eventual loss of H_2O/methanol from the reaction vials, precluded monitoring of the disappearance of PLM-B for multiple half-lives and thus an accurate assessment of the rate of decomposition. This problem was overcome by carrying out incubations at 50°C which resulted in an elevated rate of decomposition. Under these conditions solvent loss was not a significant issue as a shorter incubation time was required to monitor the disappearance of PLM-B for at least 2~3 half-lives.

The rate of reaction for the decomposition of PLM-B was determined by regression analysis at all pH and temperatures. The rate of disappearance of PLM-B (k_{OBS}), half-life and correlation coefficient at 50°C and 30°C from this analysis are indicated in Table 1.

pH Effects on PLM-B Degradation

The k_{OBS} values for degradation of PLM-B at 30°C and 50°C were used to construct a pH-rate constant profile (Figure 2). The resulting U-shaped profile showed a marked-pH dependence with the fastest rates of degradation occurring at either pH extreme (See Table 1,

Table 1 Pseudo first-order degradation rate constants and half lives for acid and basic degradation of PLM-B at (A) 30°c and (B) 50°c. * $t_{1/2}$ calculated based on the k_{OBS} by regression analysis

(A)

pH	k_{OBS} (hr^{-1})	$t_{1/2}$ (hr)	r^2
2.0	8.01×10^{-2}	8.65	0.99
5.0	8.0×10^{-4}	8662*	0.37
7.4	1.0×10^{-4}	6930*	0.56
8.0	2.0×10^{-4}	3465*	0.03
9.0	1.4×10^{-3}	495	0.87
10.0	2.02×10^{-4}	34	0.98

(B)

pH	k_{OBS} (hr^{-1})	$t_{1/2}$ (hr)	r^2
2.0	5.19×10^{-1}	1.33	0.98
3.0	4.49×10^{-2}	15.4	0.99
4.0	6.30×10^{-3}	110	0.95
5.0	7.00×10^{-4}	990	0.92
6.0	4.00×10^{-4}	1730	0.98
7.0	7.00×10^{-4}	990	0.91
8.0	2.30×10^{-2}	301	0.99
9.0	2.22×10^{-2}	31.2	0.94
10.0	4.30×10^{-2}	9.40	0.99

Figure 2). In the pH range 5~7, PLM-B exhibited relatively slow degradation. At pH 6 there appeared to be maximum stability with $k_{OBS}=0.0004\,hr^{-1}$ (50°C) ($t_{1/2}=72$ days); approximately three orders of magnitude slower than at either pH 2.0 or 10.0.

For the U shaped graphs, k_{OBS} at a given pH can be defined as $k_{OBS}=k_H\times10^{-pH}+k_{OH}\times10^{pH-14}$ where k_H and k_{OH} are the rate constants in base and acidic conditions. Using this formula and k_{OBS} values, regression analysis provided $k_H=8.4\pm0.1\ M^{-1}h^{-1}$; $k_{OH}=132\pm3\ M^{-1}h^{-1}$ (30 °C), $k_H=45\pm7\ M^{-1}h^{-1}$; $k_{OH}=448\pm73\ M^{-1}h^{-1}$ (50°C). The pH at the minimum of the U shaped graph ($pH_{min}=1/2\ pk_w-1/2\log k_{OH}/k_H$) provided the pH of maximum stability for PLM-B at 50°C (6.6) and 30°C (7.3), consistent with the observation of slowest degradation in the pH range 5 to 7.

HPLC Analysis of PLM-B Degradation Products

As described above, PLM-B was found to degrade in solution at both acidic and basic pH. As shown in Figure 3, HPLC analysis revealed three major degradation products **2**, **3**, and **4** were formed under basic pH conditions (pH >7). Under acidic conditions (pH 2.0 to 5.0), three different degradation products **5**, **6** and **7** were evident in the HPLC chromatograms. When PLM-B was incubated at 50°C at pH of 6.0 all six degradation products were eventually observed.

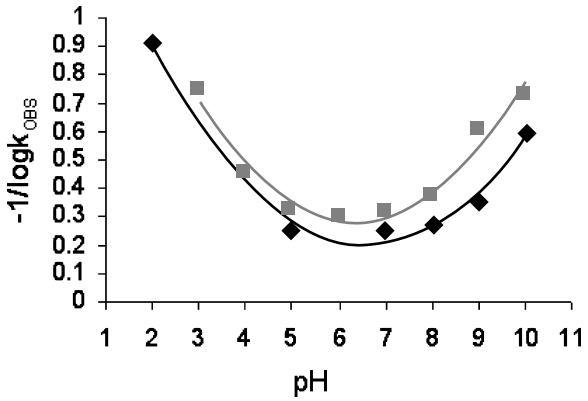

Fig. 2 Plot showing the rate constant-pH profile for the degradation of PLM-B (**1**) at 30°C (◆) and 50°C (■).

Relative Rates of Basic and Acid Degradation Products

The rate of formation of all three basic degradation products **2**, **3** and **4** increased with the increase in pH. Furthermore, the ratio of the degradation products was **2**>**3**>**4** under all basic pH conditions (Figure 4A). These analyses were based on peak areas in the HPLC chromatogram. The molar ratio of **2** relative to **3** and **4** is likely to be even greater as it retains the α,β-double bond of PLM-B (as described below this is lost in **3** and **4**) and have the largest extinction coefficient.

The rate of formation of the acidic degradation products **5**, **6** and **7** was found to increase with the decrease in pH. Towards the end of the degradation under all acid pH conditions the amount of products based on HPLC area was consistently **6**>**5**>**7**. However, the ratio **5**>**6**>**7** was seen in the initial stages of degradation. This observation can be clearly seen in Figure 4B which represents the HPLC peak areas associated with the degradation products **5**, **6** and **7** after incubation at pH 3.0, 4.0 and 5.0 for 24 hours. At pH 3 with the faster and more complete degradation the ratio is **6**>**5**>**7**. The ratio of **5** and **6** is more comparable at pH 4, and at pH 5, where there was much less PLM-B degradation, the ratio is very clearly **5**>**6**>**7**.

Chemical Analysis of PLM-B

The structure of PLM-B isolated from the NP1 mutant was confirmed by MS, ^{1}H NMR, ^{1}H-^{1}H COSY and HSQC analysis. The ^{1}H and ^{13}C NMR assignments (Tables 2 and 3) were consistent with previous assignments [2, 4]. Similarly the LC/MS analysis of the PLM-B was obtained

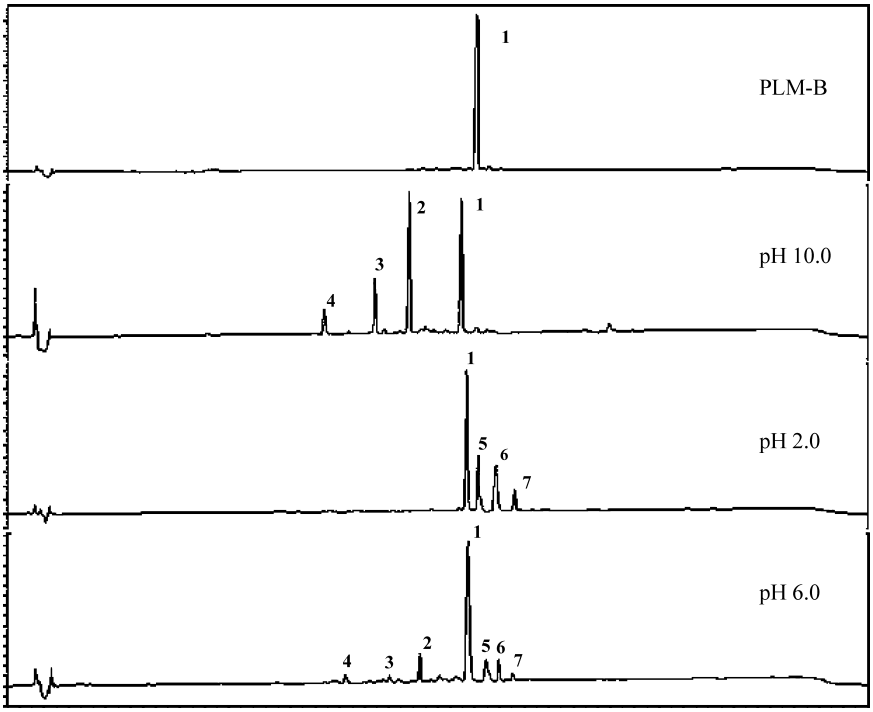

Fig. 3 Typical HPLC chromatograms of PLM-B (**1**) and its degradation at pH 10.0, 2.0 and 6.0 respectively. The degradation products are denoted by **2**, **3** and **4** (basic pH) and **5**, **6** and **7** (acidic pH).

in both positive and negative ESI mode (*m/z* 514 $[M+H]^+$, *m/z* 512 $[M-H]^-$) was consistent with both the structure and previous analyses [2].

Chemical Analysis of PLM-B Base Degradation Products

LC/MS spectra of the basic degradation product **2** gave a parent $[M+H]^+$ at *m/z* 532 and a parent $[M-H]^-$ at *m/z* 530. This product indicates an increase in mass of 18 Da, consistent with addition of one water molecule. The ^{1}H-NMR spectrum of **2** was very similar to PLM-B except for the significant shifts in resonances associated with the lactone ring (Table 2, notably an upfield shift for 5-H) and consistent with base-catalyzed lactone hydrolysis (see Figure 1). Resonances for olefinic protons at 2-H and 3-H indicate that the α,β-double bond was retained in **2**. The purified product 2 on incubation at pH 10.0 (50°C) for 72 hours was found to be stable, demonstrating that is not an intermediate in the formation of products **3** and **4**.

The LC/MS of product **3** showed an increase in mass by 50 Da (*m/z* 564 $[M+H]^+$, *m/z* 562 $[M-H]^-$) compared to PLM-B, consistent with addition of both water and methanol. Similar to product **2**, this product showed significant shifts in the ^{1}H NMR resonances associated with the lactone ring, consistent with opening of the lactone (notably an upfield shift of the 5-H signal and shifts in the 6-H and 7-H resonances). In contrast to **2** and PLM-B, the downfield resonances for 2-H and 3-H are shifted upfield (~4 ppm) as a result of hydration of the α,β-saturated double bond. This analysis suggests product **3** being formed by an initial hydration of this double bond and subsequent methanolysis of the lactone ring (Figure 1) (an alternative product formed by hydrolysis on the lactone ring and addition of methanol across the double bond cannot be ruled out). The resonance for the proposed C-3 methoxy group was not observed and is likely masked by the solvent (CD_3OH) signal (3.24~3.38).

Product **4** showed an increase in mass by 36 Da (*m/z* 550 $[M+H]^+$, *m/z* 548 $[M-H]^-$) consistent with addition of two water molecules. It is likely that **4** is formed initially by hydration of the α,β-double bond of the unsaturated lactone, and subsequent lactone hydrolysis (Figure 1), although there was insufficient material to verify this by ^{1}H-NMR analysis.

Proposed Pathways for Base-Catalyzed Degradation Mechanism of PLM-B

Under base conditions ring opening of the unsaturated lactone ring of PLM-B is the major product (product **2**) (Figure 1). This appears to be a stable end product and prolonged incubation with base did not lead to additional changes (notably there was no loss of the double bond of

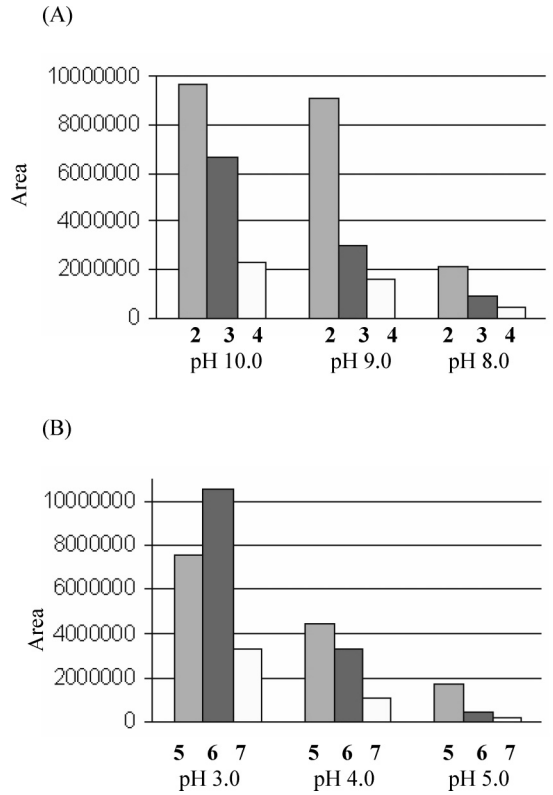

Fig. 4 Histogram representing the HPLC peak areas for (A) degradation products **2**, **3** and **4** formed at pH 10.0, 9.0 and 8.0 after 24 hours incubation at 50°C and (B) degradation products **5**, **6** and **7** formed at pH 3.0, 4.0 and 5.0 after 24 hours incubation at 50°C.

Table 3 Assignment of ^{13}C-NMR resonances for PLM-B and degraded product **7**

Assignment	δ (ppm) PLM-B	δ (ppm) **7**
C-2	120.08	120.08
C-3	151.32	151.68
C-4	39.54	39.65
C-5	81.34	81.68
C-6	126.71	127.22
C-7	136.20	136.43
C-8	ND	ND
C-9	77.60	79.30
C-10	39.60	37.50
C-11	63.69	77.0
C-12	133.47	130.97
C-13	123.68	132.86
C-14	121.88	126.67
C-15	139.41	141.84
C-16	36.70	41.20
C-17	25.97*	26.13*
C-18	26.00*	26.14*
C-19	25.99*	26.18*
C-20	26.05*	26.20*
C-21	33.40*	33.07*
C-22	21.81	21.85
C-23	10.42	10.35
C-24	33.33	35.43
C-25	36.14	35.93

* Overlapping signals for C17 - C21. ND- Not Detected.

Table 2 Partial ^{1}H-NMR assignments for PLM-B and degradation products **2**, **3** and **7**

Assignment	δ (ppm) PLM-B	δ (ppm) **2**	δ (ppm) **3**	δ (ppm) **7**
2-H	6.09	6.03	1.72 (H_a) 2.28 (H_b)	6.07
3-H	7.09	5.51	3.13	7.10
4-H	2.55	3.03	2.59	2.61
5-H	5.11	4.14	3.81	5.12
6-H	6.04	5.91	6.02	5.69~5.60
7-H	5.97	5.62	5.63	
9-H	4.28	4.25	4.29	4.61
11-H	4.95	5.10	5.10	4.54~4.48
12-H	5.41	5.42	5.41	5.69~5.60
15-H	5.33	5.32	5.28	
13-H, 14-H	6.32~6.20	6.29~6.20	6.29~6.19	6.22~5.98
16-H	2.48	2.48	2.37	1.97
25-H	3.04	3.06	3.08	2.99

the α,β unsaturated lactone). Products **3** and **4** involve an addition reaction across the α,β-double bond and it can be concluded (based on the stability of **2**) that this addition reaction must occur prior to the hydrolysis of the lactone. The proposed intermediate product **8** (Figure 1) could not be clearly identified in the LC-MS analyses. However, small peaks were observed in the HPLC traces of PLM-B under base degradation (these peaks were relatively small compared to **2**, **3** and **4** and did not increase during the time course of the degradation study) and one of these could be the proposed intermediate. Thus subsequent lactone hydrolysis and/or methanolysis step must proceed at a comparable rate to the addition reaction (conversion of **3** to **8**) (see Figure 1). The two step reaction for the formation of products **3** and **4** is slower than direct one step hydrolysis for the formation of **2** (the ratio of **2**>**3**>**4** was observed at all stages during base-catalyzed PLM-B degradation). Product **3** was found to be unstable and reverted back to PLM-B on storage in deuterated methanol.

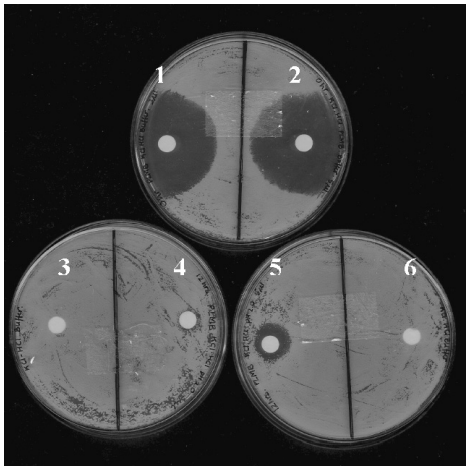

Fig. 5 Assaying antifungal activity of PLM B and acid degradation products generated at two different concentrations.1 and 2: 0.05 mg and 0.08 mg of PLM-B, 4 and 5: 0.05 mg and 0.08 mg of PLM-B degraded at pH 2.0 for 24 hours, 3 and 6 are negative controls.

Chemical Analysis of PLM-B Acid Degradation Products

LC/MS analysis of PLM-B incubated at pH 2.0 indicated the formation of 3 degradation products. All three acidic degradation products showed a decrease in mass of 18 Da (m/z 496 $[M+H]^+$) but with different retention times consistent with formation of dehydration products. The UV spectral analysis of all the acid degradation products revealed λ_{max} from 235~238 nm that was identical to PLM-B. This observation would preclude the dehydration of the C-11 hydroxyl group to form a double bond between C-10 and C-11 and formation of a conjugated triene (resulting in a shift in λ_{max} to a longer wavelength) for all of these cases. Further characterization of the acid degradants was carried out by NMR analysis. The ^{1}H-NMR and ^{1}H-^{1}H COSY spectra of the decomposition product **7** showed an intact α,β-unsaturated lactone ring (2-H, 3-H and 5-H resonances appeared essentially unchanged from observed for PLM-B, see Table 2), and all of the other resonances for PLM-B (including the vinyl protons 12-H, 13-H, 14-H and 15-H). These observations preclude **7** from being a dehydration product which involves generation of a new carbon–carbon double bond. A small upfield and downfield chemical shift was seen for 11-H and 9-H, respectively (Table 2). HSQC analysis revealed that most of the ^{13}C resonances were unchanged with the exception of a 14 ppm shift for C-11 (Table 3). These analyses are consistent with **7** being a C-9/C-11 cyclic phosphorinane dehydration product of PLM-B (Figure 1). Compound **7** differs from a C-8/C-9 cyclic phosphorinane previously obtained by treatment of phospholine with dicyclohexylcarbodiimide. A similar C-8/C-9 phosphorinane derivative of fostriecin has also been reported [23].

The ^{1}H NMR spectral analysis indicated that **5** and **6**, which were well-resolved sharp peaks in HPLC analysis, were comprised of mixtures of related compounds. All appeared to contain the α,β-unsaturated lactone but additional double bonds in the central carbon core consistent with dehydration reactions involving the C-8 OH group of PLM-B. As discussed above the product ratio in the initial stages of degradation (**5**>**6**>**7**) was different to that observed for the latter stages (**5**>**6**>**7**). This observation would be consistent with degradation product **5** containing one or intermediates in pathway to forming components of degradation product **6**. The presence of a potential multistep process and closely related products which we were unable to resolve by HPLC precluded a facile delineation of the acid degradation process.

Antifungal Activity of PLM-B and PLM-B Degradants

An antifungal assay demonstrated potent activity of PLM B against the fungus *Rhodotorula glutinitis*, as indicated by a zone of inhibition. No significant antifungal activity was observed in degradation products of PLM B (Figure 5); a significantly reduced zone was seen for 0.08 mg of the combined acid degradants, and no clear zone was seen for 0.05 mg of this mixture (5 μl of 0.08 mg/ml and 0.05 mg/ml of PLM-B degraded in 50 μl of buffer at pH 2.0 and 10.0 for 24 hours). Similar observations of poor antifungal activity were made with the base degradants.

Conclusion

PLM-B decomposition was observed to exhibit a U-shaped pH-rate profile, described by the equation $k_{OBS}=k_H\times10^{-pH}+k_{OH}\times10^{pH-14}$ (where $k_H=45\pm7\,M^{-1}\,h^{-1}$ and $k_{OH}=448\pm73\,M^{-1}\,h^{-1}$ at 50°C). Based catalyzed-degradation was faster than acid-catalyzed degradation. The rate of degradation was faster at either pH extreme. PLM-B was most stable in solution at a pH 6.6 (close to neutrality).

PLM-B was found to degrade to three major products in basic aqueous conditions and more than three different products in acidic aqueous conditions. In base hydrolysis of the lactone ring is the major decomposition product. The two other products also involve opening of the lactone but are preceded by hydration of the α,β-double bond. Multiple acid dehydration productions are obtained, including a C-9/C-11 cyclic phosphorinane derivative of PLM-B.

Previous reports have suggested that the various substituents at C-18 of PLM-B moiety yielding PLM analogs A and C-F do not contribute to the antifungal activity [1]. In contrast, none of the PLM-B degradation products had significant antifungal activity, demonstrating that an intact lactone ring, the phosphate group and the C-8 hydroxyl group all contribute to the antifungal activity. These groups have also been shown to be important for the selective protein phosphatase activity of fostriecin [23].

The apparent stability of PLM-B along with a mutant strain NP1 [10] which exclusively produces this product provides an exciting basis for the continued development of this class of compounds.

Achnowledgmments This work was funded by a grant AI51629 form the National Institutes of Health. We are grateful to Professor Hiroyuki Osada for providing the original *Streptomyces* sp. HK803 strain and to Dr. Michael Hindle for help with the LC-MS analyses.

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