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Phosphonic acid analogue of forfenicine – synthesis, antibacterial activity and degradation by *Pseudomonas fluorescens*

Short Communication

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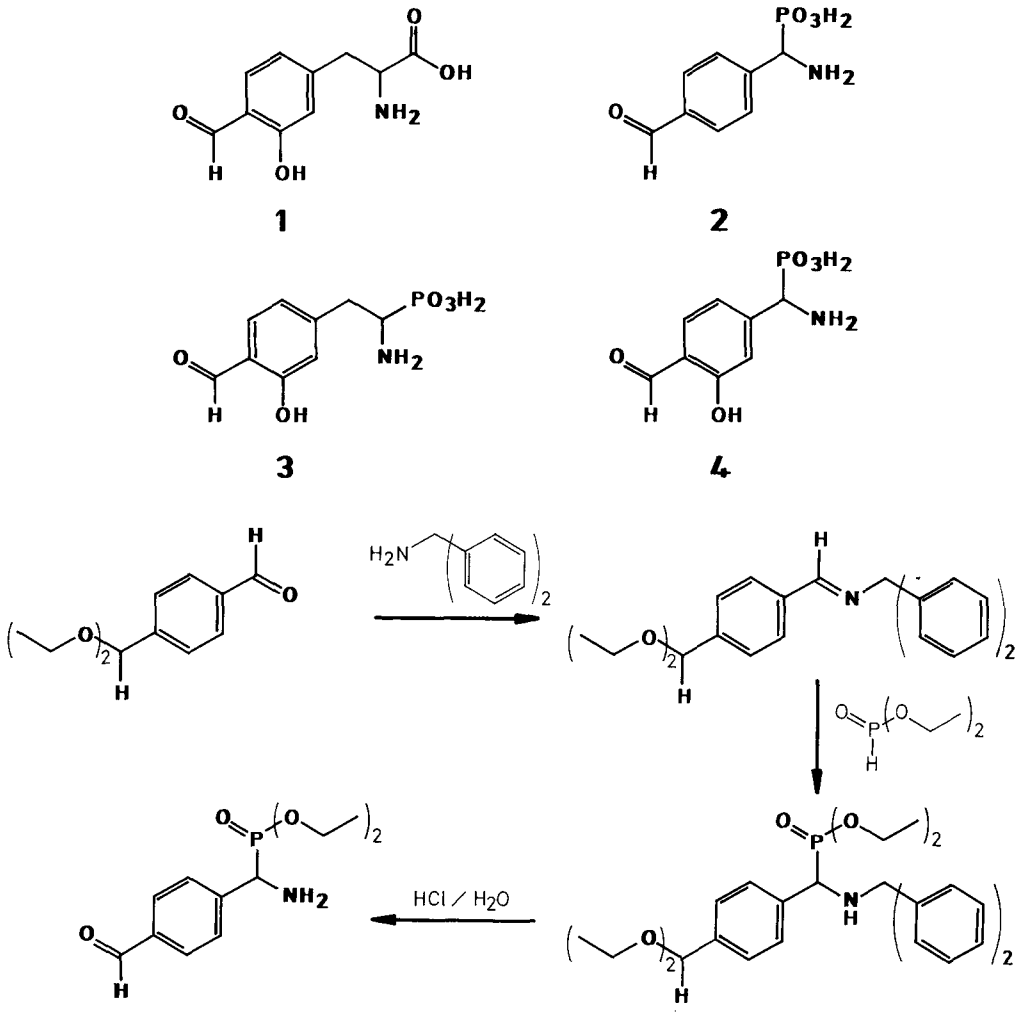
Summary. Phosphonic acid analogue of forfenicine, amino(*p*-formylbenzyl)-phosphonic acid, was synthesized and evaluated as antibacterial agent. As indicated by disc diffusion test this compound was found to inhibit significantly the growth of *Bacillus subtilis* and *Staphylococcus aureus* and moderately the growth of *Escherichia coli*. Resistance of *Pseudomonas fluorescens* to the action of the aminophosphonate may result from the ability of the strain to degrade this compound.

Keywords: Amino acid analogue – Phosphorus-to-carbon bond breakage – Biodegradation – Antibiotic

Introduction

Forfenicine (**1**), a non-protein amino acid produced by many *Actinomycetes*, and its homologues deserve attention since they act as an antibacterial and immunostimulating agents (Aoyagi et al., 1978; Umezawa et al., 1978, 1980a,b). Phosphonic acid analogues of substituted phenylglycines have also received considerable interest because some representatives of this class were found to display herbicidal and antibacterial activity (Linfield et al., 1961; Kafarski and Lejczak, 1991; Maier and Diel, 1994). Amino(*p*-formylbenzyl)phosphonic acid (**2**), although differs from the parent compound in the length of side chain may be considered as an forfenicine analogue, since similarly substituted phenylglycines were found to exhibit similar biological properties as forfenicine (Umezawa et al., 1978). In this paper we continue our studies on the synthesis of aminoalkylphosphonic acids and evaluation of their biological activity and describe synthesis of compound **2**, evaluation of its antibacterial activity and studies on its degradation by wild-type strain of *Pseudomonas fluorescens*.

Results and discussion



Scheme 1

Compound **2** was obtained using modification of the standard procedure outlined in the Scheme 1. The use of benzhydrylamine instead of benzylamine enabled the removal of the group masking amino moiety, hydrolysis of phosphonate esters and hydrolysis of acetal in single step. The trials to obtain formal forfenicine analogues (**3** and **4**) by means of Vilsmayer reaction were totally unsuccessful.

The results of the disc diffusion assay carried out on solid RST medium are shown in Table 1. Compound **2** was moderately or weakly effective against *Staphylococcus aureus* and *Bacillus subtilis*. The activity against *B. subtilis* was practically non-dependent on the presence or absence of aromatic amino acids in the growth medium. Only in the medium deficient in all three amino acids (phenylalanine, tyrosine and tryptophan) the unexpected reversal of the toxic effect was observed. This may result from the ability of this strain to utilize compound **2** for growth under deprivation of all three aromatic amino

Table 1. Sensitivity of bacterial strains to compound 2 determined on solid RST medium

Medium	Diameter of inhibition zone [mm]			
	Bacillus subtilis PCM 1949	*Staphylococcus aureus* PCM 2054	*Escherichia coli* PCM 2057	*Pseudomonas fluorescens*
RST	7.0	6.0	0.0	0.0
RST−Tyr[a]	7.0	7.0	12.0	0.0
RST−Phe	7.0	7.0	6.0	0.0
RST−Trp	7.0	7.0	7.0	7.0
RST−Aar[b]	0.0	7.0	13.0	0.0
RST+Phe	8.0	0.0	7.0	0.0
RST+Tyr	7.0	0.0	0.0	0.0
RST+Aar	7.0	7.0	12.0	0.0

[a]sign "−" means that broth is deficient, whereas "+" that it is supplemented with specified amino acid; [b]Aar – all three amino acids (Phe+Tyr+Trp)

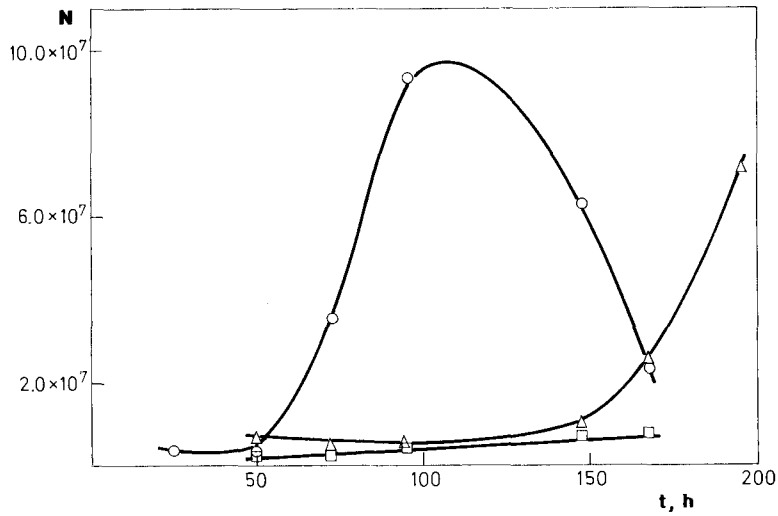


Fig. 1. Kinetics of the growth of *Pseudomonas fluorescens* taken from exponential phase of growth in the broth containing: inorganic phosphate (○), broth deficient in phosphorus (□) and on compound **2** as the sole source of phosphorus (△)

acids. In the case of *S. aureus* supplementation of the broth with phenylalanine or tyrosine caused drastic decrease of antibacterial activity of this compound, suggesting that it might act as antimetabolite of aromatic amino acids. *Pseudomonas fluorescens* appeared to be resistant to action of compound **2**, despite of the composition of growth medium. The only exception is antibacterial activity recorded when this strain was grown in the medium deficient in tryptophan. The most complicated pattern of the activity was observed in the case of *Escherichia coli*. The observed effects are quite difficult to rationalize, but suggest that compound **2** disturbs metabolism of aromatic amino acids in this strain.

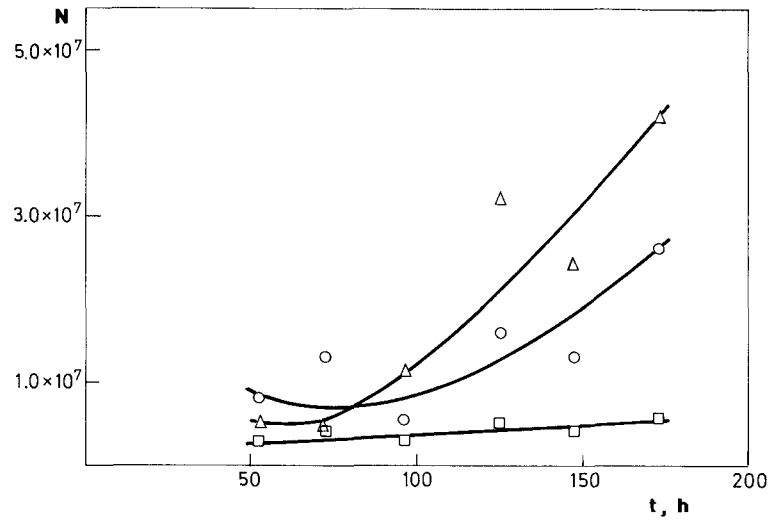


Fig. 2. Kinetics of the growth of *Pseudomonas fluorescens* taken from logarithmic phase of growth in the broth containing: inorganic phosphate (○), broth deficient in phosphorus (□) and on compound **2** as the sole source of phosphorus (△)

Since the resistance of *P. fluorescens* may result from the ability of this strain to degrade toxic aminophosphonate we have also studied the ability of this strain to utilize compound **2** as the sole source of phosphorus or nitrogen for growth. *P. fluorescens* failed to utilize the phosphonic acid analogue of forfenicine as the sole source of nitrogen whereas it grew quite well when this compound was used as a sole source of phosphorus. Rate of utilization of compound **2** as a sole source of phosphorus for growth was strongly dependent on the strain growth phase, namely if bacteria were taken from exponential or stationary phase. The presence of a huge lag phase observed if bacteria from exponential phase (Fig. 1) were applied indicate that this process is induced upon phosphate deprivation. Bacteria taken from stationary phase fastly, and nearly to the same extent as those growing on inorganic phosphate, utilized compound **2** as the sole source of phosphorus (Fig. 2). Anyway, the ability of *P. fluorescens* to degrade the studied analogue of forfenicine may be a cause of the resistance of this strain upon the action of compound **2**.

Materials and methods

Chemical synthesis

Solution of 8.4 g (40 mmol) of diethyl acetal of *p*-formylbenzaldehyde and 7.4 g (40 mmol) of benzhydrylamine in 100 ml of toluene was refluxed for 2 h, then cooled to room temperature and 10 g of anhydrous magnesium sulphate was added. After several minutes sulphate was filtered off and 5.6 g (40 mmol) of diethyl phosphite was added. The solution was then refluxed for 3 h, cooled to room temperature and the solvent was evaporated under reduced pressure. Obtained dense oil (19.7 g, 92% of yield) was dissolved in 100 ml of concentrated hydrochloric acids and refluxed for 4 h. The two-layered product was then extracted with chloroform (100 ml) and aqueous phase was evaporated in vacuo. Ob-

tained semisolid was dissolved in 100 ml of water, decolorized with charcoal and water was removed under reduced pressure. White crystals of crude product were purified by recrystallization from 90% ethanol. Yield 65%, product decomposes at 350°C before melting. Elemental analysis calculated for $C_8H_{10}NO_4P$ (215.14): 6.51% N; 14.40% P; found: 6.32% N and 14.73% P. ^{1}H-n.m.r. (in D_2O), δ in ppm: 4.46 (d, J_{PCH} = 16.5 Hz, 1H, CHP), 7.54 and 7.88 (d, J = 8.1 Hz, 2H each, aromatic protons), 9.84 (s, 1H, CHO); P-n.m.r. (in D_2O) δ in ppm: 10.35.

Microorganisms

The wild type-strain of *P. fluorescens* was isolated from a soil sample collected in industrial vegetable oil plant near Brzeg, Poland (Sztajer et al., 1988). All the other microorganisms were obtained from Polish Collection of Microorganisms (PCM) and are indicated by the appropriate accession number. All the strains were maintained on appropriate standard bacteriological media.

Determination of antibacterial activity by disc diffusion assay

Sensitivity of bacterial strains to compound **2** was elucidated using disc diffusion method on RST minimal medium (Payne and Nisbet, 1980) or the same medium deficient in aromatic amino acids, or supplemented with double amounts of them. Each plate was inoculated with a 0.1 ml sample (about 10^6 cells) of an exponential-phase culture. Paper discs of 5 mm in diameter were saturated with 15 μl portions of forfenicine analogue solution (500 μg/ml) and placed at the surface of solid medium. After incubation for 18 h at 37°C (30°C in the case of *P. fluorescens*) zones of growth inhibition were measured.

*Pseudomonas fluorescens utilization of compound **2** as a sole source of phosphorus*

Growth rate determinations were performed with 250 Erlenmeyer flasks containing 25 ml of the previously described medium (Zboińska et al., 1992) which received 0.25 μl of inoculum (77×10^5 cells) from exponential-phase of growth or 0.1 ml of inoculum ($38,8 \times 10^5$ cells in physiological salt) from stationary-phase of growth. Cultures were incubated at 28°C on a shaking platform at 100 rpm. The growth of the culture was determined either by tubrbidometric method (at 600 nm) or by counting the number of cells by dilution method.

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