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Phosphonic drugs: Experimental and theoretical spectroscopic studies of fosfomycin

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ABSTRACT

pH and time-dependant changes of fosfomycin molecular structure in an aqueous solution are studied by Raman, NMR, and generalized 2D correlation spectroscopies. Interpretation of the experimental spectra is based on the assumption of formation of different species running on applied physicochemical conditions. Geometries of all possible structures were entirely optimized with the 6-311++G(2df,p) basis set at the B3LYP theoretical level using procedures implemented in the Gaussian '03 set of programs. Harmonic frequency calculations verified the nature of the studied structures and allowed to simulate obtained Raman spectra. The theoretical NMR shielding was calculated using the GIAO method at the same computational level. In addition, in some cases PCM model was used to monitor the influence of water molecules on the NMR spectra. It is shown that in the pH range of 1–2 of fosfomycin aqueous solution oxirane ring is open sequent to nucleophilic attack and forms 1,2-dihydroxyphosphonic acid with small content of its monodeprotonated species. On the other hand, in pH 7 and higher it appears either as 1,2-epoxypropylphosphonic or 1,2-dihydroxyphosphonic dianion depending upon whether hydrolysis took place or not. It is also discussed that Raman marker bands originating from the individual species of fosfomycin can be used to detect and/or to monitor this antibiotic in an aqueous medium (for example urine samples). Hence, depending upon the structure found in urine one can tell about metabolic processes of this antibiotic in the body.

1. Introduction

In recent decades it has been observed steadily growing family of natural and synthetic biologically active products containing P–C bond that is resistant to cleavage by strong acids and bases and also by most of enzymes. For example, the heterocyclic phosphonic acids and their analogues show biological activity towards herbicides, bactericides, or biocides [1–3]. The pyridine and alkyl hydroxybisphosphonates such as etidronian, alendronian, clodronian, pamidronian, tiludronian, or recently risedronian are very powerful inhibitors of osteoclastic resorption [4–6] and their derivatives and model compounds are currently of great interest in devising extremely sensitive and specific nanosensors in immunobiological assay and drugs [7,8]. In the last decade, many natural products and so called phosphonomono- and phosphonodipeptides with the C–P bond, in which the *C*-terminal amino acid is replaced by its phosphonic acid analogue, have been synthesized. Their physiological activity ranges from herbicidal, plant hormone growth, antibacterial to anticancer, as well inhibition of enzymes such as phosphatases, glutamine synthetase, cathepsin C, or zinc proteinases [9–13]. It has to be mentioned that our knowledge of the biosynthesis of these compounds is still fragmentary especially with regard to their catabolic processes [14].

Fosfomycin, (*1R,2S*)-(−)-1,2-epoxypropylphosphonic acid (only this enantiomer is bioactive), also belongs to this family since it possesses the C–P bond in its structure. It is a low molecular weight antibiotic that was first isolated in 1969 from *Streptomyces fradiae* assisted in fermented broths [15,16]. It can be synthesized by stereospecific *cis*-epoxidation of (*Z*)-1-propenylphosphonic acid derivatives followed by optical separation from the racemic epoxide with optically active amines [15,17] or by the asymmetric synthesis based on the use of tartaric acid as a chiral auxiliary in directing an appropriate bifunctionalization of prochiral (*Z*)-1-propenylphosphonic acid [18]. This antibiotic is not toxic. It shows only few side effects and is very effective in prevention of recurrent lower urinary tract infections. It exhibits a very broad spectrum of antimicrobial activity against Gram positive and Gram negative bacteria such as *Escherichia coli*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Serratia*, *Streptococcus pneumonia*, and *Staphylococcus aureus*. When adhibit it acts at very early stage in synthesis of bacterial cell wall by inhibiting the UDP-GlcNAc-3-*O*-enolpyruvyltransferase (MurA)

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enzyme that catalyses the condensation of phosphoenolpyruvate with N-acetylglucosamine to form N-acetylmuramic acid [19]. Most likely, the active form of this antibiotic is associated with epoxy fragment of fosfomycin [20,21]. However, there is a group of enzymes that catalyse the hydration of fosfomycin to generate the vicinal diol. Thus, the bacteria's like *Mesorhizobium loti*, *Desulfitobacterium hafniense*, *Brucella melitensis*, or *Clostridum botulinum* are fosfomycin resistant [22,23]. At present, this antibiotic is commercially available in pharmacies under the name MONOURA® or MONOURIL® and contains rather fosfomycin *tris*(hydroxymethyl)ammonium salt than fosfomycin disodium salt as an active compound. Since this medicine dissolves very well in water showing high bioavailability among other fosfomycin derivatives is orally administrated [24,25]. However, it has to survive encounters with various pH (2 in stomach and above 8 in duodenum) and degrading enzymes. Thus, observation of different species that are formed in the pH range of 1–9 and their stability in these conditions is essential for better understanding of the physiological process associated with this antibiotic.

In this work we define all possible molecular structures of fosfomycin species, i.e. 1,2-epoxypropylphosphonic (*epoxy* form) or 1,2-dihydroxyphosphonic (*open*, *diol* form) acids and their mono- and dianions that can be formed in different physicochemical conditions based on the Raman and NMR spectra. The Raman spectra are interpreted with the help of generalized 2D correlation analysis (2DCA) as well as by employing quantum-chemical calculations by using 6-311++G(2df,p) basis set at the B3LYP level of theory, while theoretical NMR shielding was calculated using the GIAO method at the same computational level. In addition, in some cases polarizable continuum model (PCM) was used to monitor the influence of water molecules on the NMR spectra. To our best knowledge, density functional theory (DFT) calculations for fosfomycin have not been published thus far. Only charge distribution and conformational analysis were performed by Smeyers et al. [26] using the standard semiempirical CNDO/2 procedure and *ab initio* calculations with the STO-4G basis set [27]. These calculations were compared to the NMR experimental data to define final structures of obtained fosfomycin derivatives and the products of its enzymatic reactions [18–22,28]. The Raman spectra of fosfomycin in an aqueous solution were compared to these of fosfomycin disodium salt and its monometylated analogue [29] and to three derivatives of stereoisomer of fosfomycin, (*1R,2R*)-1,2-epoxypropylphosphonic [30]. In this field we have extensive experience [31–36] by applying NMR, Raman, and surface-enhancement Raman (SERS) to study such compounds. Despite such research, it has to be mentioned, that there is no single work describing molecular structures of the species that can be formed from fosfomycin in different pH conditions. It is known that neutral fosfomycin after administration may go, depending upon the way of administration and specific for each patient biochemical processes, through several structural changes till final excretion from the organism with urine. Thus, vibrational and NMR analysis of the possible fosfomycin species that are expected to be formed in different conditions are studied in this work and in turn can be useful, especially for Raman identification of them in urine samples. Simply, it looks like this method can be fast and reliable method for fosfomycin analysis.

2. Materials and methods

2.1. Compounds

Fosfomycin disodium salt was purchased from Fluka. MONURAL® (Zambon Group, Italy) containing fosfomycin *tris*(hydroxymethyl)ammonium salt as an active compound and some garnish such as saccharin, sucrose and mandarin or grapefruit flavour (in "trace" concentration) was bought from a local pharmacy. Samples were dissolved in deionized H_2O, according to a drug information leaflet to give 0.4 mol/L solution of this antibiotic. The samples were measured in the pH (pD) range from 1 to 13. pH (pD) was adjusted with concentrated NaOH or HCl to achieve final values.

2.2. Spectroscopic measurements

^1H and ^{31}P NMR spectra were measured on the Mercury VX 300 Hz spectrometer (Varian). The ^1H NMR spectra were measured with TMS as a standard with resonance frequency of 300.08 MHz. ^{31}P NMR spectra were recorded with the 85% H_3PO_4 as an external standard with the 121.47 MHz resonance frequency. ^{13}C NMR spectra were measured on the Bruker AMX 500 MHz spectrometer with proton coupling and decoupling using dioxin as an external standard with resonance frequency of 125.8 MHz. For recording the one-bond and long-bond heteronuclear ^1H–^{13}C correlation spectra gHMQC and gHMBC techniques were used (Mercury VX 300 MHz, Varian).

Raman spectra were recorded using a FT-Raman spectrometer 'MultiRam' (Bruker, Germany) and a FT-Raman Spectrometer Nicolet NXR 9650. These spectrometers were equipped with Nd:YAG laser, emitting at 1064 nm, and a germanium detector cooled with liquid nitrogen. Laser power at the sample was set at 0.5 mW. All spectra were acquired with a spectral resolution of 4 cm^{-1} in the 100–4000 cm^{-1} spectral range. Depending upon S/N 2048 or 4096 scans was collected.

2.3. Calculations

Geometries of all studied structures were fully optimized with the 6-311++G(2df,p) basis set [37] and at the B3LYP [38] theoretical level using procedures implemented in the Gaussian '03 set of programs [39]. Harmonic frequency calculations verified the nature of the studied structures. All of them are minima at the Potential Energy Surface (all calculated frequencies are real). The theoretical NMR shielding was calculated using the GIAO method [40] at the same computational level. In addition, some calculations were done for the molecules in an aqueous solution (NMR spectra) by using Polarisable Continuum Model (IEF-PCM), a default method of PCM implemented to Gaussian [41]. Performed harmonic frequency calculations allowed us to introduce simulations of the theoretical Raman spectra of the studied species. The Raman intensities were calculated from Raman activities estimated by Gaussian using the methodology presented by Michalska and Wysokinski [42]. The computed IR and Raman spectra were not scaled. To provide the unequivocal assignments of these spectra (for diol form of fosfomycin), the potential energy distribution (PED) was performed according to the Pulay, Forgasi, and coworkers [43,44] internal coordinates. In the case, where it was not possible (epoxy structure), characteristic group frequencies, GaussView (implemented to Gaussian) and Gar2ped [44] were used for band assignment.

Generalized 2D correlation analysis of the Raman spectra obtained in five different pHs was performed using software SpectraCorr 1.1 SP1 (Thermo Fisher Scientific, Inc.).

3. Results and discussion

3.1. Conformational analysis of fosfomycin and its ionic species

*pK*a1 and *pK*a2 have been determined for *epoxy* form of fosfomycin to be 1.8 and 6.5, respectively [26]. This is an expected value for monophosphonic compounds [7]. No special changes in *pK*a1

and *pK*a2 for a diol (so-called *open*) form of fosfomycin is expected based on the extensive research of similar phosphonic compounds [7].

Calculations conducted at the B3LYP/6-311++G** level of theory were used to explore the conformational landscapes of all discussed species: fosfomycin (*epoxy* and *open* forms) and their anions. The interaction of the title molecule with the solvent (water) was taken into account by using the PCM method. The conformational analysis of the *epoxy* form of fosfomycin was conducted by rotation of the methyl, phosphonic, and hydroxylic groups. In that way several conformers for neutral, monoanionic, and dianionic forms of fosfomycin were found and the structures of their lowest-lying conformers are displayed in Fig. 1. Relative energies (including zero point vibrational contribution) of *fosfomycin*(0) conformers are 1.3–5.1 kJ/mol above the global minimum. The stability sequence obtained for *fosfomycin*(−1) conformers indicated that the closest (0.6 kJ/mol) to the global minimum is a conformer obtained by ∼180° rotation along the P–O_7 bond. So in fact, these two alternative structures should be considered as equivalent. The relative energies of the other conformers are 2.6–6.2 kJ/mol above the global minimum. The structure of *fosfomycin*(−2) with the lowest energy is similar to that of *fosfomycin*(0) with a relatively rigid structure. The same Fig. 1 presents the lowest possible conformations of three species of the *open* form of fosfomycin. They structures are stabilized by hydrogen bonding either between two adjacent molecules or with the water molecules. The most important thought is that this hydrogen bonding is the strongest for the dianion and the weakest for the neutral *open* conformer.

3.2. Theoretical geometrical parameters (bond lengths and valence angles) of fosfomycin and its anionic forms

The optimized bond lengths and valence angles of the all discussed species are given in Table 1. No imaginary harmonic frequencies were obtained. For comparison experimental data received for phenethylammonium(+)-*cis*-(*1S,2R*)-(1,2-epoxypropyl)phosphonate monohydrate are also included in Table 1 [45]. No crystallographic data for proper diol has been found.

3.2.1. 1,2-Epoxypropylphosphonic acid

It has to be noted that the calculated in this work bond lengths of the oxirane ring of *fosfomycin*(−1) are in very good agreement with the literature data [45]. As is seen in Table 1, the C_2–O_1 bond elongates, while the C_3–O_1 bond shortens when deprotonation takes place. The C_2–C_3 and C_3–C_4 bonds do not practically change during consecutive deprotonation. It is expected, and our calculations follow expectations, that C_2–P_5 and P_5–O_8 bonds become longer upon deprotonation. On the other hand, our calculations show that deprotonation of H_{15} provokes simultaneous shortening of the P_5–O_6 bond and elongation of the P_5–O_7 bond. However, the second deprotonation causes equalization of the length of these two P–O bonds. These changes follow those reported earlier [45]. Only slight changes in length are observed for the C–H bonds.

Discussed deprotonation practically does not change valence angles, except O_1–C_2–P_5, where the changes reach up to around 7°.

3.2.2. 1,2-Dihydroxyphosphonic acid

During opening of the oxirane ring two hydroxyl groups are attached to the C_2 and C_3 atoms and the C_2–C_3 bond becomes longer than that in *epoxy* form of fosfomycin. The length of this latter bond is not influenced by deprotonation. Thus, the founded C_3–O_1 and C_2–O_{16} bonds have almost the same length and, as in the case of C_2–C_3, are not affected by deprotonation. Similar, as discussed in the case of the *epoxy* form, changes in the bond lengths and valence angles are observed only within the phosphonic group.

3.3. ^1H, ^{13}C and ^{31}P NMR spectra

When fosfomycin is dissolved in pH below 3 and kept for more than 30 min the oxirane ring opens, the most probably due to a nucleophilic attack, and forms the respective diol. The data shown in this paragraph are obtained from fosfomycin dissolved in D_2O at different pH (acidity is expressed in pH) and incubated for an hour before measurement. The recorded NMR spectra are presented in Fig. 2, while in Table 2 chemical shifts of fosfomycin and its *open* form are gathered.

The proton decoupled ^{13}C NMR spectra obtained in aforementioned conditions are consistent with the presence of the two structures of fosfomycin: *epoxy* and *diol*. At pH 1, four resonances are observed: one doublet at 73.75 ppm and two singlets at 68.80 and 20.10 ppm. The doublet comes from coupling of the carbon C_2 to the adjacent phosphorus atom P_5 with the coupling constant $^1J(PC)$ 175.7 Hz. Remaining two carbon atoms do not couple that is opposite situation to that observed, for example, in phosphonic pyridine derivatives [34]. They are assigned to the C_3 and C_4 carbon nuclei, respectively. In this pH ^1H NMR spectrum shows one doublet (1.20 ppm), double doublets (3.45 ppm), and sextet

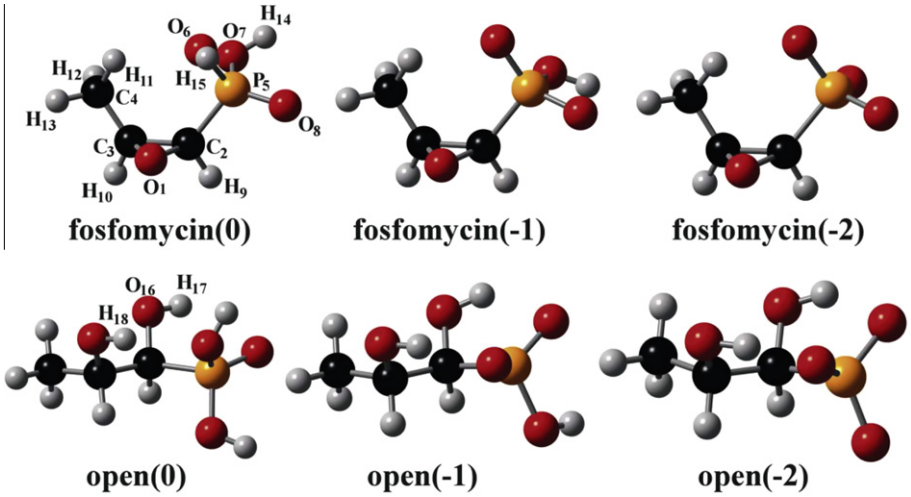

Fig. 1. The most stable structures (B3LYP/6-311++G**) of *epoxy* and *open* forms of fosfomycin.

Table 1
Selected geometrical parameters (bond lengths (Å) and angles (degrees)) of different forms of fosfomycin (*epoxy* form) and its *open* form (diol) at the B3LYP/6-311++G(2df,p) level.

Parameter	Open(0)	Open(−1)	Open(−2)	Fos(0)	Fos(−1)	Fos(−1)–exp (Ref. [45])	Fos(−2)
O_1–C_2				1.4334	1.4484	1.446	1.490
O_1–C_3	1.4174	1.4166	1.4143	1.4332	1.4294	1.436	1.426
C_2–C_3	1.5411	1.5383	1.5384	1.4732	1.4724	1.466	1.471
C_3–C_4	1.5216	1.5233	1.5303	1.5013	1.5039	1.477	1.508
C_2–P_5	1.8354	1.8796	1.9275	1.8153	1.8523	1.809	1.903
P_5–O_6	1.6198	1.4993	1.5512	1.6048	1.4900	1.490	1.533
P_5–O_7	1.5983	1.6557	1.5429	1.6009	1.6720	1.580	1.536
P_5–O_8	1.4735	1.5054	1.5221	1.4700	1.4988	1.497	1.529
C_2–H_9	1.1002	1.1013	1.1030	1.0893	1.0918	1.010	1.094
C_3–H_{10}	1.0994	1.1039	1.1083	1.0896	1.0940	0.976	1.101
C_4–H_{11}	1.0896	1.0939	1.0967	1.0874	1.0897	0.927	1.096
C_4–H_{12}	1.0919	1.0906	1.0924	1.0928	1.0952	1.027	1.098
C_4–H_{13}	1.0904	1.0930	1.0974	1.0926	1.0959	1.006	1.101
O_6–H_{15}	0.9664			0.9657			
O_7–H_{14}	0.9659	0.9636		0.9662	0.9636	1.069	
O_1–H_{18}	0.9646	0.9832	1.0234				
C_2–O_{16}	1.4166	1.4285	1.4397				
O_{16}–H_{17}	0.9659	0.9737	0.9901				
C_2–O_1–C_3				61.849	61.541	61.16	60.530
O_1–C_2–C_3				59.070	58.591	59.10	57.574
O_1–C_3–C_2	113.582	113.629	113.877	59.081	59.868	59.74	61.896
O_1–C_3–C_4	106.789	107.504	107.783	118.684	117.472	118.56	116.818
O_1–C_2–P_5				117.225	120.505	119.27	125.550
C_2–C_3–C_4	111.081	112.149	112.701	126.159	124.634	125.51	122.599
C_3–C_2–P_5	116.201	113.912	114.502	127.229	127.293	125.08	127.819
C_2–P_5–O_6	107.727	109.471	103.724	106.100	110.280	111.96	106.023
C_2–P_5–O_7	101.899	103.352	107.183	101.573	96.860	103.46	98.094
C_2–P_5–O_8	113.375	104.560	99.208	114.094	107.433	105.84	103.418
C_3–C_2–O_{16}	110.299	111.641	111.801				
P_5–C_2–O_{16}	107.672	108.110	107.580				

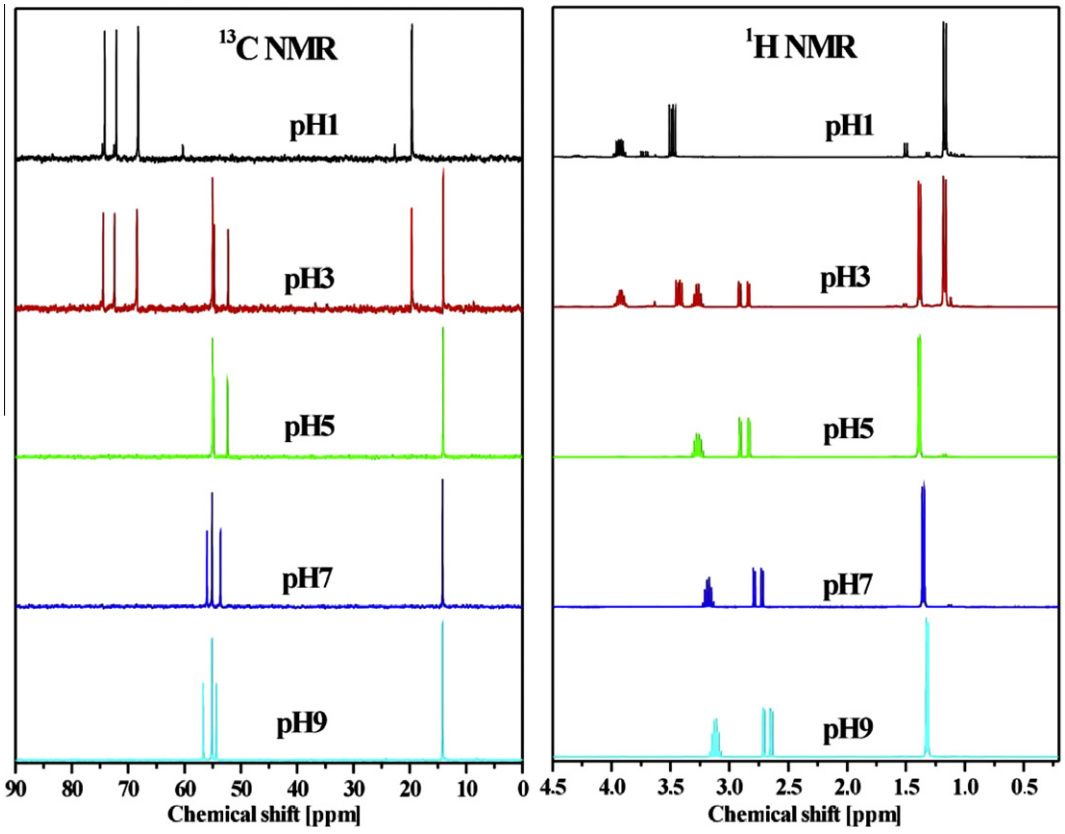

Fig. 2. Experimental 13C and 1H NMR spectra of fosfomycin.

(3.90 ppm). These are due to the methyl protons, and nuclei H_9 and H_{10}, respectively. In addition, ^{31}P resonance is observed at 19.82 ppm. Our calculations show that in those conditions fosfomycin exists in its *diol* form. This data agree quite well with these

Table 2
Comparison of experimental (D_2O solution expressed in pH) and theoretical (GIAO, B3LYP/6-311++G(2df,p)) chemical shifts (ppm) of fosfomycin (*epoxy* form) and its *open* form (diol).

Nucleus	Experiment					Theory					
	pH 1	pH 3	pH 5	pH 7	pH 9	Open(0)	Open(−1)	Open(−2)	Fos(0)	Fos(−1)	Fos(−2)
C_2	73.75d	73.75d				73.64	76.23	80.31			
		54.25d	54.25d	55.20d	52.30d				51.40	52.77	66.26
C_3	68.80	68.80				67.07	69.09	71.52			
		55.25	55.25	55.25	55.25				53.44	56.77	55.42
C_4	20.10	20.10						19.03			
		14.20	14.20	14.20	14.20	15.58	16.08	16.11	10.68	11.79	15.74
H_9	3.45dd	3.42dd				3.73	3.23				
		2.75dd	2.75dd	2.75	2.70			2.79	2.70	2.32	2.44
H_{10}	3.90s	3.90s				4.18	3.81				
		3.15s	3.18s	3.20	3.15			3.83	2.89	2.42	2.51
H_{met}	1.20d	1.20d				1.12	0.79			1.39	1.74
		1.34d	1.33d	1.33d	1.32d			0.63	1.28		
P	19.82	18.24				31.78	23.23	24.74			
		13.21	12.97	11.51	10.78				16.91	14.44	12.46
Interpretation	Open(0) and/or Open(−1)	Open(0) Open(−1) Fos(0) Fos(−1)	Fos(−1) Fos(−2)	Fos(−2) Fos(−1)	Fos(−2)						

Abbreviations: d – doublet, dd – double doublet, s – sextet.

published previously. However, there are some changes in the resonance frequencies up to 20% probably due to solution conditions [19–24]. In pH 3 two species exist. One is characterized by ^1H, ^{13}C, and ^{31}P resonances described above, whereas the other set of resonances is typical for the *epoxy* form of fosfomycin. Briefly, in the ^{13}C NMR spectrum again four resonances are observed: one doublet at 54.25 ppm that comes from C_2–P_5 coupling and two singlets at 55.25 and 14.20 ppm. ^1H resonances of H_9 (two doublets at 2.75 ppm), H_{10} (sextet at 3.20 ppm), and methyl protons (doublet at 1.35 ppm) are observed at expected frequencies [19–24]. On the other hand, ^{31}P resonance is much down-shifted to 13. 21 ppm in comparison to the *open* form. It has to be noticed that in pH 5 only the *epoxy* form is present in solution. It is also noticed that increase of pH causes some effect on the resonance frequency of the ^{31}P nucleus, i.e. it shifts to lower ppm.

Adoption of the PMC model that includes four water molecules did not improve parameters presented in Table 2 (based on GIAO, B3LYP/6-311++G(2df,p) calculations).

3.4. Experimental and calculated pH-dependent Raman spectra of fosfomycin (disodium salt or MONURAL®)

pH-dependent Raman spectra of *MONURAL*® in an aqueous solutions are shown in Fig. 3 in the spectral range of 1550–600 cm^{-1}. These spectra were measured just after dissolving of tablets in order to protect fosfomycin from hydrolysis (*vide supra*). Practically, the same spectra are obtained from fosfomycin disodium salt solutions. The observed and calculated band positions together with proposed band assignments are listed in Table 3. Theoretical Raman spectra are shown in Fig. 4. Since there was not possible to use Pulay normal coordinates [43] for DFT calculations of the Raman spectra (to obtain PED), characteristic group frequencies [46–48], GaussView (implemented to Gaussian), and Gar2ped [44] were used to proposed assignment. It has to be mentioned that the Raman bands from *MONURAL*® additives seen as week bands at 766 and 1467 cm^{-1} (trometamol), at 834 and 1130 cm^{-1} (sucrose), and at 708 cm^{-1} (saccharin) do not impede the interpretation of the spectra.

The bands observed at 1453, 1415, 1379, 1265, 846, and 720 cm^{-1} (Fig. 3) are characteristic for this antibiotic despite the stage of deprotonation and type of fosfomycine species. Thus, they can be used as the marker bands to detect and/or monitor this

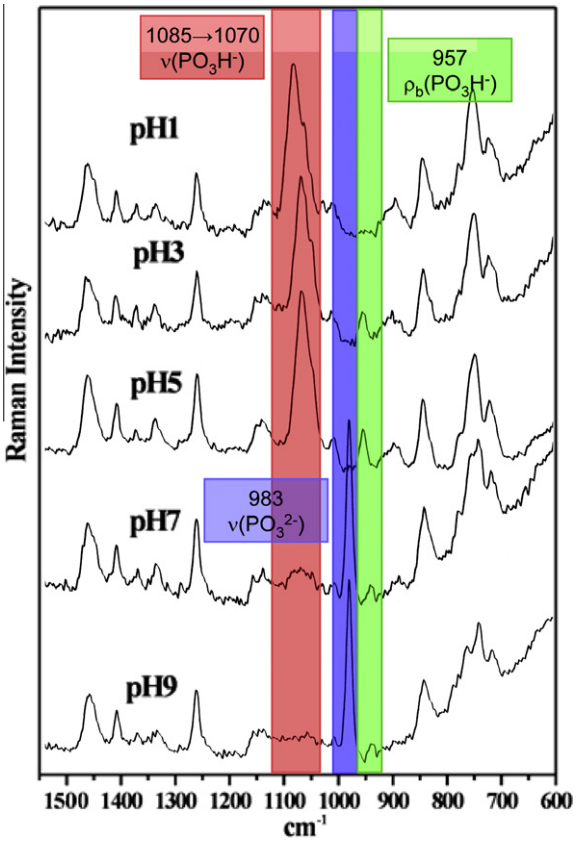

Fig. 3. Raman spectra of *MONURAL*® in aqueous solutions in the pH range from 1 to 9.

medicine in an aqueous medium. Unsubstituted oxirane ring (ethane oxide) belongs to the C_{2v} point symmetry group. Thus, 15 normal vibrations, divided among $5A_1 + 3A_2 + 3B_1 + 4B_2$ classes of symmetry, are expected to be enhanced in its Raman spectrum. In the collected spectra (Fig. 3) three bands originate from the fosfomycin oxirane ring. These are at around 1270 cm^{-1} (the symmetric ring breathing (A_1)), at around 880 cm^{-1} (the symmetric ring

Table 3
Proposed band assignments and frequencies of the observed bands in the Raman spectra of fosfomycin (*epoxy* form) and its ionized forms.

Fosfomycin(0)			*Fosfomycin*(−1)			*Fosfomycin*(−2)		
Experimental (cm^{-1})	Calculated (cm^{-1})	Assignment	Experimental (cm^{-1})	Calculated (cm^{-1})	Assignment	Experimental (cm^{-1})	Calculated (cm^{-1})	Assignment
1453	1461	$\delta(CH_3)$	1452	1459	$\delta(CH_3)$	1455	1459	$\delta(CH_3)$
	1454	$\delta(CH_3)$		1458	$\delta(CH_3)$		1452	$\delta(CH_3)$
1415	1413	$\delta(C_3–H)$ $\delta(CH_3)$ ν_{ring}	1415	1413	$\delta(C_3–H)$ $\delta(CH_3)$ ν_{ring}	1416	1410	$\delta(C_3–H)$ $\delta(CH_3)$ ν_{ring}
1376	1378	$\delta_s(CH_3)$	1379	1373	$\delta_s(CH_3)$	1381	1363	$\delta_s(CH_3)$
1342	1335	$\delta(C_3–H)$ $\delta(C_2–H)$	1342	1331	$\delta(C_3–H)$ $\delta(C_2–H)$	1344	1323	$\delta(C_3–H)$ $\delta(C_2–H)$
1265	1257	Ring breathing	1265	1260	Ring breathing	1265	1259	Ring breathing
846	828	Ring deformation	846	834	Ring deformation	846	835	Ring deformation
724	688	ν(P–C)	722	683	ν(P–C)	718	669	ν(P–C)
1186	1210	$\nu(P{=}O_8)$	1156	1182	$\nu_{as}(PO_2)$	1052	1027	$\nu_{as}(PO_3)$
1005	997	$\rho_b(P–O_7H)$	1070	1027	$\nu_{as}(PO_2)$	1009	1010	$\nu_{as}(PO_3)$
966	981	$\rho_b(P–O_6H)$	957	976	$\rho_b(P–O_7H)$	983	893	$\nu_s(PO_3)$
924	836	$\nu_{as}(PO_2)$	747	720	$\nu(P–O_7)$			
791	804	$\nu_s(PO_2)$						

Abbreviations: ν – stretching, δ – deformations, ρ_b – bending, s – symmetric, and as – asymmetric vibrations.

deformation (A_1)), and at around 900 cm^{-1} (asymmetric ring deformation (B_1)). It is worth to mention that the medium–strong relative intensity and the position of the symmetric ring breathing mode at 1265 cm^{-1} are distinctive of (*1R,2S*)-1,2-epoxypropylphosphonic acid, an active form of fosfomycin. In the case of its stereoisomer (*1R,2R*)-1,2-epoxypropylphosphonic acid the frequency of this band shifts down to around 1246 cm^{-1} [29,30].

The main changes in the spectra presented in Fig. 3 illustrate deprotonation of the fosfomycin phosphonic group that takes place with increasing pH of the solution. The vibrations of the $–PO_3H_2$ group in monophosphonic acids and their anionic forms are very characteristic and obey the local group symmetry [49,50]. Consequently, the *fosfomycin*(0) (at pH 1) spectrum exhibits three bands at 1186, 924, and 791 cm^{-1} due to the $\nu(P{=}O)$, $\nu_{as}(PO_2)$, and $\nu_s(PO_2)$ vibrations, respectively. In the spectrum of *fosfomycin*(−1) (at pH 3) the bands at 1156 and 1070 cm^{-1} are assigned to ν(P=O) mixed with the asymmetric $\nu_{as}(PO_2^-)$ and symmetric $\nu_s(PO_2^-)$ modes, respectively. Additionally, the stretching mode of P–OH (ν(P–OH)) is observed at 747 cm^{-1}. The spectra of *fosfomycin*(−2) (at pH's 7 and 9) are in a good agreement with those presented in literature [29] and reveals two asymmetric $\nu_{as}(PO_3^{2-})$ modes at 1052 and 1009 cm^{-1} and one very strong symmetric $\nu_s(PO_3^{2-})$ vibration at 983 cm^{-1}.

Above discussed changes are clearly seen in Fig. 5 where generalized 2D correlation maps (synchronous (on left) and asynchronous (on right)) in the frequency range of 1550–680 cm^{-1} generated from the above mentioned pH-dependent Raman spectra (Fig. 3) are presented. Briefly, the intensity of the synchronous 2D correlation map represents the simultaneous or coincidental changes of spectral intensity measured at two different (in this case) Raman frequencies that are perturbed by pH changes [51,52]. Auto-peaks located at the diagonal positions represent the extent of dynamic variations of spectral intensity at different frequencies. Synchronous cross-peaks appear at off-diagonal positions if the basic trends of dynamic changes observed at these two different frequencies of the cross-peak spectral coordinate are similar. Positive cross-peaks (in this case red[1] colored) indicate that both investigated bands decrease or increase their intensities together, while negative peaks (in this case blue colored) show that the intensity of one band increases when the other one decreases. On the other hand, an asynchronous 2D correlation map consists exclusively of off-diagonal cross-peaks and is antisymmetric with respect to the diagonal line providing information that is complementary to the synchronous spectrum. The intensity of the asynchronous map represents sequential changes of spectral intensities measured at two different frequencies. Asynchronous cross-peaks appear only if the basic trends of changes observed at two different frequencies of the cross-peak spectral coordinate are different [51,52].

The synchronous 2D correlation map (Fig. 5, on left) contains at (983, 983) cm^{-1} one very strong and at (1070, 1070) cm^{-1} (pH 5,

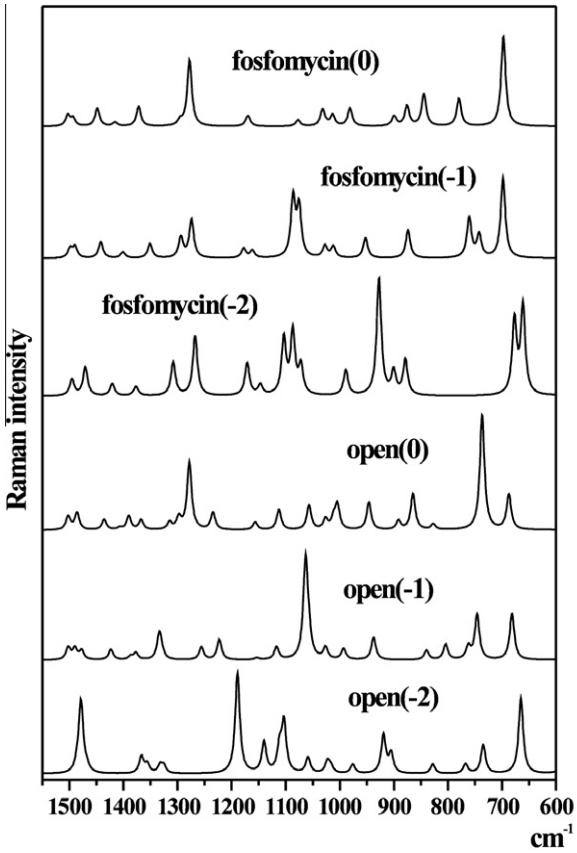

Fig. 4. Theoretical Raman spectra of epoxy and diol forms of fosfomycin.

[1] For interpretation of color in Figs. 1–3, and 5, the reader is referred to the web version of this article.

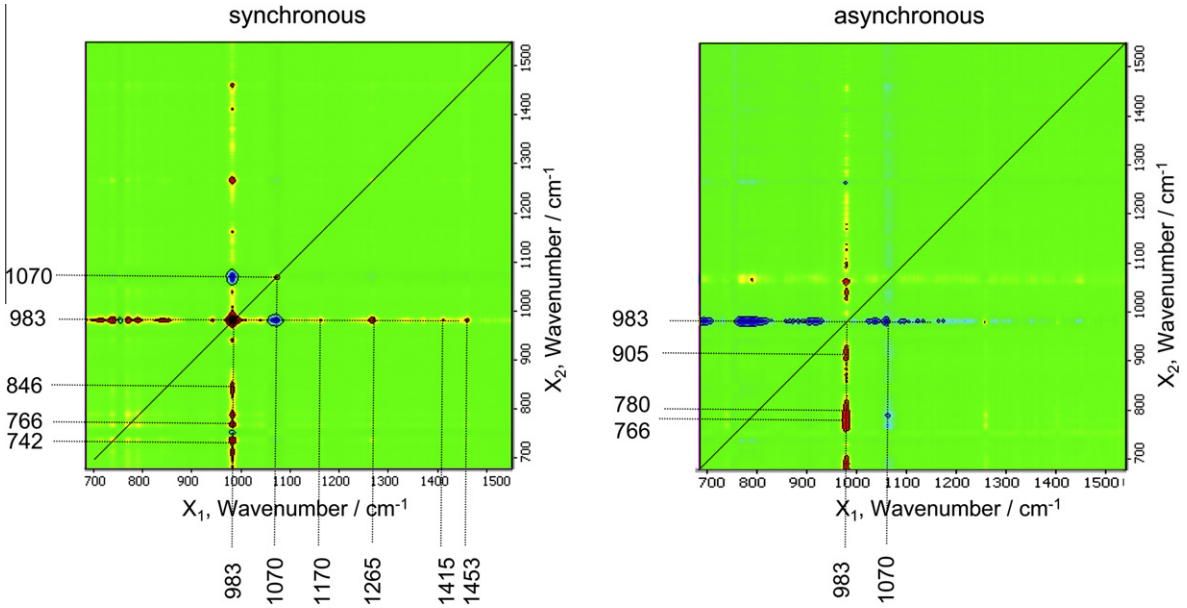

Fig. 5. Generalized 2D correlation maps (synchronous (on left) and asynchronous (on right)) in the frequency range of 1550–680 cm^{-1} generated from the pH-dependent Raman spectra of *MONURAL*®.

$\nu(P{=}O) + \nu_s(PO_2^-)$) one low intensity auto-peaks. The strong intensity of the former auto-peak suggests that the enhancement of this bands changes most significantly with the changes of the solution pH, in our case increases. In addition to the auto-peaks, several less intense positive cross-peaks; for example, at (1265, 983), (983, 742), and (1453, 983) cm^{-1} are present in the synchronous 2D correlation map. The positive sign of these cross-peaks indicates that all these Raman signals gain enhancement when the solution pH become more basic. On the other hand, medium–strong negative cross-peaks at (1070, 983) cm^{-1} implies that at the same time when the relative intensity of the 983 cm^{-1} band increases the 1070 cm^{-1} spectral feature loses enhancement. Also, the asynchronous 2D correlation map develops several cross-peaks. The appearance of these cross-peaks points out that the directions of the transition moments of these modes are different. For instead, the positive sign of the (983, 780) cm^{-1} peak indicates that pH-induced spectral changes take place earlier at 983 cm^{-1} than that at 780 cm^{-1}. On the other hand, the negative sign of the two example peaks at (1070, 780) and (1070, 983) cm^{-1} suggests that spectral changes at 1070 cm^{-1} follow those at 780 and 983 cm^{-1}.

pH-dependent Raman spectra, in the 1550–600 cm^{-1} range, of 1,2-dihydroxyphosphonic acid (hydrolyzed fosfomycin, the *open* form) in an aqueous solutions are shown in Fig. 6. As can be seen the spectra are different from those presented in Fig. 3. The theoretical Raman spectra of the *open* form structure (open(0)) and its mono- (open(−1)) and dianion (open(−2)) are shown in Fig. 4. (*vide supra*). Calculated frequencies, adequate to above species, together with proposed bands assignment based on the PED analysis are gathered in Tables 4–6 (see Supplementary Material). At pH 1, the strongest band at 1420 cm^{-1} band is mainly due to the bending vibrations of the methyl group, while to other bands at 1357 and 1322 cm^{-1} are dominated by the δ(C–C–H) and δ(O–C–H) modes that proves investigated structure. The medium strong band at 1242 cm^{-1} has to be assigned rater to ν(C-O(H)) than to ν(P═O) since its frequency stays invariant from pH changes. It has to be noticed that this frequency is down-shifted from this of A_1 ring vibration discussed earlier in the paper. According to our calculations, the bands at around 1050 –990 and at 925 cm^{-1} are mainly due to the P–O–H stretching and bending vibrations, while the Raman signals below 1101 cm^{-1} are mainly associated with the

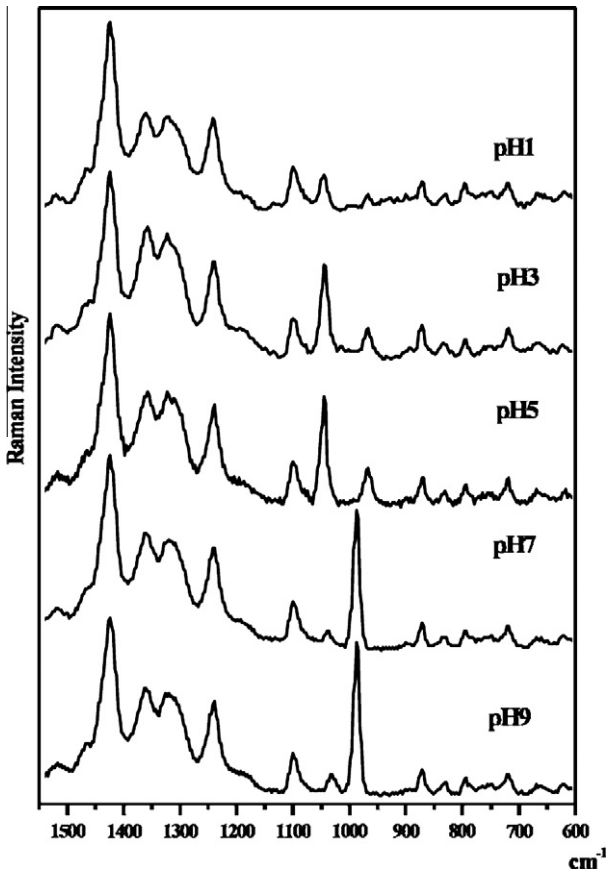

Fig. 6. Raman spectra of 1,2-dihydroxyphosphonic acid (hydrolyzed fosfomycin) in aqueous solutions in the pH range from 1 to 9.

C–C–H bending and C–C and C–O stretching vibrations and show very complex PED (Table 5, Supplementary Material). Examination of the spectra at pH 1, 3 and 5 and Table 5 allow to propose reliable assignments. Thus, increase in the relative intensity of Raman

bands at 1048 and 972 cm^{-1} at higher pH show that these bands should be assign to ($\nu(PO_3H^-)$ and (δ(P–O–H)) spectral features. At pH 7 these bands disappear and a new band at 992 cm^{-1} emerges that has to be assign to the $\nu(PO_3^{2-})$ vibration of dianion. It has be noted that the frequencies of phosphonic moiety vibrations (at the same level of deprotonation) are slightly different between epoxy and open (diol) forms that represent different PED of proper modes. In other words vibrations of tetrahedral phosphonic moiety are influenced by the alkyl chain.

It is known that after an oral administration of *MONURAL®* fosfomycin is excreted by the renal route and its mean urinary concentration reaches 2.5 g/L level from 2 to 4 h [52,25]. It is worth to notice that at this concentration fosfomycin can be easily detected by using Raman spectroscopy. Moreover, presented here data (*epoxy* vs. *diol*) can shed the light on the physiological pathway of individual patient. Therefore, the use of this technique for fosfomycin direct detection in urine samples should be a very fast and reliable analytical tool. The proper protocol will be developed in our laboratory.

4. Conclusion

Raman spectroscopy is a very useful method that can be utilized to extract information about structural changes occurring in the process of drug deprotonation that play a key role in understanding its metabolism. Furthermore, it is easy to distinguish between fosfomycin and its hydroxy product (1,2-dihydroxyphosphonic acid) as well as recognize between neutral and ionic forms of this drug based on the characteristic marker bands. Also, as suggested, FT- Raman spectroscopy due to lack of possible fluorescence background coming from natural sources, i.e. urine) can be used as an analytical method for drug analysis occurring in low concentration.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molstruc.2010.11.033.

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