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Quantum Mechanical Calculations Useful For Determining the Mechanism of Action of Fosfomycin

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Abstract □ CNDO/2 calculations were performed to determine at the molecular level the mechanism of action of the antibiotic fosfomycin, (−)-(1*R*,2*S*)-(1,2-epoxypropyl)phosphonic acid. Fosfomycin, a bacterial cell wall inhibitor, is known to act as a competitive inhibitor of *N*-acetylglucosamine-3-*O*-enolpyruvyl transferase, the normal substrate of which is phosphoenolpyruvate. Both compounds were studied, and the theoretical calculations revealed that the preferred conformations of phosphoenolpyruvate and fosfomycin presented the same spatial charge distributions on the active sites, the values of which are in complete agreement with the experimental observations. These results permit the projection of some details of the receptor, with implications for the modification of fosfomycin to increase its antibiotic activity.

Keyphrases □ Fosfomycin—preferred molecular conformation for biological activity, quantum mechanical calculations □ Phosphoenolpyruvate—preferred molecular conformation for biological activity, quantum mechanical calculations □ Structure–activity relationship—fosfomycin and phosphoenolpyruvate, molecular level mechanism of action, preferred conformation

Fosfomycin, (−)-(1*R*,2*S*)-(1,2-epoxypropyl)phosphonic acid, a relatively new low molecular weight antibiotic, contains both an epoxide ring and a carbon–phosphorus bond (Fig. 1) [found for the first time among natural products (1)]. Despite the presence of the epoxide ring, fosfomycin is quite stable, and its activity seems to be limited to the inhibition of *N*-acetylglucosamine-3-*O*-enolpyruvyl transferase, resulting in the formation of an irreversible adduct with the enzyme. The reaction seems to be stereospecific; (+)-(1*R*,2*S*)-, (−)-(2*R*,1*S*)-, and (+)-(2*R*,1*S*)-(1,2-epoxypropyl)phosphonic acids do not form stable adducts, as shown by their lack of biological activity. The absolute configuration of fosfomycin has been found to be (−)-(1*R*,2*S*) (2).

Fosfomycin is structurally similar to phosphoenolpyruvate (Fig. 2), an important substance for both bacterial and animal cells (3). Kahan *et al.* (4) have studied the mechanism of action of fosfomycin, which appears to be a competitive inhibitor of phosphoenolpyruvate in the cell wall biosynthesis of bacteria. The reactive sites on the enolpyruvyl transferase are a nucleophilic sulfur of a cysteine residue and a proton donor. The reaction is interpreted by the authors as a sulfhydryl addition across the C(2)—O(1) bond, analogous with the assumed sulfhydryl addition across the C(2)=C(3) double bond of phosphoenolpyruvate in the bacterial cell wall. In the present work, quantum mechanical conformational and charge distribution calculations for fosfomycin and phosphoenolpyruvate were performed to determine the mimetic action of fosfomycin at the molecular level.

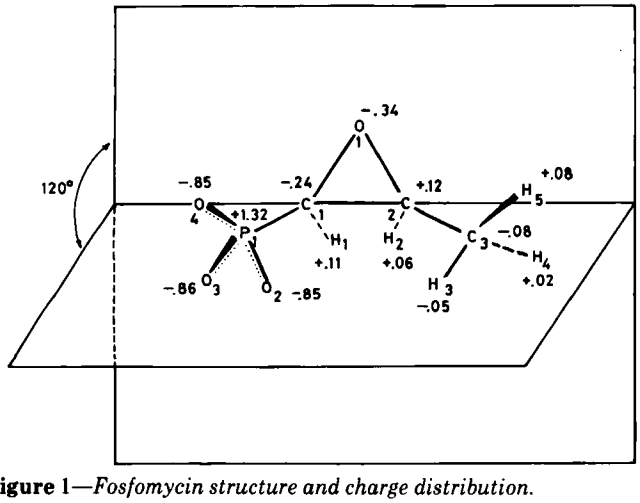

Figure 1—*Fosfomycin structure and charge distribution.*

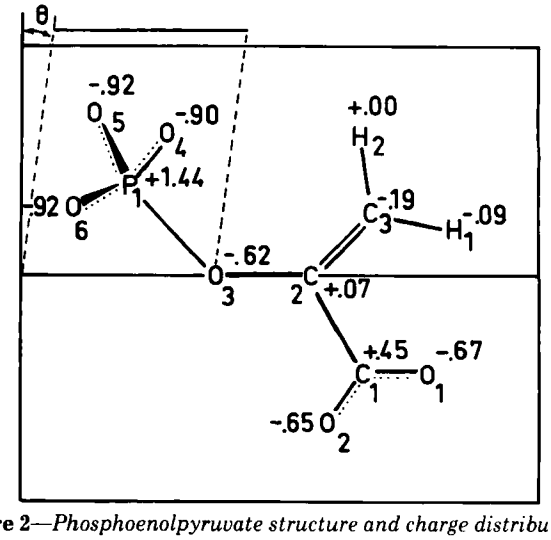

Figure 2—*Phosphoenolpyruvate structure and charge distribution.*

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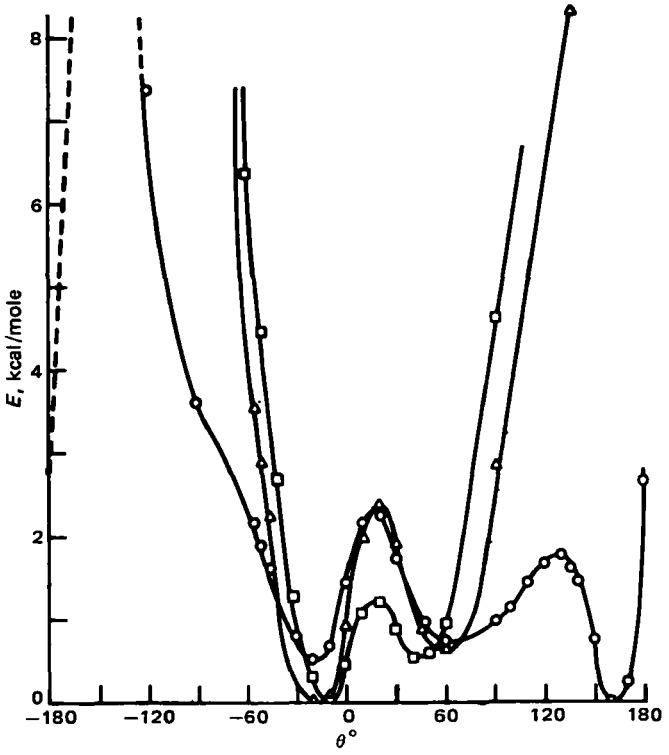

Figure 3—*Calculated relative potential energy curves for the one-* (○), *two-* (△), *and threefold* (□) *ionized species of phosphoenolpyruvate as a function of the rotation angle around the C—O bond of the ester bridge.*

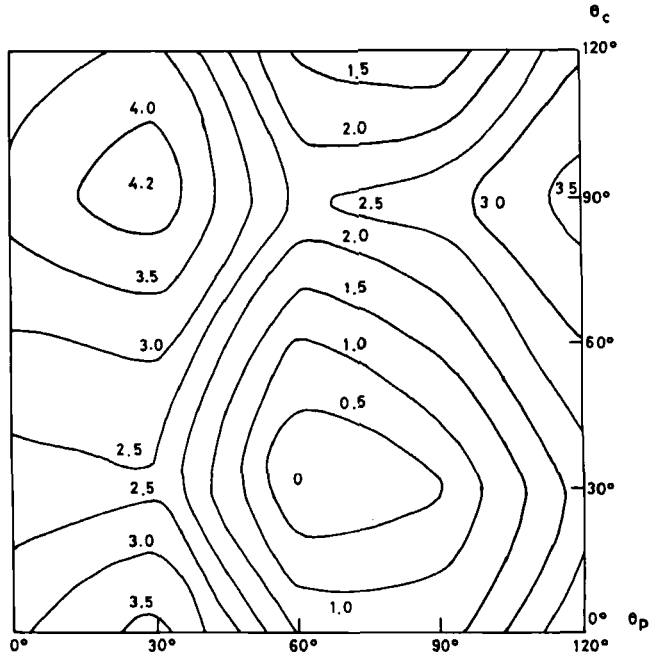

Figure 4—*Calculated potential energy (kcal/mole) map for the internal rotation in fosfomycin.* θ_p *and* θ_c *are the rotation angles of the phosphonium and carboxylic groups, respectively.*

THEORETICAL

Since fosfomycin and phosphoenolpyruvate are relatively large molecules to be approached by an *ab initio* method, the charge distribution calculations and conformational analysis were performed using the standard semiempirical CNDO/2 procedure (5). This method considers the system under study in the gas phase and needs only knowledge of the atomic number and the position coordinates of the atoms. Solvent effects can be introduced (6). These effects usually appear as a small increase in the charges, which will not modify the overall conclusions of this work. Therefore, solvent effects will not be considered explicitly.

Although the CNDO/2 method must be used with some caution in the conformational calculations (7), this procedure usually yields reasonable charge distributions. However, since it resorts to the zero differential overlap approximation (ZDO), the electronic charge distributions must be recalculated conveniently to obtain more reliable values (8).

In the ZDO approach, the electric charge of a given atom, A, with core charge Z_A, is expressed as:

$$Q_A = Z_A - 2\sum_{a}^{N}\sum_{i}^{n} C_{ia}^2 \qquad \text{(Eq. 1)}$$

where C_{ia} are the coefficients of the ath basis function in the ith molecular orbital, N is the number of basis functions centered on atom A, and n the number of occupied molecular orbitals. In this approximation, the basis functions may be regarded as atomic Slater orbitals orthogonalized by the Löwdin procedure. This orthogonalization involves a delocalization of the basis functions. To undo this transformation seems to be necessary in order to obtain localized charges on the atoms. So, according to the Löwdin transformation, the new coefficients are written as:

$$C' = CS^{-1/2}$$

where S is the overlap matrix between the Slater orbitals, and C and C' are the coefficient matrices before and after deorthogonalization, respectively. After this operation, the electric charges are given by the Mulliken population analysis formula:

$$Q_A = Z_A - 2\sum_{a}^{N}\sum_{b}^{M}\sum_{i}^{n} C'_{ia}C'_{ib}S_{ab} \qquad \text{(Eq. 2)}$$

where the summation on the index b must be extended to all the nonorthogonal basis orbitals.

EXPERIMENTAL

Phosphoenolpyruvic acid and fosfomycin should be ionized in a physiological medium. Essentially complete ionization of both compounds at pH ~7.4 is shown by the pK_a values of the carboxyl group (3.5) and the phosphoric acid groups (<2 and 6.4) of phosphoenolpyruvate acid and the phosphonic moiety (1.85 and 6.40) of fosfomycin (9). One-, two-, and threefold ionized species for fosfomycin were considered in the calculations.

To determine the coordinates of the ionized species, the bond lengths and bond angles were taken from the available crystallographic data, *i.e.*, X-ray measurements in solid phase on the monocyclohexylammonium salt of phosphoenolpyruvate (10) and monophenethylammonium salt of fosfomycin (11). Because the ions may show some conformational modifications in liquid phase, various possible conformations were taken into account.

Phosphoenolpyruvate Calculations—It appears that phosphoenolpyruvate has not been studied from a quantum point of view, except for two previous works on bond energy wealth (12, 13). X-ray measurements (10) show a roughly planar structure except for the phosphate group, which extends out from the plane of the enolpyruvate moiety. The P(1)—O(3) bond forms a tetrahedral angle (~109°) with the C(2)—O(3) bond of the ester bridge, and the P(1)—O(3)—C(2) plane forms a dihedral angle of 90° with the molecular planar frame. Because of the tetrahedral character of the P(1)—O(3) bond, the lack of steric effect, and its high symmetry, the phosphate group is expected to be able to rotate in the free molecule. Among the small irregularities of the planar frame, the carboxylic group appears somewhat twisted, and the oxygen atom (O-3), lies somewhat under the enolpyruvate plane. From a quantum point of view, it may be expected that the more ionized the molecule, the more planar it is because of a clear increasing of the conjugation of the π-electron system. This feature is especially true for the carboxylic group, which may be considered as rigorously coplanar in its ionized form.

Concerning the phosphoric rest, the question is whether the P(1)—O(3)—C(2) plane remains vertical up the enolpyruvic plane. Therefore, conformational calculations were performed around the O(3)—C(2) bond, taking as origin of rotations the conformation in which the phosphorus atom lies in the pyruvic plane side of the methylenic group. In these calculations, however, the O-3 atom is assumed to remain in its original location, slightly under the molecular plane.

The conformational dependencies of the three ionized species of phosphoenolpyruvic acid are reported in Fig. 3; they show a potential asymmetric double-well behavior, due to the out-of-plane O-3 atom. Minima are found at 60° and −20°, 60° and −20°, and 45° and −10° in the one-, two-, and threefold ionized species, respectively. The one-ionized

species has an extra minimum at 160°. As expected, at least to some extent, the more ionized the compound the more planar the ion. In the liquid phase, however, it may be expected that the solvent effect will damp this trend, because of its dielectric nature (6).

The preferred conformations appear also at lower angles. However, since the energy differences between the two branches of double wells are not too large (0.27, 0.66, and 0.54 kcal/mole), it may be expected that the ionized species will exist in both conformations at the physiological temperature. Furthermore, the barrier heights between the two branches are relatively low (1.8, 2.4, and 1.3 kcal/mole) so that the interconversion should be relatively easy through a switching of the O-3 atom.

The localized charge distributions were determined using Eq. 2 as a function of the torsion angle for the three ionized species. The distributions look qualitatively similar in the three species, although the more ionized the phosphoenolpyruvate species, the sharper the charge distribution, probably because of the increasing conjugation of the π-system. Their conformational dependencies, however, are not too large in the region of the minima. In Fig. 3, the charge distribution of the preferred conformation of the threefold ionized species in which the P(1)—O(3)—C(2) plane lies at −10° from the molecular plane is given.

Fosfomycin Calculations—Compared with phosphoenolpyruvate, fosfomycin presents a relatively rigid structure. In the X-ray measurements (11), fosfomycin may be regarded as a planar epoxide bearing a phosphonate and methyl groups in *cis* positions. Taking as rotation axis the C(1)—C(2) ring side and as origin of rotation the conformation in which the P atom lies in the epoxide half-plane, the phosphonate and methyl groups point rigidly out ~120° from the molecular plane. Only hindered internal rotations of the phosphonate and methyl radicals are possible. Therefore, the conformational calculations on the one- and twofold ionized species of fosfomycin were restricted to these two possibilities.

The conformational map for the twofold ionized species is given in Fig. 4, in which the threefold symmetry of the rotors is taken into account. In Fig. 4, the preferred conformation has the methyl and phosphonate groups rotated at 30° and 60°, respectively, taking as origin of rotation the conformation in which one atom of the rotating group lies in the plane formed by the rotation axis and the ring side. The conformational map, however, shows a valley corresponding to the single rotation of the methyl group, with a relatively low saddle point at 2 kcal/mole. This result reveals that the methyl group is able to rotate, though with some hindrance, regardless of the position of the phosphonate group.

The localized charge distributions of the two ionized species were calculated as a function of the two internal rotation angles. These distributions look very similar in the two species, and their conformational dependencies appear to be very small. In Fig. 1, the charge distribution of the twofold ionized species for the preferred conformation is also given.

RESULTS AND DISCUSSIONS

The results below are discussed assuming a neutral pH in the bacterial cell. These results also are valid, however, at lower pH values where local variations could occur in the reactive medium.

As has been seen, phosphoenolpyruvate and fosfomycin possess a central frame to which the PO_3^{2-} moiety is attached. Fosfomycin has a rigid structure; in contrast, phosphoenolpyruvate is a more flexible molecule which can adopt different conformations. The question arises whether fosfomycin may be considered as a rigid analogue of the natural compound.

As it is seen in Fig. 3, phosphoenolpyruvate has essentially two preferred conformations. To adopt the structure of fosfomycin ($\theta \sim 120°$) the fully ionized species needs more than 20 kcal/mole, which is a large energy requirement. On the other hand, in this conformation some distances between active sites such as P-1 and C-3 appear to be too large when compared with the corresponding distance between P-1 and O-1 in fosfomycin. In the more stable conformation of phosphoenolpyruvate (which appears for lower angles), all the distances between the active sites coincide approximately with those of fosfomycin (Fig. 5).

When considering the charges of the active sites, *i.e.*, the proton acceptor centers (the C-3 atom in phosphoenolpyruvate and the O-1 atom in fosfomycin) and the sulfhydryl acceptor centers (the C-2 atoms in both molecules), it is seen that the proton acceptor sites have a negative charge in both molecules. The sulfhydryl acceptor centers, C-2, have a positive charge in both molecules. Furthermore, the second carbon atom of the epoxide ring of fosfomycin (C-1), which could be a competitive acceptor center for the sulfhydryl residue, appears to be negatively charged, analogous with the oxygen atom (O-3) of the ester bridge in phosphoenolpyruvate. The charge distributions are similar in the PO_3^{2-} groups of both molecular systems.

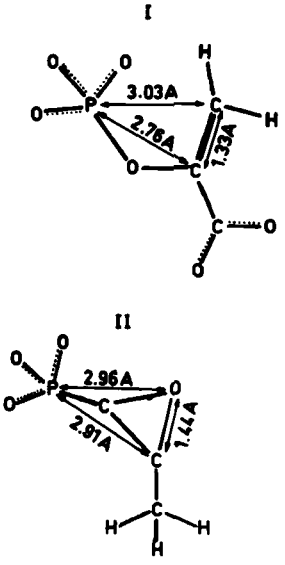

Figure 5—*Spatial distributions of the active sites (proposed receptor pattern) in the preferred conformation of the phosphoenolpyruvate (I) and fosfomycin (II).*

Therefore, fosfomycin should not be considered a rigid analogue of phosphoenolpyruvate. Nevertheless, this compound and fosfomycin appear to possess some local isosterism with a similar electric charge distribution at the active sites. This isosterism does not extend to the carboxylic group of phosphoenolpyruvate or methyl group of fosfomycin, which probably do not enter directly into the addition reaction. Fosfomycin, however, shows a high stereospecificity regarding the methyl group. This feature may be attributed to some steric hindrance, assuming that the reaction takes place in two steps: (*a*) fosfomycin is first adsorbed sideways by the PO_3^{2-} on the receptor and (*b*) the sulfhydryl addition reaction takes place on the opposite free face of the epoxide ring. Finally, it may be underlined that the knowledge of the charge distribution in fosfomycin suggests the possibility of selecting some structural modifications to increase the antibiotic activity of fosfomycin.

The present theoretical calculations confirm the assumed mechanism of action for the sulfhydryl and proton additions in fosfomycin as well as in phosphoenolpyruvate (4). The conformational analysis and electric charge distribution calculations indicate the same spatial charge distribution on the active sites in both molecular systems, the values of which are in accordance with the experimental data. Furthermore, taking into account the rigidity of fosfomycin and the stereospecificity involved, it may be stated that the active centers are probably located in a very small area on the receptor, limited approximately by the positions of three adjacent active centers, PO_3^{2-}, O-1, and C-2 (Fig. 5).

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In Vitro Method for Detecting Precipitation of Parenteral Formulations After Injection

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Abstract □ Many injectable formulations currently on the market, including diazepam and alprazolam, utilize one or more cosolvents to solubilize the active constituents. On injection into an aqueous medium, some of these components tend to precipitate. A simple procedure is described for measuring the degree of precipitation that occurs when a solubilized drug is injected. This *in vitro* technique was used to show that alprazolam injection shows less precipitation than diazepam injection under all tested conditions, and that the precipitation observed with diazepam can be controlled by ensuring that the formulation is injected very slowly. This simple technique also can be used during preformulation development to evaluate the relative potential for precipitation of various formulations.

Keyphrases □ Diazepam—*in vitro* detection of precipitation for injectable formulations □ Alprazolam—*in vitro* detection of precipitation for injectable formulations □ Formulations, injectable—potential precipitation in aqueous media, *in vitro* detection using diazepam and alprazolam

It is often necessary to administer a drug parenterally at a concentration which exceeds its aqueous solubility. The use of water-miscible cosolvents is by far the most versatile means of increasing the solubility of drugs. Cosolvents in concentrations up to 50% v/v can produce solubility increases of several orders of magnitude for very insoluble drugs (1, 2).

In some cases, the injection of a formulation (in which the drug is solubilized by a cosolvent) into blood or some other aqueous fluid can result in precipitation of the drug (2–7). This precipitation, in turn, can result in erratic or reduced drug bioavailability, pain on injection, and/or thrombophlebitis (3–7). The amount of precipitation, and thus the severity of the above problems, often is related to the rate at which the drug is injected (3–7).

The elimination of precipitation on dilution not only can lead to a safer and more effective formulation, it can also

Table I—Some Parenteral Products Formulated with Cosolvents

Generic Name	Cosolvent Composition
Hydralazine HCl[a]	10% propylene glycol
Lorazepam[b]	80% propylene glycol 20% polyethylene glycol
Deslanoside[c]	9.8% ethanol 15% glycerin
Phenytoin sodium[d]	40% propylene glycol 10% ethanol
Dihydroergotamine mesylate[e]	6.1% ethanol 15% glycerin
Dimenhydrinate[f]	50% propylene glycol
Digoxin[g]	40% propylene glycol 10% ethanol
Chlordiazepoxide HCl[h]	20% propylene glycol
Phenobarbital sodium[i]	67.8% propylene glycol
Multiple vitamin infusion[j]	30% propylene glycol
Pentobarbital sodium[k]	40% propylene glycol 10% ethanol
Methocarbamol[l]	50% polyethylene glycol
Reserpine[m]	10% dimethylacetamide 5% polyethylene glycol
Diazepam[n]	40% propylene glycol 10% ethanol

[a] Apresoline (Ciba). [b] Ativan (Wyeth). [c] Cedilanid (Sandoz). [d] Dilantin (Parke-Davis). [e] DHE 45 (Sandoz). [f] Dramamine (Searle). [g] Lanoxin (Burroughs Wellcome). [h] Librium (Roche). [i] Luminal (Winthrop). [j] MVI (USV). [k] Nembutal (Abbott). [l] Robaxin (Robins). [m] Serpacil (Ciba). [n] Valium (Roche).

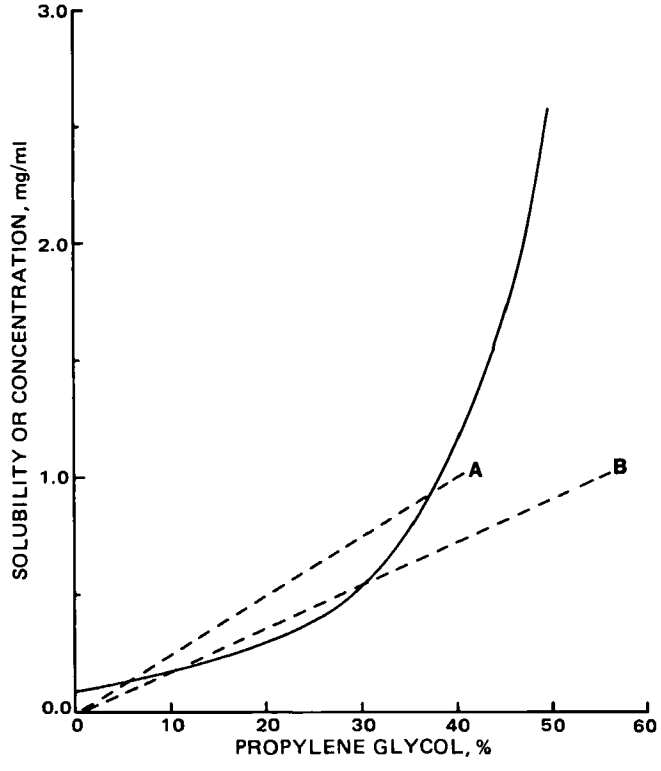

Figure 1—*Solubility (——) of alprazolam in propylene glycol–water mixtures containing 40% (A) and 55% (B) propylene glycol. The dilution lines (- - -) above the solubility curve represent conditions under which precipitation can occur.*

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