Antitumor Agents LXIII: The Effects of Microlenin on Nucleic Acid and Protein Syntheses of Ehrlich Ascites Cells

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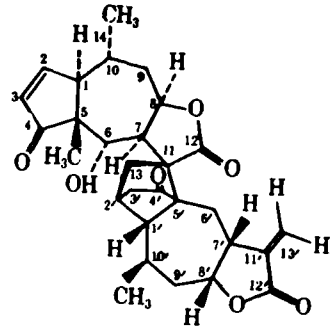
Abstract □ Microlenin, a novel dimeric sesquiterpene lactone isolated from Texas *Helenium microcephalum*, was shown to inhibit Ehrlich ascites carcinoma growth. Metabolic studies demonstrated that DNA synthesis and protein synthesis were significantly inhibited by two doses of microlenin at 5 mg/kg/day. DNA synthesis appeared to be blocked at several sites including DNA polymerase, purine synthesis, and dihydrofolate reductase. Thymidine nucleotide pools were significantly reduced by microlenin. Protein synthesis inhibition by microlenin appeared to occur during the initiation step of polypeptide synthesis. The metabolic effects of microlenin were similar to other sesquiterpene lactones in the Ehrlich ascites carcinoma cells. However, a lower dose of microlenin was required to bring about these metabolic effects when compared with other sesquiterpene lactones. Thus, microlenin may be a more likely therapeutic agent than helenalin which has demonstrated cellular toxicity.

Keyphrases □ Microlenin—sesquiterpene lactone, inhibition of DNA and protein syntheses, inhibition of Ehrlich ascites carcinoma growth □ Protein synthesis—effects of microlenin, Ehrlich ascites carcinoma growth

Microlenin was originally isolated from Texas *Helenium microcephalum* (1–3). The molecule appears to arise from a Diels-Alder type condensation involving the 11,13-double bond of helenalin and the enol form of the cyclopentenone ring of a norpseudoguaianolide. Preliminary antineoplastic screening demonstrated that microlenin was active against rat Walker 256 carcinosarcoma growth at 2.5 mg/kg/day affording a T/C% value equal to 172%. Further testing against the mouse P-388 lymphocytic leukemia growth demonstrated that at 12.5 mg/kg/day, a T/C% = 167 was obtained. Sesquiterpene lactones of varying structures have been shown to be potent inhibitors of nucleic acid and protein synthesis of tumor cells. Reported at this time are the effects of microlenin on the cellular metabolism of Ehrlich ascites cell carcinoma.

EXPERIMENTAL

Ehrlich Ascites Screen—Male CF_1 mice (30 g) were implanted with 2×10^6 Ehrlich ascites cells intraperitoneally on day 0. Microlenin was suspended by homogenization in 0.05% polysorbate 80–water and administered intraperitoneally at 5 and 10 mg/kg/day. The mice were sacrificed on day 10, and the ascites fluid was collected from the peritoneal cavity. The volume and ascrit (packed cell volume) were determined for each animal, and the percent inhibition of tumor growth was calculated (4).



Structure of Microlenin

In vitro incorporation of [^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine was determined using 10^6 Ehrlich ascites cells, 1-μCi labeled precursor, minimum essential medium, and varying final concentrations of drug from 0.125 to 2.0 m*M* (5). The tubes were incubated at 37° for 60 min and inactivated by trichloroacetic acid. The acid-insoluble, labeled DNA, was collected on GF/F glass filter disks[1], and RNA and protein were precipitated on nitrocellulose filters[2] by vacuum suction. Results are expressed as dpm of incorporated precursor/hr/10^6 cells. For *in vitro* studies, cells were collected on day 10, and the microlenin was incubated at 0.25–1.0 m*M* concentration.

Ehrlich ascites cells (10^6) were injected intraperitoneally into CF_1 male mice (~22 g) on day 0. On days 8 and 9, microlenin (5 mg/kg/day in 0.05% polysorbate 80–water) was injected intraperitoneally. Incorporation of thymidine into DNA was determined by the method of Chae *et al.* (6). One hour prior to sacrifice on day 10, 10 μCi of [6-^{3}H]thymidine (21.5 Ci/mmole) was injected intraperitoneally. The DNA was isolated and the tritium content was determined in a toluene-based scintillation fluid[3]. The DNA concentration was determined by the diphenylamine reaction using calf thymus DNA as a standard. Uridine incorporation into RNA was determined using 10 μCi of [5,6-^{3}H]uridine (22.4 Ci/mmole). RNA was extracted by the method of Wilson *et al.* (7). Using yeast RNA as a standard, the RNA content was assayed by the orcinol reaction. Leucine incorporation into protein was determined by the method of Sartorelli (8) using 10 μCi of [4,5-^{3}H]leucine (52.2 Ci/mmole). Extracted protein was determined by the Lowry procedure using bovine albumin as a standard. *In vitro* and *in vivo* nuclear DNA polymerase activity was determined on isolated Ehrlich ascites cell nuclei (9). The incubation method was that described by Sawada *et al.* (10) except that [methyl-^{3}H]deoxythymidine triphosphate (82.4 Ci/mmole) was used. The acid-insoluble nucleic acid was collected on GF/F filters and counted.

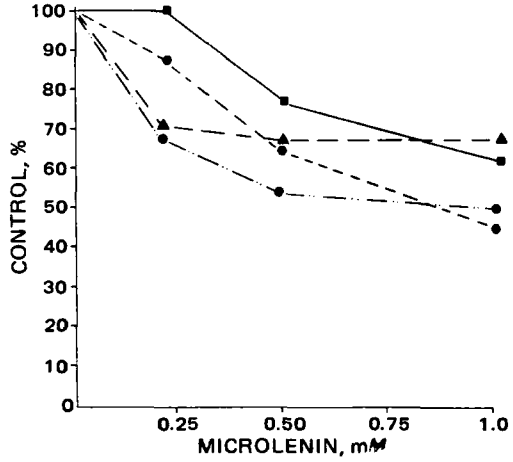


Figure 1—*The* in vitro *effects of microlenin on the incorporation of radiolabeled precursors into DNA, RNA, protein and purine of Ehrlich ascites cells. Key: (■) [^{3}H]leucine incorporation into protein; (▲) [^{3}H]uridine incorporation into RNA; (●) [^{3}H]thymidine incorporation into DNA; and (◆) [^{14}C]formate incorporation into purine.*

[1] Whatman GF/F.
[2] Millipore.
[3] Fisher ScintiVerse.

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Table I—Antineoplastic Activity of Microlenin Against the Growth of Ehrlich Ascites Carcinoma Cells [a]

Compound	Survival at Day 10	Volume of Ascites	Ascrit Packed Cells Volume	Inhibition, %
Control (0.05% polysorbate 80)	6/6	6.68	43.1	—
Microlenin (5 mg/kg/day)	6/6	0.02	5.1	99.9
Microlenin (10 mg/kg/day)	6/6	0.75	36.8	90.4
Mercaptopurine	6/6	0.10	2.5	99.9

[a] Number of mice = 6.

Nuclear RNA polymerase activities were determined on enzymes isolated from nuclei (9). Messenger, ribosomal, and transfer RNA polymerases were isolated using 0.3 *M*, 0.04 *M*, and 0.0 *M* concentrations of ammonium sulfate in magnesium chloride, respectively. The incubation medium was that of Anderson *et al.* (11) using [^{3}H]uridine triphosphate (23.2 Ci/mmole). The acid-insoluble RNA was collected on nitrocellulose filters and counted.

Deoxythymidine as well as deoxythymidylate monophosphate and diphosphate kinase activities were measured spectrophotometrically at 340 nm for 20 min using reduced nicotinamide adenine dinucleotide (0.1 μmole) (12). [6-^{3}H]Thymidine (21.5 Ci/mmole) incorporation into the nucleotides was also measured using the reaction medium of Maley and Ochoa (12) and then plating the ether extract of the reaction medium on PEI cellulose F plates. The plates were eluted with 0.5 *N* formic acid–0.6 *N* LiCl (1:1). After identifying R_f values consistent with the standards, thymidine, thymidylate monophosphate, and thymidylate diphosphate, the areas on the plates were scraped and counted. Carbamyl phosphate synthetase activity was determined using the reaction medium of Kalman *et al.* (13) in the presence of ornithine and the enzyme ornithine transcarbamylase. Citrulline formed from ornithine was measured at 490 nm by the method of Archibald (14). Aspartate transcarbamylase activity was assayed using the incubation medium of Kalman *et al.* (13). The colorimetric determination of carbamyl aspartate was conducted by the procedure of Koritz and Cohen (15). Orotidine monophosphate decarboxylase activity was assayed by the method of Appel (16) using 0.1 μCi of [^{14}C]orotidine monophosphate (34.9 mCi/mmole). The $^{14}CO_2$ generated in 15 min was trapped in 1 *M* methanolic base[4] and counted. Thymidylate synthetase activity was determined using a postmitochondrial supernatant (9000×*g* for 10 min) and 5 μCi of [5-^{3}H]deoxyuridine monophosphate (14 Ci/mmole) according to the method of Kampf *et al.* (17). [^{14}C]Formate incorporation into purines was determined by the method of Spassova *et al.* (18), using 0.5 μCi of [^{14}C]formic acid (52.0 mCi/mmole). Purines were separated on silica gel TLC plates eluted with 1-butanol–acetic acid–water (4:1:5). After identifying R_f values consistent with the standards, adenine and guanine, the plates were scraped and the radioactive content determined. Phosphoribosyl-1-pyrophosphate amidotransferase activity was determined on a supernatant fraction (600×*g*, for 10 min) measuring the reduction of 0.6 μmole of nicotinamide adenine dinucleotide at 340 nm for 30 min (19). Inosinic acid dehydrogenase activity was determined by the method of Becker and Löhr (20) using a supernatant 7000×*g*, for 10 min and [8-^{14}C]inosine-5′ monophosphate (61 mCi/mmole). After plating on PEI cellulose F plastic precoated plates and eluting with 0.5 *M* $(NH_4)_2SO_4$, the spot corresponding to xanthine monophosphate was scraped and counted. Dihydrofolate reductase activity was determined at 340 nm for 30 min as the oxidation of reduced nicotinamide adenine dinucleotide phosphate (21). Ribonucleotide reductase activity was determined by the method of Moore and Hurlbert (22) using [5-^{3}H]cytidine-5-diphosphate (25 Ci/mmole). Ribose and deoxyribose nucleotide were separated on PEI cellulose F plastic precoated plates eluted with 4% boric acid–4 *M* LiCl (4:3) and scraped at the R_f values consistent with the standard deoxycytidine diphosphate. An *in vitro* method[5] using a lysate of Ehrlich ascites cells was used to determine if microlenin was an initiation or elongation inhibitor of protein synthesis by using the standards pyrocatechol violet (an initiation inhibitor) and emetine (an elongation inhibitor) using 1 μCi [^{3}H]leucine (24.7 Ci/mmole). Aliquots of the reaction medium were removed every 2 min, spotted on dried filter paper[5], treated for 10 min in boiling 5% trichloroacetic acid, and washed with cold 5% trichloroacetic acid, ether–ethanol (4:1), and ether. The disks were dried and counted[3].

[4] Hyamine Hydroxide, New England Nuclear.

[5] Whatman #3.

Table II—*In Vivo* Effects of Microlenin at 5 mg/kg/day ip on Ehrlich Ascites Carcinoma of CF_1 Male Mice

Biochemical Parameter or Enzyme (n = 6)	Control) (0,05%) Polysorbate 80 $\bar{X} \pm SD$	Microlenin (5 mg/kg/day) on Days 8 & 9 $\bar{X} \pm SD$
[^{3}H]Thymidine incorporation into DNA	100 ± 8	48 ± 5[a]
[^{3}H]Uridine incorporation into RNA	100 ± 9	98 ± 8
[^{3}H]Leucine incorporation into protein	100 ± 8	59 ± 7[a]
[^{14}C]Formate incorporation into purines	100 ± 12	58 ± 8[a]
DNA polymerase activity	100 ± 6	38 ± 3[a]
Messenger RNA polymerase activity	100 ± 8	104 ± 9
Ribosomal RNA polymerase activity	100 ± 9	82 ± 9[b]
Transfer RNA polymerase activity	100 ± 10	72 ± 9[a]
Ribonucleotide reductase activity	100 ± 6	102 ± 8
Thymidylate monophosphate levels	100 ± 10	47 ± 5[a]
Thymidylate diphosphate levels	100 ± 12	45 ± 6[a]
Thymidylate triphosphate levels	100 ± 9	52 ± 7[a]
Phosphoribosyl pyrophosphate amidotransferase activity	100 ± 9	37 ± 5[a]
Inosinic acid dehydrogenase activity	100 ± 10	86 ± 8
Dihydrofolate reductase activity	100 ± 8	62 ± 7[a]
Carbamyl phosphate synthetase activity	100 ± 10	104 ± 11
Aspartate transcarbamylase activity	100 ± 9	108 ± 10
Orotidine monophosphate decarboxylase activity	100 ± 10	82 ± 9
Thymidylate synthetase activity	100 ± 9	107 ± 10
Number of cells × 10^6/ml ascites fluid	100 ± 9	41 ± 4[a]

[a] $p \leq 0.001$. [b] $p \leq 0.010$.

Protein for enzymatic assay was determined by the Lowry *et al.* technique (23).

Probable (p) significant differences were determined by the Student's t test. Data are expressed in Tables I–II as percent of control with standard deviations.

RESULTS

Microlenin has potent activity against Ehrlich ascites carcinoma growth at 5 and 10 mg/kg/day with 99.9 and 90.4% inhibition, respectively (Table I). Preliminary *in vitro* studies demonstrated that DNA, RNA, and protein syntheses are effectively reduced by microlenin. An ID_{50} value of 1.0 μ*M* was obtained for DNA synthesis and protein synthesis at 1.42 m*M*. Formate incorporation into purine was significantly suppressed by microlenin with an ID_{50} value of 783 μ*M*. Phosphoribosyl pyrophosphate amido transferase activity was markedly reduced in the presence of microlenin, affording an ID_{50} value of 611 μ*M* concentration and for dihydrofolate reductase activity an ID_{50} value of 520 μ*M* was obtained. Marginal inhibition (28%) was observed by 100 μ*M* of microlenin on orotidine monophosphate decarboxylase activity. The thymidine nucleotide pools were reduced during *in vitro* incubation with microlenin. Thymidine monophosphate pools were reduced 45% at 25 μ*M*. DNA polymerase activity was suppressed with microlenin resulting in an ID_{50} value of 498 μ*M*. Deoxyribonuclease activity *in vitro* was inhibited 48% at 100 μ*M* of microlenin. A number of enzyme activities were not affected by microlenin. Those include messenger, ribosomal, and transfer RNA polymerases, ribonucleotide reductase, carbamyl phosphate synthetase, aspartate carbamyl transferase, thymidylate synthetase, and RNA synthesis. *In vivo* studies after 2 days dosing with microlenin at 5 mg/kg/day showed that DNA synthesis was markedly reduced. The control DNA synthesis rate is 107,533 dpm/hr/mg of isolated DNA, which was inhibited 52% by microlenin. Uridine incorporation into RNA for the 10-day control was 51,193 dpm/hr/mg of isolated RNA which was unaffected by drug treatment. Leucine incorporation for the control was 19,181 dpm/hr/mg of isolated protein, which was suppressed 41% by drug therapy. Formate incorporation into purines was 28,786 dpm/mg of protein, which was inhibited 42% by microlenin. Nuclear DNA polymerase activity for the control was 76,528 dpm/hr/mg of nucleoprotein, which was inhibited 62% by drug therapy. Messenger RNA polymerase activity for 10-day cells was 4867 dpm/hr/mg of nucleoprotein, ribosomal RNA polymerase activity was 8751 dpm/hr/mg of nucleoprotein, and transfer RNA polymerase activity was 10,792 dpm/hr/mg of nucleoprotein, which were inhibited 0, 28, and 18%, respectively, by administration of microlenin. Ribonucleotide reductase activity for the control was 153,791 dpm/mg of protein, which was not affected by drug treatment. Thymidine nu-

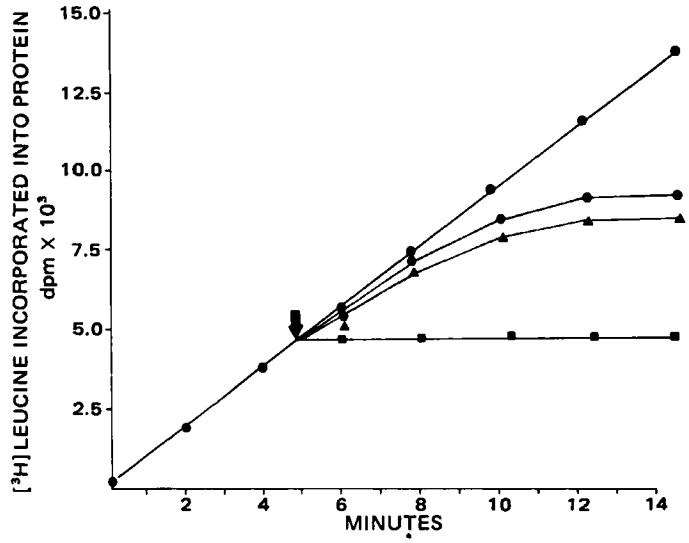

Figure 2—*Effects of microlenin on the initiation and elongation of protein synthesis of Ehrlich ascites carcinoma cells. Key: (●) control, (●) pyrocatechol violet, (■) emetine, and (▲) microlenin, (↓) addition of drug at 100 μM concentration.*

cleotide pools were altered by microlenin treatment. Thymidylate monophosphate pools were reduced 53%, thymidylate diphosphate pools 55%, and thymidylate triphosphate pools 48%.

Examination of the regulatory enzymes of *de novo* purine synthesis phosphoribosyl amido transferase activity for the control was 0.544 optical density units change/hr/mg of protein, which was reduced 63% by two doses of microlenin. Inosinic acid dehydrogenase activity for the 10-day control was 36,530 dpm/mg of protein, which was reduced 14% by microlenin administration. Dihydrofolate reductase activity for the untreated cells afforded a change of 0.514 optical units/hr/mg of protein which was suppressed 38% by drug administration. Examination of pyrimidine synthesis, the regulatory enzyme carbamyl phosphate synthetase activity for the control was 0.128 mg of carbamyl phosphate formed/hr/mg of protein and aspartate carbamyl transferase activity for the control was 7.526 mg of carbamyl aspartate formed/hr/mg of protein, which were not affected by drug treatment. Orotidine monophosphate decarboxylase activity for the control was 10,775 dpm of CO_2 generated for 15 min/mg of protein. Drug treatment reduced the activity 18%. Thymidylate synthetase activity for the control was 103,328 dpm/mg of protein, which was unaffected by drug therapy. Two days of treatment with microlenin at 5 mg/kg/day reduced the number of ascites cells/ml from 226×10^6 to 93×10^6.

DISCUSSION

Microlenin effectively suppressed Ehrlich ascites carcinoma growth as well as DNA synthesis and protein synthesis. Similar findings have also been observed for other sesquiterpene lactones. For example, helenalin at 33.3 mg/kg totally inhibited Ehrlich ascites growth (24) and at 12.5 mg/kg/day for 3 days significantly reduced DNA synthesis 88% (24–26). Tenulin produced a 97.3% reduction of Ehrlich ascites growth and a 91% inhibition of DNA synthesis at the same dose (24–26). Germacranolides [*i.e.*, eupahyssopin (27), eupaformosanin (28), and molephantinin (29)] produced similar effects in the Ehrlich tumor growth. However, the dose microlenin required to bring about the same degree of suppression of tumor growth was much lower: 6 mg/kg/day, compared with the higher doses of drugs, *i.e.*, 33.3 and 12.5 mg/kg/day, for the other derivatives. DNA polymerase activity was significantly reduced by microlenin. A number of pseudoguaianolides and germacranolides have been shown to inhibit both *in vivo* and *in vitro* DNA polymerase activity (24–29), basically at a higher dose than required by microlenin. Thymidylate synthetase activity was also suppressed by the pseudoguaianolides and germacranolides (24–26), but it should be noted that microlenin did not have any effect on thymidylate synthetase activity. These latter two enzymes contain sulfhydryl groups, which are supposedly alkylated by the α,β-unsaturated carbonyl moiety through a rapid Michael-type addition (25). The α,β-unsaturated carbonyl system in a ketone unit exists as the β-unsubstituted cyclopentenone moiety and as the α-methylene-γ-lactone in the structure of microlenin.

Formate incorporation into purines was significantly suppressed by microlenin. The activity of the regulatory enzyme of purine synthesis, phosphoribosyl pyrophosphate amido transferase, was also significantly reduced by microlenin. The magnitude of inhibition of the regulatory enzyme by the drug would account for the degree of reduction of purine synthesis. Nevertheless, microlenin marginally suppressed inosinic acid dehydrogenase activity, another regulatory enzyme of the purine pathway, and dihydrofolate reductase activity was moderately inhibited by microlenin. The latter enzyme plays a major role in the transfer of one carbon unit for purine and pyrimidine synthesis. The earlier regulatory enzymes of pyrimidine synthesis were not affected by drug treatment; however, a later regulatory enzyme, orotidine monophosphate decarboxylase, was marginally inhibited by *in vivo* administration. The nucleotide pools were reduced by microlenin with ~50% reduction. The reductions of monophosphate, diphosphate, and triphosphate pools will severely limit DNA synthesis of cells. Inhibition of formate incorporation, regulatory enzyme activities of the purine pathway, and nucleotide pools has been demonstrated previously with molephantinin in Ehrlich ascites cells.

Analysis of the protein data suggests that microlenin is an initiation inhibitor of protein synthesis of Ehrlich ascites carcinoma cells. Protein synthesis inhibition has been shown for helenalin, eupahyssopin, molephantinin. Recent studies in P-388 lymphocytic leukemia cells and rabbit reticulocyte cells demonstrated that helenalin and bis(henalinyl)malonate inhibit the formation of a 48S ribosomal complex during the initiation step of protein synthesis.

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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.

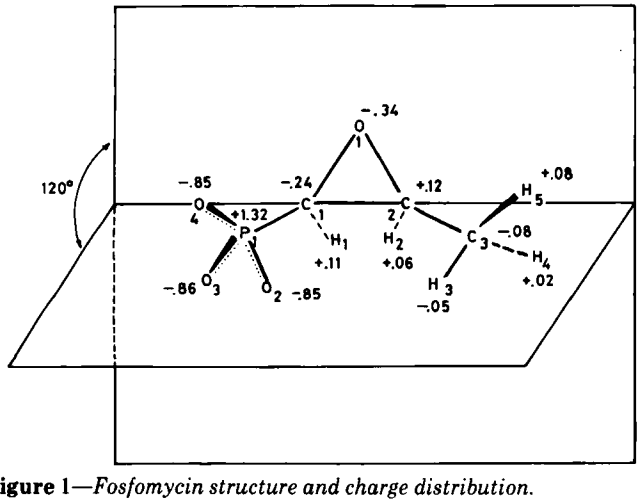

Figure 1—*Fosfomycin structure and charge distribution.*

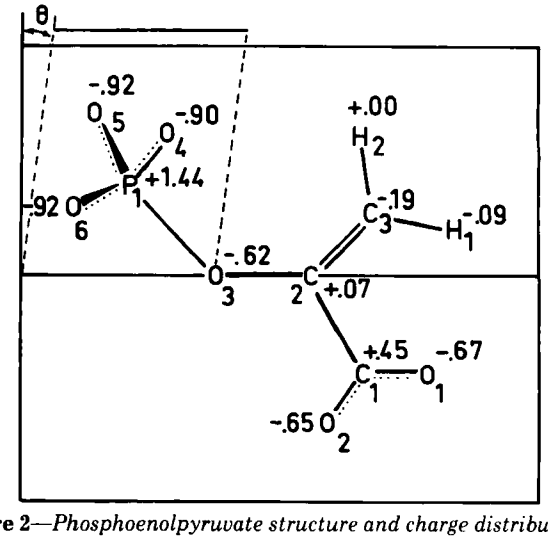

Figure 2—*Phosphoenolpyruvate structure and charge distribution.*

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