Microbial Epoxidation of cis-Propenylphosphonic to (−)-cis-1,2-Epoxypropylphosphonic Acid

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Eighteen species of Penicillium, one of Oidium and one of Paecilomyces were found to effect a stereospecific conversion of cis-propenylphosphonate to fosfomycin which was identified by paper chromatography and gas-liquid chromatography (GLC) of the trimethylsilyl esters. Penicillium spinulosum carried out the epoxidation only after the glucose substrate had been utilized. Glucose controlled the epoxidation since its residual concentrations in the broth severely depresses the reaction. At optimum levels of glucose, an epoxidation efficiency approaching 90% of olefin charged (0.2 g/liter) was obtained after 10 days of incubation. The olefin concentration could be increased to 0.5 g/liter when glucose was replaced by glycerol, whereby a 90% conversion to fosfomycin was attainable in 6 days. The high conversion efficiency, a good agreement between the GLC assay and bioactivity, are indicative of the levorotatory nature of the product.

Hendlin et al. (9) reported the discovery of a new broad-spectrum orally active antibiotic, phosphonamycin, produced by a Streptomyces. The antibiotic, subsequently renamed fosfomycin, was found to be nontoxic and highly potent and is potentially of great clinical value (8). It was identified as (−)-(1R,2S)-1,2-epoxypropylphosphonic acid (4).

Its synthesis was accomplished by Christensen and co-workers (4) by chemical epoxidation of cis-propenylphosphonic acid which gives racemic mixture of (±)-cis-1,2-epoxypropylphosphonate. Since the active isomer was obtained by resolution via the quinine salt in less than 50% yields, it was desirable to seek other more practical methods for the epoxidation step. To this end, a fermentation process was developed which effected a stereo-specific conversion of the olefin to the desired (−)-cis-1,2-epoxypropylphosphonate.

The structure is as follows.

\[
\begin{align*}
\text{CH}_3 & \quad \text{C} \equiv \text{C} \quad \text{H} \\
\text{CH}_3 & \quad \text{P}_3 \text{O}_3 \text{H}_2
\end{align*}
\]

Olefin

\[
\begin{align*}
\text{H} & \quad \text{C} \quad \text{O} \quad \text{C} \quad \text{H} \\
\text{CH}_3 & \quad \text{P}_3 \text{O}_3 \text{H}_2
\end{align*}
\]

Fosfomycin

MATERIALS AND METHODS

Screen methodology. Microorganisms contained in the Merck Microbiology Department culture collection were screened for the ability to transform the biologically inactive cis-propenylphosphonate into biologically active fosfomycin (−)-cis-1,2-epoxypropylphosphonic acid. The lyophilized organism was transferred aseptically to 5 ml of SM-1 medium containing technical grade of glucose (cerelose), 30 g; nutrient broth, 8 g; yeast extract, 2 g; malt extract, 3 g; adjusted to pH 7.0 and made with water to 1 liter. This suspension was inoculated in duplicate into 10 ml of the same medium with and without cis-propenylphosphonate (250 µg/ml final concentration). The tubes were incubated on a rotary shaker (220 rev/min 2-inch throw) at 28 C until adequate growth was obtained. The cells were removed by centrifugation, and the supernatant fluid was examined for the presence of fosfomycin by disc assay against fosfomycin-sensitive and -resistant strains of Proteus vulgaris (strains MB-838 and MB-2146, respectively). Those organisms which were grown with cis-propenylphosphonate and excreted a substance that inhibited the growth of the fosfomycin-sensitive P. vulgaris (but not the resistant strain) were selected for further study. In SM-1 medium lacking the olefin, they did not produce any antibiotic substances active against either assay organism. This observation provided presumptive evidence for the conversion of the inactive olefin to the L-form of fosfomycin.

Shaken-flask studies. Many of the organisms that were active in the screen (Table 1) were tested for transformation of cis-propenylphosphonate in shaken flasks. A loopful of the organism was used to inoculate 40 ml of seed medium per 250-ml Erlenmeyer flask which contained, in grams per liter: cornsteep liquor, 40; invert molasses, 26; CaCO₃, 5; and distiller’s dry solubles, 20; adjusted to final pH 5.2. Incubation was with agitation at 220 rev/min (2-
TABLE 1. Fungi found to epoxidize cis-propenylphosphonate to fosfomycin

| Organism            | Strain | Inhibition zone (mm) (sensitive to P. vulgaris) |
|---------------------|--------|-------------------------------------------------|
| Penicillium charceps | MF-4315 | 35                                              |
| P. flavo-glauces    | MF-115 | 24                                              |
| P. aculeatum        | MF-2771| 31                                              |
| P. corylphinus      | MF-2779| 25                                              |
| P. martis-mear      | MF-819 | 42                                              |
| P. funiculomus      | MF-4384| 23                                              |
| P. pessoides        | MF-946 | 16                                              |
| P. multicolor       | MF-2806| 26                                              |
| P. pinophilum       | MF-1180| 33                                              |
| P. flavidosum       | MF-1262| 20                                              |
| P. pfefferantian    | MF-1005| 32                                              |
| P. roseo-maculatum | MF-1096| 41                                              |
| P. spinulosum       | MF-2843| 37                                              |
| P. frequentsens     | MF-4072| 34                                              |
| P. palitons         | MF-1169| 32                                              |
| P. purpurogenum     | MF-2796| 15                                              |
| P. gelmanii         | MF-4355| 26                                              |
| P. purpurrescens    | MF-999 | 25                                              |
| Paecilomyces varioti| MF-3741| 27                                              |
| Oidium sp.          | MF-3091| 26                                              |

Organisms were grown as described in the text. Discs (7-mm; Whatman no. 1) were dipped into the cell-free broth and spotted on petri dishes containing nutrient broth agar (Difco) seeded with P. vulgaris MB-838 (sensitive) or P. vulgaris MB-2146 (resistant). The dishes were incubated overnight at 37 C, and zones of inhibition were recorded. No activity against P. vulgaris MB-2146 was seen.

The column was washed in sequence with 3 ml of water, 3 ml of methanol-water (3:1), and 3 ml of methanol-1 M ammonium hydroxide (3:1). The washes contained negligible activity and were discarded. The column was eluted with 25 ml of 1 M ammonium hydroxide. The first 15 ml of eluate containing most of the bioactivity was lyophilized, the solids were slurried in 3.0 ml of methanol, and the mixture was filtered through a sintered glass funnel. A 2-ml amount of methanol was used to complete the transfer and to wash the precipitate. The filtrate and wash were concentrated to dryness. The residue was trimethylsilylated with 0.25 ml of REGISIL (Regis Chemical Co., Ill.) at 70 C for 10 min, transferred to a 1.0-ml volumetric flask with 0.25 ml of REGISIL, adjusted to volume with dry dimethylformamide, and 10 µl of this solution was used for analysis. The gas-liquid chromatography (GLC) was carried out on a Varian Aerograph 1700 with a 5 by ½ inch (12.7 by 0.64 cm) stainless-steel column containing 3% OV-17 on 80/100 Chromasorb WHP at 105 C with a helium flow of 90 ml/min. Under these conditions, silylated cis-propenylphosphonic acid and trimethylsilylated fosfomycin had retention times of 4.0 and 7.2 min, respectively.

Assays. Fosfomycin was quantitated by microbiological assay as described by Stapley et al. (19). Residual glucose in the production medium was determined by a ferricyanide-sulfuric acid assay for reducing sugars automated with a Technicon Autoanalyzer. Growth was measured as dry weight of cells per milliliter of culture.

Chemicals. The phosphonic acids used in this study were synthesized in the Process Research Department of Merck & Co., Inc., Rahway, N.J.

RESULTS

Screening. Of 325 bacteria, 150 actinomycetes, and 150 yeasts screened, none was found to convert cis-propenylphosphonate to biologically active material. Of 600 fungi, however, 20 organisms (Table 1) yielded from the olefin a product which inhibited the growth of the fosfomycin-sensitive strain of P. vulgaris, did not affect the resistant strain, and identified as fosfomycin. Eighteen of the active species belonged to the genus Penicillium, one to Oidium, and one to Paecilomyces. The frequency of positive cultures in the entire screen was 1.6%, whereas the frequency for fungi was 3.3%. When only the penicillia are considered, the frequency of positive cultures is 5.6%. Apparently, the ability to transform cis-propenylphosphonate is not an unusual event among the penicillia.

Identification of fosfomycin. A 50-ml amount of clarified broth was passed through a 28-cm-high column containing 25 ml of Dowex 50W-X8, 200 to 400 mesh, on the NH4+ cycle. A forerun (10 ml) containing a negligible amount of antibiotic was discarded, and 42 ml of effluent was collected. The column was washed with 15 ml of water. The combined effluent and wash were adjusted to pH 5.7 by the addition of 0.45 ml of 2.5 N HCl and was charged to a 15-cm-high column containing 3.0 g of acid-washed alumina (Merck). The fine particles had been removed from the alumina by three slurries with water (20 ml per slurry) followed by decantation.
EPOXIDATION OF PROPENYLPHOSPHONATE

chromatography on Whatman paper no. 3 with propanol-2-N-methylamine (7:3). The chromatograms were dried and subjected to bioautography in large Pyrex dishes containing nutrient agar seeded with *P. vulgaris* MB-838. In both cases, the *Rf* of the bioactive spots of the broth were coincident with that of authentic fosfomycin. Co-chromatography of both broths with authentic 1,2-epoxypropylphosphonate produced only a single spot, further supporting the conclusion that the observed activity was from fosfomycin. Final proof was GLC of several active broths.

The antibiotic in a filtered broth of *P. purpureascens* was concentrated by ion-exchange and alumina chromatography to yield a fraction (253 mg) containing 2.3 mg of fosfomycin (by bioassay). The solids were treated with methanol and filtered to remove insoluble neutral salts. After concentrating the filtrate to dryness, the residue was allowed to react with *O,N*-bis-trimethylsilyl trifluoroacetamide in dimethylformamide. GLC gave a peak indistinguishable from the *bis*-trimethylsilyl ester of 1,2-epoxypropylphosphonic acid. Mass spectral examination of the peak showed a fragmentation pattern identical to trimethylsilylated 1,2-epoxypropylphosphonic acid. As frequently found with trimethylsilyl derivatives, the molecular ion was not observed; the ion of highest mass at *m/e* 267 corresponds to the loss of methyl from the molecular ion (18).

A broth from *P. martis-mear* was concentrated and silylated in the same way. Measurement of the area of the GLC peak indicated 0.7 mg of 1,2-epoxypropylphosphonic acid in the broth concentrate, a lower value than the 1.0 mg found by bioassay. Better agreement of the two assay methods was obtained in a broth concentrate of *P. spinulosum*; GLC indicated 4.0 mg and bioassay 4.5 mg of epoxide in the concentrate. Since racemic 1,2-epoxypropylphosphonic acid would give a GLC result twice that of the bioassay, the agreement of the two assays indicates that optically active levorotatory isomer was formed by epoxidation of *cis*-propenylphosphonic acid.

To substantiate this result, the optical properties of the fermentation product were examined. The *bis*-trimethylsilyl ester of fosfomycin was isolated from a broth concentrate by preparative GLC, and a synthetic sample of (-)-1,2-epoxypropylphosphonic acid was treated similarly for comparison. The optical rotatory dispersion curve of the ester isolated from broth agreed in sign and slope with the synthetic sample but the absolute rotations for the broth sample ([α]₂₀₀ nm\(^{2\text{b}+}\) + 956, [α]₁₉₅ nm\(^{4\text{b}+}\) + 1,690) were higher than the rotations for the synthetic sample, ([α]₂₀₀ nm\(^{2\text{b}+}\) + 590, [α]₁₉₅ nm\(^{4\text{b}+}\) + 1,470). The numerical discrepancy is thought to indicate an inaccuracy of the instrument at the low wavelength used rather than the presence of some optically inactive epoxide in the synthetic sample.

**Shaken flask studies.** Although broths from the initial screen of *P. martis-mear* MF-819 and *P. roseo-maculatum* MF-1006 (Table 1) gave the largest zones of inhibition on agar plates of *P. vulgaris*, *P. spinulosum* MB-2843 gave the most consistent results in shaken flasks; therefore, this organism was chosen for further study. Figure 1

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**FIG. 1.** Growth, sugar utilization, and conversion of *cis*-propenylphosphonate to fosfomycin by *P. spinulosum* MF-2843. Production medium (SM-1) as described in text. *cis*-Propenylphosphonate (90% pure) was added at 200 μg/ml as the free acid.

**FIG. 2.** Effect of sugar concentration on the synthesis of fosfomycin by *P. spinulosum* MF-2843. Incubation time was 10 days. *cis*-Propenylphosphonate (90% pure) added at 200 μg/ml as the free acid.
shows a time course of growth, glucose utilization, pH profile, and conversion of cis-propenylphosphonate to fosfomycin. A lag in the growth and sugar utilization occurred during the first 12 hr, after which a rapid increase in both parameters took place. The pH fell to 5.5 during the first 33 hr, began to rise slowly, and reached pH 8.0 at 96 hr. The stationary phase of growth began at 36 to 48 hr postinoculation. At this time, no fosfomycin was detected in the broth. Epoxidation of the olefin commenced immediately after sugar had been exhausted (56 hr). Although this experiment was terminated at the 96th hr, other data revealed that fosfomycin synthesis continues past 96 hr, and, at 8 to 10 days, 80 to 90% molar conversion of olefin to 1,2-epoxypropylphosphonate was observed.

The fact that the epoxidation reaction was not initiated until the glucose had been exhausted suggested that the sugar concentration in the fermentation was critical. Various levels of glucose were, therefore, added to the SM-1 production medium containing 0.2 g of olefin per liter (Fig. 2). When 1% or less of glucose was supplied, no fosfomycin was synthesized. The optimum sugar range for epoxidation was 6 to 10%. Above 10%, residual sugar remained in the medium throughout the fermentation, and the epoxidation reaction was inhibited. At an initial level of 7.5 to 10%, glucose did not result in a significant level of residual glucose in the medium, and fosfomycin synthesis was reduced by 11.6% (90% pure olefin added at 200 μg/ml with the formation of 180 μg of fosfomycin per ml by microbiological assay). There was no significant effect of sugar concentration on the growth of the organism.

Glycerol could effectively replace glucose as a carbon source in the SM-1 production medium. Figure 3 shows that efficient epoxidation was obtained with an initial 0.5 g/liter concentration when glycerol was used as the carbon source. Above this 0.5 g/liter concentration of cis-propenylphosphonate, the efficiency of epoxidation fell off markedly. With glucose as the carbon source, conversion efficiencies approaching 90% were obtained only at much lower (0.1 to 0.2 g/liter) levels of olefin. At 3 g of cis-propenylphosphonate per liter, little or no epoxidation was obtained regardless of the sugar source. There was no effect on growth yield or rate with any olefin concentration.

Sucrose, fructose, raffinose, ribose, arabinose, and starch can all replace glucose or glycerol for growth and epoxidation, but less effectively. Maltose, galactose, xylose, sorbitol, trehalose, and dulcitol were completely ineffective for epoxidation although they fully supported growth of the organism.

The importance of the three other components of the SM-1 production medium, nutrient broth and yeast and malt extracts, were also investigated (Table 2). The deletion of nutrient broth almost completely prevented fosfomycin synthesis. Deletion of yeast extract also decreased the conversion but to a lesser extent than did omission of nutrient broth. The malt extract component had little effect. Growth of the organism was not effected by any of the deletions.

Specificity of epoxidation. Substitution of the olefin by 1,2-dihydroxypropylphosphonic acid or trans-propenylphosphonate did not yield any bioactive material in the broths of P. spinulosum. Since the trans-isomer of fosfomycin is not biologically active, it was possible that the trans-olefin was epoxidized to the trans-isomer of fosfomycin, but it could not be detected by bioac-

![Fig. 3. Effect of glycerol, glucose, and cis-propenylphosphonate on the efficiency of epoxidation bit P. spinulosum MF-2843. Glucose (cerelose) at 30 g/liter (○); glycerol at 30 g/liter (●). Six days of incubation at 28°C.](http://aem.asm.org/)
tivity. In this regard the broth of \( P. \) spinulosum grown with \( \text{trans} \)-propenylphosphonate was subjected to GLC.

The purified, trimethylsilylated broth indicated that all the \( \text{trans-1} \)-propenylphosphonic acid remained unchanged and that no epoxidation had occurred. Peaks corresponding to trimethylsilylated fosfomycin and to trimethylsilylated \( \text{trans-1,2} \)-epoxypropylphosphonic acid were absent from the chromatogram.

**DISCUSSION**

Many investigators have reported the epoxidation of steroids by microorganisms (2, 3, 11). Simpler ring structures containing epoxides also have been isolated from fermentation broths of actinomycetes (6, 14), fungi (5, 17), and bacteria (21). Besides ring structures, epoxides have been found on the olefinic side chains of quinones; Friis, Daves, and Folkers (7) described mono- and diepoxyubiquinones isolated from \( \text{Rhodospirillum rubrum} \). Mills and Turner (13) reported the isolation of a new metabolite from \( \text{Aspergillus melleus} \) which contained an epoxypetyl side chain on a methyl pyranone ring. A similar material with an epoxypetyl side chain was described by Argoudelis and Zieserl (1) after isolation from broths of \( \text{Aspergillus nidulans} \). A report from van der Linden (20) stated that heptane-grown cells of \( \text{Pseudomonas aeruginos}a \) oxidize octene-1 to octene-1,2-epoxide. A polysaturated long-chain antibiotic substance containing an epoxide at carbons 2 and 3 also has been reported (15, 16). Thus, the appearance of epoxide functionalities in long-chain saturated or unsaturated compounds, in side chains to ring structures, and directly on cyclic structures occurs fairly frequently in microbial products. Much rarer, however, is the appearance of the epoxide in simple natural compounds, although Martin and Foster (11) reported that \( \text{Aspergillus fumigatus} \) produced \( \text{trans-L-epoxy-succinate} \) from glucose in a 21\% yield. The epoxidation of \( \text{cis-propenyl} \)-phosphonate reported here is then the second such example. This epoxidation reaction has as its product the simplest known biologically active epoxide.

The oxidation of \( \text{cis-propenylphosphonate} \) to fosfomycin by \( P. \) spinulosum is a very efficient microbial epoxidation since 90\% of the \( \text{cis-propenylphosphonate} \) (200 \( \mu \) g/ml) was converted to fosfomycin in 10 days when glucose was used as the carbon source. When glycerol was used instead of glucose, a higher olefin concentration was possible (0.5 g/liter) and 90\% epoxidation was attained in 6 days.

The efficiency of epoxidation, the recovery of 90\% of the theoretical bioactivity, and the good agreement between bioactivity and GLC assay show that the reaction is stereo-specific and that only the levorotatory isomer was synthesized. The epoxidizing systems show substrate specificity since neither the 1,2-dihydroxypropylphosphonate nor \( \text{trans-propenylphosphonate} \) were epoxidized. Because of the high efficiency and the stereospecificity, the microbial epoxidation of \( \text{cis-propenylphosphonate} \) might be of value in the commercial synthesis of fosfomycin.

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