STUDIES ON THE BIOSYNTHESIS OF FOSFOMYCIN

4. THE BIOSYNTHETIC ORIGIN OF THE METHYL GROUP OF FOSFOMYCIN†

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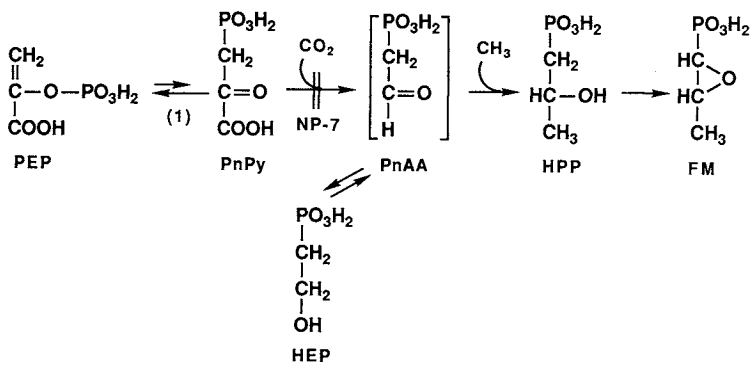
Fosfomycin (FM) is a clinically useful antibiotic produced by several *Streptomyces* species[2,3], *Pseudomonas syringae*[4] and *Pseudomonas viridiflava*[5]. This small compound has a unique structure, including a carbon-phosphorus bond (C-P bond) and an epoxide[6]. Recently we proved that the C-P bond in FM was formed by intramolecular rearrangement of phosphoenolpyruvate (PEP) to phosphonopyruvate catalyzed by PEP phosphomutase (Fig. 1 (1))[1] utilizing a mechanism common to the formation of other natural C-P compounds such as bialaphos[7], 2-aminoethylphosphonic acid[8], 2-hydroxyethylphosphonic acid (HEP)[9] and FR-33289[7].

By bioconversion experiments using two FM non-producing mutants of *Streptomyces wedmorensis*, NP-7 and A16, we also showed that the epoxide ring of FM was formed by dehydrogenation of 2-hydroxypropylphosphonic acid (HPP)[10,11]. Fig. 1 summarizes the biosynthetic pathway of FM and the blocked step of NP-7 based on our experiments reported previously[10,12]. Since mutant A16, which was defective in the biosynthesis of hydroxocobalamin, could convert HPP to FM, it is suggested that incorporation of the methyl group into the FM molecule should occur during a stage between HEP and HPP, and that hydroxocobalamin is required for methylation of a putative C_2 biosynthetic intermediate, possibly phosphonoacetaldehyde (PnAA) or its analogue. Thus it is proposed that PnAA is methylated by methylcobalamin to generate HPP.

To elucidate the biosynthetic origin of the methyl group of FM, we examined incorporation of [*methyl*-^{14}C]methylcobalamin into FM using mutant A16. [*Methyl*-^{14}C]methylcobalamin was prepared by reduction of hydroxobalamin followed by reaction with [^{14}C]methyl iodide according to SCHRAUZER[13]. Mutant A16 was cultivated as reported previously in the FM-production medium containing starch 4%, salad oil 1.5%, Sungrain 5%, wheat germ 2%, K_2HPO_4 0.1% and $CoCl_2$ 0.0001% (pH 8.0). The ^{14}C-labeled precursor was added at 48 hours after inoculation and after a further 24 hours at 27°C, ^{14}C labeled FM was partially purified according to the method reported previously[4]. The broth filtrate was adsorbed on a Dowex-1 (Cl^- form) column and FM was eluted with 5% NaCl solution.

Fig. 1. The proposed biosynthetic pathway of fosfomycin.

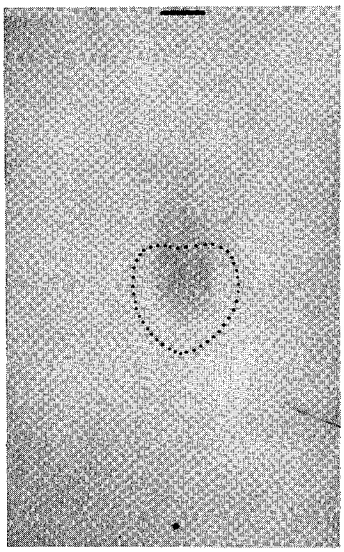


PEP = phosphoenolpyruvic acid, PnPy = phosphonopyruvic acid, PnAA = phosphonoacetaldehyde, HEP = 2-hydroxyethylphosphonic acid, HPP = 2-hydroxypropylphosphonic acid, FM = fosfomycin, ‖: indicates the blocked step of NP-7.

† For part 3: See ref 1.

Fig. 2. Autoradiogram and bioautogram of partially purified fosfomycin on silica gel TLC plate developed with MeOH-2% aq NaCl solution (75:25).

A dotted line indicates bioactivity of fosfomycin against *Proteus* sp.



The eluate was concentrated and extracted with methanol twice. The extract was concentrated and analyzed by silica gel and cellulose TLC developed with MeOH-2% aq NaCl solution (75:25) and BuOH-AcOH-H_2O (3:1:1), respectively. FM was detected by bioautography against *Proteus* sp. and its radioactivity was detected by autoradiography. The radioactive spot coincided with bioactivity on silica gel (Fig. 2) and cellulose TLC plates. Thus the methyl group of FM appears to be derived from methylcobalamin.

ROGERS and BIRNBAUM reported that the methyl carbon of L-methionine was efficiently incorporated into the methyl carbon of FM[3]. However, failure of mutant A16 to produce FM by addition of DL-methionine (data not shown) suggested that methionine was not utilized as the direct methyl donor for methylation of PnAA.

We have recently shown that methylcobalamin served as the direct methyl donor for methylation of the phosphorus atom of the phosphinic acid function in a bialaphos biosynthetic intermediate using a cell free system[14]. Since the carbonyl carbon of PnAA is positively charged as is the phosphorus atom in the phosphinic acid function, it is likely that PnAA is methylated by a nucleophilic attack of the methyl anion derived from methylcobalamin.

OKUMURA *et al.* reported high incorporation of [*methyl*-^{3}H]methylcobalamin into fortimicin A indicating the direct incorporation of the methyl group of the labeled compound into the antibiotic[15]. TESTA *et al.* also reported the involvement of cobalt-dependent methylation in the gentamicin fermentation[16]. Since the cobalt atom is a central component of the cobalamin molecule, it seems likely that cobalamin is involved in methylation reactions in the biosynthetic pathway of gentamicin. The detailed mechanisms of these cobalamin-mediated methylations, however, remain unclear. In order to define the direct methyl group donor for methylating PnAA, it is necessary to establish a cell free system for this reaction.

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