STUDIES ON THE BIOSYNTHESIS OF BIALAPHOS (SF-1293) 4. PRODUCTION OF PHOSPHONIC ACID DERIVATIVES, 2-HYDROXYETHYLPHOSPHONIC ACID, HYDROXYMETHYLPHOSPHONIC ACID AND PHOSPHONOFORMIC ACID BY BLOCKED MUTANTS OF *STREPTOMYCES HYGROSCOPICUS* SF-1293 AND THEIR ROLES IN THE BIOSYNTHESIS OF BIALAPHOS[1)]

Sir:

Bialaphos (formerly called SF-1293[2,3)]) is a metabolite produced by *Streptomyces hygroscopicus* SF-1293 and is presently being developed as a herbicide. As an approach to improve the production yield of bialaphos and thereby cutting down its production cost, we have been engaged in the biosynthetic studies of this compound[1,4,5)]. As a result we isolated phosphinic acid derivatives such as MP-101, MP-102, MP-103, MP-104 and MP-105 from the fermentation broth of the parent strain cultivated in the absence of cobalt ion[5)] or of a blocked mutant[1)]. Some of these metabolites, MP-101, MP-102 and MP-103, in particular, proved to be true biosynthetic intermediates by their very efficient transformation to the final product[1,5)]. MP-103 was converted to MP-102 *via* MP-101 and methylation of the phosphinic acid moiety in MP-102 took place at the final stage of bialaphos biosynthesis. Thus, it turned out that the reduction of phosphonic acid (H_2O_3P-C-) to phosphinic acid (H_2O_2P-C-) is the key step for the formation of the C-P-C bond in bialaphos. The detailed mechanism, however, remains as yet to be clarified. Further screening for biosynthetic intermediates resulted in the isolation of phosphonic acid derivatives situated in the earlier biosynthetic steps.

We wish to report herein the isolation, structural elucidation and biological conversion of these metabolites, 2-hydroxyethylphosphonic acid, hydroxymethylphosphonic acid and phosphonoformic acid which were produced by cultivation in a similar way as reported previously[4,5)] of blocked mutants (NP-46, NP-213 and NP-221) obtained by treatment with nitrosoguanidine of the parent strain. Although these compounds had been prepared by chemical synthesis[6~8)], they have never been found in nature.

Throughout the isolation process, fractions containing C-P compounds were detected by ^{31}P NMR spectroscopy. Since the chemical shifts of the phosphorus atom in C-P compounds are very characteristic, ^{31}P NMR enabled us to detect C-P compounds selectively and efficiently.

2-Hydroxyethylphosphonic Acid (HEP)

Filtered broth (470 ml, pH 3.5) of the mutant NP-46 was passed through a Dowex-50 (H^+ form) column and then adsorbed on a Dowex-1 (Cl^- form) column which was, after washing with water, eluted with 2% NaCl solution. Fractions showing ^{31}P signals (δ_P *ca.* 20) were combined and purified by Sephadex G-10 column chromatography. The combined fraction rich in a C-P compound was further purified by cellulose column chromatography (BuOH - AcOH - H_2O, 2: 1: 1) to give the monosodium salt of HEP (210 mg), $C_2H_6O_4PNa$, FD-MS (*m/z*) 149 $(M+H)^+$, 171 $(M+Na)^+$.

The ^1H NMR spectrum of HEP showed the presence of two contiguous methylene units adjacent to a phosphorus atom [δ_H 3.74 (2H) and 1.89 (2H), J_{vic}=8.3 Hz, $^3J_{P-H}$=18.1 Hz and $^2J_{P-H}$=1.89 Hz]. The downfield shift of one of the methylene signals suggested its linkage to a hydroxyl group. This partial structure is supported by the ^{13}C and ^{31}P NMR spectral data (δ_C $-CH_2-OH$ 58.4 and $P-CH_2-$ 32.5, J_{C-P}= 126.2 Hz, δ_P 19.0). Since this compound showed no doublet proton signal at δ_H *ca.* 7.0 characteristic to phosphinic acid derivatives[4,5)], it is apparent that the phosphorus is present as a phosphonic acid function in this molecule. Thus the structure of HEP has been established as shown in Fig. 1 and is supported by identity with a synthetic sample[6)].

Hydroxymethylphosphonic Acid (HMP)

This compound was produced by the blocked mutant NP-221. Filtered broth (2 liters) was passed through a Dowex-50 (H^+ form) column and the effluent was adsorbed on an Amberlite IRA-45 (OH^- form) column. After washing with 0.5 N AcOH, HMP was eluted with 0.5 N HCl and a rich fraction was further purified by Sephadex G-10 (developed with H_2O) and Toyopearl HW-40 (developed with H_2O) to give 50 mg of HMP, CH_5O_4P, FD-MS (*m/z*) 135 (M+Na). The structure of HMP has been determined by ^1H NMR (δ_H 3.70, J_{H-P}=7.3 Hz), ^{13}C NMR (δ_C 56.7, J_{C-P}=158.4 Hz) and ^{31}P NMR (δ_P 18.0) spectral data. The structure of this compound

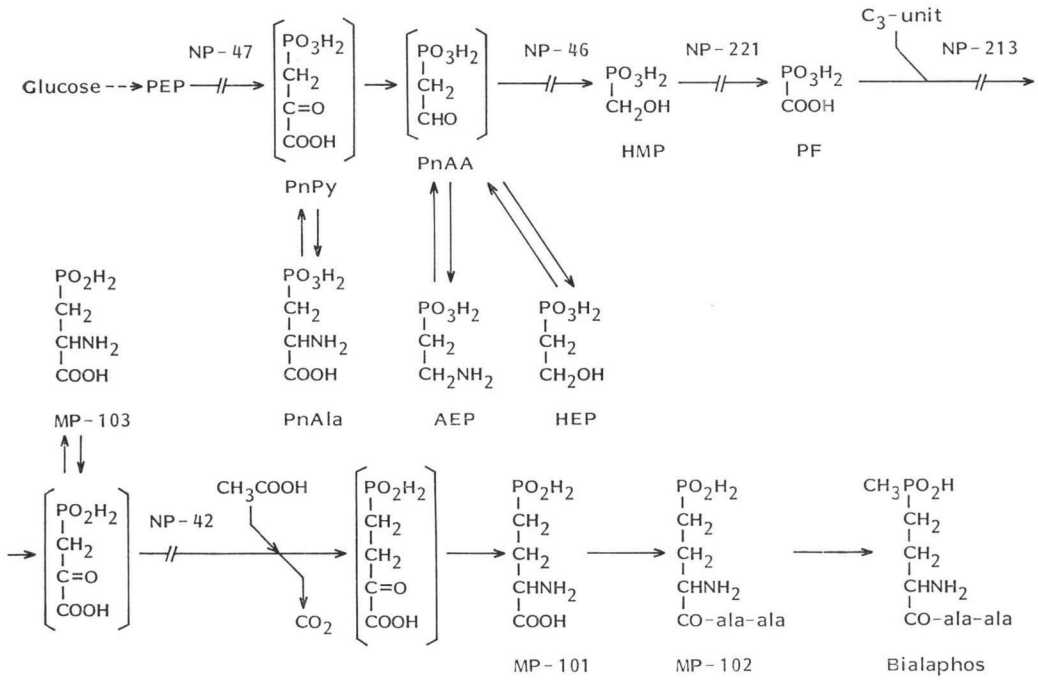
Fig. 1. Biosynthetic pathway of bialaphos.

MP-101=2-amino-4-phosphinobutyric acid, MP-102=2-amino-4-phosphinobutyrylalanylalanine, MP-103=2-amino-3-phosphinopropionic acid.

Abbreviations; PEP=phosphoenolpyruvic acid, PnPy=phosphonopyruvic acid, PnAla=phosphonoalanine, PnAA=phosphonoacetaldehyde, AEP=aminoethylphosphonic acid, HEP=2-hydroxyethylphosphonic acid, HMP=hydroxymethylphosphonic acid, PF=phosphonoformic acid, ala-ala=alanyl alanine.

// Represents blocked points of mutants.

Compounds in blackets represent hypothetical intermediates or related compounds as yet to be isolated.



was confirmed by chemical synthesis according to the method reported by PAGE[7].

Phosphonoformic Acid (PF)

Since PF is unstable, all the isolation procedures were made in a cold room at 0°C. Filtered broth (2 liters) of a mutant NP-213 (formerly called NTG-213[1]) was passed through a column of Dowex-50 (H^+ form) and the effluent was, after neutralization to pH 7.0 with NaOH, adsorbed on a Dowex-1 (Cl^- form) column which was developed with 0.5% NaCl and then with 3% NaCl solution. The latter fraction was further purified by repeated column chromatography on Sephadex G-10 to afford a pure sample of the trisodium salt of PF (150 mg), CO_5PNa_3, FD-MS (*m/z*) 193 $(M+H)^+$, 215 $(M+Na)^+$. Its structure was established by ^{13}C NMR (δ_C 183.6, J_{C-P}=223 Hz) and ^{31}P NMR (δ_P 0) spectroscopy and by direct comparison with a synthetic sample (Sigma Chemical Co.) known as an antiviral agent[9].

It is important to note that PF can be regarded as a potential phosphinic acid derivative since it is very easily converted to phosphorous acid with evolution of CO_2 under acidic condition[8].

Transformation of the Isolated Phosphonic Acid Derivatives to Bialaphos

In order to know whether these metabolites are true biosynthetic intermediates, they were subjected to transformation experiments using appropriate blocked mutants (NP-46, NP-221 and NP-213) and another blocked mutant NP-47 which can not produce any C-P compound (S. IMAI *et al.* unpublished results). Although HEP, HMP and PF were not as good precursors as MP-103, they were clearly transformed to bialaphos as shown in Table 1. Thus NP-47 converted HEP, HMP and PF, NP-46 changed HMP

Table 1. Biological transformation of isolated phosphonic acid derivatives by mutants of *S. hygroscopicus* SF-1293.

Precursor (500 μg/ml)	Bialaphos produced by mutants (μg/ml)			
	NP-47	NP-46	NP-221	NP-213
None	0	0	0	0
2-Hydroxyethylphosphonic acid	1.0	0	0	0
Hydroxymethylphosphonic acid	0.9	2.4	0	0
Phosphonoformic acid	2.5	4.5	2.7	0
MP-103	18.0	24.0	7.5	93.0

Experiments were carried out as reported previously[1].

Table 2. Biological transformation of phosphonic acid derivatives by blocked mutant (NP-47) of *S. hygroscopicus* SF-1293.

Precursor (500 μg/ml)	Bialaphos produced by NP-47 (μg/ml)
None	0
2-Aminoethyl phosphonic acid	13.0
Phosphonoalanine	18.0
Phosphonopyruvic acid	2.6
MP-103	18.0

Experiments were carried out as reported previously[1].

and PF, and NP-221 transformed PF to bialaphos. On the other hand, NP-213 could not utilize these phosphonic acid derivatives as substrates. Based on these experimental results, the reaction sequence for the biosynthesis of bialaphos together with the blocked steps of the mutants are summarized as shown in Fig. 1.

It has been generally accepted that the C-P bond in naturally occurring C-P compounds is formed by intramolecular rearrangement of phosphoenolpyruvate to phosphonopyruvate which is then metabolized to phosphonoalanine and phosphonoacetaldehyde[10]. However, since these phosphonic acids were not utilized at all by another blocked mutant (NP-213), we postulated in a previous paper[1] that phosphonic acid derivatives would not be involved in the biosynthesis of bialaphos and that direct reduction of phosphoenolpyruvate to (hypothetical) phosphinoenolpyruvate precedes the C-P bond formation. Therefore, it is significant that NP-47 could transform phosphonoalanine, phosphonopyruvic acid and aminoethylphosphonic acid, a plausible biological equivalent of phosphonoacetaldehyde to bialaphos (Table 2). With these results in hand, phosphonic acid derivatives are concluded to play important roles for the formation of the phosphinic acids.

It is also interesting to note that HEP was converted to fosfomycin by its producing organism[11]. Therefore, it may be concluded that there exists a common biosynthetic pathway which forms phosphonic acid derivatives possessing a two carbon unit such as HEP from phosphoenolpyruvate.

The mechanism converting phosphonoformic acid to α-keto MP-103 still remains to be clarified.

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