STUDIES ON THE BIOSYNTHESIS OF BIALAPHOS (SF-1293) 5. PRODUCTION OF 2-PHOSPHINOMETHYLMALIC ACID, AN ANALOGUE OF CITRIC ACID BY *STREPTOMYCES HYGROSCOPICUS* SF-1293 AND ITS INVOLVEMENT IN THE BIOSYNTHESIS OF BIALAPHOS[1]

Sir:

In a previous paper[2], we reported that the carbon skeleton of bialaphos (BA), a herbicide produced by *Streptomyces hygroscopicus* SF-1293, is build up from two C_2 units originating from glucose and acetic acid (Fig. 1). Further investigation resulted in the isolation of MP-103 with a C_3 unit, which was converted to BA *via* MP-101 with a C_4 skeleton[3] by a blocked mutant of the producing organism (Fig. 1). A plausible mechanism to explain this transformation would be to assume the involvement of an enzyme system identical with or similar to TCA cycle.

As an approach to shed light on this reaction sequence, we investigated the effect of monofluoroacetic acid (MFA) which is known as a strong inhibitor of aconitase. We wish to report herein the isolation of 2-phosphinomethylmalic acid (PMM) from the fermentation broth of *S. hygroscopicus* SF-1293 supplemented with MFA and its roles in the biosynthesis of BA.

As shown in Table 1, addition of the inhibitor caused marked decrease in the production of BA with a slight increase of sugar metabolism rate. Analysis of these fermentation broths by ^{31}P NMR revealed the presence of an unknown signal at *ca.* 24 ppm suggesting the accumulation of a new metabolite, which was isolated as follows. Fermentation broth prepared as reported previously[3] with the exception of the addition of 0.1% MFA, was adjusted to pH 2 and centrifuged to remove mycelia. The supernatant was passed through a Dowex-50 (H^+ form) and then adsorbed on a Dowex-1 (Cl^- form) column. After washing with water, the column was eluted with 2% NaCl. Fractions containing PMM were combined and concentrated to a small volume. After removal of precipitated NaCl by filtration, the filtrate was subjected to Dowex-50 (H^+ form) column chromatography developed with water. The combined fraction containing PMM was concentrated and then applied to a cellulose column equilibrated with BuOH - AcOH - H_2O, 2:1:1. Development with the same solvent mixture gave a fraction rich in PMM, which was then passed through a Dowex-

Table 1. Inhibition of the production of bialaphos by monofluoroacetic acid.

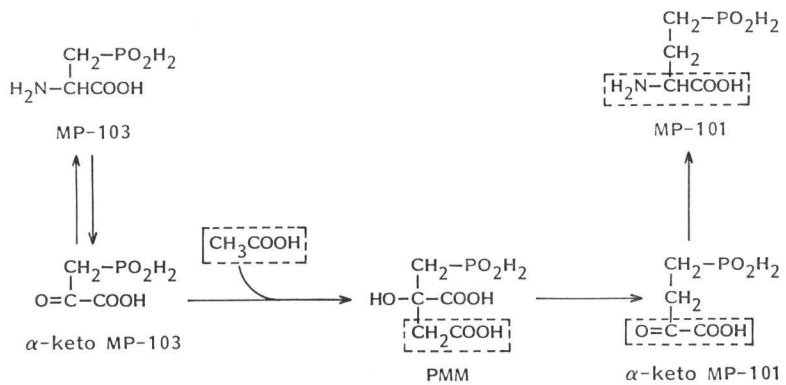
Concentration of CFH_2COONa (%)	Bialaphos produced (%)	pH (final)	Residual sugar
0	100	7.26	4.53
0.001*	81.9	7.04	4.50
0.003	82.8	6.93	4.20
0.01	62.5	6.40	4.08
0.03	35.8	6.50	4.11
0.1	16.1	6.28	3.60
0.1**	26.1	6.19	3.69

The fermentation was carried out as reported previously[4] using the parent strain of *S. hygroscopicus*.

* The inhibitor was added to the medium prior to the initiation of the fermentation.

** Added on the 2nd day.

Fig. 1. Biosynthetic pathway from MP-103 to MP-101 *via* 2-phosphinomethylmalic acid (PMM).



50 (H^+ form) column. Concentration of the effluent gave 112 mg of an oily substance of PMM free acid; $C_5H_9O_7P$, FD-MS (*m/z*) 213 $(M+H)^+$. This compound was characterized as its sodium salt as follows.

Fifty mg of this sample was dissolved in a small amount of water and 3 equivalents of NaOH was added. Concentration gave a white powder of trisodium salt of PMM. Its physico-chemical properties are as follows; $C_5H_6O_7PNa_3 \cdot H_2O$, found; C 20.35, H 2.67, O 42.91, P 10.39, Na 23.15, calcd; C 20.28, H 2.72, O 43.23, P 10.46, Na 23.30. MP 105~107°C (dec), $[\alpha]_D^{20}$ −18.1° (*c* 1, H_2O), FD-MS (*m/z*) 279 $(M+H)^+$.

The ^1H NMR spectral data of PMM revealed the presence of a phosphinic acid (δ_H 7.12, J_{H-P}= 558 Hz and δ_P 24.5)[4] and two isolated methylene units (2.90 and 3.07, AB quartet, and 2.20 and 2.45, AB quartet), the latter being long range coupled to the phosphinic acid proton (J=1.5 Hz). The ^{13}C NMR spectral data of PMM showed the presence of two carboxylic acids (δ_C 173.7 and 176.8), two methylene units (δ_C 44.9 and 40.2) and one quaternary oxycarbon (δ_C 73.6). The very large coupling constant of one methylene carbon to phosphorus (J_{C-P}=89.5 Hz) indicated the partial structure, $-CH_2-HP(=O)-OH$. Since the two methylene protons were not coupled each other, they must be separated by the quaternary oxycarbon. Thus its structure has been established as shown in Fig. 1. This conclusion is also supported by comparison of the ^{13}C NMR spectral data of PMM and citric acid [δ_C C-1(–COOH) 174.2, C-2 ($-CH_2-$) 44.1, C-3 (HO–C–) 74.2 and C-6 (–COOH) 177.5][5].

Supplement of this compound into the fermentation broth of *S. hygroscopicus* stimulated the production of BA. Thus, addition of 300 μg/ml of the substrate on the 2nd day caused the production of BA at the level of 670 μg/ml with the control (no addition) being 390 μg/ml. When 100 μg/ml of PMM was added on 2nd, 3rd and 4th days, the amount of BA reached to 760 μg/ml. Therefore, PMM is believed to be a biosynthetic intermediate of BA.

In order to reveal the action mechanism of MFA, transformation experiments were carried out using MP-103, MP-101 and phosphinothricin. As shown in Table 2, MFA inhibited the conversion of MP-103, while it did not affect the transformation of MP-101 and phosphinothricin to BA. Therefore MFA inhibits selectively the step from a C_3 compound to a C_4 metabolite. Since MP-103 is a biological equivalent of α-keto MP-103[3], which may be regarded as an analogue of oxalacetic acid, it may be reasonably assumed that MP-103 reacts with acetic acid to give PMM, an analogue of citric acid which would then be metabolized to MP-101 in a similar way to produce glutamic acid.

It is interesting to know whether the enzyme system involved is related to that of TCA cycle. In this regard, it is very important to determine the absolute stereochemistry of PMM which remains as yet to be established.

HARUO SETO
SATOSHI IMAI†
TORU SASAKI
KUMIKO SHIMOTOHNO
TAKASHI TSURUOKA††
HIROSHI OGAWA†
ATSUYUKI SATOH†
SHIGEHARU INOUYE††
TARO NIIDA††
NOBORU ŌTAKE
Institute of Applied Microbiology,
The University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan

Table 2. Inhibition of transformation of several biosynthetic intermediates to bialaphos by the addition of monofluoroacetic acid.

Concentration of CFH_2COONa (%)	Amount of bialaphos produced by the addition of			
	None	MP-103*	MP-101*	Phosphinothricin*
0	0	24	32	75
0.01	0	18	32	75
0.03	0	11	30	75
0.1	0	10.5	32	75

* Concentration of substrate; 100 μg/ml.
Transformation experiments were carried out by using a blocked mutant (NP-213) of *S. hygroscopicus*[1].

†Pharmaceutical Development Laboratories,
Meiji Seika Kaisha Ltd.,
Saiwai-ku, Kawasaki 210, Japan
††Central Reseach Laboratories,
Meiji Seika Kaisha Ltd.,
Kohoku-ku, Yokohama 222, Japan

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