STUDIES ON THE BIOSYNTHESIS OF BIALAPHOS (SF-1293) 7. THE ABSOLUTE CONFIGURATION OF 2-PHOSPHINOMETHYL-MALIC ACID, A BIOSYNTHETIC INTERMEDIATE OF BIALAPHOS[1]

Sir:

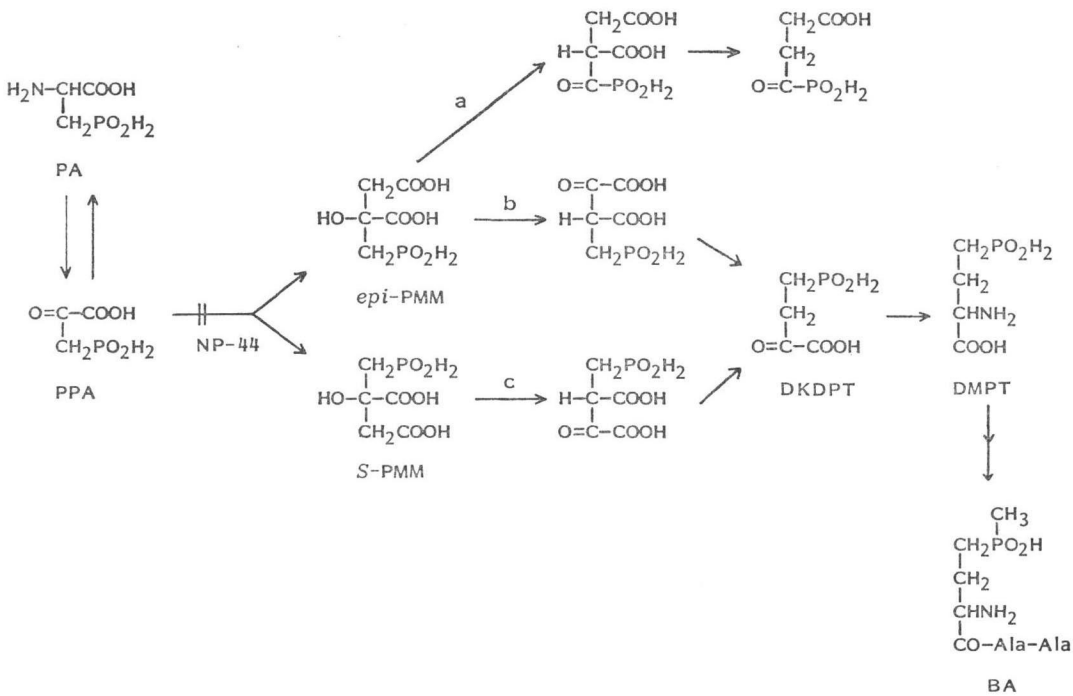
Bialaphos (BA) is a metabolite produced by *Streptomyces hygroscopicus* SF-1293[2,3] and is now being in use as a herbicide. This metabolite is very unique among the natural products in possessing two unique C-P bonds[4] and has attracted our attention with regard to its mechanism of biosynthesis. As a result of extensive investigations utilizing ^{13}C-labeled precursors[5], blocked mutants[6] and metabolic inhibitors[7], we have revealed the detailed biosynthetic pathway of bialaphos.

During these studies, 2-phosphinomethylmalic acid (PMM, Fig. 1) was isolated as a biosynthetic intermediate when the fermentation broth of *S. hygroscopicus* SF-1293 was supplemented with monofluoroacetic acid[7]. Its stereochemistry, however, remains to be established due to failure to obtain good crystalline materials suitable for X-ray analysis.

This compound is structurally related to citric acid and proved to be an intermediate during the transformation of phosphinopyruvic acid (PPA)[6] to demethylphosphinothricin (DMPT)[8]. Since PPA and DMPT can be regarded as analogs of oxalacetic acid and glutamic acid, respectively, the enzymes catalyzing the conversion of PPA to DMPT may be closely related to those of TCA cycle as shown in Fig. 1.

However, if PPA were metabolized by the TCA cycle enzymes (pathway a), the structure of the final product would be totally unrelated to the biosynthesis of BA. Therefore, the conversion of PPA to DMPT may be explained by either one of the two metabolic pathways which are somewhat different from the TCA cycle. If the condensation between AcOH and PPA were catalyzed by citrate synthase, the absolute configuration of PMM should be *R* and the following reactions must be catalyzed by enzymes different from those of TCA cycle (pathway b). On the

Fig. 1. Conversion of phosphinoalanine to demethylphosphinothricin *via* phosphinomethylmalic acid.
Abbreviations: PA; phosphinoalanine, PPA; phosphinopyruvic acid, PMM; 2-phosphinomethylmalic acid, DKDPT; deamino-α-keto-demethylphosphinothricin, DMPT; demethylphosphinothricin, BA; bialaphos.



other hand, if the absolute configuration of PMM is *S*, the first condensation step must be carried out by the enzyme different from citrate synthase and the following reactions may be catalyzed by the TCA cycle enzymes (pathway c). Consequently, it is very important to determine the absolute configuration of PMM to study the detailed mechanism of transformation of PPA to DMPT. We wish to report herein the absolute stereochemistry of PMM determined by ORD spectral comparison with *epi*-PMM formed from PPA and acetyl-CoA by citrate synthase of porcine heart origin (Sigma Chem. Co.).

Preliminary experiments using a reaction system comprising acetyl-CoA and citrate synthase showed that the substrate, PPA, was not utilized at all at 25°C, while oxalacetic acid was transformed to citric acid almost quantitatively. Use of radioactive acetyl-CoA, however, revealed the formation of a trace amount of a metabolite corresponding to PMM. The reaction was carried out as follows; a reaction mixture containing 17 μl of 0.1 M Tris-HCl buffer (pH 7.9), 2.5 μl of 0.1 M PPA, 5 μl of 1 mM [1-^{14}C]acetyl-CoA (0.24 mCi/mmol) and 7.6×10^{-2} units of porcine citrate synthase was incubated at 30°C for 2 hours. The reaction was stopped by heating in a boiling water bath for 3 minutes and the products were analyzed by cellulose TLC developed with BuOH - AcOH - H_2O (3:1:1) followed by autoradiography. The Rf value of one of the labeled products was in accord with that of authentic PMM. This metabolite was determined to be *epi*-PMM as follows.

In order to determine its chemical structure including the absolute configuration, a similar procedure was repeated in a larger scale using unlabeled acetyl-CoA as follows; 28 mg of PPA was added into 15 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 2,000 units of citrate synthase and 150 mg of acetyl-CoA. The reaction was carried out at 37°C and monitored by the increase of CoA with 5,5′-dithiobis(2-nitrobenzoic acid)[9]. After exhausting the bulk of the acetyl-CoA (about 10 hours), the mixture was boiled for 3 minutes and then passed through Diaion HP-20 and Dowex-50 (H^+ form) columns. After being adjusted to pH 8 with 1 N NaOH, the effluent was subjected to Sephadex G-10 column chromatography developed with H_2O. The combined fraction containing *epi*-PMM was concd to a small volume and purified by paper chromatography (Toyo Roshi, No. 51) developed with BuOH - AcOH - H_2O (3:1:1). *epi*-PMM extracted with H_2O was concd and applied to a Sephadex G-10 column. The column was developed with H_2O and appropriate fractions were combined and concd to give 11.8 mg of an amorphous powder of *epi*-PMM.

Fig. 2. ORD spectra of 2-phosphinomethylmalic acid (PMM) and *epi*-PMM.

The ORD spectra of PMM and *epi*-PMM were measured at 4 mg/ml and 5 mg/ml, respectively, dissolved in H_2O at 23°C.

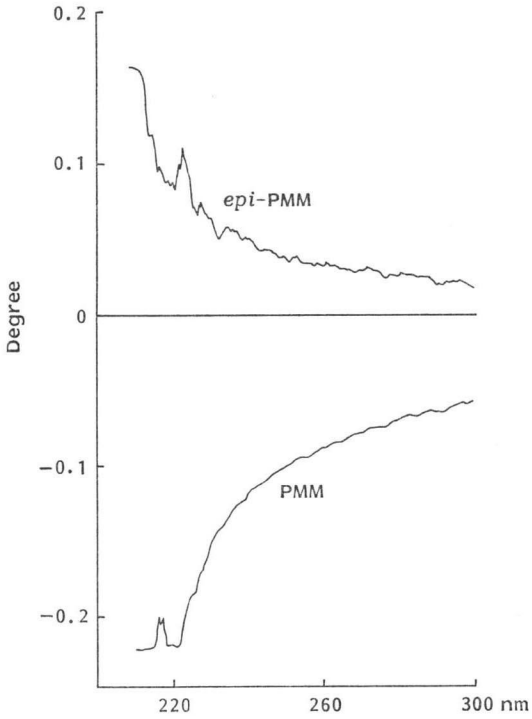


Table 1. Biological transformation of 2-phosphinomethylmalic acid (PMM) and *epi*-PMM by a blocked mutant (NP-44) of *S. hygroscopicus*.

Substrate	Amount of substrate (μg/ml)	Bialaphos produced by NP-44 (μg/ml)
PMM	5	6.5
PMM	10	14.5
epi-PMM	50	0

Physico-chemical properties of *epi*-PMM trisodium salt are as follows; mp 106~107°C (dec), FD-MS (*m/z*) 279 $(M+H)^+$, soluble in H_2O and MeOH, insoluble in $CHCl_3$, Me_2CO and EtOAc, negative to ninhydrin reaction. Its ^{1}H NMR

spectrum was identical with that of authentic PMM. Thus, this compound proved to be chemically identical with PMM. However, the ORD curve of *epi*-PMM was the mirror image of the latter as shown in Fig. 2. In agreement with this, *epi*-PMM was not converted to BA by the previously reported procedure[6] using a BA non-producing mutant, NP-44, which was blocked between PPA and PMM (unpublished data), while PMM was transformed efficiently to BA by the same mutant as shown in Table 1.

Based on the well established stereochemical reaction mechanism of citrate synthase, that the enzyme catalyzes the condensation of acetic acid with oxalacetic acid from *si*-face[10], it is very reasonable to assume that *epi*-PMM has *R* configuration. This assumption was fortified by the finding that the amination of deamino-α-ketodemethylphosphinothricin (DKDPT) to DMPT was catalyzed by glutamic oxalacetic transaminase of porcine heart suggesting the stereochemical course of the citrate synthase to be unaffected by the substitution of γ-carboxylic acid in oxalacetic acid by phosphinic acid (unpublished data). Therefore, the absolute configuration of PMM is concluded to be *S* as shown in Fig. 1.

The enzyme converting PPA to PMM in *S. hygroscopicus* SF-1293, which is hereafter called as PMM synthase, is stereo-specifically similar to *R*-citrate synthase purified from a few obligate anaerobic bacteria[11].

With the clarified stereochemistry of PMM, it may be reasonably assumed that the conversion from PMM to DKDPT probably *via* phosphinic acid analogs of *cis*-aconitic acid, isocitric acid and oxalsuccinic acid is catalyzed by the relevant TCA cycle enzymes (pathway c). In this regard, it may be very important to note that this conversion and the following transamination to form DMPT were accomplished by washed mycelium of *Streptomyces lividans* and a cell free system of *Brevibacterium lactofermentum* ATCC 13869 (details will be published elsewhere). Experiments to reveal the reaction mechanism leading to DKDPT from PMM and to purify PMM synthase are now under way.

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KUMIKO SHIMOTOHNO
HARUO SETO*
NOBORU ŌTAKE
SATOSHI IMAI†
ATSUYUKI SATOH†

Institute of Applied Microbiology,
The University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan
†Pharmaceutical Development Laboratories,
Meiji Seika Kaisha Ltd.,
Saiwai-ku, Kawasaki 210, Japan

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References

1) For part 6, see IMAI, S.; H. SETO, T. SASAKI, T. TSURUOKA, H. OGAWA, A. SATOH, S. INOUYE, T. NIIDA & N. ŌTAKE: Studies on the biosynthesis of bialaphos (SF-1293). 6. Production of *N*-acetyldemethylphosphinothricin and *N*-acetylbialaphos by blocked mutants of *Streptomyces hygroscopicus* SF-1293 and their roles in the biosynthesis of bialaphos. J. Antibiotics 38: 687~690, 1985
2) KONDO, Y.; T. SHOMURA, Y. OGAWA, T. TSURUOKA, H. WATANABE, K. TOTSUKAWA, T. SUZUKI, C. MORIYAMA, J. YOSHIDA, S. INOUYE & T. NIIDA: Studies on a new antibiotic SF-1293. I. Isolation and physico-chemical and biological characterization of SF-1293 substance. Sci. Reports of Meiji Seika Kaisha 13: 34~41, 1973
3) OGAWA, Y.; T. TSURUOKA, S. INOUYE & T. NIIDA: Studies on a new antibiotic SF-1293. II. Chemical structure of antibiotic SF-1293. Sci. Reports of Meiji Seika Kaisha 13: 42~48, 1973
4) For reviews of C-P compounds, see HORI, T.; M. HORIGUCHI & A. HAYASHI (*Ed.*): Biochemistry of Natural C-P Compounds. Maruzen Ltd., Kyoto, 1984
5) SETO, H.; S. IMAI, T. TSURUOKA, A. SATOH, M. KOJIMA, S. INOUYE, T. SASAKI & N. ŌTAKE: Studies on the biosynthesis of bialaphos (SF-1293). 1. Incorporation of ^{13}C- and ^{2}H-labeled precursors into bialaphos. J. Antibiotics 35: 1719~1721, 1982
6) SETO, H.; S. IMAI, T. TSURUOKA, H. OGAWA, A. SATOH, T. SASAKI & N. ŌTAKE: Studies on the biosynthesis of bialaphos (SF-1293). Part 3. Production of phosphinic acid derivatives, MP-103, MP-104 and MP-105, by a blocked mutant of *Streptomyces hygroscopicus* SF-1293 and their roles in the biosynthesis of bialaphos. Biochem. Biophys. Res. Commun. 111: 1008~

1014, 1983

7) SETO, H.; S. IMAI, T. SASAKI, K. SHIMOTOHNO, T. TSURUOKA, H. OGAWA, A. SATOH, S. INOUYE, T. NIIDA & N. ŌTAKE: 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. J. Antibiotics 37: 1509~1511, 1984

8) SETO, H.; T. SASAKI, S. IMAI, T. TSURUOKA, H. OGAWA, A. SATOH, S. INOUYE, T. NIIDA & N. ŌTAKE: Studies on the biosynthesis of bialaphos (SF-1293). 2. Isolation of the first natural products with a C-P-H bond and their involvement in the C-P-C bond formation. J. Antibiotics 36: 96~98, 1983

9) ELLMAN, G. L.: Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82: 70~77, 1959

10) SPECTOR, L. B.: Citrate cleavage and related enzymes. *In* The Enzymes. Vol. 7. *Ed.*, P. D. BOYER, pp. 357~368, Academic Press, New York, 1972

11) GOTTSCHALK, G. & H. A. BARKER: Synthesis of glutamate and citrate by *Clostridium kluyveri*. A new type of citrate synthase. Biochemistry 5: 1125~1133, 1966