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[1]

Sir:

Compounds possessing a C-P or C-P-C bond are very rare among the natural products and their formation mechanisms remain as yet to be investigated in detail. We report here the first isolation of compounds with a H-P-C bond and their involvement in the C-P-C bond formation.

Bialaphos (formerly called SF-1293)[2] is a tripeptide, phosphinothricylalanylalanine (Fig. 1) produced by *Streptomyces hygroscopicus* SF-1293[2] and *S. viridochromogenes*[3] and its use as a herbicide is now being actively investigated. The metabolite is characterized by the presence of a C-P-C bond in the phosphinothricin moiety. During biosynthetic studies on this compound in order to shed light on the formation mechanism of carbon-phosphorous bonds[1], we found the accumulation in the fermentation broth of two new metabolites named MP-101 and MP-102 containing a H-P-C bond which, to the best of our knowledge, has never been found in nature.

When *S. hygroscopicus* was cultivated in the absence of Co^{++}, accumulation of a new type of compounds was detected by direct analysis of the broth filtrate by ^{31}P NMR spectroscopy (Fig. 2). Thus, new signals were observed at δ_P 27.4 and 29.7 in the proton noise decoupled spectra; these chemical shifts are characteristic to –C–P(O)OH structures[4]. Without proton decoupling these signals collapsed to doublets with very large coupling constants (J_{H-P} = 525 Hz) suggesting the direct linkage of the phosphorous to a hydrogen atom. The ^{31}P NMR analysis of the fermentation broth also showed that the amount of MP-102 (δ_P 27.4) reached its maximum level

Fig. 1. The structures of bialaphos, phosphinothricin, MP-101 and MP-102.

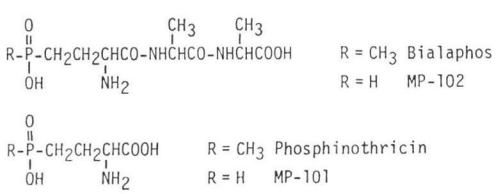
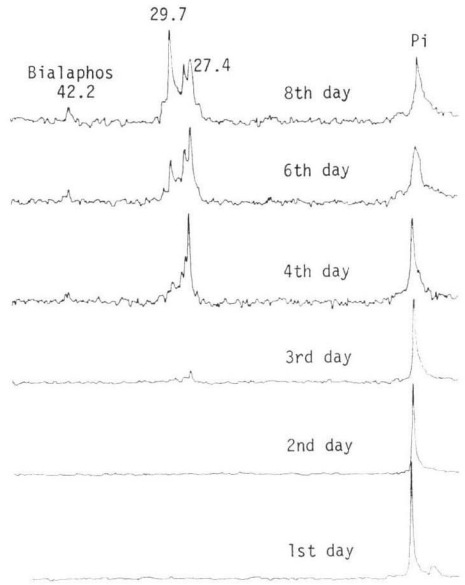


Fig. 2. ^{31}P NMR spectral analysis of the fermentation broths of *Streptomyces hygroscopicus* SF-1293.

Chemical shifts are expressed in ppm downfield from internal inorganic phosphate.



4~5 days after the initiation of the fermentation and that it gradually changed to MP-101 (δ_P 29.7). The maximum yield of MP-101 was obtained after a further 6~8 days.

These two compounds were isolated as follows. The broth filtrate of *S. hygroscopicus* was passed through a column of Dowex-50 (H^+) and the column was washed successively with water. Appropriate washing fractions containing C-P compounds (detected by ^{31}P NMR) were passed through a column of Dowex-1 (CH_3COO^-). After washing the column with water, C-P compounds were eluted with 0.3 N acetic acid. Concentration of the eluate and crystallization of the residue from water gave MP-102 (from the fermentation broth of 4th day) or MP-101 (from the fermentation broth of 11th day). Their physicochemical properties are as follows.

MP-101, $C_4H_{10}O_4NP$, $(M+H)^+$ 168 (FD-MS), $[\alpha]_D^{20}$ +28.9° (*c* 1, 1 N HCl), mp 221~222°C (dec.). *Anal.* Calcd.: C 28.74, H 5.99, N 8.38, P 18.56, Found: C 29.20, H 6.06, N 8.21, P 18.60, positive to ninhydrin.

MP-102, $C_{10}H_{20}O_6N_3P$, $(M+H)^+$ 310 (FD-MS), $[\alpha]_D^{20}$ −37.7° (*c* 1, 1 N HCl), mp 226~228°C (dec.). *Anal.* Calcd.: C 38.83, H 6.47, N 13.59, P 10.03, Found: C 39.02, H 6.62, N 13.70, P 9.95, positive to ninhydrin.

Fig. 3. The 400 MHz ^1H NMR spectra of MP-101 and 2-amino-4-phosphonobutyric acid taken in ^2H_2O.

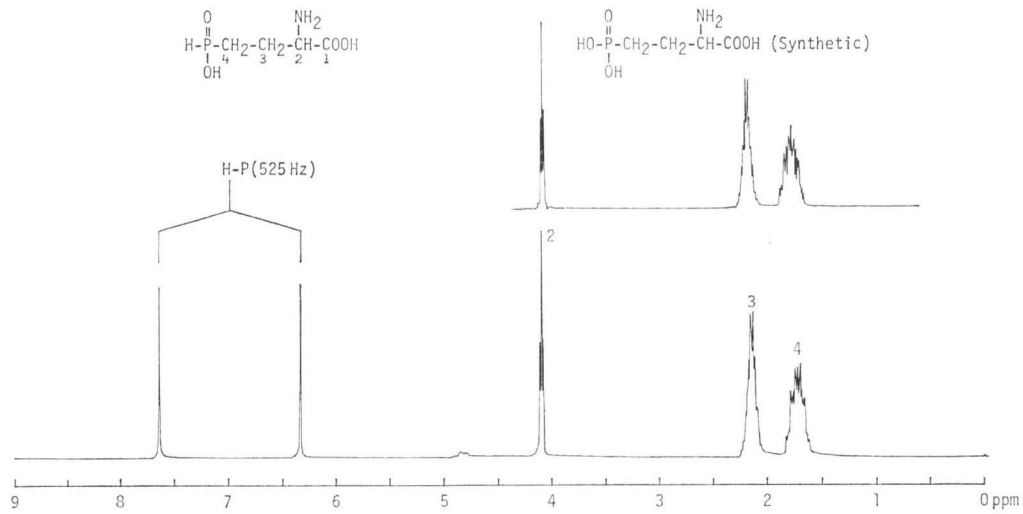
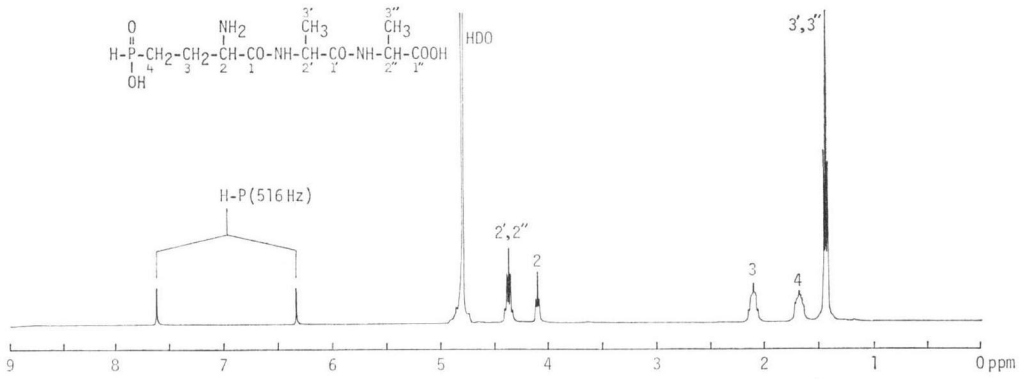


Fig. 4. The 400 MHz ^1H NMR spectrum of MP-102 taken in ^2H_2O.



In the ^1H NMR spectrum taken in D_2O, MP-101 showed a very characteristic doublet signal at δ_H 7.00 (J_{H-P}=525 Hz) due to a H–P(O)OH–C partial structure. The remaining part of MP-101, $-CH_2CH_2CH(NH_2)COOH$, including its L-configuration was proved by spin decoupling experiments (δ_H H-2:4.20, H-3:2.15 and H-4:1.70) and by comparison to 2-amino-4-phosphonobutyric acid[5] (Fig. 3) and phosphinothricin[3]. $[\alpha]_D^{20}+27°$ (c 1, 1 N HCl).

Thus, the structure of MP-101 has been determined as shown in Fig. 1.

MP-102 gave two moles of L-alanine and one mole of MP-101 on acid hydrolysis. Treatment of MP-102 with dansyl chloride followed by acid hydrolysis gave alanine and dansylated MP-101. The ^1H NMR spectrum of MP-102 also showed a very characteristic signal at δ_H 6.98 (J_{H-P}= 516 Hz) and the remaining signals were almost completely identical with those of bialaphos (Fig. 4). Consequently MP-102 is a tripeptide comprising MP-101 and two moles of L-alanine as shown in Fig. 1.

MP-101 and MP-102 did not show any biological activity tested so far except that the former inhibited only the growth of the producing organism at the concentration of 10 μg/ml.

Both the compounds were quantitatively converted to bialaphos as shown in Table 1 by a mutant (NTG-213) of the producing organism of bialaphos which is blocked at an early step of the biosynthetic pathway. This experimental result clearly shows that the formation of a H-P bond is a prerequisite for the methylation of phos-

Table 1. Transformation of MP-101 and MP-102 to bialaphos by washed mycelia of a mutant (NTG 213) of *Streptomyces hygroscopicus* SF-1293.

Precursor added	Concentration (mM)	Amount of bialaphos produced (mM)	Conversion rate (%)
MP-101	0.143	0.173	121
	0.057	0.053	93
	0.029	0.034	117
	0.014	0.016	114
MP-102	0.077	0.071	92
	0.031	0.025	81
	0.016	0.012	75
	0.008	0.008	100

The reaction was carried out in phosphate buffer (pH 6.5, 50 mM) at 28°C for overnight. The amount of bialaphos was determined by biological activity against *Bacillus subtilis*. The transformation product was confirmed to be bialaphos by TLC analysis.

phorous to take place. As far as our knowledge is concerned, this is the first report to show the involvement of H-P metabolites in the formation of a C-P bond. The accumulation of MP-101 was also found in the fermentation broths of several other mutants obtained from *S. hygroscopicus* SF-1293 by nitrosoguanidine or acriflavine treatment.

It has been generally accepted that phosphonic acid derivatives such as fosfomycin[6] and FR-33289[7] are formed by the intramolecular rearrangement of phosphoenolpyruvate[8]. In the case of bialaphos, however, several experimental evidences in our hand (to be published elsewhere) are in favor of the hypothesis that prior to the formation of the C-P bond, the reduction of a phosphate ester (most probably phosphoenolpyruvate) takes place to give a phosphite ester which would then rearrange to form a phosphinic acid intermediate for MP-101 and MP-102.

The purification of the enzyme catalyzing this reduction which may be named "phosphate reductase" is now under way.

Acknowledgment

This work was supported in part by a Grant-in- Aid for Special Project Research, The Ministry of Education, Science and Culture, Japan.

Haruo Seto
Toru Sasaki
Satoshi Imai*
Takasi Tsuruoka**
Hiroshi Ogawa*
Atsuyuki Satoh*
Shigeharu Inouye**
Taro Niida**
Noboru Ōtake

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
**Central Research Laboratories,
Meiji Seika Kaisha,
Kohoku-ku, Yokohama 222, Japan

(Received August 27, 1982)

References

1) For Part 1 see, 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
2) 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
3) Bayer, E.; K. H. Gugel, K. Hagele, H, Hagenmaier, S. Jessipow, W. A. Konig & H. Zahner: Stoffwechselprodukte von Mikroorganismen. Phosphinothricin und Phosphinothricyl-Alanyl-Alanin. Helv. Chim. Acta 55: 224~239, 1972
4) Crutchfield, M. M.; C. H. Dungan, J. H. Letcher, V. Mark & J. R. Van Wazer: ^{31}P Nuclear Magnetic Resonance. Wiley Interscience, New York, 1967
5) Chambers, J. R. & A. F. Isbel: A new synthesis of amino phosphonic acid. J. Org. Chem. 29: 832, 1964
6) Rogers, T. O. & J. Birnbaum: Biosynthesis of fosfomycin by *Streptomyces fradiae*. Antimicrob. Agents Chemother. 5: 121~132, 1974
7) Okuhara, M.: Studies on new antibiotics containing phosphonic acid. Ph. D. Thesis (in Japanese), The University of Tokyo, 1980
8) Horiguchi, M.: Biosynthesis of 2-aminoethylphosphonic acid in cell-free preparations from tetrahymena. Biochim. Biophys. Acta 261: 102~113, 1972