STUDIES ON THE BIOSYNTHESIS OF BIALAPHOS (SF-1293) 6. PRODUCTION OF *N*-ACETYL-DEMETHYLPHOSPHINOTHRICIN AND *N*-ACETYLBIALAPHOS BY BLOCKED MUTANTS OF *STREPTOMYCES HYGROSCOPICUS* SF-1293 AND THEIR ROLES IN THE BIOSYNTHESIS 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. Based on the structures of biosynthetic intermediates such as phosphinic or phosphonic acid derivatives, which were produced by blocked mutants[4,5], by the addition of monofluoroacetic acid[1], or accumulated under fermentation conditions minus cobalt ion[6], its biosynthesis has been assumed to proceed as follows; phosphoenolpyruvate→phosphinopyruvic acid (formerly called α-keto MP-103)→2-phosphinomethylmalate→deamino α-keto demethylphosphinothricin (formerly called α-keto MP-101)→demethylphosphinothricin (DMPT, formerly called MP-101)→demethylbialaphos (DMBA, formerly called MP-102)→BA.

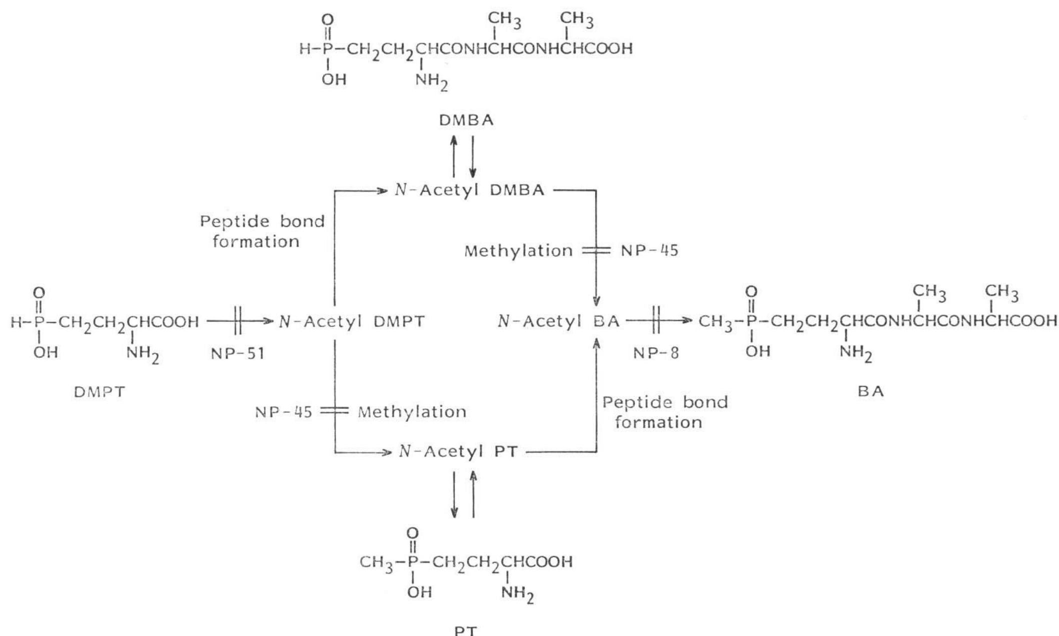
Recently we have succeeded by mutagenesis with nitrosoguanidine in the isolation of three mutants (NP-51, NP-45 and NP-8) with blocks after DMPT (Fig. 1). We wish to report herein their accumulation products and abilities as converters. These data provide detailed information about the late steps in the biosynthetic pathway of BA.

These three mutants produced BA by cosynthesis with each other. Based on transformation experiments using washed mycelia (Table 1) the sequence of their blocked sites has been determined as follows; NP-51→NP-45→NP-8.

Accumulation of *N*-Acetyl DMPT, DMPT and DMBA by NP-45

NP-45 is believed to lack the ability to catalyze methylation of phosphinic acid derivatives, since it can transform only *P*-methyl derivatives such as phosphinothricin (PT) and *N*-acetyl PT, but not demethyl derivatives such as DMPT or DMBA to BA (Table 2). Mutant NP-51 can produce BA using the fermentation broth of NP-45 as a substrate (Table 1). Therefore, we tried to isolate an unknown biosynthetic intermediate present in the fermentation broth of

Fig. 1. Proposed biosynthetic pathway of bialaphos from demethylphosphinothricin.



Abbreviations used; BA=bialaphos, DMBA=demethylbialaphos, PT=phosphinothricin, DMPT=demethylphosphinothricin.

Table 1. Production of bialaphos by blocked mutants of *Streptomyces hygroscopicus* SF-1293 using broth filtrates of secretors.

Converter	Bialaphos produced (μg/ml) Broth filtrates of secretors		
	NP-51	NP-45	NP-8
NP-51	Trace	18	55
NP-45	Trace	Trace	55
NP-8	Trace	Trace	Trace

Experiments were carried out as reported previously[1].

NP-45 by employing the transformation ability of NP-51 and ^{31}P NMR as assay methods[6].

Filtered broth of NP-45 (200 ml) which was prepared as reported previously[4], was passed through Dowex-50 (H^+ form) and then adsorbed on a Dowex-1 (CH_3COO^- form) column. After washing with 0.5 N AcOH, an active fraction was eluted with 0.5 N HCl. The eluate was concentrated *in vacuo* to a small volume and after being adjusted to pH 0.8, applied to a column of HP-20 (400 ml). Concentration of the active fraction gave a sample which was identical with *N*-acetyl DMPT prepared by acetylation of DMPT[7]. Its properties are as follows; $C_6H_{12}O_5NP$, *Anal* found: C 34.40, H 5.85, O 38.30, N 6.67%, calcd: C 34.46, H 5.78, O 38.25, N 6.70%; ^{1}H NMR (100 MHz) δ_H 1.60~2.20 (4H, m, PCH_2CH_2–), 2.03 (CH_3CON, s), 4.41 (1H, dd, –CH(NH)CO–), 7.04 (1H, d, J_{P-H}=552 Hz, HP–).

In addition to *N*-acetyl DMPT, DMPT and DMBA were also isolated from the fermentation broth of NP-45 by the procedures described previously[6] and their structures were confirmed by comparing with authentic specimen. Thus the metabolic pattern in NP-45 seems to be similar to that of the parent strain when methylation is inhibited by the absence of cobalt ion[6].

Acetyltransferase Activity of Mutant NP-51

Since NP-51 could transform *N*-acetyl DMPT and PT but not DMPT to BA (Table 2) as mentioned above, its blocked site is related to the formation of *N*-acetyl derivatives. Therefore, the acetyltransferase activity of NP-51 was compared with that of NP-45. These two mutants were cultivated at 28°C for 4 days in 250-ml Erlenmeyer flasks containing the following fermentation medium; glucose 7.0, Bacto-soyton 4.4, KH_2PO_4 0.327, Na_2HPO_4 0.0852, TES 1.15 and $CoCl_2\cdot 6H_2O$ 0.0001%, pH being adjusted to 6.0. Mycelium collected from 100-ml fermentation broth was washed with 50 mM phosphate buffer (pH 6.5) containing 0.5 mg/ml dithiothreitol, suspended in 20 ml of the same

Table 2. Transformation of biosynthetic intermediates to bialaphos by blocked mutants of *Streptomyces hygroscopicus* SF-1293.

Intermediate (250 μg/ml)	Bialaphos produced by mutants (μg/ml)		
	NP-51	NP-45	NP-8
Demethylphosphinothricin	Trace	Trace	Trace
N-Acetyldemethylphosphinothricin	7.5	Trace	Trace
Demethylbialaphos	Trace	Trace	Trace
Phosphinothricin	31	150	Trace
N-Acetylphosphinothricin	41	131	Trace

Experiments were carried out as reported previously[1].

Table 3. Acetyltransferase activity of mutants of *Streptomyces hygroscopicus* SF-1293, NP-45 and NP-51 as measured by the decrease of demethylphosphinothricin.

Mutant	Remaining concentration of demethylphosphinothricin (μg/ml)*		Consumption of demethyl-phosphinothricin (μg/ml)
	− Acetyl CoA	+ Acetyl CoA	
NP-45	71.7	9.7	62.0
NP-51	79.0	75.3	3.7

* The concentration of demethylphosphinothricin in the starting reaction mixture was 75 μg/ml. The reaction was carried out at 30°C for 1 hour. For details, see in the text.

solution and sonicated by a Kubota insonator 200M for 10 minutes at 1.5 A. The supernatant obtained by centrifugation at 10,000 rpm for 15 minutes was used as a crude enzyme solution. DMPT (0.05 ml, final 75 μg/ml) was treated with this solution (0.9 ml) in the presence of acetyl CoA (0.05 ml, final 500 μg/ml) at 30°C for 1 hour and the amount of the remaining DMPT was determined by an amino acid analyzer. Acetyltransferase activity of the enzyme solution was expressed as a decrease of DMPT using the reaction mixture containing no acetyl CoA as a control. As shown in Table 3, NP-45 transformed about 86% of DMPT to its *N*-acetyl derivative under this experimental condition, while NP-51 could hardly catalyze the formation of *N*-acetyl DMPT. Similar results were obtained when PT was utilized as a substrate. Thus, NP-51 is considered to be a blocked mutant which lacks acetyltransferase activity. This finding suggests that *N*-acetyl DMPT is a biosynthetic intermediate of BA biosynthesis.

Accumulation of *N*-Acetyl BA and *N*-Acetyl PT by NP-8

Isolation of a biosynthetic intermediate accumulated by mutant NP-8 was carried out using the ability of NP-45 and NP-51 as converters and ^{31}P NMR as assay systems.

Filtered broth of NP-8 (100 ml) prepared as reported previously[4] was passed through Dowex-50 (H^+ form) and then adsorbed on a Dowex-1 X2 (Cl^- form) column. After developing with 0.5% NaCl, an active fraction was eluted with 2% NaCl. The eluate was concentrated *in vacuo* to a small volume and precipitated NaCl was removed by filtration. The filtrate was applied to a column of Sephadex G-10 (4× 77 cm, in fractions of 10 ml each) and the combined active fractions (38~42) were concentrated to dryness to give *N*-acetyl BA which was in complete agreement with a synthetic sample prepared by acetylation of BA. Its physico-chemical properties are as follows; mp 212~213°C, $[\alpha]_D^{25}$ −65.1° (*c* 1.0, H_2O); $C_{13}H_{24}O_7N_3P$, *Anal* found: C 42.24, H 6.94, N 11.36, P 8.55%, calcd: C 42.74, H 6.62, N 11.50, P 8.47%; ^1H NMR (400 MHz in D_2O) δ_H 1.394 (CH_3, d, 7.0 Hz), 1.428 (CH_3, d, 7.1 Hz), 1.494 (CH_3P–, d, 13.7 Hz), 1.8~2.05 (4H, m), 2.032 (CH_3CON, s), 4.3~4.4 (3H, m); ^{13}C NMR (100 MHz) δ_C 14.4 (CH_3P, J_{C-P}=90 Hz), 16.8 (CH_3–), 17.1 (CH_3–), 22.4 (CH_3CON), 24.7 (CH_2–), 26.1 (CH_2P, J_{C-P}=92 Hz), 49.3 (CHN), 50.2 (CHN), 54.6 (CHN), 173.6, 175.2 and 177.0.

Following the fractions containing *N*-acetyl BA was eluted *N*-acetyl PT (fractions 43~48), which was further purified by cellulose column chromatography (BuOH - AcOH - H_2O=2:1:1) to give a pure sample identical with chemically prepared *N*-acetyl PT. Its physico-chemical properties are as follows: mp 145°C; $[\alpha]_D^{25}$ +8.5° (*c* 1.0, H_2O), $C_7H_{14}O_5NP$, *Anal* found: C 38.06, H 6.59, N 6.44, P 13.84%, calcd: C 37.67, H 6.38, N 6.28, P 13.88%; ^1H NMR (200 MHz in D_2O) δ_H 1.508 (CH_3P–, d, 14.2 Hz), 1.8~2.2 (4H, m), 2.048 (CH_3CON, s), 4.434 (1H, dd, *J*=5.0 and 2.9 Hz).

Thus, NP-8 is concluded to lack the enzyme system to remove the *N*-acetyl group from *N*-acetyl BA.

Based on the results reported above, the biosynthetic steps of BA following the formation of DMPT is summarized as shown in Fig. 1.

N-Acetyl derivatives were also reported to be biosynthetic intermediates of kanamycin[8] and leupeptin[9]. It is interesting to note that *N*-acetylation is a prerequisite step for the formation of peptide bonds in leupeptin, which, like BA, is a tripeptide substance.

The detailed reaction sequence from *N*-acetyl DMPT to *N*-acetyl BA remains to be clarified. Formally at least two reactions are required to convert *N*-acetyl DMPT to *N*-acetyl BA, *i.e.*, methylation of the phosphinic acid moiety and peptide bond formation with the alanylalanine residue. Since DMBA was produced when the methylation reaction was inhibited[6], it may be assumed that *N*-acetyl DMBA is the direct precursor of *N*-acetyl BA. Accumulation of *N*-acetyl DMPT by mutant NP-45 may be ascribed to the action of a strong enzyme system catalyzing the cleavage of the dipeptide moiety from BA (S. IMAI, unpublished data) on *N*-acetyl DMBA which might otherwise accumulate in the fermentation broth of this blocked mutant.

On the other hand, since *N*-acetyl phosphinothricin was isolated together with *N*-acetyl BA from the fermentation broth of NP-8 (*vide supra*), another pathway may also be postulated, *i.e.* *N*-acetyl DMPT is methylated to give *N*-acetyl PT which is then involved in the peptide

formation to afford *N*-acetyl BA. If this sequence is correct, DMBA must be regarded as an abnormal metabolite accumulated under the condition where methylation of the phosphinic acid moiety is not possible. Accumulation of *N*-acetylphosphinothricin, however, may be explained by the removal of the alanylalanine residue from *N*-acetyl BA by the same enzyme system described above. Thus further investigations are required to make clear the detailed formation mechanism of bialaphos.

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(Received October 25, 1984)

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