CONVERSION OF BIALAPHOS TO OTHER OLIGOPEPTIDES CONTAINING PHOSPHINOTHRICIN BY *Streptomyces hygroscopicus*

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Two oligopeptides containing phosphinothricin were accumulated in the culture of bialaphos (BA) producer *Streptomyces hygroscopicus* SF1293 when large amount of BA was added to the culture at idio phase. One of the oligopeptides was a new substance, BA dimer (phosphinothricyl-alanyl-alanyl-phosphinothricyl-alanyl-alanine), and the other was a known substance called **If** (phosphinothricyl-alanyl-alanyl-phosphinothricin). Though BA none producing mutants which were blocked at the steps 1, 10 and 13, respectively in BA biosynthesis also converted BA to BA dimer and **If**, the mutants blocked at the step 11 (alanylation) could not curry out the conversion. Antibiotic activities of BA dimer and **If** were lower than 1/100 of BA.

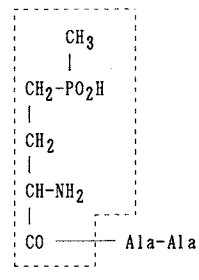
Bialaphos (BA): (L-phosphinothricyl-L-alanyl-L-alanine)[1~3] (Fig. 1) is a herbicidal secondary metabolite produced by *Streptomyces hygroscopicus* SF1293 (SF1293) and its structural component phosphinothricin (PPT) (Fig. 1) which has a unique $R-CH_2-PO_2H-CH_3$ bonding is a strong glutamine synthetase (GS) inhibitor[4,5].

The biosynthetic pathway of BA was proposed by SETO *et al.*[6~9], and IMAI *et al.*[10~12] on the basis of the identification of the intermediates accumulated by various blocked mutants and their conversion by the mutants (see Fig. 2). Though BA is the main product of SF1293, it is produced as one of a number of products which have PPT in common in their structure, *i.e.*, several analogous substances, **Ia** (PPT-Gly-Ala), **Ib** (PPT-Ala-Gly), **Ic** (PPT-Ala-α-amino butylic acid (ABA)), **Id** (PPT-Ala-Val), **Ie** (PPT-Ala-Ser), **If** (PPT-Ala-Ala-PPT), were known as minor components so far[13].

We show in this paper the pathway for the biosynthesis of one such minor components, **If**, and a novel substance, BA dimer, and discuss the physiological significance of their production.

Fig. 1. Structure of BA.

Ala-Ala: Alanyl alanine. PPT moiety is circled in the dotted line.



Materials and Methods

Strains and Media

BA producer *S. hygroscopicus* SF1293 strain HP5-2ϑ and BA none producing mutants NP47, NP2007-1, NP2008-1, Bar⁻/pGSC1, NP61, NP62, MNP17, MNP18, NP8 were obtained from the Meiji Seika collection[6~12,14]. Media (S_1, BA production and minimum medium) and culture conditions are described by OGAWA *et al.*[2], and MURAKAMI *et al.*[15].

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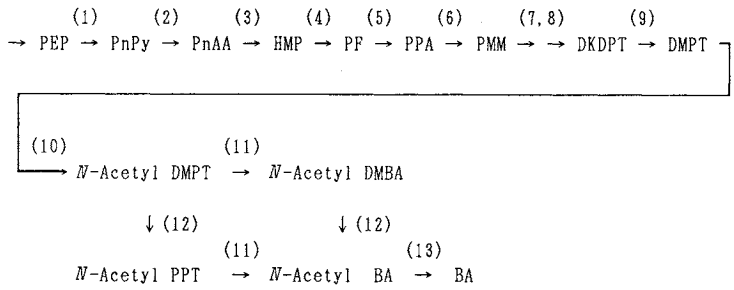
Fig. 2. BA biosynthesis pathway.



The pathway shown above is based on the work of SETO *et al.*[6~9], and IMAI *et al.*[10~12]. Numbers in parenthesis show the step of biosynthesis. PEP: Phosphoenolpyruvic acid, PnPy: phosphonopyruvic acid, PnAA: phosphonoacetaldehyde, HMP: hydroxymethylphosphonic acid, PF: phosphonoformic acid, PPA: phosphinopyruvic acid, PMM: phosphinomethyl malic acid, DKDPT: deamino-α-keto-demethylphosphinothricin, DMBA: demethylbialaphos.

Cultivation for Conversion of BA

The stock culture of BA producer *S. hygroscopicus* SF1293 or its mutants was subcultured in 10 ml of S_1 medium at 28°C for 24 hours. One ml of the seed was transferred to 30 ml of production medium in 250 ml conical flask and cultured by rotary shaker (220 rpm) at 30°C. BA was added at 3rd ~ 5th day.

Assay of Products

The culture broth was diluted with water, heated for 5 minutes in boiling water to extract the intracellular substances and centrifuged. The resulting supernatant was used for the assay. BA dimer, **If** and BA were analyzed by HPLC as described in the previous paper[14]. An amino acid analyzer (ATTO MLC-703) was also used for the assay of the above three substances and PPT.

Purification of BA Dimer and **If**

Sample for Purification: The nonproducing mutant NP47 was cultivated for 3 days and BA was added to the culture (final conc 35 mg/ml). The culture added BA was further cultivated for 3 days and was used for the purification. The whole broth (240 ml) was adjusted to pH 2.0 and agitated for 30 minutes to extract the compound in the cell. After centrifugation to separate mycelia, the supernatant was used for the isolation of BA dimer and **If**.

Purification (See Table 1): Purification of BA dimer and **If** is summarized in Table 1.

Structural Analysis

NMR: NMR spectrophotometer JNM-GX400 was used.

SI-MS Spectrum: Mass spectrophotometer M-80B (Hitachi) was used.

Amino Acid Composition: A sample (73 μg) was dissolved in 50 μl of distilled water and 50 μl of 12 N HCl was added to the solution and the mixture was poured into an ampoul (8 × 100 mm) and sealed after degassing under low pressure. Hydrolysis was carried out at 110°C for 17 hours, after which the sample was evaporated completely and 500 μl of 20 mM HCl was added. Amino acid composition was analyzed by an automatic amino acid analyzer L-8500 (Hitachi).

Amino Acid Sequence Analysis: A sample (7.3 μg) was dissolved in 5 μl of distilled water and used for amino acid sequence analysis. The analysis was carried out by using a protein sequencing system 450A (Applied Bio System).

Measurement of Antiboitic Activity

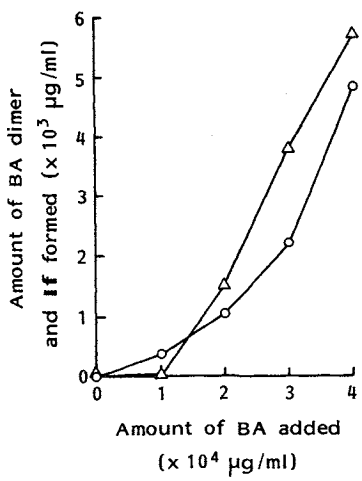
Antibiotic activity was assayed using *Bacillus subtilis* 8193 and *S. hygroscopicus* SF1293 Bar^-[14]. The strain *S. hygroscopicus* SF1293 Bar^- were streaked on the minimal medium[2] supplemented with BA, BA dimer and **If**, respectively, and were incubated at 28°C for 3 days. In the case of *B. subtilis* 8193 as the indicator, paper discs containing 1 μg of BA dimer, **If** and BA, respectively, were plated on the nutrient

Table 1. Purification of BA dimer and **If**.

Step	Resin	Procedure	Components	Yields (mg)
—	—	Initial culture broth (240 ml)	BA	6,480
			BA dimer	912
			If	552
			PPT	120
1st	Dowex 50W (H^+)	The ninhydrin positive fraction eluted with water was collected and was further purified by next step.	BA	5,832
			BA dimer	866
			If	524
			PPT	116
2nd	Dowex 1X2 (CH_3COO)	BA was eluted with 0.5 N CH_3COOH and BA dimer, **If** and PPT by 0.5 N HCl. The fraction containing BA dimer, **If** and PPT was further purified by next step.	Purified BA	5,540
			BA dimer	797
			If	493
			PPT	110
3rd	Dowex 1X2 (CH_3COO)	Elution was carried out with continuously increasing concentration (0.5~4.0 N) of CH_3COOH. The order of the eluates was PPT→BA dimer→**If**. **If** fraction was separated and the fraction containing PPT and BA dimer was further purified by next step.	Purified **If**	463
			BA dimer	478
			PPT	22
4th	Sephadex G-10	PPT and BA dimer were separated.	Purified BA dimer	287
			PPT	<1

Fig. 3. Addition of BA and formation of BA dimer and **If**.

○ BA dimer, △ **If**.



BA was added to 3 days cultures and the cultures were further cultivated for 2 days.

Table 2. The conversion of BA to BA dimer and **If** by various blocked mutants.

Strain	Blocked point	Conversion of BA to	
		BA dimer	**If**
NP47	Step 1	+	+
NP2007-1	Step 1	+	+
NP2008-1	Step 1	+	+
Bar^-/pGSC1*	Step 10	+	+
NP61	Step 11	−	−
NP62	Step 11	−	−
MNP17	Step 11	−	−
MNP18	Step 11	−	−
NP8	Step 13	±	±

* Bar^- mutant is very sensitive to BA because its blocked point step 10 (acetylation) is also involved in self-defence. Therefore, pGSC1 that is a high copy number plasmid containing a GS gene from *Streptomyces coelicolor*[18] was introduced into the Bar^- mutant to confer BA resistance to the mutant[13]. The converting ability of the mutants is expressed by the ratio of BA converted as +: ≧5%, ±: <5% and −: not detected.

agar medium supplemented with the strain and the plate was incubated at 37°C for 1 day. Antibiotic activity was estimated from the diameter of the growth inhibitory zones.

Results

Conversion of BA to BA Dimer and **If**

A large amount of BA (final conc 35 mg/ml) was added to the idio phase culture of BA producer

SF1293 strain HP5-29. As the result, a considerable amount of BA dimer and **If** were accumulated. As shown in Fig. 3, notable accumulation of both substances was recognised when over 20 mg of BA was added to the culture. The same results were obtained in the case of various blocked mutants, NP47 (step 1 blocked), Bar^-/pGSC1* (step 10 blocked) and NP8 (step 13 blocked) (Table 2). On the other hand, mutants (NP61, NP62, MNP17 and MNP18) that were blocked at the alanylation step of BA could not accumulate either substance (Table 2).

Structure of BA Dimer

BA dimer was obtained as a colorless powder which was soluble in water and positive to ninhydrin. The molecular formula of BA dimer was determined to be $C_{22}H_{42}N_6O_{11}P_2$ from SI-MS data (*m/z* 629 $(M+H)^+$, 651 $(M+Na)^+$) and ^1H and ^{13}C NMR data (Tables 3 and 4). On acid hydrolysis, BA dimer gave alanine and PPT which was detected by amino acid analyzer and their molar ratio was 2 : 1. Considering the molecular formula, it is concluded that BA dimer consists of 4 mol of alanine and 2 mol of PPT. The structure of BA dimer was determined to be phosphinothricyl-alanyl-alanyl-phosphinothricyl-alanyl-alanine by amino acid sequence analysis.

Table 3. ^1H NMR data of BA dimer, **If** and BA.

BA dimer		**If**		BA	
4.35	4H m	4.43	1H dd	4.34	2H m
4.16	1H dd	4.35	1H q	4.08	1H t
4.08	1H t	4.31	1H q	2.10	2H m
2.12	2H m	4.06	1H t	1.70	2H m
1.98	2H m	2.20~1.74	8H	1.41	3H d
1.68	2H m	1.52~1.36	12H	1.43	3H d
1.62	2H m			1.30	3H d
1.48~1.22	18 H				

Structure of **If**

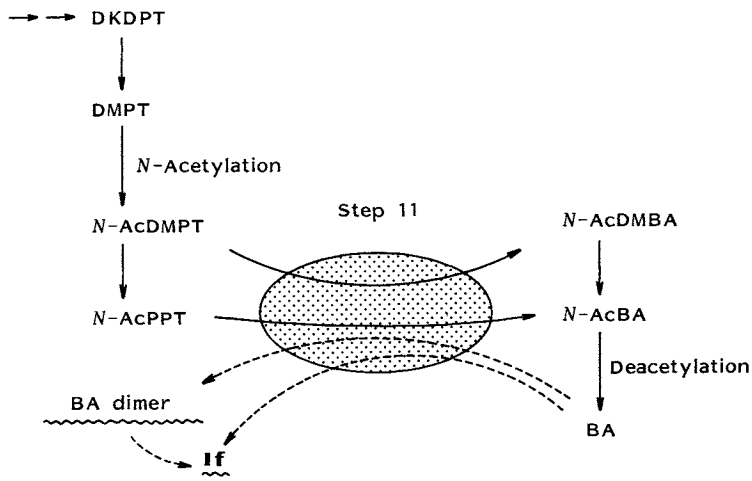
If was obtained as a colorless powder. The molecular formula of **If** was determined to be $C_{16}H_{32}N_4O_9P_2$ by SI-MS data (*m/z* 487 $(M+H)^+$, 509 $(M+Na)^+$) and ^1H and ^{13}C NMR data (Tables 3 and 4). Acid hydrolysis of **If** followed by amino acid analysis revealed that **If** consisted of 2 mol of alanine and 2 mol of PPT. The structure of **If** was determined to be phosphinothricyl-alanyl-alanyl-

Table 4. ^{13}C NMR data of BA dimer, **If** and BA.

BA dimer		**If**		BA
180.9 s		175.9 s		179.9 C-1″
175.9 s		175.6 s		173.9 C-1′
175.2 s		175.2 s		160.6 C-1
174.5 s		169.8 s		
174.0 s				
170.3 s				
55.5 d	$^3J_{cp}=15.9$ Hz	54.0 d ×2	$^3J_{cp}=15.9$ Hz	53.9 C-2
54.4 d	$^3J_{cp}=14.3$ Hz			
52.0 d		50.7 d ×2		51.2 C-2′
50.7 d				
50.6 d				50.4 C-2″
50.5 d				
28.6 t	$^1J_{cp}=92.2$ Hz	27.1 t	$^1J_{cp}=93.8$ Hz	27.2 C-4
27.7 t	$^1J_{cp}=90.6$ Hz	26.5 t	$^1J_{cp}=90.6$ Hz	
25.9 t		25.2 t		25.3 C-3
25.8 t		24.8 t		
18.6 q		17.5 q		17.9 C-3′
17.7 q		17.4 q		17.1 C-3″
17.6 q	×2			
16.3 q	$^1J_{cp}=93.8$ Hz	15.3 q	$^1J_{cp}=92.3$ Hz	15.9 C-2
16.2 q	$^1J_{cp}=92.2$ Hz	14.8 q	$^1J_{cp}=92.2$ Hz	

Fig. 4. Conversion pathway of BA to BA dimer and **If**.

—— De novo synthesis pathway, ------ presumptive conversion pathway.



DKDPT: Deamino-α-keto-demethylphosphinothricin, DMBA: demethylbialaphos.

phosphinothricin by amino acid sequence analysis.

Antibiotic Activities of BA Dimer and **If**

Antibiotic activities of BA dimer and **If** using *B. subtilis* and Bar$^-$ mutant defecting detoxication modification (*N*-acetylation) as the indicators were lower than one hundredth of that of BA, *e.g.* 100 μg/ml of BA dimer or **If** did not inhibit the growth of the Bar$^-$ mutant, on the other hand, 1 μg/ml of BA inhibited it.

Discussion

The results of the accumulation of BA dimer and **If** when a large amount of BA was added to the various blocked mutants show that these substances were not generated by *de novo* synthesis but by the conversion of BA. The result also showed that the blocked mutants defective in step 11 (alanylation) could not convert BA to these substances, indicating that the enzyme catalysing the alanylation step in BA *de novo* biosynthesis is also involved in this conversion.

The proposed BA biosynthetic pathway is shown in Fig. 2. As shown in this figure, the substrate of alanylation step is the *N*-acetylated intermediate (*N*-acetyl demethylphosphinothricin (DMPT) or PPT). On the contrary, the acetylation step (step 10) is not necessary in the case of BA dimer or **If** formation because the Bar$^-$ mutant defective in the *N*-acetylation step[14] also can convert BA to BA dimer and **If**. Presumably, though the enzyme involved in the alanylation of DMPT or PPT requires strictly that the amino group of the substrate be acetylated, the same enzyme can convert BA into BA dimer or **If** without the acetylation of BA. The presumptive process of BA dimer and **If** formation is illustrated in Fig. 4.

PPT is a structural component of BA and is a strong GS inhibitor[4,5]. PPT also inhibits SF1293 GS (S. IMAI; personal communication). Though the primary self defence mechanism of SF1293 in the production of BA is known to be the acetylation of PPT[5], quantitative change of GS is also involved, since an increase of GS activity accompanied by BA accumulation is observed during BA production[16]. Qualitative change of the GS is also supposed to be involved, since SF1293 has two GSs and one of them GS II-Sh is more resistant to PPT than the other GS, GS I-Sh[17], but the crucial proof is not obtained because the expression of GS II-Sh in SF1293 was not well recognised so far.

Finally, we will discuss the physiological significance of the conversion in relation to the self defence. In this experiment, no further increasing of BA concentration was observed when 40 mg/ml or lower BA was loaded to BA producer. And further, BA dimer and **If** did not have antibiotic toxicity. These results

suggest that the high concentration of BA affects to the physiological activity of the BA producer and the conversion of BA to these oligopeptides may diminish the toxic effect of BA and may play a role of a self defense in the presence of high concentration of BA.

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