EFFECT OF B-FACTOR AND ITS ANALOGUES ON RIFAMYCIN BIOSYNTHESIS IN *NOCARDIA* SP.

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(Received for publication October 21, 1987)

B-Factor, 3′-(1-butylphosphoryl)adenosine, which was isolated from yeast extract, is an inducer of rifamycin production in a rifamycin non-producing *Nocardia* mutant. Feeding of B-factor to the mutant culture demonstrated that the induction process was triggered during early stationary phase. Rifamycin production in the mutant was also induced by an exogenous supply of 3-amino-5-hydroxybenzoic acid, an intermediate of the antibiotic pathway, suggesting that a step upstream from the intermediate is regulated by B-factor. B-Factor analogues, *i.e.*, alkylesters of 3′-AMP with alkyl side chains of C(2)~C(12) and *n*-butyl esters of 3′-GMP and 2′-AMP all showed the B-factor activity. Among these *n*-octyl ester of 3′-AMP showed the lowest effective concentration of approximately 3×10^{-10} M. An intrinsic substance of the *Nocardia* sp. with potent B-factor activity and a UV absorption maximum at 260 nm was isolated from the cells of the parental strain.

Involvement of self-provided regulatory factors in the induction of secondary metabolism in streptomycetes was first demonstrated with A-factor for streptomycin production in *Streptomyces griseus*[1,2]. The related γ-lactone compounds, L-factor[3] and inducing factor[4], were also reported as self-regulatory factors for leucaemomycin production in *S. griseus* and virginiamycin production in *Streptomyces virginiae*, respectively.

B-Factor, 3′-(1-butylphosphoryl)adenosine, is an additional example of such diffusible factors. It was isolated from yeast extract as an inducer of rifamycin B production in a mutant strain of *Nocardia* sp.[5]. The mutant deficient in antibiotic synthesis was reversed in this characteristic by the exogenous supplement of approximately 10^{-8} M of B-factor. Although it was found in yeast extract, B-factor or its analogues seem to play a regulatory role in the *Nocardia* cells. This paper deals with several features of the effect of B-factor as well as activities of B-factor analogues on the production of rifamycin in the *Nocardia* mutant. Purification of an intrinsic substance with B-factor activity from the parental strain of *Nocardia* sp. is also described.

Materials and Methods

Strain and Medium

A rifamycin non-producing mutant of *Nocardia* sp. KB-993 was used[5]. Storage of the mutant frequently resulted in a high proportion of revertants producing the antibiotic. Purification of the mutant from such cultures was performed by homogenization of cell suspension with a Waring blender and plating for single colony isolation.

Medium for the seed culture contained Nutrient broth (Difco) 0.8%, glucose 0.5% and NaCl 0.3%, pH 7.0. The medium containing glycerol 2%, soy bean meal (Esusan Meat, Ajinomoto Co. Inc.) 1% and NaCl 0.2%, pH 7.0, was used for rifamycin production by the mutant.

Assay of B-Factor Activity

Activities of various B-factor analogues and others were assayed in liquid cultures of the *Nocardia* mutant by measuring induced production of rifamycins according to the absorption at 410 nm of the EtOAc extracts of the cultured broth at pH 2.0[5]. For the determination of the effective concentrations, various amounts of the samples were added to the mutant cultures in 50 ml of liquid medium in 500-ml Erlenmeyer flasks and the amounts of the antibiotic produced during 10 days cultivation with shaking at 26.5°C were measured spectrophotometrically.

Biological assay on solid medium using the *Nocardia* mutant[5] was also used.

Chemical Synthesis of B-Factor Analogues

Analogues of B-factor with various lengths of acyl moieties at the 3′-phosphate position were synthesized by the method described previously[5]. The first step of purification was performed by Diaion HP-20 column chromatography except for the ethyl analogue which did not adsorb to this resin. The adsorbed substances were eluted with a linear gradient of MeOH (0~50%) in water and then applied to reversed phase preparative HPLC (Senshu SSC-ODS-763). An ethyl analogue was purified by direct application of the latter.

Chemicals

3-Amino-5-hydroxybenzoic acid was provided by O. GHISALBA, Ciba-Geigy Limited. 2′-(1-Butylphosphoryl)adenosine was provided by S. TESHIBA, Kyowa Fermentation Industry Co.

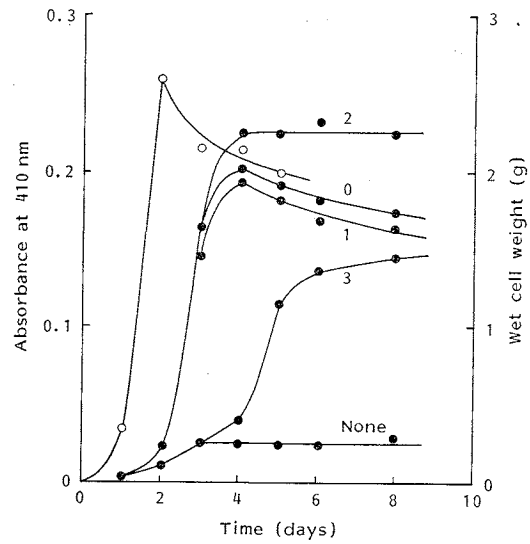
Results and Discussion

Effect of B-Factor on Rifamycin Production in the Mutant

A seed culture of the rifamycin non-producing *Nocardia* mutant was inoculated into the production medium and a sufficient amount of B-factor (5×10^{-7} M) was added at 0~3 days after inoculation. As shown in Fig. 1, rifamycin production began at early stationary phase (2 days after the inoculation), and addition of B-factor at this time caused maximum rifamycin production which reached up to 70% of that by the parental strain. Growth and cellular morphology of the mutant were identical to those of the parent and the mutant in the absence of B-factor. Earlier addition of B-factor (0 and 1 day) gave slightly lower production probably due to degradation of B-factor before initiation of the antibiotic production. Addition of B-factor 3 days after the inoculation caused decreased production of the antibiotic. These data suggest that the critical point of induction by B-factor is during the limited period at the early stationary growth phase. We have not yet tested an additivity of B-factor, if any, to enhancement of rifamycin production thereafter.

Fig. 1. Time course of rifamycin production induced by B-factor in the mutant of *Nocardia* sp.

B-Factor (final concentration 5×10^{-7} M) was added to the culture at 0 (curve 0), 1 (1), 2 (2), or 3 (3) days after the inoculation and their rifamycin production (●) and growth (expressed by wet cell weight including soy bean meal in the media) (○) were measured. Control (none) is the culture of the mutant without B-factor.



The biosynthetic pathway of rifamycins and

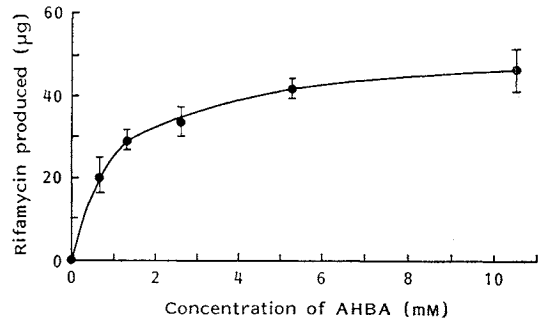
other ansamycin antibiotics branches from the shikimate pathway to produce 3-amino-5-hydroxybenzoic acid (AHBA) as the starter unit to form their chromophores[6]. In order to determine the blocked point of rifamycin biosynthesis in the mutant, feeding experiments with AHBA was carried out. As shown in Fig. 2, the mutant produced rifamycin upon the addition of AHBA in the absence of B-factor. The concentration of AHBA to cause half maximum production of the antibiotic was 0.9 mM. Shikimate showed no effect in inducing antibiotic production and the mutant had no nutritional requirement for aromatic amino acids. These results indicate that induction by B-factor occurs at a point before AHBA in the rifamycin biosynthetic pathway after branching from the shikimate pathway.

Although barbiturate was reported to stimulate rifamycin production in *Streptomyces mediterranei*[7], no effect of barbiturate was observed in the *Nocardia* mutant in this assay system.

Effects of B-Factor Analogues on Rifamycin Production in the Mutant

Neither naturally occurring nucleosides, nucleotides nor their derivatives previously examined showed B-factor activity for rifamycin production in the mutant[5]. Therefore we synthesized various B-factor analogues in which the butyl moiety linked to 3′-phosphate was replaced with other alkyl groups with different chain lengths, and these were examined for their ability to induce rifamycin production in the mutant. In addition, butyl esters of 3′-GMP [3′-(1-butylphosphoryl)guanosine] and 2′-AMP [2′-(1-butylphosphoryl)adenosine] were also examined. As shown in Fig. 3, all the alkyl esters of 3′-AMP and 3′-GMP showed B-factor activity. The highest activity was observed with the *n*-octyl

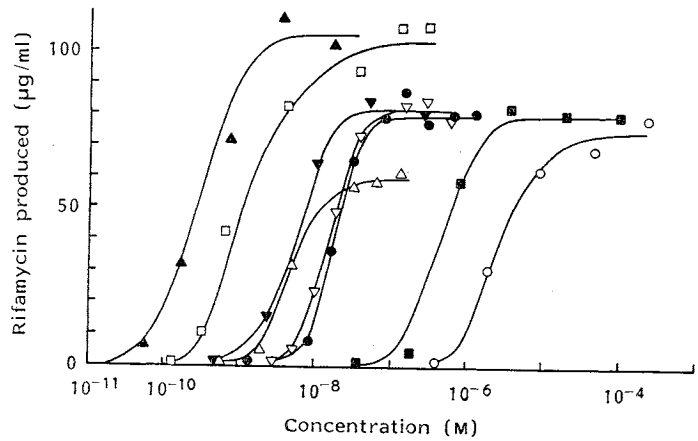
Fig. 2. Effect of 3-amino-5-hydroxybenzoic acid to induce rifamycin production in the mutant.



Rifamycin production was measured after 10 days cultivation.

Fig. 3. Effect of B-factor analogues on rifamycin production in the mutant.

Rifamycin production induced by 3′-AMP esters with alkyl moieties of ethyl (○), butyl (B-factor, ●), *iso*-butyl (■), *n*-amyl (▼), *n*-hexyl (□), *n*-octyl (▲) and *n*-lauryl (△), and by the butyl ester of 3′-GMP (▽) were measured in the liquid medium.



ester of 3′-AMP which caused half maximum induction at 3.1×10^{-10} M (Table 1). The maximum rifamycin production induced by saturated concentrations of the *n*-octyl and *n*-hexyl esters were distinctly higher than that by B-factor. The butyl ester of 2′-AMP showed one tenth lower activity than that of B-factor (Fig. 4). Activity of the *n*-butyl ester of 2′-AMP might be due to translocation of a butyl group in the mutant cells leading to production of B-factor. These results indicate that the alkyl esters of 3′-purine nucleotides possess potent inducing activity almost comparable with that of 3′,5′-cyclic AMP in various regulatory systems.

Purification of Intrinsic B-Factor from the *Nocardia* Cells

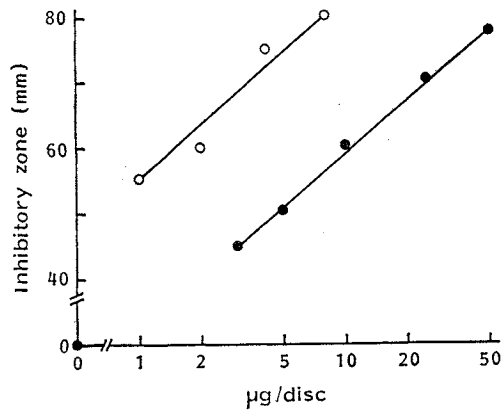
The activity to induce rifamycin production in the *Nocardia* mutant was detected with a hot water extract of the parental *Nocardia* cells, which suggested the presence of an intrinsic B-factor. The active substance was purified as follows.

About 300 g of wet cells of the parental *Nocardia* sp. KB-993 were washed twice with 2 liters of methanol and then extracted twice with

Table 1. Effective concentrations of B-factor analogues to give half maximum induction of rifamycin production.

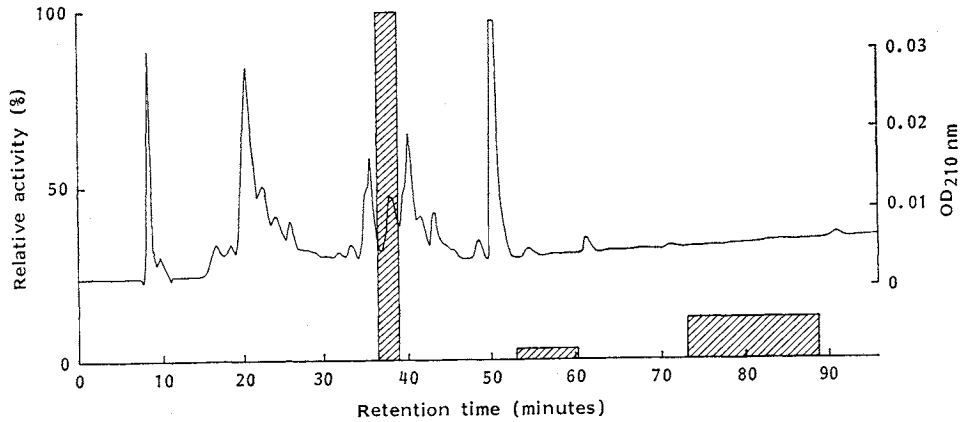
Analogues		Effective concentration (M)
Base	Alkyl chain	
Adenine	Ethyl	2.5×10^{-6}
Adenine	*iso*-Butyl	5.2×10^{-7}
Adenine	*n*-Butyl	1.9×10^{-8}
Adenine	*n*-Amyl	6.8×10^{-9}
Adenine	*n*-Hexyl	8.8×10^{-10}
Adenine	*n*-Octyl	2.3×10^{-10}
Adenine	*n*-Lauryl	5.0×10^{-9}
Guanine	*n*-Butyl	1.4×10^{-8}

Fig. 4. Effect of the butyl ester of 2′-AMP on rifamycin production in the mutant.
B-Factor (○), butyl ester of 2-AMP (●).



Various amounts of B-factor and the butyl ester of 2′-AMP contained in paper discs were applied to the *Nocardia* mutant on the assay plates and growth inhibiting zones due to the induced production of rifamycin were measured.

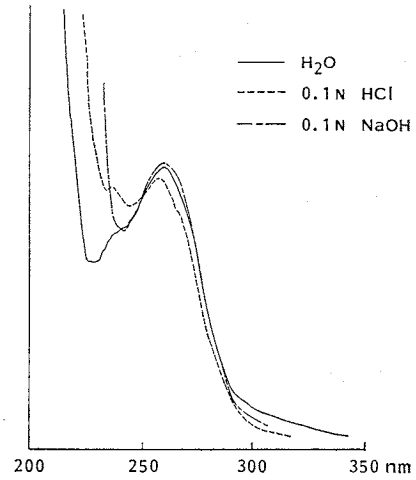
Fig. 5. Reversed phase liquid chromatography of the intrinsic substances having the B-factor activity.



Relative activity was calculated according to the amounts of rifamycin inducibly synthesized by each fraction, which was estimated by the plate assay.

2 liters of hot water (60°C). The extract was concentrated to 500 ml *in vacuo* and applied onto a Diaion HP-20 column (bed volume, 500 ml) and eluted with 2 liters of methanol - water with a linear gradient of 0~100%. The active fraction was eluted with 10~30% methanol in water and was concentrated and applied onto a silica gel column (30×300 mm) and eluted with 200 ml each of 5:1, 2:1, 1:1 and 0:1 (ethyl acetate - methanol), successively. The activity was recovered from the eluate with 2:1 (ethyl acetate - methanol). Then the active fraction was applied to reversed phase preparative liquid chromatography (Senshu Pak ODS-5251U, 20× 250 mm) using a solvent system of 0.1% trifluoroacetic acid - water (A) and 0.06% trifluoroacetic acid - methanol (B) with a linear gradient from A+B (9:1) to 100% B. The highest activity was found in a fraction which had a UV peak, and minor activity was also detected in two other fractions with no UV absorption (Fig. 5). Rechromatography of the main active fraction gave a single UV absorbing peak exactly coinciding with the activity. The UV spectra of the purified fraction in 0.1 N HCl, H_2O and 0.1 N NaOH showed the absorption maxima at 260 nm (Fig. 6). The maximum rifamycin production induced by a sufficient amount of the active fraction was identical to that by B-factor. Assuming the molecular absorption coefficient at 260 nm of the active substance identical to that of B-factor (ε=12,700), we estimated its saturating concentration to be less than 5×10^{-7} M. These results suggest that a nucleotide derivative possibly being a B-factor analogue is present in the *Nocardia* cells and might play a regulatory role in rifamycin production.

Fig. 6. UV spectra of the purified intrinsic B-factor.



Acknowledgments

We thank O. GHISALBA and S. TESHIBA for their providing us AHBA and 2′-(1-butylphosphoryl)adenosine, respectively.

References

1) KHOKHLOV, A. S.; I. I. TOVAROVA, L. N. BORISOVA, S. A. PLINER, L. A. SHEVCHENKO, E. YA. KORNITSKAYA, N. S. IVKINA & I. A. RAPOPORT: A-factor responsible for the biosynthesis of streptomycin by a mutant strain of *Actinomycetes streptomycini*. Dokl. Akad. Nauk SSSR 177: 232~235, 1967
2) HARA, O. & T. BEPPU: Mutants blocked in streptomycin production in *Streptomyces griseus* — The role of A-factor. J. Antibiotics 35: 349~358, 1982
3) GRÄFE, U.; G. REINHARDT, W. SCHADE, D. KREBS, I. ERITT, W. F. FLECK, E. HEINRICH & L. RADICS: Isolation and structure of novel autoregulators from *Streptomyces griseus*. J. Antibiotics 35: 609~614, 1982
4) YAMADA, Y.; K. SUGAMURA, K. KONDO, M. YANAGIMOTO & H. OKADA: The structure of inducing factors for virginiamycin production in *Streptomyces virginiae*. J. Antibiotics 40: 496~504, 1987
5) KAWAGUCHI, T.; T. ASAHI, T. SATOH, T. UOZUMI & T. BEPPU: B-Factor, an essential regulatory substance inducing the production of rifamycin in a *Nocardia* sp. J. Antibiotics 37: 1587~1595, 1984
6) GHISALBA, O. & J. NÜESCH: A genetic approach to the biosynthesis of the rifamycin-chromophore in *Nocardia mediterranei*. IV. Identification of 3-amino-5-hydroxybenzoic acid as a direct precursor of the

seven-carbon amino starter-unit. J. Antibiotics 34: 64~71, 1981
7) KLUEPFEL, D.; G. C. LANCINI & G. SARTON: Metabolism of barbital by *Streptomyces mediterranei*. Appl. Microbiol. 13: 600~604, 1965