B-FACTOR, AN ESSENTIAL REGULATORY SUBSTANCE INDUCING THE PRODUCTION OF RIFAMYCIN IN A *NOCARDIA* SP.

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"Curing" treatment of a rifamycin-producing *Nocardia* sp. resulted in a mutant deficient in the synthesis of antibiotics. This deficiency was reversed in a medium containing yeast extract. The active substance, named B-factor, which induced rifamycin production in the mutant was purified from yeast extract, and its structure, 3′-(1-butylphosphoryl) adenosine, was determined by structural analysis and chemical synthesis. An extremely low concentration of B-factor (10 ng/ml) caused recovery of rifamycin B synthesis in the mutant and stimulated synthesis of the antibiotic in the parental strain.

Complex pathways of secondary metabolism in actinomycetes are under the control of various exogenous and endogenous factors. The most characteristic finding is that synthesis of several antibiotics as well as morphological differentiation is stimulated by these regulatory substances.[1] Among these substances, A-factor for streptomycin production and sporulation in *Streptomyces griseus* has been extensively studied.[2~9] Mutants deficient for A-factor biosynthesis were easily obtained by plasmid-curing treatment.[8] These mutants showed simultaneous loss of streptomycin productivity and sporulation, and exogenous supplement of extremely small amounts of A-factor caused complete recovery of both defects. Genetic studies with cloning experiments of A-factor genes have revealed involvement of an unstable genetic determinant in *S. griseus*[10,11] as well as complex regulatory networks in streptomycetes.[11~13] Similar effector substances have been reported such as the "inducing material" for staphylomycin synthesis in *S. virginiae*,[14] and 4,5-dihydroxy-decanoyl-γ-lactone for leukaemomycin production in *S. griseus*.[15,16] These activator substances seem to act as a primary molecular signal regulating production of secondary metabolites as well as cellular differentiation.

During the course of genetic studies on rifamycin-producing *Nocardia*, a mutant deficient for the antibiotic synthesis was derived by "curing". This deficiency was reversed by the addition of yeast extract. The effective substance in yeast extract, designated B-factor, was purified and identified as 3′-(1-butylphosphoryl)adenosine. B-factor or its analogue seems to play an essential regulatory role for secondary metabolism in this *Nocardia* strain, like A-factor in *S. griseus*.

Materials and Methods

Microorganisms

Nocardia sp. KB-993 isolated from soil produces rifamycin B as a sole antibiotic in the media. A mutant, blocked in its ability to synthesize rifamycin, was also studied.

Bacillus subtilis ATCC 6633 was used for bioassay of rifamycin B.

Media

Medium for the seed culture contained 0.8% nutrient broth (Difco), 0.5% glucose and 0.3% NaCl,

pH 7.0. The production medium of rifamycin B contained 2% glycerol, 1% soy bean meal and 0.2% NaCl, pH 7.0. Several media described in Table 1 were also used.

Biological Assay

The following method was used for quantitative assay of B-factor.

The rifamycin B non-producing mutant isolated from *Nocardia* sp. KB-993 was cultured in liquid seed medium at 30°C with shaking. After 3 days, the culture broth was homogenized with a glass homogenizer to obtain a uniform cell suspension. Glycerol was added to the suspension to give 20% concentration, and then frozen at −80°C. An aliquot of the frozen cell suspension was mixed with a 100-fold volume of the melted soft agar containing 0.5% agar in the production medium at a temperature as low as possible, and the mixture was overlaid on the production medium base plate (agar, 2%). A paper disc containing a sample was placed onto the agar plate. After incubation at 26.5°C for 3 days, nutrient soft agar solution (agar, 1%) containing *B. subtilis* ATCC 6633 was overlaid. The plate was further incubated at 37°C for 18~24 hours and then diameter of inhibitory zone was measured. Linear relationship between the log of the amounts of B-factor and the diameter was obtained at the range of 1~30 μg/disc. Determination was performed by comparing with a standard curve obtained from a control performed simultaneously.

Chemical Assay

The following method was used for the quantitative assay of rifamycin B.

Erlenmeyer flask (500 ml) containing 50 ml of the production medium was inoculated with 0.5 ml of the cell suspension obtained as described above and shake-cultured at 26.5°C. The culture broth was centrifuged to remove mycelia and 1 ml of the supernatant, added to an equal volume of glycine buffer (pH 2.0), was extracted with 3 ml of EtOAc. Thin-layer chromatography and other analyses showed that the strain produced rifamycin B as a sole colored substance with absorption at 410 nm. The absorbance of the organic phase at 1 cm light path was measured at 410 nm, one of the characteristic absorption maxima of rifamycin B at pH 2.0. Direct reading of the absorption was used as a relative amount of rifamycin B.

Thin-layer Chromatography

B-Factor could be extracted from yeast extract with MeOH. Crude extracts were applied to a thin-layer silica gel plate (silica gel 60 F_{254}, Merck), and developed with EtOAc - MeOH (1: 1). After 10 cm development, the dried silica gel plate was divided into ten equal sections and extracted with 2 ml of MeOH. This sample was assayed for B-factor by the same procedure as described above.

Chemical Synthesis of B-factor

Adenosine 3′-monophosphate (200 mg) was dehydrated by repeated evaporation in dry pyridine and suspended in 30 ml of dry pyridine. Benzoyl chloride (6 equiv) was added to the suspension at 0°C and the sealed reaction mixture stirred at room temperature. After 1 hour, the solution was treated with 1-BuOH (4.4 equiv) and stirred at room temperature for 15 minutes. The reaction mixture was treated with dicyclohexylcarbodiimide (DCC) (10 equiv) and kept in the dark at room temperature for 5 hours. The reaction was poured into cold 50% aq pyridine (30 ml) containing sodium bicarbonate (0.5 g). Hexane (90 ml) was added, the precipitate of dicyclohexylurea was filtered off, and washed with 60 ml of 50% aq MeOH. After combining the filtrates, the aq layer was separated, washed with three 90 ml portions of hexane, and dried under reduced pressure. The residue was dissolved with 100 ml of 28% NH_4OH and kept at 55°C for 5 hours. The solution was concentrated to dryness, and the crude product was applied to reverse phase preparative high performance liquid chromatography column (Senshu Scientific Co., SSC-ODS-763; 20×250 mm) using a solvent system composed of MeOH - H_2O (2: 8). Thus, chemically synthesized B-factor was obtained with yield of 10.3%.

Physico-chemical Measurements

Melting point was determined with a Yazawa micro melting point type BY-1. The UV spectra were measured using a Jasco UVIDEC-610 double beem spectrophotometer. The IR spectrum was in KBr tablet measured using a Jasco A-202 infrared spectrophotometer. The secondary ion mass spectrum was measured using a Hitachi M-80A mass spectrometer. The ^1H NMR spectra were

measured at room temperature in a Jeol JNM-MH-100 spectrometer, with D_2O as a solvent using 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt as an internal standard. The ^{13}C NMR spectra were measured at room temperature in a Jeol JNM-FX100 spectrometer, with D_2O as a solvent using MeOH as the internal standard.

Results

Isolation of Rifamycin-negative Mutant

Rifamycin-negative mutants were observed during cultivation of *Nocardia* sp. KB-993 at elevated temperature (42°C) or after long storage of the cultures on agar. One of these mutants was found to lose not only its antibiotic productivity but also the ability to form aerial mycelia. Loss of rifamycin productivity was confirmed by measuring both antimicrobial activity and spectral analysis of the ethyl acetate extracts of culture filtrates. Rifamycin B shows a characteristic absorption maximum at 410 nm.

Recovery of Rifamycin Production by Yeast Extract in the Mutant

The mutant obtained above was deficient for the synthesis of rifamycin in the production medium; however, the addition of yeast extract to the medium was found to result in reversal of this loss (Table 1). Rifamycin B was identified to be sole antibiotic produced by the mutant in the presence of yeast extract. This effect of yeast extract was not observed with the addition of meat extract or Polypepton. Yeast extract had no effect on aerial mycelia formation of the mutant.

In order to confirm the presence of some essential factor in yeast extract for rifamycin biosynthesis, yeast extract was suspended in methanol, and the methanol solution was applied to thin-layer chromatography plates as described in Materials and Methods. An activity which induced rifamycin biosynthesis in the mutant was found which coincided with a UV-absorbing spot with Rf 0.4~0.5. The active substance was designated B-factor and isolated as follows.

Isolation and Purification of B-factor

The purification process of B-factor is shown in Fig. 1. B-factor was assayed by observing the stimulation of rifamycin biosynthesis in the mutant strain. UV absorption at 260 nm was also used for monitoring at the later steps of purification.

Yeast extract (Difco, 454 g) was extracted with 5 liters of methanol at 40°C, and the suspension was clarified by filtration. The filtrate was concentrated under reduced pressure to about 2 liters and

Table 1. Reversal of loss of rifamycin biosynthesis in a mutant by the addition of yeast extract.

Media	Parental strain		Non-producing mutant	
	A_{410}	Diameter of inhibitory zone (mm)	A_{410}	Diameter of inhibitory zone (mm)
1)	0.73	22.0	0	0
2)	1.18	27.3	0.21	15.5
3)	0.43	20.0	0.18	20.0
4)	0.04	17.5	0	0
5)	0	0	0	0

The medium contained 2.0% glycerol, 0.2% NaCl and; 1) 1.0% soy bean meal, 2) 1.0% soy bean meal and 0.1% yeast extract (Difco), 3) 1.0% yeast extract (Difco), 4) 1.0% meat extract, 5) 1.0% Polypepton (Daigo Eiyo Kagaku).

The samples were prepared with 4 days culture broth as described in Materials and Methods.

Fig. 1. Isolation and purification of B-factor.

Yeast extract (Difco)
454 g
↓ Extracted with 5 liters hot MeOH
↓ Filtration
Filtrate
↓ Changed to aq solution
Amberlite XAD-2 column chromatography
↓ (H_2O - MeOH, linear gradient)
Approximately 6 g
↓
Silica gel column chromatography
↓ ($CHCl_3$ - MeOH, step-wise)
$CHCl_3$ - MeOH, 1 : 1 fraction
Approximately 900 mg
↓
Water soluble fraction
↓
ODS preparative HPLC
↓
46 mg

Table 2. Physico-chemical properties of B-factor.

Appearance	White powder
Formula	$C_{14}H_{21}N_2O_7NaP$
MP (°C)	182~186
Solubility	
Soluble	H_2O, pyridine, MeOH
Insoluble	Acetone, EtOAc, $CHCl_3$
Color reactions	
Positive	Ammonium molybdate - perchloric acid reagent
Negative	2,4-DNPH, ferric chloride, ninhydrin
SI-MS	426 ($M+H^+$), 448 ($M+Na^+$), 464 ($M+K^+$)
UV $\lambda_{max}^{H_2O}$ nm ($\varepsilon \times 10^{-3}$)	207 (16.7), 260 (12.7)
IR ν_{max}^{KBr} cm^{-1}	3400, 3000, 1650, 1230, 1080, 800

evaporated further with continuous addition of water to substitute methanol for water as solvent. The aqueous solution was adsorbed to a column of Amberlite XAD-2 (60×700 mm). After washing with distilled water, the column was eluted with 8 liters of aqueous methanol using a linear gradient of methanol from 0 to 100%. The active fractions eluted with approximately 20~50% methanol were combined and concentrated under reduced pressure to give a brown powder. The crude powder (about 6 g) was dissolved in a small amount of methanol and adsorbed onto a silica gel column (20×650 mm). The column was developed successively with 600 ml of chloroform, 600 ml of chloroform - methanol (3 : 1) and 600 ml of chloroform - methanol (1 : 1). B-factor eluted with the last solvent. The active eluate was evapo-

Fig. 2. IR spectrum of B-factor (KBr).

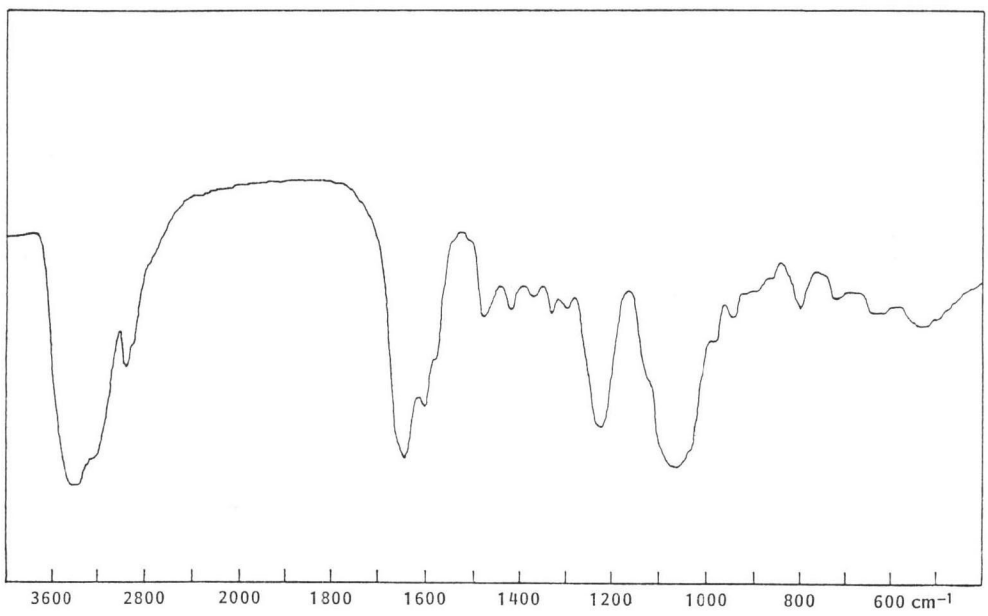
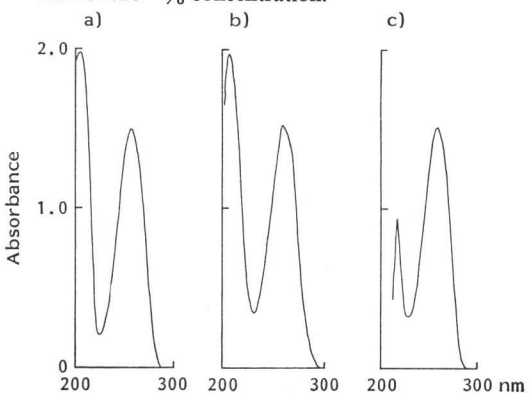


Fig. 3. UV spectra of B-factor.
a) H_2O, b) 0.1 N HCl, c) 0.1 N NaOH. Samples were 5×10^{-3}% concentration.



rated under reduced pressure to dryness and subjected to reverse phase preparative liquid chromatography column (Senshu Scientific Co., SSC-ODS-763; 20×250 mm) using a solvent system composed of methanol - water (2: 8). The eluate was evaporated to give a white powder, which gave a single UV-absorbing spot on the thin-layer chromatogram.

Physico-chemical Properties of B-factor

B-factor was isolated as white powder which was soluble in water, pyridine and methanol but

Fig. 4. ^1H NMR spectra of B-factor and 3′-AMP (in D_2O, 100 MHz).
a) 3′-AMP, b) B-factor.

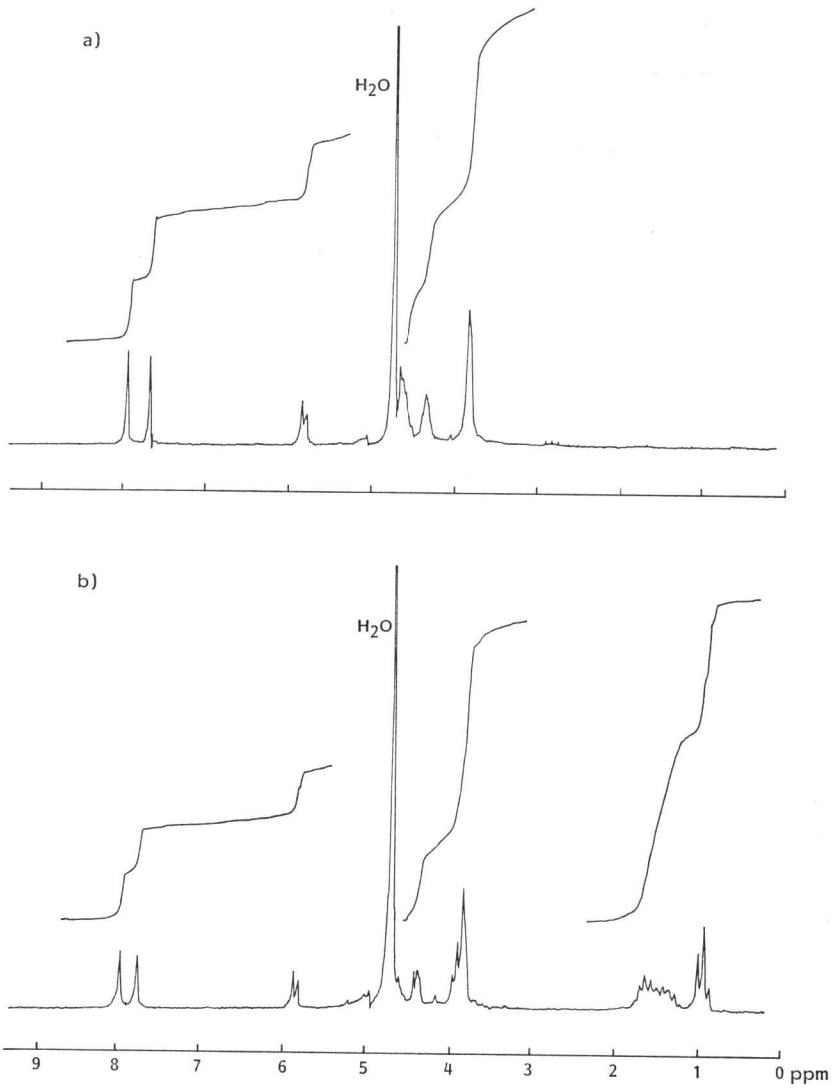
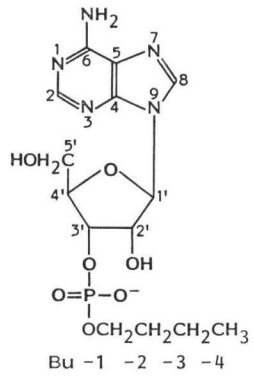


Table 3. ^{13}C NMR spectral data of B-factor and 3′-AMP (in D_2O, 25 MHz).

Carbon	Chemical shift and coupling constant*			
	B-factor		3′-AMP	
	(ppm)	(Hz)	(ppm)	(Hz)
2	153.2	—	152.5	—
4	149.1	—	148.2	—
5	119.8	—	118.7	—
6	156.2	—	155.1	—
8	141.4	—	140.8	—
1′	89.1	—	89.0	
2′	74.0	4.9	74.1	3.7
3′	75.5	4.9	74.5	2.4
4′	86.1	2.4	86.0	3.7
5′	62.3	—	62.4	—
Bu-1	67.3	6.1		
-2	32.9	7.3		
-3	19.3	—		
-4	13.9	—		

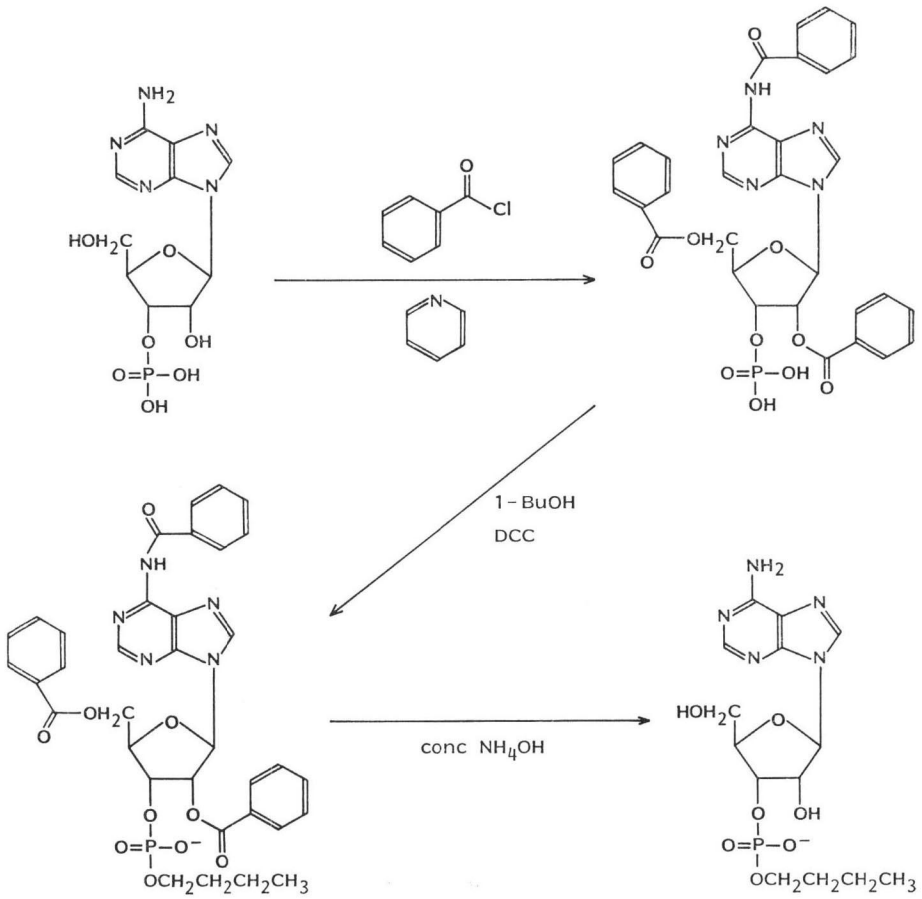
* ^{13}C-^{31}P coupling

Fig. 5. Chemical structure of B-factor.



insoluble in acetone, ethyl acetate and chloroform. It gave a positive color reaction on TLC plate with ammonium molybdate - perchloric acid reagent[17], suggesting that phosphate ester(s) might exist in the B-factor molecule. Secondary

Fig. 6. Chemical synthesis of B-factor.



ion mass spectrometry (SI-MS) on B-factor gave a peak at m/z 426 ($M+H^+$), 448 ($M+Na^+$) and 464 ($M+K^+$). Based on these data together with those of the ^{13}C NMR spectrum, the molecular formula of B-factor was determined to be $C_{14}H_{21}N_5O_7NaP$ (MW 425). Physico-chemical properties of B-factor are summarized in Table 2. The IR spectrum of B-factor (in KBr tablet) is shown in Fig. 2. The UV spectra of B-factor in water, 0.1 N HCl and 0.1 N NaOH solution were almost identical with those of adenosine 3′-monophosphate (3′-AMP) (Fig. 3).

Structure Elucidation of B-factor

The structural elucidation of B-factor was performed by comparing the ^1H NMR and ^{13}C NMR spectra with those of 3′-AMP. The spectral data of B-factor and of 3′-AMP are shown in Fig. 4 (^1H NMR, in D_2O, 100 MHz), and Table 3 (^{13}C NMR, in D_2O, 25 MHz).

^1H NMR spectrum of B-factor showed all signals found in the spectrum of 3′-AMP with the addition to several new signals; these were assigned to be OCH_2 (2H, 3.9 ppm), CH_2 (4H, 1.3~1.8 ppm) and CH_3 (3H, 0.9 ppm). In the ^{13}C NMR spectrum, 10 signals corresponding to the carbons of 3′-AMP were found in B-factor at almost the same chemical shift values as those of 3′-AMP. The additional 4 signals in the spectrum of B-factor were assigned to be OCH_2 (67.3 ppm), CH_2 ×2 (32.9 and 19.3 ppm) and CH_3 (13.9 ppm) based on their chemical shifts and the multiplicity in an off-resonance spectrum. Thus, B-factor was concluded to contain 3′-AMP and a 1-butyl moiety bound to some oxygen atom. Furthermore, the signals assigned to C-1 and C-2 of the butyl moiety and those of 2′-, 3′- and 4′-carbons of the ribose moiety appeared as doublets, which were reasonably elucidated as results of coupling with the phosphorus atom bound to the 3′-carbon.

From the results described above, the chemical structure of B-factor was concluded to be 3′-(1-butylphosphoryl) adenosine as shown in Fig. 5.

Chemical Synthesis of B-factor

In order to confirm the structure of B-factor, chemical synthesis was performed according to the procedure shown in Fig. 6. The final product was purified by reverse phase preparative liquid chromatography. The chemically synthesized preparation showed the identical activity of B-factor from yeast extract by inducing rifamycin

Fig. 7. Effect of B-factor on rifamycin synthesis by a *Nocardia* mutant.

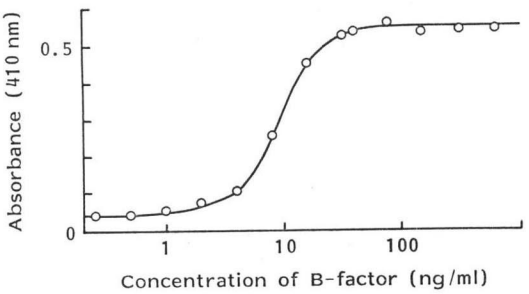
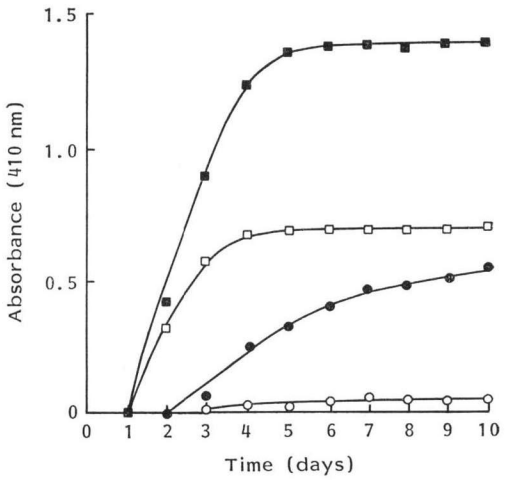


Fig. 8. Time course of rifamycin production in the parental strain and the non-producing mutant.

B-factor (purified preparation) was added to the production liquid medium at a concentration of 0.3 μg/ml.

■ Parental strain (+ B-factor), □ parental strain (— B-factor), ● non-producing mutant (+ B-factor), ○ non-producing mutant (— B-factor).



biosynthesis in the *Nocardia* mutant. All of the spectral data (UV, IR, ^{1}H NMR and ^{13}C NMR) of the synthesized preparation were completely identical with those of natural B-factor (data not shown).

Effect of B-factor and Related Compounds on Rifamycin Biosynthesis

The activity of B-factor was detected on the plate assay at a minimum of 1 μg per disc. When the rifamycin-negative mutant of *Nocardia* was cultured in the liquid medium containing various amounts of B-factor, distinct antibiotic production was observed at a concentration above 2 ng/ml. Additional stimulation was not seen above 30 ng/ml (Fig. 7). Rifamycin B account for more than 90% of the 410 nm-absorbing material produced by the mutant in the presence of B-factor. Rifamycin B production induced by 30 ng/ml of B-factor exceeded 75 μg/ml.

The time course of antibiotic synthesis induced by B-factor is shown in Fig. 8. When sufficient amount of B-factor was added to the culture, the rifamycin-negative mutant recovered synthetic capacity up to 70% of that of the parental strain. Furthermore, addition of B-factor was found to cause marked stimulation of rifamycin productivity in the parental strain by almost 200%.

Compounds structurally related to B-factor as follows showed no activity: adenosine, adenine, adenosine 5′-triphosphate, adenosine 3′-monophosphate, adenosine 3′,5′-cyclic monophosphate, N^{6},$O^{2'}$-dibutyryl adenosine 3′,5′-cyclic monophosphate, guanine, guanosine, cytosine, cytidine, inosine, xanthine, hypoxanthine, xanthosine and tubercidine.

Discussion

The production of rifamycin induced by 30 ng/ml of B-factor in the mutant exceeded 75 μg/ml, which corresponds to an almost 1,500-fold amplification at the molar concentration ratio. It is evident that B-factor plays a role not as an intermediate of rifamycin biosynthesis but as an essential stimulating factor similar to A-factor for streptomycin biosynthesis in *S. griseus* and its relative, *S. bikiniensis*.[1,8]

Structure of B-factor is somewhat similar to those of 3′,5′-cyclic AMP and adenosine oligophosphates both of which are substances with general significance in the regulation of multiple cellular functions in a wide variety of organisms. Although B-factor was isolated from yeast extract, a stimulatory activity for rifamycin production in the B-factor-requiring mutant was observed in cell-free extract of *Nocardia* sp. KB-993. (unpublished data). It seems highly possible that B-factor itself or its analogue is present in the *Nocardia* cells and is involved in the regulation of secondary metabolism in this organism.

Addition of B-factor induces production of mainly rifamycin B in the mutant, which does not accumulate any precursor compounds with ansamycin structure in the absence of B-factor. The factor seems to induce biosynthesis of ansamycin structure in this organism. The fact that B-factor causes stimulation of rifamycin synthesis even in the parental strain of *Nocardia* suggests its possible usefulness in the improvement of antibiotic productivity.

The *Nocardia* mutant requiring B-factor for rifamycin biosynthesis was obtained by the treatment known to cause plasmid curing. We have reported that a gene for A-factor biosynthesis in *S. griseus* is possibly located on a plasmid or an unstable genetic determinant.[8,10,11] The possible involvement of an unstable genetic element in B-factor biosynthesis in *Nocardia* requires additional study.

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