IDENTIFICATION OF BINDING PROTEIN OF VIRGINIAE BUTANOLIDE C, AN AUTOREGULATOR IN VIRGINIAMYCIN PRODUCTION, FROM *STREPTOMYCES VIRGINIAE*

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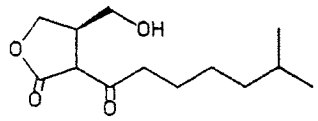
In *Streptomyces virginiae*, production of virginiamycin is triggered by signal molecules named virginiae butanolide A, B or C (VB-A, B or C: YAMADA, Y. *et al.* J. Antibiotics 40: 496~504, 1987). We have found a specific VB-C binding protein from *S. virginiae*, and characterized it by using a tritium-labeled VB-C analogue as a ligand. By equilibrium dialysis in the absence and presence of radio-inert VB-C, a crude extract from 1 g of wet mycelia specifically bound 3.5 pmol of [^{3}H]VB. The binding disappeared after pronase digestion and showed ligand specificity toward *cis* VB-C (*cis* VB-C>*trans* VB-C≫A-factor type), indicating that binding was due to a *cis* VB-C specific binding protein. Scatchard analysis of the binding demonstrated a single class of high affinity binding sites (K_d 1.1 nM) and low number of the binding sites (30~40 sites/genome DNA). By gel filtration on Sephadex G-75 and molecular sieve HPLC, the binding protein was shown to have an M_r of about 20,000. These results indicate that the substance is a novel VB-C binding protein and suggest that it is a VB-receptor mediating the pleiotropic signal transmitted by VBs in *S. virginiae*.

Streptomycetes are Gram-positive bacteria characterized by their versatile ability to produce secondary metabolites as well as their morphological complexity. One of the most interesting features of these microorganisms is their production of endogenous and exogenous signal molecules. These have sometimes been called "autoregulators"[1,2], and the best studied among them is A-factor from *Streptomyces griseus*, which triggered production of streptomycin and the formation of aerial mycelia[3] in some antibiotic- and aerial mycelia-minus mutants. Besides A-factor, additional signal molecules have been isolated and their structures elucidated: Anthracycline-inducing factors from *Streptomyces viridochromogenes*[4], *Streptomyces bikiniensis* and *Streptomyces cyaneofuscatus*[5], B-factor which induces rifamycin production in *Nocardia* sp.[6,7], C-factor which induces conidia formation in *S. griseus*[8], pamamycin which stimulates aerial mycelia formation in *Streptomyces alboniger*[9], and a sporulation pigment from *Streptomyces venezuelae*[10].

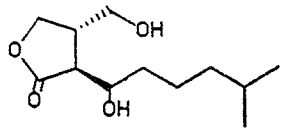
Recently, we have isolated similar signal molecules named virginiae butanolides A, B and C (VBs-A, B and C, Fig. 1) from *Streptomyces virginiae*[11], which are effective even at 1 ng/ml in initiating virginiamycin production. VBs are 2,3-disubstituted γ-butyrolactones and thus share a common skeleton with A-factor and anthracycline-inducing factors. A-factor has been shown to be present in about 15% of *Streptomyces* species[3] and anthracycline-inducing factors in about 26%[12]. Therefore, γ-butyrolactone – type signal molecules are distributed widely in *Streptomyces* species. However, little is known of the fundamental mechanism by which they exert their pleiotropic effects. In

Fig. 1. Structures of signal molecules isolated from *Streptomyces*.

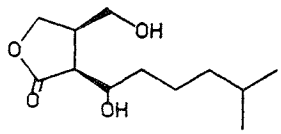
A-factor from *S. griseus*.

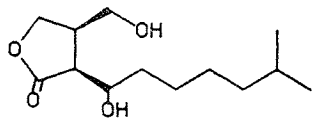


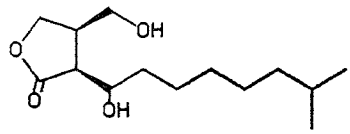
Factor I from *S. viridochromogenes*.



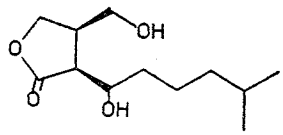
Factors from *S. bikiniensis* and *S. cyaneofuscatus*.



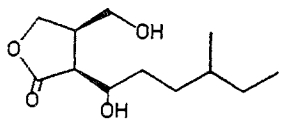




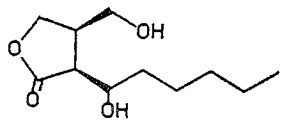
Virginiae butanolides from *S. virginiae*.



VB-A



VB-B



VB-C

eucaryotic cells where many signal molecules, such as peptide- or steroid-hormones, are known, almost all of them seem to function through binding to specific receptors[13,14]. By analogy, we suspect that signal molecules in procaryotes, such as VBs, may function through binding to specific receptors. In this report, we establish that a novel VB-C binding protein is present in *S. virginiae*. The binding protein shows clear specificity toward a compound having novel 2,3-*cis* stereochemistry and its affinity (K_d 1.1 nM) is comparable to that of hormone receptors.

Materials and Methods

Strain and Cultivation Conditions

S. virginiae of YANAGIMOTO and TERUI[15,16] was used throughout this study. The medium for *S. virginiae* contained Bacto-casitone (Difco) 7.5 g, yeast extract (Difco) 7.5 g, glycerol 15 g and NaCl 2.5 g per liter, pH 6.5. For seed culture preparation, 20 ml of the medium in a 100-ml Erlenmeyer flask was inoculated from slants and cultured at 28°C for 36 hours on a reciprocating shaker (120 strokes/minute). The cells were collected aseptically by centrifugation (6,000×*g*) for 10 minutes, resuspended in a 20-ml portion of fresh medium, and used to inoculate the main culture. The main culture was grown in 70-ml portions of the medium in 500-ml Erlenmeyer flasks. Each flask was inoculated with 2.1 ml of the 36-hour cell suspension, and incubated at 28°C on a recriprocating shaker (120 strokes/minute) for 10 hours. The cells were collected by centrifugation (6,000×*g*) for 10 minutes at 4°C, washed with cold NaCl 0.9% and triethanolamine-HCl buffer 0.05M (pH 7.0) containing KCl 0.5 M, EDTA-K_2 5 mM, phenylmethyl sulfonylfluoride (PMSF) 0.1 mM, 2-mercaptoethanol 5 mM and glycerol 10% (buffer A), and stored at −80°C until use.

Preparation of Cell-free Extract

Cells (1 g wet mycelia) were suspended in 10 ml of buffer A, and disrupted by sonication for 1 minute×2 at 4°C. Cell debris was removed by centrifugation (28,000×*g*) for 20 minutes at 4°C. To the supernatant, solid ammonium sulfate was added with stirring to give 60% saturation while the pH was kept at 7 by adding 3 N KOH. After 30 minutes at 4°C, precipitated protein was collected

by centrifugation as described above, dissolved in a minimum amount of buffer A, and dialyzed against 100-fold volumes of buffer A for 6 hours at 4°C.

Synthesis of 2-(1′-Hydroxy-[6′,7′-^{3}H]heptyl)-3-(hydroxymethyl)butanolide ([^{3}H]VB-C_7)

Tritium labeled VB-C_7, which is present as a natural inducer (VB-D)[17] and has the strongest VB activity among VB-C analogues[18], was chemically synthesized for use as a ligand in the binding assay (Scheme 1).

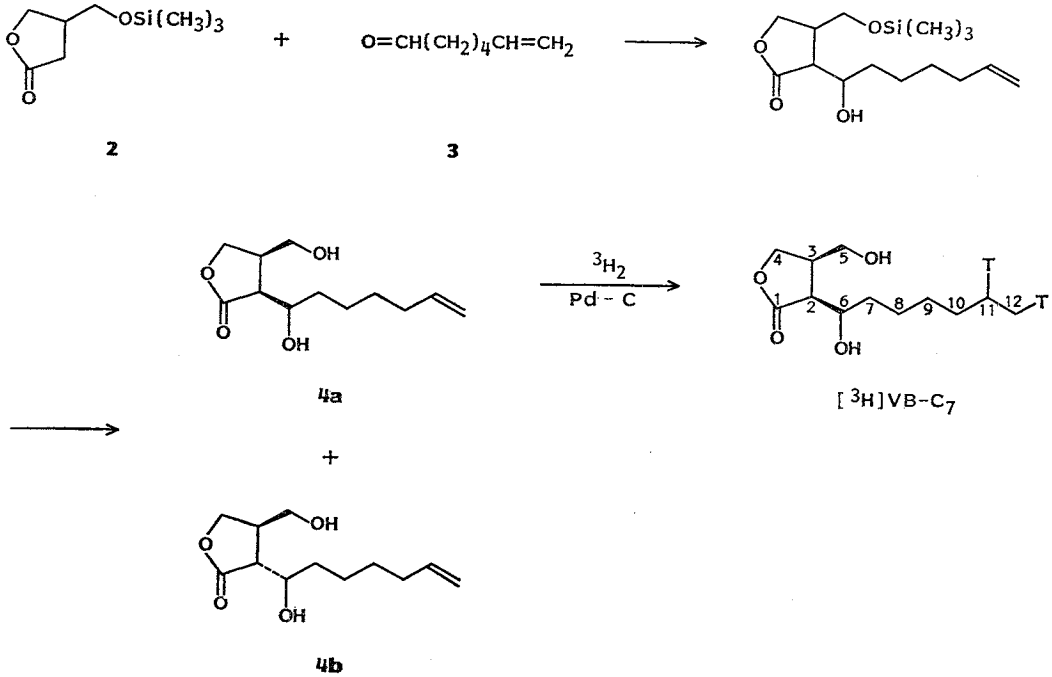
1) 3-(Hydroxymethyl)butanolide (**1**): **1** was synthesized by reduction of diethylformylsuccinate with $NaBH_4$, and trimethylsilylated to give 3-(trimethylsilyloxymethyl)butanolide (**2**) as previously described[11].

2) 6-Heptenal (**3**): To a solution of 7-octene-1,2-diol (Aldrich Chemical Co.) (1.44 g) dissolved in a mixture of ethanol - water (5 ml/10 ml), $NaIO_4$ (2.2 g) was added gradually with stirring at 25°C. After the addition, CH_2Cl_2 (15 ml) was added, and the reaction continued for 1 hour. The reaction mixture was extracted with CH_2Cl_2; the solvent layer was washed with water and dried over anhydrous sodium sulfate. Evaporation of the solvent gave 1.1 g of **3**.

^{1}H NMR (60 MHz, $CDCl_3$) δ 1.3~1.8 (4H, m), 1.8~2.2 (2H, m), 2.2~2.6 (2H, m), 4.7~5.2 (2H, m), 5.4~6.0 (1H, m), 9.6~9.8 (1H, t); IR (film) cm^{-1} 3070, 2940, 2860, 2720, 1730, 1640.

3) 2-(1′-Hydroxy-6′-heptenyl)-3-(hydroxymethyl)butanolide (**4**): Lithium diisopropylamide was prepared in 50 ml of dry THF from 3.5 ml of diisopropylamine and *n*-butyl lithium (1.29 M *n*-hexane solution, 19.08 ml) at −78°C. To this solution, 4.22 g of **2** in THF was added dropwise. After the addition, the reaction mixture was stirred for 1 hour at −78°C. Then 2.76 g of **3** was added dropwise at −78°C and the reaction mixture was kept at −78°C for 1 hour. The temperature of the reaction was then allowed to rise to 0°C. The reaction mixture was poured into 100 ml of ice-water and 15 ml of AcOH and extracted with CH_2Cl_2. The CH_2Cl_2 layer was washed with 10% sodium bicarbonate solution and water, and dried over anhydrous sodium sulfate. Evaporation of the solvent gave 7.96 g of crude material. The crude oil was refluxed in 160 ml of EtOH and 40 ml of water for 30 minutes to remove the trimethylsilyl group. The reaction mixture was concentrated and the resulting crude oil (5.26 g) was purified on a silica gel (50 g) column developed with *n*-hexane -

Scheme 1. Synthetic route to tritium-labeled VB-C_7.



EtOAc (1 : 1), yielding 4.27 g of racemic **4**. The *cis* isomer of **4** (**4a**) was obtained by reverse-phase HPLC using MeOH - H_2O (55 : 45). Yield: 96 mg. IR and NMR signals were as described[18], and supported the expected structure resonably well.

4) *cis*-2-(1′-Hydroxy-[6′,7′-^{3}H]heptyl)-3-(hydroxymethyl)butanolide ([^{3}H]VB-C_7): *cis* **4a** (96 mg) dissolved in 2 ml of EtOH was catalytically reduced in the presence of 25 mg of 5% Pd - C under a tritium atmosphere (25 Ci) for 2 hours at room temperature (New England Nuclear). Removal of the catalyst and evaporation of the solvent gave pure [^{3}H]VB-C_7. The chemical and radiochemical purity was determined by TLC using ether as a solvent and radio-inert VB-C_7 as a standard. The specific radioactivity of [^{3}H]VB-C_7 was 54.6 Ci/mmol.

Specific Binding of [^{3}H]VB-C_7 to Protein

The ammonium sulfate-fraction was incubated for 1 hour at 25°C with [^{3}H]VB-C_7 (54.6 Ci/mmol) at a final concentration of 34.8 nM in the presence or absence of an 3,600-fold excess of non-radioactive VB-C (0.125 mM). Each 100 μl of the solution was then dialyzed against 10 ml of buffer A for 5 hours, and a 50 μl-portion was assayed for radioactivity with a liquid scintillator (Beckman LS 7500) in the presence of 10 ml toluene containing Omnifluor (4 g/liter, New England Nuclear) and Triton X-100 (500 g/liter). Specifically bound [^{3}H]VB-C_7 represents the difference in [^{3}H]VB-C_7 binding in the presence and absence of non-radioactive VB-C.

Gel Filtration on Sephadex G-75

Gel filtration was performed at 4°C with a Sephadex G-75 (super fine) column (1.1 × 21 cm) pre-equilibrated with 0.05 M triethanolamine-HCl buffer (pH 7.0) containing KCl 0.5 M, EDTA-K_2 5 mM, 2-mercaptoethanol 5 mM and PMSF 0.1 mM. The sample (500 μl) preincubated with [^{3}H]VB-C_7 in the presence or absence of nonlabeled VB-C was applied, and 0.5 ml fractions were collected. From each, 50-μl portions were taken to measure radioactivity. A calibration curve was obtained by using ferritin (M_r 450,000), albumin (M_r 68,000), chymotrypsin (M_r 25,000) and cytochrome C (M_r 12,500) (Boehringer Mannheim GmbH. Biochemica Co., Ltd.).

Molecular Sieve HPLC

A Trirotar V (Japan Spectroscopic Co., Ltd.) chromatograph equipped with a TSK-gel G2000SW_{XL} (Tosoh Manufacturing Co., Ltd.) (M_r<160,000) column using 0.1 M potassium phosphate buffer (pH 7.0) containing 0.2 M NaCl at a flow rate of 1 ml/minute was used. Samples were monitored by UV (210 nm) and fluorescence (Ex 280 nm, Em 340 nm) detectors. Fractions of every 15 seconds (*ca.* 250 μl) were collected, and radioactivity in each 50 μl was measured. Standard proteins were obtained from Oriental Yeast Co., Ltd.

DNA Content Determination

The 10-hour cells of *S. virginiae* were first suspended in cold 0.5 N perchloric acid, centrifuged (1,500 × *g*) for 15 minutes, and resuspended in cold 0.5 N perchloric acid. The cells were disrupted by sonication (4°C, 6 minutes), and DNA was extracted by heating at 70°C for 45 minutes. After centrifugation, the DNA content in the supernatant was determined by BURTON's chemical method[19] using purified *Escherichia coli* B DNA (Sigma Chemical Company) as a standard. From the average genome size for *Streptomyces* species ($4.28 \pm 0.45 \times 10^9$)[20], 1 g of wet mycelia was calculated to contain $(4.01 - 3.25) \times 10^{11}$ genomes.

Results and Discussion

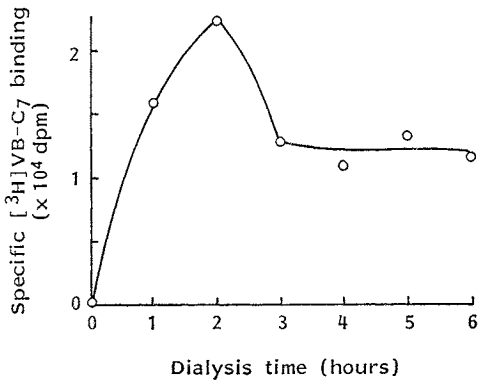
Existence of VB-C Specific Binding Protein

Under our cultivation conditions, production of endogenous VBs starts at 12 hours and VBs trigger virginiamycin production from 14 hours of cultivation. Addition of exogenous VBs at 8 hours resulted in the production of virginamycin from 10 hours. Therefore, the hypothetical binding protein should be present in 8-hour cells. To avoid the effect of endogenous VBs which should compete with [^{3}H]VB-C_7 during binding assay and may show down-regulation on the amount of the bind-

ing protein, we harvested cells at 10 hours at which no endogenous VBs were present. Then, cell-free extracts were prepared from the 10-hour cells, and we investigated the presence of the hypothetical VB binding protein by equilibrium dialysis (Fig. 2). Differences in [^{3}H]VB-C_7 bound in the absence and presence of 3,600-fold excess nonlabeled VB-C, *i.e.* specific [^{3}H]VB-C_7 binding, reached a plateau after 3-hour dialysis and remained constant until 6 hours, clearly indicating VB-binding capacity of the cell-free extract (3.5 pmol bound/extract from 1 g-wet mycelia).

Next, to confirm the macromolecular nature of the [^{3}H]VB-C_7 binding activity, we performed gel filtration on a Sephadex G-75 column (Fig. 3). Unbound [^{3}H]VB-C_7 was eluted at around fraction 45 and protein-bound [^{3}H]VB-C_7 appeared at around fraction 21 (Fig. 3A). In contrast, when [^{3}H]VB-C_7 binding was blocked by the presence of nonlabeled VB-C (Fig. 3B), the peak at fraction 21 almost disappeared, supporting the specific nature of VB-C binding. Minor radioactivity in fractions 15~19 is attributed to nonspecific binding. Calibration of the column indicated that fraction 21 corresponded to M_r 20,000~25,000. To confirm the molecular weight of the binding protein, we used a molecular sieve column for HPLC (Fig. 4). Typically 4 peaks eluting at 5.75, 9.75, 14.5 and 16.25 minutes, respectively, were observed (Fig. 4A). A peak eluting later than 20 minutes represented unbound [^{3}H]VB-C_7. For the sample blocked by nonlabeled VB-C, the peak at 9.75 minutes significantly decreased, while the other 3 peaks did not show any difference (Fig. 4B), indicating that the 9.75 minutes-peak was due to a VB-C-specific binding protein and other peaks represented nonspecific binding proteins. Actually, fraction 21 in Fig. 3A showed only the 9.75-minute protein (Fig. 4C). The 9.75 minute-peak corresponded to M_r of 20,000, which was in good agreement with the data from Sephadex G-75 gel filtration.

Fig. 2. Time course of specific [^{3}H]VB-C_7 binding during equilibrium dialysis.

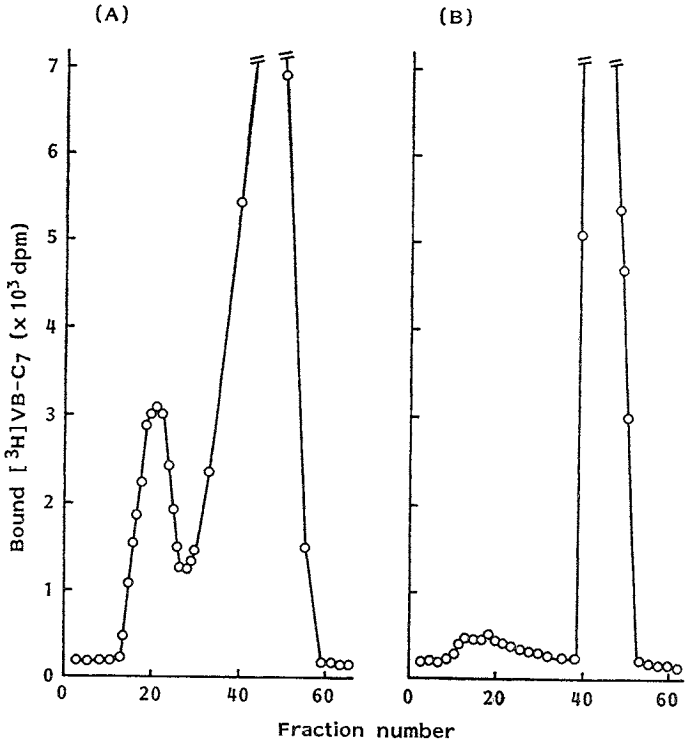


Cell-free extract (100 μl) was incubated with 34.8 nM of [^{3}H]VB-C_7 (0.2 μCi) in the presence and absence of nonlabeled VB-C (125 μM) for 1 hour at 28°C, and dialyzed against 10 ml of buffer A. At the indicated time, each 50 μl of inner and outer solution of the dialysis tube was taken for radioactivity measurements to determine differences in the amount of [^{3}H]VB-C_7. The specific [^{3}H]VB-C_7 binding represents the difference between the values obtained in the absence and presence of nonlabeled VB-C.

Characterization of the VB-Binding Protein

From the evidence described above, *S. virginiae* has a VB-C binding protein of M_r 20,000. To determine the ligand specificity of binding, we investigated the effectiveness of several VB analogues as a competitor of [^{3}H]VB-C_7 (Table 1). In *S. virginiae*, all the natural VBs have *cis* stereochemistry, and the effectiveness of several VB-C analogues as signal molecules was as follows[18]: *cis* VB-C>*trans* VB-C≫A-factor type. If the binding protein participates in transducing the VB signal, it should show similar ligand specificity. Indeed, the effectiveness of VB-C analogues as competitive ligands correlated well with their ability to induce virginiamycin production: *cis* VB-C>*trans* VB-C≫A-factor type. To know more about the VB binding protein, we investigated the concentration dependence of [^{3}H]VB-C_7 binding (Fig. 5A). Scatchard plots of the data (Fig. 5B) revealed that the binding protein

Fig. 3. Sephadex G-75 column chromatography of cell-free extracts preincubated with [^{3}H]VB-C_7 alone (A) and [^{3}H]VB-C_7+nonlabeled VB-C (B).



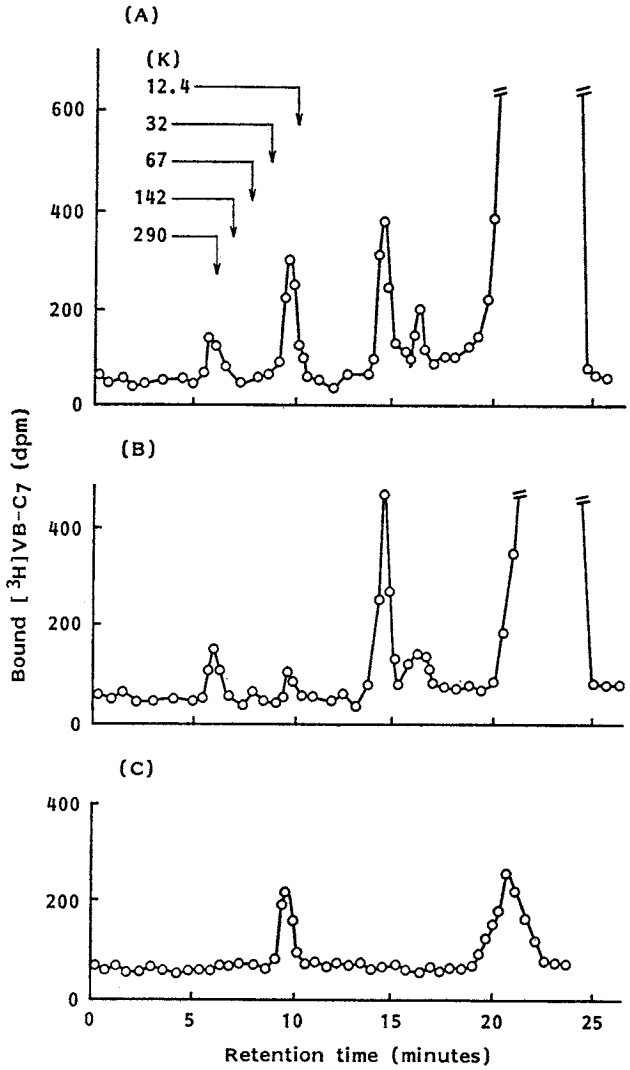
Fractions of 0.5 ml were collected, and aliquots (50 μl) were taken for radioactivity measurements. Radioactivity per 50 μl of each fraction was indicated in the figure. Other experimental conditions are similar to those described in Fig. 2.

had very high affinity for VB-C_7 (K_d 1.1 nM), which was in good agreement with the minimum effective concentration of VB-C_7 (3.5 nM)[18] as an inducer of virginiamycin production. Therefore, it can be concluded that the protein of M_r 20,000 is a specific VB binding protein.

From the Scatchard plots, we could also estimate the maximum number of binding sites per genome DNA of *S. virginiae* (30~40 binding sites/genome DNA) by chemical determination of the DNA content. The number of the binding sites is much lower than those for human platelet-derived growth factor receptor (3×10^5 sites/cell)[21] or α-pheromone receptor from *Saccharomyces cerevisiae* (9×10^5 sites/cell)[22], but similar to those for murine granulocyte macrophage-colony stimulating factor receptor (10~60 sites/cell)[23]. The small number of VB binding sites may indicate high efficiency in the signal transducing pathway.

In eucaryotic cells, RNA has been found to associate with a variety of steroid hormone receptors such as estrogen receptor[24] and glucocorticoid receptor[25], and is believed to modulate the DNA-binding ability of the receptors. Thus, the interaction of RNA with steroid hormone receptors seems to be important for receptor-mediated regulation of gene expression[26]. In order to check the similarity of VB binding protein to steroid receptors, we checked the effect of digestion by DNase, RNase and pronase. Neither binding assay (Table 2) nor gel filtration analyses on Sephadex G-75 (Figs.

Fig. 4. Separation of [^{3}H]VB-C_7-protein complex by molecular sieve HPLC.



Cell-free extracts were incubated with [^{3}H]VB-C_7 in the absence (A) and presence of nonlabeled VB-C (B); each 20 μl sample, treated as in Fig. 2 was injected, and fractions were collected every 15 seconds. Values represent the radioactivity in 50-μl portions. Fraction 21 from Sephadex G-75 chromatography (Fig. 3A) (C); 20 μl of the concentrated fraction was injected into HPLC and fractionated as above. Marker proteins used were glutamate dehydrogenase (M_r 290,000), lactate dehydrogenase (M_r 142,000), yeast enolase (M_r 67,000), yeast adenylate kinase (M_r 32,000) and cytochrome c (M_r 12,400), respectively.

6A and 6B) showed any effect of DNase or RNase treatment; only 5% and 20% decrease in [^{3}H]VB-C_7 binding activity, respectively. In contrast, the sample treated with pronase completely lost [^{3}H]VB-C_7 binding activity (Table 2) and macromolecule-bound [^{3}H]VB-C_7 on gel filtration (Fig. 6C). From these results, it can be concluded that the VB binding protein is mainly proteinaceous and does not associate with DNA or RNA under our experimental conditions, although we cannot exclude the pos-

sibility that removal of DNA or RNA from the VB binding protein has little effect on its binding activity.

In conclusion, *S. virginiae* possesses a specific VB-C binding protein with high affinity (K_d 1.1 nM). The affinity is comparable to those of receptors for hormones in eucaryotic cells. The binding protein exists in small amount (30~40 binding sites/genome DNA) and has a molecular weight of 20,000. It may mediate the pleiotropic signal conveyed by VBs in *S. virginiae*. To our knowledge, this is the first report of the presence and character of a binding protein for pleiotropic signal molecules in procaryotic cells.

Table 1. Effect of several VB-C analogues as competitive ligands on the binding of [^{3}H]VB-C_7 to the VB binding protein.

Nonlabeled compound	Bound [^{3}H]VB-C_7			
	500-fold molar excess		1,000-fold molar excess	
	10^3 dpm	%	10^3 dpm	%
A-Factor type	2.55	17.4	5.40	35.0
trans VB-C	10.48	71.7	10.81	70.1
cis VB-C	14.60	100	15.40	100

Cell-free extract (100 μl) was incubated with 34.8 nM of [^{3}H]VB-C_7 (0.2 μCi) in the absence and presence of nonlabeled VB-C analogues; the values shown represent the difference in amounts of radioactivity bound. The concentrations of VB-C analogue used were 17.4 μM (500-fold excess) and 34.8 μM (1,000-fold excess). The A-factor type analogue, *trans* VB-C and *cis* VB-C were 2-(1'-hexanoyl)-3-(hydroxymethyl)butanolide, 2,3-*trans* VB-C and 2,3-*cis* VB-C, respectively. They were synthesized as described previously[18].

Fig. 5. (A) Concentration dependence of the [^{3}H]VB-C_7 binding in the absence (○) and presence of nonlabeled VB-C (●), (B) Scatchard plots of specific [^{3}H]VB-C_7 binding.

The concentration of nonlabeled VB-C was 2,000-fold in excess of [^{3}H]VB-C_7.

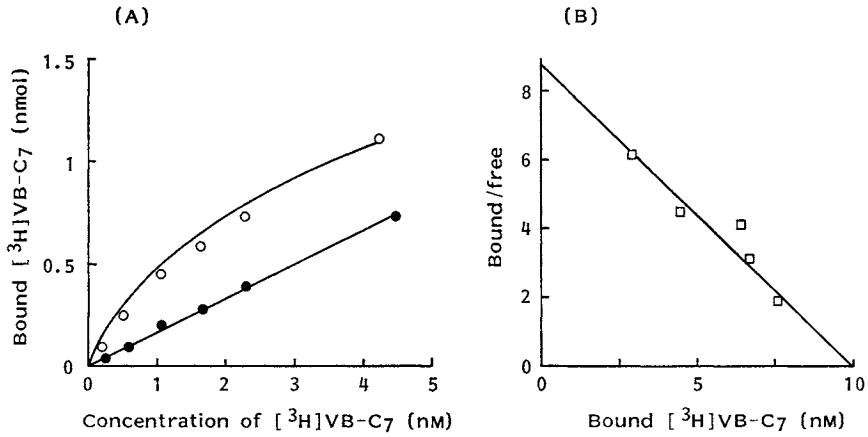


Table 2. Effect of enzyme treatment on [^{3}H]VB-C_7 binding activity of the cell-free extract.

Enzyme used	[^{3}H]VB-C_7 binding (10^3 dpm/50 μl)			Relative activity (%)
	Total[a]	Nonspecific[b]	Specific[c]	
None	23.20	9.15	14.05	(100)
DNase I	24.25	10.85	13.40	95
RNase A	18.85	7.55	11.30	80
Pronase	5.35	5.95	0	0

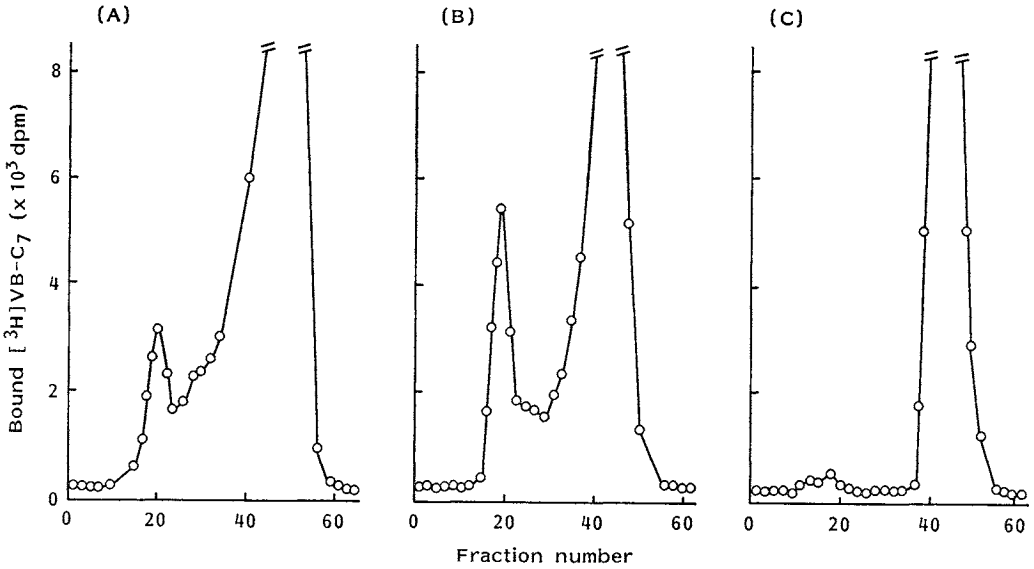
Cell-free extract was digested either with DNase I, RNase A or pronase as described in the legend of Fig. 6. [^{3}H]VB-C_7 binding was measured by equilibrium dialysis as described in Materials and Methods.

[a] Cell-free extract was incubated with [^{3}H]VB-C_7 alone.

[b] Cell-free extract was incubated with [^{3}H]VB-C_7+non-radiolabeled VB-C.

[c] Specific binding represents the difference between total and nonspecific binding.

Fig. 6. Sephadex G-75 gel filtration of cell-free extracts treated with (A) DNase, (B) RNase and (C) pronase.



Cell-free extracts were digested either with DNase I (10 μg/ml, 37°C, 20 minutes), RNase A (40 μg/ml, 30°C, 30 minutes) or pronase (0.2 mg/ml, 30°C, 5 minutes), and 500-μl portions were applied. Other experimental conditions are identical to those described in Fig. 3.

Acknowledgment

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