THE EFFECT OF SINEFUNGIN AND SYNTHETIC ANALOGUES ON RNA AND DNA METHYLTRANSFERASES FROM *STREPTOMYCES*

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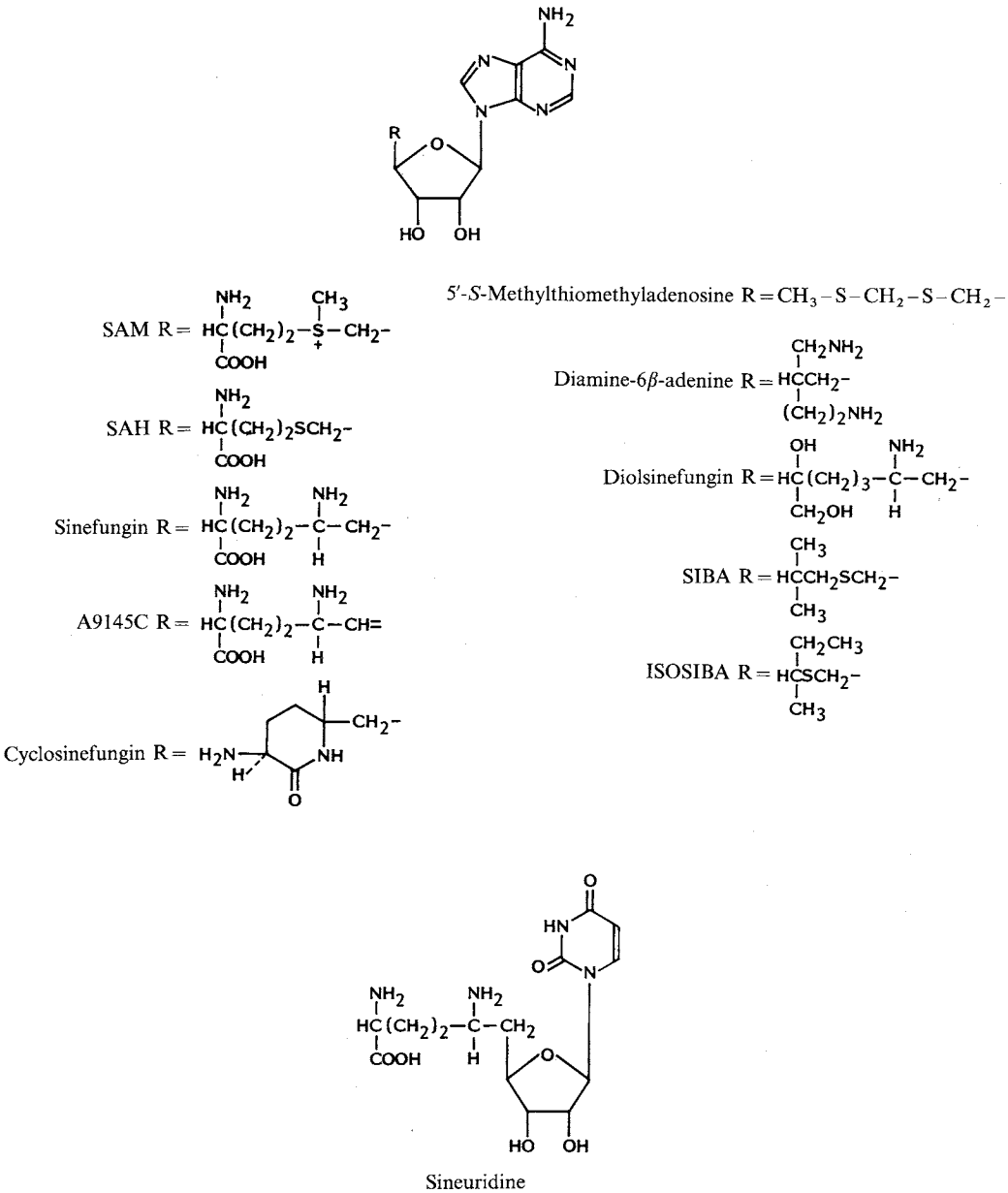
Sinefungin is an antibiotic structurally related to *S*-adenosylmethionine. It has been described as an inhibitor of RNA transmethylation reactions in viruses and eukaryotic organisms, but not in bacteria. We show here that sinefungin strongly inhibits RNA methyltransferase activity, but not the biosynthesis of these enzymes in *Streptomyces*. All the methylated bases found in *Streptomyces* RNA (1-methyladenine, N^6-methyladenine, N^6,N^6-dimethyladenine and 7-methylguanine) are inhibited by this antibiotic. Experiments with sinefungin analogues show that specific changes in the ornithine radical of the molecule still preserve its inhibitory capability. The substitution of the adenine radical by uridine causes the loss of the inhibitory effect. These results and our former studies on *Streptomyces* DNA methylation, suggest that nucleic acid modification is the main target of sinefungin in *Streptomyces*.

Sinefungin is a natural nucleoside produced by *Streptomyces incarnatus* NRRL 8089 and *Streptomyces griseolus* NRRL 3739[1]. It is a structural analog of *S*-adenosylmethionine (SAM) (Fig. 1), which is known as an inhibitor of transmethylation reactions related to DNA[2], proteins[3], phospholipids[4] and other molecules[5]. Inhibition of RNA methyltransferase activities by sinefungin has been reported with viruses and eukaryotic organisms[6~10], but has not been shown in bacteria. Sinefungin also inhibits viral mRNA methylation[6,7], tRNA methylation[8,9] and eukaryotic rRNA methylation[10].

Sinefungin is described as an antifungal[11], antiviral[6] and antiparasitic[3,12] agent. This antibiotic is nephrotoxic "*in vivo*" and several laboratories have synthesized structurally related analogues. Some of these analogues are listed in Fig. 1. A9145C is a natural analogue isolated from cultures of *S. griseolus* NRRL 3739[13]. *S*-Adenosylhomocysteine (SAH) (Fig. 1) is one of the products and a known inhibitor of SAM mediated methylation reactions. The 5′-*S*-(2-methylpropyl)adenosine (SIBA) analogue inhibits the tRNA methylation during transformation of chick embryo fibroblasts (CEF) by Rous sarcoma virus (RSV)[14]. Sinefungin and SAH inhibit the same processes but only sinefungin inhibits RSV induced formation of foci, SAH being inactive[8]. Sinefungin inhibits the monomethylation of guanine in positions 2 and 7 and adenine in positions 1 and 6, more powerfully than SAH. This may suggest a role for these methylations in the transformation process[9].

Streptomyces antibioticus ETHZ 7451 is capable of sporulating in a submerged culture (I.S. NOVELLA *et al.*; submitted for publication) and sinefungin inhibits this phenomenon (M. J. YEBRA *et al.*; submitted for publication). We previously showed that sinefungin inhibits the activity of DNA methyltransferases but not that of protein methyltransferases of *Streptomyces*[2]. Here, we describe its effect on *S. antibioticus*, *S. incarnatus* and *S. griseolus* RNA methyltransferase activity. Furthermore, in an attempt to identify those structural features of sinefungin which are essential for its inhibitory effect, we assayed the effect of several analogues on RNA and DNA methyltransferase activities of *Streptomyces*. Our results show that

Fig. 1. Structure of SAM, SAH, sinefungin and analogues.



sinefungin strongly inhibits RNA methyltransferase activities in *Streptomyces* and that several synthetic analogues have variable effects on these methyltransferases.

Materials and Methods

Strains, RNA and DNA Sources

S. antibioticus ETHZ 7451 (Eidgenössische Technische Hochschule, Zürich). *S. incarnatus* NRRL 8089 (Northern Regional Research Laboratory), and *S. griseolus* NRRL 3739. tRNA and 16*S* and 23*S* rRNA from *Escherichia coli* MRE 600 and DNA from bacteriophage λ were provided by Boehringer Mannheim.

Chemicals

S-Adenosyl-L-[*methyl*-^{3}H]methionine (specific activity 11.9 Ci/mmol) was obtained from DuPont, Nuclear Iberica SA., SAH was from Boehringer Mannheim. Sinefungin, A9145C, cyclosinefungin, diamine-6β-adenine, diolsinefungin, SIBA, 5'-*S*-(1-methylpropyl)adenosine (ISOSIBA), 5'-*S*-methylthiomethyladenosine and sineuridine were generous gifts from Dr. M. ROBERT-GERÓ (Institut de Chimie des Substances Naturelles, C.N.R.S., Gif-Sur-Yvette, France).

Growth of *Streptomyces* and Preparation of Enzymatic Extracts

S. antibioticus, *S. incarnatus* and *S. griseolus* were grown at 30°C in a liquid complex medium[15] with vigorous shaking for 19 hours and cells were harvested by centrifugation. In each case 10 g of cells (wet weight) were resuspended in 40 ml of a buffer containing Tris-HCl 20 mM (pH 8.0), EDTA 1 mM, 2-mercaptoethanol 7 mM and NaCl 100 mM. The cells were disrupted by sonication and the extracts clarified by centrifugation at 12,100 × *g* for 20 minutes followed by ultracentrifugation at 150,000 × *g* for 2.5 hours at 4°C. Proteins in the high speed supernatant were precipitated by addition of solid ammonium sulfate (70% of saturation)[16]. The precipitate was dissolved in 5 ml of the above buffer and dialyzed against this buffer. The cell-free extracts were stored at −70°C in buffer containing Tris-HCl 50 mM (pH 8.0), EDTA 1 mM, dithiothreitol (DTT) 1mM, NaCl 100 mM and glycerol 50%. This preparation was subsequently used as a source of methylase activity.

Isolation of rRNA and tRNA from *S. incarnatus*

rRNA was isolated by two methods (LiCl-Urea or phenol extraction) as previously described[17,18], from the 70S ribosome particle. Total tRNA was isolated from the supernatant obtained after ultracentrifugation at 100,000 × *g* in the preceding methods. This supernatant was precipitated with ethanol in the presence of 0.3 M NaCl. Proteins were removed by phenol extraction and DNA by DNase (RNase free, Boehringer). After subsequent precipitation, the tRNA was redissolved in 10 mM HEPES-KOH pH 7.6, 10 mM $MgCl_2$ and 5 mM 2-mercaptoethanol, and stored at −70°C. The purity of RNA was tested by the A_{260}/A_{280} relationship and by visualization in agarose gels.

Effect of Sinefungin on RNA Methyltransferase Biosynthesis by *S. antibioticus*

In order to investigate if sinefungin inhibits biosynthesis of RNA methyltransferases in *S. antibioticus* ETHZ 7451, this strain was cultivated in a liquid complex medium[15] in the absence and presence of sinefungin (1.5 mM) during 19 hours at 30°C. In both cases RNA methyltransferase activity was determined before and after ammonium sulfate precipitation in order to analyze the effect of sinefungin on enzyme biosynthesis, as detailed in Results. The cell-free extracts were obtained as described above.

Enzymatic Assay for RNA Methyltransferases

Methylation assays (30 μl, final volume) were carried out at 37°C for 1 hour and contained 5 μg of tRNA or rRNA from *E. coli* as the substrate, 3 μl of crude methylase (about 20 μg of protein) and 12 μM [*methyl*-^{3}H]SAM in the buffer previously described for DNA methylation[19]. After stopping the methylation reaction at 70°C, for 10 minutes, DNA from cell-free extracts was removed by DNase (RNase free, Boehringer) 0.05 μg/μl during 30 minutes at 37°C. The methylated RNA was detected by two different methods: precipitation of RNA with ethanol and NH_4 acetate after protein extraction with phenol and by a method of retention on DEAE-cellulose filters (DE-81)[20]. The labeled RNA was quantified on a toluene scintillation medium.

Sinefungin and analogues were added to the reaction at different concentrations. The inhibitory effect of these compounds was calculated on the basis that the activity of the control free from inhibitors was 100%.

Analysis of Methylated Bases

The methylated bases were analyzed by TLC, using a method previously described for DNA[19], except that 5 μg of labeled *methyl*-^{3}H tRNA was hydrolyzed by incubation in 1.5 ml formic acid (90%) at 100°C for 2 hours. 5-Methylcytosine (5 mC), 3-methylcytosine (3 mC), 1-methyladenine (1 mA), N^6-methyladenine (N^6mA), N^6,N^6-dimethyladenine (N^6,N^6mA) and 7-methylguanine (7 mG) were used as controls.

Enzymatic Assay for DNA Methyltransferases

The *in vitro* DNA methylation reaction used has been already described[19]. RNA from cell-free extracts was removed by RNase (Boehringer) 0.15 μg/μl during 30 minutes at 37°C after stopping the methylation reaction at 70°C, for 10 minutes. The methylated DNA was detected as described above for RNA.

The inhibitory effect of sinefungin analogues was calculated as mentioned above for RNA methyltransferases.

Results

The Effect of Sinefungin on RNA Methyltransferase Activities

Sinefungin strongly inhibited RNA methyltransferase activities from *S. antibioticus*, *S. incarnatus* and *S. griseolus* (Table 1). Endogenous RNA methylation in crude cell-free extracts in absence of added substrate, amounted to about 34% for *S. antibioticus*, 50% for *S. incarnatus* and 17% for *S. griseolus*. rRNA and RNA from *S. incarnatus* were isolated and their capability to accept methyl groups *in vitro* with the homologous RNA methyltransferase investigated. No incorporation was observed (not shown).

The methylated bases detected on RNA are shown in Table 2. The major base present is 1 mA for *S. antibioticus* and *S. incarnatus*. Only *S. antibioticus* crude cell-free extract has activity for N^6,N^6mA. 7mG base is produced in low concentration by the three strains. Sinefungin inhibits 95~100% of the activity of every base residue methylated (not shown). When *E. coli* 16*S* and 23*S* rRNA was used as a substrate, lower exogenous methylation levels were observed, which amounted to about 10% of those obtained with tRNA (not shown).

The Effect of Sinefungin on RNA Methyltransferase Synthesis by *S. antibioticus*

Sinefungin inhibits *in vitro* RNA methyltransferases in *S. antibioticus* as described above. However, sinefungin does not inhibit synthesis of these methylases (Table 3), as when RNA methylase activity was determined before ammonium sulfate precipitation, we observed methylase inhibition in the crude cell-free extract from the culture with sinefungin (Table 3). This indicated that sinefungin remain active in the cells, and is eliminated from the samples after ammonium sulfate precipitation. Overall, the data suggest that sinefungin inhibits the activity *in vivo*, but not the synthesis of RNA methyltransferases.

The Effect of Sinefungin Analogues on RNA Methyltransferase Activities

Sinefungin analogues (Fig. 1) showed variable effects on these methyltransferase activities from

Table 1. The effect of sinefungin (50 μM) on RNA methyltransferase activity from *Streptomyces*.

Strain	Activity (cpm)[a]		Inhibition (%)[b]
	Control	Sinefungin	
S. antibioticus	14,102	564	96
S. incarnatus	4,850	≤100	100
S. griseolus	10,952	1,533	86

[a] tRNA from *Escherichia coli* (5 μg) was used as the exogenous substrate.

[b] Inhibition values correspond to the average of at least three different experiments. Deviation from the values given did not exceed 3%.

Table 2. Analysis by TLC of the RNA residues methylated by crude cell-free extracts of *Streptomyces*.

Strain	Bases	cpm
S. antibioticus	1 mA	10,961
	N^6 mA	397
	N^6N^6mA	1,279
	7 mG	275
S. incarnatus	1 mA	3,354
	N^6 mA	484
	7 mG	312
S. griseolus	1 mA	5,083
	N^6 mA	5,209
	7 mG	208

The data corresponds to the average of at least three different experiments. *Escherichia coli* tRNA was used as exogenous substrate.

Table 3. Effect of sinefungin (1.5 mM) on RNA methyltransferase synthesis in *Streptomyces antibioticus*.

	Activity (cpm)[a]		Inhibition (%)[b]
	Control	Sinefungin	
(A)	6,432	6,390	0
(B)	6,359	2,053	68

The activity was tested after (A) and before (B) ammonium sulfate precipitation.

[a] tRNA from *Escherichia coli* (5 μg) was used as the exogenous substrate.

[b] The data corresponds to the average of three experiments. Deviation from the values given did not exceed 3%.

Streptomyces (Table 4). The most active compound is A9145C, since at 6 μM it inhibits the RNA methyltransferase activities of crude cell-free extracts from *S. incarnatus* by 94%. Diamine-6β-adenine and sineuridine do not inhibit any RNA methyltransferase.

The Effect of Sinefungin Analogues on DNA Methyltransferase Activities

Sinefungin inhibits DNA methyltransferase activities in crude cell-free extract of *S. antibioticus*[2] but does not inhibit those from *S. incarnatus*[2], however SAH inhibits both[2]. Sinefungin (100 μM) inhibits about 80% and SAH (100 μM) about 57% of DNA methyltransferase activity in the crude cell-free extract of *S. griseolus*. The effects of sinefungin analogues on DNA methyltransferases are shown in Table 5. The DNA methyltransferases of *S. griseolus* are very sensitive to sinefungin analogues (Table 5).

Table 4. The effect of sinefungin analogues on RNA methyltransferase activities from *Streptomyces*.

Compound (100 μM)	Inhibition (%)[a]		
	S. antibioticus	*S. incarnatus*	*S. griseolus*
SAH	88	97	100
A9145C[b]	95	94	90
Cyclosinefungin	50	62	40
Diamine-6β-adenine	0	0	0
Diolsinefungin	0	17	0
SIBA	55	0	0
ISOSIBA	52	14	16
5′-*S*-Methylthio-methyladenosine	65	79	88
Sineuridine	0	0	0

[a] The data is the average of at least three experiments. Deviation from the values given did not exceed 5%.

[b] Assayed at concentration of 50 μM (*S. antibioticus*), 6 μM (*S. incarnatus*) and 25 μM (*S. griseolus*).

Table 5. The effect of sinefungin analogues on DNA methyltransferase activities from *Streptomyces*.

Compound (100 μM)	Inhibition (%)[a]		
	S. antibioticus	*S. incarnatus*	*S. griseolus*
A9145C[b]	90	57	79
Cyclosinefungin	0	61	65
Diamine-6β-adenine[b]	0	0	31
Diolsinefungin	0	0	28
SIBA[b]	46	0	87
ISOSIBA	41	0	75
5′-*S*-Methylthio-methyladenosine	84	28	78
Sineuridine	0	0	0

[a] The data is the average of at least three experiments. Deviation from the values given did not exceed 5%.

[b] Assayed at a concentration of 25 μM (*S. griseolus*).

Some of the analogues were assayed *in vivo* on the above strains. Cyclosinefungin (1.5 mM) did not inhibit sporulation of any of the strains. SIBA (150 μM) delayed growth but did not inhibit sporulation. Interpretation of these results compared to the observed effects *in vitro* are difficult, as permeability of the bacteria to these compounds, or their intracellular modification were not investigated.

Discussion

We have shown that sinefungin inhibits strongly the RNA methyltransferase activities from *Streptomyces*. The DNA methylation is also inhibited in *S. antibioticus*[2], but not the protein methyltransferase activity[2]. These results suggest that nucleic acid methylation could be the main target of sinefungin in *Streptomyces*. We found 1 mA, N^6 mA, N^6,N^6 mA and 7 mG base residues methylated on RNA of *Streptomyces*. We did not find RNA methyltransferases for the cytosine bases. The synthesis of

all the former methylated residues was inhibited strongly by sinefungin. This result is analogous to that obtained with sinefungin for methylation of *E. coli* tRNA by RNA methyltransferases from mammalia cells; adenine or guanine methylation seem to be inhibited to a greater degree than that of cytosine[9]. Similar results were obtained for DNA methylation in *Streptomyces* and other bacteria[2].

The lack of methylation on added, homologous rRNA or tRNA substrates and on heterologous *E. coli* rRNA, could indicate that the effect of sinefungin on RNA in *Streptomyces* is exercised on mRNA methylation rather than on rRNA or tRNA. However, due to the instability of the mRNA, it is more conceivable that the modification affected was on precursors of mature rRNA and/or tRNA molecules during the process of formation.

Sinefungin inhibits the sporulation of *S. antibioticus* (M. J. YEBRA *et al.*; submitted for publication). It does not inhibit the biosynthesis of DNA methyltransferases (M. J. YEBRA *et al.*; submitted for publication) nor RNA methyltransferases in that strain. This suggests that sinefungin inactivates the enzymes when it is in contact with them. The analogue does not appear to join covalently to the methyltransferases.

Our studies with sinefungin analogues on RNA and DNA methyltransferases, suggest that the adenosine residue of sinefungin is necessary for its inhibitory effect and that only some changes in the ornithine moiety preserve its activity. It is conceivable that the interaction of sinefungin with these enzymes could be carried out through a specific spatial conformation of the entire molecule. More detailed research with these and other analogues will allow us to understand more exactly the physiological and biochemical mechanism of the action of sinefungin on *Streptomyces* and other bacteria.

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References

1) HAMILL, R. L. & M. M. HOEHN: A9145, a new adenine-containing antifungal antibiotic. I. Discovery and isolation. J. Antibiotics 26: 463~465, 1973
2) BARBES, C.; J. SANCHEZ, M. J. YEBRA, M. ROBERT-GERÓ & C. HARDISSON: Effects of sinefungin and S-adenosylhomocysteine on DNA and protein methyltransferases from *Streptomyces* and other bacteria. FEMS Microbiol Lett. 69: 239~244, 1990
3) PAOLANTONACCI, P.; F. LAWRENCE & M. ROBERT-GERÓ: Differential effect of sinefungin and its analogs on the multiplication of three *Leishmania* species. Antimicrob. Agents Chemother. 28: 528~531, 1985
4) MCCAMMON, M. T. & L. W. Parks: Inhibition of sterol transmethylation by *S*-adenosylhomocysteine analogs. J. Bacteriol. 145: 106~112, 1981
5) FULLER, R. W. & R. NAGARAJAN: Inhibition of methyltransferases by some new analogues of *S*-adenosylhomocysteine. Biochem. Pharmacol. 27: 1981~1983, 1978
6) PUGH, C. S. G.; R. T. BORCHARDT & H. O. STONE: Sinefungin, a potent inhibitor of virion mRNA (guanine-7-)methyl-transferase, mRNA (nucleoside-2'-)methyltransferase, and viral multiplication. J. Biol. Chem. 253: 4075~4077, 1978
7) PUGH, C. S. G. & R. T. BORCHARDT: Effects of *S*-adenosylhomocysteine analogues on vaccinia viral messenger ribonucleic acid synthesis and methylation. Biochemistry 21: 1535~1541, 1982
8) VEDEL, M.; F. LAWRENCE, M. ROBERT-GERÓ & E. LEDERER: The antifungal antibiotic sinefungin as a very active inhibitor of methyltransferases and of the transformation of chick embryo fibroblasts by Rous sarcoma virus. Biochem. Biophys. Res. Commun. 85: 371~376, 1978
9) VEDEL, M. & M. ROBERT-GERÓ: Comparative effect of *S*-adenosylhomocysteine (SAH) and sinefungin on tRNA-base methylation in whole cells and *in vitro*. FEBS Lett. 128: 87~89, 1981
10) LI, A. W.; R. A. SINGER & G. C. JOHNSTON: Effects of sinefungin on rRNA production and methylation in the yeast *Saccharomyces cerevisiae*. Arch. Biochem. Biophys. 240: 613~620, 1985
11) LI, A. W.; R. A. SINGER & G. C. JOHNSTON: Cell cycle effects in yeast cells of the antifungal agent sinefungin. FEMS Microbiol. Lett. 23: 243~247, 1984
12) FERRANTE, A.; I. LJUNGSTROM & E. LEDERER: Antitoxoplasmosis properties of sinefungin in mice. C. R. Acad. Sci. Paris. 306: 109~113, 1988

13) BOECK, L. D.; G. M. CLEM, M. M. WILSON & J. E. WESTHEAD: A9145, a new adenine-containing antifungal antibiotic: Fermentation. Antimicrob. Agents Chemother. 3: 49～56, 1973
14) VEDEL, M.; M. ROBERT-GERÓ, M. LEGRAVEREND, F. LAWRENCE & E. LEDERER: Inhibition of tRNA methylation *in vitro* and in whole cells by an oncostatic *S*-adenosylhomocysteine (SAH)-analogue: 5'-deoxy-5'-*S*-isobutyladenosine (SIBA). Nucleic Acids Res. 5: 2979～2989, 1978
15) SÀNCHEZ, J.; C. BARBÈS, A. HERNANDEZ, C.-R. G. DE LOS REYES GAVILÀN & C. HARDISSON: Restriction-modification systems in *Streptomyces antibioticus*. Can. J. Microbiol. 31: 942～946, 1985
16) SURI, B.; J. C. W. SHEPHERD & T. A. BICKLE: The *Eco*A restriction and modification system of *Escherichia coli* 15T: Enzyme structure and DNA recognition sequence. EMBO J. 3: 575～579, 1984
17) FAHNESTOCK, S.; V. ERDMANN & M. NOMURA: Reconstitution of 50S ribosomal subunits from *Bacillus stearothermophilus*. Methods Enzymol. 30: 554～562, 1974
18) ZIMMERMANN, R. A.: Protein-RNA interactions in the bacterial ribosome. Methods Enzymol. 59: 551～583, 1979
19) BARBES, C.; C. HARDISSON, I. S. NOVELLA, M. J. YEBRA & J. SANCHEZ: DNA-methyltransferase activities in *Streptomyces antibioticus*. FEMS Microbiol. Lett. 55: 59～64, 1988
20) LACKS, S. A. & S. S. SPRINGHORN: Cloning in *Streptococcus pneumoniae* of the gene for *Dpn*II DNA methylase. J. Bacteriol. 157: 934～936, 1984