BIOSYNTHESIS OF THIOPEPTIDE ANTIBIOTIC A10255 IN STIRRED REACTORS USING A CHEMICALLY DEFINED MEDIUM SUPPLEMENTED WITH CONTINUOUS NUTRIENT FEEDS

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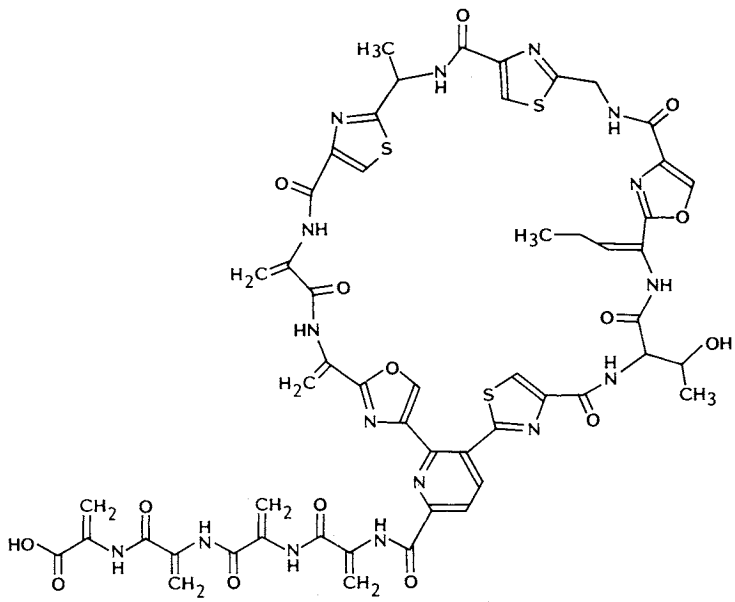
A10255 is a complex of new thiopeptide antibiotics produced by *Streptomyces gardneri*. When stirred reactors were operated in batch mode using a defined medium with a glucose feed, 250 μg/ml of A10255 were produced during a four-day fermentation cycle. The linear growth phase of *S. gardneri* was extended through seven days by supplementing the defined medium with continuous feeds of hydrolyzed casein and methyl caprate. With the supplementary feeds, antibiotic biosynthesis paralleled growth during the extended cycle and attained levels of 1,750 μg/ml. Increasing the standard glucose feed rate increased titers principally by increasing cell mass. Supplementing the standard glucose feed with lipids such as caprylate or caprate, and decyl alcohol, affected cell mass minimally but produced higher titers by increasing the specific biosynthesis of A10255 per unit of biomass.

A10255 is a complex of novel thiopeptide antibiotics that contains a cyclic peptide core with a dehydroalanine side chain. Members of the complex differ in the number of dehydroalanine residues present in the side chain and/or in the number of methyls situated on one of the amino acids in the peptide core[1]. The chemical structure of factor B (Fig. 1)[2] assigns A10255 to the sulfur-containing peptide antibiotic family that includes thiostrepton[3], nosiheptide[4], sulfomycin I and berninamycin A[5], and thioxamycin[6]. A distinguishing feature of family members is the presence of dehydroalanine, which has been shown to

Fig. 1. Structure of A10255B.



arise from serine[7].

Taxonomic data and preliminary fermentation studies with initial strains of the producing culture, *Streptomyces gardneri*, have been reported[8]. In those studies, yields of approximately 325 μg/ml were obtained by using a complex nutrient medium supplemented with continuous feeds of glucose and acid-hydrolyzed casein.

Complex media are commonly employed in fermentation because of their characteristic productivity advantage over defined media. Despite this benefit, complex media possess limitations. For example, trace elements can be present as impurities of varying quantity in one or more primary components. Because their level is difficult to control but is potentially critical for metabolism, they may enhance variability. While defined media may not support adequate cell growth and frequently fail to support high product yields, their composition can be formulated more precisely than complex media. This is an advantage for studying the effects of specific nutrients on growth and the biosynthesis of secondary metabolites like antibiotics. Accordingly, the use of a chemically defined medium for growth of *S. gardneri* and production of A10255 in stirred reactors was examined. The results of this study, along with glucose feed rate studies and the effects of continuous supplementary feeds such as casein and lipids, are reported here.

Materials and Methods

Fermenter Inoculum

Stock cultures of NRRL 18260, a higher-producing strain of *S. gardneri* derived from NRRL 15922 by nitrosoguanidine mutagenesis, were maintained in the vapor phase of liquid nitrogen after growth in submerged culture under the conditions used for fermenter inoculum. The latter was grown in wide-mouth 250-ml Erlenmeyer flasks containing 50 ml of a medium composed of glucose 0.75%, dextrin 1.0%, glycerol 0.5%, Trypticase Soy Broth (Gibco) 3.0%, enzyme-hydrolyzed casein 0.5% and technical grade $CaCO_3$ 1.0% in tap water, pH 7.0 before autoclaving. Inoculated flasks were incubated 72 hours at 30°C on a gyratory shaker orbiting at 250 rpm in a 5.1-cm diameter circle. The culture was then transferred serially into 400 ml of the same medium in wide-mouth 2-liter flasks and incubated an additional 48 hours on shaker boards inclined at a 10° angle from the horizontal plane. The resulting culture was used to provide a 2% (v/v) level of inoculum to stirred reactors.

Fermentation Medium

The defined (SYN) medium was formulated to contain glucose 0.2%, NH_4Cl 0.1%, Na_2SO_4 0.2%, $MgCl_2 \cdot 6H_2O$ 0.0304%, $FeCl_3$ 0.0062%, $CaCl_2 \cdot 2H_2O$ 0.006%, $MnCl_2 \cdot 4H_2O$ 0.0035%, $ZnCl_2$ 0.0019%, $CuCl_2 \cdot 2H_2O$ 0.0005%, SAG 471 (Union Carbide) 0.02% and P-2000 (polypropylene glycol, MW ~2,000) 0.01%. Sterilization was accomplished through the application of 22~25 heating units by the F_0 method[9], after which the medium was supplemented with KH_2PO_4 0.067%, that had been adjusted to pH 6.1 with aqueous KOH and autoclaved separately. A continuous glucose feed was initiated at the rate of $\sim 2.2 \times 10^{-1}$ mg/ml/hour immediately after inoculation unless otherwise noted. Lipids and related compounds, purchased from Aldrich Chemical Company, Inc., were of 96~99.5% purity.

Fermenters

Fermentations were conducted in fully baffled, stirred reactors of conventional design with two 6-bladed turbine impellors, a total capacity of 165 liters and a height-diameter ratio of approximately 1 : 1 for the post-inoculation medium volume of 115 liters. Dissolved oxygen levels were monitored with galvanic sensors and computer-controlled at 45% of air saturation under an internal head pressure of 0.34 atmospheres. The pH was controlled at 6.9~7.1 with aqueous H_2SO_4 and NH_4OH. Continuous nutrient feeds were individually sterilized by autoclaving and metered into the vessel by peristalic pumps from calibrated glass vessels adjacent to the reactors. Feed rates were regulated by adjusting duration of the computer-controlled

"On"/"Off" cycles for the feed pumps. Continuous addition was simulated by brief "Off" periods, typically 5~15 seconds. Nominal feed rates were calculated as the mean rate fed from initiation of the feed until the fermentation was terminated. Exhaust gases from the reactors were monitored continually with a Perkin-Elmer MGA-1200 mass spectrometer and the data were employed for calculations of oxygen uptake (OU) and respiration quotient (RQ). This unit was interfaced with the same Hewlett-Packard 2113E/A900 computers employed for all aspects of process analysis and control.

Analytical Measurements

Residual glucose levels in the fermentation medium were determined by an automated glucose oxidase assay method (Biodynamics/Boehringer Mannheim Corporation, Indianapolis, IN). Because >99% of the antibiotic was cell-associated, A10255 was extracted from the biomass with four volumes of anhydrous methanol per volume of fermented broth and assay values were corrected for dilution. Individual components of the A10255 complex were identified and quantitated with an isocratic analytical HPLC system. This analytical technique utilized a Model 710B WISP autosampler and 6000A delivery pump (both from Millipore/Waters, Milford, MA), a Nova C_{18} Rad-PAK column, a mobile phase composed of acetonitrile - water (38 : 62) with 0.5% ammonium acetate (w/v), a flow rate of 2.0 ml/minute and detection at 247 nm with a Spectroflow SF770 variable wavelength UV detector (Schoeffel Instrument Corporation)[8]. Biomass was measured gravimetrically after drying the washed mycelia to constant weight.

Results and Discussion

Growth and Productivity in the Chemically Defined Medium

After inoculation of *S. gardneri* into the standard SYN medium, oxygen uptake (OU) began to increase almost immediately and typically peaked at 0.25~0.3 mmol/liter/minute within 12~14 hours. The OU thereafter plunged sharply to about 0.1 mmol/liter/minute, where it stabilized. Biomass multiplied rapidly during the first 24 hours (Fig. 2), grew more slowly during the next 72 hours, and increased only slightly thereafter. The maximum level attained was 4.2 mg/ml. A10255 production was detectable by 17 hours and levels increased with biomass to peak on the fourth day at 260 μg/ml. The antibiotic complex during this study consistently contained 93~96% factor G, 3~5% factor B and <1% factor E. Although the analytical HPLC system was capable of identifying A10255C, D, F, H and J, these factors were not detected in unconcentrated samples.

Fig. 2. Time-course profile of *S. gardneri* biomass and A10255 titers in medium SYN.

□ A10255, ▲ biomass.

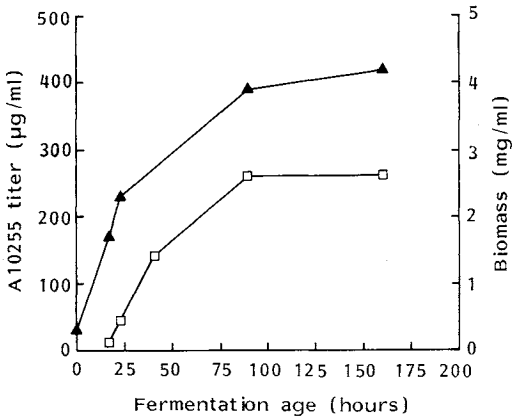
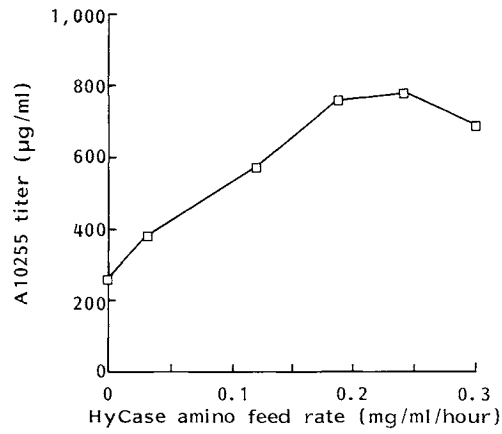


Fig. 3. Effect of HyCase Amino feed rate on A10255 titers in medium SYN.



Effect of Additional Nitrogen

The addition of NH_4OH on a demand basis regulated by acid production was beneficial in the basal medium. However, initial levels of ammonium salts higher than 0.1% or continuous feeds of ammonium salts depressed A10255 yields. HyCase Amino (Sheffield Products), an acid-hydrolyzed casein whose high solubility makes it amenable to continuous feeding, had been shown in an earlier study to be a preferred nitrogen source in complex media[8]. Fig. 3 shows the effect on antibiotic yields when HyCase Amino was fed at different rates to *S. gardneri* in the defined medium. Initiation of a continuous feed at the rate of $1.8 \sim 2.4 \times 10^{-1}$ mg/ml/hour approximately 18 hours post-inoculation enhanced growth and increased A10255 production 200%, to nearly 800 μg/ml. From this study the HyCase Amino feed rate of $\sim 2 \times 10^{-1}$ mg/ml/hour was selected for the medium designated SYN-1.

Effect of Glucose Feed Rates

Glucose is frequently employed as a fermentation carbon source because it is widely metabolized and is relatively inexpensive. However, high levels can be required during extended cycles and incorporation of high levels into the medium at make-up may repress synthesis of secondary metabolites. For example, carbon catabolite regulation is a major control mechanism in rebeccamycin biosynthesis, with the antibiotic being formed only after glucose is depleted from the defined medium[10]. Similarly, glucose levels as low as 5 mM inhibit ACV-synthetase activity by 70% in *Cephalosporium acremonium*[11]. Avermectin production is inhibited when glucose is added during the early stages of fermentation but is increased when glucose is added later[12]. Glucose is a preferred carbon source for the A10255 fermentation but supports good antibiotic yields in a complex medium only when supplied as a limiting nutrient by continuous feeding[8]. The low, standard glucose feed rate of 2.2×10^{-1} mg/ml/hour used with medium SYN had been selected somewhat arbitrarily on the basis of data from the complex medium. Therefore, the effect of various glucose feed rates on growth of *S. gardneri* and A10255 biosynthesis was examined with medium SYN-1.

During the brief lag and early log phases of growth, while the standard glucose feed rate of 2.2×10^{-1} mg/ml/hour exceeded the catabolic rate, glucose accumulated in the medium. The level of free glucose then declined and stabilized at $25 \sim 50$ μg/ml prior to 17 hours, after which it again increased slowly but did not surpass 200 μg/ml. Therefore, all glucose feeds were initiated at the standard rate and modified

Fig. 4. Effect of glucose feed rate on A10255 titers (medium SYN-1 minus the standard glucose feed).

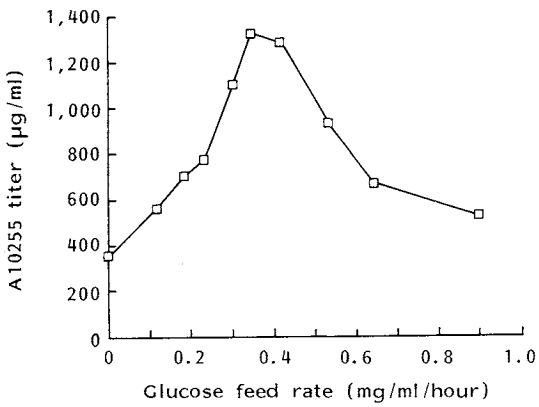


Table 1. Effect of glucose feed rate on growth of *S. gardneri* and biosynthesis of A10255[a].

Glucose feed rate (mg/ml/hour)[b]	Total biomass[c] (mg/ml)	A10255 biosynthesis (μg/mg biomass)
0	4.2	84.5
1.21	9.3	60.2
1.85	11.2	62.9
2.17	12.2	63.9
3.04	15.6	71.2
3.48	17.0	77.6
4.21	17.2	75.0
5.08	17.3	41.6
6.42	17.7	37.9
9.00	18.4	28.8

[a] Medium SYN-1.
[b] $\times 10^{-1}$.
[c] 161 hours data.

at 17~20 hours when other rates were to be evaluated. Neither the maximum nominal rate tested, 9.0×10^{-1} mg/ml/hour, nor an overnight rate of 10.8×10^{-1} mg/ml/hour, resulted in free glucose levels exceeding 450 μg/ml. This is in sharp contrast to the maximum glucose oxidation rate of 3.75×10^{-1} mg/ml/hour exhibited in the A54145 fermentation by *S. fradiae*[13], and suggests that *S. gardneri* is capable of metabolizing glucose at an even higher rate.

Fig. 4 demonstrates the effect of various glucose feed rates on productivity of the A10255 fermentation. Table 1 shows how the glucose feed rate affected growth of *S. gardneri* and specific antibiotic biosynthesis per unit of cell mass. Although biomass formation was restricted when glucose was not fed, the highest antibiotic biosynthesis per unit of biomass was obtained during glucose starvation. The lowest glucose feed rate examined reduced specific productivity over 25% but enhanced cell mass 120%, resulting in a 55% increase in total antibiotic production. Linear increases in both biomass and A10255 biosynthesis accompanied feed rate increases up to $\sim 3.5 \times 10^{-1}$ mg/ml/hour. Higher feed rates increased growth only slightly while sharply curtailing both the total antibiotic level and specific biosynthesis.

Effect of Continuous Lipid Feeds

Feeding studies with putative precursors of the acyl side chains of A54145, the lipopeptide antibiotic complex produced by *S. fradiae*, demonstrated that supplementing the glucose feed with $C_{10} \sim C_{18}$ lipids increased total antibiotic biosynthesis by 36~107%[14]. Although A10255 does not contain an acyl side chain potentially precursable by lipids, higher glucose feed rates had been shown to promote the growth of *S. gardneri* while reducing specific antibiotic productivity. Therefore, supplementary lipid feeds were examined as a technique for supplying the culture additional carbon while circumventing the known inhibition by higher levels of glucose. Respiration quotient (RQ) data indicated lipids were not oxidized by *S. gardneri* during the first day of fermentation when glucose was present. Consequently, lipid feeds were not initiated until approximately 24 hours postinoculation. Because some alkyl compounds are extremely toxic[14], feeds were initiated at the rate of $2 \sim 3 \times 10^{-1}$ μmol/ml/hour and increased to $5 \sim 6 \times 10^{-1}$ μmol/ml/hour when respiration data confirmed metabolism of the compound, usually prior to 42 hours. The effect of lipids and related compounds on production of A10255 is shown in Table 2.

Table 2. Effect of lipids and related compounds on A10255 production by *S. gardneri*.

Supplementary substrate	RQ[b]		A10255 titer (%)	Supplementary substrate	RQ[b]		A10255 titer (%)
	Calculated	Observed			Calculated	Observed	
None[a]	1.0	0.97~1.0	100[c]	Undecanoate[e]	0.76	0.89	201
Glycerol	0.85	0.91	168	Undecylenate	0.71	0.90	184
Acetate[d]	1.0	0.98	94	Myristate[e]	0.70	0.91	186
Propionate[d]	0.88	0.94	133	Oleate[e]	0.70	0.87	163
Butyrate	0.80	0.94	109	Decane	0.65	0.96	81
Hexanoate	0.75	0.89	177	Decyl aldehyde	0.69	0.88	119
Caprylate	0.73	0.88	207	Decyl alcohol	0.67	0.84	161
Caprate[e]	0.71	0.88	229				

[a] Medium SYN-1.
[b] Respiration quotient. Calculated value is based on complete oxidation when serving as the sole carbon source. Observed value is mean nominal value.
[c] 780 μg/ml. The maximum level obtained by feeding glucose at the higher, optimum, level of 3.48×10^{-1} mg/ml/hour without supplementary lipid was 170%.
[d] Sodium salt.
[e] Methyl or ethyl ester.

Except for acetate, whose RQ cannot be distinguished from the RQ of glucose because their calculated values are identical, respiration data for each compound tested indicated co-metabolism with glucose. Every compound except acetate, butyrate and decane increased the synthesis of A10255. However, only glycerol and $C_6 \sim C_{18}$ compounds produced increases of a magnitude equaling or exceeding those obtained by feeding glucose at the optimum rate in the absence of lipid. $C_8 \sim C_{12}$ compounds were superior.

The time-course profiles of A10255 biosynthesis shown in Fig. 5 demonstrate the enhancement of antibiotic production by the supplementary feeds, HyCase Amino (medium SYN-1) and HyCase Amino plus methyl caprate (medium SYN-2). In the latter medium, the continuous supply of nutrients supported linear growth of *S. gardneri* during the entire fermentation time-course with antibiotic biosynthesis paralleling biomass after the initial 17 hours (Fig. 5). A similar parallel between growth and biosynthesis occurred in the SYN medium (Fig. 1), but neither growth nor synthesis was sustained in the absence of the additional nutrients.

The apparent glucose catabolite regulation resulting in reduced A10255 yields could be avoided by feeding glucose continuously, but only if the feed rate did not exceed 4×10^{-1} mg/ml/hour. Higher feed

Fig. 5. Time-course profile of A10255 titers produced by modification of medium SYN.

● SYN-2, ▲ SYN-1, □ SYN.

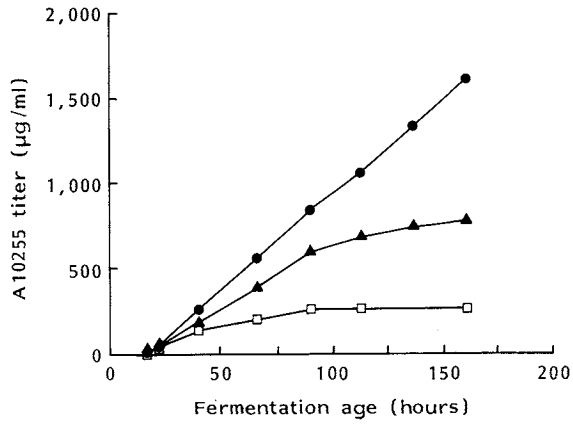


Fig. 6. Time-course profiles of *S. gardneri* biomass and A10255 titers in medium SYN-2.

□ A10255, ▲ biomass.

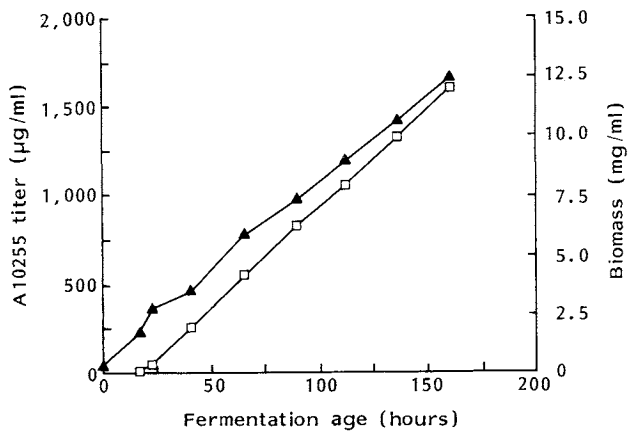


Table 3. Effect of supplementary carbon feeds on growth of *S. gardneri* and biosynthesis of A10255.

Supplementary feed	Biomass		A10255 (μg/ml)	A10255 synthesized per unit biomass	
	(mg/ml)	% Change		(μg/ml)	% Change
None[a]	12.2[b]		780[b]	64	
Glucose[c]	17.0	+39	1,320	78	+ 22
Caprylate[d]	12.8	+ 5	1,610	126	+ 97
Caprate[d]	12.5	+ 2	1,780	143	+123
Decyl alcohol[d]	13.3	+ 9	1,590	120	+ 88

[a] Medium SYN-1.
[b] 161 hours data.
[c] 1.275×10^{-1} mg/ml/hour in addition to the standard feed of 2.2×10^{-1} mg/ml/hour.
[d] Initiated at ~24 hours at $2 \sim 3 \times 10^{-1}$ μmol/ml/hour, increased to $5 \sim 6 \times 10^{-1}$ μmol/ml/hour at ~42 hours.

rates did not increase biomass appreciably but, although still metabolically limiting, substantially reduced antibiotic biosynthesis per unit of biomass. The yield increases obtained by increasing the glucose feed rate from the initial rate to the optimum rate were clearly produced by a different mechanism than those achieved with the non-carbohydrate feeds. An optimized glucose feed increased biomass 39% over the standard glucose feed rate while increasing specific antibiotic productivity 22% (Table 3). Caprate, caprylate and decyl alcohol each increased growth <10% while increasing specific antibiotic productivity 123%, 97% and 88%, respectively. Therefore, the enhancement attributable to additional glucose resulted principally from increased cell mass while the enhancement attributable to the lipid and alcohol feeds resulted from increased biosynthesis per unit of biomass.

Acknowledgment

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References

1) MICHEL, K. H.; M. M. HOEHN, L. D. BOECK, J. W. MARTIN, M. A. ABBOTT, O. W. GODFREY & F. P. MERTZ: A10255, a new thiopeptide antibiotic complex. 1. Discovery, fermentation, isolation, and characterization. Program and Abstracts of the 29th Intersci. Conf. on Antimicrob. Agents Chemother., No. 409, p. 169, Houston, Sept. 17~20, 1989
2) DEBONO, M.; R. M. MOLLOY, J. L. OCCOLOWITZ, J. W. PASCHAL, K. H. MICHEL & J. W. MARTIN: A10255, a new thiopeptide antibiotic complex produced by *Streptomyces gardneri*. 2. Structure determination of A10255B. Program and Abstracts of the 29th Intersci. Conf. on Antimicrob. Agents Chemother., No. 410, p. 169, Houston, Sept. 17~20, 1989
3) MOCEK, U.; J. M. BEALE & H. G. FLOSS: Reexamination of the ^{1}H and ^{13}C NMR spectral assignments of thiostrepton. J. Antibiotics 42: 1649~1652, 1989
4) MOCEK, U.; L.-C. CHEN, P. J. KELLER, D. R. HOUCK, J. M. BEALE & H. G. FLOSS: ^{1}H and ^{13}C NMR assignments of the thiopeptide antibiotic nosiheptide. J. Antibiotics 42: 1643~1648, 1989
5) ABE, H.; K. KUSHIDA, Y. SHIOBARA & M. KODAMA: The structure of sulfomycin I and berninamycin A. Tetrahedron Lett. 29: 1401~1404, 1988
6) MATSUMOTO, M.; Y. KAWAMURA, Y. YASUDA, T. TANIMOTO, K. MATSUMOTO, T. YOSHIDA & J. SHOJI: Isolation and characterization of thioxamycin. J. Antibiotics 42: 1465~1469, 1989
7) RINEHART, K. L.: Biosynthetic studies of antibiotics. *In* Chemistry of Natural Products. *Ed.*, Y. WANG, pp. 62~70, Gordon and Breach Science Publishers, Inc., 1982
8) BOECK, L. D.; D. M. BERRY, F. P. MERTZ & R. W. WETZEL: A10255, a complex of novel growth-promoting thiopeptide antibiotics produced by a strain of *Streptomyces gardneri*. Taxonomy and fermentation studies. J. Antibiotics 45: 1222~1230, 1992

9) BOECK, L. D.; R. W. WETZEL, S. C. BURT, F. M. HUBER, G. L. FOWLER & J. S. ALFORD, Jr.: Sterilization of bioreactor media on the basis of computer-calculated thermal input designated as F_0. J. Ind. Microbiol. 3: 305~310, 1988

10) LAM, K. S.; J. MATTEI & S. FORENZA: Carbon catabolite regulation of rebeccamycin production in *Saccharothrix aerocolonigenes*. J. Ind. Microbiol. 4: 105~108, 1989

11) ZHANG, Y.; S. WOLFE & A. L. DEMAIN: Carbon source regulation of ACV-synthetase in *Cephalosporium acremonium* C-10—cephalosporin and adicillin biosynthesis by *Acremonium chrysogenum*; effect of C-source. Curr. Microbiol. 18: 361~367, 1989

12) IKEDA, H.; H. KOTAKI, H. TANAKA & S. ŌMURA: Involvement of glucose catabolism in avermectin production by *Streptomyces avermitilis*. Antimicrob. Agents Chemother. 32: 282~284, 1988

13) BOECK, L. D.; H. R. PAPISKA, R. W. WETZEL, J. S. MYNDERSE, D. S. FUKUDA, F. P. MERTZ & D. M. BERRY: A54145, a new lipopeptide antibiotic complex: Discovery, taxonomy, fermentation and HPLC. J. Antibiotics 43: 587~593, 1990

14) BOECK, L. D. &. R. W. WETZEL: A54145, a new lipopeptide antibiotic complex: Factor control through precursor directed biosynthesis. J. Antibiotics 43: 607~615, 1990