EURYSTATINS A AND B, NEW PROLYL ENDOPEPTIDASE INHIBITORS

III. FERMENTATION AND CONTROLLED BIOSYNTHESIS OF EURYSTATIN ANALOGS BY *Streptomyces eurythermus*

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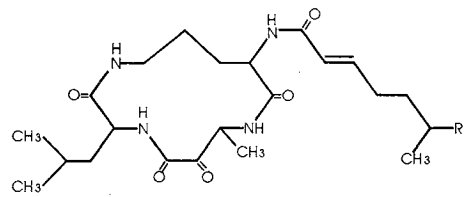
Accurate and precise component analysis of eurystatin analogs in fermentation broth was devised by HPLC methods with and without 2,4-dinitrophenylhydrazonation. Detailed optimization of fermentation conditions and strain improvement by HPLC analysis significantly increased the eurystatin productivity of *Streptomyces eurythermus*. Chemically defined fermentation media which produced eurystatins A and B at fermentation yields comparable to complex media were elaborated for radio-isotope fermentation studies and controlled biosynthesis. Radio-isotope incorporation study using ^{14}C-labeled amino acids in chemically defined medium demonstrated that L-leucine and L-ornithine were the direct precursors for the L-leucine and L-ornithine moieties of eurystatins A and B, respectively. Based on this finding, L-valine and L-isoleucine were supplemented to the growing culture of *S. eurythermus* in chemically defined medium, which resulted in the controlled biosynthesis of new eurystatin analogs named eurystatins C, D, E and F.

As described in previous papers,[1,2] eurystatins A and B (Fig. 1), new prolyl endopeptidase (PED) inhibitors, are acyl peptides whose PED-inhibitory activity depends on the β-amino-α-keto group. With this interesting inhibitory functional group kept intact, several chemical and biological approaches have been attempted for derivation of more specific and potent inhibitors against a variety of enzymes, but to date without success. In the continued series of biological approaches, assumed that these peptide antibiotics were produced by the so-called thiotemplate elongation mechanism,[3] the current authors considered it highly probable and worth testing to purposefully produce new eurystatin analogs by controlled fermentation of *Streptomyces eurythermus* R353-21.

Before detailed fermentation studies, it was essential to devise more reliable analyses of eurystatin analogs for identification and quantitation of eurystatin components in fermentation broth. The PED inhibition assay has been proved very specific and sensitive for qualitative analysis of eurystatins A and B, but clearly found to be least reliable for quantitative component analysis of eurystatins A and B in the fermentation time course study of the streptomycete. After a variety of analytical methods were evaluated, HPLC methods with and without

Fig. 1. Structures of eurystatins A and B.



Eurystatin A R=CH_3
B R=C_2H_5

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dinitrophenylhydrazonation were concluded to be currently most suitable and convenient for quantitative component analysis of eurystatins A and B in fermentation broth. By combination of the single spore isolation technique with media studies, the fermentation conditions of *S. eurythermus* R353-21 B121 were optimized in complex as well as chemically defined media for large-scale production and controlled fermentation of eurystatin analogs.

The radio-isotope incorporation study in chemically defined medium revealed that L-leucine and L-ornithine were the precursor amino acids for the L-leucine and L-ornithine moieties of eurystatins A and B. It was therefore reasonable to expect that exogenous supplementation of a leucine-related amino acid such as valine, isoleucine or norleucine, for example, might result in at least partial replacement of the endogenous pool of leucine, leading to production of new eurystatin analogs.

The current paper describes the quantitative HPLC analysis of eurystatin analogs, fermentation optimization and controlled biosynthesis of new eurystatin analogs.

Materials and Methods

Streptomycete and Seed Culture

Streptomyces eurythermus R353-21 was subjected to repeated single spore isolations for productivity improvement, yielding a high producer strain numbered B121 which was employed throughout the current studies.

One loopful of mature spores of the strain were inoculated into a 500-ml Erlenmeyer flask containing 100 ml of seed medium (2% soluble starch, 0.5% glucose, 0.3% NZ-case, 0.2% yeast extract, 0.5% fish extract D30X (Banyu Nutrient Co.) and 0.3% $CaCO_3$, pH 7.0), and cultivated at 28°C for 3 days on a rotary shaker (throw 5 cm; rotation 200 rpm).

Radio-chemicals

The following uniformly ^{14}C-labeled amino acids were purchased from Dai-Ichi Pure Chemicals Co., Ltd., Japan: L-alanine (171.7 mCi/mmol), γ-aminobutyric acid (150 mCi/mmol), L-arginine (339.4 mCi/mmol), L-aspartic acid (224.8 mCi/mmol), L-glutamic acid (281.4 mCi/mmol), glycine (110.5 mCi/mmol), L-leucine (368 mCi/mmol), L-ornithine (257.2 mCi/mmol) and L-valine (272.8 mCi/mmol).

Quantitative Analyses of Eurystatins A and B in Fermentation Broth

Culture broth (3 ml) of strain B121 was vigorously mixed with 3 ml of *n*-BuOH for 20 minutes. The organic layer was collected by centrifugation and evaporated to dryness. The evaporation residue was taken in 3 ml of 75% acetonitrile and subjected to quantitative analyses as follows:

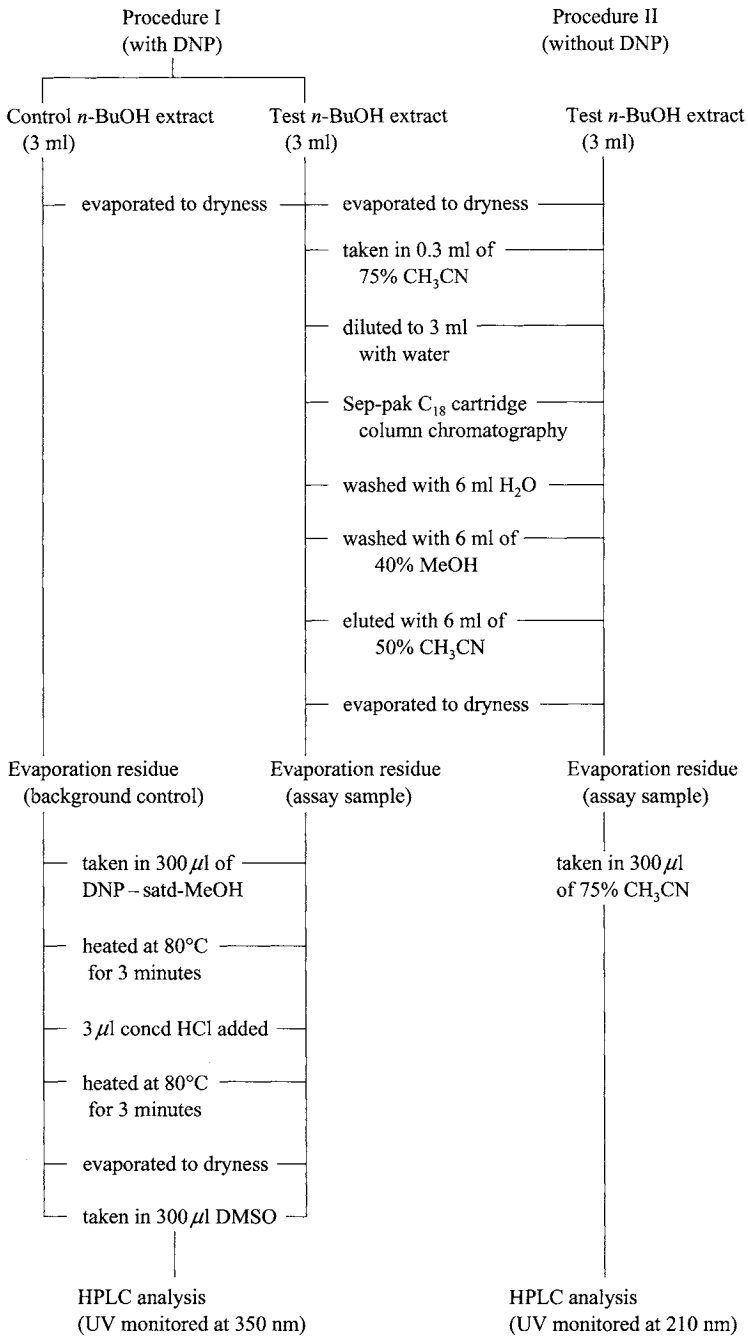
(1) PED Inhibition Assay Analytical conditions for the PED inhibition assay were described in a previous paper.[1] The PED inhibition assay was standardized by using eurystatin A as reference compound. The total amount of eurystatins A and B in broth is expressed in eurystatin A equivalent. This qualitative enzyme inhibition assay, however, was used without component separation and accordingly was semi-quantitative, as eurystatins A and B differ in the specific PED inhibitory activity.

(2) HPLC Analyses

(2-a) HPLC analysis with 2,4-dinitrophenylhydrazonation (Scheme 1, Procedure I). For satisfactory component separation as well as improved analytical accuracy and precision, a mixture of eurystatins A and B was labeled with 2,4-dinitrophenylhydrazine (DNP) before HPLC. More particularly, the *n*-BuOH extract of eurystatins A and B in 75% acetonitrile was purified by quick column chromatography and then 2,4-dinitrophenylhydrazonated as summarized in Scheme 1 (assay sample) for improvement of component separability and assay accuracy and precision.

As background control, the same procedure with eurystatin-deleted broth was simultaneously run for monitoring nonspecific background absorption and, if necessary, for compensation of the HPLC chromatogram of DNP-eurystatins A and B (Scheme 1, Procedure I, background control).

Scheme 1. Quantitative eurystatin component analysis.



Analytical HPLC conditions employed for DNP-eurystatins A and B were as follows: YOKOGAWA LC100 HPLC System; Excelpak SIL-C18 5B column (YOKOGAWA, 4.6 i.d. × 150 mm); mobile phase 65% acetonitrile in 22 mM phosphate buffer, pH 7.0; flow rate 1.0 ml/minute; UV monitoring at 350 nm.

(2-b) Direct HPLC analysis without 2,4-dinitrophenylhydrazonation (Scheme 1, Procedure II) In some cases where the component quantitation was urgently required at a fermentation level above 1 μg/ml, the quickly column chromatographed eurystatins A and B sample skipped 2,4-dinitrophenylhydrazonation

and was directly submitted to HPLC using 60% MeOH in 22 mM phosphate buffer, pH 7.0, as eluent with a UV-monitor at 210 nm. Under such conditions, eurystatins A and B showed retention times of 11.8 and 18.8 minutes, respectively (Fig. 4).

Preparation of Eurystatin A or B 2,4-Dinitrophenylhydrazone as Analytical Standard

Eurystatin A (5.5 mg) was mixed with 5 ml of 2,4-dinitrophenylhydrazine-saturated MeOH and heated at 80°C for 3 minutes in a sealed tube. After a drop of concd HCl was added, the content was heated at 80°C for a further 3 minutes. On standing at room temperature, the reaction mixture produced pure eurystatin A 2,4-dinitrophenylhydrazone crystals. Yield 4.0 mg; MP 270°C (dec); IR (KBr) 3300, 1660, 1640, 1620, 1520, 1430, 1340, 1140, 840 cm^{-1}; UV max ($E^{1\%}_{1cm}$) (dissolved in 0.1 ml of DMSO and then diluted to 3.0 ml with MeOH) 350 nm (320); FAB-MS (pos.) *m/z* 631 $(M+H)^+$; HPLC (described above) Rt = 7.9 minutes.

Eurystatin B 2,4-dinitrophenylhydrazone was similarly prepared. MP 270°C (dec); IR (KBr) 3300, 1660, 1640, 1620, 1520, 1430, 1340, 1140, 840 cm^{-1}; UV max ($E^{1\%}_{1cm}$) (in MeOH containing a small amount of DMSO) 350 (314); FAB-MS (pos.) *m/z* 645 $(M+H)^+$; HPLC (described above) Rt = 10.3 minutes.

Media Studies

(1) Complex Media Table 1 summarizes the compositions of representative complex production media studied in the current paper. Each production medium (100 ml) was inoculated by 5 ml of the seed culture.

(2) Chemically Defined Media For cancellation of possibly significant effects of residual nutrients in the seed culture, the mycelia were recovered by centrifugation, washed twice in sterile saline and resuspended in the original volume of the culture of saline. The washed mycelium suspension (5 ml) was transferred into a 500 ml Erlenmeyer flask containing 100 ml of chemically defined medium (for chemical

Table 1. Compositions of complex media.

Code No. Ingredient (%)	50R	12	48E	53	84b	101	117B	118C	122E	132	FR19
Soluble starch	3.0	2.0		3.0				2.0	2.0	2.0	4.0
Lactose				1.0							
Glucose					3.0		2.5				
Glycerol			3.0								
Mannitol						1.0					
Bacto-liver	1.0										
Beet molasses									1.0		
C.S.L.							1.5				
Corn meal						2.0					
Distillers' sol.			1.5				1.0				
Fish meal			1.0	1.0				1.0	1.0		1.0
Pharmamedia		1.0	1.0		0.5		0.5				
Soybean meal					3.0					3.0	
Tomato paste									2.0		
Polypeptone	0.5					0.5					
Yeast extract					0.1						
Polyglycol							0.25				
$CaCO_3$	0.6	0.4	0.6	0.6	0.3	0.3	0.3	0.5		1.0	0.6
$MgSO_4 \cdot 7H_2O$										0.33	
$CoCl_2 \cdot 6H_2O$							0.001		0.001		
NaCl						0.2					0.1
$(NH_4)_2SO_4$	0.1			0.1							
$ZnSO_4 \cdot 7H_2O$		0.003									
Titer (μg/ml)	0.6	1.1	<0.3	1.7	1.4	1.6	0.3	1.5	<0.3	<0.3	4.6

pH of medium adjusted to 7.0 prior to autoclaving.
C.S.L. = Corn Steep Liquor.

composition, refer to Table 2) and cultivated at 28°C and 200 rpm.

Incorporation of Radio-labeled Amino Acid Precursors into Eurystatins A and B

Four day-old culture of strain B121 in 10 ml of chemically defined medium MGAT was pre-incubated with 10 μg/ml of a cold test amino acid for 60 minutes at 30°C, in expectation that the incorporation efficiency of that radio-active amino acid might be improved. The radio-labeled amino acid was then supplemented to the culture at a radio-activity of 0.2 μCi/ml and incubated for a further 22 hours. Eurystatins A and B were extracted with 10 ml *n*-BuOH, and the *n*-BuOH extract was concentrated to dryness under reduced pressure. The evaporation residue was taken in 1 ml of 75% CH_3CN containing 100 μg eurystatin A. A 100 μl aliquot of the solution was applied on a preparative silica gel 60 F_{254} TLC sheet (HPTLC-Alufolien Kieselgel 60 F_{254}, 20×20 cm, 0.2 mm thick; E. Merck Co.) and developed with a solvent system of CH_2Cl_2-MeOH-EtOAc (10:1:3 in volume; Rf=0.35 for eurystatins A and B). In a separate run, all the amino acids tested were found to remain at the origin of the TLC sheet under the same analytical conditions. Each chromatographic lane was horizontally devided into 1 cm-high bands and each band was scraped off from the TLC sheet for radioactivity measurement in an ALOKA LSC-700 liquid scintillation counter using NEN Aquasol-2 as scintillator.

Hydrolysis of Radioactive Eurystatins A and B

Radioactive eurystatins A and B samples were developed on preparative silica gel TLC sheets as described above, and the eurystatins A and B fractions were recovered from the silica gels with a 10:3 mixture of CH_2Cl_2 and MeOH. After the solvent was removed by evaporation, the radioactive eurystatins A and B mixture was dissolved in 200 μl of MeOH and divided into two halves.

For measurement of the radioactivities of the L-leucine and L-ornithine moieties in eurystatins A and B, one half of the radioactive eurystatins A and B mixture was transferred in a tube and hydrolysed at 110~120°C for 17 hours in a mixture of 1 ml 6N HCl and 0.4 ml 1,4-dioxane.[2]

As β-amino-α-ketobutyric acid easily decomposes in 6N HCl, the other half of the radioactive eurystatins A and B mixture was first reduced with $NaBH_4$ and then subjected to acid hydrolysis under the same conditions as stated above. Radioactivity in the β-amino-α-ketobutyric acid fraction, if any, is detectable as β-amino-α-hydroxybutyric acid (AHBA).

The acid hydrolysate was extracted four times with 2 ml each of ethyl ether so that lipophilic products might be completely removed. For easy location by ninhydrin visualization, cold marker amino acids such as L-leucine, L-ornithine, γ-amino-α-hydroxybutyric acid, alanine, arginine, glutamic acid, glycine and valine were added to the hydrolysate. Amino acids in the acid hydrolysate were developed by two-dimensional silica gel TLC (HPTLC-Alufolien Kieselgel 60 F_{254}; solvent systems CH_2Cl_2-MeOH-17% NH_4OH, 4:4:2 in volume for the first dimension and phenol-water, 4:1 in volume for the second dimension); visualized with 0.2 M ninhydrin in MeOH; and scraped off from the TLC sheet for radioactivity measurement.

Results and Discussion

Quantitative HPLC Analyses of Eurystatin Analogs

As the fermentation titer of the eurystatin producer was initially very low (<1 μg/ml), and as several eurystatin components were supposed to be produced, more accurate and precise analyses other than the PED inhibition assay were prerequisite for fermentation improvement and biosynthetic studies. After several physico-chemical methods such as silica gel and cellulose TLC were preliminarily tested, HPLC was concluded to be most appropriate for quick and reproducible component analysis of fermentation broths.

(1) HPLC Analysis with 2,4-Dinitrophenylhydrazonation

As the fermentation titer of the streptomycete after isolation from a soil sample was far below 1 μg/ml, the minimum assay precision for each eurystatin component was assumed to be required as low as 0.01 μg/ml,

provided that eurystatin analogs were well separated into individual components. Accordingly, both assay sensitivity improvement and component separation were attempted by labeling the keto group of eurystatin analogs with 2,4-dinitrophenylhydrazine. Using 2,4-dinitrophenylhydrazonated eurystatins A and B, HPLC analysis conditions were improved in details, resulting in establishment of the routine analysis conditions as described in the Materials and Method section. In comparison with the PED inhibition assay and the below-described direct HPLC, this HPLC analysis with 2,4-dinitrophenylhydrazonation showed a more than 10 times higher assay sensitivity, when chemically pure eurystatin A or B was employed. In addition, 2,4-dinitrophenylhydrazonation served to improve HPLC separability of eurystatin components. Practical use of 2,4-dinitrophenylhydrazonation, however, depends on urgency of the assay, the required assay accuracy and precision, the fermentation level of eurystatin analogs and the assay reproducibility and reliability, as such pretreatment is time-consuming and laborious and the assay accuracy is counterbalanced to a more or less extent by background yellowing associated with 2,4-dinitrophenylhydrazonation.

(2) HPLC Analysis without 2,4-Dinitrophenylhydrazonation

Particularly when a variety of complex media were comparatively studied, the assay accuracy and reliability of the HPLC analysis with 2,4-dinitrophenylhydrazonation was found to decrease to a more or less extent on account of nonspecific background yellowing presumably by keto compounds other than eurystatins A and B. In addition, the time course study of eurystatin fermentation often required quick assays which allowed no time for 2,4-dinitrophenylhydrazonation. In practice, although the UV absorption at 210 nm is nonspecific, the HPLC analysis of eurystatins A and B above 1 μg/ml was generally shown to be more convenient without 2,4-dinitrophenylhydrazonation than with 2,4-dinitrophenylhydrazonation, as the former procedure requires fewer steps of treatment than the latter.

Fermentation Studies

(1) Fermentation Conditions

Using complex medium 50R defined in a previous paper,[1] fermentation conditions were optimized for strain B121. As a result, the strain was concluded to be best fermented at pH 7.0 and 28°C for 4 days in a medium volume of 100 ml (data not shown).

(2) Complex Media

As the fermentation titer of the parent strain (R35-21) was initially far below 1 μg/ml in medium 50R, complex media were optimized for carbon, nitrogen and mineral sources for large-scale fermentation. Compositions of representative complex media are summarized in Table 1. In conclusion, the productivity of the streptomycete is improved to 5 μg/ml by replacing medium 50R with medium FR19.

To medium FR19, possible amino acid precursors such as leucine, ornithine, alanine, threonine and isoleucine were supplemented at concentrations of 0.1 and 0.3%, but without significant increase in the fermentation titer. Detailed time course analysis suggested a strong likelihood that supplementation of such precursor amino acids might have little effect on the intracellular pools of related amino acids (data not shown), as far as complex media were employed.

(3) Chemically Defined Media

For biosynthetic studies, chemically defined media seemed more preferable than complex media,

because the streptomycete possessed a comparatively poor fermentation productivity even in complex media which was assumed to make it difficult to accurately and precisely analyze possible effects of additives on eurystatin analogs. Using medium SM-1 as base medium, carbon, nitrogen and mineral sources were optimized. As a result, medium MGAT which contained 2% mannitol, 1% γ-aminobutyric acid, 0.3% L-arginine, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.04% $MgSO_4 \cdot 7H_2O$, 0.03% $CaCl_2 \cdot 2H_2O$, 0.0004% $FeSO_4 \cdot 7H_2O$, 0.0004% $MnCl_2 \cdot 4H_2O$, and 0.0004% $ZnSO_4 \cdot 7H_2O$ in 0.1 M TES buffer, pH 7.2, was established for biosynthetic studies.

Time course of eurystatins A and B fermentation by *S. eurythermus* R-353-21 B121 in medium MGAT was followed by the PED inhibition assay as well as the direct HPLC analysis and is illustrated in Fig.

Table 2. Compositions of chemically defined media.

Code No. Ingredient (%)	SM1*	SM2*	SM3*	SM5	MGT	MGAT
Glucose	4.0	4.0	4.0	2.0		
Mannitol					2.0	2.0
GABA[a]			1.0	1.0	1.0	1.0
L-Arginine		0.3	0.3	0.3		0.3
$(NH_4)_2SO_4$	0.1	0.1	0.1	0.1	0.1	0.1
$(NH_4)_2HPO_4$	0.4	0.4				
$NH_4H_2HPO_4$	0.3	0.3				
KH_2PO_4	0.1	0.1	0.1	0.1	0.1	0.1
$CaCl_2 \cdot 2H_2O$				0.03	0.03	0.03
$MgSO_4 \cdot 7H_2O$	0.04	0.04	0.04	0.04	0.04	0.04
$CaCO_3$	0.6	0.6	0.6			
Mineral soln.[b]	4.0	4.0	4.0	4.0	4.0	4.0
1M TES, pH 7.2[c]				10	10	10
Titer (μg/ml)	2.6	3.4	3.0	2.9	2.6	4.8

* pH of medium adjusted to 7.0 prior to autoclaving.
[a] γ-Amino-butyric acid.
[b] 0.01% $FeSO_4 \cdot 7H_2O$ + 0.01% $MnCl_2 \cdot 4H_2O$ + 0.01% $ZnSO_4 \cdot 7H_2O$; added in volume percent.
[c] added in volume percent.

Fig. 2. Time course of eurystatin fermentation in medium MGAT.

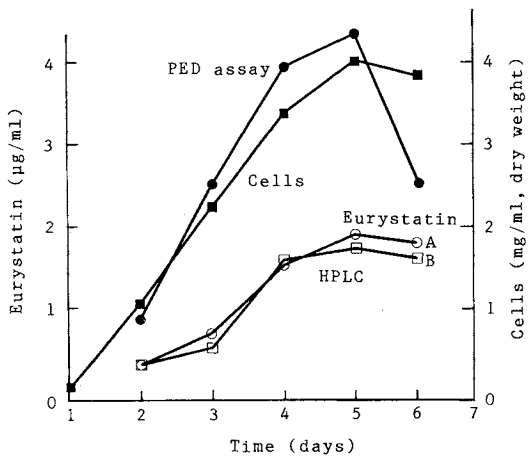


Table 3. Effects of 0.1% amino acids on eurystatins A and B production in chemically defined medium MGAT.

Amino acid	Titer (μg/ml)*	% increase
None (control)	3.2	+/−0
L-Alanine	4.4	+ 37
AHBA[a]	7.2	+125
L-Arginine	5.9	+ 84
L-Aspartic acid	5.4	+ 68
L-Glutamic acid	5.6	+ 75
Glycine	4.4	+ 37
L-Isoleucine	3.4	+ 6
L-Leucine	6.7	+109
L-Ornithine	4.1	+ 28
L-Proline	2.5	− 22
L-Threonine	1.8	− 44
L-Valine	2.4	− 25

* Fermentation titer was determined on day 8 by the PED inhibition assay.
[a] β-Amino-α-hydroxy-butyric acid.

2. It is probable that eurystatins A and B which differ only in the acyl side chain (see Fig. 1) are simultaneously produced in equimolar fashion over the fermentation time course.

Using medium MGAT, effects of exogenously added amino acids were examined at 0.1% on the total fermentation titer of eurystatins A and B. Results in Table 3 show that, unlike complex medium FR19, chemically defined medium MGAT evidently responds to the addition of exogenous amino acids, among which β-amino-α-hydroxybutyric acid, L-leucine, L-arginine, L-glutamic acid and L-aspartic acid seem worth further study. Although not yet examined in details, it is likely that β-amino-α-hydroxybutyric acid is the direct precursor for the β-amino-α-ketobutyric acid moiety.

Like other antibiotic-producing streptomycetes, *S. eurythermus* favorably responded to L-arginine in a fairly wide range of concentrations from 0.025 to 2%, presumably through growth stimulation (data not shown), where the production of eurystatins A and B increased depending on the arginine concentration upto 1%.

Incorporation and Intramolecular Distribution of Radioactive Amino Acids in Eurystatins A and B

Because the controlled biosynthesis of peptide antibiotics depends on the competitive incorporation between endogenous precursor amino acids and exogenously added amino acids, it was essential to determine direct precursor amino acids for eurystatins A and B, if possible. As described above, fermentation studies using cold amino acids and complex media provided no useful information about the direct precursor amino acids for eurystatins A and B. It seemed probable, however, that, as the intracellular pools of direct precursor amino acids in the streptomycete might be large enough to supply their demands for production of eurystatins A and B at a level as low as 1 μg/ml, significant effects of exogenously added amino acids were hardly observed in complex media. If this assumption were correct, supplementation of possibly direct precursor amino acids to the culture of strain B121 in chemically defined medium was considered to be worth examination. Table 4 summarizes the incorporation rates of exogenously added radioactive amino acids into the TLC fractions of eurystatins A and B, which indicates that alanine,

Table 4. Incorporation rates of radioactive amino acids into eurystatins A and B.

Amino acid	Incorporation (%)
U-^{14}C-L-Alanine	0.46
U-^{14}C-L-Arginine	1.02
U-^{14}C-L-Aspartic acid	0.13
U-^{14}C-γ-Aminobutyrate	0.17
U-^{14}C-L-Glutamic acid	0.13
U-^{14}C-Glycine	0.11
U-^{14}C-L-Leucine	2.80
U-^{14}C-L-Ornithine	3.34
U-^{14}C-Valine	0.25

Table 5. Radioactivity distribution in the constituent amino acids of eurystatins A and B.

^{14}C-Amino acid	Constituent amino acid (dpm)			
	AHBA*	Ornithine	Leucine	Valine
None (control)	14	22	26	
L-Alanine	201	66	52	
L-Arginine	44	674	30	
L-Glutamic acid	38	66	50	
L-Leucine	57	34	1,056	
L-Ornithine	58	1,349	40	
L-Valine	105	78	123	113

* β-Amino-α-hydroxy-butyric acid.

arginine, leucine, ornithine and valine are worth further analysis for their intramolecular distribution.

Results in Table 5 show the distribution of radioactivity among the constituent amino acids of eurystatins A and B. It is obvious that L-leucine and L-ornithine are incorporated into the L-leucine and L-ornithine moieties, respectively (Fig. 3). L-Arginine, after deguanidylation, seems to serve as ornithine source. L-Alanine is most likely to be incorporated into the β-amino-α-ketobutyric acid moiety. L-Valine, which was expected to give new analogs by competition with L-leucine, suggests the possibility that L-valine not only replaces L-leucine but is also converted to L-leucine.

Controlled Biosynthesis of New Eurystatin Analogs by Supplementation of L-Valine and L-Isoleucine

As the radio-isotope experiment described above suggested the possible formation of new eurystatin congeners, 0.1% L-valine was supplemented to chemically defined medium SM-2 and fermented. The PED inhibitory fraction of the culture was recovered by *n*-BuOH extraction and analysed by HPLC without 2,4-dinitrophenylhydrazonation. The assay results of standard eurystatins A and B and the L-valine-fed batch in Fig. 4 clearly indicate the presence of two new eurystatin analogs designated eurystatins C and

Fig. 3. Incorporation of radio-active L-leucine, L-ornithine and L-alanine into eurystatins A and B.

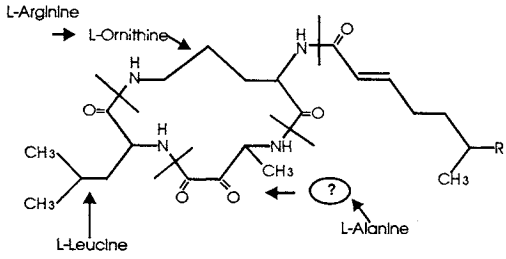


Fig. 4. HPLC chromatograms of eurystatins A, B, C and D.

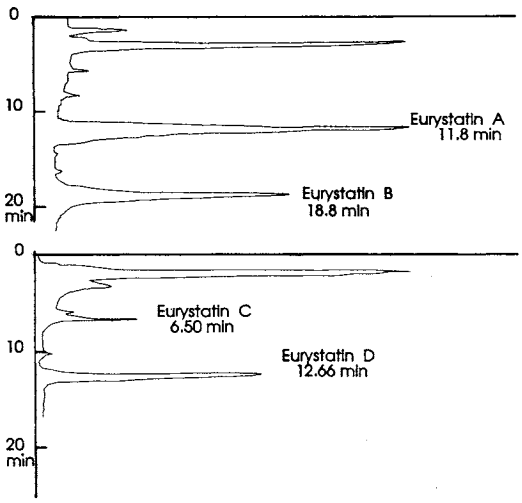
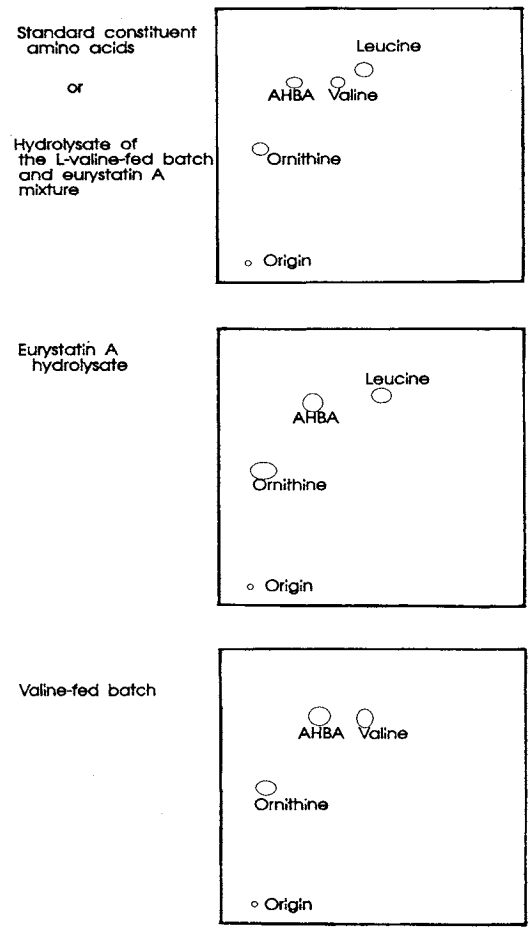
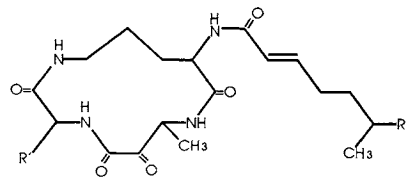


Fig. 5. Silica gel TLC analysis of the L-valine-fed batch.



D at retention times of 6.50 and 12.66 minutes, respectively. The PED inhibition assay of the HPLC eluate fractions demonstrated that both of the new peaks had PED-inhibitory activities comparable to eurystatins A and B. Under the fermentation conditions employed, eurystatins A and B seem to be completely replaced by the new analogs presumably through high preference of L-valine to L-leucine (Figs. 4 and 5). Identification of valine without leucine in the acid hydrolysate reveals that eurystatins C and D probably differed only in the acyl side chain, as is the case in eurystains A and B.

Fig. 6. Proposed structures of eurystatins C, D, E and F.



	R	R'
Eurystatin C	$-CH_3$	$-CH(CH_3)_2$
D	$-C_2H_5$	$-CH(CH_3)_2$
E	$-CH_3$	$-CH(CH_3)C_2H_5$
F	$-C_2H_5$	$-CH(CH_3)C_2H_5$

Supplementation of L-isoleucine to chemically defined medium SM-2 similarly resulted in formation of new congeners designated eurystatins E and F, which showed retention times of 7.4 minutes and 16.3 minutes, respectively, by HPLC without 2,4-dinitrophenylhydrazonation. Complete replacement of eurystatins A and B by eurystatins E and F was again observed, as is the case in eurystatins C and D.

Chemical structures of the new eurystatin analogs are proposed in Fig. 6.

Although not yet studied, L-norvaline, L-norleucine and L-aminobutyric acid are assumed to give corresponding analogs by replacement of the intracellular L-leucine pool.

To date, no replacement of the L-ornithine and β-amino-α-ketobutyric acid moieties has yet been experimentally observed.

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