New Cineromycins and Musacins Obtained by Metabolite Pattern Analysis of *Streptomyces griseoviridis* (FH-S 1832)

I. Taxonomy, Fermentation, Isolation and Biological Activity†

KATJA BURKHARDT and HANS-PETER FIEDLER*

Biologisches Institut, LB Mikrobiologie/Antibiotika, Universität Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany

SUSANNE GRABLEY and RALF THIERICKE*

Hans-Knöll-Institut für Naturstoff-Forschung e.V., Beutenbergstraße 11, D-07745 Jena, Germany

AXEL ZEECK

Institut für Organische Chemie, Universität Göttingen, Tammannstraße 2, D-37077 Göttingen, Germany

(Received for publication November 24, 1995)

Chemical screening using thin layer chromatography and various staining reagents offers the opportunity to visualize a nearly complete picture of a microbial secondary metabolite pattern (metabolic finger-print). This approach can be used advantageously for both, the detection of so-called "talented" strains, and for qualifying microbial strain collections, especially as a fundamental step of efficiently applied biological high-throughput assays. Based on their metabolic finger-print, microbial isolates can be classified in: (i) non-producing organisms, which gave no indication of the formation of secondary metabolites up to a defined detection limit, (ii) organisms of narrow productivity, which produce one or two secondary metabolites as main products with a restricted dependance to alteration of the culture conditions, and (iii) talented organisms, which are able to synthesize an array of structurally different secondary metabolites. As an example, the talented strain, *Streptomyces griseoviridis* (FH-S 1832), was studied in detail. Investigations in its taxonomical characterization, fermentation, as well as the isolation and purification procedures leading to 14-membered macrocyclic lactones of the cineromycin-type (cineromycin B and three new congeners) and to the musacins A to F are reported. Musacin C exhibits anthelminthic and weak antiviral activities.

In comparison to target-directed screening attempts the chemical screening strategy (reviewed by NAKAGAWA[2]) applied to culture concentrates of microorganisms was found to be an efficient supplemental and alternative method, especially with the aim to discover new secondary metabolites[3~6]. In order to apply chemical screening in a reproducible way, standardized procedures were used to prepare defined extracts (50-fold concentrated) from both, the culture filtrate (adsorption chromatography on Amberlite XAD-16; elution with methanol-water), and the mycelium (extraction with acetone)[5~7]. In our routine, the obtained concentrates were analyzed by thin-layer chromatography using different solvent systems [*e.g.* $CHCl_3$ - MeOH, 9 : 1, and 1-butanol - acetic acid - water, 4 : 1 : 5, upper phase] and visual detection under the support of UV-extinction (254 and 366 nm) and colorization reactions obtained by staining with different reagents (*e.g.* anisaldehyde-, orcinol-, blue tetrazolium and Ehrlich's reagent). This procedure mainly focuses on the chemical behaviour and reactivity of the natural products and renders a good visualization of the secondary metabolite pattern produced by each strain. It is worth highlighting the fact that defined classes of metabolites such as the agistatines[1], decarestrictines[5,8], waraterpols[7], oasomycins[6] or the gabosines[9] can easily be recognized by this method.

As a consequence, chemical screening resulted in a characteristic metabolic finger-print, which gives rise for a classification of the analyzed strains *via* the secondary metabolite pattern produced. Besides non-producing

† Art. No. 31 on secondary metabolites by chemical screening. Art. No. 30: see ref. 1.

organisms and strains with a narrow productivity, we observed organisms, which were able to synthesize a broad array of structurally diverse metabolites based on variations of both, culture media and cultivation conditions. ZÄHNER[10] has introduced the term "talented" to the latter sub-class of organisms, on which we focused our interest.

The present paper deals with the description and evaluation of our chemical screening strategy, the selection of so-called "talented" microorganisms with the example *Streptomyces griseoviridis*, FH-S 1832, taxonomical and fermentation investigations, as well as with the isolation and purification procedures leading to new secondary metabolites of the cineromycin and musacin types. Physico-chemical properties and structural elucidation studies of the new metabolites will be the subject of an accompanying paper[11].

Taxonomy

Strain FH-S 1832 was isolated from a soil sample collected near Aiu Musa, Egypt, according to common isolation procedures for streptomycetes strains and has been characterized by morphological and chemo-taxonomical methods [*e.g.* by the standards of the International Streptomycetes Project (ISP)[12,13]]. The isolate FH-S 1832 shows a light-brown substrate mycelium and a reddish-brown aerial mycelium. Scanning electron microscopy analysis presented helical spore chains and round ellipsoidal spores with a smooth surface ($1 \sim 1.4$ μm in diameter). When the strain was grown on calcium-caseinate-tyrosine agar and peptone-iron agar no melanine production was observed. Growth was not inhibited by concentrations up to 1% lysozyme and 5% NaCl, respectively. The cell wall contained L,L-diaminopimelic acid. In combination with physiological parameters[12~14], which are summarized in Table 1, the strain FH-S 1832 seems to be an isolate of *Streptomyces griseoviridis*. However, this has not been proven by DNA-hybridization techniques.

Table 1. Taxonomical characteristics of *Streptomyces griseoviridis* (strain FH-S 1832).

Melanoid formation	−
Hydrolysis of starch	−
Liquefaction of gelatine	+
Utilization of urea	−
Lysozyme tolerance	1%
NaCl tolerance	5%
LL-Diaminopimelic acid	+
Production of	
nitrate reductase	−
β-galactosidase	+
tryptophanase	−
catalase	+
cytochrome oxidase	+
H_2S	+
acetoin	−
Carbon utilization	
Glycerol	+
D-Xylose	−
D-Arabinose	−
D-Glucose	+
D-Mannose	−
D-Galactose	+
D-Fructose	+
L-Rhamnose	+
D-Mannitol	−
D-Sorbitol	+
i-Inositol	+
Maltose	−
Sucrose	+
Raffinose	+
Starch	−
Cellulose	−

Detection of Secondary Metabolites

In the primary screening routine[7,9] strain FH-S 1832 was cultivated in 300 ml Erlenmeyer flasks containing 100 ml of medium A (soybean meal/mannitol), medium B (oat flakes), and medium C (glycerol/casopeptone), respectively, at 30°C for 5 days. Concentrates of the mycelia and the culture filtrates, which were obtained by extraction with acetone and adsorption chromatography on XAD-16 resins, respectively, were analyzed *via* the chemical screening routine on TLC plates (detection limit: 0.1 μg/liter culture broth). This procedure resulted in the observation of a number of different colored spots typical for a so-called talented strain.

Fermentation and Isolation

In a 50-liter fermentor, *Streptomyces griseoviridis* (strain FH-S 1832) was cultivated with medium C (glycerol/casopeptone) at 30°C. The logarithmic growth phase extended from 3 to 70 hours after inoculation, and the cell mass reached its maximum after about 80 hours, resulting in a content of 6 g per liter culture. The maximal amount of DNA was obtained after a fermentation period of 160 hours, showing a value of 170 μg/ml. Glycerol was exhausted after 180 hours. During the initial 20 hours of incubation, the ammonium concentration increased from 18 mM to 47 mM and decreased to zero after 30 hours. The phosphate content showed a maximum value of 6.7 mM after 30 hours and was diminished to 1.5 mM after 190 hours of fermentation. During lysis of the cells, which was apparent after about 200 hours, phosphate and ammonium concentrations increased. On the basis of TLC analysis, time for harvesting was fixed at 120 hours of fermentation.

Scheme 1. Isolation and purification of the cineromycins and musacins.

Fermentation broth (50 liters)

Filtrate | Mycelium

MCI-gel chromatography
(elution with MeOH - H_2O, 4 : 1)

Oily crude material (59 g)

silica gel chromatography
(gradient: $CHCl_3$ - MeOH from 9 : 1 to 4 : 1)

Fraction 1 | Fraction 2 | Fraction 3 | Fraction 4 | Fraction 5

Fraction 1: silica gel chromatography (gradient: $CHCl_3$ - MeOH from 95 : 5 to 9 : 1); Sephadex LH-20 (MeOH); Sephadex LH-20 ($CHCl_3$)

Fraction 2: silica gel chromatography (EtOAc - *n*-hexane, 3 : 1); Sephadex LH-20 (MeOH)

Fraction 3: silica gel chromatography (gradient: EtOAc - MeOH from 99 : 1 to 95 : 5); silica gel chromatography (EtOAc); Sephadex LH-20 (MeOH)

Fraction 4: Sephadex LH-20 (MeOH)

Fraction 5: Sephadex LH-20 (MeOH, twice)

Dehydro-cineromycin B (3.4 mg/l) | Musacin D (3.0 mg/l) | Musacin E (0.4 mg/l) | Cineromycin B (18.3 mg/l) | 2,3-Dihydro-cineromycin B (8.3 mg/l) | Musacin F (1.0 mg/l) | Musacin B_1 (4.5 mg/l) | Oxycinero-mycin B (2 mg/l) | Musacin B_2 and C (50.7 mg/l) | Musacin A (4.0 mg/l)

Table 2. Rf values and color reactions of the cineromycins and the musacins.

Compound	Rf-value	Rf-value	Anisaldehyde-H_2SO_4	Orcinol reagent	Ehrlich's reagent	Blue tetrazolium reagent
Cineromycin B	0.59[c]	0.72[d]	Blue to grey	Brown	Blue	n.c.
Dehydrocineromycin B	0.80[c]	0.95[d]	Green	Orange	Pink	n.c.
Oxycineromycin B	0.18[c]	0.40[d]	Blue	Brown	Violet	n.c.
2,3-Dihydrocineromycin B	0.40[c]	0.72[d]	Blue	Brown	Blue/violet	n.c.
Musacin A	0.54[a]	0.52[e]	Brown to grey	Orange	n.c.	Blue/violet
Musacin B_1	0.74[a]	0.74[e]	Brown	Grey	Violet	Blue/violet
Musacin B_2	0.70[a]	0.67[e]	Brown	Grey	Violet	Blue/violet
Musacin C	0.70[a]	0.67[e]	Brown	Green	n.c.	Blue/violet
Musacin D	0.86[a]	0.59[b]	Blue	Green	n.c.	Blue/violet
Musacin E	0.86[a]	0.55[b]	Blue	Green	n.c.	Blue/violet
Musacin F	0.77[a]	0.41[b]	Blue	Green	n.c.	Blue/violet

Solvent systems: [a] $CHCl_3$ - MeOH (4:1), [b] Ethyl acetate - *n*-hexane (4:1), [c] Ethyl acetate - *n*-hexane (3:1), [d] $CHCl_3$ - MeOH (9:1), [e] Ethyl acetate - MeOH (9:1), n.c. no colorization.

After harvesting, the culture filtrate of the 50-liter fermentation was adsorbed on MCI-gel and the metabolites were eluted with MeOH - H_2O (4:1). The oily concentrate obtained was separated on a silica gel column with $CHCl_3$ - MeOH gradient elution, resulting in a number of different metabolites (Rf-values see Table 2). Further purifications were performed by column chromatography on silica gel with ethyl acetate-*n*-hexane or $CHCl_3$ - MeOH mixtures as eluents, and on Sephadex LH-20 gel permeation chromatography with MeOH or $CHCl_3$. The purification and isolation procedures are summarized in Scheme 1. The yields of the purified metabolites ranged from 50.7 mg/liter to 0.4 mg/liter.

Characterization and Properties

Initially, the isolated and purified compounds were roughly classified *via* their reactivity due to different staining reagents on TLC. The color reactions and Rf-values are summarized in Table 2. These investigations led us to assume the presence of two different structural

types of secondary metabolites. (i) Four metabolites, which showed no reaction with tetrazolium blue reagent, but a striking variability due to anisaldehyde-H_2SO_4, orcinol and Ehrlich's reagent. (ii) Seven metabolites, which are characterized by a brown or blue colorization after staining with anisaldehyde-H_2SO_4, and a blue to violet reaction with tetrazolium blue reagent. Based on high resolution mass spectroscopy, detailed NMR investigations as well as derivatization reactions, the structures of the isolated compounds were determined (see accompanying paper[11]). Class (i) compounds were identified to be 14-membered macrocyclic lactones of the cineromycin-type (cineromycin B[15] and three new congeners) and (ii) as new γ-lactones and open ring derivatives, named musacins A to F. Musacin E [5-(1-hydroxy-but-2-enyl)-tetrahydrofurane-2-one] is identical with nigrosporolactone[16].

Biological Activities

In preliminary studies the isolated metabolites have been examined in various biological tests. The musacins exhibit no significant antibacterial, antifungal, antiprotozoal, herbicidal and insecticidal activity, while in an anthelminthic pre-screening with *Caenorhabditis elegans* musacin C was conspicuous. This result was verified *in vivo* using *Trichostrongylus colubriformis* in jird (10 mg/kg per os, about 95% reduction of the nematodes; subcutan application showed no effect). In addition, musacin C resulted in a weak antiviral activity against adenovirus, HSV-1, HSV-2, and influenza virus (44 μg/ml, dosis tolerata: 400 μg/ml). The cineromycin-type metabolites showed weak activity against Gram-positive bacteria, *e.g. Staphylococcus aureus* (20 to 6 μg/ml). No further biological activities have yet been observed.

Discussion

Analysing the secondary metabolite pattern of *Streptomyces griseoviridis*, FH-S 1832, we illustrated that it is worthwhile to focus screening investigations on biosynthetically talented strains. These organisms are characterized by a large number of the spots in our chemical screening, each of which showed a typical colorization behaviour due to staining with reagents, while usually no distinct main product could be observed. In combination with the aim to get further insight into the variability of secondary metabolism in general we investigated in the isolation and structure elucidation of the secondary metabolite pattern of *Streptomyces griseoviridis* (FH-S 1832), which has been selected on the number and colorization behaviour of TLC spots as well as on the variability of the produced metabolite pattern to alteration of the cultivation conditions. In a first attempt the producing organism has been cultivated on glycerol/casopeptone medium, which resulted in the detection and isolation of three new congeners of the cineromycin type as well as in six new musacins. Only two compounds were identified as known secondary metabolites, namely cineromycin B[15] and nigrosporolactone[16].

As a consequence of the detailed analysis of the results obtained from our chemical screening project it seems reasonable to comment on the secondary metabolism of the analyzed strains in a more general manner. Regarding their metabolic finger-print visualized by the chemical screening method after fermentation on different cultivation media streptomycetes strains can be classified in: (i) non-producing organisms, which gave no indication of the formation of any secondary metabolite up to a detection limit of 0.1 to 1 mg/liter culture broth, (ii) organisms of narrow productivity, which produce one or two secondary metabolites as main products with a restricted dependance to the alteration of the culture conditions, and (iii) talented organisms, which produce a large variability of structurally diverse secondary metabolites, usually with a stronger dependance to the applied cultivation conditions. According this terminology, we observed nearly a third of the screened strains belonging to the category of non-producing organisms, while about 1% could be highlighted as talented strains. In order to eliminate secondary metabolites, which seemed to be of non interest or have been discovered more frequently in the screening routine, we usually favoured stronger selection criterea.

Based on our acquired expertise in qualifying the spots obtained *via* the TLC-method, about 50% of all metabolites isolated and structurally characterized appeared to be new. It is worth mentioning, that these findings strongly correlate to the quality of the isolation protocols and the selection criterea leading to the strains, as well as the routine cultivation conditions and ingredients of the culture media.

Based on the above findings the chemical screening method can be used as a screening strategy, leading to unknown secondary metabolites. Advantageously, chemical screening allows a qualification of microbial strain collections, which may become a center of interest in pharmaceutical companies in the evaluation of high-thoughput assay systems. In order to highlight quality against quantity in biological screening approaches non-producing strains as well as strain dublicates should be eliminated and the screening should be focused on organisms with narrow productivity and talented strains. With the use of computer-based scanning and analysis techniques in combination with an automatic application of concentrates onto TLC plates our chemical screening is easy-to-handle, and can be integrated in biological screening strategies advantageously.

Experimental

General

Morphological observations were done using a scanning electron microscope, Jeol Model JSM T20. Drying of the strain samples at the critical point was performed by using Technics Inc. CPA II, and covering with a gold layer by a Humer Junior chamber (Technics Inc.). All chemicals were purchased from Riedel de Haen, additional cultivation media from Difco Ltd. Fermentations were carried out in a 50-liter fermentor (Biostat P) from Braun Diessel (Melsungen, Germany). TLC was performed on silica gel plates (Merck, HPTLC-ready-to-use-plates, silica gel $60F_{254}$ on aluminium foil or glass). Column chromatography was done with MCI-gel CHP2OP (Mitsubishi Kasei), silica gel 60 ($0.040 \sim 0.063$ mm, Merck) and Sephadex LH-20 (Pharmacia). Staining reagents are described in reference 7.

Cultivation Media

Medium A: Soy bean meal (degreased) 2%, mannitol, 2% in deionized water, pH=7.2 prior to sterilization.

Medium B: Oat flakes 2% in deionized water and addition of 5 ml trace element concentrate per liter, pH=7.0 prior to sterilization.

Medium C: Glycerol 3%, caseinpeptone 0.2%, K_2HPO_4 0.1%, NaCl 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% in tap water and addition of 5 ml trace element concentrate per liter, pH=7.0 prior to sterilization.

Trace element concentrate: 3 g $CaCl_2 \cdot 2H_2O$, 1 g Fe-III-citrate, 0.2 g $MnSO_4$, 0.1 g $ZnCl_2$, 0.025 g $CuSO_4 \cdot 5H_2O$, 0.02 g $Na_2B_4O_7 \cdot 10H_2O$, 0.004 $CoCl_2$, 0.01 g $NaMoO_4 \cdot 2H_2O$, ad 1 liter of deionized water.

For morphological and strain conservation, 2% of agar were added to the different media.

Fermentation

Strain FH-S 1832 was cultivated in 300 ml Erlenmeyer flasks containing 100 ml of the media on a rotary shaker at 180 rpm and 30°C over a period of 5 days. These cultures were used to analyse both, the secondary metabolite pattern by the chemical screening method, and the time course of a fermentation of *Streptomyces griseoviridis* (FH-S 1832). Growth was determined by measurement of DNA[17] and dry weight. The nitrogen concentration was measured by means of an ammonium electrode, and the amount of glycerol was determined by HPLC analysis. The phosphate concentration was analyzed *via* phosphor-molybdato complexes and subsequent UV-measurement.

In order to produce larger amounts of the secondary metabolites, strain FH-S 1832 was cultivated in 50-liter fermentors using medium C for 5 days at 30°C with an aeration rate of 0.5 v/v/m and an agitation of 200 rpm. The fermentor was inoculated with 800 ml of cultures grown in Erlenmeyer flasks on medium A for 3 days.

TLC Analysis

100 ml of broth obtained from cultivations in Erlenmeyer flasks on different media for 5 days were separated by centrifugation (4000 rpm, 10 minutes). 50 ml of the supernatant were loaded on an Amberlite XAD-16 column (2.5×5 cm), washed with water (100 ml) and eluted with 50 ml of methanol - water (4 : 1). The eluates were concentrated to dryness and dissolved in 1 ml of methanol - water (1 : 1). The mycelium was extracted with 50 ml of acetone. The extract was concentrated to dryness and dissolved in 1 ml of methanol - water (1 : 1). 5 μl of the concentrates were separated on tlc (HPTLC-silica gel plates) using $CHCl_3$ - MeOH (9 : 1) and 1-butanol - acetic acid - water (4 : 1 : 5, upper phase), respectively, and analyzed by UV extinction at 254 and 366 nm as well as by colorization with staining reagents (*e.g.* anisaldehyde-H_2SO_4, orcinol reagent, Ehrlich's reagent, and blue tetrazolium reagent).

Isolation

The cineromycins and musacins were isolated from a 50-liter fermentation using medium C. The fermentation broth was separated by multiple sheet filtration. The culture filtrate was applied on a MCI-gel column (7.5×46 cm), which was washed with 10 liters of deionized water and eluted with methanol - water (4 : 1). The MCI eluate was concentrated to dryness to yield 59 g of an dark brown oily crude product. This residue was chromatographed on a silica gel column (7.5×40 cm) by elution with $CHCl_3$ - MeOH (gradient from 9 : 1 to 4 : 1) to result in five main fractions containing cineromycins and musacins. Further purification was carried out with column chromatography on silica gel using ethyl acetate or gradient elution with mixtures of MeOH, $CHCl_3$, ethyl acetate and/or *n*-hexane. Further purification was performed by Sephadex LH-20 column chromatography (column size 2.5×100 cm) with MeOH or $CHCl_3$ as eluent (see also scheme 1).

Acknowledgment

We would like to thank Dr. J. Wink for microbial work, E. Faust, A. Godawa, M. Klingebiel, D. Kornblüh, M. Oswald, M. Sonnentag, S. Sauerbrei, P. Stapf and P. Wahl for excellent technical assistance. This work was done in the course of a collaboration project between the Hoechst AG and Prof. A. Zeeck (University of Göttingen) and was supported by a grant from the Bundesministerium für Forschung und Technologie (BMFT, grant 0319311B).

References

1) Kind, R.; A. Zeeck, S. Grabley, R. Thiericke & M. Zerlin: Secondary metabolites by chemical screening. 30. Helmidiol, a new macrodiolide from *Alternaria* sp. J. Nat. Prod., in press.
2) Nakagawa, A.: Chemical screening. *In* The search for bioactive compounds from microorganisms. *Ed.*: S. Ōmura, pp. 263 ~ 280, Springer Verlag, New York, 1992
3) Zähner, H. & H. Drautz: Methods in the search

for new secondary metabolites from microorganisms—a comparison. *In* VIII International Symposium on Medicinal Chemistry, Proceedings Vol. 2. *Ed.*, R. DAHLBOHM & J. L. G. NILSSON, pp. 49~68, Swedish Pharmaceutical Press, Stockholm, 1985

4) GRABLEY, S.; J. WINK & A. ZEECK: Chemical screening as applied to the discovery and isolation of microbial secondary metabolites. *In* Biotechnology Focus 3. *Eds.*, R. K. FINN & P. PRÄVE, pp. 359~370, Hanser Publishers, Munich, 1992
5) GÖHRT, A.; A. ZEECK, K. HÜTTER, R. KIRSCH, H. KLUGE & R. THIERICKE: Secondary metabolites by chemical screening. 9. Decarestrictines, a new family of inhibitors of cholesterol biosynthesis from *Penicillium*. II. Structure elucidation of the decarestrictines A to D. J. Antibiotics 45: 66~73, 1992
6) GRABLEY, S.; G. KRETZSCHMAR, M. MAYER, S. PHILIPPS, R. THIERICKE, J. WINK & A. ZEECK: Secondary metabolites by chemical screening. 24. Oasomycins, new macrolactones of the desertomycin family. Liebigs Ann. Chem. 1993: 573~579, 1993
7) HENNE, P.; R. THIERICKE, S. GRABLEY, K. HÜTTER, J. WINK, E. JURKIEWICZ & A. ZEECK: Secondary metabolites by chemical screening. Part 23. Waraterpols, new *Penicillium* metabolites and their derivatives. Liebigs Ann. Chem. 1993: 565~571, 1993
8) GRABLEY, S.; P. HAMMANN, K. HÜTTER, R. KIRSCH, H. KLUGE, R. THIERICKE, M. MAYER & A. ZEECK: Secondary metabolites by chemical screening. 20. Decarestrictines, a new family of inhibitors of cholesterol biosynthesis from *Penicillium*. III. Decarestrictines E to M. J. Antibiotics 45: 1176~1181, 1992
9) BACH, G.; S. BREIDING-MACK, S. GRABLEY, P. HAMMANN, K. HÜTTER, R. THIERICKE, H. UHR, J. WINK & A. ZEECK: Secondary metabolites by chemical screening. Part 22. Gabosines, new carba-sugars from *Streptomyces*. Liebigs Ann. Chem. 1993: 241~250, 1993
10) ZÄHNER, H.: personal comunication
11) SCHNEIDER, A.; J. SPÄTH, S. BREIDING-MACK, A. ZEECK, S. GRABLEY & R. THIERICKE: Secondary metabolites by chemical screening. 32. New cineromycins and musacins obtained by metabolite pattern analysis of *Streptomyces griseoviridis* (FH-S 1832). II. Structure Elucidation. J. Antibiotics 49: 438~446, 1996
12) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. System. Bacteriol. 16: 313~340, 1966
13) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. Int. J. System. Bacteriol. 18: 169~189, 1968
14) HÜTTER, R.: Systematik der Streptomyceten. Bibl. Microbiol. 6: 1~21, 1967
15) MIYAIRI, N.; M. TAKASHIMA, K. SHIMIZU & H. SAKAI: Studies on new antibiotics. J. Antibiotics 19: 56~62, 1966
16) EVANS, R. H.; G. A. ELLESTAD & M. P. KUNSTMANN: Two new members from an unidentified *Nigrospora* species. Tetrahedron Lett. 22: 1791~1795, 1969
17) HERBERT, D.; J. P. PHIPPS & R. E. STRANGE: Chemical analysis of microbial cells. *In* Methods of Enzymology, *Eds.* J. R. NORRIS & D. W. RIBBONS, pp. 209~344, Academic Press, London, 1971