on alumina. Besides α-spinasterol, three sterols, Δ^7-stigmastenol, Δ^{22}-stigmastenol, and stigmasterol, were isolated and identified. Although Δ^{22}-stigmastenol was already known as synthetic material, this work is the first example of its isolation from plants.

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108. **Shoshiro Nakamura**: Studies on Structure of Griseolutein B, a Streptomyces Antibiotic. I. Characterization and Degradation.

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Umezawa, Hayano, Maeda, and Okami[1] isolated a new antibiotic from *Streptomyces griseoluteus* and the antibiotic was named griseolutein. Later, Osato, Maeda, and Umezawa[2] isolated another antibiotic from the same strain and they designated the first one griseolutein-A, and the second one, griseolutein-B. Both griseolutein-A and -B inhibit gram-positive and negative bacteria and are low in toxicity. According to pharmacological studies made by Ogata,[3] griseolutein-B gives a relatively high blood level when administered subcutaneously or orally. These observations suggested a possible usefulness of this antibiotic and elucidation of the structure of griseolutein-B was undertaken which is described in this and subsequent papers.

The cultured broth of *Streptomyces griseoluteus* was filtered and griseoluteins were extracted with butyl acetate at pH 2.0. Evaporation of the solvent under vacuum gave a brownish orange crude powder containing griseolutein-A and -B. This was recrystallized from dioxane and crude crystals chiefly containing griseolutein-B were obtained. The crude crystals were further purified by the 60-tube counter-current distribution between phosphate buffer of pH 5.8 and ethyl acetate. Griseolutein-B was present in tubes No. 1 and 2 and a small amount of A was found in tubes No. 44~46. Acidification of the aqueous layer of tubes No. 1~2 precipitated griseolutein-B as crystals. Further recrystallization from pyridine-dioxane by the addition of ether gave yellow prisms of griseolutein-B, $C_{17}H_{16}O_6N_2$ (mol. wt., 344.31). It did not give a clear melting or decomposition point, but it began to brown near 160°, darkened at about 180°, and charred at about 220°. Griseolutein-B is insoluble in toluene, ether, or butyl acetate, sparingly soluble in dioxane or methyl

Fig. 1. Ultraviolet Absorption Spectra (in MeOH)

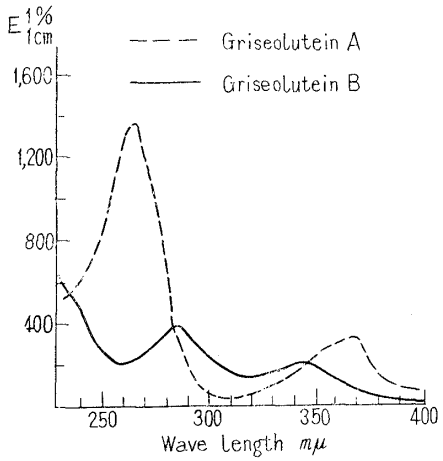
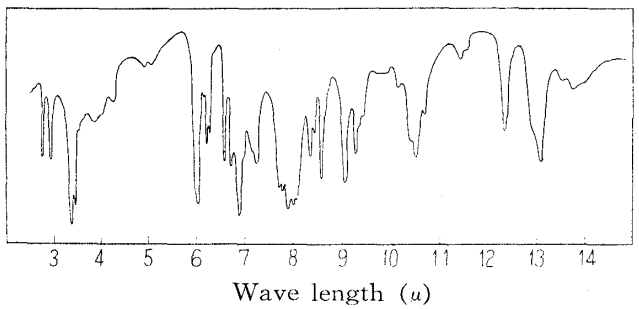


Fig. 2. Infrared Spectrum of Griseolutein B (in Nujol.)



* Yayoi-cho, Bunkyo-ku, Tokyo (申村昭四郎).

1) H. Umezawa, S. Hayano, K. Maeda, Y. Ogata, Y. Okami: J. Antibiotics(Japan), **4**, 34(1951).
2) T. Osato, K. Maeda, H. Umezawa: *Ibid.*, **7**, 15(1954).
3) Y. Ogata, K. Nitta, S. Yamazaki, O. Taya, T. Takeuchi, H. Umezawa: *Ibid.*, **6**, 139(1953).

ethyl ketone, and soluble in sodiun hydrogen carbonate solution or pyridine. $[\alpha]_D^{23}$ −6° (c=0.7, pyridine). U. V. λ_{max}^{MeOH} mμ($E_{1cm}^{1\%}$): 281~283(296), 342~344(170) (Fig. 1). The infrared absorption spectrum is shown in Fig. 2.

Tubes No. 44~46 of the counter-current distribution described above gave orange crystals of griseolutein-A, $C_{17}H_{14}O_6N_2$ (mol. wt., 342.3), m. p. 194~197°(decomp.). U. V. λ_{max}^{MeOH} mμ ($E_{1cm}^{1\%}$): 267(1326), 368(330) (Fig. 1).

Acetylation of griseolutein-B with pyridine and acetic anhydride, and recrystallization from hydrous methanol gave light yellow prisms, diacetylgriseolutein-B, $C_{21}H_{22}O_9N_2$(mol. wt. 446.40), double m. p. 117°/180~181°. U. V. λ_{max}^{MeOH} mμ ($E_{1cm}^{1\%}$): 285(472), 342(287). The titration with 0.01*N* sodium hydroxide gave equivalence value of 447 and one mole of water was lost by heating at 120° for three hours. One methoxyl group (Zeisel), two acetyl groups (Kuhn-Roth), no C-methyl group (Kuhn-Roth), and one carboxyl group (titration) were found. There was no hydrogen absorption by catalytic reduction over platinum in methanol or acetic acid. The molecular weight of its anhydride by Rast method with camphor was 398.8(calcd., 428.39). Titration[4] of diacetyl griseolutein-B with perchloric acid in glacial acetic acid and acetic anhydride gave no equivalence value. Diacetylgriseolutein-B did not change on heating with zinc in acetic acid and the absence of N-oxide[5] was confirmed. Methylation with diazomethane gave a monomethyl ester of diacetylgriseolutein-B as yellow plates, $C_{22}H_{22}O_8N_2$, m. p. 158~159°; U. V. λ_{max}^{MeOH} mμ($E_{1cm}^{1\%}$): 284(248), 357(166). Diacetylgriseolutein-B was easily crystallizable and therefore, pure sample of diacetylgriseolutein-B could be obtained through acetylation of crude crystals of griseolutein-B containing a small amount of griseolutein-A without application of the counter-current distribution.

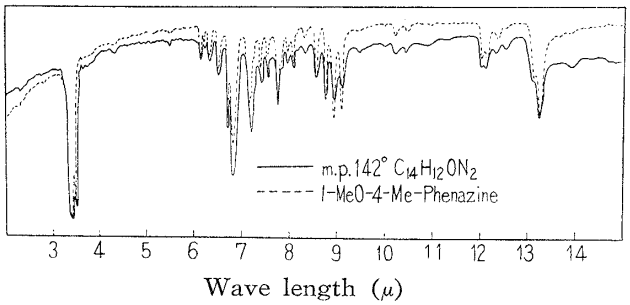
Rrefluxing of diacetylgriseolutein-B in 3*N* sodium hydroxide in an oil bath for one hour and neutralization of the reaction mixture with hydrochloric acid gave a reddish brown precipitate of an acid. It was named griseoluteic acid. Griseoluteic acid failed to crystallize but its methyl ester obtained by methylation with diazomethane crystallized as orange yellow needles, $C_{16}H_{14}O_4N_2$, m. p. 189°; U. V. λ_{max}^{MeOH} mμ($E_{1cm}^{1\%}$): 267(1442), 362(292). Acetylation of griseoluteic acid with pyridine and acetic anhydride and further methylation with diazomethane gave monomethyl monoacetylgriseoluteate as orange yellow needles, $C_{18}H_{16}O_5N_2$, m. p. 148°.

Griseoluteic acid was distilled with 5 times its weight of zinc dust and the reaction products were subjected to alumina chromatography.

A fraction eluted with hexane yielded yellow prisms, $C_{12}H_8N_2$, m. p. 170°; U. V.[6] λ_{max}^{MeOH} mμ($E_{1cm}^{1\%}$): 248(8800), 362(1100). This substance was identified as phenazine[7] on admixture with an authentic sample and by comparison of their infrared absorption spectra.

A fraction obtained by elution with hexane-ether mixture gave two yellow substances: Yellow needles, $C_{14}H_{12}ON_2$, m. p. 142°; U. V. λ_{max}^{MeOH} mμ($E_{1cm}^{1\%}$): 265(1980), 362(330); and yellow plates, m. p. 165°. These substances were considered to be 1-methoxyphenazine derivatives on the basis of their ultraviolet absorptions, isolation of phenazine from the other fraction, and methoxyl group in diacetylgriseolutein-B as described above. Moreover, the fact that both gave violet coloration in conc. hydrochloric acid[8] suggested that they are 1-methoxyphenazine derivatives. The identity of the yellow

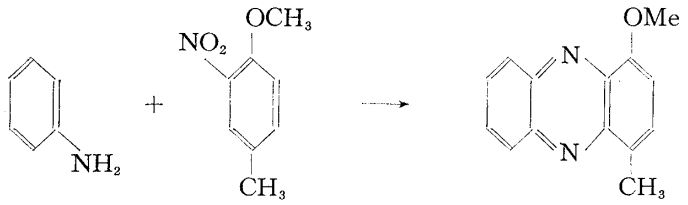
Fig. 3. Infrared Spectra (in Nujol)



4) H. Brockmann, E. Meyer: Chem. Ber., **86**, 1514(1953).
5) Y. Kidani, H. Otomasu: This Bulletin, **4**, 319(1956).
6) G. M. Badger, R. S. Pearce: J. Chem. Soc., **1951**, 3203
7) A. Wohl, W. Aue: Ber., **34**, 2446(1901).
8) I. Yoshioka: Yakugaku Zasshi **72**, 1128(1952).

plates of m.p. 165° with 1-methoxyphenazine was confirmed by comparing the mixed melting point and infrared spectra with those of authentic sample. The substance which formed yellow needles, m.p. 142°, is a new compound. The out-of-plane CH-vibrations in its infrared absorption spectrum[9] (Fig. 3) suggested the presence of four adjacent free hydrogen atoms (13.22 μ) and two adjacent hydrogen atoms (12.00, 12.10 or 12.32 μ). On the other hand, most of the natural phenazine compounds are known to have substituent in their 1-position. Therefore, this substance was considered to be 1-methoxy-4-methylphenazine.

1-Methoxy-4-methylphenazine was synthesized by the Wohl-Aue reaction[7,8] from aniline and 3-nitro-4-methoxytoluene as shown in the following route:



It was found that two kinds of crystal forms existed in 1-methoxy-4-methylphenazine. The one obtained by recrystallization from hexane came as yellow needles, m.p. 151°, and the other obtained by recrystallization from a mixture of hexane and benzene came as yellow prisms, m.p. 153°. These crystals showed no melting point depression on admixture, but they were differentiated by infrared spectra in Nujol. However, both gave the same spectra in carbon disulfide solution.

TABLE I. Bacteriostatic Effect of Griseoluteins

Bacteria	Minimum inhibitory concentration (γ/cc.) Griseolutein-A	Griseolutein-B
Micrococcus pyogenes var. *aureus* 209P	0.8	0.4
Micrococcus pyogenes var. *aureus* TERAJIMA	0.2	0.2～0.4
Bacillus anthracis	0.4	0.2
Bacillus subtilis B558	0.1	1～2
Escherichia coli	6.3	5
Shigella dysenteriae	1.6	1～2
Shigella paradysenteriae KOMAGOME BIII	0.8	2～4
Salmonella typhosa	3.1	5～10
Salmonella paratyphi	3.1	2
Salmonella schottmuelleri	—	2～4
Salmonella typhi murium	25	25～50
Proteus vulgaris OX19	0.2	25
Pseudomonas aeruginosa	12.5	25～50
Pneumococcus Type I	—	25
Pneumococcus Type II	—	25
Pneumococcus Type III	—	10
Mycobacterium tuberculosis 607	—	20
Mycobacterium avium	—	1～2

The substance forming yellow needles, obtained by zinc-dust distillation of griseoluteic acid, was confirmed to be identical with synthetic 1-methoxy-4-methylphenazine by mixed melting point test and comparison of their infrared spectra as shown in Fig. 3.

The author expresses his sincere gratitude to Prof. H. Umezawa and Prof. Y. Sumiki of this Institute for their constant guidance and directions in the course of this study. He also expresses deep thanks to Prof. S. Sugasawa for his valuable advices. The author is also greatly indebted to Mr. Maeda and Mr. Osato, National Institute of Health, Tokyo, for their generous supply of the material of griseolutein-B, and to Dr. I. Yosioka and Mr. Y. Kidani for their generous supply of phenazine and 1-methoxyphenazine used in this work.

9) L. J. Bellamy: "The Infrared Spectra of Complex Molecules," Methuen & Co., London, 54(1954).

Experimental

Aerated Tank Culture and Extraction—The medium of 170 L. was placed in a stainless steel fermenter of 400 L. capacity and sterilized for 20 mins. at 120° by blowing high pressure steam. Two litres of the shake-cultured broth of *St. griseoluteus* was incubated for 35~40 hrs. During the fermentation, the stirrer was turned at 200 r.p.m. and the rate of aeration was 200 L./min. While under cultivation 500 cc. of soybean oil was used as an antifoamer. At the beginning, pH of the broth increased to 7.2~7.6 and thereafter it gradually decreased to 5.4~5.6 after 60~70 hrs. After 65 hrs., the fermentation was stopped and the cultured broth was filtered. The filtrate was adjusted to pH 2.0 with HCl and extracted with 60 L. of AcOBu. The extract was concentrated *in vacuo* and after standing in a refrigerator, 9.5 g. of crude crystals (520 γ/mg.) precipitated.

Isolation and Purification.—When 9 g. of the crude crystals was placed in 700 cc. dioxane at 60°, 3.9 g. of it dissolved and 5.1 g. remained insoluble. After the solution was concentrated *in vacuo* and cooled, 3.2 g. of crystals containing griseolutein-B and a trace of grieseolutein-A was obtained. 200 mg. of these crystals was dissolved in phosphate buffer (pH 5.8) and distributed between this buffer and AcOEt, using Craig's counter-current distribution apparatus of 60 tubes. The aqueous layer of tubes No. 1 and 2 were combined, acidified with HCl, and 120 mg. of griseolutein-B crystals precipitated. It was dissolved in a pyridine-dioxane mixture, ether was added, and placed in a refrigerator for about 1 day. Griseolutein-B was obtained as yellow crystals decomposing at about 220°. *Anal.* Calcd. for $C_{17}H_{16}O_6N_2$: C, 59.30; H, 4.68; N, 8.14. Found: C, 59.49; H, 4.90; N, 7.86.

Griseolutein-A was present in tubes No. 44~46 and was recrystallized from MeOH to orange needles, m.p. 194~197°(decomp.). *Anal.* Calcd. for $C_{17}H_{14}O_6N_2$: C, 59.65; H, 4.12; N, 8.18. Found: C, 59.51; H, 4.36; N, 8.02.

Diacetylgriseolutein-B—To 500 mg. griseolutein-B crystallized from dioxane, 10 cc. of pyridine and 50 cc. of Ac_2O were added and dissolved with warming. After standing overnight, the solvent was evaporated *in vacuo* and the residue was extracted with hot MeOH, removing the insoluble matter. MeOH solution was treated with activated carbon to remove colored impurities and 0.625 g. of yellow prisms was obtained on addition of about 2 volumes of water. Recrystallized from hydr. MeOH to yellow prisms, double m. p. 117°/181°. *Anal.* Calcd. for $C_{21}H_{22}O_9N_2$: C, 56.50; H, 4.97; N, 6.28; OCH_3, 6.94. Found: C, 56.60; H, 4.85; N, 6.07; OCH_3, 6.58.

Diacetylgriseolutein-B Monomethyl Ester—Ether solution of CH_2N_2 was added to 100 mg. of diacetylgriseolutein-B dissolved in MeOH (10 cc.), the solvent was evaporated, and 85 mg. of yellow plates (from MeOH) were obtained. m. p. 158~159°. *Anal.* Calcd. for $C_{22}H_{22}O_8N_2$: C, 59.72; H, 5.01; N, 6.33. Found: C, 59.82; H, 5.28; N, 6.20.

Alkali Hydrolysis—Diacetylgriseolutein-B (290 mg.) was dissolved in 20 cc. of 3*N* NaOH and the mixture was heated in an oil bath for 1 hr. at 110°, under reflux. After cool, the solution was acidified with HCl and 175 mg. of reddish brown precipitate of griseoluteic acid was obtained. This acid was methylated with CH_2N_2 in MeOH and purified by alumina column chromatography. Methyl griseoluteate was eluted with ether-AcOEt and was recrystallized from MeOH to orange yellow needles, m.p. 189°. *Anal.* Calcd. for $C_{16}H_{14}O_4N_2$: C, 64.42; H, 4.73; N, 9.39. Found: C, 64.20; H, 4.81; N, 9.52.

Griseoluteic acid was acetylated with pyridine-Ac_2O and subsequently methylated with CH_2N_2. The reaction product, monomethyl monoacetylgriseoluteate, was purified by alumina chromatography and obtained as orange yellow needles (from MeOH), m. p. 148°. *Anal.* Calcd. for $C_{18}H_{16}O_5N_2$: C, 63.52; H, 4.74; N, 8.23. Found: C, 63.74; H, 4.62; N, 8.21.

Zinc-dust Distillation of Griseoluteic Acid—One gram of griseoluteic acid was mixed with 5 g. of Zn dust and the mixture was divided into 20 parts. Each part was distilled in the range of 300~400°; the reaction mixtures were extracted with ether, the solvents were combined, concentrated, and purified through alumina column chromatography. Yellow needles (5 mg.), m. p. 170°, obtained from the effluent with hexane, was identified as phenazine on admixture with an authentic sample and also by infrared spectra. *Anal.* Calcd. for $C_{12}H_8N_2$: C, 79.98; H. 4.48; N, 15.55. Found: C, 79.66; H. 4.48; N. 15.42.

Yellow needles (7 mg.), m. p. 142°, obtained from the first effluent with hexane-ether, were identified as 1-methoxy-4-methylphenazine on admixture with the synthesized sample and by comparison of their infrared spectra. *Anal.* Calcd. for $C_{14}H_{12}ON_2$: C, 74.99; H, 5.38; N, 12.49. Found: C, 74.93; H, 5.24; N, 12.71.

Yellow plates (2 mg.), m.p. 165°, obtained from the second effluent with hexane-benzene, were identified as 1-methoxyphenazine on admixture with an authentic sample.

Synthesis of 1-Methoxy-4-methylphenazine—A mixture of aniline (8 g.), 3-nitro-4-methoxytoluene (10 g.), toluene (60 cc.), and powdered KOH (30 g.) was heated in a three-necked flask equipped with a stirrer and a condenser, in an oil bath for 4 hrs. under reflux. The hot reaction mixture was filtered, the filtrate was distilled *in vacuo*, and the residue was steam distilled. The residue was dissolved in benzene and extracted with 10% HCl. The aqueous layer was neutralized with NH_4OH, crude crystals precipitated, which were dissolved in benzene, purified on alumina, and eluted with benzene.

Yellow needles (0.2 g.), m.p. 149~151° (from hexane). Yellow prisms (1.3 g.), m. p. 152~153° (from hexane-benzene). *Anal.* Calcd. for $C_{14}H_{12}ON_2$: C, 74.99; H, 5.38; N, 12.49. Found (needles): C, 75.02;

H, 5.51; N, 12.28. Found (prisms): C, 74.82; H, 5.40; N, 12.41. The two kinds of substances gave no melting point depression on admixture.

Summary

Griseolutein-B and -A were purified by the counter-current distribution method and their properties were examined. Diacetylgriseolutein-B was easily purified and crystallized. It had one methoxyl group, two acetyl groups, and one carboxyl group, but no C-methyl group or N-oxide group. Its methyl ester was obtained. Alkaline hydrolysis of diacetylgriseolutein-B gave griseoluteic acid which was crystallized as its methyl ester or as monomethyl ester of monoacetylgriseoluteic acid. Distillation of griseoluteic acid with zinc dust gave phenazine, 1-methoxyphenazine, and another compound. This compound was identified as 1-methoxy-4-methylphenazine by comparison with the synthesized sample. 1-methoxy-4-methylphenazine was synthesized from aniline and 3-nitro-4-methoxytoluene and its two crystal forms were observed.

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109. Shoshiro Nakamura: Studies on the Structure of Griseolutein-B, a Streptomyces Antibiotic. II[1]. Decarboxylation and Periodic Acid Oxidation.

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As described in the preceding paper,[1] griseolutein-B, $C_{17}H_{16}O_6N_2$, was easily purified and crystallized as diacetylgriseolutein-B, $C_{21}H_{20}O_8N_2 \cdot H_2O$, in which one methoxyl group, two acetyl groups, one carboxyl group, and no C-methyl group were determined. Alkaline hydrolysis of diacetylgriseolutein-B gave an acid, named griseoluteic acid. Monomethyl ester of griseoluteic acid, $C_{16}H_{14}O_4N_2$, and monomethyl ester of monoacetylgriseoluteic acid, $C_{18}H_{16}O_5N_2$, were crystallized. The zinc-dust distillation of griseoluteic acid gave phenazine, 1-methoxyphenazine, and 1-methoxy-4-methylphenazine. In this paper, results of decarboxylation of diacetylgriseolutein-B and periodic acid oxidation of griseolutein-B are described, and on the basis of these results partial structures of griseoluteic acid and griseolutein-B are presented.

Diacetylgriseolutein-B was decarboxylated in quinoline by heating at 230° for 2 hours, using copper dust as a catalyst. The reaction product was subjected to alumina chromatography for purification and the fraction obtained by elution with benzene gave yellow crystals, $C_{16}H_{14}O_3N_2$, m.p. 139°, U.V. λ_{max}^{MeOH} $m\mu(E_{1cm}^{1\%})$: 260~261 (1660), 361~362 (265) (Fig. 1). Another crop was obtained from the effluent with ethyl acetate as yellow crystals, $C_{14}H_{12}O_2N_2$, m. p. 196°, U.V. λ_{max}^{MeOH} $m\mu(E_{1cm}^{1\%})$: 263 (1945), 362 (324) (Fig. 1). No difference was found between the former and the acetylated product of the latter on admixture and by comparison of their infrared spectra. The latter was presumed to be 1-methoxy-4-hydroxymethylphenazine because of the following facts: 1-Methoxy-4-methylphenazine[1] was obtained as one of zinc-dust distillation

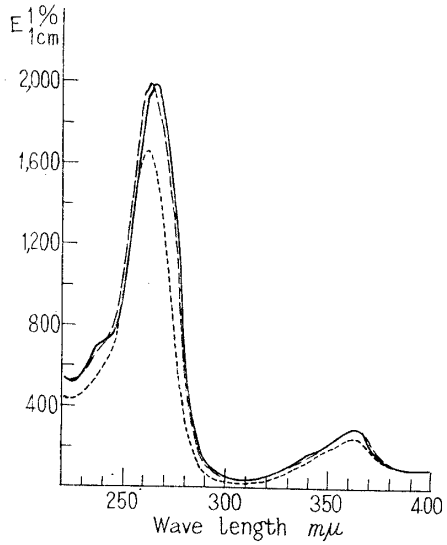

Fig. 1. Ultraviolet Absorption Spectra (in MeOH)
——— 1-Methoxyl-4-methylphenazine
— — — Decarboxylation product $C_{14}H_{12}O_2N_2$, m. p. 196°
- - - - - Decarboxylation product $C_{16}H_{14}O_3N_2$, m. p. 139°

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1) Part I. S. Nakamura: This Bulletin, 6, 539(1958).