identified as 1-methoxy-4-methylphenazine (IX) on admixture with an authentic specimen. The second crop of yellow crystals (VIII), m.p. 196° (13 mg.), obtained from the fractions eluted with AcOEt was recrystallized from hexane. *Anal.* Calcd. for $C_{14}H_{12}O_2N_2$: C, 69.99 ; H, 5.03. Found : C, 70.22 ; H, 4.98.

Periodic Acid Oxidation of Griseolutein-B—i) Determination of periodic acid[5]: To a solution of griseolutein-B (49.60 mg.$=0.1440\times10^{-3}M$) dissolved in N $NaHCO_3$ (5 cc.) and water (15 cc.), $0.1N$ HIO_4 solution (10 cc.) was added and the mixture was allowed to stand for 1 hr. at room temperature. From this reaction mixture 5.0 cc. aliquot was pipetted out, 5 cc. of a saturated $NaHCO_3$ solution, 5 cc. of $0.048M$ arsenite solution, and 1 cc. of $NaHCO_3$ solution containing 20% KI were added with 1 cc. of a 1% soluble starch solution as indicator. 3.06 cc. of $0.045M$ iodine solution was required to titrate the excess arsenite. On the other hand, 2.53 cc. of $0.045M$ iodine solution was consumed by the blank test carried out by the same procedure. Therefore,

$$(3.06-2.53)\times0.045\times30/5\times10^{-3}M=0.1431\times10^{-3}M$$

ii) Determination of formaldehyde[5] and griseoluteic acid[1]: Griseolutein-B (64.32 mg.$=0.1868\times10^{-3}M$) was dissolved in N $NaHCO_3$ (0.7 cc.) and water (4.5 cc.), $0.1N$ HIO_4 solution (5 cc.) was added and the mixture was allowed to stand 1 hr. at room temperature. After the solution was adjusted to pH 7.4, standard arsenite solution (6 cc.) was added, and the mixture was allowed to stand at room temperature for 1 hr. The solution was acidified with $2N$ AcOH to pH 5.6 and AcONa-HCl buffer (10 cc.) was added. The brown red precipitate was removed by filtration. 0.4% dimedone solution (6 cc.) was added and the mixture was allowed to stand for 24 hrs. The white needles of methylene-bismethone were collected, washed with water, and dried. m.p. 186°. Yield, 48.5 mg. (Calcd. for $0.1868\times10^{-3}M=55.10$ mg.). It was proved to be methylene-bismethone by admixture with authentic sample. The reddish brown precipitate (47 mg.) (Calcd. for $0.1868\times10^{-3}M=52.05$ mg.) was methylated with CH_2N_2 and orange yellow needles of m.p. 188~189° were obtained. It was identified as methyl griseoluteate on admixture and by comparison of their infrared spectra.

iii) Determination of formic acid[5]: Griseolutein-B (62.45 mg.$=0.1814\times10^{-3}M$) was dissolved in N $NaHCO_3$ (0.7 cc.) and water (4.5 cc.), $0.1N$ HIO_4 solution (5 cc.) was added, and the mixture was allowed to stand for 1 hr. at room temperature. 0.04 cc. of ethylene glycol was added to the solution to decompose the excess HIO_4, and the mixture was allowed to stand for 1 hr. The solution was adjusted to pH 7.4~7.6 and lyophilized. The residue was dissolved in water (15 cc.) and was added with 1 cc. of a saturated AcONa solution and 0.2 cc. of $4N$ HCl. After the precipitate of griseoluteic acid was filtered off, 2 cc. of 10% $HgCl_2$ solution was added to the filtrate, and the mixture was heated in a boiling water bath in the dark for 1 hr. After cooling to 5°, Hg_2Cl_2 was collected, washed with water, and dried. Yield, 35 mg. (Calcd. for $0.1814\times10^{-3}M=85.44$ mg.).

Summary

Decarboxylation of diacetylgriseolutein-B in qinoline with copper gave 1-methoxy-4-hydroxymethylphenazine. The periodic acid oxidation of griseolutein-B gave griseoluteic acid, formic acid, and formaldehyde. From these results a partial structure of griseolutein-B was presented. The reduction of 1-acetoxy-4-methoxycarbonylphenazine with lithium aluminum hydride which gave 1-hydroxy-4-hydroxymethylphenazine and 1-hydroxy-4-methylphenazine was described.

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110. Shoshiro Nakamura: Studies on Structure of Griseolutein-B, a Streptomyces Antibiotic. III.[2] The Complete Structure.

(*Institute of Applied Microbiology,* University of Tokyo*)

Diacetylgriseolutein-B[1] ($C_{21}H_{20}O_8N_2\cdot H_2O$) can be easily purified and crystallized. It has one methoxyl, two acetyl, and one carboxyl groups but no C-methyl group. Its alkaline hydrolysis gave an acid, named griseoluteic acid.[1] which was crystallized as its methyl ester ($C_{16}H_{14}O_4N_2$) or as a monomethyl ester of its monoacetyl derivative ($C_{18}H_{16}O_5N_2$). Zinc dust

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

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

[2] Part II. S. Nakamura: This Bulletin, **6**, 543 (1958).

distillation[1)] of griseoluteic acid gave phenazine, 1-methoxyphenazine, and 1-methoxy-4-methylphenazine. Decarboxylation[2)] of diacetylgriseolutein-B in quinoline with copper gave 1-methoxy-4-hydroxymethylphenazine and its acetate. Periodic acid oxidation[2)] of griseolutein-B ($C_{17}H_{16}O_6N_2$) gave griseoluteic acid, formic acid, and formaldehyde after consumption of 1 mole of periodic acid. From these results, partial structures for griseoluteic acid and griseolutein-B were proposed in the preceding paper.[2)] In the present work the position of carboxyl group in griseolutein-B was determined and the total structure for griseolutein-B is presented.

Reduction of diacetylgriseolutein-B monomethyl ester[1)] with lithium aluminum hydride gave orange yellow needles, $C_{16}H_{14}O_4N_2$, m. p. 189°, which was identified as methyl griseoluteate on admixture and by comparison of their infrared spectra. It is probable that during this reduction, the coordinated product of methyl griseoluteate and lithium aluminum hydride precipitated immediately and the reduction did not proceed further. This fact indicates that the carboxyl in griseoluteic acid was not newly produced by alkali hydrolysis or periodic acid oxidation of griseolutein-B, but was present originally in griseolutein-B. Strong bands[3)] due to the out-of-plane C-H deformation vibration of three adjacent free hydrogen atoms (13.02 μ) and two adjacent free hydrogen atoms (11.88, 12.20, or 12.50 μ) in aromatic rings were observed in the infrared spectrum[2)] of methyl griseoluteate. As described in a previous paper,[2)] 1-methoxy-4-hydroxymethylphenazine was obtained by decarboxylation of diacetylgriseolutein-B. It is suggested from these facts that the structure of griseoluteic acid would be 1-methoxy-4-hydroxymethyl-9-(or 6)-carboxyphenazine.

It had been confirmed[2)] that 1-acetoxy-4-methoxycarbonylphenazine is reduced to 1-hydroxy-4-hydroxymethylphenazine and further to 1-hydroxy-4-methylphenazine with lithium aluminum hydride. Methyl ester of diacetylgriseolutein-B was reduced on refluxing with lithium aluminum hydride in tetrahydrofuran and yellow needles, $C_{15}H_{14}ON_2$, m. p. 190°, were obtained. Its infrared spectrum (Fig. 1) indicated the absence of hydroxyl or carboxyl and therefore, the needle crystals were suggested to be 1-methoxy-4, 9 (or 4, 6)-dimethylphenazine. The identity of the yellow needle crystals with 1-methoxy-4, 9-dimethylphenazine was confirmed by comparison with the synthetic sample described below, on admixture and also by comparison of their infrared spectra.

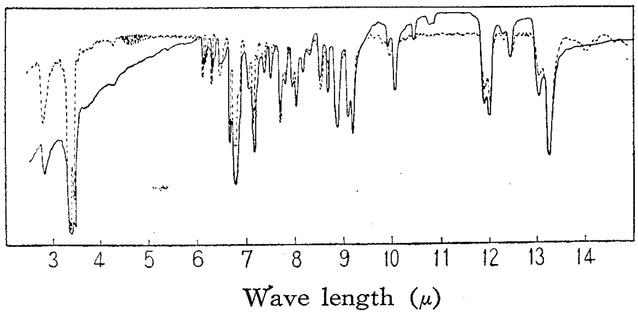


Fig. 1. Infrared Absorption Spectra (in Nujol)

——— Reduction product, $C_{15}H_{14}ON_2$, m. p. 190°

- - - - - 1-Methoxy-4, 9-dimethylphenazine

1-Methoxy-4, 9-dimethylphenazine was prepared by the Wohl-Aue reaction[4,5)] of *o*-toluidine and 2-nitro-4-methoxytoluene, as shown in Chart 1. The reaction product was purified by

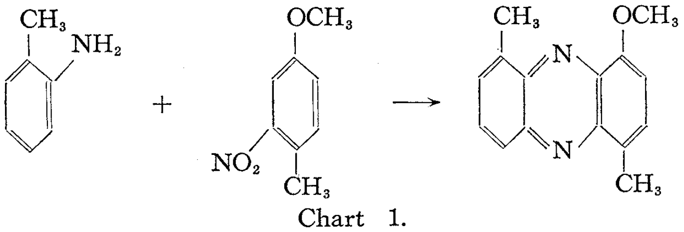


Chart 1.

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

[4)] A. Wohl, W. Aue: Ber., **34**, 2446(1901).

[5)] I. Yosioka: Yakugaku Zasshi, **72**, 1128(1952).

alumina chromatography. Thus, yellow needles, $C_{15}H_{14}ON_2$, m. p. 190°, were obtained, whose infrared spectrum is shown in Fig. 1.

1-Methoxy-4, 6-dimethylphenazine was obtained by the Wohl-Aue reaction of *o*-toluidine and 3-nitro-4-methoxytoluene, as shown in Chart 2.

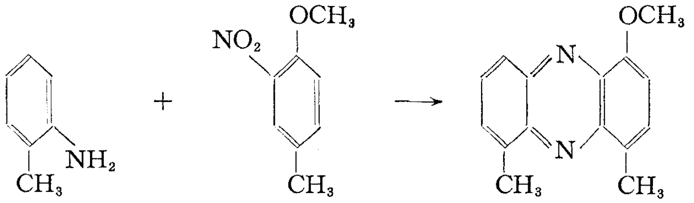
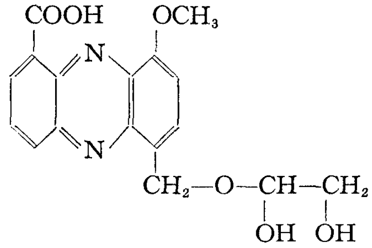


Chart 2.

Purification of the reaction product by alumina chromatography gave yellow crystals, m. p. 164°.

As described above, the product obtained by drastic reduction of griseolutein-B with lithium aluminum hydride was confirmed to be identical with 1-methoxy-4, 9-dimethylphenazine, and not 1-methoxy-4, 6-dimethylphenazine.

As described in the previous paper, the periodic acid oxidation of griseolutein-B ($C_{17}H_{16}O_6N_2$) gave griseoluteic acid, formic acid, and formaldehyde after consumption of one mole of periodic acid. This result indicated that 1, 2-dihydroxyethyl group is linked to hydroxymethyl group of griseoluteic acid. Therefore, griseolutein-B can be represented as 1-methoxy-4-[(1, 2-dihydroxyethoxy) methyl]-9-carboxyphenazine.



It can be attributed to steric hindrances of the substituents that diacetylgriseolutein-B was not reduced to 5, 10-dihydrophenazine[6] compound by catalytic hydrogenation with platinum and did not give a titration value by perchloric acid in nonaqueous solvent. There are no evidence against the structure of griseolutein-B presented here.

Iodinine,[7] pyocinine,[8,9] 1-hydroxyphenazine,[10] 1-carboxyphenazine, and its acid amide[11] have been isolated from cultured broth of some bacteria, but there has been no report on phenazine compounds produced by Actinomycetes. Griseolutein-B is the first phenazine antibiotic found in metabolic products of Actinomycetes. As indicated by the molecular formula and the ultraviolet spectrum shown in the first paper,[1] griseolutein-A must also be a phenazine antibiotic closely related to griseolutein-B. Griseolutein-B exhibits an extremely strong antimicrobial effect compared to phenazine antibiotics derived from bacteria. Diacetylgriseolutein-B inhibited the growth of *Bacillus anthracis, Bacillus subtilis,* and *Micrococcus pyogenes* var. *aureus* at a concentration of 50 γ/cc. but did not inhibit *Escherichia coli, Sarcina lutea, Klebsiella pneumoniae, Salmonella typhi, Proteus vulgaris, Mycobacterium* 607, and *Mycobacterium phlei* even at a concentration of 100 γ/cc.

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[6] L. Birkofer, A. Birkofer: Chem. Ber., **85**, 286 (1952).
[7] G. R. Clemo, H. McIlwain: J. Chem. Soc., **1938**, 479.
[8] F. Wrede, E. Strack: Z. physiol. Chem., **140**, 1 (1924).
[9] F. Wrede, E. Strack: Ber., **62**, 2051 (1929).
[10] J. Zweig: Vet. J., **102**, 55 (1946) [C. A. **42**, 5950 (1948)].
[11] L. Birkofer, A. Birkofer: Klin. Wochschr., **26**, 528 (1948) [C. A. **44**, 8400 (1950)].

This fact indicates that the free hydroxyls in griseolutein-B are essential for antimicrobial activity.

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Experimental

Reduction of Diacetylgriseolutein-B Monomethyl Ester with $LiAlH_4$ in Ether—In a 100-cc. three-necked flask equipped with a stirrer, a dropping funnel, and a condenser, a solution of $LiAlH_4$ (30 mg.) in 10 cc. of ether was placed, cooled in water, and a solution of diacetylgriseolutein-B monomethyl ester (186 mg.) in tetrahydrofuran (10 cc.) and ether (50 cc.) was added dropwise with stirring. After completion of the addition, the reaction mixture (colored reddish brown) was refluxed gently for 1 hr. After decomposition of excess hydride with water, the aqueous phase was extracted twice with benzene. The extracts were combined, purified by alumina chromatography, eluted with AcOEt, and orange-yellow needles (35 mg.), m.p. 188~190° (from benzene-hexane), were obtained. *Anal.* Calcd. for $C_{16}H_{14}O_4N_2$: C, 64.42; H, 4.73; N, 9.39. Found: C, 64.54; H, 4.79; N, 9.29.

No difference was found between these crystals and methyl griseoluteate in both m.p. and infrared absorption spectra.

Reduction of Diacetylgriseolutein-B Monomethyl Ester with $LiAlH_4$ in Tetrahydrofuran—A solution of $LiAlH_4$ (200 mg.) in tetrahydrofuran (10 cc.) was placed in the apparatus as described above and a solution of diacetylgriseolutein-B monomethyl ester (400 mg.) in tetrahydrofuran (20 cc.) was slowly dropped in with stirring. After the addition, the reaction mixture was heated for 5 hrs. under reflux, cooled, and a small amount of water was added to decompose the hydride. The white precipitate of metal hydroxides was filtered off and the filtrate was distilled off *in vacuo* to dryness. The residue was dissolved in benzene, purified by alumina chromatography, and eluted with benzene, and yellow needles (53 mg.), m. p. 189~190° (from benzene-hexane), were obtained. *Anal.* Calcd. for $C_{15}H_{14}ON_2$: C, 75.60; H, 5.92; N, 11.76. Found: C, 75.43; H, 5.95; N, 12.24.

This substance was identified as 1-methoxy-4, 9-dimethylphenazine on admixture with a synthesized sample and by comparing their infrared spectra.

Synthesis of 1-Methoxy-4,9-dimethylphenazine—A mixture of 2-nitro-4-methoxytoluene (10 g.), *o*-toluidine (10 g.), and powdered $NaNH_2$ (8 g.) in toluene (100 cc.) was refluxed for 4 hrs. in an oil bath. After the reaction mixture was filtered, toluene was distilled off *in vacuo* and the residue was steam-distilled. The brown yellow precipitate deposited was dissolved in benzene and extracted 3 times with 10% HCl. The precipitate obtained by neutralization of the acidic solution was dissolved in benzene and purified on alumina. Yellow needles (0.95 g.) were obtained from the fraction eluted with benzene, m.p. 189~190° as recrystallized from hexane-benzene. *Anal.* Calcd. for $C_{15}H_{14}ON_2$: C, 75.60; H, 5.92; N, 11.76. Found: C, 75.67; H, 5.86; N, 11.87.

Synthesis of 1-Methoxy-4,6-dimethylphenazine—A mixture of 3-nitro-4-methoxytoluene (15 g.), *o*-toluidine (15 g.), and finely powdered KOH (40 g.) was refluxed in toluene (60 cc.) for 4 hrs. with stirring. The reaction mixture was treated in the same way as above, and purified on alumina. It gave 1-methoxy-4, 6-dimethylphenazine (0.9 g.) as yellow crystals (from benzene), m.p. 164°. *Anal.* Calcd. for $C_{15}H_{14}ON_2$: C, 75.60; H, 5.92; N, 11.76. Found: C, 75.49: H, 5.94; N, 11.57.

Summary

A drastic reduction of diacetylgriseolutein-B monomethyl ester gave 1-methoxy-4, 9-dimethylphenazine. Griseoluteic acid was assumed to be 1-methoxy-4-hydroxymethyl-9-carboxyphenazine. From these results and the periodate oxidation, griseolutein-B was concluded to be 1-methoxy-4-[(1, 2-dihydroxyethoxy) methyl]-9-carboxyphenazine.

The synthesis of 1-methoxy-4, 6-dimethylphenazine and 1-methoxy-4, 9-dimethylphenazine is described.

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