N-Methyl-N'-(*p*-anisyl)phenylacetamidine Hydrochloride——Obtained from the reaction with *p*-anisidine, mp 136—140° (from acetone–ethanol). Yield, 77.3%. *Anal.* Calcd. for $C_{16}H_{19}N_2OCl$: C, 66.08; H, 6.58; N. 9.63; Cl, 12.19. Found: C, 66.13; H, 6.64; N, 9.82; Cl, 12.51. IR ν_{max}^{KBr} c^{-1}: 1650, 1212, 1023, 825, 750, 720.

N-Methyl-N'-(*p*-chlorophenyl)phenylacetamidine Hydrochloride——Obtained from the reaction with *p*-chloroaniline, mp 243—245° (from acetone–ethanol). Yield, 22%. *Anal.* Calcd. for $C_{15}H_{16}N_2Cl$: C, 61.03; H, 5.46; N, 9.46; Cl, 24.08. Found: C, 61.53; H, 5.59; N, 9.55; Cl, 23.41. IR ν_{max}^{KBr} cm^{-1}: 1650, 810, 742, 704.

N,N'-Diethyl-N-(phenethyl)phenylacetamidine——To a boiling solution of 16.3 g (0.1 mole of N-ethylphenylacetamide in 98 ml of chloroform, 15.4 g (0.1 mole) of phosphoryl chloride was dropwise added in 5 min. The resulting mixture was washed several times with concd. HCl containing 5% $FeCl_3$. The separated chloroform layer was dried over $MgSO_4$. Removal of the drying agent and chloroform gave a viscous liquid, which hardly crystallized. This was dissolved in 100 ml of ethanol and hydrogenated with 3.2 g of 5% palladium-on-charcoal as catalyst under atmospheric hydrogen pressure and at room temperature. After up-take of nearly two molar equiv. of hydrogen, the catalyst was filterd off and the filtrate was concentrated to dryness. The oily residue was treated with cold saturated KOH solution to liberate the free amidine, which were extracted with benzene and the benzene solution was dried over K_2CO_3. Removal of the drying agent and benzene afforded a brown liquid, which was distilled under reduced pressure to give an oil, bp 151—153° (0.018 mmHg), 3.7 g (25% yield) of the product, N,N'-diethyl-N-(phenethyl)phenylacetamide. *Anal.* Calcd. for $C_{20}H_{26}N_2$: C, 81.58; H, 8.90; N, 9.52. Found: C, 81.34; H, 8.77; N, 9.54. NMR (τ in $CDCl_3$): 8.98 (3H, t, J=7.5 Hz, $CH_3CH_2N<$), 8.84 (3H, t, J=7.5 Hz, $CH_3CH_2N=$), 7.14 (2H, q, J=7.5 Hz, $CH_3CH_2N<$), 6.69 (2H, q, J=7.5 Hz, $CH_3CH_2N=$), 6.64 (2H, t, J=6.15 Hz, $C_6H_5CH_2CH_2N$), 6.61 (2H, t, J=6.15 Hz, $C_6H_5CH_2CH_2N$). 6.32 (2H, s, $C_6H_5CH_2$), 2.68 (10H, s, aromatic).

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Fusidic Acid, a Steroidal Antibiotic from *Isaria kogane*[1]

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In an attempt to obtain physiologically active metabilites from Basidiomycetes, we have carried out submerged culture of a number of Basidiomycetes. The fermentation brothes were filtered and the filtrates extracted with ethyl acetate. The extracts from the water and ethyl acetate layers were submitted to screeing tests for the antibacterial activity by the paper disc method using *Staphylococcus aureus* 209P, *Sarcina lutea*, *Bacillus subtilis*, and *Escherichia coli*. As a result, it was found that the ethyl acetate extract of the fermented broth of a Basidiomycetes, *Isaria kogane* Hasegawa et Koyama (IFO 5299) (Hypocreaceae), a mushroom parasitic mainly on larvae and imagos of beetles,[3] showed the strongest antibacterial activity against the Gram-positive bacteria and, in particular, against a strain of

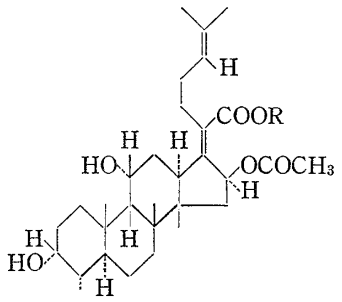
1) This paper is Part II in the series on Fungal Metabolites. Part I: H. Hikino, D. Kuwano, and T. Takemoto, *Yakugaku Zasshi*, **89**, 1149 (1969); This also forms Part XVI in the series on Steroids. Part XV: H. Hikino, Y. Ohizumi, and T. Takemoto, *Yakugaku Zasshi*, in press.
2) Location: Aoba-yama, Sendai.
3) K. Hasegawa and R. Koyama, Ringyo Shikenjo Hokoku, **4**, 1 (1941).

S. aureus, though no activity was noted against the Gram-negative bacterium, *E. coli*.

The organism, *I. kogane* (IFO 5299), was subsequently cultured at 27° under shake condition. Periodical assay of the antibiotic activity by the agar dilution method using *Staphylococcus aureus* as test organism demonstrated that the maximum production of an antibiotic is achieved in about two weeks.

Mycelia were removed from the fermentation broth by filtration and the filtrate was extracted with ethyl acetate, the antibiotic substance in the filtrate being transfered to the organic layer. After evaporating the solvent, the ethyl acetate extract was subjected to chromatography twice over silica gel. From fractions which showed the antibacterial activity, a crystalline antibiotic (I) was obtained, which gave a unique pattern on summarized paper chromatography.[4]

The antibiotic gave a positive Liebermann-Burchard reaction, indicating it to be a steroid or a triterpenoid. Its molecular formula was determined as $C_{31}H_{48}O_6$ by elemental analysis and mass spectroscopy. The nuclear magnetic resonance (NMR) spectrum demonstrates the presence of one secondary methyl and five tertiary methyls. The facts that it behaved as an acid on thin-layer chromatography (TLC) (a positive test with bromothymol blue spray reagent), that the infrared (IR) spectrum exhibits an absorption band at 1715 cm^{-1} and that on treatment with diazomethane it afforded a monomethyl ester (II), show it to contain a carboxyl group. Further, an ultraviolet (UV) absorption at 220 nm defines it to be an α,β-unsaturated acid. IR bands at 1725 and 1250 cm^{-1}, NMR signals at 1.94 (3H) and 5.84 ppm (1H) reveal the presence of a secondary acetoxyl group. That the antibiotic possesses two secondary hydroxyl groups was established by the findings that the IR spectrum discloses a band at 3400 cm^{-1} and the NMR spectrum displays signals at 3.73 (1H), 4.30 (1H), and ~4.1 ppm (2H); the last signal disappeared on addition of deuterium oxide. The accumulated data indicated that the antibiotic was similar in properties to fusidic acid which is produced by a Deuteromycetes, *Fusidium coccineum*.[5] Direct comparison was subsequently performed to corroborate the identity.



I : R=H
II : R=CH_3

All of known steroidal antibiotics such as fusidic acid, cephalosporin P_1,[6] and helvolic acid,[7] are metabolites of only Deuteromycetes and Ascomycetes. Therefore, it is of interest that a Basidiomycetes has now been found to produce a steroidal antibiotic, fusidic acid.

After the identification with fusidic acid of the antibiotic obtained from a strain of *I. kogane*, it has naturally become an interesting subject to examine whether the antibiotic is produced by some other strains of the same genus. Each of the three strains of the *Isaria* genus, such as *I. kogane* (IFO 5711), *I. farinosa* (IFO 7866), and *I. fumoso-rosea* (IFO 7072), was subsequently grown in the same medium in shake cultures for ten days, at which time the culture filtrates were assayed for activity against *S. aureus*. However, no activity was noted in each fermented broth.

4) N. Ishida and J. Miyazaki, *J. Antibiotics Ser. A*, **5**, 481 (1952); J. Miyazaki, K. Omachi, and T. Kamata, *Ibid. Ser. A*, **6**, 6 (1953).
5) W.O. Godtfredsen, S. Jahnsen, H. Lorck, K. Roholt, and L. Tybring, *Nature*, **193**, 987 (1962); W.O. Godtfredsen and S. Vangedal, *Tetrahedron*, **18**, 1029 (1962); W.O. Godtfredsen, W. von Daehne, S. Vangedal, A. Marquet, D. Arigoni, and A. Melera, *ibid.*, **21**, 3505 (1965).
6) *cf.* T.S. Chou, E.J. Eisenbraun, and R.T. Rapala, *Tetrahedron Letters*, **1967**, 409.
7) *cf.* S. Iwasaki, M.I. Sair, H. Igarashi, and S. Okuda, *Chem. Commun.*, **17**, 1119 (1970).

Experimental[8]

Fermentation of *Isaria kogane* and Isolation of Fusidic Acid——Mycelium of *Isaria kogane* HASEGAWA et KOYAMA was cultured at 27° for 2 weeks in a medium containing wood dust (30 g) immersed in a solution (90 ml) containing 2.0% glucose and 0.5% dried beer yeast. The mycelium grown on the surface of the wood dust medium was inoculated to a 500 ml volume flask charged with a medium for submerged culture (100 ml) which contains 5.0% glucose, 0.2% pepton, 0.5% dried beer yeast, 0.2% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, and 1.6% $CaCO_3$.[9] The cultures were grown at 27° for a period of two weeks under shake conditions, the shaker, of the reciprocating type with a stroke length of 7 cm, being operated at rate of 140 strokes per min. The filtrate of the culture broth (11.7 l) was then extracted with AcOEt and the extract was evaporated to give a fermentation product (1.72 g). The harvested fermentation product (1.72 g) was chromatographed over silica gel twice. Fractions showing the activity were combined to afford a crystalline paste (140 mg) which on crystallization from benzene giving fusidic acid (I) as colorless needles (105 mg), mp 133.5—135°. *Anal.* Calcd. for $C_{31}H_{48}O_6 \cdot 1/2\ H_2O$: C, 69.94; H, 9.40. Found: C, 70.12; H, 9.34. Mass Spectrum *m/e*: 516 (M^+), 456, 438, 420, 405, 351, 283, 232, 189, 95, 93, 69, 60, 45, 43, 41. UV λ_{max}^{EtOH} nm (log ε): 220 (3.86). IR ν_{max}^{KBr} cm^{-1}: 3400 (hydroxyl), 1725, 1250 (acetoxyl), 1715 (carboxyl). NMR ($CDCl_3$): 3H s's at 0.90, 0.96, 1.36 ($C_{(18)}\underline{H}_3$, $C_{(19)}\underline{H}_3$, $C_{(32)}\underline{H}_3$), 3H d at 0.91 (J=6, $C_{(30)}\underline{H}_3$), 3H d's at 1.58, 1.66 (J=4, $C_{(26)}\underline{H}_3$, $C_{(27)}\underline{H}_3$), 3H s at 1.94 ($C_{(16)}$-OCOC$\underline{H}_3$), 1H m at 3.73 ($C_{(3)}\underline{H}$), 1H m at 4.30 ($C_{(11)}\underline{H}$), 1H m at ~5.1 ($C_{(24)}\underline{H}$), 1H d at 5.84 (J=9, $C_{(16)}\underline{H}$). The identity was confirmed by comparison of TLC behaviors, and IR and NMR spectra, and by mixed melting point test with an authentic sample.

Methylation of Fusidic Acid——To a solution of fusidic acid (I) (10 mg) in ether was added anethereal solution of CH_2N_2. After 10 min, evaporation of the solvent gave a crystalline mass (10 mg) which was crystallized from ether–hexane to furnish methyl fusidate (II) as colorless prisms (8 mg). mp 136—138°. IR ν_{max}^{KBr} cm^{-1}: 3450 (hydroxyl), 1710, 1230 (ester). Identification was carried out by comparison of TLC behaviors and IR spectra and by mixed fusion test with an authentic sample.

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8) Melting points are uncorrected. NMR spectrum was recorded at 60 MHz. Chemical shifts are expressed in ppm downfield from TMS as internal reference and coupling constants (J) in Hz. Abbreviations: s=singlet, d=doublet, and m=multiplet.

9) T. Takeuchi, H. Iinuma, J. Iwanaga, S. Takahashi, T. Takita, and H. Umezawa, *J. Antibiotics*, **22**, 215 (1969).