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STUDIES IN THE BIOCHEMISTRY OF MICROORGANISMS

Part IX.—Structure of Amudane, Amudene and Amujane Metabolic Products of Penicillium martinsii Biourge

AHMAD KAMAL, SHAHEEN A. HUSAIN, NAJMA MURTAZA, RADIA NOORANI, IZHAR H. QURESHI and ASAF A. QURESHI

P.C.S.I.R. Laboratories, Karachi 39

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The structures of amudane (m.p. 219–220°C), amudene (m.p. 270–72°C) and amujane (m.p. 252°C), the metabolic products of *Pennicillium martinsii* Biourge, have been established to be griseofulvin $C_{17}H_{17}O_6Cl$ (m.p. 220–222°C), dehydrogriseofulvin $C_{17}H_{15}O_6Cl$ (m.p. 270–72°C) and dihydrogriseofulvin $C_{17}H_{19}O_6Cl$ (m.p. 196°C), respectively, through physical methods. Besides these, mannitol has also been isolated.

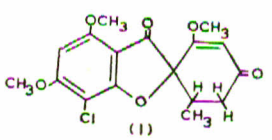
Pennicillium martinsii Biourge[1] grown on semisynthetic media produced six crystalline compounds:[2] amudol, $C_7H_7O_3Cl$, m.p. 146–47°C; sterol, m.p. 156-7°C; amudane, $C_{17}H_{17}O_6Cl$, m.p. 220–222°C; amudene, $C_{17}H_{15}O_6Cl$, m.p. 270–272°C; amujane, $C_{19}H_{17}O_6Cl$, m.p. 196°C; and mannitol, $C_6H_{14}O_6$, m.p. 165°C.

The mold was grown on Moyers medium[3] enriched with carrot extract.[4] Extraction of the broth gave a crystalline product amudol. The structure of amudol has already been reported to be 2,5-dihydroxy-4-chlorobenzyl alcohol.[2] No other crystalline metabolic product was obtained from the broth. The dried mycelium was extracted with petroleum ether, chloroform and finally with ethanol. The petroleum ether extract gave a crystalline product, m.p. 156–7°C. This gave positive Liebermann-Burchard test for sterol.*

The chloroform extract gave a solid residue (1.69 g from 100 g mycelium), m.p. 244–45°C. This solid material gave three compounds by preparative thin-layer chromatography with ammoniacal silver nitrate impregnated plates,[5] the solvent system being benzene–ether (95:5).

Amudane (R_f 0.32, m.p. 220–222°C) crystallized from MeOH–H_2O in colourless needles and analysed for $C_{17}H_{17}O_6Cl$ by high resolution mass measurement. It was found to be optically active $[\alpha]_D^{20°}$ + 350. The UV absorption showed λ_{max} 324, 291, 252, 230 mμ ($\log\varepsilon$ 3.72, 4.31, 4.10 and 4.30). The IR spectrum showed bands at 1705 cm^{-1} (O—C=O) and 1685 cm^{-1} and 1660 cm^{-1} (—CH=CH—). There were two more bands at 1605 cm^{-1} and 1580 cm^{-1}, indicating the presence of benzene ring in the molecule. The PMR spectrum in deutroacetone showed a doublet centred at τ 9.08 (3H; J_{AB_3} 7 c/s) indicating the presence of CH_3CH in the molecule. One multiplet appeared at τ 7.4 (3*H*, one methyl group). Another ill-defined doublet appeared at τ 7.28; (2H; due to AB_2 system) indicative of >CH—CH_2— group in the molecule.

A singlet appeared at τ 6.3 (3H; one vinyl —OCH_3 group). Two more signals appearing at τ 6.02 and τ 5.94 pointed to the presence of two aromatic—OCH_3. There was a sharp singlet at τ 4.5 (1H; due to one vinyl proton in the molecule). In the lower field there was a sharp singlet at τ 3.85 due to one aromatic proton. All these physical and spectral evidence safely accounted for structure I, which is that of a known mold metabolite grisofulvin,[6] first isolated from *Penicillium griseofulvum*, Dierckx,[7] and subsequently from a variety of other Penicillium species.



(I)

The above structure was further confirmed from its mass spectral studies, which showed the molecular ion at m/e 352^+, corresponding to the molecular formula $C_{17}H_{17}O_6Cl$ by high-resolution mass measurement.

The second intense peak appeared at m/e 337^+ (m* 322) was due to loss of one methyl group. This underwent fragmentation to give ions at m/e 310^+ (m* 292) due to the loss of C_2H_3. This was followed by the loss of

*Work on this product is in progress and will be reported later.

carbon monoxide: m/e 282+ (m* 254). Other prominent peaks were at m/e 321+, 319+, 291+, 284+, 267+, 254+, 239+, 215+, 214+, 198+, 197+, 169+, 168+, 138+, 123+, and 83+. The general breakdown sequence is outlined in Chart I.

Amudene (R_f 0.45; m.p. 270–272°C) analysed for $C_{17}H_{15}O_6Cl$, showed the same UV and the IR absorption bands as were observed in the case of griseofulvin with a slight difference. Its UV absorption bands appeared at a higher

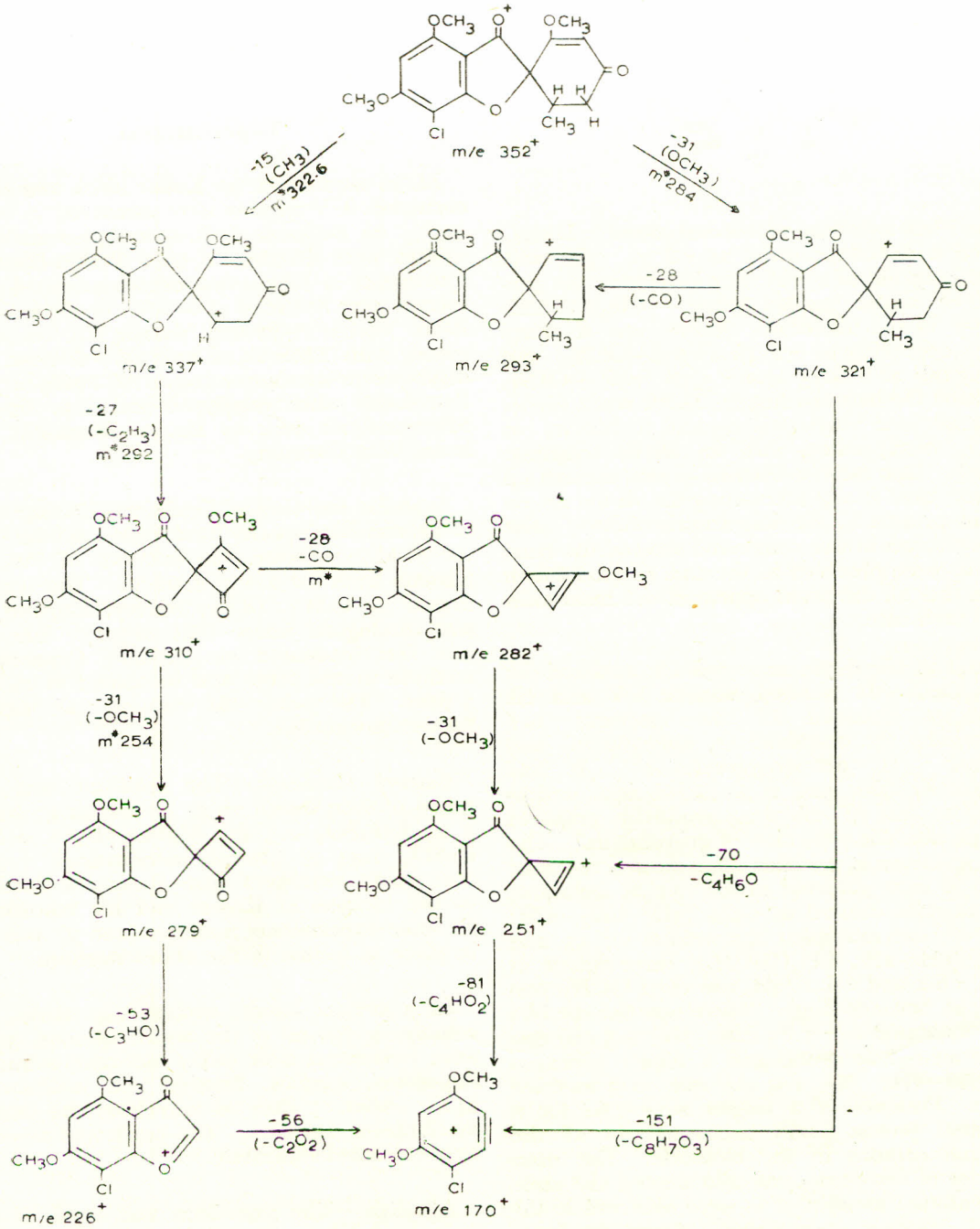
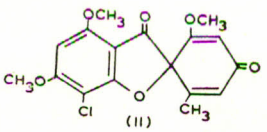


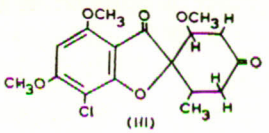
Chart I

wavelength (λ_{max}, 331, 296, 257, 237 mμ (log 3.45, 4.16, 4.02 and 4.18), indicative of the presence of α,β-unsaturated ketone system in the molecule. It could therefore, safely be assumed that amudene was dehydrogriseofulvin (II).[8] This was confirmed by its PMR and mass spectral studies.


(II)

The PMR spectrum of this compound in DMSO showed a doublet centred at τ 8.33, (3H, with allylic coupling of 2 c/s) thus indicating the presence of allylic methyl group —(CH_3)C=CH—O—) in the molecule. There was a sharp singlet appearing at τ 6.48 (vinyl—OCH_3) and two signals at τ 6.15 and τ 5.96 (two —OCH_3 groups on the benzene ring). There was a singlet at τ 4.48 (one vinyl proton) and a doublet at τ 4.26 (vinyl proton with an allylic coupling, J 2 c/s). One more benzenoid proton showed up at τ 3.8, thus finally accounting for all the fifteen protons present in the dehydrogriseofulvin. The mass spectrum of this compound showed the same losses as were observed in the case of griseofulvin except that all the peaks appeared two mass units less in each case.

Amujane (R_f 0.78; m.p. 196°C) analysed for $C_{17}H_{19}O_6Cl$. It showed similar UV and IR spectra as obtained for the previous two compounds, viz. amudane (I, griseofulvin) and amudene (II, dehydrogriseofulvin). It had identical R_f value and m.p. (mixed m.p. undepressed) as that of dihydrogriseofulvin prepared through catalytic reduction of griseofulvin.[7] The PMR spectrum in pyridine (d_6) showed the same signals as were observed in the case of amudane, viz. a doublet centred at τ 9.0 (3H; CH_3—CH; J_{AB_3} 7 c/s) two multiplets appeared at τ 7.55 and τ 7.23 (1H+2H; CH_3CHCH_2), three signals at τ 6.55, τ 6.2 and τ 6.1 (9H, one vinyl OCH_3, two OCH_3 on benzene ring). There was one doublet which appeared at τ 3.85 (2H; J_{AB_2} 1.5 c/s) due to one methylene group and a triplet appeared at τ 3.68 (1H; J_{AB_2} 1.5 c/s) due to a methine proton. Presence of a singlet at τ 3.62 for a benzenoid proton finally accounted for all the seventeen protons in the molecule. The mass spectrum of this compound also showed the same fragmentation ion-species as were observed in the case of amudane (griseofulvin) except the losses which appeared with the two higher mass units than amudane (griseofulvin). These findings support the structure for amujane to be dihydrogriseofulvin[7] (III).


(III)

Experimental

M. ps were taken on Kofler block and are uncorrected. UV spectra were measured in ethanol (95%) on Beckman D.B. spectrophotometer. IR spectra were determined on a Perkin-Elmer 137 instrument in KBr unless otherwise stated. PMR spectra were recorded at 60 Mc/s on Perkin-Elmer R-10, Varian A-60 and Varian HA 100 instruments, with TMS as an internal reference. The mass spectra were measured on AE IM89 at 70CV. Petroleum ether employed had b.p. 65–86°C. Microanalyses were by Dr. A. Bernhardt, Mulheim, West Germany.

Organism and Cultural Condition.—During studies of Pennicillium species from West Pakistan one of us (N.M.) reported the isolation of *Penicillium martinsii* from the soil of Karachi. This was confirmed by C.M.I. (Kew, Surrey, England) and was catalogued under IMI 125917. The mold was first incubated on ordinary Czapeck–Dox medium in test tubes and incubated at 24°C for 9 days. The 9-day old culture was used for further inoculation.

Cultural Conditions.—The medium was composed of corn starch 20 g; lactose 44 g; $NaNO_3$ 3 g; KH_2PO_4 0.5 g; $MgSO_4.7H_2O$ 0.25 g; $ZnSO_4$ 0.44 g; glucose monohydrate 2.75 g; and carrot extract 1 litre (prepared according to the method of Kamal and his coworkers).[4] In some experiments molasses (120 g) was used in place of lactose in the above medium.

In a typical batch, twelve 1-l. flasks, each containing 350 ml of the above medium (pH 5) were inoculated with 9-day old tube cultures of *Penicillium martinsii* Biourge and incubated at 24°C. After 17 days the mycelium was removed by filtration (suction). The broth and mycelium were extracted separately.

Mycelium.—The mycelium was dried at 60°C (204 g of dried material from 12 flasks). In a typical experiment the dried mycelium (100 g)

was continuously extracted in a Soxhlet extractor using: (a) petroleum ether (2 days), (b) chloroform (2 days) and (c) ethanol (4 days).

(a) *Petroleum Ether (65–86°C) Extract.*—After extraction (2 days) and removal of solvent a colourless product was obtained which crystallized from ethyl ether to yield colourless prismatic needles, m.p. 156–57°C, 0.51 g, which gave a positive Liebermann-Burchard test for sterols.

(b) *Chloroform Extract.*—After extraction with petroleum ether the mycelium was extracted with chloroform (2 days). Removal of the solvent gave a colourless solid; m.p. 244–45°C, 1.67 g, which gave a positive Beilstein test for chlorine.

The solid material was separated into three fractions, viz. amudane (R_f 0.32), amudene (R_f 0.45) and amujane (R_f 0.78), using ammoniacal silver nitrate (5%) impregnated keiselgel G plates[5] (solvent system: benzene–petroleum ether 95:5).

Amudane.—Amudane was crystallized from ethyl acetate–ether in colourless needles, m.p. 221°C, 0.96 g. It was soluble in ether, ethyl acetate, methanol and ethanol, sparingly soluble in chloroform and carbon tetrachloride and insoluble in petroleum ether and water.

It was identified as griseofulvin by its mixed m.p. (undepressed) and comparison with IR and UV spectra of authentic sample of the material and their identical R_f values.

Found: C, 58.05; H, 4.98; O, 27.24; Cl, 10.07%. Calc. for $C_{17}H_{17}O_6Cl$: C, 57.95; H, 4.8; O, 27.2; Cl, 9.98%.

Amudene.—Amudene was crystallised from chloroform–ether from which it was obtained in colourless prismatic needles, m.p. 270–72°C, 0.5 g. It had almost the same solubility as griseofulvin and was found to be dehydrogriseofulvin from its mixed m.p. (undepressed) and comparison with the IR and UV spectra of an authentic sample of the material and their identical R_f values.

Found: C, 57.99; H, 4.32; O, 27.51; Cl, 10.21%. Calc. for: $C_{17}H_{15}O_5Cl$: C, 58.28; H, 4.2; O, 27.4; Cl, 10.12%.

Amujane.—Amujane was purified by repeated preparative thin-layer chromatography. (R_f 0.78, ammoniacal silver nitrate (5%) keiselgel G plates,[5] benzene–petroleum ether 95:5) m.p. 196°C, 16 mg, was identified as dihydrogriseofulvin from its mass spectrum and P.M.R. studies and also from its identical R_f value and m.p. undepressed on admixture with a synthetic sample of the material prepared through catalytic reduction of griseofulvin.[7]

Mannitol.—After the extraction with chloroform, the mycelium was exhaustively extracted with ethanol (30 hr) in a Soxhlet apparatus. On removal of the solvent a colourless crystalline residue was obtained. On repeated recrystallisations from ethanol it was obtained as colourless needles, m.p. 164–65°C (0.6 g). It gave positive tests for carbohydrates and was identified as mannitol through its mixed m.p. (undepressed) and comparison of its *Rf* value with an authentic samples of the sugar.

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