

MICROBIAL CHEMISTRY

Part III. Isolation and Identification of the Metabolic Products of *Penicillium funiculosum* Thom*. The Chemistry of Funiculosic Acid

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Penicillium funiculosum Thom, grown on a semisynthetic medium has been shown to produce diethyl phthalate and a phthalaldehydic acid, $C_9H_8O_5$, for which the name funiculosic acid has been proposed. The chemistry of funiculosic acid (II) has been investigated and its synthesis achieved.

INTRODUCTION

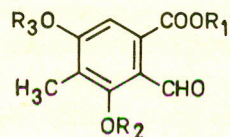
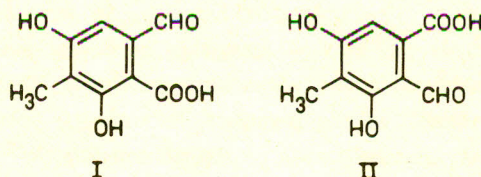
During screening of indigenous mycoflora, a strain of *Penicillium* series was isolated by one of us (N.M.) which was identified and confirmed as *Penicillium funiculosum* Thom (IMI-157575). In the earlier reports this mould has been shown to produce heleine [1], mitorubrin [2] and related compounds, funiculosin [3], funicone [4] and deacetoxymannin [5].

In the present studies, the culture filtrate of *Penicillium funiculosum*, grown on Czapeck-Dox medium enriched with carrot extract [6] was extracted with ethyl acetate. The extract was then separated into acidic and neutral fractions. The neutral fraction furnished an oily product with pleasant smell, b.p. 295° . If analysed for $C_{12}H_{14}O_4$. In the IR spectrum it showed bands at ν_{max} 1723 (C=O, ester), 1600, 1580 cm^{-1} (aromatic stretching vibrations). It gave a positive hydroxamic acid test for esters and on alkaline hydrolysis furnished phthalic acid, m.p. 206° (dec). The neutral oily product was, therefore, identified as diethyl phthalate, confirmed by superimposed IR spectrum with that of authentic sample. Isolation of phthalic acid esters from microbial sources is not very common. However, in an earlier report Cross *et al.* [7] have described the isolation of dimethyl phthalate from *Gibberella fugikuroi*.

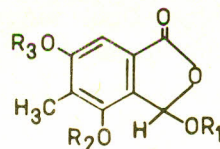
From the acidic fraction a crystalline acid, m.p. 260° (dec) was obtained for which the trivial name funiculosic acid has been proposed.

Funiculosic Acid. Funiculosic acid, obtained as yellow rods from n-propanol-water m.p. 260° (dec), is optically inactive. It analysed for $C_9H_8O_5$ (M^+ 196). The proposed structure II follows from its chemical degradation and spectral studies. Funiculosic acid contains one -COOH group. A positive silver mirror test and formation of 2,4-

DNP was indicative of the presence of -CHO group. A violet to red colouration with ferric chloride was compatible with a *meta*-diphenol system. The PMR spectrum of funiculosic acid revealed the presence of a methyl group attached to a benzene ring, δ 2.05 (3H, s. Ar-CH₃) and only one aromatic proton δ 7.1 (1H, s.).



- III $R_1=CH_3$ $R_2=R_3=H$
 IV $R_1=C_2H_5$ $R_2=R_3=H$
 V $R_1=C_2H_5$ $R_2=R_3=Ac$



- VI $R_1=R_2=R_3=H$
 VII $R_1=R_2=R_3=Ac$
 VIII $R_1=H$ $R_2=R_3=CH_3$
 IX $R_1=R_2=R_3=CH_3$

Esterification of funiculosic acid (EtOH/ H_2SO_4) afforded the corresponding ethyl ester (IV), m.p. 165° . Acetylation of IV with acetic anhydride-pyridine furnished O,O-diacetyl derivative V, $C_{15}H_{16}O_7$, m.p. 92° . However, when funiculosic acid was acetylated under similar

*Pakistan J. Sci. Ind. Res. 19, 120 (1976) may be regarded as Part II of the series.

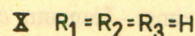
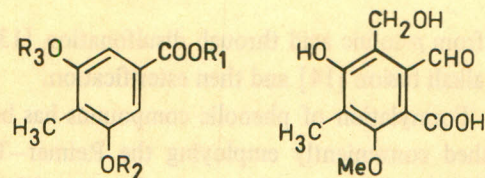
condition, instead of the expected diacetoxy derivative, a triacetate VII $C_{15}H_{14}O_8$ (M^+ 322) m.p. 195° was obtained. The IR and PMR spectra of VII showed absence of $-CHO$ and $-COOH$ groups. The presence of absorption bands at ν_{max} 1789 cm^{-1} ($C=O$, lactol) and particularly the signal due to the methine proton $-O-CH-OAc$ appearing at δ 7.4 (1H,s) in the PMR spectrum were compatible with structure VII.

Similarly methylation of funiculosic acid with methyl iodide and potassium carbonate afforded an O,O-dimethyl derivative VIII, m.p. 173° and an oily product believed to be IX. However, due to scarcity of material IX could not be purified and hence was not studied further. The IR spectrum of VIII showed absence of $-CHO$ and $-COOH$ groups. The presence of bands at ν_{max} 3401 ($-OH$) and 1764 cm^{-1} ($C=O$, lactol) were compatible with structure VIII.

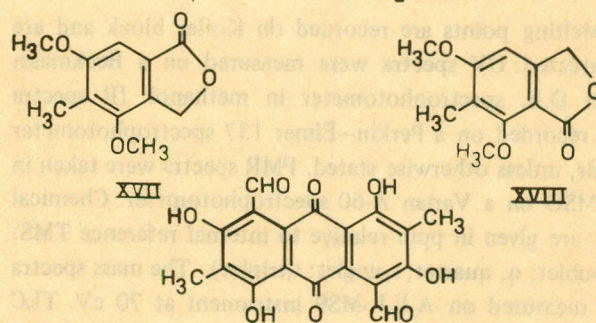
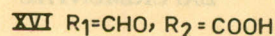
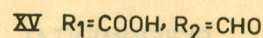
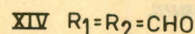
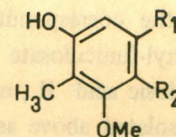
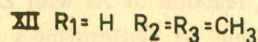
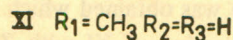
The formation of lactols VII and VIII implies that the $-CHO$ and $-COOH$ groups are located *ortho* to each other. Also there exists strong intramolecular hydrogen bonding between the $-CHO$ and the adjacent phenolic $-OH$ in funiculosic acid, II, which inhibits the formation of lactol and accounts for the stability of the molecule. Removal of hydrogen bonding in II by acetylation or methylation favours the formation of lactol derivatives VII and VIII, respectively.

The reaction of aldehydes with fused alkali resulting in disproportionation or cleavage has been successfully employed in structural elucidation of natural products. Birkinshaw and his colleagues [8] reported the isolation of 3,5-dihydroxy-*p*-toluic acid X by reacting cyclopic acid XIII with fused alkali. Similarly in our hands funiculosic acid on reaction with fused alkali also furnished 3,5-dihydroxy-*p*-toluic acid X. The later compound proved very useful since, with the exception of $-CHO$ group, it clearly indicated the relative positions of all the substituents in the benzene ring of funiculosic acid. Placing the remaining formyl function *ortho* to the carboxyl group will give structure II for funiculosic acid. However, the evidence elaborated so far can not exclude the possibility of the alternate structure I, because on reaction with fused alkali it would also give X as in the case of cyclopic acid XIII.

Structurally related to I and II, are the aldehydic acids XV and XVI, reported by Birkinshaw and his co-workers [9] as the selective oxidation products of quadri-lineatin XIV. On reduction followed by methylation, XV furnished 4,6-dimethoxy-5-methyl phthalide XVII, m.p. 157° , while the isomeric acid XVI afforded 5,7-dimethoxy-6-methyl phthalide XVIII, m.p. 172° . Both the phthalides have been independently synthesised by Birch *et al.* [10].



XIII



XIX

Similarly the O,O-dimethyl derivative, 4,6-dimethoxy-3-hydroxy-5-methyl phthalide VIII on reduction with zinc - sulfuric acid furnished a compound m.p. 156° identified as phthalide XVII and confirmed by direct comparison with an authentic sample of XVII prepared by the action of chloromethyl ether on XII according to the procedure of Birkinshaw *et al.* [9]. The formation of 4,6-dimethoxy-5-methyl phthalide XVII suggests that funiculosic acid is a derivative of *para*-toluic acid and hence structure II was assigned to it. Incidentally Sassa *et al.* [11] reported isolating from an unidentified mould a methyl ester $C_{10}H_{10}O_5$ which on alkaline hydrolysis afforded an acid m.p. 260° (dec) identical with our funiculosic acid.

Funiculosic acid when heated above its melting point dimerises to a reddish compound, λ_{max} 535 nm which gives a reddish colour in alkali and an orange colour in sulfuric acid. The dimer is believed to have structure XIX which is consistent with the observation of Mody and Shah [12].

Synthesis of Funiculosic Acid. The key step in the synthesis of funiculosic acid is the formylation of 3,5-dihydroxy-*p*-toluic acid methyl ester XI which was obtain-

ed from *p*-toluic acid through disulfonation [13] followed by alkali fusion [14] and then esterification.

Formylation of phenolic compounds has been accomplished conveniently employing the Reimer–Tiemann or Gattermann reaction. In our hands, when XI was treated with CHCl_3 – NaOH , under Reimer–Tiemann conditions, a coloured resinous residue was obtained which could not be crystallised. However, reaction of XI with $\text{Zn}(\text{CN})_2$ – AlCl_3 – HCl (dry) according to Gattermann's procedure readily furnished the corresponding 2-formyl derivative, identical with methyl-funiculosate III, which on hydrolysis afforded funiculosic acid II, m.p. 260° (dec) identical with the sample isolated above as the metabolic product of *Penicillium funiculosum*.

EXPERIMENTAL

Melting points are recorded on Kofler block and are uncorrected. UV spectra were measured on a Beckmann model D.B. spectrophotometer in methanol. IR spectra were recorded on a Perkin–Elmer 137 spectrophotometer in KBr, unless otherwise stated. PMR spectra were taken in d_6 -DMSO on a Varian A-60 spectrophotometer. Chemical shifts are given in ppm relative to internal reference TMS: (d, doublet; q, quartet; s, singlet; t, triplet). The mass spectra were measured on A.E.I.-MS9 instrument at 70 eV. TLC was carried out on silica gel-PF254. The petroleum ether used refers to the fraction with b.p. 60 – 80° .

Organism. *Penicillium funiculosum* Thom was isolated by one of us (N.M.) from the Karachi soil and was confirmed by the Commonwealth Mycological Institute, England and catalogued under No: IMI-157575.

Penicillium funiculosum was first inoculated on ordinary Czapeck–Dox medium in test tubes and incubated at 27° for 9 days. The nine-day old culture was then used to inoculate flasks containing the culture medium.

Culture Conditions. The modified Czapeck–Dox medium used was composed of glucose, 50.0 g; NaNO_3 , 3.0g; KH_2PO_4 1.0 g; KCl, 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g and aqueous carrot extract [6], 1 liter.

In a typical batch 50 (1 l) conical flasks containing 350 ml of the above medium were autoclaved at 10 lb pressure for 20 min then inoculated with the nine-day tube culture of *Penicillium funiculosum* till there was no more consumption of glucose, determined polarimetrically. The mycelia were separated by filtration and the broth (13.8 l; pH 6) was repeatedly extracted with ethyl acetate. The combined extracts were dried (an Na_2SO_4), concentrated under reduced pressure and extracted with sodium bicarbonate solution. The neutral ethyl acetate layer (Fraction A) and the alkaline aqueous layer (Fraction B) were worked up

separately.

Isolation of Diethylphthalate. The neutral ethyl acetate layer (Fraction A) was evaporated to dryness under reduced pressure and the resulting oily residue was thoroughly extracted with petroleum-ether by refluxing. The combined extracts were washed with dil sodium bicarbonate solution, then with water and finally dried (an Na_2SO_4). Removal of solvent furnished diethyl phthalate as pleasant smelling oil (300 mg), b.p. 295° . ν_{max} 1723 (C=O, ester), 1600, 1580 cm^{-1} (aromatic). (Found: C 64.55, H 6.4%. $\text{C}_{12}\text{H}_{14}\text{O}_4$ requires: C 64.85, H 6.35%).

Hydrolysis: Diethylphthalate (100 mg) in ethanol (5 ml) was added to a solution of KOH (140 mg) in ethanol (2.5 ml) and the mixture refluxed for 2 hr. On usual work-up, it afforded white crystals of phthalic acid, (25 mg) m.p. 206° lit [7] m.p. 212 – 213° .

Isolation of Funiculosic Acid. The alkaline aqueous layer (Fraction B) was acidified (pH 1) with 2N HCl and extracted with ethyl acetate. The organic layer was washed with water, dried (an Na_2SO_4), and solvent removed. The resulting solid on crystallisation from *n*-propanol–water, gave yellow rods of funiculosic acid, (600 mg), m.p. 260° (dec) λ_{max} 225 (log ϵ 4.314), 290 nm (log ϵ 4.302). ν_{max} 3425 (–OH, phenolic), 1695 (C=O, aldehyde and carboxyl groups), 1626, 1587 cm^{-1} (aromatic). δ 2.05 (3H, s, Ar-CH_3); δ 5.6 (1H, broad –COOH), δ 7.1 (1H, s, aromatic), δ 10.4 (1H, s, –CHO), δ 12.8 (2H, broad singlet $2 \times$ –OH phenolic); m/e 196 (M^+), 178, 168, 167, 150 (base peak), 123, 122. (Found: C 54.76, H 4.1%. $\text{C}_9\text{H}_8\text{O}_5$ requires: C 55.11, H 4.11%).

Funiculosic acid is soluble in methanol, ethanol, ethyl acetate and acetone; sparingly soluble in benzene, ether and hot water; insoluble in chloroform and petroleum ether.

Methyl Funiculosate (III). Funiculosic acid (100 mg), methanol (10 ml) and concd sulfuric acid (0.1 ml) were refluxed on a water-bath for 5 hr. On working up in the usual manner and crystallisation of the resulting solid from benzene furnished methyl funiculosate III (40 mg), m.p. 170° (M^+ 210), ν_{max} 3400 (–OH), 1680 (C=O, ester), 1638 cm^{-1} (C=O, aldehyde), λ_{max} 230, 250 and 299 nm. δ 2.36 (3H, s, aromatic CH_3), δ 3.77 (3H, s, –CO– OCH_3) δ 11.56 (1H, s, CHO), δ 14.0 (2H, broad, $2 \times$ –OH).

Ethyl Funiculosate (IV). Funiculosic acid (50 mg), ethanol (5 ml) and concd sulfuric acid (0.1 ml) were refluxed on a water-bath for 4 hr. On working up in the usual manner crystallisation of the gummy solid from aqueous ethanol furnished ethyl funiculosate IV, (25 mg) m.p. 165° , ν_{max} 3165 (–OH), 1718 (C=O, ester); 1706 cm^{-1} (C=O, aldehyde). δ 1.4 (3H, t, J 7.5 Hz, CH_2CH_3). δ 2.1 (3H, s, Ar-CH_3); δ 4.35 (2H, q, J 7.5 Hz, – CH_2 – CH_3); δ 7.04 (1H, s, aromatic); δ 10.32 (1H, s, –CHO). δ 12.82

(2H, s, 2x-OH), m/e 224 (M^+), 196, 195 (base peak), 179, 177, 151, 150, 149, 123, 122, 121.

Ethyl O,O-Diacetyl Funiculosate (V). A solution of ethyl funiculosate (IV, 25 mg) in acetic anhydride (2.5 ml) and pyridine (2.5 ml) was refluxed for 2 hr and worked up in the usual manner to give ethyl O,O-diacetyl funiculosate. (V, 20 mg), m.p. 92° (from benzene-petroleum ether). ν_{\max} 1754 ($-\text{OCOCH}_3$); 1706 cm^{-1} (C=O, ester and aldehyde). (Found: C 58.24, H 5.51%. $\text{C}_{15}\text{H}_{16}\text{O}_7$ requires: C 58.44, H 5.23%).

3,4,6-Triacetoxy-5-methyl Phthalide (VII). A solution of funiculosic acid (II, 50 mg), acetic anhydride (5 ml) and pyridine (5 ml) was allowed to stand at 37° for 6 days. The reaction mixture was then poured into ice-water and the solid obtained on crystallisation from benzene-petroleum ether afforded 3,4,6-triacetoxy-5-methyl phthalide (VII, 40 mg), m.p. 195° . λ_{\max} 280, 270, 224 nm. ν_{\max} 1789 (C=O, lactol); 1751 cm^{-1} (O-COCH₃); δ 2.13 (6H, s, Ar-CH₃ and OCOCH₃); δ 2.36 (6H, s, 2x OCOCH₃); δ 7.4 (1H, s -OCHOAc), δ 7.53 (1H, s, aromatic proton) m/e 322 (M^+), 262, 236, 220, 219, 196, 195, 194, 180, 179 (base peak), 151, 150, 84. (Found: C 55.99, H 3.9%. $\text{C}_{15}\text{H}_{14}\text{O}_8$ requires: C 55.90, H 4.38%).

4,6-Dimethoxy-3-hydroxy-5-methyl Phthalide (VIII). A mixture of funiculosic acid (II, 650 mg), anhydrous potassium carbonate (4.25 g), methyl iodide (4 ml) and dry acetone (50 ml) was refluxed for 24 hr. Acetone was removed under reduced pressure, the residue was diluted with water and extracted with ether. The aqueous phase on acidification with 2N HCl, furnished O,O-dimethyl funiculosic acid (VIII, 200 mg), m.p. 173° (from methanol-water). ν_{\max} 3401 ($-\text{OH}$); 1764 (C=O, lactol); 1629 and 1587 cm^{-1} (aromatic). (Found: C 58.64, H 5.3%. $\text{C}_{11}\text{H}_{12}\text{O}_5$ requires: C 58.8 H 5.35%).

Reduction of O,O-Dimethyl Funiculosic Acid (VIII). To a solution of O,O-dimethyl funiculosic acid (VII, 100 mg) in methanol (2.5 ml) was added zinc dust (200 mg) and sulfuric acid (50% v/v, 1.5 ml) and warmed for 15 min. The solution was filtered and the filtrate was diluted with water; chilled and the resulting solid crystallised from ethanol to give colourless needles of 4,6-dimethoxy-5-methyl phthalide (XVII, 25 mg), m.p. 154° ; lit [10] m.p. 157° . λ_{\max} (EtOH) 217 (log ϵ 4.537), 255 (log ϵ 3.834) and 296 nm (log ϵ 3.449); ν_{\max} 1727 (C=O, lactol), 1629, 1577 cm^{-1} (aromatic).

Alkali Fusion of Funiculosic Acid. Potassium hydroxide (2.5 g), water (0.5 ml) were heated to 150° (under nitrogen) in a nickel crucible and to it funiculosic acid (500 mg) was added during 15 min. The bath temperature was raised and maintained at $310\text{--}320^\circ$ for 20 min. The brownish melt was cooled, diluted with water (50 ml)

and after acidification (pH 2) with 2N HCl, calcium chloride (200 mg) dissolved in water (2 ml) was added and the solution was allowed to stand overnight. The precipitated calcium oxalate was filtered off and the filtrate was extracted with ether. Removal of solvent and sublimation of the residue afforded 3,5-dihydroxy-*p*-toluic acid (200 mg), m.p. $240\text{--}45^\circ$ (dec.); lit [14]. m.p. $245\text{--}50^\circ$ (dec).

Preparation of 4,6-Dimethoxy-5-methyl Phthalide (XVII). 3,5-Dimethoxy-*p*-toluic acid [14] (100 mg), glacial acetic acid (1.5 ml) and chloromethyl ether (1 ml) were heated on water bath for 10 hr. After addition of 1N HCl (25 ml), the reaction mixture was refluxed for another 1 hr. The resulting solid on crystallisation from ethanol furnished white needles of 4,6-dimethoxy-5-methyl phthalide (XVII, 20 mg), m.p. 156° (lit [10] m.p. 157°).

*Preparation of Methyl-3, 5-dihydroxy-*p*-toluate (XI)*. 3,5-Dihydroxy-*p*-toluic acid (500 mg), methanol (25 ml) and concd sulfuric acid (0.25 ml) were refluxed under anhydrous conditions for 6 hr. On working up in the usual manner, a solid was obtained which on crystallization from benzene furnished methyl-3, 5-dihydroxy-*p*-toluate (XI, 170 mg), m.p. 188° (lit [8] m.p. $189\text{--}190^\circ$).

Formylation of (XI). Into a 2-necked (50 ml) round-bottomed flask, kept in freezing mixture and fitted with a reflux condenser and a gas bubbler, were added methyl-3, 5-dihydroxy-*p*-toluate (XI) [9,14] 100 mg dissolved in dry ether (10 ml) and freshly prepared zinc cyanide (150 mg). The reaction mixture was stirred magnetically and to it was added aluminium chloride (150 mg) in chilled dry ether (25 ml). The dry hydrogen chloride gas was then bubbled through this mixture for 45 min. The reaction mixture was then allowed to stand overnight, the supernatant liquid was decanted off and to the pasty residue, water (2 ml) was added and the mixture was boiled for 5 min. On cooling, a solid was separated which on crystallisation from benzene gave methyl-2-formyl-3, 5-dihydroxy-*p*-toluate (30 mg), m.p. 170° identical with methyl funiculosate III.

Hydrolysis. Methyl-2-formyl-3,5-dihydroxy-*p*-toluate (III, 15 mg) and 1N NaOH solution (0.2 ml) were kept together for 20 hr at room temperature. The reaction mixture was diluted with water (5 ml), acidified and extracted with ethyl acetate. Removal of solvent and crystallisation of the solid obtained from *n*-propanol-water furnished 2-formyl-3, 5-dihydroxy-*p*-toluic acid (8 mg), m.p. 260° (dec) identical with funiculosic acid (II).

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