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MICROBIAL CHEMISTRY

Part IV. Isolation and Identification of the Metabolic Products of *Aspergillus ustus* (Bainier) Thom and Church NMR Studies of Cyclopaldic Acid

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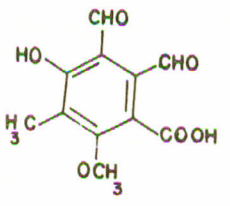
Aspergillus ustus grown on semi-synthetic medium has been shown to elaborate cyclopaldic acid, fumaric acid, succinic acid, ergos-terol, stearic acid, mannitol and a yellow solid which was resolved into seven crystalline compounds through preparative tlc. Additional evidence based on NMR is presented to show that cyclopaldic acid exists predominantly in the lactol Form I-a.

INTRODUCTION

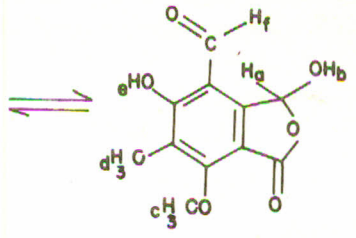
Aspergillus ustus, (Bainier) Thom & Church[1], first isolated in 1926 is a very versatile mold and has been shown to elaborate several metabolites under different cultural conditions. In 1951 Raistric and Sticking first reported the isolation of ustic acid [2] from this mold grown on Czapek-Dox medium. Later reports describe the isolation of dioxopiperazines[3], austadiol[4], versicolorine, averufin and autocystins [5,6] from this mold grown on maize meal.

In the present studies metabolic products of *Aspergillus ustus* grown on Czapeck-Dox medium enriched with carrot extract[7] have been investigated. The ethylacetate extract of the culture filterate on removal of solvent afforded a gummy solid shich was repeatedly extracted with ether. The ether soluble fraction on removal of solvent furnished a colorless crystalline compund, mp 216 – 7°, $[\alpha]_D^{28}$ + 116. It analysed for $C_{11}H_{10}O_6$ (M^+ 236) and gave a purple colour with $FeCl_3$ indicating the presence of a phenolic (-OH) group. Effervescence with $NaHCO_3$ solution suggested the presence of a (-COOH) group while a positive silver mirror test and formation of 2,4-DNP was indicative of the presence of a (-CHO) group. The compound was indentified as cyclopaldic acid I, a known metabolite of *Penicillium cyclopium*[8]. The presence of an absorption band at ν max 1740 cm^{-1} (C = O, lactol) and particularly the signal due to the methine proton Ha, δ 6.92 (IH, S) and the tertiary alcoholic -OHb, δ3.36 (IH, hump) in the NMR spectrum supports the view that cyclopaldic acid exists predominantly in the lactol form I-a. It may be noted that funiculosic acid II,[9] exists in the open form. The intra-molecular hydrogen bonding between the -CHO and the adjacent phenolic -OH in II inhibits lactolization and accounts for the stability of the molecule. On the other hand in cyclopaldic acid the -CHO group located *ortho* to -COOH group is free from hydrogen bonding which fascilitates lactolization. The strong intramolecular hydrogen bonding between the -CHO located *meta* to -COOH group and the adjacent phenolic -OH is evident from the fact that the signal due to phenolic $-OH_e$ moved down field appearing at δ8.36 in the NMR spectrum (See Table -1).

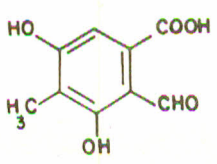
The ether insoluble fraction on fractional crystallization from ethanol afforded colorless crystals m.p, 200° (Sublimed). It was identified as fumaric acid by direct comparision with an authentic sample of fumaric acid.



(I)



(I-a)



(II)

The mother liquor of fumaric acid furnished another crop of crystals m.p. 180° indentified as succinic acid by direct comparision with an authentic sample.

Mycelium: The petroleum ether extract of mycelium on removal of solvent gave a yellow solid and an oily material. This oily material on saponification, extraction of the unsaponifiable fraction with ether and removal of solvent afforded colorless crystals, m.p. 165 – 6° $[\alpha]_D^{28}$ – 171, λ max 290, 268 nm. It gave positively Lieberman Burchard test for sterols and was identified as ergosterol by direct comparision with an authentic sample.

The saponified fraction on acidification (pH 1) and subsequent work up gave colorless crystalline solid m.p 56-7°. It gave tests for fatty acids and was identified as stearic acid, confirmed by identical R_f values and undepressed mixed m.p. with an authentic sample.

The methanol extract of mycellium on removal of

Table 1. NMR Spectrum of cyclopaldic acid.

S.No.,	Chemical shift	Multiplicity	Protons	
1.	2.06	Singlet	Ar–CH_3	(d)
2.	4.10	Singlet	–OCH_3	(c)
3.	3.36*	Hump	–OH	(b)
4.	6.92	Singlet	–CH	(a)
5.	8.36*	Singlet	–OH	(e)
6.	10.2	Singlet	–CHO	(f)

*These Signals disappear on addition of D_2O.

Table 2. Physical and spectral data of the fractions obtained from preparative t1c of the yellow solid.

S.No.	Fractions	mp. °C	R_f	Chlorine test	IR ν max	UV λ max
1.	Compound I Yellow needles.	245	0.85	Positive	3000,1650,(γ-pyrone) 1625,1610,1585, 1275 Cm^{-1}	365,325,272,268 245,235 nm.
2.	Compound II Yellow prisms.	224	0.76	Negative	3280,3029,1650, (γ–pyrone), 1610, 1585, 1270 Cm^{-1}	360,320,275,263 255,219 nm.
3.	Compound III Yellow needles.	255	0.68	Positive	3150,1656, (γ–pyrone) 1610,1585,1270 Cm^{-1}	322,300,280,263, 243,216 nm.
4.	Compound IV Colorless needles.	196–7	0.50	Positive	1667, (γ– pyrone), 1620 1610,1587, 1270, Cm^{-1}	335,280,247, 219 nm.
5.	Compound V Yellow prisms.	233–4	0.42	Negative	3465,3180,1650, (γ–pyrone), 1625,1610 1585,1270 Cm^{-1}	360,320,263,255, 247,219 nm.
6.	Compound VI Yellow needles	186–7	0.31	Positive	3080,1655, (γpyrone) 1610,1585,1270 Cm^{-1}	320, 300,280,263, 243,219 nm.
7.	Compound VII Yellow needles.	229	0.20	Positive	3080,1650, (γ–pyrone) 1605,1575,1275 Cm^{-1}	322,300,280,263 243,219 nm.

solvent furnished a colorless crystalline compound, m.p. 165°. It was identified as mannitol by direct comparision with an authentic sample. It formed hexaacetate, m.p. 126°.

The crude yellow solid on purification through preparative tlc. afforded seven crystalline compounds (see Table-2). Their IR and UV spectra are comparable with xanthone like structures. It was interesting to note that five of these compounds bear a covallently bonded chlorine atom. Structure elucidation of these compounds will form the subject of a separate communication.

EXPERIMENTAL

Melting points were taken on Gallenkamp melting point apparatus and are uncorrected. IR spectra were recorded on Perkin Elmer 137 spectrophotometer in KBR, unless other wise stated. UV spectra were measured on Beckmann model DB spectrophotometer in methanol. NMR spectra were taken in $CDC1_3$ on a Varian A-60 spectrophotometer. Chemical shifts are given in ppm relative to internal reference TMS. The mass spectra were measured on A.E.I.-MS9 instrument at 70eV. The petroleum ether used refers to the fraction with b.p. 45-60°.

Aspergillus ustus was first inoculated on ordinary Czapeck-Dox agar salants in test tubes and incubated at 24° for nine days. This 9-day-old culture was then used to inoculate flasks containning the culture medium.

Cultural Conditions: The modified Czapeck-Dox medium used was composed of glucose, 50.0g; $NaNO_3$, 3.Og; KH_2PO_4, 1.Og; KC1, 0.5g; $MgSO_4$, $7H_2O$, 0.5g; $FeSO_4$, $7H_20$, 0.01g and aqueous carrot extract [7] to make a volume of one litre.

In a typical batch 30 (1 litre) conical flasks containing 350 ml of the above medium (pH= 5.0) were autoclaved at 10 1b psi for 20 min then inoculated with the 9-day-old tube culture of *Aspergillus ustus* and incubated at 24° for 21 days until, the residual glucose was exhausted. During incubation, broth (10 ml) was drawn asceptically, first after a week and then every third day, for pH and optical rotation measurements. The data is recorded in Table 3.

After 21 days incubation, the mycelium was separated by filtration and the broth (8.3 litre) was acidified (pH 1) with 2N-HC1 and repeatedly extracted with ethylacetate. The combined extracts were dried over Na_2SO_4 (anhyd) and solvent was removed *invacuo*. The resulting gummy solid (1.0 g) was thoroughly extracted with ether to give (a) ether soluble fraction and (b) ether insoluble fraction.

Isolation of Cyclopaldic Acid: The ether soluble fraction on removal of solvent and crystallization of the resulting solid from ethanol furnished colorless fluffy needles of cyclopaldic acid 1 (25 mg) m.p. 216–7° $[\delta]_D^{28}$ + 116 (C, 0.5 in $CHCl_3$) .ϒ max 3540, 3029, 1740, 1690, 1656, 1610, and 1587 Cm^{-1}. λ max 290 (log ε 3.2814) and 250 nm (log ε 2.3651). δ 2.06 (3H, S, Ar-CH_3). δ 4.1 (2H, S, -OCH_3), δ 3.36 (H, hump-OH alcoholic), δ 6.92 (IH, S, methine proton), δ 8.36 (IH, S, -OH Phenolic) δ 10.2 (IH, S, -CHO); *m/e* 236 (M+). Found: C, 55.3: H, 4.3% $C_{11}H_{10}0_6$ requires C, 55.47; H, 4. 27%.

Isolation of Fumaric Acid and Succinic Acid: The ether insoluble fraction on crystallization from ethanol afforded colorless needless of fumaric acid (110 mg), m.p. 200° (sublimed).

The mother liquor on concentration furnished another crop of crystals which on recrys-tallization from ethanol yielded succinic acid 200 mg, m.p. 180°.

The mycelium (100 g from 30 flasks) was dried overnight in the oven at 60°, powdered and extracted in a soxhlet apparantus with: (a) Petroleum ether, (b) Ethylacetate. (c) Methanol.

Petroleum Ether Extract: The mycelium was extracted with petroleum ether for 30 hr. Removal of solvent and filtration of the residue yielded (a) an oily material (8.5g) and (b) a yellow solid (3g).

Isolation of Ergosterol: The oily material (2.0g) and 15 % alcohol in potassium hydroxide (30 ml) were refluxed for 2 hr in an atmosphere of nitrogen. The solvent was removed *in vacuo,* the residue was diluted with water and extracted with ether. Removal of solvent and crystallization of the unsaponifiable residue with ethanol afforded color-

Table 3. Measurement of pH and residual glucose during incubation period.

S.No.	Days of incubation	pH	Angle of rotation	Residual glu %
1.	1 st	5.0	2.56	4.86
2.	8 th	5.7	1.12	2.10
3.	15 th	7.8	0.85	1.60
4.	17 th	8.0	0.5	0.90
5.	19 th	8.0	0.2	0.28
6.	21 st	8.0	0.0	0.00

less needles of ergosterol (65 mg), mp 165-6°, $[\delta]_D^{28}$ – 171 (C, 2.0 in $CHCl_3$), ν max 3333 (–OH), 1640 (CH =CH) λ max 290 (log ϵ 3.57), 278 (log ϵ 3.82) and 268 nm (log ϵ 3.80).

Isloation of Stearic Acid: The aqueous saponified fraction was acidified (pH 1) and the resulting solid on filtration (log ϵ 3.80).

Isloation of Stearic Acid: The aqueous saponified fraction was acidified (pH 1) and the resulting solid on filtration and crystallization from aqueous methanol afforded colorless needless of stearic acid (50 mg) m?. 57-7°. ν max 3154 (–OH) and 1709 (C=O, acid).

Ethylacetate Extract: Removel of solvent gave a small amount of the same yellow solid which was obtained from petroleum ether extract and hence they were mixed together for purification.

Methanol Extract: The mycelium was extracted with methanol for 22 hr. Removal of solvent furnished a solid which on recrystallization form ethanol gave crystals of mannitol (200 mg) m.p. 165° ν max 3356 Cm^{-1} (–OH). It formed hexa-acetate, m.p. 126°.

Purification of Yellow Soild: Preparative thin layer chromatography (Silica gel) of the yellow compound, using petroleum ether-benzene mixture as eluant furnished seven crystalline compounds as detailed in Table 2, in the text.

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