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STUDIES IN THE BIOCHEMISTRY OF MICROORGANISMS

Part XVIII.—Isolation of Kamusol and Structure of Kojic Acid, Mannitol, Kojic Acid Methyl Ether, Methyl Octa-2,4,6-triene-1-carboxylate and Tetronic Acid, Metabolic Products of Aspergillus sulphureus

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Isolation of six metabolites of *Aspergillus sulphureus* is described. Structures of five of these, viz. kojic acid, mannitol, kojic acid monomethyl ether, methyl octa-2,4,6-triene-1-carboxylate, and tetronic acid, are established while the sixth metabolite *kamusol*. $C_7H_{14}O_7$ has been characterised as a pentahydroxy compound.

Aspergillus sulphureus Thom and Church has been isolated from the soil of different parts of Pakistan. However, no attempt was made uptil now, to study its metabolic products. In the present communication we have described the isolation of a number of metabolites when this particular mold was grown on Czapek–Dox medium enriched with carrot extract as described earlier.[1] The mycelium was golden yellow on the top, while the reverse side was slightly brown in colour. The spores multiplied very fast, were intense yellow in colour but did not give any pigment. It is interesting to note that when this mold was grown on media like Findlay's, Raulin and Thom with or without carrot extract, or Czapek–Dox medium without carrot extract, it hardly produced any metabolites.

Broth

Kojic acid.—The broth on extraction with ethyl acetate gave a crystalline residue, which through repeated crystallizations yielded a product which analysed for $C_6H_6O_4$ and melted at 152°C. Its UV spectrum showed λ_{max} at 265 nm (ε 80230) and with a drop of NaOH it showed a bathochromic shift of 43 nm, giving a band at λ_{max} 308 nm, which indicated the presence of a diketone system in the molecule. The original spectrum was regained with a drop of acid.

The IR spectrum showed an absorption band at 1660 cm^{-1} for the carbonyl stretching of a γ-pyrone system. Two sharp bands appeared at 1630 and 1608 cm^{-1} for the double bonds present in the molecule. Other bands appeared at 3260 and 3180 cm^{-1} for the hydrogen-bonded—OH.

The PMR spectrum in DMSO (d_6) showed a doublet and a triplet at τ 5.6 (2H, —C*H*$_2$OH, J_{AB_2} 7 Hz) and τ 4.3 (1H, —CH$_2$O*H*, J_{AB_2} 7 Hz) respectively. Two sharp singlets appeared at τ 3.4 (1H) and τ 2.0 (1H) indicating the presence of a methine proton. A sharp singlet appeared at τ 1.2 for phenolic OH which alongwith the triplet at τ 4.3 disappeared on deuteration, indicating two hydroxyl protons. The above data, leads to the structure of kojic acid, which is a known metabolite and has been isolated from a number of molds, such as *Aspergillus flavus*,[2] *Aspergillus indicus*,[3] and *Aspergillus stellatus* Curzi.[4]

This structure was further confirmed by taking mixed melting point with an authentic sample which was undepressed and by its mass spectral studies. Its mass spectrum showed a molecular ion peak at m/e 142$^+$, which underwent fragmentation to lose 16 (O), 17 (OH), 18 (H_2O) and 29 (CHO) mass units to give the ionic species at m/e 126$^+$, 125$^+$, 124$^+$, and 113$^+$ respectively. The ionic species m/e 113$^+$ underwent a loss of 28 (—CO) mass units to give the ionic species m/e 85$^+$. This ion further underwent a loss of 13 (CH) giving rise to a species m/e 72$^+$. The rest of the significant peaks were at 97$^+$, 96$^+$, 86,$^+$ 83$^+$, 69$^+$, 68$^+$, 57$^+$, 43$^+$, and 39$^+$. All these fragmentation ions are shown in Chart 1 which are supported by their appropriate metastable peaks.

The mother liquor after the removal of kojic acid on standing gave mannitol m.p. 164°C, mixed m.p. with an authentic sample undepressed. The UV, IR spectra were found to be identical.

O-*Methyl Ether of Kojic Acid.*—The mother liquor obtained after the removal of mannitol, was subjected to preparative TLC to obtain four other compounds. Three of the above compounds were in such minute quantities that they could not be isolated sufficiently pure for spectroscopic studies. The fourth compound obtained in a somewhat major quantity had m.p. 160°C and analysed for $C_7H_8O_4$. Its UV spectrum showed absorption bands at λ_{max} 260 nm(ε 8003). On addition of a drop of a drop of aq. NaOH there was no bathochromic shift, indicating the absence of acidic hydroxyl group. Its IR spectrum showed strong absorptions bands at 3180 cm^{-1} (free OH group) and 1660 cm^{-1} (—O—$\overset{|}{C}$=O). Two sharp bands appeared at 1620 and 1586 cm^{-1} for the double

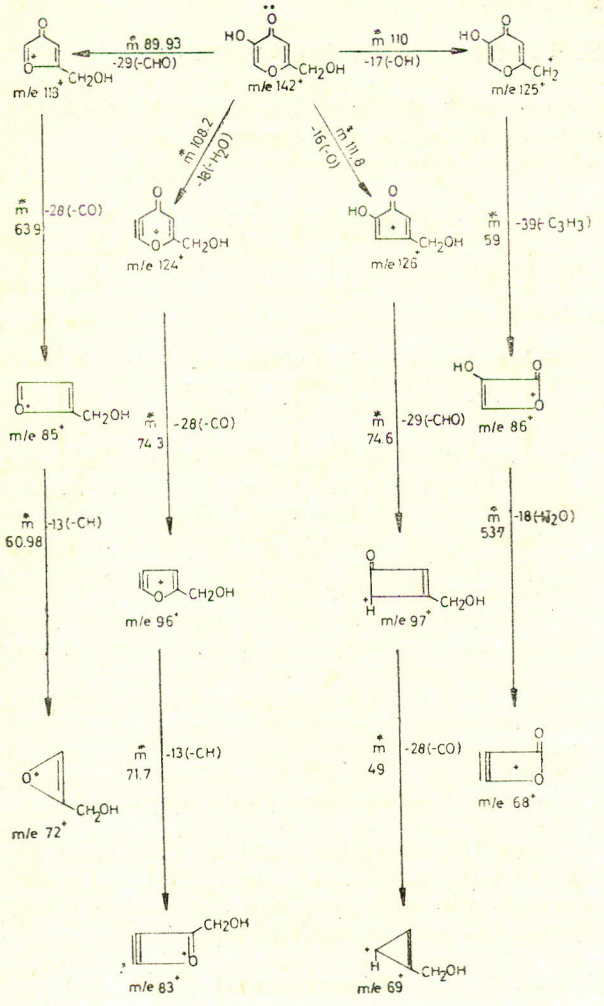

Chart 1.—Mass fragmentation pattern of kojic acid.

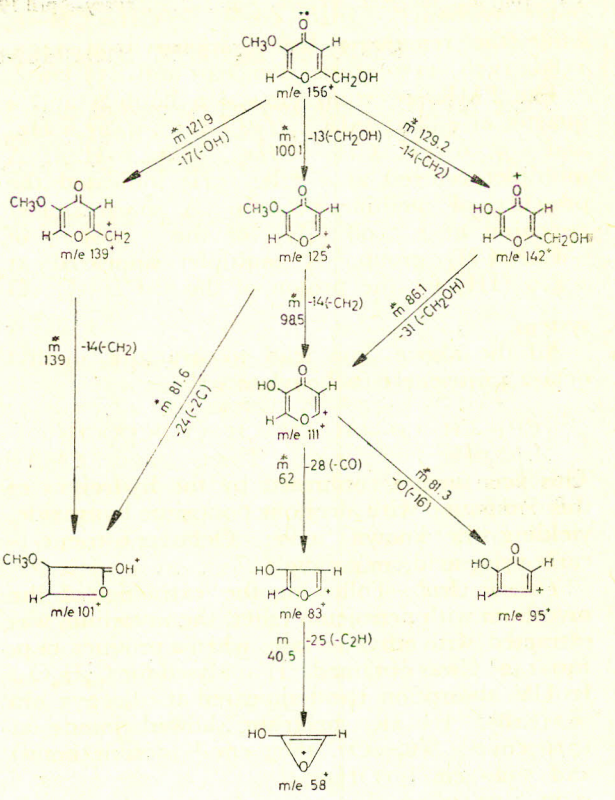

Chart 2.—Mass fragmentation pattern of kojic acid methylether.

bonds present in the molecule. The PMR spectrum in DMSO (d_6) showed a sharp singlet at τ 6.3 (3H) indicating the presence of a methoxyl group in the molecule. There was a doublet at τ 6.8 (2H, —C*H*$_2$OH, J_{AB_2} 7 Hz) and a triplet at τ 4.4 (1H, —CH$_2$O*H*, J_{AB_2} 7 Hz). Two singlets at τ 3.7 (1H) and τ 1.9 (1H) showed the presence of two olefinic protons. The lower value for these protons suggested the presence of some hetero atom or a double bond in adjacent environments. All these signals lead to the structure of *O-methylkojic acid*,[5] not so far reported as a metabolite. This was further confirmed by its mass spectral studies. It showed a molecular ion peak at m/e 156^+ which underwent fragmentation to lose 14 (—CH$_2$) and 17 (—OH) mass units to give peaks corresponding to the molecular ion species $C_6H_6O_4^+$ (m/e 142^+) and $C_7H_7O_3^+$ (m/e 139^+). This is a common feature of —OCH$_3$ group that when it is bombarded with an electron beam, only 14 mass units are lost leaving behind a proton on the oxygen. The molecular ion m/e 156^+, also loses 31 mass units (—CH$_2$OH), forming ionic species at m/e 125^+ (base peak). Other peaks appeared at m/e 138^+, 126^+, 122^+, 112^+, 111^+, 110^+, 101^+, 95^+, 83^+, 72^+, 70^+, 69^+ and 55^+. The whole fragmentation pattern is outlined in Chart 2. The structure of *O*-methylkojic acid was further confirmed by methylating an authentic sample of kojic acid with diazomethane and the product obtained was found to be identical to the isolated metabolite reported above.

Myeclium

Methyl Octa-2,4,6-triene-1-carboxylate.—The dried mycelium on extraction with petroleum ether gave a crystalline product, m.p. 155–157°C which analysed for $C_9H_{12}O_2$. Its UV absorption spectrum gave bands at λ_{max} 290 nm (ε42220), 278 nm (ε 10550), 268 nm (ε 60320) and a shoulder at 257 nm (ε 35720), which are characteristic bands for octatriene.[6] Its IR spectrum showed bands at 2870 cm^{-1} (—CH$_2$—CH<), 1735 cm^{-1}

(ester carbonyl), 1640 cm^{-1} (—CH=CH—), while the remaining bands appeared at 1450, 1380, 1370, 1150, 1050, 960, 940 and 850 cm^{-1}.

The PMR spectrum showed a doublet and a quartet at τ 8.62 (3H, C*H*$_3$—CH<, J_{AB_3} 6 Hz) and τ 5.50 (1H, CH$_3$—C*H*<, J_{AB_3} 6 Hz). A multiplet centred at τ 7.82 (5H) indicated the presence of methine protons. A sharp singlet appeared at τ 5.98 (3H) for the protons of —COOCH$_3$ group. A multiplet appeared at τ 4.0 (1H) for the proton of the =C*H*—C=O system.

All the above data lead to structure methyl octa-2,4,6-triene-1-carboxylate:-

$$CH_3—CH=CH—CH=CH—CH=CH—COOCH_3$$

This was further confirmed by the hydrolysis of this compound with alcoholic potassium hydroxide, yielding the known acid: Octa-2,4,6-triene-1-carboxylic acid, m.p. 189°C.[7]

Tetronic Acid.—Following the extraction of the mycelium with petroleum ether, the mycelium was extracted with ethyl acetate, when a product m.p. 140–142° Cwas obtained. It analysed for $C_4H_4O_3$. Its UV absorption band appeared at λ_{max} 275 nm (ε 1818). Its IR spectrum showed bands at 1710 cm^{-1} (>C=O), 1663 cm^{-1} (double bond) and 3320 cm^{-1} (OH).

The PMR spectrum gave a sharp singlet at τ 4.97 (1H) for a methine proton and another singlet at τ 6.00 (2H) for methylene protons, the compound was identified as tetronic acid.[8] The mass spectrum showed a molecular ion peak at m/e 100$^+$ which readily lost 17 OH mass units, giving rise to an ionic species at m/e 83$^+$, which further underwent fragmentation to lose 28 mass units (>C=O) to give a stable four membered unsaturated cyclic ether at m/e 55$^+$. A further loss of 14 mass units (—CH$_2$) gave a three-membered cyclic ether species at m/e 41$^+$.

γ-Lactone characteristically loses 28 mass units (>CO) when subjected to electron bombardment and this was observed in this case also, giving rise to the ionic species at m/e 72$^+$. The rest of the fragmentation ions appeared at m/e 71$^+$, 57$^+$ and 56$^+$ as shown in Chart 3.

Final extraction of the mycelium with methanol gave colourless rectangular plates, m.p. 158–59°C. It analysed for $C_7H_{14}O_7$ and ad $[\alpha]_D^{25}$— 9.09 in water. Its UV spectrum in water showed λ_{max} at 260 nm(ε 30). Its IR spectrum showed very strong absorption bands in the region 3140~3320 cm^{-1} for OH groups. Total absence of any bands in the carbonyl region and the region above 1500 cm^{-1} was clearly indicative of a simple aliphatic type of compound. We have named this compound *kamusol*. It gave a penta-acetyl derivative $C_{17}H_{24}O_{12}$, which melted at 107–108°C and gave

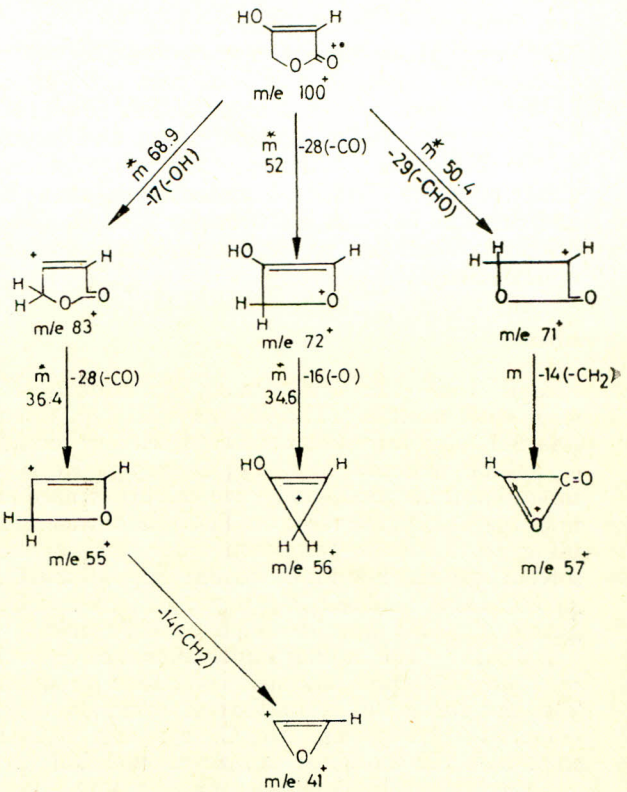

Chart 3.—Mass fragmentation of tetronic acid.

a sharp IR absorption band at 1740 cm^{-1} for O.CO.CH$_3$ and 3410 cm^{-1} (OH). Further work on the structureof this compoundis continuing, and will be reported later.

Experimental

M. ps were taken on Kofler block and are uncorrected. UV spectra were measured in methanol on a Beckman DB-spectrophotometer. IR spectra were determined with a Perkin-Elmer 237 E instrument in KBr unless otherwise stated. PMR spectra were recorded at 60 MHz on a DP-60 Varian Instrument containing TMS as an internal reference. Mass spectra were measured on AE1, MS9 at 70 eV. Petroleum ether used had b.p. 66–86°C.

Cultural Conditions.—Aspergillus sulphureus (Thom & Church) was first grown on ordinary Czapek–Dox medium in test tubes at 24°C for 7 days. The seven-day old culture was then used to inoculate the culture medium. The medium used was composed of glucose 50 g; KH_2PO_4 1g; KCl, 0.5g; $MgSO_4.7H_2O$, 0.5g; $FeSO_4.7H_2O$, 0.1g; $NaNO_3$, 3g; aqueous extract of 200 g of carrots prepared according to the method of Kamal *et al.*[1] and volume made upto 1 l.

In a typical batch, conical flasks (1-l capacity) each containing 350 ml of the above medium

and autoclaved at 10 lb/in^2 pressure (20 min, pH 5) were inoculated with seven-day old culture of *Aspergillus sulphureus* and incubated at 24–27°C for three weeks. A thick mycelial felt developed which was dark yellow in colour on top and brown on the reverse side. After 3 weeks the mycelium was removed by filtration from the broth (12.25 l) and dried at 60°C in the oven for 4 days. The dried mycelium (215 g) was powdered and worked up separately.

Broth

Kojic Acid.—The broth 12.25 l was extracted with ethyl acetate. The extract was then dried (Na_2SO_4) and the solvent removed, leaving behind crystalline material m.p. 135°C, 3.5 g. Recrystallisation from methanol, gave colourless needles m.p. 152°C, 2.5 g. R_f value on TLC plates was 0.11 using ethyl acetate–petroleum ether (3:1) as the solvent system. (Found: C, 50.88; H,4 .46 and O, 44.66%. Calc: $C_6H_6O_4$, C, 50.71; H, 4.26 and O, 45.03%.) This was identified as kojic acid.

Mannitol.—The mother liquor after the removal of kojic acid gave another crystalline product. This was purified by crystallisation from methanol; colourless plates, m.p. 164°C, 250 mg. Mixed m.p. with the authentic sample of mannitol was undepressed. Hexaacetate, m.p. 121°C, was identical to the hexaacetate of the authentic sample of mannitol both from its m.p. and mixed m.p. (undepressed).

Kojic Acid Methyl Ether.—The solvent in the mother liquor left after the removal of mannitol was removed. The residue was then subjected to TLC on Merck's Kieselgel PF 254 with ethyl acetate–petroleum ether (2:1) as the solvent system. Four bands appeared on the plates, three of which could not be isolated. A fourth product was isolated through extraction with methanol. Removal of solvent gave a crystalline material, 320 mg. It was crystallised by dissolving it in methanol and adding ether to it. Kojic acid methyl ether crystallised out from the solution, m.p. 158°C, 290 mg. Mixed m.p. taken with an authentic sample of kojic acid methyl ether was undepressed. (Found: C, 52.58; H, 5.45; O, 41.97%. Calc. for: $C_7H_8O_4$: C, 53.85; H, 5.16; O, 40.99%.)

Mycelium.

Methyl Octa-2,4,6-triene-1-carboxylate.—The mycelium was removed and dried in an oven at 40–50°C. This was then powdered and extracted with petroleum ether in a soxhlet apparatus. On evaporation of the solvents a semisolid residue was obtained which on crystallisatio from heexane gave rectangular crystals, m.p. 155–157°C, 80 mg. (Found: C, 70.89; H, 7.65 and O, by diff. 46%. Calc. for $C_9H_{12}O_2$: C, 71.03; H, 7.95 and O (by diff) 21.02%.)

Octa-2, 4, 6-triene-1-carboxylic Acid.—Methyl octa-2,4,6-triene-1-carboxylate (200 mg) was taken up in ethanolic KOH (10%, 20 ml) and heated (2hr) on a water bath (N_2 atmosphere). Removal of solvent, acidification with 2N HCl, extraction with ethyl acetate, drying (Na_2SO_4) and removal of solvent gave octa-2,4,6-triene-1-carboxylic acid. Colourless needles from methanol–ether mixture. M.p. 189°C (152 mg). Lit.[7] m.p. 188–188.5°C.

Tetronic Acid.—The mycelium already extracted with petroleum ether as above was subsequently extracted with ethyl acetate (Soxhlet) (54 hr.) Removal of the solvent yielded a crystalline compound in the for mof colourless needles. M.p. 140–141°C, 54 mg. (Found: C, 48.08; H, 4.01; O, 47.91%. Calc. for: $C_4H_4O_3$; C, 48.28; H, 4.13; O, 47.96%.) It was identified as tetronic acid.

Kamusol.—After the extraction of the mycelium with ethyl acetate it was finally extracted with methanol (48 hr). Evaporation of the solvent yielded a reddish brown oil which was taken up in water and then methanol was added to it. A crystalline material was obtained which was recrystallised in the same manner from water and methanol. Colourless needles, m.p. 158–59°C, 60 mg; $[\alpha]_D^{25}$—9.09 (0.6% in methanol). (Found: C, 39.73; H, 7.49; O, (by diff) 52.78%. $C_7H_{14}O_7$ requires: C, 40.00; H, 6.71; O, 53.28%.)

Pentaacetyl Kamusol.—Kamusol (50 mg) was taken up in freshly distilled pyridine (2 ml) containing acetic anhydride (4 ml) and heated on a water bath (½ hr) and left overnight. The product was poured over crushed ice, extracted several times with ethyl acetate. The combined ethyl acetate extract was washed with 2N HCl and dried (Na_2SO_4). Removal of solvent gave *pentaacetyl kamusol* which on recrystallisation from ether–petroleum ether came out in colourless diamond—shaped crystals, m.p. 107–8°C, 55 mg. (Found: C, 48.80; H, 6.08 and O, 45.12%, $C_{17}H_{24}O_{12}$ requires: C, 48.57; H, 5.76 and O, 45.68%.)

Methylation of Kojic Acid.—Kojic acid (100 mg) was taken up in ether treated with excess of diazomethane and left overnight. Kojic acid methyl ether which separated out was recrystallised by taking it up in methanol and adding ether to the solution. M.p. 160°C. 80 mg (Found: C, 53.31; H, 5.25; O, (by diff) 41.44%. Calc. for $C_7H_8O_4$: C, 53.85; H, 5.16 and O, 40.99%.)

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