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STUDIES IN THE BIOCHEMISTRY OF MICROORGANISMS

Part XX.—Isolation and Characterization of 2, 5-Dimethoxybenzoquinone, Tartronic acid, Itaconic Acid, Succinic Acid, Pyrocalciferol, Epifriedlinol, Lanosta-7,9(11)-24-triene-3-β-21-diol, Trichodermene-A*, Methyl 2,4,6-octa-trienecarboxylate, Cordycepic Acid and Mannitol—Metabolic Products of Trichoderma pseudokoningii

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The isolation of eleven metabolites from *Trichoderma pseudokoningii* (IMI No. 130748) are described. They are characterized as 2,5-dimethoxy-benzoquinone (I), $C_8H_6O_4$, m.p. 250°C; tartronic acid (II), $C_3H_4O_5$, m.p. 163°C; itaconic acid (III), $C_6H_6O_4$, m.p. 162–164°C; succinic acid (IV), $C_4H_6O_4$, m.p. 185–187°C; pyrocalciferol (V), $C_{28}H_{44}O$, m.p. 93–95°C, $[\alpha]_D^{25}$ +506°; epifriedlinol (VI), $C_{30}H_{52}O$, m.p. 280°C, $[\alpha]_D^{25}$ + 24°; lanosta-7,9(11)-24-triene-3β-21-diol (VII), $C_{30}H_{48}O_2$, m.p. 194–197°C, $[\alpha]_D^{25}$ +72°; *trichodermene-A* (VIII), $C_{19}H_{28}$, b.p. 85°C; methyl 2,4,6-octatrienecarboxylete (IX), $C_9H_{12}O_2$, m.p. 158C; cordycepic acid, $C_7H_{12}O_6$, X m.p. 168°C, $[\alpha]_D^{25}$ +6.8°; and D-mannitol(XI) mainly through physical methods using UV, IR, PMR and mass spectral analyses.

During screening of several molds isolated from soil for their metabolites, *Trichoderma pseudokoningii* (IMI 130748) was obtained. The mold, when grown on different synthetic media like Czapek–Dox[1] modified Czapek–Dox with diammonium tartarate as nitrogen source,[2] and corn starch media enriched with carrot extract,[3] yielded eleven metabolites in different quantities. The best yield was obtained when the mold was grown on corn starch medium enriched with carrot extract for 4 weeks at 24–27°C. The mycelium was separated from the broth and dried in an oven at 50°C for 24 hr. The broth was extracted thoroughly from ethyl acetate. The combined ethyl acetate extract was separated into sodium bicarbonate-insoluble and sodium bicarbonate-soluble fractions.

Metabolites from Broth

(a) *Sodium Bicarbonate-insoluble Fraction*

The sodium bicarbonate-insoluble fraction gave a yellow compound; m.p. 250°C (20 mg), which analysed for $C_8H_8O_4$. It showed visible and UV absorption band at λ_{max} 375 nm (ε 1608) and 282 nm (ε 4057), which is a typical spectrum for quinones, closely resembling that of 4-methoxy-benzoquinone. Its IR absorption bands appeared 2924 cm^{-1}, 1698 cm^{-1} (> CO vibrations), 1645 and 1608 cm^{-1} for the presence of two double bonds.

The PMR spectrum in $CDCl_3$ showed sharp singlets at τ 6.1 (6H) and τ 4.12 (2H) for protons of two $-OCH_3$ groups and two vinylic protons repectively. The above data can be easily accounted for 2,5-dimethoxybenzoquinone (I). This is a known metabolite isolated from *Polyporus fumosus*.[4] The above structure was confirmed by its detailed mass spectral studies, which showed a molecular ion peak at m/e 168^+. It readily losses 28 (—CO) and 30 ($—CH_2O$) mass units to give rise to ionic species at m/e 140^+ and 138^+ respectively, which again underwent fragmentation to lose 30 ($—OCH_2$) and 13 (>CH—) mass units, giving rise to ionic species at m/e 110^+ and 125^+ respectively. It is interesting to note that *O*-methyl ether group loses 15 ($—CH_3$) mass units giving rise to ionic species at m/e 153^+ from the molecular ion. This ion readily loses 28 (—CO) mass units giving rise to ionic species at m/e 125^+.

The ionic species formed at m/e 138^+ underwent successive losses of 26 ($—C_2H_2$), 28 (—CO) and 31 ($—OCH_3$) mass units, giving rise to charged species at m/e 112^+, 84^+ and 53^+ respectively. Similarly the ionic species formed at m/e 140^+ loses 30 ($—OCH_2$), 13 (—CH), 17 (—OH) and 18 (H_2O) mass units, giving rise to peaks at m/e 110^+, 97^+, 80^+ and 79^+ respectively. The last species being formed due to the migration of one proton. The other prominent peaks were at m/e 111^+, 97^+ and 66^+. The mass fragmentation pattern is shown in the Chart 1. Most of the speciess. were followed by their appropriate metastable peaks.

(b) *Sodium Bicarbonate-soluble Fraction from Broth*

The aqueous bicarbonate extract after removal of the neutral fraction above, gave on acidification

* This compound has been named trichodermene-A to distinguish it from trichodermene of Abrahamsson and Nilsson [13] who have reported its isolation from a strain of *Trichoderma*'. They however do not mention the name of the species.Trichodermene-A has no structural relationship with trichodermene.

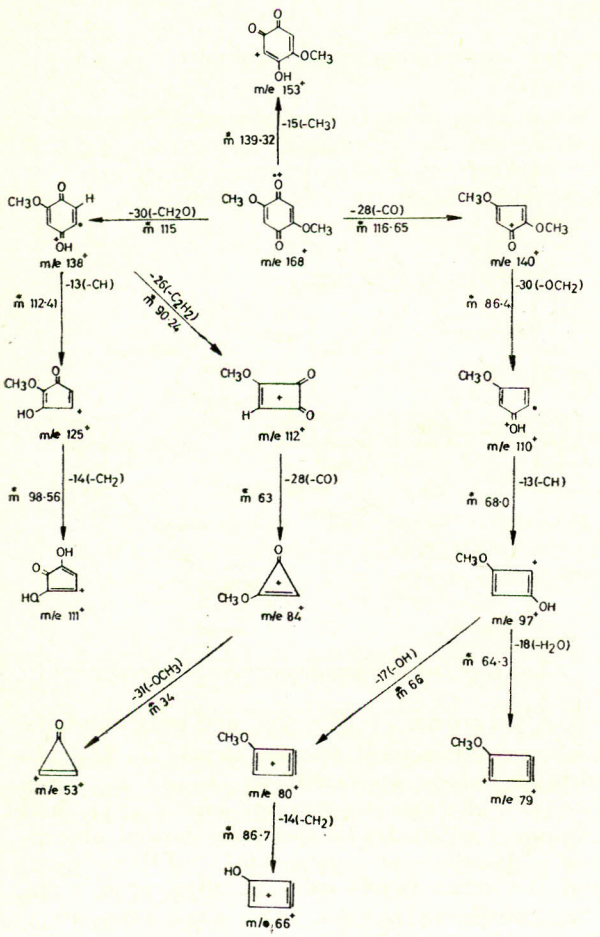


Chart 1.—Mass fragmentation pattern of 2, 5–dimethoxy benzoquinone.

a mixture of three acids, which were separated through fractional crystallization from ether. The first fraction on recrystallization from ether yielded colourless needles (II), m.p. 163°C (dec), 102 mg, which analysed for $C_3H_4O_5$.

The mother liquor on standing gave another crop of crystals, which on repeated crystallization from ether gave colourless crystalline compound III, m.p. 162–164° (dec); 158 mg, which analysed for $C_5H_6O_4$. The remaining mother liquor gave another crystalline product (IV), m.p. 185–187°C, 306 mg, which analysed for $C_4H_6O_4$.

The compound $C_3H_4O_5$ (II), m.p. 163°C, showed no UV absorption bands except for a slight hump at λ_{max}. 265 nm (ε 549). The IR absorption bands appeared at 2941 cm^{-1} (—OH); 2703, 2500, 1739 and 1709 cm^{-1} (CO vibrations). The other bands appeared at 1473, 1429, 1408, 1389, 1299, 1227 and 1111 cm^{-1}. The structure of this compound was established through its PMR studies. It showed a singlet at τ 6.24 (1H), indicating a methine proton next to a hetero-atom. Another singlet appeared at τ 3.2 (1H) for the proton of one —O*H* group. The remaining two protons appeared as a broad hump centred at τ 0.84 (2H) which were obviously in a similar environment and were due to the protons of carboxyl groups.

The above structure is that of tartronic acid as was also evident from its m.p., which was identical to the m.p. recorded in literature (163°C dec). Tartronic acid has been isolated as a metabolic product of a number of bacteria like *Acetobacter acetosum* and *Glucanoacetobacter liquefaciens*.[5]

The esterification of tartronic acid with diazomethane, gave dimethyl tartronate which analysed for $C_5H_8O_5$. Its IR spectrum gave absorption bands at 3500 cm^{-1} (OH), 1739 cm^{-1} (ester carbonyl) and other bands were at 1433, 1370, 1351, 1212, 1071 and 1026 cm^{-1}. The PMR spectrum in $CDCl_3$ gave two singlets at τ6.24) (3H) and τ 6.26 (3H) both for the two methyls of the ester groups. It showed two doublets one at τ 5.08 (1H) for the methine proton and the other at τ 4.95 (1H) for the hydroxyl proton: (J_{AB}, 2.5 Hz) due to an AB system. These doublets are absent in parent compound II due to the hydrogen bonding between the acid carbonyls and the hydroxyl group which disappeared on methylation.

Compound III, $C_5H_6O_4$ m.p. 162–164°C also showed no absorption in UV region and its IR spectrum gave absorption bands at 2899 cm^{-1} 2740, 2532 and 1724 cm^{-1} (acid carbonyl). Other bands appeared at 1429, 1299, 1235, 1111, 862, 781, 749 cm^{-1}.

The PMR spectral studies provided an answer to the structure of this acid. It gave a singlet at τ 6.25 (2H methylene protons), and a broad hump centred at τ 0.9 (2H) which could be assigned to carboxylic protons. Another singlet at τ 2.25 (2H) was indicative of a methylene proton next to a double bond. On the basis of this evidence the structure of this acid is that of itaconic acid (III).[6]

The proposed structure was confirmed through mass spectral studies, which showed a very weak molecular ion peak at m/e 130.$^+$ The most intense peak appeared at m/e 113^+, M^+17, due to the loss of one OH from the carboxyl group. It also loses a molecule of water to form anhydride at m/e 112^+, which was 50% in intensity than that of the previous one. This ion underwent fragmentation to form a four-membered lactone with the loss of 28 (—CO) mass units at m/e 84^+ and this loses readily 16 (—O) mass units forming a base peak at m/e 68^+. The other prominent peaks appeared at m/e 99^+, 98^+, 95^+, 86^+, 85^+ and 40^+, all the fragment ions are outlined in Chart 2.

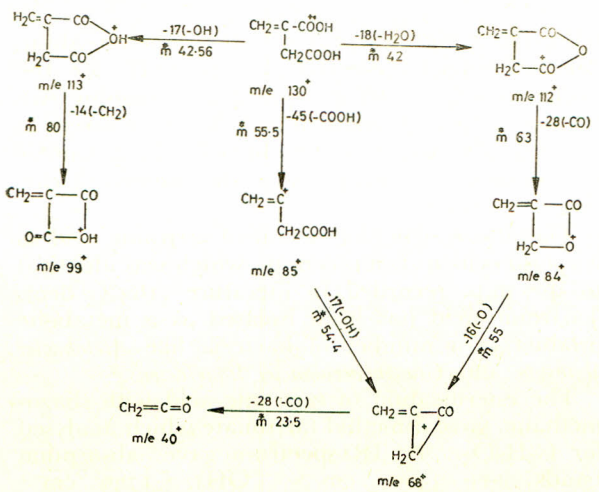


Chart 2.—Mass fragmentation pattern of itaconic acid.

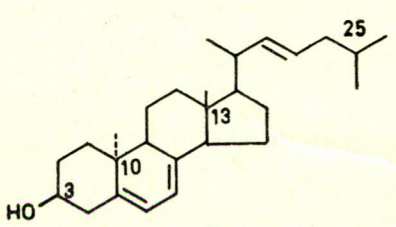
The constitution of this compound, in the absence of an authentic sample of itaconic acid, was confirmed by its esterification with diazomethane when it gave dimethyl itaconate which analysed for $C_7H_{10}O_4$. Its UV spectrum showed no absorption bands. The IR bands appeared at 1748 cm^{-1} (ester carbonyl), 1575 cm^{-1} (C=C); and the other bands were at 1449, 1385, 1290, 1220, 1099, 1057 cm^{-1}. Its PMR spectrum (in $CDCl_3$) showed a multiplet at τ 5.75 (2H) for methylene protons next to a double bond. A singlet at τ 6.02 (2H) for methylene next to a carbonyl group. There were two singlets one at τ 6.19 (3H) and another at τ 6.30 (3H) for two methyls of ester groups.

The compound $C_4H_6O_4$ (IV), m.p. 185–187°C was found to be succinic acid from its identical IR spectrum and mixed m.p. (undepressed) with an authentic sample. Its PMR spectrum (in DMSO) gave a sharp singlet at τ 6.05 (4H) which could be assigned to two methylene groups in identical environment. Another singlet at τ 3.26 (2H) was due to the two O*H* of the carboxyl groups.

Metabolites from Mycelium

(c) *Petroleum Ether Extract*

The mycelium on extraction with petroleum ether and removal of solvent gave a crystalline product, which analysed for $C_{28}H_{44}O$ (V), m.p.



V

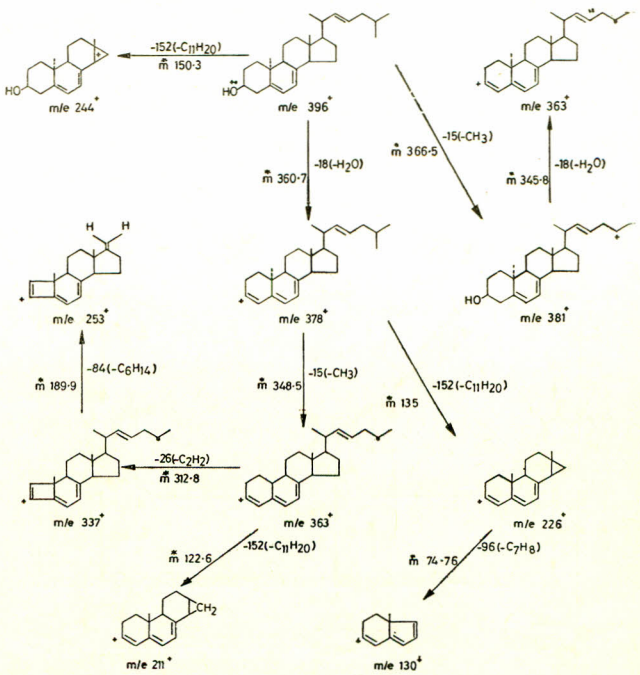


Chart 3.—Mass fragmentation pattern of pyrocalciferol.

93–95°C (110 mg), $[\alpha]_D^{25}$ +502° and gave a positive Lieberman–Burchard test for sterol. Its UV absorption bands appeared at λ_{max} 295 nm (ε 3111), 285 nm (ε 4375) 271 nm (ε 4733) and 263 nm (ε 4368). Its IR spectrum showed absorption bands at 3436 cm^{-1} (OH), 3322, and the other bands were at 2873, 1736, 1724, 1643, 1618, 1471, 1390, 1370, 1242, 1163, 1073, 1058, 1042, 975, 383, 806, 727 cm^{-1}.

The general appearance of the IR spectrum confirmed that the compound was a sterol and it was found that the compound had the same characteristics as those of pyrocalciferol. (V).[7] The above conclusion was further confirmed by its PMR spectrum ($CDCl_3$), which showed two singlets at τ9.35 (3H) and τ 9.22 (3H) for angular methyl groups substituted at carbons 10 and 13. The *gem*-dimethyls and two C-methyls (at 25) oft he side chain appeared as a doublet centred at τ 9.1 (12H). The proton of hydroxyl group (at 3) appeared at τ 5.75 (1H) as a broad hump which disappeared on deuteration. The two protons of the B ring substituted at carbons 6 and 7 appeared as singlet at τ 4.78 (2H). The remaining protons of the ring and the side chain (23H) appeared as a broad multiplet in the region τ 7.5~8.8, thus accounting for all the protons in the molecule.

The above structure was further confirmed by its mass spectral studies, which showed typical fragmentation pattern as are observed for sterols. The mass spectrum showed an intense molecular ion peak at m/e 396^+, which readily loses 18 and

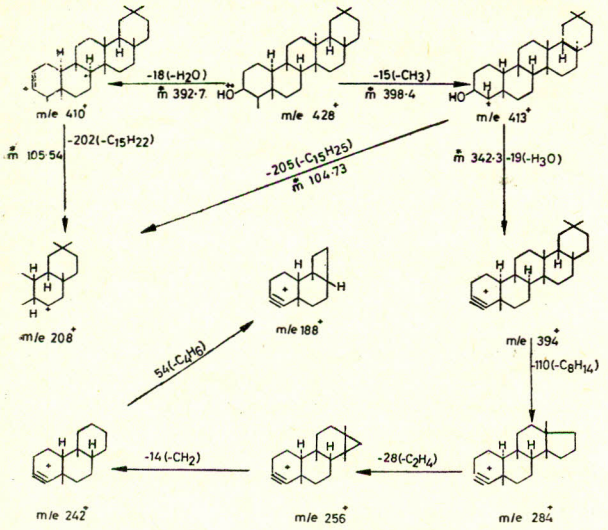


Chart 4.—Mass fragmentation pattern of epi-friedlinol.

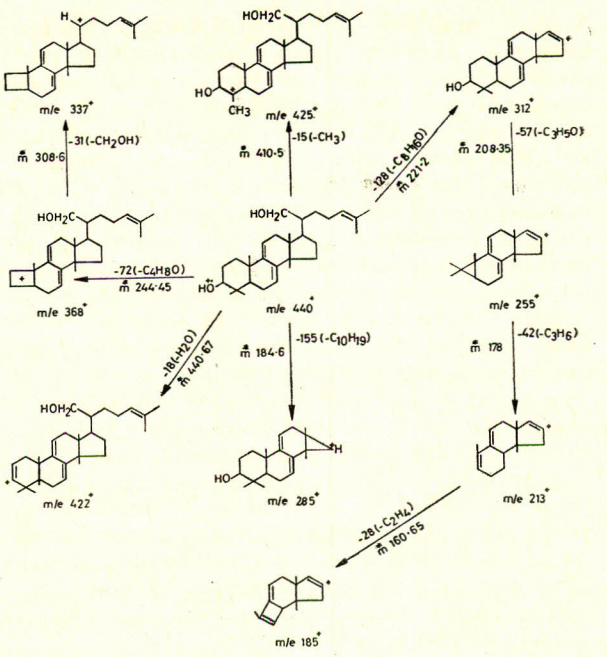


Chart 5.—Mass fragmentation pattern of lanosta-7,9 (11)-24-triene-3β,-21 diol.

15 mass units giving rise to peaks at m/e 378^+ and 363^+ formed due to the successive losses of water and one methyl group. The rest of the peaks appeared at m/e 337^+, 231^+, 211^+, 199^+, 197^+, and 147^+ as outlined in Chart 3. Most of the ions were formed in a predictable and rational manner by no less than four carbon–carbon bond homolyses and hydrogen transfer in the manner known to follow in the case of sterols.

After removal of pyrocalciferol (V) the remaining mother liquor was subjected to preparative thin layer chromatography over Merck's silica-gel PF-254 which gave three more compounds, viz. VI, R_f 0.35; VII, R_f 0.5; VIII, R_f 0.65.

Compound VI (203 mg) analysed for $C_{30}H_{52}O$, had m.p. 280°C, and was optically active: $[\alpha]_D^{25}$ +24° (in chloroform). Its UV absorption band showed λ_{max} at 290 nm (ε 4448). The IR spectrum showed strong absorption for OH group at 3846 cm^{-1} and the rest of the spectra showed typical absorption for sterols. The PMR spectrum (in $CDCl_3$) was also indicative of a sterol type of compound which was identified as epifriedlinol.[8]

The above structure was confirmed by the fragmentation pattern of its mass spectrum. It showed a molecular ion peak at m/e 428^+ which underwent fragmentation to lose 15 and 18 mass units for the loss of (—CH_3) and (—H_2O) giving rise to peaks at m/e 413^+ and 410^+ respectively. The rest of the main peaks appeared at m/e 394^+, 284^+, 256^+, 242^+, 208^+ (base peak) 188^+. All these ionic species are shown in Chart 4.

Compound VII (20 mg) analysed for $C_{30}H_{48}O_2$, m.p. 194–197°C; $[\alpha]_D^{25}$ + 72° (in chloroform). Its UV spectrum showed two bands at λ_{max}. 325 nm (ε 31152) and λ_{max} 275 nm (ε 20157). The IR spectrum gave absorption bands at 3448 cm^{-1} (OH) 2941 cm^{-1} and 2857 cm^{-1}, 1645, 1604, CH=CH 1471 and 1379 cm^{-1}. Literature revealed that the characteristics of this compound were comparable to those of lanosta-7,9 (11)-24-triene-3β 21-diol, isolated from *Polyporous pinicola Fr.*[9]

The PMR spectrum (in $CDCl_3$) of this compound did not reveal much to substantiate the assumption regarding the structure of the compound. It showed a singlet at τ 8.40 (15 H, $5\times CH_3$), a multiplet at τ 6.68 (8H, $4\times CH_2$), second multiplet at τ 5.15 (3H, 3x C*H*–) and yet another at τ 4.58. However, the structure of this compound was confirmed by mass spectral studies. It showed an intense molecular ion peak at m/e 440^+. The main significant peaks appeared at m/e 425^+, 422^+, 408^+, 394^+, 368^+, 346^+, 339^+, 338^+, 313^+, 312^+, 311^+, 285^+, 280^+, 264^+, 255^+, 231^+, 222^+, 213^+, 207^+, 199^+, 185^+, 171^+, 129^+ and 110^+, as outlined in Chart 5.

Compound VIII analysed for $C_{19}H_{28}$, b.p. 85°C, 128 mg, is not known in the literature, and has been named trichodermene-A by us. It is a simple hydrocarbon, having six degrees of unsaturation. Its IR spectrum gave absorption bands at 1645, 1604 and 1471 cm^{-1}. The first two bands were indicative of a *cis* and *trans* system of hydrogen atoms in this molecule.

The PMR spectrum did not provide any clear clue to the structure of the compound. It showed two broad multiplets centred at τ 8.78 (16H; —CH_2) and at τ 9.17 (6H; $2\times CH_3$)