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STUDIES IN THE BIOCHEMISTRY OF MICROORGANISMS

Part X.—Isolation, Structure and Stereochemistry of Yasimin and other Metabolic Products of Aspergillus unguis Emile-Weil and Gaudin

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The structure of *yasimin* is shown to be tri-dechloronornidulin. The other three metabolites have been identified as nidulin, nornidulin and mannitol. These structures have been established mainly through physical methods.

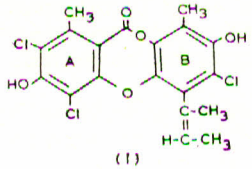
Aspergillus unguis (I.M.I. 138767) was first described by Emile-Weil and Gaudin in 1939.[1] However, no attempt was made to investigate the metabolic products. This paper concerns the isolation and structures of the various metabolites.

The mold was grown on different standard media, viz. Czapek–Dox medium with yeast extract enriched with carrot extract, malt extract agar[2] and Czapek–Dox medium enriched with carrot extract with diammonium tartrat- as nitrogen source. The same metabolic products were isolated from all the three mediums but the first one gave the highest total yield of the various metabolites.

The mold was grown on Czapek–Dox medium with yeast extract enriched with carrot extract for 20 days at 24–27°C. The mycelium was separated from the broth, and the broth when extracted with ethyl acetate and ether gave a crystalline mixture which on fractional crystallisation from ether–petroleum ether gave a crystalline product: m.p. 185°C which analysed for $C_{19}H_{15}O_5Cl_3$. The UV spectrum showed only a shoulder at 262 mμ. The IR spectrum showed a sharp band at 3448 cm^{-1} (free OH), 1701 cm^{-1} (>CO) and two bands at 1603 cm^{-1} and 1585 cm^{-1} (benzene stretching). The PMR spectrum in $CDCl_3$ showed a doublet and an octet centred at τ 8.24 and τ 4.58 due to AB_3 system (J_{AB3} 6 c/s) indicating the presence of CH_3—CH=group in the molecule.

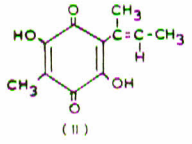
Then there was another sharp singlet appearing at τ 8.1 for methyl group on benzene ring. Another ill-defined doublet was present at τ 7.8 for CH_3—C(|)=which showed a coupling-constant of 2 c/s due to allylic coupling. This coupling constant indicates the presence of two C—methyl groups in *cis* configuration with each other in the side chain: CH_3—CH=C(|)—CH_3. There was one more singlet at τ 7.5 (CH_3—) present on the benzene ring. This unusually low chemical shift could be safely assumed to be indicative of the presence of a neighbouring electronegative element (like chlorine).

A broad hump appeared at τ 4.2 for two hydroxyl groups which disappeared on deuteration. The above data can be easily accounted by the following structure I which is already known as nornidulin.[3]



(I)

This was further confirmed by the isolation of 2,4-dihydroxy-3-methyl-6-(2-but-2-enyl) benzoquinone (II) through degradation according to the method of Beach and Richards:[4] heating with HBr and red phosphorus followed by heating with methanolic KOH and then isolating the product.



(II)

The structure of nornidulin was further confirmed from its mass spectral fragmentation ions.

From the ether and petroleum ether soluble fraction, another crystalline compound (m.p. 180°C) was obtained, which analysed for $C_{20}H_{17}O_5Cl_3$ and was found to be nidulin.[5]

The extraction of the dried mycelium with petroleum ether gave nornidulin. Subsequent sox-

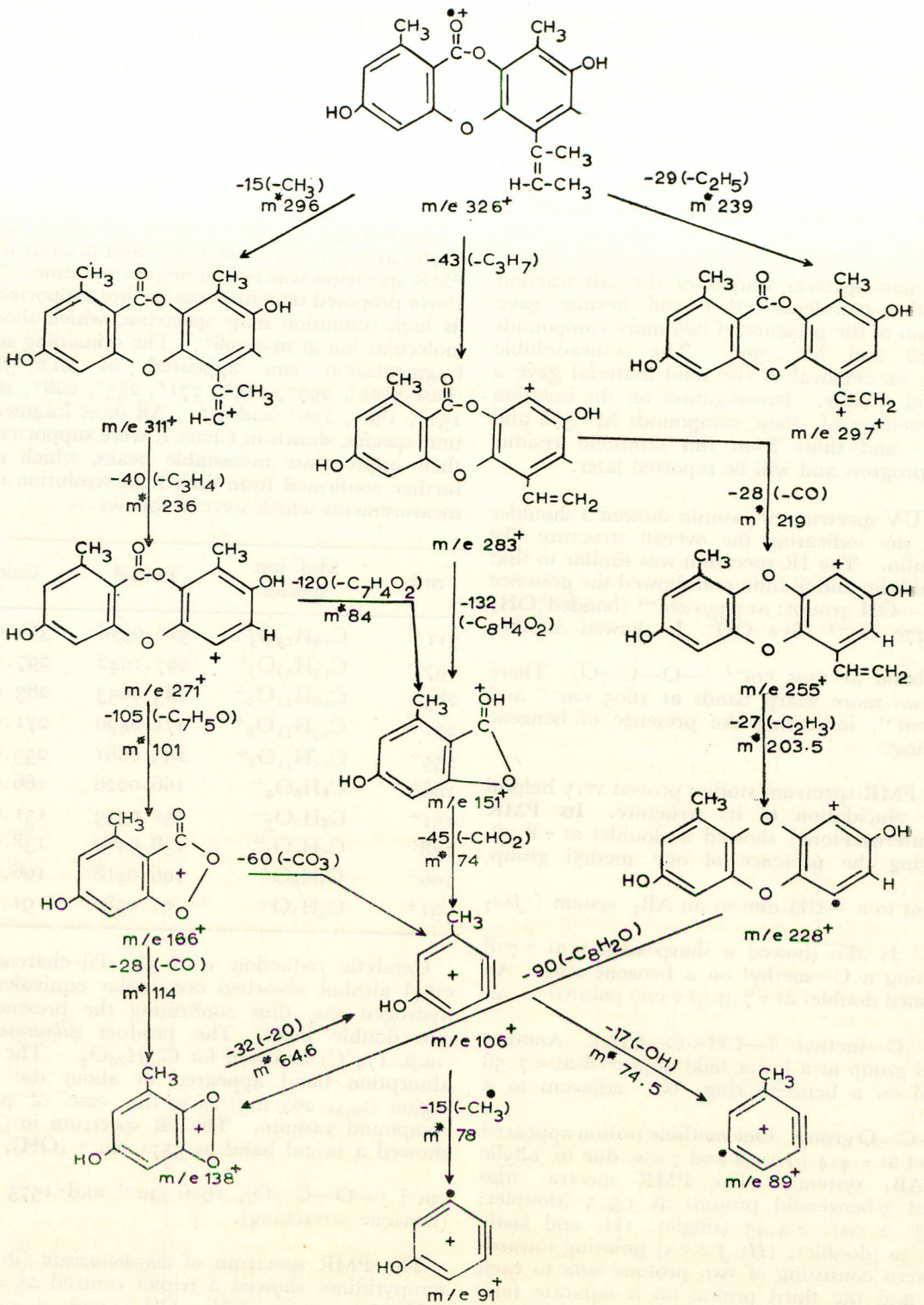


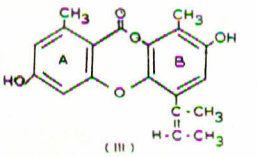
Chart I.—Mass fragmentation pattern of yasimin.

hlet extraction with ether gave a solid which on fractional crystallisation from ether–petroleum ether and then finally from hexane yielded a pure crystalline colourless compound (m.p. 200°C) as the major component. It analysed for $C_{19}H_{18}O_5$, established on the basis of high resolution mass measurement. (Found M^+ 326.1131 calculated 326.1154). This compound is not reported in literature and we have named it *yasimin*.

The mass spectral studies of the tail fraction from ether–petroleum ether and hexane gave indication of the presence of two more compounds M^+ 378 and M^+ 392. The ether-soluble fraction on removal of the solid material gave a semisolid residue. Investigation on the isolation and structures of these compounds M^+ 378 and M^+392 and those from this semisolid residue are in progress and will be reported later.

The UV spectrum of yasimin showed a shoulder at 262 mμ indicating the overall structure like nornidulin. The IR spectrum was similar to that of nornidulin and nidulin and showed the presence of two —OH groups: at 3350 cm^{-1} (bonded OH) and 3470 cm^{-1} (free OH). It showed another sharp band at 1705 cm^{-1} (—O—C(=O)—). There were two more sharp bands at 1605 cm^{-1} and 1590 cm^{-1}, indicating the presence of benzene stretching.

The PMR spectrum studies proved very helpful in the elucidation of its structure. Its PMR (in deuteroacetone) showed a doublet at τ 8.18, indicating the presence of one methyl group, adjacent to a —CH— due to an AB_3 system (J_{AB_3} 7 c/s). It also showed a sharp singlet at τ 7.8 indicating a C—methyl on a benzene ring. An ill-defined doublet at τ 7.9 (J 2 c/s) pointed to an allylic C—methyl (—CH=C(—)—CH_3). Another methyl group at a lower field appeared at τ 7.58 located on a benzene ring but adjacent to a —O—C(=O)— group. One methine proton appeared as octet at τ 4.4 (J 2 c/s and 7 c/s; due to allylic and AB_3 systems). The PMR spectra also showed 3-benzenoid protons at τ 3.5 (doublet; 1H; J: 2 c/s); τ 3.45 (singlet; 1H) and lastly at τ 3.32 (doublet; 1H; J 2 c/s) pointing towards a pattern consisting of two protons *meta* to each other and the third proton on a separate fully substituted benzene ring. The presence of two broad humps centred at τ 6.6 and τ 0.5 indicated two OH groups. These disappeared on shaking with deuterium oxide. The above data gave the undernoted possible structure of yasimin (III).



(III)

Interestingly enough yasimin showed a sharp singlet at τ 3.18 for three benzenoid protons when PMR spectrum was run in deuteropyridine. The above proposed structure was further supported by its high resolution mass spectrum, which showed molecular ion at m/e 326^+. The remaining main fragmentation ions appeared at m/e 311^+, (base-peak), 297^+, 283^+, 271^+, 255^+, 228^+, 166^+, 151^+, 138^+, 106^+ and 91^+. All these fragmentation species, shown in Chart I, were supported by their appropriate metastable peaks, which were further confirmed from their high resolution mass measurements which were as follows:—

m/e	Mol. ion species	Found	Calc.
311^+	$C_{18}H_{15}O_5^+$	311.0918	311.0920
297^+	$C_{17}H_{13}O_5^+$	297.1042	297.1062
283^+	$C_{16}H_{11}O_5^+$	283.0653	283.0606
271^+	$C_{15}H_{11}O_5^+$	271.0490	271.0442
255^+	$C_{15}H_{11}O_4^+$	255.0681	255.0657
166^+	$C_8H_6O_4^+$	166.0226	166.0266
151^+	$C_8H_7O_3^+$	151.0333	151.0395
138^+	$C_7H_6O_3^+$	138.0460	138.0316
106^+	$C_7H_6O^+$	106.0418	106.0418
91^+	$C_6H_3O^+$	91.0580	91.0183

Catalytic reduction with 5% Pd–charcoal in ethyl alcohol absorbed one molar equivalent of hydrogen gas, thus confirming the presence of one double bond. The product *dihydroyasimin* (m.p. 174°C) analysed for $C_{19}H_{20}O_5$. The UV absorption band appeared at about the same region (λ_{max} 267 mμ) as in the case of parent compound yasimin. The IR spectrum in (KBr) showed a broad band at 3571 cm^{-1} (OH), 1724 cm^{-1} (—O—C(=O)—), 1626 cm^{-1} and 1575 cm^{-1} (benzene stretching).

The PMR spectrum of *dihydroyasimin* (in deuteropyridine) showed a triplet centred at τ 9.22 (3H; $J_{A_2B_3}$: 7 c/s; CH_3—CH_2) and a quartet centred at τ 8.5 (2H; $J_{A_2B_3}$ 7 c/s; CH_3—CH_2—). There was a doublet and a quartet centred at

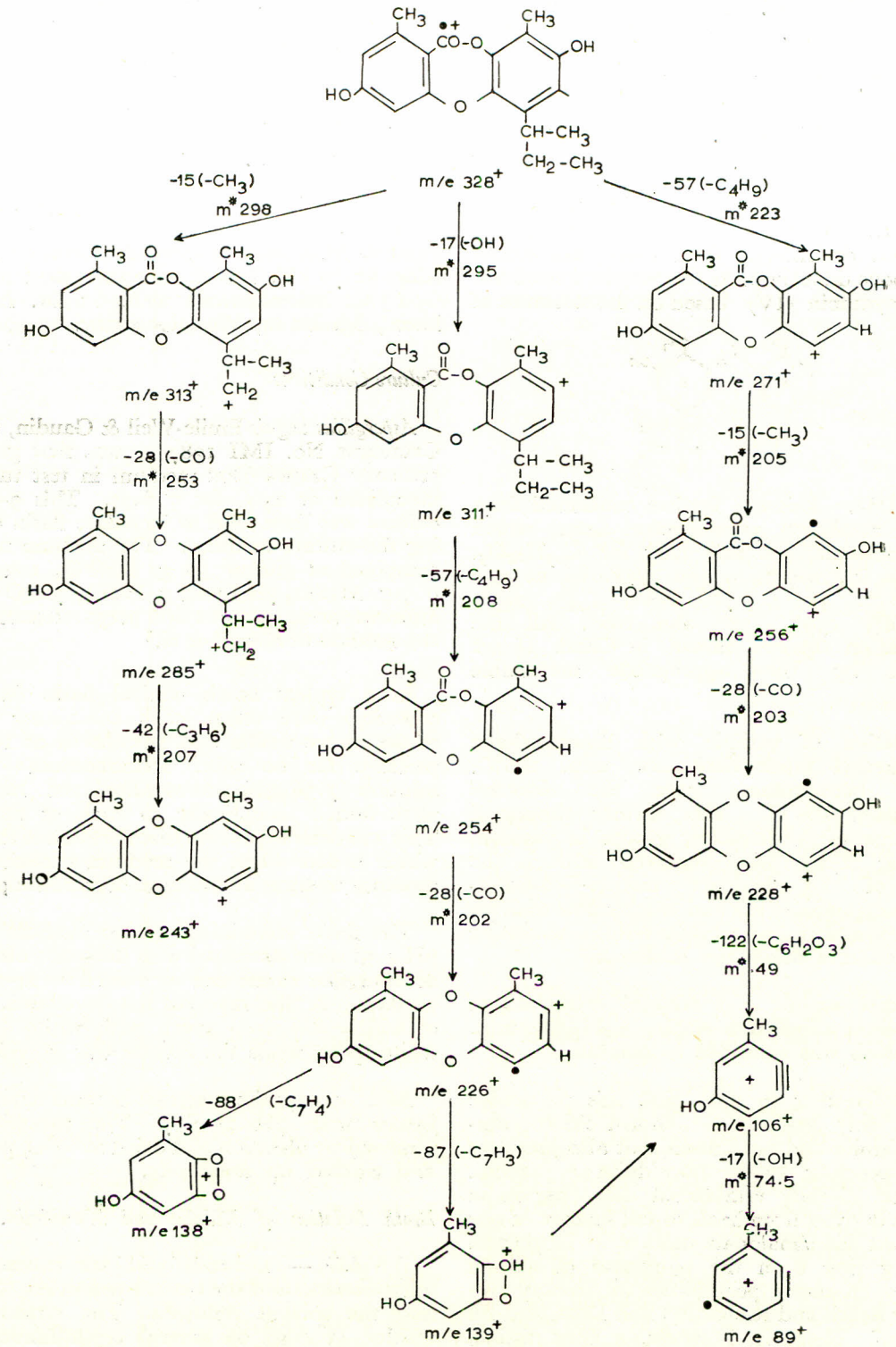
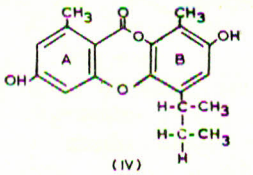


Chart II.—Mass fragmentation pattern of dihydroyasimin.

τ 8.89 (3H; J_{AB_3} 7 c/s, CH_3—CH—) and τ 6.52 (1H; J_{AB_3} 7 c/s, —CH—CH_3) respectively. Then there were two sharp singlets at τ 7.55 and τ 7.45 (two methyl groups on benzene rings). In the benzenoid region there were two doublets appearing at τ 3.17 and τ 3.06, indicating the presence of two protons *meta* to each other. (J 2 c/s). There was a sharp singlet at τ 3.2 for one isolated benzenoid proton. A broad singlet appeared at τ 0.25 for two phenolic O*H*, which disappeared on shaking with deuterium oxide. All the above data supports the proposed structure of dihydroyasimin (IV) based on the structure of



(IV)

yasimin (III). This was further confirmed from its high resolution mass-spectral data, which showed molecular ion m/e 328^+ for $C_{19}H_{20}O_5$. The main fragmentation ions appeared at m/e 313^+, 311^+, 285^+, 271^+, 256^+, 254^+, 245^+, 228^+, 226^+, 139^+, 138^+ and 106^+. All the fragmentation species shown in Chart II are supported by their appropriate metastable peaks.

Methylation of yasimin with diazomethane gave dimethyl ether derivative, m.p. 160°C. Similarly benzoylation gave the *dibenzoyl yasimin*, m.p. 100°C. This further supported the presence of two hydroxy groups in yasimin. Remaining doubts were removed through the isolation of 2,5-dihydroxy-3-methyl-6-(2-but-2-enyl) benozquinone (II) and comparison with an authentic sample prepared from nidulin according to the method of Beach and Richards.[4]

The ethyl acetate and ethanol extraction of the mycelium afforded a sugar, $C_6H_{14}O_6$, m.p. 166°C, which was identified as mannitol.

It is interesting to note that this particular mold not only produces the known chloro compounds nidulin and nornidulin, but also produces the corresponding dechloronornidulin i.e. yasimin. Birch[6] has already established that ring A in nidulin is derived from head to tail linkage of one acetate and three malonate units in its biogenesis. Regarding ring B in the biogenesis of nidulin there can be many possibilities as already discussed by Beach and Richards,[4] and Dean and his coworkers.[7] There is, however, a very distinct possibility that it may be derived from the condensation of two isoprenoid units with the transmethylation of one methyl group from methionine.

Experimental

Melting points were taken on Kofler block and are uncorrected. UV spectra were measured in ethanol (95%) on a Beckman D.B. spectrophotometer and IR spectra were determined with 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 containing TMS as internal reference. Mass spectra were measured on an AEI MS9 at 70 eV. Petroleum ether used had b.p. 65–85°C. Microanalyses were by Dr. A. Bernhardt, Mullheim, West Germany.

Culture Conditions

Aspergillus unguis Emile-Weil & Gaudin, (C.M.I. Catalogue No. IMI 138767) was first grown on ordinary Czapek–Dox medium in test tubes and inoculated at 24°C for 9 days. This 9-day old culture was then used to inoculate flasks containing the culture medium. The medium used was composed of glucose 50 g, $KHPO_4$ 1.0 g, KCl 0.5 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.01 g, aqueous carrot extract 1 litre prepared according to the method of Kamal *et al.*[8]

In a typical batch conical flasks (each 1-l. capacity) were taken, each containing 340 ml of the above media and autoclaved at 10 lb/in^2 pressure for 20 min. Diammonium tartrate solution (7.56 g/l.) was sterilised and 10 ml of it were added asceptically to each of the flasks. This was done to prevent discoloration of the media which occurs when the medium contains diammonium tartrate at the time of sterilisation.

The flasks containing culture medium (3.6 l., pH 4.5) were incubated with the 9-day old culture of *Aspergillus unguis* and incubated at 24–27°C for 20 days. A thin mycelial felt developed, the top of which was deep green with white and pale white spots, while the reverse was grayish green.

After 20 days the substrate became dark reddish brown with pH at 6–7. The mycelium was removed by filtration and dried at 60°C, powdered and worked up separately.

Broth Isolation of Nidulin and Nornidulin.

(i) *Nidulin.*—The broth (3.6 l.) was extracted with ethyl acetate, and the extract was dried (Na_2SO_4) and the solvent removed. The semicrystalline residue (1.4 g) on several crystallisations from ether–petroleum ether gave colourless prismatic needles; m.p. 170–176°C. The pure product (40 mg) (R_f 0.8) was obtained through preparative

thin-layer chromatography on keiselgel HF254 (Merck's) 0.5 mm, 20×20 cm plates; the solvent system being ethanol–chloroform 95:5, m.p. 180°C. It was identified to be nidulin.

Found: C, 54.1; H, 4.3; O, 17.8; Cl, 23.6%. Calc. for $C_{20}H_{17}O_5Cl_3$: C, 54.3; H, 3.9; O, 18.5; Cl, 23.5% (by difference).

(ii) *Nornidulin.*—On concentrating the combined mother liquors after the removal of nidulin, impure crystals of nornidulin (m.p. 168–170°C) were obtained, which were recrystallised several times from ether–petroleum ether and finally purified through preparative thin layer chromatography as above. The pure product (R_f 0.85) in ethanol–$CHCl_3$ 95:5) melted at 185°C; (0.7 g). It was found to be nornidulin.

Found: C, 52.9; H, 4.4; O, 18.5; Cl, 24.7%. Calc. for $C_{19}H_{15}O_5Cl_3$: C, 53.2; H, 3.5; O, 18.6; Cl, 24.5%.

Isolation of Metabolites from the Mycelium.

(iii) *Yasimin.*—The mycelium was removed and dried at 50–60°C for 7 days and then powdered (69 g from 12 flasks). The dried mycelium was extracted (54 hr) in a Soxhlet apparatus, first with petroleum ether which gave a small quantity of nornidulin. Subsequent extraction with ether yielded a semisolid residue (3.1 g), which gave crystalline material on standing. Several crystallisation from ether–petroleum ether and finally from hexane gave pure yasimin in colourless needles, m.p. 200°C (2.4 g).

Found: C, 69.37; H, 5.71; O, 24.42%. $C_{19}H_{18}O_5$ requires C, 69.93; H, 5.56 O, 24.51%.

It gave a positive ferric chloride test. Its UV spectrum in 95% ethanol showed a shoulder at 262 mμ (ε 11807). IR absorption bands were at: 3475 cm^{-1} (OH); 2900 cm^{-1} and 2950 cm^{-1} (CH_2—CH—); 1705 cm^{-1} (—CO—O—); 1605 cm^{-1} and 1590 cm^{-1} (benzene ring stretching); 1420 cm^{-1} and 1450 cm^{-1} (CH_3—). The remaining bands were at 1375, 1348, 1325, 1275, 1252, 1210, 1175, 1151, 1050, 995, 975, 900, 868, 855, 825, 765, and 700 cm^{-1}.

The mass spectrum showed peaks at m/e 326^+, 311^+, 298^+, 297^+, 283^+, 271^+, 270^+, 269^+, 255^+, 241^+, 229^+, 228^+, 217^+, 215^+, 201^+, 190^+, 178^+, 175^+, 161^+, 151^+, 138^+, 106^+, 105^+, 103^+, 91^+, 83^+, 77^+, 74^+, 59^+, 45^+, 43^+ and 41^+.

The mother liquor remaining after removal of yasimin gave two more products (Rf 0.24 and 0.74) by preparative thin layer chromatography (ether–petroleum ether 2:1).

The ethereal filtrate, after removal of the above solid products, gave a semisolid residue.

(iv) *Mannitol.*—The mycelium which was first extracted with ether was subsequently extracted with ethyl acetate. Removal of combined extracts yielded a crystalline compound which on recrystallisation from ethanol melted at 166°C. It was identified to be mannitol both by its identical R_f value (0.59; ethanol–chloroform 95:5) and mixed m.p. (undepressed) with an authentic sample of the sugar (m.p. 166°C).

Dihydroyasimin.—Yasimin (50 mg) in ethyl alcohol (15 ml) containing 5% Pd–charcoal (10 mg) was shaken in an atmosphere of hydrogen gas. Absorption ceased after 1 molecular equivalent of the gas had been absorbed (determined through mass spectrometry), filtration and removal of solvent gave a solid residue which on purification by preparative thin-layer chromatography (R_f 0.56; ethanol–ether 4:1) and subsequent isolation and crystallisation from ethyl acetate–petroleum ether gave *dihydroyasimin*, m.p. 174°C 40 mg; $[\alpha]_D^{22}$ −3.5 (conc. 2% in 95% ethanol).

Found: C, 69.67; H, 6.21; O, 24.42%. $C_{19}H_{20}O_5$ requires: C, 69.50; H, 6.14; O, 24.36%.

Its UV spectrum showed a shoulder at 267 mμ (ε 8148). IR absorption bands were at 3571 cm^{-1} (OH). 1704 cm^{-1} (—O—C(=O)—), 1603 cm^{-1}, 1580 cm^{-1} (benzene ring stretching); 1471 cm^{-1} and further bands appearing at 1445, 1342, 1290, 1266, 1170, 1122, 1070, 1002, 892 and 865 cm^{-1}.

Mass spectrum showed peaks at m/e 328^+, 313^+, 311^+, 301^+, 300^+, 299^+, 285^+, 284^+, 281^+, 271^+, 258^+, 256^+, 254^+, 244^+, 243^+, 229^+, 228^+, 227^+, 226^+, 217^+, 215^+, 201^+, 189^+, 151^+, 141^+, 138^+, 106^+, 105^+, 104^+, 103^+, 91^+ and 83^+.

O,O-*Dimethyl Yasimin.*—Yasimin (50 mg) in ether was methylated with excess diazomethane and allowed to stand (4 days). Removal of the solvent from the reaction mixture gave a residue, which was taken up in ether and shaken with 2N NaOH to remove any unreacted material. Drying (Na_2SO_4) and removal of ether gave *O,O*-dimethyl yasimin m.p. 160°C, 39 mg.

Found: C, 71.72; H, 6.26; O, 22.57%. $C_{21}H_{22}O_5$ requires: C, 71.17; H, 6.26; O, 22.57%.

UV absorption spectrum gave a shoulder 265 mμ (ε10974). It did not show any hydroxyl absorption in the IR spectrum. PMR spectrum (in pyridine d_6) showed an extra sharp singlet at τ 6.2 ($2 \times CH_3$) alongwith other signals as in the case of yasimin.

O,O-*Dibenzoylyasimin.*—Yasimin (50 mg) was taken in pyridine (5 ml) and freshly purified benzoyl chloride in excess (250 mg) was added and shaken overnight (room temperature). The reaction mixture was poured over ice. The precipitate isolated with chloroform yielded viscous residue of O,O-*dihenzoylyasimin* which was purified by preparative thin-layer chromatography (solvent system: ether–petroleum ether 3:1; R_f 0.4). Crystallisation from ether–petroleum ether gave *O,O*-dibenzoylyasimin in colourless prismatic needles; m.p. 100°C; 55 mg.

Its UV absorption spectrum showed λ_{max} 260 mμ (ε 6642). Its IR spectrum also showed absence of hydroxyl groups. It showed bands at 1818, 1754, 1724, 1626, 1613 and 1471 cm^{-1}.

Found: C, 74.4; H, 4.67; O, 21.04%. $C_{33}H_{26}O_7$ requires: C, 74.15; H, 4.90; O, 20.95%.

2,5-*Dihydroxy*-3-*methyl*-6-(2-*but*-2-*enyl*) *benzoquinone.*—To yasimin (500 mg) in glacial acetic acid (10 ml) was added red phosphorus (1 g) and constant boiling hydrobromic acid (8 ml) and the mixture boiled (1 hr). The reaction mixture was then poured over crushed ice (150 g) and the resulting slurry was extracted with ether several times. Residual red phosphorus was removed from the combined ether extracts through filtration over Celite bed. The ethereal filtrate was successively washed with water, aquous sodium bicarbonate and again with water. Removal of ether (nitrogen atmosphere) gave a residue which was taken up in 1% methanolic KOH (20 ml) and refluxed (2 hr), under nitrogen atmosphere.

The solution was poured into water and the pH adjusted to 6.3 with aqueous KOH. Extracted with ether 2–3 times and the aqueous phase acidified with sulphuric acid (pH 1.0) and the acidified aqueous solution extracted with ether. The ethereal solution of the acidic products on drying and removal of the solvent gave 2,5-dihydroxy-3-methyl-6-(2-but-2-enyl) benzoquinone.[6] It was purified through preparative thin layer chromatography: (R_f 0.72; ethanol–chloroform 3:1); m.p. 197°C.

Acknowledgement.—We are indebted to Professor A.I. Scott, Sterling Chemistry Laboratories, Yale University, Conn., U.S.A., for granting the facilities for the use of PMR and mass spectrometers and Dr. W. J. McMurray for the computarised high resolution mass-spectral data.

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