Biological Sciences Section

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STUDIES IN THE BIOCHEMISTRY OF MICROORGANISMS

Part XII.—Isolation and Structures of Haiderin, Rubinin, Shirin and Nasrin Metabolic Products of Aspergillus unguis Emile-Weil and Gaudin

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The isolation of four new depsidone metabolites from *Aspergillus unguis* Emile-Weil and Gaudin viz. *Haiderin* (IV), $C_{19}H_{19}O_6Cl$, m.p. 170°C; *Rubinin* (VII), $C_{20}H_{21}O_6Cl$, m.p. 162°C; *Shirin* (VIII), $C_{19}H_{17}O_5Cl_3$, m.p. 118°C; and *Nasrin* (XI), $C_{22}H_{21}O_5Cl_3$, m.p. 232°C have been described and their structures established.

In an earlier communication we have described the isolation and structure of a new metabolite, yasimin,[1] and three other metabolites, nornidulin, nidulin and mannitol, from the broth and mycelium of *Aspergillus unguis* (I.M.I. 138767) when grown on Czapek–Dox medium enriched with carrot extract. The present communication describes the isolation and structures of two new metabolites *haiderin* and *rubinin* from the tail fraction of the yasimin mother liquors,[1] and isolation and structures of *shirin* and *nasrin*, out of the nine new metabolites obtained from the ethereal filtrate after removal of sparingly soluble yasimin, haiderin and rubinin from the ether extract of the mycelium, through preparative TLC.

Aspergillus unguis was grown on Czapek–Dox medium enriched with carrot extract for 22 days at 24–26°C. The broth was filtered off, the mycelium was dried *in vacuo* at 60°C, powdered and exhaustively extracted in a Soxhlet apparatus with petroleum ether and finally with ether. The ethereal extract on standing yielded practically all the sparingly soluble yasimin mixed with two new products. Yasimin was separated through fractional crystallisation and its mother liquor on concentration afforded a semisolid material which showed the presence of three compounds. These were isolated pure by preparative TLC, using ether–petroleum ether (2:1). One of the compounds was yasimin (R_f 0.45, m.p. 200°C), while the other two were new entities not yet described in literature and we have named them as *haiderin*, R_f 0.24, $C_{19}H_{19}O_6Cl$ (M^+ 378), m.p. 170°C, and *rubinin*, R_f 0.74, $C_{20}H_{21}O_6Cl$ (M^+ 392), m.p. 162°C. The ethereal filtrate on exhaustive investigation gave the following nine metabolites obtained through repeated preparative TLC; using ether–petroleum ether (3:1).

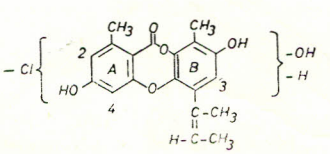
Metabolites	Ether-pet. ether (3:1) R_f	Methanol-ether (2:98) R_f	M.p.
Nazirin	0.05	—	
Sarwin	0.07	—	
Khalinin	0.14	—	
Aminin	0.16	—	
Altamashin	0.30	—	
Shirin	0.33	0.24	118
Nasrin	0.36	0.38	232
Shahidin	0.50	—	—
Yazidin	0.72	—	—

The structures of only four metabolities viz. haiderin, rubinin, shirin and nasrin are described in this communication. The work on the remaining metabolites is in progress and will be reported later.

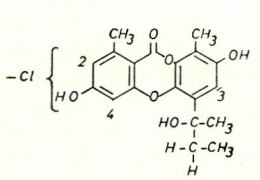
Haiderin (IV).

Haiderin (m.p. 170°C) had the molecular formula $C_{19}H_{19}O_6Cl$ by high resolution mass measurements. Its UV spectrum gave λ_{max} at 265 mμ (ε 10108) and a shoulder at 320 mμ (ε 1026). Its IR absorption bands were at 3401 cm^{-1} (–OH), 1722 cm^{-1} (>C=O) and two bands at 1610 cm^{-1} and 1590 cm^{-1} (benzene ring stretching). Both the UV and the IR absorption bands were the same as observed in the cases of yasimin,[2] nornidulin[5] and nidulin.[5] Its mass spectrum showed a strong molecular ion peak at m/e 378^+ which lost 18 mass units, indicating the loss of one molecule of water, giving rise to an ionic

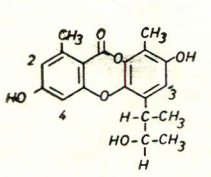
species at m/e 360$^+$ (M^+–18; m* 343). This ion underwent fragmentation loosing —C_4H_7 to give an ion at m/e 305$^+$ (*a*; m* 258.4) indicative of the presence of the same butyl side chain as was observed for yasimin. The ionic species *a* after loosing 105 mass units (—C_7H_5O) gave rise to an ion at m/e 200$^+$ (*b*; m* 207.8) which is similar in pattern to the mass spectrum of yasimin, except that it contained one atom of chlorine somewhere in the benzene ring A. The rest of the significant peaks were at 361$^+$, 345$^+$, 317$^+$, 289$^+$, 262$^+$, 200$^+$, 184$^+$, 172$^+$, 149$^+$ (base peak), 140$^+$, 125$^+$, 123$^+$, 121$^+$, and 99$^+$. All these fragmentation ions are shown in Chart 1 which were supported by their appropriate metastable peaks. The general fragmentation pattern was very similar to that of yasimin and dihydroyasimin, thus indicating the similar gross structure of haiderin, except for the ring A which contains one chlorine atom located at one of the two position, viz., 2 or 4, and for the ring B in which there are one additional hydrogen atom and a hydroxyl group. All these findings are shown in structure I. This hydroxyl group could be either substituted at position 3 in ring B or in the butyl side chain. The ease of loss of one molecule of water in the mass spectrum of this compound was clearly indicative of hydroxyl group being located in the side chain. This was further confirmed by heating this compound with concentrated sulphuric acid resulting in the loss of one molecule of water, yielding *dehydrohaiderin*, $C_{19}H_{17}O_5Cl$, m.p. 180°C. Dehydrohaiderin on ozonisation and reduction followed by steam-distillation into 2,4-dinitrophenylhydrazine solution gave acetaldehyde dinitrophenylhydrazone, thereby confirming the presence of hydroxyl group in the butyl side chain. The above chemical evidence coupled with the evidence provided by mass spectrum narrowed down the structure of haiderin to II or III.



(I)



(II)



(III)

The PMR spectrum (in pyridine d_6) of haiderin showed a triplet centred at τ 9.1 (3H, $J_{A_2-B_3}$ 7 c/s, CH_3—CH_2—) and a quartet at τ 8.2 (2H, $J_{A_2-B_3}$ 7 c/s, CH_3—CH_2) confirming the presence of —OH group in structure II. The location of the hydroxyl group on the α- and not on the β-position was further supported by the negative iodoform test.

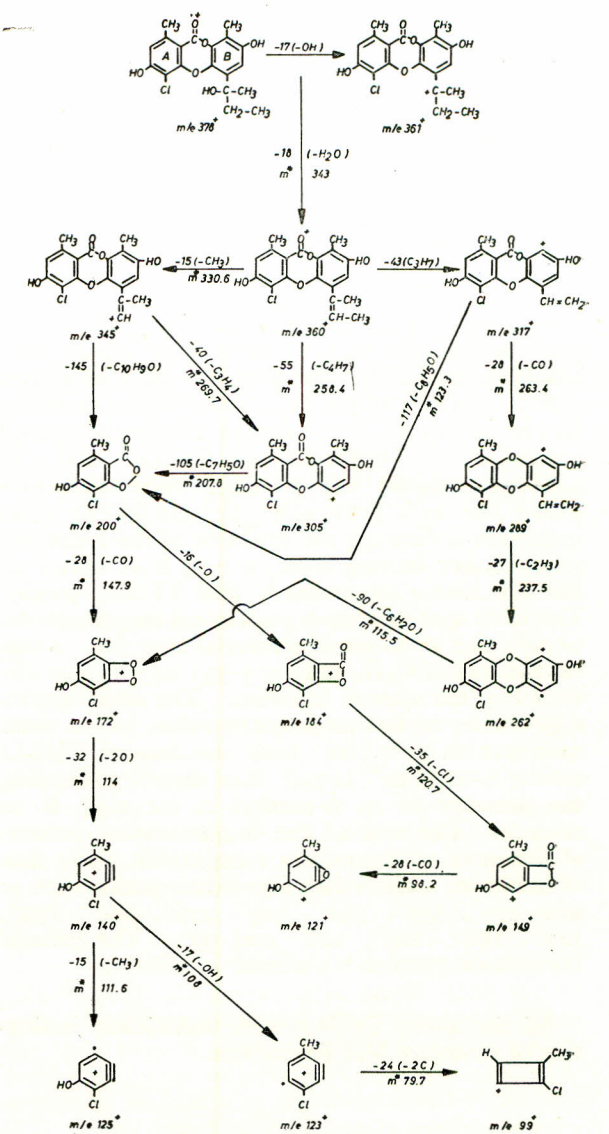


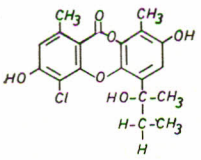
Chart 1.—Mass spectrum fragmentation pattern of haiderin.

The exact position of chlorine atom in ring A was decided by treating haiderin with a base and then decarboxylating the generated acid with copper-bronze in quinoline and subsequently breaking the ether linkage with a mixture of hydroiodic and nitric acids yielding 4-chloro-orcinol,[3] m.p. 104°C. This confirmed the presence of chlorine atom at position 4 in ring A, finally establishing structure IV for haiderin.

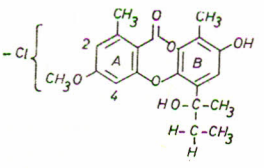
Rubinin (VII).

Rubinin (m.p. 162°C) had the molecular formula $C_{20}H_{21}O_6Cl$ by high resolution mass measurements. Its UV spectrum gave λ_{max} at 268 mμ (ε 10170) and a shoulder at 320 mμ (ε 1285). Its IR absorption bands were at 3448 cm^{-1} (—OH), 1734 cm^{-1} (>C=O) and two bands at 1606 cm^{-1} and 1580 cm^{-1} (benzene ring stretching) as were observed in the cases of yasimin, nornidulin and nidulin. Its mass spectrum showed a strong molecular ion peak at m/e 392^+, which was only 14 mass units higher than haiderin, indicating the presence of a methoxyl group in this compound. This *O*-transmethylation can occur on any of the three hydroxyl groups present in the molecule. As in the case of haiderin, rubinin lost 18 mass units with ease giving rise to a peak m/e 374^+ (*c*) in the mass spectrum, indicating the presence of the methoxyl group either in ring A or in ring B as shown in the alternative structures V and VI for rubinin. The ionic species *c* again underwent fragmentation to give rise to an ionic species at m/e 319^+ *d* due to the loss of C_4H_7 moiety (–55) as was also observed in the case of haiderin. The ionic species *d* gave rise, on further fragmentation, to the same species *b* at m/e 200^+ with the loss of C_8H_7O moiety (–119; m* 125.4) thus clearly indicating the presence of an *O*-methyl in the ring B of rubinin. The rest of the fragmentation pattern of the mass spectrum was comparable with that of haiderin. The other significant peaks were at m/e 375^+, 359^+, 331^+, 303^+, 276^+, 184^+, 172^+, 147^+, 140^+, 123^+, 121^+ and 99^+. The general breakdown pattern is outlined in Chart 2.

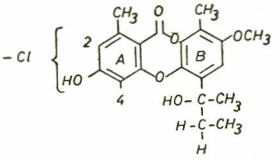
All the above findings were sufficiently indicative of structure VII for rubinin.



(IV)

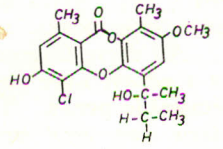


(V)



(VI)

However, to remove any remaining doubts with regard to the structure of rubinin, it was subjected to dehydration which gave dehydrorubinin, (m.p. 140°C). On ozonolysis of dehydrorubinin, acetaldehyde was obtained (confirmed from its dinitrophenyl hydrozone derivative) confirming the presence of the hydroxybutyl side chain as indicated in VII.



(VII)

Rubinin was subjected to alkaline hydrolysis and the acid so obtained was decarboxylated. The decarboxylated product on treatment with a hydroiodic acid–nitric acid mixture gave 4-chlororcinol confirming the location of chlorine atom in position 4 of ring A. The reaction

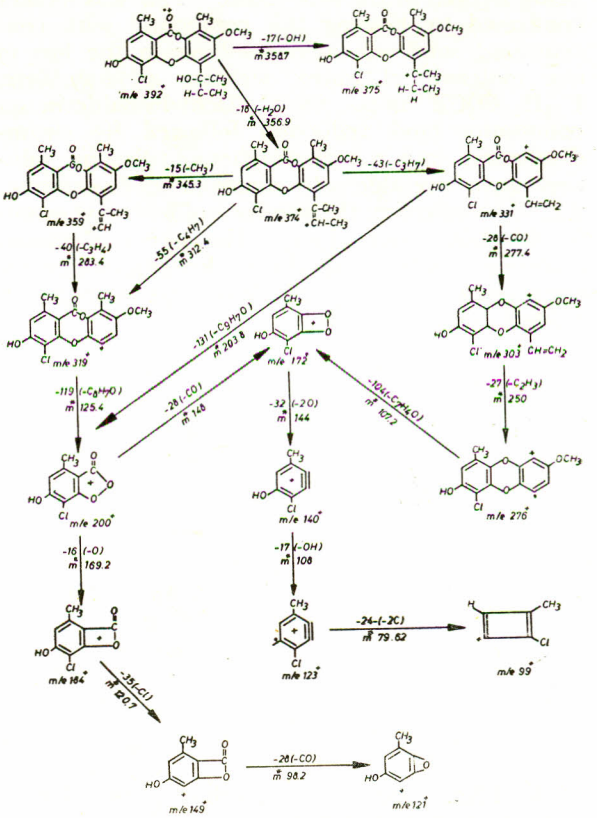


Chart 2.—Mass spectrum fragmentation pattern of rubinin.

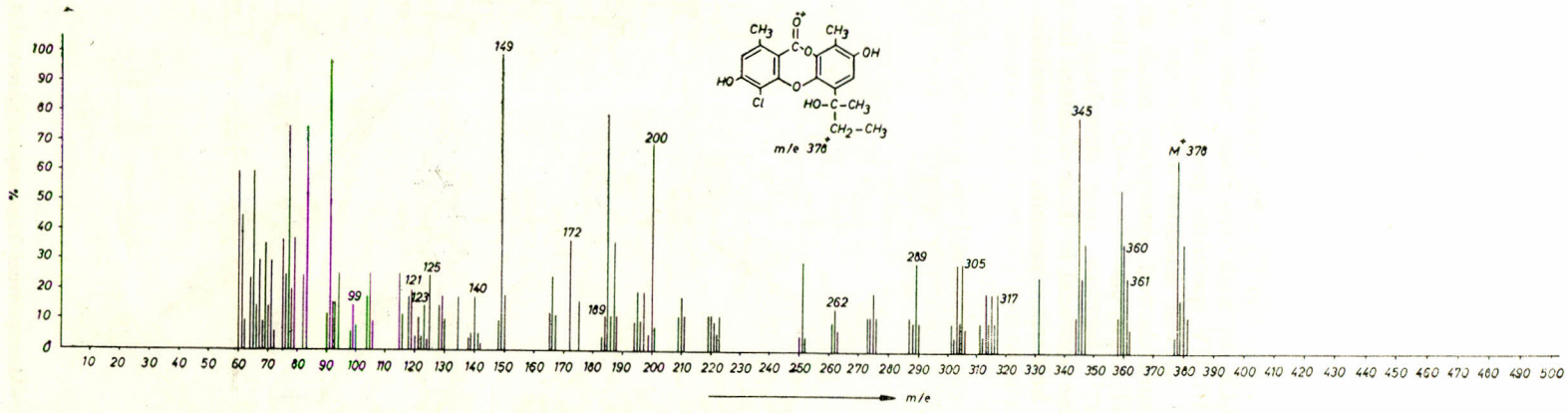


Fig. 1.—Mass spectrum of haiderin.

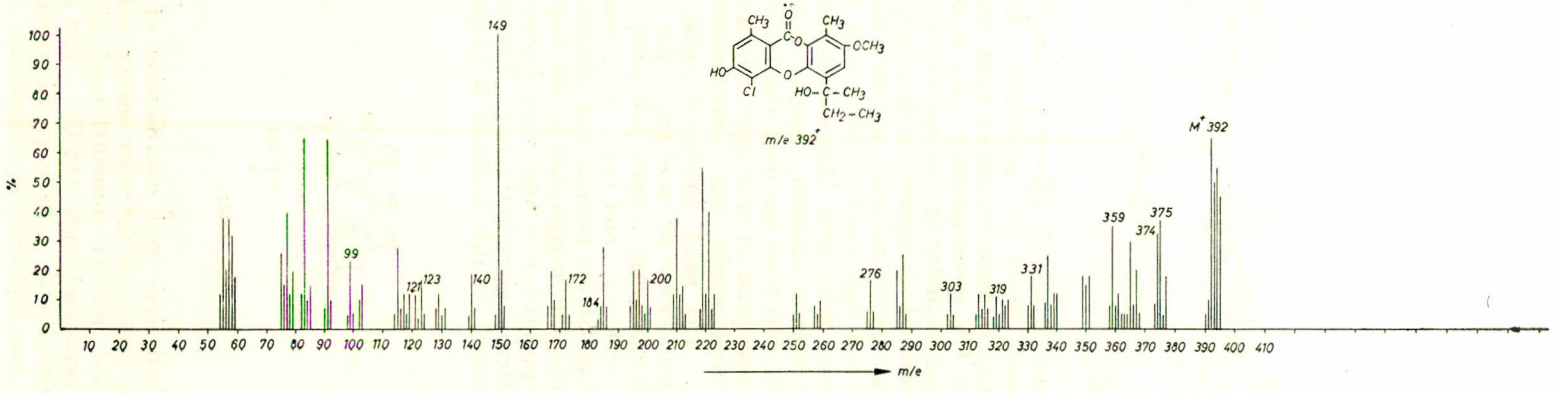


Fig. 2.—Mass spectrum of rubinin.

sequence is given in Chart 3. These reactions finally confirmed the structure of rubinin as shown in VII.

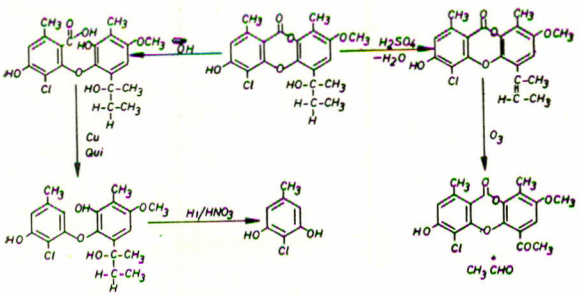
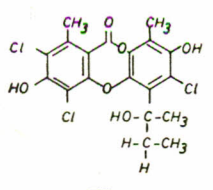


Chart 3.

Shirin (VIII).

Shirin m.p. 118°C, $[\alpha]_D^{25}$ −3.5 (0.057% soln in methanol), gave the molecular formula: $C_{19}H_{17}O_5Cl_3$, by high resolution mass measurements. Its UV spectrum gave λ_{max} at 265 mμ (ε 9708) and shoulder at 320 mμ (ε 929). IR spectrum showed absorption bands at 3333 cm^{-1} (OH); 1724 cm^{-1} (>C=O) and two bands at 1608 cm^{-1} and 1590 cm^{-1} (benzene ring stretching). These values were identical to those obtained for rubinin, haiderin, yasimin, nornidulin and nidulin. Its mass spectrum showed a strong molecular ion peak at m/e 430^+ which clearly indicated that shirin was dihydronornidulin (M^+ 428). The most significant peaks in its mass spectrum were at m/e 415^+, 413^+, 387^+, 373^+, 368^+, 356^+, 345^+, 330^+, 328^+, 207^+, 174^+, 149^+ (base peak) 157^+ and 133^+. Its general mass spectrum breakdown pattern was identical with that of nornidulin, except that all the fragmentation ions appeared at 2 mass units higher. The general pattern is given in Chart 4. The structure of shirin based on this evidence is proposed as VIII.



(VIII)

Alkaline hydrolysis and esterification of shirin, followed by rupture of the ether linkage with nitric acid yielded methyl 2-methyl-3,5-dichloro-4,6-dihydroxybenzoate[4] (m.p. 115°C). The identity of this methyl ester (R_f 0.4; ether–petroleum ether 3:1) was confirmed by comparison with an authentic sample (m.p. 115°C) of the material also obtained from nornidulin under similar conditions.

Shirin was hydrolysed and this was followed by decarboxylation of the liberated acid which on reaction with a mixture of hydroiodic and nitric acids gave 2, 4-dichloroorcinol[5] (m.p. 121°C).

Nasrin (X).

Nasrin (m.p. 232°C) gave molecular formula $C_{22}H_{21}O_5Cl_3$ by high resolution mass measurements. Its UV spectrum gave λ_{max} at 270 mμ (ε 10112) with a shoulder at 320 mμ (ε 587). IR spectrum showed absorption bands at 3448 cm^{-1} (OH), 1713 cm^{-1} (—C—O) and 1610 cm^{-1} 1585 cm^{-1} (benzene ring stretching). These values were identical to those obtained for shirin, rubinin, haiderin, yasimin, nornidulin and

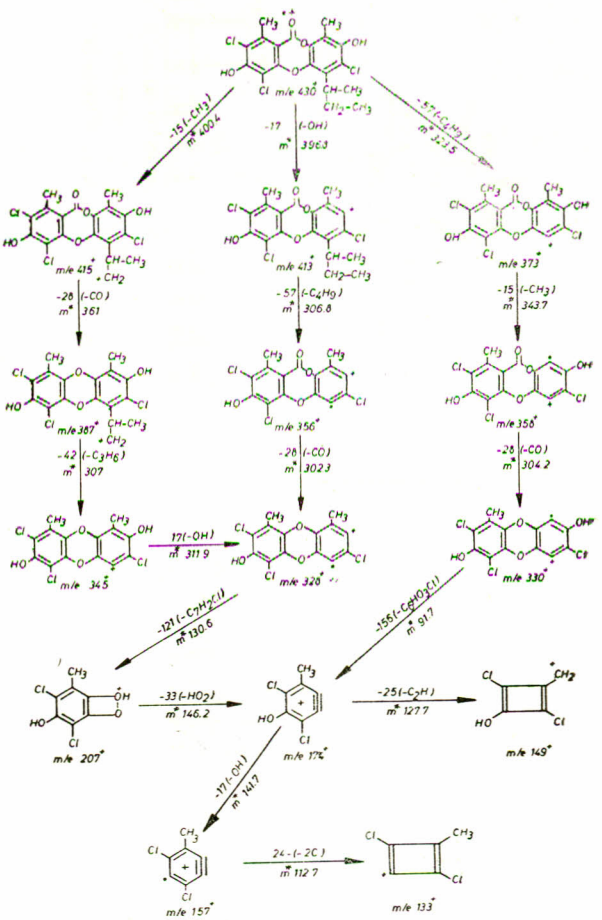
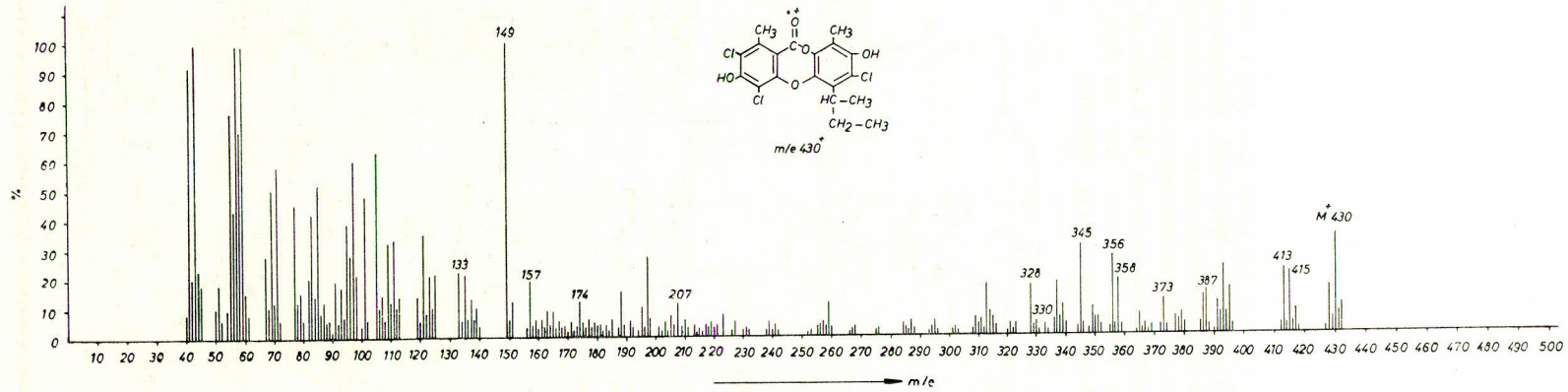


Chart 4.—Mass spectrum fragmentation pattern of shirin.



Flg. 3.—Mass spectrum of shirin.

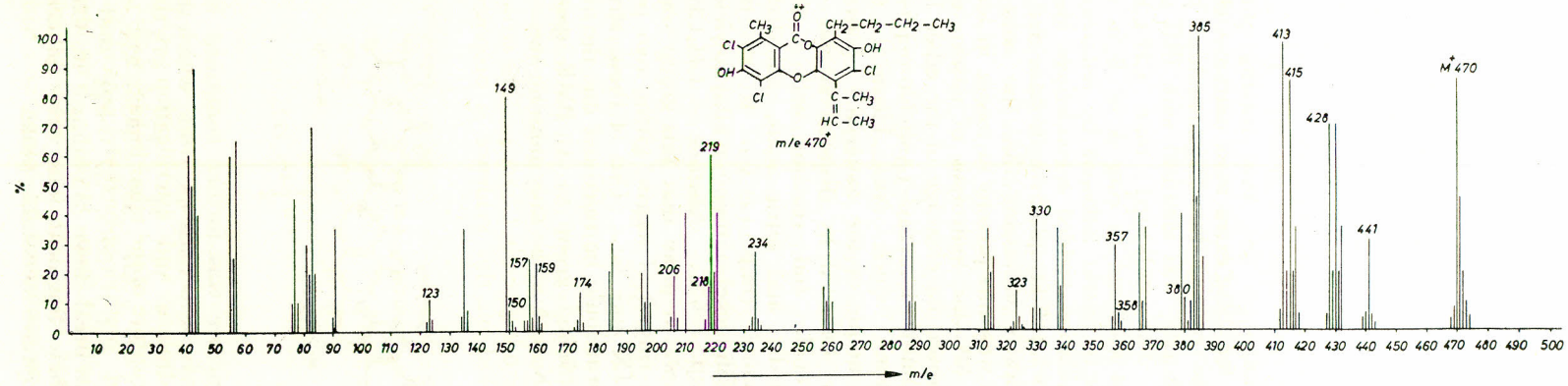
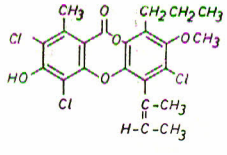
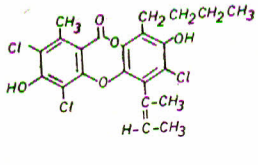


Fig 4.—Mass spectrum of nasrin.

nidulin. Its mass spectrum showed a strong molecular ion peak at m/e 470^+. This molecular ion underwent fragmentation to give a strong peak at m/e 428^+, which was due to the loss of 42 mass units, followed by a metastable peak at m* 389.6, indicating a unified loss of 42 mass units. This could only happen either by the loss of—$CO.CH_3$ or of $CH_2CH_2CH_3$. The presence of $COCH_3$ group in nasrin was ruled out due to its molecular formula. Moreover, the absorption band in its IR spectrum for $COCH_3$ group in the region of 1640–1680 cm^{-1} was not present. It also gave negative iodoform test, thus leaving only one possibility for these extra mass units as CH_2CH_2-CH_3. The rest of the fragmentation pattern below m/e 428^+ was identical with that of nor-nidulin. This indicated that $CH_2CH_2CH_3$ group was attached to ring A or B in nasrin. Ozonolysis of nasrin followed by reduction and steam distillation yielded acetaldehyde identified through its 2,4-dinitrophenylhydrozone derivative, thereby clearly indicating that the same butyl side chain was also present in nasrin in identical position. Alkaline hydrolysis of nasrin, esterification followed by cleavage of the ether linkage yielded methyl 2-methyl-3,5-dichloro-4,6-dihydroxybenzoate which was identical to an authentic sample of the material.[4] Nasrin on hydrolysis, followed by decarboxylation of the generated acid, and reaction with a mixture of hydroiodic and nitric acids, afforded 2, 4-dichloroorcinol[5] (m.p. 121°C). It was dechlorinated[6] with nickel–aluminium alloy to yield pure orcinol, m.p. 106°C. Absence of $CH_2CH_2CH_3$ in ring A was a proof that this group was substituted in ring B of nasrin, giving two possible structures IX and X. Out of these, structure IX for nasrin could be ruled out due the absence of any methoxyl group in its PMR spectrum. Structure X is thus the only possible structure of nasrin.



(IX)



(X)

This structure was further confirmed by the detailed study of its mass spectrum, which showed significant ions at m/e 441^+, 428^+, 415^+, 413^+, 323^+, 385^+, 380^+, 357^+, 330^+, 325^+, 234^+, 219^+, 218^+, 206^+, 174^+, 159^+, 157^+, 150^+ and 133^+. The general break-down is outlined in Chart 5. Most of these fragment ions were followed by their appropriate metastable peaks.

The biogenesis of this compound is very interesting to follow. Rings A and B most probably were derived from head-to-tail condensation of four acetate–malonate units and two isoprene units respectively, the side chain $CH_2CH_2CH_2CH_3$ was derived from two extra acetate units. Biogenesis of these compounds is under study and will be reported later.

Experimental

Mps. were taken on Kofler block and are uncorrected. UV spectra were measured in methanol on a Beckman D.B. spectrophotometer and IR spectra were determined with a Perkin-Elmer 137 instrument, in KBr unless otherwise stated. Mass spectra were measured on an AE-I MS9 at 70 eV. Petroleum ether used had b.p. 65–85°C. The Keisel gel used was PF-254 (Merck).

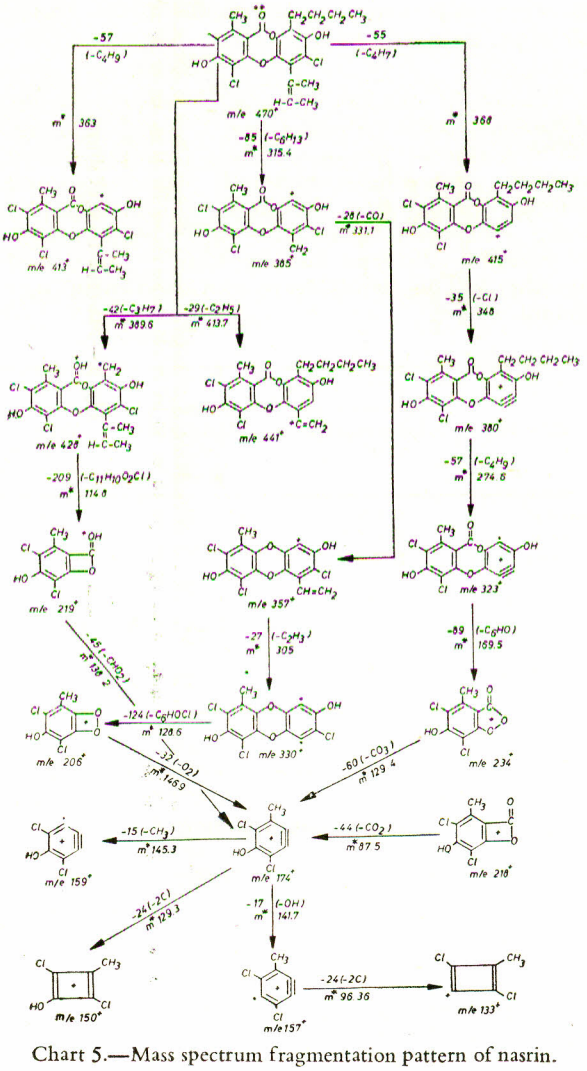


Chart 5.—Mass spectrum fragmentation pattern of nasrin.

Isolation of Haiderin, Yasimin and Rubinin.—The mold *Aspergillus unguis* was grown on modified Czapek–Dox medium (5 l.) enriched with carrot extract, for 22 days at 24–26°C. The mycelium was separated from the broth and dried at 60°C for 48 hr *in vacuuo*. The powdered mycelium (70 g) was extracted with petroleum ether for 72 hr in a Soxhlet apparatus, and followed by extraction with ether for 96 hr. On cooling, the ether extract yielded solid material (3.1 g) which gave yasimin[2] (2.2 g) on fractional crystallisation from ether–petroleum ether and finally from hexane as colourless needles, m.p. 200°C. The combined mother liquors on concentration afforded a semisolid residue which resolved into three components when subjected to repeated preparative thin-layer chromatography using ether–petroleum ether (2:1) as solvent system. The components were named haiderin, $C_{19}H_{19}O_6Cl$, R_f 0.21, m.p. 170°C; yasimin,[2] $C_{19}H_{18}O_5$, R_f 0.45, m.p. 200°C; and rubinin, $C_{20}H_{21}O_6Cl$, R_f 0.74, m.p. 162°C.

Isolation of Nazirin, Sarwin, Khalinin, Aminin, Altamashin, Shirin, Shahidin and Yazidin.—The remaining ethereal filtrate was freed from the solvent and the following compounds were separated through preparative thin-layer chromatography using ether–petroleum ether as solvent system (2:1) and finally repurified by using 2% methanol in ether. The bands were extracted with methanol.

Compounds	R_f value		M.p.
	Ether–pet. ether (3:1)	Methanol–ether (2:98)	
Nazirin	0.05		
Sarwin	0.07		
Khalinin	0.16		
Aminin	0.16		
Altamashin	0.30		
Shirin	0.33	0.24	118°C
Nasrin	0.36	0.38	232°C
Shahidin	0.50		
Yazidin	0.72		

Dehydrohaiderin.—Haiderin (IV) (110 mg) was treated with concd H_2SO_4 (1 ml) and heated on water bath (2 hr). The reaction mixture was then poured over crushed ice (10 g) and extracted with ethyl acetate. The extract on drying (Na_2SO_4) and removal of solvent gave dehydrohaiderin which was crystallised from ethyl acetate and petroleum ether, colourless prismatic needles, m.p. 152°C (35 mg), R_f 0.68 (ether–pet. ether 2:1). Its IR spectrum showed presence of a double bond at 1620 cm^{-1}.

Dehydrorubinin.—Rubinin (VII) (110 mg) gave dehydrorubinin under the above experimental conditions, colourless prismatic needles from ethyl acetate, m.p. 140°C (39 mg), R_f 0.92, (ether–petroleum ether 2:1). Its IR spectrum showed the presence of a double bond at 1618 cm–1.

Ozonolysis of Dehydrohaiderin and Dehydrorubinin.—Dehydrohaiderin (20 mg) and dehydrorubinin (20 mg) were taken separately in glacial acetic acid (10 ml) and ozone was passed through (3 hr). To the reaction mixture water (4 drops) and Zn dust (100 mg) were added. After completion of the reduction (2 hr) the products were steam-distilled into a saturated solution of 2,4-dinitrophenylhydrazine (in 2N HCl). Both hydrozones crystallised from ethanol and had identical m.p. 146°C, undepressed with an authentic sample of acetaldehyde 2,4-dinitrophenylhydrazone (wt 5 mg in each case).

Iodoform Test of Haiderin and Rubinin.—Haiderin and rubinin (20 mg each) were separately dissolved in dioxane (5 ml) and treated with aqueous NaOH (10%, 1 ml). A solution prepared from KI (20 mg) and I_2 (10 mg) in water (80 ml) was added till absorption of I_2 was complete and the mixture left on a water bath (1 hr). It did not give any smell of iodoform.

Isolation of 4-Chloroorcinol from Haiderin and Rubinin.—Haiderin and rubinin (60 mg each) were dissolved in dioxane (2 ml) and then NaOH (2N; 5 ml) was added under nitrogen. The reaction mixture thus obtained was heated on a water bath (½ hr), poured over crushed ice and acidified with 2N H_2SO_4 and the product isolated with ethyl acetate. On drying (Na_2SO_4) and removal of the solvent, a semisolid residue was obtained in each case which was then dissolved in quinoline (0.5 ml) and copper–bronze (100 mg) was added. The reaction mixture was heated in a metal bath (2 hr) at 180–220°C. After cooling, the mixture was extracted with ethyl acetate. The extract was filtered and the solvent removed *in vacuo*. The resulting oily residue was dissolved in glacial acetic acid (3 ml) and then HI (1 ml) and concd HNO_3 (0.5 ml) were added and the mixture left overnight. The reaction mixture was taken up in ethyl acetate and repeatedly washed with water, saturated $NaHCO_3$ solution and then finally with water. The ethyl acetate extract was then shaken with aqueous 2N NaOH solution. The alkali extract was acidified with 2N HCl and then extracted several times with ethyl acetate. The combined ethyl acetate extract was washed with a solution of $Na_2S_2O_3$ and then with water. The ethyl acetate extract was finally dried (Na_2SO_4) and filtered. Removal of solvent *in vacuo* gave 4-chloroorcinol which was crystallised

from ether–petroleum ether mixture, 18 mg, m.p. 104°C undepressed on admixture with an authentic sample of the material.

Reduction of Nornidulin.—Nornidulin (20 mg) dissolved in ethanol (10 ml) was reduced with H_2 over Pd–C catalyst (5% 10 mg). The reaction mixture was filtered. The filtrate on removal of the solvent gave dihydronornidulin, m.p. 118°C, R_f 0.24 (2% methanol in ether). It was found identical to shirin both in its R_f value (0.24) and m.p. (118°C) undepressed on admixture with shirin (m.p. 118°C).

Isolation of 2-Methyl-3,5-Dichloro-4,6-Dihydroxy Benzoic Acid as its Methyl Ester from Shirin, Nasrin and Nornidulin.—Shirin (40 mg), nasrin (30 mg) and nornidulin (50 mg) were degraded according to the method of Dean and his coworkers.[4]

In all the three cases, methyl 2-methyl-3,5-dichloro-4,6-dihydroxybenzoate was obtained, m.p. 115°C, undepressed with an authentic sample of the material. R_f 0.4 (ether–petroleum ether 3:1) were also identical. Yields were from shirin 7 mg, from nasrin 5 mg, and from nornidulin 5 mg.

Isolation of 2,4-Dichlororcinol from Shirin and Nasrin.—Shirin (40 mg.) was dissolved in dioxane (2 ml), aqueous NaOH (2N, 5 ml) was added under N_2. The reaction mixture was heated on water-bath (2hr), and then poured over ice, acidified with 2N H_2SO_4 and the product isolated with ethyl acetate. The product obtained on drying and removal of solvent was dissolved in quinoline (3 ml) containing copper–bronze (100 mg) and heated at 190–225°C (metal bath) till decarboxylation was complete. The mixture was extracted with ethyl acetate several times. The combined extracts were washed with 2N HCl followed by water. The extract was dried and the solvent removed. The resulting oily residue was taken up in acetic acid (2 ml), HI (1 ml) and concd HNO (0.5 ml), and left overnight. The reaction mixture was taken up in ethyl acetate and repeatedly washed with water, $NaHCO_3$ (saturated aq soln) and finally with aqueous NaOH (2N). The NaOH extract was acidified and repeatedly extracted with ethyl acetate. The combined ethyl acetate extract was washed with Na_2SO_3 solution and water respectively, dried (Na_2SO_4) and filtered. Removal of the solvent gave colourless needles of 2,4-dichlororcinol, m.p. 121°C (11 mg), R_f 0.27 (ether–petroleum ether 2:1), identical to the authentic sample similarly obtained from nornidulin, in both its m.p. (undepressed) and R_f value.

Nasrin (40 mg) treated as above also yielded the same 2,4-dichlororcinol, giving the same R_f 0.27 and m.p. 121°C, undepressed on admixture with an authentic sample of 2,4-dichlororcinol.

Orcinol from 2,4-Dichlororcinol.—2,4-Dichlororcinol (20 mg) obtained from the above reaction was dissolved in 2N NaOH (10 ml) and Ni–Al alloy (160 mg) was added in 20 mg portions at ½-hr intervals while the solution was being heated (mild reflux). The reaction mixture was filtered and the filtrate acidified with 2N HCl and extracted with ethyl acetate. Orcinol was obtained, 8 mg, m.p. 106°C, undepressed with an authentic sample of the material.

Ozonlysis of Nasrin.—Nasrin (60 mg) was dissolved in glacial acetic acid (10 ml) and ozone was passed for 4 hr. The reaction mixture was reduced with Zn dust (20 mg) in presence of 4 drops of water for 2 hr and steam-distilled into 2,4-dinitrophenylhydrazine solution (in 2N HCl); acetaldehyde 2,4-dinitrophenyl, m.p. 146°C, 7 mg was obtained.

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