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Fungus Pigments

XIII *. Tramesanguin, the Pigment of *Trametes cinnabarina* var. *sanguinea* (L.) Pilat

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Tramesanguin, a pigment isolated from the fungus *Trametes cinnabarina* var. *sanguinea* (L.) Pilat is shown to be 2-amino-1-formylphenoxazin-3-one-9-carboxylic acid (III). The distribution of cinnabarin, cinnabarinic acid and tramesanguin is discussed.

In 1955 a shipment of the fungus *Trametes cinnabarina* (Jacq.) Fr. (*Polystictus sanguineus* L.) was received from the Belgian Congo. This shipment also contained a small amount of another fungus, which has now been identified as *Trametes cinnabarina* var. *sanguinea* (L.) Pilat (*Polyporus sanguineus* L. ex Fr.).

Schatz *et al.*[1] investigated the extract of this fungus spectrophotometrically and compared it with the extract of *Polyporus cinnabarinus* Jacq., and found them to be very similar. They suggested that the pigment might be the same in both species.

Extraction of the fungus yielded a crystalline pigment, which is called tramesanguin. Paper chromatography of the crude pigment revealed the presence of small amounts of cinnabarin, but no cinnabarinic acid could be detected. Tramesanguin can be freed from accompanying cinnabarin by chromatography on cellulose powder, but as this procedure involves the use of large amounts of solvent, it was used only for the preparation of a sample for analysis. All the reactions described below were carried out with the cinnabarin-containing material. The amount of cinnabarin is estimated to be about 5 %.

Analytical data for tramesanguin indicated a formula $C_{14}H_8O_5N_2$, although $C_{14}H_{10}O_5N_2$ could not be completely excluded. No OCH_3 or CCH_3 groups were detected.

The ultraviolet spectrum of tramesanguin is very similar to the spectra of cinnabarin (I) and cinnabarinic acid (II) [2]

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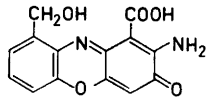
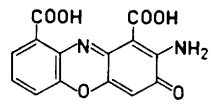


I



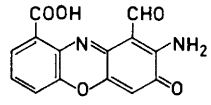
II

suggesting that tramesanguin is also a derivative of phenoxazin-3-one. This is further supported by the infrared spectrum, in which strong bands occur at 1660 and 1580 cm^{-1}, typical of phenoxazin-3-ones[3]. A further sharp band at 978 cm^{-1} corresponds closely to similar bands in the spectra of cinnabarin (974 cm^{-1}) and cinnabarinic acid (973 cm^{-1})[4]. Tramesanguin has, furthermore, a band at 1735 cm^{-1}. Cinnabarinic acid (II) has a band in the same region (1727 cm^{-1})[4], which can be attributed to the unchelated carboxyl group in position 9.

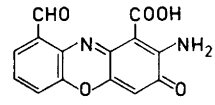
Polarographic reduction of tramesanguin shows two reduction waves, with half-wave potentials of −0.38 and −1.65 V *vs.* S. C. E., respectively. The first of these is the same as that found in cinnabarin[5]. Butenandt *et al.*[6] found two reduction waves at −1.35 and −1.55, respectively, in the polarography of 2-amino-1,9-diacetylphenoxazin-3-one, which were attributed to the reduction of the two ketonic groups. The presence of one carbonyl group in tramesanguin is therefore indicated.

Assuming that the substituents in tramesanguin are in the same positions on the phenoxazinone ring as in cinnabarin and cinnabarinic acid, and taking into account the molecular composition and lack of CCH_3-groups, the carbonyl group can only be an aldehyde group. This is further supported by the presence of weak bands at 2870—2880 cm^{-1} in the infrared spectra of tramesanguin and several of its derivatives.

Tramesanguin should thus be either III or IV.

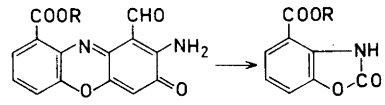


III



IV

Oxidation of tramesanguin with chromic acid gives benzoxazolone-4-carboxylic acid (V), thereby establishing the position of the substituent on the aromatic ring. It can be shown to be a carboxyl group (as in III) by the oxidation of the methyl ester of tramesanguin (VI), which gives methyl benzoxazolone-4-carboxylate (VII).



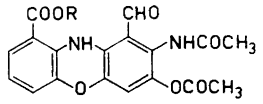
III R=H
VI R=CH_3

V R=H
VII R=CH_3

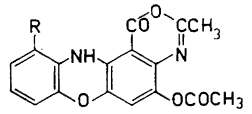
The methyl ester of tramesanguin was obtained with diazomethane, which reaction gave in addition to the desired compound a second substance which will be discussed below. The methyl ester could also be prepared by treatment

of tramesanguin with methanol and hydrochloric acid or dimethyl sulphate and potassium carbonate. However, the yield was much lower in these processes.

Further support for structure III for tramesanguin was obtained from the reductive acetylation. From the reaction mixture a compound with infrared bands at 1795, 1765, 1710, and 1675 cm^{-1} separated, which upon recrystallisation from acetic acid was transformed into a new compound, with infrared bands at 1765 and 1670 cm^{-1}. The two compounds are formulated as VIII and IX, respectively.



VIII R=$COCH_3$
IX R=H

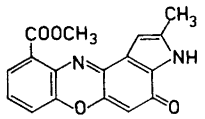


X R=$COOCOCH_3$
XI R=COOH
XII R=CH_2OCOCH_3

In IX the presence of a carbonyl group can be demonstrated by the formation of a semicarbazone. The formation of the mixed anhydride VIII in the reductive acetylation is analogous to the reductive acetylation of cinnabarinic acid, which in our hands gave (X), although Cavill *et al.*[7] obtained only the free acid (XI). Both of these, as well as the leucoacetate of cinnabarin (XII) [4] contain an oxazinone ring formed by loss of one molecule of water. In the formation of tramesanguin leucoacetate no loss of water is involved, in agreement with structure III. A compound with structure IV should have reacted in the same way as I and II.

There is no direct chemical evidence that the aldehyde and amino groups are in the positions 1 and 2, respectively (as in III), but other positions would, however, be biogenetically extremely unlikely.

As mentioned above the methylation of tramesanguin with diazomethane gave in addition to tramesanguin methyl ester a second compound. Its analysis corresponded to the formula $C_{17}H_{12}O_4N_2$, with one OCH_3 and one CCH_3. It is thus derived from tramesanguin by the addition of three CH_2-groups and loss of one molecule of water. XIII is proposed as a tentative structure for this compound.



XIII

The formation of XIII can be envisaged as the addition of two molecules of diazomethane to the group R—CHO giving R—CH_2—CO—CH_3 [8], followed by loss of water between the acetonyl group and the primary amino group. This last reaction has several precendents in the aromatic series [9-11]. The structure XIII is further supported by the infrared spectrum, in which there is only one band in the region of the NH-streching vibration (3200 cm^{-1}).

Also in agreement with this structure is the fact that the compound is less basic than tramesanguin methyl ester; it cannot be extracted from a chloroform solution by 2 N hydrochloric acid, whereas tramesanguin methyl ester can.

In an earlier paper [2] it was demonstrated that the fungus *Trametes cinnabarina* (Jacq.) Fr. (*Polystictus sanguineus* L.) grown in tropical Africa, contained in addition to cinnabarin, cinnabarinic acid, whereas from the same species from other localities no cinnabarinic acid has been isolated. As small amounts of cinnabarinic acid could easily have escaped detection, the composition of the crude pigment of the fungus from three different localities, namely tropical Africa, North America (Pennsylvania) and Finland was studied by paper chromatography. No traces of cinnabarinic acid were found in the samples from North America and Finland. However in the sample from Africa, cinnabarinic acid appeared to be a major component. None of these samples contained any tramesanguin.

There thus appear to exist three different types of phenoxazinone-producing fungi; one producing only cinnabarin, one producing cinnabarin and cinnabarinic acid, and one producing tramesanguin and cinnabarin. Whether these can be regarded as distinct species or merely varieties of one species, remains for the botanists to decide.

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EXPERIMENTAL

The melting points have been determined on a Kofler-microscope. The ultraviolet spectra have been recorded in dioxan solution by a Beckmann DK-2 instrument and the infrared spectra by a Beckmann IR-5 instrument as KBr-discs. The analyses have been carried out in the microanalytical laboratory of Dr. A. Bernhardt, Mülheim (Ruhr).

Isolation of tramesanguin. The finely ground fungal material (190 g) was extracted in a Soxhlet-apparatus with acetone. The precipitate that formed in the extraction flask was collected and washed with water. The crude product thus obtained (12.2 g) was recrystallised either from pyridine or dioxan. Paper chromatography (see below) indicated that such a preparation contained some cinnabarin but it was used for the reactions described below.

A sample for analysis was purified in the following way. It was dissolved in the minimum amount of a mixture of pyridine, butanol and water (6:2:2) and chromatographed on a column of cellulose powder (Whatman standard grade). Although no clearcut separation into different bands could be achieved, the first yellow-coloured eluates contained, according to paper chromatography, pure cinnabarin followed by a small intermediate fraction containing both components, and the last fractions, containing the bulk of material, were pure tramesanguin. Evaporation under vacuum of most of the solvent gave crystalline tramesanguin, which was further recrystallised from pyridine. It decomposed without melting above 250°. (Found: C 58.91; 59.16; H 3.30, 3.29; N 9.79, 9.51. $C_{14}H_8O_5N_2$ requires C 59.16; H 2.84; N 9.86). U.V. spectrum: λ_{max} 238 mμ (log ε 4.55), 430 mμ (log ε 4.41), 450 mμ (infl.) (log ε 4.38), λ_{min} 318 mμ (log ε 3.57). Main I.R.-maxima: 3380(8), 3280(6), 3100(4), 2870(4), 2710(4), 1735(8), 1660(9), 1580(10), 1485(8), 1433(6), 1373(5), 1320(7), 1293(5), 1280(5), 1203(8), 1178(4), 1148(4), 1136(5), 1063(1), 978(4), 856(4), 822(3), 810(3), 763(6), 744(4) and 715(5) cm^{-1}.

Reductive acetylation of tramesanguin. Tramesanguin (150 mg) was suspended in acetic anhydride (2 ml). Two drops of pyridine were added followed by zinc-powder in small

portions. The mixture was warmed to about 30°. When all of the original tramesanguin had disappeared the yellow precipitate that had been formed was filtered off (100 mg). This was recrystallised from acetic anhydride giving the mixed anhydride of acetic acid and tramesanguin leucoacetate (VIII). The product decomposed without melting above 250°. (Found: C 58.06; H 3.99. $C_{20}H_{16}O_8N_2$ requires C 58.25; H 3.91). U.V.-spectrum: λ_{max} 220 mμ (log ε 4.60), 452 mμ (log ε 4.12), λ_{min} 312 mμ (log ε 2.80). Main I.R.-maxima: 3240(4), 2880(1), 1795(8), 1765(7), 1710(4), 1675(8), 1600(4), 1570(1), 1495(10), 1465(8), 1397(6), 1370(6), 1287(7), 1250(9), 1200(8), 1165(8), 1092(9), 1028(7), 1007(8), 962(6), 885(3), 752(5) and 714(4) cm^{-1}.

When the compound was recrystallised from acetic acid it was converted into tramesanguin leucoacetate (IX). IX was also obtained, when the mother liquor obtained in the reductive acetylation, was treated with water. It had no m.p. (Found: C 58.14; H 4.04; N 7.61. $C_{18}H_{14}O_7N_2$ requires C 58.38; H 3.81; N 7.57). U.V.-spectrum: λ_{max} 219 mμ (log ε 4.53), 376 mμ (log ε 3.61), 445 mμ (log ε 4.02), λ_{min} 309 mμ (log ε 2.93), 386 mμ (log ε 3.59). Main I.R.-maxima: 3220(6), 2990(5), 2870(5), 1765(7), 1670(9), 1603(6), 1567(2), 1500(9), 1450(8), 1405(8), 1383(7), 1294(5), 1245(10), 1190(9), 1163(7), 1114(5), 1034(4), 1021(4), 972(2), 940(2), 887(5), 853(2), 810(1), 755(6), 742(4), 716(4) and 688(5) cm^{-1}.

The semicarbazone was prepared in the usual way and recrystallised from acetic acid. It decomposed without melting. (Found: C 53.01; H 4.28; N 16.15. $C_{19}H_{17}O_7N_5$ requires C 53.39; H 4.00; N 16.39). U.V.-spectrum: λ_{max} 242 mμ (log ε 4.32), 301 mμ (log ε 4.07), 418 mμ (log ε 3.90), λ_{min} 278 mμ (log ε 3.91), 340 mμ (log ε 3.19).

Methylation of tramesanguin. Tramesanguin (300 mg) was suspended in chloroform and an ethereal solution of diazomethane was added. After standing for 2 h most of the material had dissolved. The filtered solution was evaporated under vacuum and chromatographed in a chloroform solution on aluminium oxide. Two major bands were formed, a fast moving orange-red band and a slower moving red one. These were eluted separately. Evaporation of the orange coloured band gave tramesanguin methyl ester. After recrystallisation from benzene/chloroform it had m.p. 245—250°. (Found: C 60.33; H 3.66; N 9.45; OCH_3 10.02. $C_{15}H_{10}O_5N_2$ requires C 60.40; H 3.38; N 9.39; 1 OCH_3 10.41). U.V.-spectrum: λ_{max} 238 mμ (log ε 4.59), 418 mμ (log ε 4.45), 438 mμ (log ε 4.45), λ_{min} 310 mμ (log ε 3.56), 428 mμ (log ε 4.43). Main I.R.-maxima: 3400(4), 3280(6), 2940(2), 2870(1), 1740(5), 1720(5), 1650(8), 1585(10), 1502(5), 1477(5), 1435(4), 1374(2), 1312(8), 1294(7) 1208(7), 1147(7), 1012(3), 981(3), 930(2), 921(2), 844(5), 804(3), 774(3), 757(5), 746(3), 725(1) and 712(2) cm^{-1}.

The slower moving red band gave, on evaporation and recrystallisation from benzene/chloroform, red needles, m.p. 292—293°. (Found: C 65.86; H 4.08; N 9.19; OCH_3 9.93; CCH_3 3.76. $C_{17}H_{12}O_4N_2$ requires C 66.23; H 3.92; N 9.09; 1 OCH_3 10.03; 1 CCH_3 4.92). U.V.-spectrum: λ_{max} 222 mμ (log ε 4.47), 252 mμ (log ε 4.36), 393 mμ (log ε 4.43), 413 mμ (log ε 4.37), λ_{min} 242 mμ (log ε 4.31), 297 mμ (log ε 3.50), 405 mμ (log ε 4.34). Main I.R.-maxima: 3200(6), 2950(3), 1730(7), 1635(9), 1605(10), 1567(6), 1538(6), 1502(6), 1470(6), 1433(4), 1340(2), 1314(5), 1292(7), 1256(5), 1223(2), 1191(7), 1173(5), 1159(6), 1140(5), 1085(4), 1010(3), 982(2), 920(1), 831(4), 801(4), 762(6), 754(5), 738(2), 723(2) and 676(2) cm^{-1}.

Oxidation of tramesanguin. Tramesanguin (100 mg) was suspended in acetic acid, and chromium trioxide dissolved in a small amount of water was added. The mixture was allowed to stand over night, after which time the tramesanguin had disappeared. Excess of chromic acid was destroyed with methanol and the acetic acid removed under vacuum. The residue was extracted with ether. Evaporation of the ether and sublimation of the residue gave benzoxazolone-4-carboxylic acid. Its I.R.-spectrum with maxima at 3210(5), 3060(4), 2840(3), 2550(3), 1765(10), 1685(9), 1635(6), 1485(4), 1458(6), 1415(4), 1330(3), 1302(5), 1282(6), 1255(8), 1164(2), 1142(7), 1058(2), 975(1), 940(7), 916(3), 880(3), 813(1), 797(6), 742(5), 712(5) and 698(6) cm^{-1} was identical with that of an authentic sample [2].

Oxidation of tramesanguin methyl ester. This was carried out in the same way as described above for the oxidation of tramesanguin. After sublimation and recrystallisation from light petroleum the methyl benzoxazolone-4-carboxylate had p.m. 218—220°. Main I.R.-maxima: 3250(4), 2940(2), 2870(1), 1785(10), 1730(10), 1635(4), 1493(3), 1465(6), 1433(5), 1320(7), 1277(5), 1260(7), 1210(2), 1192(2), 1159(1), 1130(7), 1056(2), 998(2), 949(4), 933(3), 882(3), 814(2), 807(2), 761(4), 744(6), 734(6) cm^{-1}. Enough material for an analysis was not available. Its I.R.-spectrum as well as its R_F-values in thin-layer

chromatography on silicagel in a number of different solvents were completely identical with those of an authentic sample prepared by esterification of benzoxazolone-4-carboxylic acid with methanol and sulphuric acid.

Reductive acetylation of cinnabarinic acid. Cinnabarinic acid was reduced by zinc and acetic anhydride in the presence of a little pyridine. The yellow precipitate of the mixed anhydride (X) was filtered off and recrystallised from acetic anhydride. It had no m.p. (Found: C 58.15; H 3.51; N 6.91. $C_{20}H_{14}O_8N_2$ requires C 58.54; H 3.44; N 6.83). U.V.-spectrum: λ_{max} 232 mμ (log ε 4.61), 440 mμ (log ε 4.17), λ_{min} 301 mμ (log ε 2.47). Main I.R.-maxima: 3270(2), 3090(1), 2920(1), 1795(7), 1770(7), 1730(8), 1715(6), 1660(7), 1600(3), 1570(3), 1500(10), 1477(7), 1443(8), 1370(6), 1348(4), 1318(5), 1294(9), 1256(8), 1193(10), 1166(8), 1129(7), 1083(9), 1067(10), 1026(5), 1003(8), 965(8), 940(6), 913(5), 887(3), 855(1), 893(6), 742(4), 703(3), 690(3) and 663(3) cm^{-1}.

Paper chromatography. With the solvent system pyridine: butanol:H_2O (6:2:2) using Whatman No. 1 paper and descending technique the following R_F-values were found: cinnabarin 0.73—0.75, tramesanguin 0.53—0.55, cinnabarinic acid 0.34—0.37. The cinnabarin spot was weakly yellow, that of tramesanguin strongly yellow and that of cinnabarinic acid orange yellow. The cinnabarinic acid spot usually showed quite bad tailing whereas the two others were well developed.

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