ACTA CHEMICA SCANDINAVICA 11 (1957) 1485—1492

Fungus Pigments

V*. Degradations of Cinnabarin

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Fungus material from Central-Africa, labelled as *Polystictus sanguineus*, gave, in addition to the desired cinnabarin, a second pigment, $C_{14}H_8O_4N_2$, termed cinnabarinic acid. Oxidation of cinnabarin with potassium permanganate gives benzoxazolone-4-carboxylic acid and with hydrogen peroxide oxalic or oxamic acid. No pure substances were isolated after zinc dust distillation but the spectra of some fractions indicated the occurrence of carbazole and phenoxazin-3-one derivatives. A partial formula based on the phenoxazin-3-one ring system is proposed for cinnabarin.

Shortly after publication of our first paper on cinnabarin [1], the pigment of *Trametes cinnabarina* Jacq., there appeared two papers by Australian authors [2,3] describing a pigment, polystictin, isolated from *Coriolus sanguineus* (Fr.) (*Polystictus cinnabarinus* Jacq.). However, these names are regarded as synonymous with *T. cinnabarina* [3]. Although there are some minor differences between the properties reported for cinnabarin and polystictin, both Lemberg [2] and Cavill and coworkers [3] regarded polystictin as identical with cinnabarin. This has now been confirmed by a comparison of the IR-spectrum of cinnabarin with that of polystictin published by Cavill *et al.*[3].

Although Lemberg, who had priority in the isolation of the pigment, used the name polystictin, he has proposed that the name cinnabarin should be adopted [2]. We have therefore continued to use this name.

Further work on the structure of cinnabarin was for a long time hampered by lack of material, but through kind help of Professor H. Erdtman, Stockholm, it was finally possible to secure a substantial amount of fungus material, labelled as *Polystictus sanguineus*, from tropical Africa. This material came from three different sources, namely from the Belgian Congo, Nigeria and the Gold Coast sent by Mr. J. Frasell, Dr. T. S. Robertson and Dr. M. F. Madelin, respectively.

* Part IV *Acta Chem. Scand.* **10** (1956) 1111. A preliminary account of the present work has been published in *Chemistry & Industry* **1956** 1505.

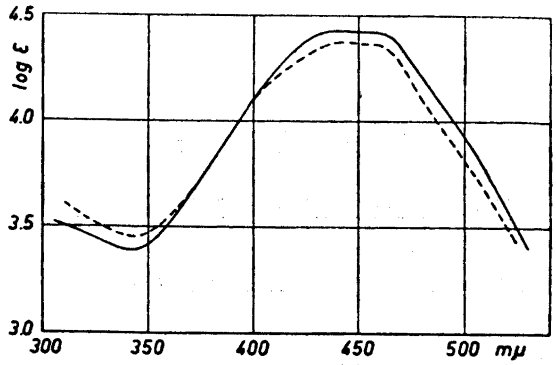


Fig. 1. Absorption curves of cinnabarin (————) and cinnabarinic acid (— — —) in pyridine.

The crude pigment obtained by extraction of this fungus material as previously described [1] was found to be a mixture of cinnabarin and another pigment, for which we propose the name cinnabarinic acid. Cinnabarinic acid differs clearly from cinnabarin in its greater acidity, being readily soluble in sodium hydrogen carbonate. The separation of the two pigments can be achieved either by using this property or by fractional extraction with hot pyridine, in which cinnabarinic acid is much more soluble.

Lemberg [2] has described a pigment, polystictinin, accompanying his polystictin. A comparison of the properties of polystictinin with those of cinnabarinic acid shows, however, that these two cannot be identical.

The analytical figures for cinnabarinic acid agree well with those required for $C_{14}H_8O_6N_2$, but it should be kept in mind that this result is from analysis of a single preparation, the purity of which is difficult to assess, owing to lack of a melting point. As cinnabarin has the composition $C_{14}H_{10}O_5N_2$ cinnabarinic acid thus differs in having one oxygen atom more and two hydrogen atoms less. A close relationship between cinnabarin and cinnabarinic acid is also indicated by the almost identical absorption curves (Fig. 1). Work is in progress aimed at relating cinnabarinic acid with cinnabarin.

Earlier work had given very little information about the structure of cinnabarin. Each group had observed that cinnabarin on alkaline hydrolysis gives one molecule of ammonia [1-3]. Cavill *et al.*[3] inferred from the IR-spectrum that cinnabarin contains a carboxamide group and also concluded that cinnabarin contains an acidic hydroxyl group and an ether oxygen, as well as a quinonoid system. Nothing was known about the basic ring system.

In the hope of obtaining some products containing a smaller or larger part of the basic skeleton, we have treated cinnabarin with different oxidants under varying conditions and submitted it to zinc dust distillation.

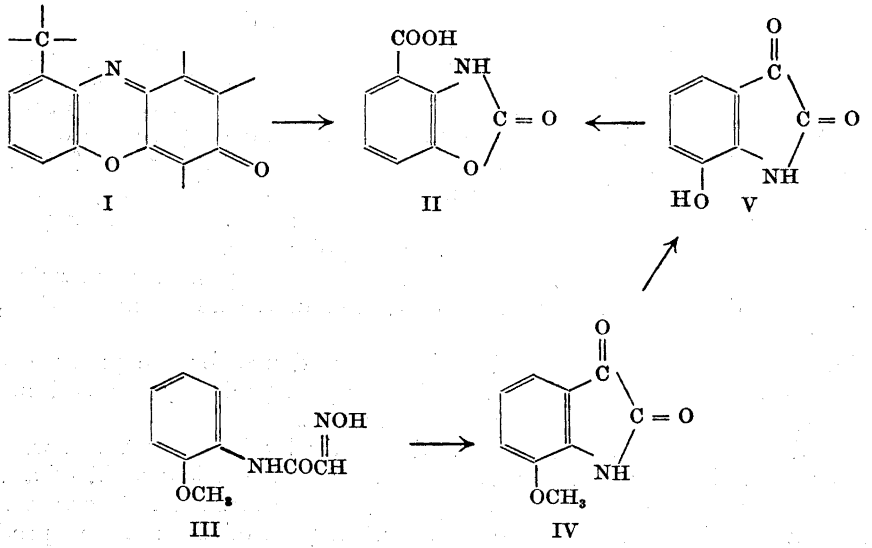
Oxidation with potassium permanganate proved to be most informative. It gave a substance, m.p. 279—281°, of the composition $C_8H_5O_4N$. With diazomethane this gave a dimethyl derivative, $C_{10}H_9O_4N$, with however, only one methoxyl. At this stage of the investigation the electronic spectrum of

cinnabarin which shows a close similarity to that of actinomycin, suggested that cinnabarin might be structurally related to the group responsible for the spectrum of the actinomycin molecule. According to Brockmann and Muxfeldt [4] this is derived from phenoxazin-3-one, and Johnson and coworkers [5,6] showed that oxidation with alkaline hydrogen peroxide followed by acid hydrolysis to remove the amino acids, gives 7-methylbenzoxazolone-4-carboxylic acid. Our oxidation product could therefore be benzoxazolone-4-carboxylic acid (II) a view further supported by the similarity of the UV-spectrum of our product with that found by Bullock and Johnson [6] for 7-methylbenzoxazolone-4-carboxylic acid.

Benzoxazolone-4-carboxylic acid was synthesised and was found to be identical with our oxidation product. The identity was established by mixed melting points of both the free acid and its dimethyl derivative with the corresponding synthetic substances. The dimethyl derivative must be methyl N-methyl-benzoxazolone-4-carboxylate; Zinner and Herbig [7] have shown that methylation of the benzoxazolone system with diazomethane leads to the N-methyl derivative.

It was therefore concluded that cinnabarin is a derivative of phenoxazin-3-one. Cavill and Tetaz [8] have also arrived at this conclusion. The recently reported observations that on oxidation phenoxazin-3-one gives benzoxazolone [6] and 2-amino-1,9-diacetylphenoxazin-3-one gives 4-acetylbenzoxazolone [9] gives further support to this view.

The benzoxazolone-4-carboxylic acid was synthesised by an analogous method to the synthesis of 7-methylbenzoxazolone-4-carboxylic acid [6]. The starting material was 7-methoxyisatin (IV). This was prepared by a modification of the Sandmeyer reaction from *o*-anisidine. Although Sandmeyer [10] explicitly states that the ring closure to an isatin derivative cannot be affected with *iso*nitroso-acet-*o*-anisidide (III) we found that use of polyphosphoric



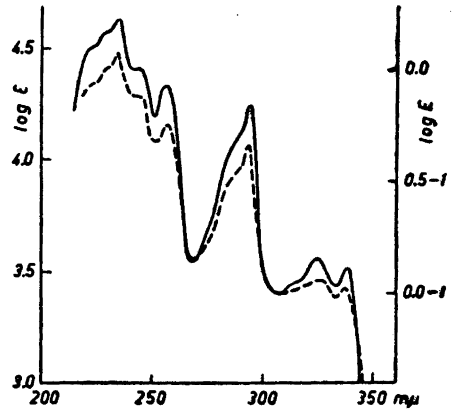


Fig. 2. Absorption curves of carbazole (————) and fraction e from zinc dust distillation (— — —) in hexane. The extinction values for the former are on the left hand side and for the latter on the right hand side.

acid instead of sulphuric acid brings about the desired ring closure, giving 7-methoxyisatin (IV). This was identified by mixed m.p. determination with an authentic sample kindly supplied by Professor A. W. Johnson, Nottingham, and originally sent to him by Professor A. Mangini, Bologna. Demethylation of 7-methoxyisatin was accomplished with pyridine hydrochloride [6] and the 7-hydroxyisatin (V) was oxidised with hydrogen peroxide in the presence of sodium hydrogen carbonate giving benzoxazolone-4-carboxylic acid (II).

The formation of benzoxazolone-4-carboxylic acid (II) places a carbon atom in position 9 in the phenoxazin-3-one nucleus leading to the partial structure I. If this carbon atom were a carboxyl group or a derivative, which would give a carboxyl group on alkaline hydrolysis, it would have been expected that benzoxazolone-4-carboxylic acid would also have been formed on oxidation with hydrogen peroxide in alkaline solution, as in the oxidation of actinomycin [5,6]. The only identifiable product from hydrogen peroxide oxidation was either oxalic acid or oxamic acid according to the alkalinity of the solution. In addition a product was formed which is very difficultly soluble in organic solvents, and is non-volatile under vacuum. It has not been possible to purify this product, which appears to be polymeric.

It is therefore concluded that the carboxyl group in benzoxazolone-4-carboxylic acid has been formed by oxidation of a side chain by the permanganate.

The formation of oxamic acid is further evidence for the presence of a carboxamide group in cinnabarin. This group is probably situated in the quinonoid ring of the phenoxazin-3-one nucleus.

Zinc dust distillation had been tried already by Lemberg [2], but without much success. Even in our hands the reaction gave a low yield of a very complex mixture. No crystalline compounds were obtained but certain fractions from the chromatography of the neutral part of the reaction product

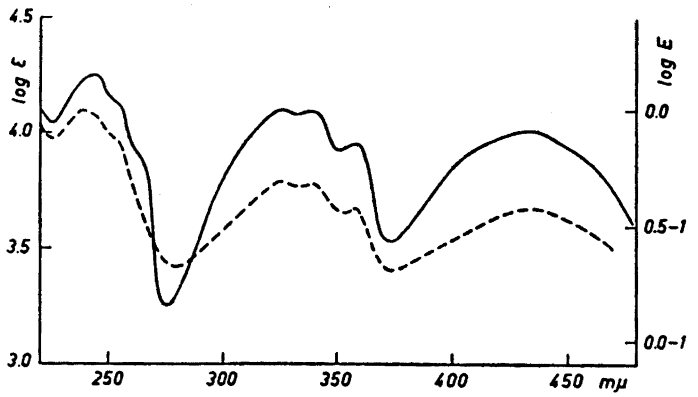


Fig. 3. Absorption curves of phenoxazin-3-one (————) and fraction 8 from zinc dust distillation (— — —) in hexane. The extinction values for the former are on the left hand side and for the latter on the right hand side.

had UV-spectra from which it has been possible to draw some conclusions about the substances present. The spectrum of one fraction was practically identical with that of carbazole (Fig. 2). This means that carbazole or a homologue (or a mixture of homologues) had been formed. Because the UV-spectra of carbazole and C-alkylated carbazoles are almost identical [11,12] it is not possible to distinguish between these alternatives.

This result was somewhat surprising. Firstly because Lemberg [2] had found no reactions typical of carbazole in his reaction product. Secondly, if cinnabarin is a derivative of phenoxazin-3-one, this means that the ring is opened at the oxygen atom which is lost, followed by ring closure again. Such an opening of the oxygen bridge during zinc dust distillation is not usually met with in simple systems such as dibenzofuran [13] and dibenzopyran [14] but there seems to be no information available about the behaviour of phenoxazines upon zinc dust distillation.

In view of the very low yield obtained, the possibility cannot be completely ruled out, that this substance was formed from some impurity in the starting material. The fact that fractions with identical UV-spectra have been obtained from two samples of cinnabarin of completely different origin can, however, be taken as evidence against such a possibility.

A further fraction had a spectrum closely similar to that of phenoxazin-3-one (Fig. 3) and this fraction evidently corresponds to the product (H) reported by Cavill and Tetaz [8].

EXPERIMENTAL

(The analyses have been performed by Dr. A. Bernhardt, Mülheim)

Separation of cinnabarin from cinnabarinic acid. a) The mixture of pigments obtained by acetone extraction of the fungus material was treated on a filter with sodium hydrogen carbonate and washed with water. This was repeated until the filtrate was no longer red.

The filtrate was run directly into dilute acid to give cinnabarinic acid as a red amorphous precipitate. This was recrystallised from pyridine giving dark brown glistening crystals, which decompose above 300° without melting. (Found: C 55.98; H 2.80; N 9.18; O 31.74. $C_{14}H_8O_6N_2$ requires C 56.01; H 2.69; N 9.33; O 31.98).

The cinnabarin remaining on the filter was purified by recrystallisation from pyridine.

b) The mixture was boiled with a small amount of pyridine, filtered hot, and allowed to crystallise. The crystals which were a mixture enriched in cinnabarinic acid, were filtered off. The mother liquor was used again for re-extraction of the undissolved material. This was repeated until the crystallisate contained pure cinnabarin, as judged by the absence of colouration upon treating a sample with sodium hydrogen carbonate. The cinnabarin was then recrystallised from fresh pyridine. Although this method readily gives cinnabarin, free from cinnabarinic acid, it is more difficult to obtain pure cinnabarinic acid and for this purpose it is better to combine it with method a).

Oxidation of cinnabarin with potassium permanganate. Cinnabarin (3.0 g) was suspended in 5 % sodium carbonate solution (120 ml) and finely powdered potassium permanganate was added with stirring until the permanganate colour persisted. The mixture was acidified with sulphuric acid and the precipitate of manganese dioxide was dissolved by adding sodium hydrogen sulphite. The solution was extracted with ten portions of ether (50 ml each). The residue from the ether extract was washed with a little water and then with benzene and sublimed (200—210°/2 mm). The sublimate was recrystallised from methanol giving benzoxazolone-4-carboxylic acid (240 mg) as slightly yellow crystals; m.p. 279—281°. (Found: C 53.17; H 2.84; N 7.74; O 34.28. $C_8H_5O_4N$ requires C 53.64; H 2.81; N 7.82; O 35.73) λ_{max} (ethanol) 239 mμ (log ε 4.05), 302 mμ (log ε 3.20); λ_{min} 224 mμ (log ε 3.80), 262 mμ (log ε 2.60); λ_{max} (0.05 N NaOH) 226 mμ (log ε 4.17), 250 mμ (log ε 4.08), 310 mμ (log ε 3.92); λ_{min} 238 mμ (log ε 3.90), 270 mμ (log ε 2.97).

Benzoxazolone-4-carboxylic acid (45 mg) was dissolved in methanol and an ether solution of diazomethane was added until the yellow colour persisted. The solution was evaporated and the residue sublimed (140—150°/2 mm) giving methyl N-methylbenzoxazolone-4-carboxylate (43 mg), m.p. 141—142° (Found: C 58.00; H 4.62; N 6.98; O 30.88; OCH_3 14.84. $C_9H_6O_3N(OCH_3)$ requires C 57.97: H 4.38; N 6.76; O 30.89; OCH_3 14.98) λ_{max}(ethanol) 240 mμ (log ε 3.90), 304 mμ (log ε 3.76); λ_{min} 226 mμ (log ε 3.74), 264 mμ (log ε 2.76).

Oxidation of cinnabarin with hydrogen peroxide. a) Cinnabarin (200 mg) was suspended in sodium hydrogen carbonate (4 %, 5 ml) and hydrogen peroxide (30 %; 5 ml) was added. More hydrogen peroxide was added from time to time until a clear almost colourless solution resulted. This took about 2 days. The solution was acidified with conc. hydrochloric acid and evaporated at room temperature in a vacuum desiccator over sodium hydroxide. The residue was extracted with several portions of hot dioxan. The dioxan solution was evaporated almost to dryness in vacuum and the crystals which formed were filtered off (*ca.* 35 mg) and recrystallised from dioxan, m.p. 210—211°. (Found N 15.40. $C_2H_3O_3N$ requires N 15.73). It gave no depression of m.p. when mixed with oxamic acid [15].

b) Cinnabarin (200 mg) was dissolved in 2 N sodium hydroxide (2 ml) and allowed to stand overnight. Hydrogen peroxide (30 %) was then added dropwise and the solution allowed to stand until it was light yellow. It was then acidified with conc. hydrochloric acid and evaporated in a vacuum desiccator over sodium hydroxide. The residue was extracted with ether and the ether evaporated giving oxalic acid.

c) Hydrogen peroxide (30 %; 2 ml) was added to cinnabarin (200 mg) followed by dropwise addition of 2 N sodium hydroxide. When the solution tended to acquire a darker colour more hydrogen peroxide was added. This was continued until a clear light yellow solution was obtained. Oxamic acid and oxalic acid were isolated from this as described in a) and b).

Zinc dust distillation of cinnabarin. A mixture of cinnabarin (252 mg) and Zn-dust (2.54 g), divided into 5 portions, was distilled in a stream of hydrogen. The volatile products were collected in a trap cooled to −70°. The distillation residue was extracted with ether which was combined with an ether solution of the volatile part. Altogether 30 mg of ether soluble material was obtained. This was divided into basic, acidic and neutral parts by extraction of the ether solution with hydrogen chloride and sodium hydroxide. Each fraction was then chromatographed on alumina using hexane, ether and methanol in that order for elution. The chromatograms on inspection by U. V., showed

several fluorescent zones. The different zones were collected in different fractions and the ultra violet spectrum of each fraction was recorded. Only the neutral part gave fractions that showed any selective absorption in the ultra-violet. Fractions Nos. 5—7 (eluted by hexane) showed two distinct maxima at 232 and 291 mμ. Further chromatography of these fractions gave finally a noncrystalline fraction with maxima at 234, 257, 293, 327 and 337 mμ, and minima at 251, 269, 307 and 332 mμ (Fig. 2).

Fraction 8 had maxima at 240, 326, 340, 358, and 528—533 mμ and minima at 225, 279, 334, 352 and 370 mμ (Fig. 3).

Fractions 9—10 had maxima at 240 and 302 mμ and minima at 227 and 280—285 mμ.

7-Methoxyisatin. (IV). *Iso*nitroso-acet-*o*-anisidide [10] (III) (0.5 g) was added to polyphosphoric acid [16] (5 ml) kept on a boiling water bath. Heating was continued until all the material had dissolved giving a dark violet-brown solution. This was cooled and a large volume of water was added. The precipitate was filtered off and treated with chloroform. The chloroform solution was chromatographed on alumina. The dark red eluate was evaporated to a small volume. 7-Methoxyisatin (0.1 g) crystallised, m.p. 242—243°, undepressed on admixture with an authentic sample.

7-Hydroxyisatin. (V). 7-Methoxyisatin (150 mg) was added to pyridine hydrochloride (1.5 g) and the mixture was heated for 20 minutes to 200° in an oil bath. Water was added to the mixture after cooling and the product was taken up in ethyl acetate. A considerable amount of dark coloured material was removed by filtering the ethyl acetate solution through a short column of silicic acid. Evaporation of the solvent gave 7-hydroxyisatin (80 mg) as brown-red crystals, which decompose around 270° without melting. (Found: C 59.01; H 3.75. $C_8H_5O_3N$ requires C 58.90, H 3.09).

Benzoxazolone-4-carboxylic acid. (II). 7-Hydroxyisatin (80 mg) was suspended in sodium hydrogen carbonate (5 %; 15 ml) and hydrogen peroxide (30 %; 2 ml) was added. After about one hour the originally dark red solution had become almost colourless. Acidification produced a crystalline precipitate of benzoxazolone-4-carboxylic acid, m.p. 279—281°. By treatment with diazomethane this was converted into methyl N-methylbenzoxazolone-4-carboxylate m.p. 140—141°. In neither case was there any depression of melting point when mixed with the corresponding substances obtained by oxidation of cinnabarin.

The authors wish to thank Mr. J. Fraselle, Yangambi, Belgian Congo, Dr. T. S. Robertson, West African Institute for Oil Research, Benin City, Nigeria, and Dr. M. F. Madelin, Botany Department, University College of the Gold Coast, Achimoto, Gold Coast, for their great kindness in collecting the fungus material used in this investigation. Our thanks are also due to Professor H. Erdtman, Stockholm, for arranging these contacts.

We wish also to thank Professor A. W. Johnson, Nottingham, for a sample of 7-methoxyisatin and for much information on the oxidation of actinomycin prior to publication.

This investigation has been financially supported by a grant from the *State Commission for Natural Sciences* (*Valtion Luonnontieteellinen Toimikunta*), which is gratefully acknowledged.

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Received June 18, 1957.