95. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

66. PENICILLIOPSIN, THE COLOURING MATTER OF *PENICILLIOPSIS CLAVARIAEFORMIS* SOLMS-LAUBACH

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IN 1936 our attention was drawn by Prof. Ch. Dhéré of the University of Fribourg to a unique fungal colouring matter, long known as "mycoporphyrin", which is present in nature in *Penicillioptis clavariaeformis* Solms-Laubach, a fungus peculiar to Java and found on the fallen and decaying fruits of *Diospyros macrophylla* Bl. [Solms-Laubach, 1887]. The whole fructification of this fungus is sulphur-yellow in colour, and Reinke [1887], in an accompanying paper, found that alcohol gives a yellow to orange extract which, on long standing, becomes purplish-red by transmitted light and shows a very striking orange-red fluorescence by reflected light. Reinke isolated from this extract red prisms which were not examined chemically but to which he gave the name mycoporphyrin since the substance gives an absorption spectrum which very closely resembles that of the true porphyrins. Other workers, e.g. Möller [1901], Hennings [1904] and Blochwitz [1931], have described investigations on the colouring matters of different species of *Penicillioptis* which did not lead to any clearer ideas as to the chemical constitution of mycoporphyrin. Fischer & Niemann [1925] showed however by a series of reactions on an alcoholic extract of the naturally occurring fungus that mycoporphyrin, in spite of its absorption spectrum, cannot be a true porphyrin. The colouring matter itself was not isolated or analysed.

When we decided to commence work on the colouring matter of *P. clavariaeformis* it immediately became obvious that, in order to obtain sufficient material for a thorough chemical investigation, it would be necessary to work with laboratory cultures. It was found that the fungus grows quite well on the well-known synthetic Czapek-Dox, NaNO_3 -glucose-mineral salts medium, and that if an orange extract be added to this medium and the NaNO_3 be replaced by ammonium citrate, approximately equivalent in nitrogen to the NaNO_3 , luxuriant and typical growth is obtained. These cultures were incubated at 24° and it is important to note that they were grown *in the absence of light*. Solvent extraction of the fungus so obtained has led to the isolation of very considerable amounts—about 14 g. per 100 flasks—of an orange crystalline substance which we propose to name *penicillioptin*. An account of our earlier work on penicillioptin and its derivatives was given to the Society in 1938 [Oxford, 1938].

Penicillioptin, m.p. about 330° , has the empirical formula $\text{C}_{30}\text{H}_{24}\text{O}_8$ and it contains 2 C-methyl groups as was shown by a Kuhn-Roth oxidation. It forms a diacetate and a bisphenylcarbamate, and analyses and molecular weight estimations on both these substances confirmed the empirical formula given. It reacts vigorously with diazomethane and diazoethane giving derivatives,

neither of which contains nitrogen, and corresponding respectively to a tetramethyl and diethyl ether. In neither case, however, could the alkoxy groups presumably present be split off as alkyl iodide in a Zeisel estimation. On heating in air either alone or mixed with zinc dust penicilliopsin gives *Frangula*-emodin anthranol and, on distillation with zinc dust in hydrogen, it yields 2-methyl-anthracene. Hence penicilliopsin must be related to the polyhydroxyanthraquinones, a conclusion which is supported by the fact that it gives tetranitro-*Frangula*-emodin and nitrococcusic acid on oxidation with HNO_3 .

Solutions of penicilliopsin in most organic solvents are yellow to orange in colour when freshly prepared, but on exposure to air and light undergo a series of colour changes which vary very much with different solvents. These colour changes involve two quite distinct reactions.

(a) *An oxidation process* dependent on the presence of atmospheric oxygen but not dependent on the presence of light. This process, which takes place very quickly in the dark in solutions of penicilliopsin in certain nitrogenous organic solvents, e.g. piperidine, or in neutral organic solvents such as ethanol or dioxane containing small amounts of organic nitrogenous bases, e.g. piperidine or brucine, results in the uptake of 3 atoms of oxygen per mol. of penicilliopsin and the formation of intensely purple non-fluorescent solutions. The oxidation product, which we propose to name *oxyenicilliopsin*, has the empirical formula $\text{C}_{30}\text{H}_{20}\text{O}_9$ and forms purplish-black aggregates which do not melt at 360° and which give a tetra-acetate on acetylation with acetic anhydride containing conc. H_2SO_4 . Oxyenicilliopsin gives colour reactions which are quite different from those given by penicilliopsin and, in striking contrast to penicilliopsin, does not give tetranitroemodin on oxidation with conc. HNO_3 .

(b) *A photochemical process*, independent of the presence of oxygen but dependent on the presence of light. This process results in the conversion of oxyenicilliopsin into an isomeric compound which we propose to name *irradiated oxyenicilliopsin*. When an alcoholic solution of oxyenicilliopsin, deep purple in colour and non-fluorescent, is exposed to bright daylight either under aerobic or anaerobic conditions the original deep purple colour soon begins to fade, a fiery red fluorescence begins to develop and irradiated oxyenicilliopsin separates in chocolate brown needles which do not melt up to 370° . Irradiated oxyenicilliopsin has the empirical formula $\text{C}_{30}\text{H}_{20}\text{O}_9$ and is, in our opinion, the pure substance which is responsible for the earlier conceptions of mycoporphyrin. A careful study of previous literature on mycoporphyrin leads to the conclusion that what has been previously understood as mycoporphyrin was, in reality, a mixture, in varying amounts, of penicilliopsin, oxyenicilliopsin and irradiated oxyenicilliopsin. Since none of these substances contains nitrogen it is obvious that, quite apart from the previously quoted evidence of Fischer & Niemann [1925], mycoporphyrin cannot be a true porphyrin and hence in agreement with Dhéré & Castelli [1937] we suggest that the misnomer mycoporphyrin be dropped from the literature. We regard this matter as one of some importance since, to quote Dhéré & Castelli [1937], "les dermatologistes désignent maintenant parfois sous le nom de 'mycoporphyrines' les porphyrines vraies extraites des champignons microscopiques agents des 'mycoses' cutanées".

There is present in the petals and stamens of St John's Wort, *Hypericum perforatum*, a red colouring matter known as hypericin or hypericum red, the photodynamic properties of which are of particular interest in comparative pathology. It has been shown by a number of workers [Ioannides, 1930; Hausmann, 1932; Quin, 1933; Horsley, 1934] that the photodynamic action of hypericin is responsible for a pathological condition caused by light and known

as hypericismus. Grazing animals which, after feeding on St John's Wort, are exposed to bright sunlight, show a characteristic photosensitization and in extreme cases die. Fischer & Hess [1930] compared the absorption spectra of crude alcoholic extracts of St John's Wort and naturally occurring *P. clavariaeformis* and concluded that hypericin is identical with mycoporphyrin. The same conclusion was reached by Dhéré [1933] who examined the fluorescence spectra of similar solutions. Later, Dhéré & Castelli [1937] examined the fluorescence spectrum at ordinary temperatures of a specimen of our oxypenicilliopsin and the same workers [1938] compared, at the temperature of liquid air, the fluorescence spectrum of our oxypenicilliopsin with that of an alcoholic extract of the petals of *Hypericum perforatum*. Finally, Dhéré & Castelli [1939] compared both the absorption and fluorescence spectra, at ordinary temperatures and in liquid nitrogen, of our irradiated oxypenicilliopsin with those of a purified specimen of hypericin from St John's Wort kindly given to us by Drs Mackinney and Pace of the University of California [Mackinney & Pace, 1939]. The continental workers could detect no differences between the very remarkable spectra of irradiated oxypenicilliopsin and hypericin, and concluded that they must either be the same substance or be very closely related to each other.

Our own purely chemical findings definitely exclude the identity of irradiated oxypenicilliopsin (mycoporphyrin) with hypericin though they certainly point to a close relationship between them. A series of comparative colour reactions, carried out early in 1939 on pure irradiated oxypenicilliopsin and Mackinney & Pace's hypericin, while showing a very close resemblance in many reactions, gave in one or two instances decisive differences.

So far as we are aware, the only work on the chemical constitution of hypericin published before 1939 is that of Cerny [1911] who assigned to it the empirical formula $C_{16}H_{10}O_5$ and suggested that it might belong to the flavones. A very recent publication by Brockmann *et al.* [1939] however records preliminary results on the chemical constitution of hypericin which support our conclusion that hypericin is not identical with irradiated oxypenicilliopsin. These workers assign the empirical formula $C_{28}H_{18}O_8$ to hypericin (M.P. about 330°), describe a hexabenzoate and advance the tentative suggestion that it may be a hexahydroxy derivative of mesodianthrone.

Our experimental work has not yet progressed sufficiently far to justify the expression of more than a very tentative opinion of the probable molecular constitution of penicilliopsin and its oxidation products. It may well be, however, that penicilliopsin ($C_{30}H_{24}O_8$) is a dimeride of emodin anthranol ($C_{15}H_{12}O_4$), which was obtained from penicilliopsin by dry distillation, in which case penicilliopsin may be regarded as a polyhydroxy derivative of a reduced mesodimethyldianthrone. We are of the opinion, however, that oxypenicilliopsin ($C_{30}H_{20}O_9$) and irradiated oxypenicilliopsin ($C_{30}H_{20}O_9$) are not dianthrone derivatives but are more probably derivatives of helianthrone or of naphthadianthrone with which they have many properties in common particularly in the colour of the crystals and in their colour reactions with many reagents.

The work on the chemical constitution of penicilliopsin is being continued.

EXPERIMENTAL

History and description of culture

We are indebted to our colleague, Mr G. Smith, for the information given in this section.

Penicillioopsis clavariaeformis Solms-Laubach was first isolated by H. Grafen zu Solms-Laubach [1887] from fallen, rotting fruits of *Diospyros macrophylla* Bl., in the Buitenzorg Botanical Gardens, Java. In its natural habitat it forms clusters of globose perithecia, similar to those of *Aspergillus glaucus* and some species of *Penicillium*, and also conidial fructifications in the form of *Clavaria*-like masses of sulphur-yellow coremia, the ultimate elements of which are essentially similar to the conidiophores of typical *Penicillia*.

The culture used throughout this work, L.S.H.T.M. Cat. No. X 12, was obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland, in June 1936 and was derived from a naturally occurring strain originating in Java. In cultural experiments on a variety of solid culture media and at various temperatures, normal coremia were formed in almost all cases but under no conditions was production of perithecia observed. The best of the media tried was orange extract agar, on which at 24° growth is very rapid, with formation of thick clusters of coremia several cm. in length, sulphur-yellow at first, then greenish-yellow and finally brown in old cultures.

Cultural conditions

P. clavariaeformis has been grown here on each of the following liquid media (a), (b) and (c). The mould grew quite well on both media (a) and (b) but gave a much more luxuriant and typical growth on medium (c). Penicilliopsin was isolated from the dried fungus grown on each of these media but as medium (c) gave a much better yield of pure penicilliopsin than either (a) or (b), it was used for the preparation of the colouring matter in quantity.

(a) Unhopped beer wort, sp. gr. 1.04.

(b) Czapek-Dox solution. Glucose, 50.0 g.; NaNO₃, 2.0 g.; KH₂PO₄, 1.0 g.; KCl, 0.5 g.; MgSO₄.7H₂O, 0.5 g.; FeSO₄.7H₂O, 0.01 g.; distilled water to 1000 ml.

(c) Orange extract medium. Glucose, 50 g.; triammonium citrate, 2.0 g.; KH₂PO₄, 1.0 g.; KCl, 0.5 g.; MgSO₄.7H₂O, 0.5 g.; FeSO₄.7H₂O, 0.01 g.; extract of one orange; *N* HCl, sufficient to adjust the pH to 4.5 (usually about 6.0 ml.); distilled water to 1000 ml. For the preparation of the orange extract, fair sized oranges, one for each litre of finished medium, were minced and the pulp boiled with water, 500 ml. per orange. The extract was strained through muslin and the pulp was further extracted with boiling water, 250 ml. per orange. The combined extracts were cleared by filtration through filter paper pulp. The luxuriance of growth varied somewhat with the variety of orange used, the best results being obtained with Brazilian oranges when in season.

For the preparation of penicilliopsin in quantity, 350 ml. of medium (c) were placed in each of 100 l.-l. conical flasks, plugged with cotton wool, which were sterilized by steaming for 1 hr. on each of 3 consecutive days. Each flask was then inoculated with a spore suspension in sterile distilled water of a mature culture of *P. clavariaeformis*, L.S.H.T.M. Cat. No. X 12, on a beer-wort agar slope, 4 flasks being sown from 1 slope. The flasks were incubated at 24° in the dark until the glucose content of the filtered metabolism solution, as measured by the polarimeter, was almost zero, the average incubation period being 14–16 days. The details of a number of typical experiments on media (a), (b) and (c) are given in Table 1.

Isolation and purification of penicilliopsin

At the end of the incubation period the mould was separated from the metabolism solution by straining through butter muslin, washed well with water, dried in a vacuum oven at 50–60° and weighed. The strained metabolism solution contains large amounts of spores of the fungus which, in one experiment with 100 flasks of medium (c), were separated, washed and dried (wt. 18 g.). Since solvent extraction of the dried spores showed that they contain practically no penicilliopsin they were discarded in subsequent experiments. The dried fungus was finely ground in a coffee mill and thoroughly extracted with light petroleum, B.P. 40–50°, in a large Soxhlet apparatus. This treatment removed small amounts of a semi-solid fatty material containing practically no penicilliopsin. The light petroleum-extracted fungus was air-dried and then exhaustively extracted with ether, 5–6 working days being necessary for complete extraction. Crude penicilliopsin separated from the ether extracts in brownish-red solid crusts which were collected daily. This crude material was purified by two crystallizations from boiling dioxane (10–12 ml. per g. of crude pigment) and was thus obtained in small, light orange, stout rectangular prisms. Details of yields obtained in a number of representative experiments are given in Table 1. The losses incurred in crystallizing penicilliopsin from dioxane were disappointingly large, and the fact that these losses are due to oxidation and irradiation was only realized at a late stage. No doubt they could be much reduced and the recovery yield of pure penicilliopsin be greatly increased by keeping all dioxane solutions and even ether solutions in the dark in almost full flasks, which should be cooled quickly and then corked immediately to prevent access of air. The only solvents in which penicilliopsin is not fairly rapidly oxidized are unfortunately those in which it is only very sparingly soluble even at the boiling point, i.e. chloroform and toluene. There was no evidence, at any time, of the presence, in the ether extracts of the fungus, of any crystalline colouring matter other than penicilliopsin.

Table 1

Medium	Number of flasks	Incubation period days	Glucose by polarimeter %	Dry wt. of fungus g.	Wt. of crude penicilliopsin g.	Wt. of pure penicilliopsin crystallized from dioxane g.
(a)	2	68	—	14.0	—	0.13
(b)	75	53	Nil	268	3.0	1.68
(c)	100	14	0.1	451	33.2	14.0
(c)	100	14	0.1	450	34.0	13.9

Penicilliopsin

(1) *General properties.* Penicilliopsin is practically insoluble in light petroleum, only slightly soluble in boiling toluene or chloroform, and rather more soluble in boiling ethanol or ethyl acetate. It crystallizes most readily from hot dioxane, in which it is moderately soluble, in microscopic, orange, stout rectangular or occasionally hexagonal prisms. The colour of the specimens prepared in this way varied from orange-yellow through orange-red to brownish-red, possibly on account of varying crystal sizes, but more probably because the more darkly coloured specimens were contaminated with small amounts of an oxidation product (see p. 799). When crystallized from ethanol it separated in very minute crystals of similar shape which were invariably orange-yellow in colour.

Penicillipsin, crystallized from dioxane, contains water and dioxane of crystallization which are lost on heating strongly, the substance becoming yellowish-brown in colour at 200° and melting sharply at about 330° without effervescence to a purplish-black liquid which shows a definite meniscus before 340°. The M.P. varies somewhat with the rate of heating. A specimen crystallized from ethanol behaved similarly on heating.

An alcoholic solution gives a dark, olive-brown colour with FeCl_3 . Penicillipsin dissolves in cold conc. H_2SO_4 with a yellowish-brown colour, which quickly changes to green on warming and then develops a strong dull red fluorescence.

Penicillipsin is insoluble in aqueous sodium acetate, slowly soluble in Na_2CO_3 and much more readily soluble in NH_4OH and very dilute NaOH and KOH giving brown solutions in each case which, on long standing or boiling for a short time become brownish-red with Na_2CO_3 , brownish-purple with NH_4OH and purple with NaOH or KOH . The characteristic behaviour with organic bases is described later (see p. 799).

(2) *Analysis of penicillipsin.* Difficulties were experienced in arriving at an empirical formula for penicillipsin since the solvents which are most suitable for its purification, especially dioxane, cling tenaciously to the substance. It is too slightly soluble in any solvent to allow an accurate mol. wt. estimation to be made but this difficulty was overcome by determining the mol. wt. of some of its derivatives (see p. 796).

Specimens crystallized from three different solvents were analysed with the following results.

(1) Crystallized from toluene containing a little acetic acid and dried to constant weight in a high vacuum at 160–170°. (Found: C, 69.87, 70.04; H, 4.66, 4.54; N, nil; CH_3O , nil; $\text{C}_{30}\text{H}_{24}\text{O}_8$ requires C, 70.28; H, 4.72%.)

(2) Crystallized from dioxane. (a) Air-dried. (Found: C, 67.12, 67.19; H, 5.24, 5.28. $\text{C}_{30}\text{H}_{24}\text{O}_8$, $\frac{1}{2}\text{H}_2\text{O}$, $\text{C}_4\text{H}_8\text{O}_2$ requires C, 66.95; H, 5.43%.) (b) Dried to constant weight at 100–110° in a high vacuum. (Found: C, 68.14, 68.20; H, 4.94, 5.15; side chain CH_3 , 4.82. $\text{C}_{30}\text{H}_{24}\text{O}_8$, $\text{C}_4\text{H}_8\text{O}_2$ requires C, 67.98; H, 5.37; 2CH_3 as CH_3 , C, 5.00%.)

(3) Crystallized from ethanol. (a) Specimen from orange extract medium dried to constant weight in a high vacuum at 100°. (Found: C, 66.99, 66.75; H, 5.09, 5.20; CH_3O , nil. $\text{C}_{30}\text{H}_{24}\text{O}_8$, $1\frac{1}{2}\text{H}_2\text{O}$ requires C, 66.76; H, 5.04%.) (b) Specimen from Czapek-Dox medium and dried as 3a. (Found: C, 67.00, 66.89; H, 5.09, 5.23; CH_3O , nil. $\text{C}_{30}\text{H}_{24}\text{O}_8$, $1\frac{1}{2}\text{H}_2\text{O}$ requires C, 66.76; H, 5.04%.)

(3) *Derivatives of penicillipsin.* Penicillipsin does not react with 2:4-dinitrophenylhydrazine hydrochloride in hot aqueous dioxane solution and was recovered unchanged when its dioxane solution was shaken with hydrogen in presence of a palladium-norite catalyst. Attempts to make the *p*-xenylcarbamate, the *p*-chlorophenylcarbamate, the *p*-bromobenzoate, the ethyl ester by the Fischer-Speier method, the methyl derivative by methylation with methyl sulphate and alkali in presence of $\text{Na}_2\text{S}_2\text{O}_4$, and the acetyl derivative by the acetic anhydride-sodium acetate method all gave uncrystallizable products, or crystalline products which were artefacts. It is remarkable that *p*-bromobenzoyl chloride has practically no action on penicillipsin even at 160–170°.

(a) *Penicillipsin bisphenylcarbamate.* Penicillipsin (1.5 g.), crystallized from dioxane and dried at 140° in a high vacuum, was refluxed with a large excess of phenyl isocyanate (5 g.) for 1.5 hr. in an oil bath at 175°. After about 1 hr. the substance had completely dissolved to an almost black solution. After cooling, the solution was diluted with much light petroleum and the greyish-brown precipitate was collected (2.0 g.). This was repeatedly extracted with

boiling benzene, the first red extract being rejected and the yellow material separating from the second and third extracts was recrystallized from boiling benzene in which the product is sparingly soluble. *Penicillioipsin* bisphenylcarbamate forms small, yellow prisms (parallelepipeds), m.p. 238–240°, with effervescence and blackening. (Found, on material dried to constant wt. in a high vacuum at 140°: C, 70.10, 70.25; H, 4.50, 4.66; N, 3.91, 3.67%. Mol. wt. (cryoscopic in dioxane), 771. $C_{44}H_{34}O_{10}N_2$, i.e. the bisphenylcarbamate of $C_{30}H_{24}O_8$, requires C, 70.36; H, 4.57; N, 3.73%. Mol. wt. 750.) The substance is readily soluble in cold dioxane, and in pyridine to give a deep red solution which exhibits a strong red fluorescence after standing in daylight for some time. It dissolves quickly but not instantly in aqueous KOH to yield a deep brown solution, hydrolysis to penicillioipsin probably taking place.

(b) *Diacetylpenicillioipsin*. Penicillioipsin (0.15 g.), treated with boiling acetic anhydride (6 ml.), dissolved in about 5 min. The red solution was boiled for a further 2 min., and on cooling deposited orange-yellow crystals (0.05 g.) which were crystallized first from ethyl acetate and then from benzene. *Diacetylpenicillioipsin* forms orange-yellow rectangular prisms which darken on heating and slowly decompose above 280°. (Found, on material dried at 110° in a high vacuum: C, 68.60, 68.60; H, 4.47, 4.42; hydrolysable CH_3CO , by alkaline hydrolysis, 14.5%; side-chain CH_3 , by Kuhn-Roth oxidation, 8.9%; mol. wt. in camphor, 636, 614; $C_{34}H_{28}O_{10}$, i.e. the diacetate of $C_{30}H_{24}O_8$, requires C, 68.45; H, 4.73; $2CH_3CO$, 14.4; $4CH_3$ as $CH_3.C$, 10.1%; mol. wt. 596.) The substance is much more soluble in the usual organic solvents, with the exception of ethanol and dioxane, than is the parent compound. Its reactions are exactly the same as those of the parent substance except that it is not immediately soluble in aqueous Na_2CO_3 . The acetyl groups are very stable to acid hydrolysis.

(4) *Action of diazomethane on penicillioipsin*. Penicillioipsin (0.4 g.) was treated with a large excess of ethereal diazomethane. There was an immediate, vigorous effervescence and a yellow, flocculent precipitate later formed which was collected and extracted with much boiling glacial acetic acid. The residue was crystallized first from nitrobenzene and then from boiling toluene in which it is very sparingly soluble. The product formed small orange-yellow platelets (parallelepipeds) which darkened above 300° and melted indefinitely at 340–360° to a black liquid. (Found on material dried to constant wt. in a high vacuum at 190–210°: C, 71.91, 71.85; H, 5.51, 5.36; N, nil; CH_3O , nil. $C_{34}H_{32}O_8$, i.e. $C_{30}H_{24}O_8 + 4CH_2$, requires C, 71.80; H, 5.55%.) The product gives a brown solution in cold conc. H_2SO_4 but is insoluble in NaOH except on very long standing when a brown solution is obtained. It gives an immediate reddish-brown colour with pyridine and the solution soon exhibits a red fluorescence on exposure to daylight.

(5) *Action of diazoethane on penicillioipsin*. This product was prepared as described above except that diazoethane was used. The product, which is much more soluble in toluene than the corresponding methyl derivative, was crystallized from dioxane in which it is very sparingly soluble in the cold. It formed minute yellow square tablets frequently having rounded corners and melted indefinitely about 310° to a dark red liquid. (Found on material dried at 140° in a high vacuum: C, 71.74, 71.66; H, 5.58, 5.48; N, nil, C_2H_5O , nil. $C_{34}H_{32}O_8$, i.e. $C_{30}H_{24}O_8 + 2C_2H_4$, requires C, 71.80; H, 5.55%.) The product is very slowly soluble in dilute NaOH to give a brown solution. It gives an immediate reddish brown colour with pyridine and the solution soon exhibits a red fluorescence on exposure to daylight.

(6) *Action of heat on penicillipsin. Formation of Frangula-emodin anthranol.* An intimate mixture of pure penicillipsin (0.2 g.) and zinc dust (3.5 g.) was placed in the closed end of a small bent test tube the open end of which was connected to a cooled receiver. The mixture was strongly heated when a yellowish-brown sublimate together with a brown liquid, which later solidified, collected in the cooler limb. The combined products from a number of similar experiments (about 25 mg. from 2 g. of penicillipsin) were crystallized from ethyl acetate in small pale yellow prisms which blackened and softened above 260°. (Found: C, 70.20, 70.21; H, 4.98, 4.73 %. $C_{15}H_{12}O_4$ requires C, 70.28; H, 4.72 %.)

The substance was shown to be *Frangula-emodin anthranol*, 4:5:7-trihydroxy-2-methylantranol (9 or 10), by direct comparison with an authentic specimen prepared by the reduction of physcion (4:5-dihydroxy-7-methoxy-2-methyl-anthraquinone) with conc. HI, sp. gr. 1.7. The two specimens crystallized from ethyl acetate in an exactly similar way and showed the same behaviour on heating, either alone or in admixture. Their colour reactions with the following reagents were also identical. (a) Dilute NaOH gave a solution which became purple on shaking with air owing to oxidation to emodin, 4:5:7-trihydroxy-2-methylantraquinone. (b) Conc. ammonia (sp. gr. 0.880) gave a solution which is dark red by transmitted light and purple in thin layers. (c) Conc. H_2SO_4 gave a solution which became green on standing and showed a deep green fluorescence on heating. (d) A solution in pyridine containing some piperidine did not develop a strong red fluorescence on standing in daylight, cf. penicillipsin (p. 799).

A similar sublimate of emodin anthranol was formed in smaller amount when penicillipsin was heated alone or mixed with kieselguhr.

Oxyenicillipsin (see p. 799) gave a trace of a yellow sublimate, possibly emodin anthranol, when similarly heated with zinc dust, but irradiated oxyenicillipsin (see p. 801) gave no sublimate at all under these conditions.

(7) *2-Methylantracene from penicillipsin.* An intimate mixture of pure penicillipsin (4 g.) and zinc dust (80 g.) was distilled at a red heat for 1 hr. in a slow stream of hydrogen. The yellowish-brown sublimate (about 0.05 g.), after resublimation at 200–250° at atmospheric pressure was crystallized from aqueous ethanol giving almost colourless plates with a bluish lustre, m.p. 196°, raised to 197–198° by a second sublimation. (Found: C, 93.64; H, 6.45 %. $C_{15}H_{12}$, i.e. methylantracene, requires C, 93.71; H, 6.30 %. $C_{14}H_{10}$, i.e. anthracene, requires C, 94.34; H, 5.66 %.) The m.p. of the substance was not depressed on admixture with an authentic specimen of 2-methylantracene and the two hydrocarbons, after sublimation, were also identical in appearance, viz. shining almost colourless plates with a fine green fluorescence. Other experiments on similar lines confirmed the formation of 2-methylantracene but failed to improve the yield.

(8) *Oxidation of penicillipsin with nitric acid. Isolation of tetranitro-Frangula-emodin, nitrococcusic acid and oxalic acid.* Many experiments were carried out in order to obtain the maximum yield of recognizable oxidation products and the following method was finally adopted.

Penicillipsin (3.0 g., crystallized from dioxane and air-dried) was suspended in conc. HNO_3 (25 ml., sp. gr. 1.4) and evaporated to dryness in a porcelain dish on the steam bath with frequent stirring, the initial reaction being very vigorous. The dark gummy residue was dissolved in hot glacial acetic acid (10 ml.) and the yellow crystals which formed on standing were separated (1.05 g.). The filtrate was freed from acetic acid *in vacuo* over solid KOH and the residue was re-treated with HNO_3 (10 ml.) and re-extracted with acetic acid yielding a further 0.78 g. of crystalline material. A third treatment with HNO_3 (5 ml.) of the oily

residue from these crystals gave a final crop of hard, brown, resinous material (0.35 g.). The total yield of solid oxidation products (2.18 g.) was fractionated as follows.

(a) *Separation and identification of tetranitro-Frangula-emodin (4:5:7-tri-hydroxy-1:3:6:8-tetranitro-2-methylanthraquinone)*. The combined products were dissolved in boiling glacial acetic acid (18 ml.) and the yellow crystalline material (0.43 g., M.P. 290°) separating after standing for 2 days was crystallized from dry benzene-light petroleum. M.P. 296°. The product was shown to be tetranitro-*Frangula-emodin* by direct comparison with a synthetic specimen prepared from *Frangula-emodin* by the method described by Oesterle [1911]. The two specimens had the following analyses. (Found: (a) on specimen from penicillipsin, C, 40.17, 40.05; H, 1.62, 1.50; N, 12.6, 12.3%; (b) on specimen from *Frangula-emodin*, C, 39.98, 40.11; H, 1.67, 1.86; N, 12.0, 12.2%. $C_{15}H_8O_{13}N_4$ requires C, 40.00; H, 1.34; N, 12.4%.) The oxidation product, on further purification, melted at 298°, the synthetic product at 302°, and a mixture of the two at 298°. They had each the same crystalline form—slender, orange prismatic rods—and they gave identical reactions as follows: (1) a deep orange-red solution in water; (2) dilute aqueous NH_4OH , a violet solution; (3) conc. NH_4OH , a deep purplish-red solution; (4) dilute aqueous Na_2CO_3 , a deep reddish-brown solution; (5) 2N $NaOH$, almost insoluble, the solid becoming almost black; (6) conc. H_2SO_4 , brownish-yellow solution; (7) dilute boiling aqueous sodium sulphide, deep violet solution.

(b) *Separation of oxalic acid as calcium oxalate*. The acetic acid filtrate from the first crystallization of the combined oxidation products was evaporated to dryness over solid KOH . The light brown solid residue (1.75 g.) contained a considerable amount of colourless material and an estimation, on a part of it (0.2843 g.), of the oxalic acid present gave calcium oxalate corresponding to 0.554 g. of anhydrous oxalic acid in the total residues.

(c) *Separation and identification of nitrococcusic acid (2:4:6-trinitro-5-hydroxy-3-methylbenzoic acid)*. The remainder of the solid from the acetic acid filtrate (1.47 g.) was treated with boiling water (8 ml.), filtered from a little insoluble coloured material (0.01 g.) and kept at 0°, when hydrated oxalic acid (0.05 g.) together with a trace of tetranitroemodin separated. The aqueous filtrate was evaporated to dryness *in vacuo* and the residue crystallized from glacial acetic acid (4 ml.). Long pale yellow needles separated (0.35 g.), M.P. 206–208° decomp., if heated slowly. The substance explodes if suddenly plunged into an oil bath at 250°. (Found on air-dried material: C, 32.01, 31.98; H, 2.58, 2.53; N, 13.4, 13.3%. $C_8H_5O_9N_3 \cdot H_2O$ requires C, 31.48; H, 2.31; N, 13.7%.) The substance is readily soluble in water giving a yellow, strongly acidic solution. It is undoubtedly nitrococcusic acid (2:4:6-trinitro-5-hydroxy-3-methylbenzoic acid) [Kostanecki & Niementowski, 1885] which is known to crystallize with 1 H_2O [Charles *et al.* 1933]. The same compound was obtained by the action of HNO_3 , sp. gr. 1.4, on *Frangula-emodin* and melted at 206–208° decomp., alone or mixed with the product from penicillipsin.

When penicillipsin was oxidized with dilute HNO_3 , sp. gr. 1.2, at 100° the main oxidation product was nitrococcusic acid, only traces of tetranitroemodin being formed while, under the same conditions, emodin yielded mainly tetranitroemodin with only traces of nitrococcusic acid, and tetranitroemodin is almost unaffected by HNO_3 of this concentration. It is therefore probable that the nitrococcusic acid formed when penicillipsin is oxidized with HNO_3 of sp. gr. 1.2 arises not from emodin or tetranitroemodin, but rather from the direct breakdown of the penicillipsin molecule itself.

Oxypenicillipsin

Solutions of penicillipsin in different organic solvents, when exposed to air and light, undergo a series of colour changes which vary with the nature of the solvent used. These changes involve, as stated in the introduction, two separate and distinct reactions; thus a saturated solution of penicillipsin in chloroform is bright yellow in colour and remains unchanged when exposed to air and light for a week, no purple colour or fluorescence being developed. On the other hand an alcoholic, ethereal or dioxane solution becomes progressively darker under these conditions and a strong red fluorescence is slowly developed; if a saturated alcoholic solution is used a slight purple deposit is formed on long standing.

(a) *The oxidation process.* The oxidation of penicillipsin to oxypenicillipsin takes place very slowly in neutral organic solvents, e.g. ethanol, ether, dioxane etc., but increases in rapidity on the addition to these solvents of certain organic bases and proceeds with great rapidity when solutions of penicillipsin in these basic organic solvents are exposed to air in the dark.

The most effective base for this purpose is piperidine, though other bases of strength comparable with that of piperidine, e.g. brucine, cinchonine, methylamine and ethylamine, were almost equally effective. Pure pyridine is not a strong enough base to bring about a quick oxidation, but samples of commercial pyridine containing piperidine, or pure pyridine to which a small amount of piperidine is added, are quite effective. The following bases are ineffective: quinoline, aniline, dimethyl- and diethyl-aniline, α -naphthylamine, caffeine and *m*-aminobenzoic acid (the last three in alcoholic solution).

Up to the present we have not been able to oxidize penicillipsin to oxypenicillipsin rapidly except by the use of a strong *nitrogenous* base. Thus while ammonia and hydrazine behave like the strong organic bases, KOH behaves in a different manner. If a few drops of alcoholic KOH are added to an alcoholic solution of penicillipsin a purple-violet colour develops on standing but no fluorescence appears on exposure to daylight. If excess of alcoholic HCl is now added and the precipitated KCl is separated by filtration the filtrate is merely yellowish-brown in colour and develops only a weak red fluorescence on exposure to daylight. In a corresponding experiment with brucine, piperidine or methylamine, the reddish-purple colour remained on addition of excess of alcoholic HCl and a very strong red fluorescence soon developed when either the original or the acidified solution was exposed to daylight.

Finally the characteristic oxidation cannot be carried out in aqueous solution and, indeed, when carried out in alcoholic solution, absolute alcohol must be used since the addition of only a small amount of water prevents the development of the characteristic deep reddish-purple colour.

The following experiment proves conclusively that the conversion of penicillipsin into oxypenicillipsin does in fact necessitate the uptake of atmospheric oxygen but is independent of light. A few ml. of pyridine containing about 5% of piperidine were placed in a long, wide specimen tube into which was inserted a smaller narrow tube filled with penicillipsin and so arranged that the smaller tube rested with its mouth well above the level of the pyridine-piperidine mixture. The two tubes were carefully inserted into a Büchner filtration flask to which was added some freshly prepared alkaline pyrogallol. The air in the Büchner flask was then removed as completely as possible by a vacuum pump and the evacuated flask was sealed from the air. After standing for some time to remove the last traces of oxygen the flask was tilted so that a small amount of penicillipsin was dissolved by the pyridine-piperidine mixture. The apparatus was

then kept in the dark for several hours when the penicillipsin solution had become merely brownish-red in colour with no trace of a purple tint. It was exposed to daylight for some time but no perceptible fluorescence developed. Air was now admitted and the flask immediately placed in the dark. The solution quickly became much darker in colour and a purple tint was evident at the air-liquid interface. After standing overnight in the dark the solution was a deep reddish-purple by transmitted light and, on exposure to daylight, quickly developed a blood red fluorescence.

(b) *Measurement of oxygen uptake in the oxidation of penicillipsin to oxypenicillipsin.* The measurement of the amount of oxygen absorbed in the oxidation of penicillipsin to oxypenicillipsin was carried out in the Barcroft differential apparatus. Pure penicillipsin, crystallized from dioxane and air-dried, was used and in both the experiments recorded dioxane was employed as solvent with piperidine as the organic base in the one experiment and brucine in the other. At the end of each experiment a good deal of oxypenicillipsin had separated from the solution. It is evident from the experimental details given below that the oxidation proceeds according to the equation

Exp. 1. Left-hand vessel contained penicillipsin (0.00883 g.), dioxane (3.0 ml.) and piperidine (0.015 ml.).

Right-hand vessel contained dioxane (3.0 ml.) and piperidine (0.015 ml.). The absorption of oxygen was virtually complete in 3 hr. Vol. of oxygen absorbed, 420.3 μl . Theor. for 3 atoms of oxygen per mol. of penicillipsin, 487 μl .

Exp. 2. Left-hand vessel contained penicillipsin (0.01014 g.), dioxane (3.0 ml.) and brucine (0.02 g.).

Right-hand vessel contained dioxane (3.0 ml.) and brucine (0.02 g.). The absorption of oxygen was much slower than in exp. 1 but was complete in 26 hr. Vol. of oxygen absorbed, 587.8 μl . Theoretical, 559.4 μl .

(c) *Preparation and properties of oxypenicillipsin.* Pure penicillipsin (1 g.) was dissolved in a mixture of pyridine (100 ml.) and piperidine (8 ml.) contained in a 1 l. conical flask the neck of which was loosely corked with a grooved cork to allow access of air. The mixture was immediately placed in the dark and kept there, with occasional shaking, for 4 days. The permanganate-coloured solution so obtained was poured into an excess of well cooled and stirred dilute HCl and, after 2 days in a dark cold room, the dark purple flocculent precipitate was collected, washed and dried (ca. 1.0 g.). The crude oxidation product proved exceptionally difficult to obtain in a satisfactory crystalline form. It separated from hot dioxane, in which it is less readily soluble than penicillipsin, in amorphous aggregates which were sparingly soluble in hot glacial acetic acid. It separated from this solvent in small aggregates which were not definitely crystalline under the microscope. Finally it separated from nitrobenzene or ethanol in large purplish-black masses, the edges of which appeared to have a crystalline structure. The product from nitrobenzene did not melt at 360° nor did it sublime appreciably at 240–250° in a high vacuum. (Found, on material from nitrobenzene dried to constant weight in a high vacuum at 200–210°: C, 68.77, 68.85; H, 3.84, 3.81; N, nil. $\text{C}_{30}\text{H}_{20}\text{O}_9$ requires C, 68.68; H, 3.85 %.)

Oxypenicillipsin dissolves sparingly in all the usual organic solvents with the formation of a deep reddish-purple colour, which is stable in the dark but fades in daylight with the development of an intense blood-red fluorescence. A basic solvent is not necessary for the development of the fluorescence; indeed it is best developed in hydrocarbon solvents like benzene or toluene. Oxypeni-

cilliopsin is insoluble in aqueous Na_2CO_3 but dissolves in NaOH to give a deep blue solution which becomes green on standing for some time and deposits a greyish-green precipitate after long standing, the supernatant liquid being pale purple. It dissolves in cold conc. H_2SO_4 giving a deep blue solution which becomes green on standing with the development of a striking dull red fluorescence; the original deep blue solution becomes green on the addition of solid NaNO_2 and pink on pouring into water. A freshly prepared alcoholic solution gives the following reactions: no marked change on the addition of aqueous NaHCO_3 or Na_2CO_3 ; an intense reddish-purple with a trace of aqueous ammonia; a bluish-purple colour with NiSO_4 solution, with precipitate on standing; solid MgO is coloured light blue and solid CaO a deep blue becoming green on standing.

It is remarkable that oxypenicilliopsin, on treatment with HNO_3 , sp. gr. either 1.2 or 1.4, failed to give either tetranitroemodin or nitrococcusic acid, cf. the action of HNO_3 on penicilliopsin, p. 797.

(d) *Acetyl (probably tetra-acetyl) derivative of oxypenicilliopsin.* A solution of oxypenicilliopsin (0.5 g.) in acetic anhydride (30 ml.) containing a few drops of conc. H_2SO_4 was boiled for 5 min., cooled and poured on to crushed ice. The brownish-red precipitate was collected, washed and dried. It separated from a mixture of acetic acid (2 parts) and acetic anhydride (1 part) in minute, light orange-red, flat rods, m.p. $308\text{--}310^\circ$ decomp. after darkening above 280° . (Found, on material dried to constant wt. in a high vacuum at 180° : C, 66.18, 66.10; H, 3.81, 3.94%; mol. wt. cryoscopic in dioxane, 698.5. $\text{C}_{38}\text{H}_{28}\text{O}_{13}$, i.e. $\text{C}_{30}\text{H}_{16}\text{O}_5 \cdot (\text{O} \cdot \text{CO} \cdot \text{CH}_3)_4$, requires C, 65.89; H, 4.07%; mol. wt. 692). Attempts to estimate accurately the acetyl content were not successful.

The acetyl derivative is only slowly soluble in cold NaOH . On warming, however, a deep violet solution results which changes to a deep pure green overnight. Its behaviour with conc. H_2SO_4 is exactly the same as that of oxypenicilliopsin. Solutions of the acetyl derivative in neutral organic solvents, e.g. ethyl acetate, dioxane, at first orange in colour, rapidly darken on exposure to bright daylight, but not if kept in the dark. There is no development of any fluorescence in daylight but a fluorescence develops if piperidine is added, probably owing to slow removal of acetyl groups.

Irradiated oxypenicilliopsin

Conversion of oxypenicilliopsin into irradiated oxypenicilliopsin. A cold saturated alcoholic solution of oxypenicilliopsin, prepared by refluxing an excess (1 g.) of the latter with absolute alcohol (250 ml.) for 10 min., filtering hot, keeping the filtrate overnight in the dark and then refiltering, was distributed in 100 6-in. test tubes, which were then left in an inclined position in front of a north window on a bright day in March 1939. After about 3 hr. irradiation, the original purple colour had visibly begun to fade, a fiery red fluorescence developed and chocolate-brown needles began to separate near the surface. After 2 days this crystalline product was collected, washed with ethanol and dried (0.20 g.). The substance was practically insoluble in boiling ethanol and was purified by recrystallization from dioxane. It had no m.p. and seemed to suffer no change, except a loss of crystalline shape, on heating to 370° . (Found, on material dried at $100\text{--}110^\circ$ in a high vacuum: C, 68.84, 68.69; H, 4.00, 3.85; CH_3O , nil. $\text{C}_{30}\text{H}_{20}\text{O}_9$, requires C, 68.68; H, 3.85%.)

No oxidation is therefore involved in the conversion of oxypenicilliopsin into irradiated penicilliopsin. This was further proved by sealing off an evacuated tube containing a cold, saturated alcoholic solution of oxypenicilliopsin and then submitting it to the action of daylight when the separation of chocolate-brown

needles and the development of a strong red fluorescence took place exactly as before when in the presence of air.

The general colour reactions of irradiated oxypenicillipsin are given in the next section where they are compared, side by side, with those of hypericin. Other points of interest in irradiated oxypenicillipsin are the following: it is very sparingly soluble in most neutral organic solvents but the solutions exhibit immediately the characteristic fiery red fluorescence associated with hypericin and "mycoporphyrin"; the red fluorescence persists even in acetic anhydride solution, but disappears on the addition of a trace of conc. H_2SO_4 , the solution becoming redder and, on standing, bluer in tone and finally taking on a mauve tint. The reaction with dilute NaOH (see below) recalls that of certain naturally occurring 2-hydroxyphenanthraquinones described by Blount [1936]. Irradiated oxypenicillipsin is however quite insoluble in sodium bisulphite solution and hence is unlikely to be a derivative of phenanthraquinone.

Comparison of the colour reactions of irradiated oxypenicillipsin and hypericin

Through the courtesy of Dr G. Mackinney of the Agricultural Experiment Station of the University of California we were kindly provided in December 1938 with a specimen of hypericin, prepared by his colleague Mr Pace from St John's Wort (*Hypericum perforatum*). This specimen had been purified by chromatographic analysis and was almost certainly the purest specimen of hypericin ever prepared up to that time. Prof. Ch. Dhéré and his collaborator, Dr V. Castelli, of the University of Fribourg, compared [Dhéré & Castelli, 1939] the absorption and fluorescence spectra of irradiated oxypenicillipsin and the above specimen of hypericin, both at ordinary temperatures and at the temperatures of liquid air and liquid nitrogen. The very remarkable spectra given by both substances were found to be indistinguishable from each other.

This evidence, which apparently points to the identity of hypericin and irradiated oxypenicillipsin is, however, not supported by their colour reactions, carried out side by side with a number of reagents, the results of which are given below. It is evident from these reactions that hypericin and irradiated oxypenicillipsin are closely related substances but the reaction with alcoholic methylamine is sufficient in itself to prove their non-identity.

Reagent	Hypericin	Irradiated oxypenicillipsin
Cold conc. H_2SO_4	Green with strong, dull red fluorescence. Stable	Green, with strong dull red fluorescence. Stable
2N NaOH	Green. Fades to dirty brown in 10 min. and later gives a flocculent precipitate	Stable green. Green flocculent precipitate overnight
2N NH_4OH	Blue with slight green tinge. Becomes brown overnight	Pale mauve. Quite stable
2N Na_2CO_3	Slowly dissolves to a blue solution which becomes pale brown overnight	Absolutely insoluble. No colour even overnight
Pyridine	The very dilute solution is definitely purplish in thin layers. Intense red fluorescence	The very dilute solution is brownish-pink in thin layers. Intense red fluorescence
Acetone	Cerise. Intense red fluorescence	Cerise but less blue in tone than with hypericin. Intense red fluorescence
33% wet wt. solution of methylamine in ethanol	Deep green. Becomes brown overnight. No marked fluorescence	Pale violet. Becomes cherry-red overnight and an intense fiery red fluorescence develops

SUMMARY

The colouring matter present in laboratory cultures, grown in the dark at 24°, of the fungus *Penicillioopsis clavariaeformis* Solms-Laubach has been isolated in good yield and has been named *penicilliopsin*. Penicilliopsin, $C_{30}H_{24}O_8$, orange prisms m.p. 330° (decomp.), is oxidized in air in suitable solvents to *oxypenicilliopsin*, $C_{30}H_{20}O_9$, purple-black crystalline aggregates, m.p. above 360°, solutions of which, on exposure to light, are converted into the isomeric *irradiated oxypenicilliopsin*, $C_{30}H_{20}O_9$, chocolate-brown needles m.p. above 370°, which give intensely fluorescent solutions. Reasons are given for the belief that the colouring matter from naturally occurring specimens of *P. clavariaeformis*, which was formerly known as mycoporphyrin, was a mixture of penicilliopsin, oxypenicilliopsin and irradiated oxypenicilliopsin. It is shown that irradiated oxypenicilliopsin is very closely related to, but is not identical with, hypericin the colouring matter of St John's Wort, *Hypericum perforatum*. A number of derivatives and breakdown products of penicilliopsin are described, and since this substance gives *Frangula*-emodin anthranol on heating alone and tetranitroemodin on oxidation with HNO_3 the view is tentatively advanced that penicilliopsin may be a polyhydroxy derivative of a reduced mesodimethyldianthrone.

We desire to thank Prof. Ch. Dhéré for much friendly correspondence on the spectra of penicilliopsin; oxypenicilliopsin and irradiated oxypenicilliopsin; Dr G. Mackinney for a gift of hypericin; and our colleagues Mr G. Smith and Dr C. T. Calam for valued assistance during certain parts of the work.

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