Studies in the Biochemistry of Micro-organisms

95. RUGULOSIN, A CRYSTALLINE COLOURING MATTER OF *PENICILLIUM RUGULOSUM* THOM

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Raper & Thom (1949) in their *Manual of the Penicillia* separate species in this genus into four major sections, Monoverticillata, Asymmetrica, Biverticillata-Symmetrica and Polyverticillata. They subdivide each of the sections into a number of series so that the Biverticillata-Symmetrica section, for example, includes six such series among which, for the purpose of this communication, the following are of interest: the *P. luteum* series including *P. wortmanni* Klöcker; the *P. funiculosum* series including *P. islandicum* Sopp; and the *P. rugulosum* series including *P. rugulosum* Thom. In common with other series in the Biverticillata-Symmetrica, strains in the three series mentioned produce yellow to orange or reddish colours in the aerial vegetative mycelium with a colony reverse in similar colours or occasionally dark purple-red to almost black.

A number of crystalline colouring matters have been isolated here from strains of *P. islandicum* (Howard & Raistrick, 1949, 1950, 1954*a, b*), and we now report similar investigations on the colouring matters of *P. rugulosum* and *P. wortmanni*. This work was commenced early in 1939 by one of us (J.B.) and resulted in the isolation, from a highly pigmented strain of *P. rugulosum*, strain H.Z., recently isolated from natural sources and cultivated in the laboratory on Czapek-Dox solution, of considerable quantities of a beautifully crystalline, bright yellow colouring matter which was named rugulosin, since it had not been described previously. The work was discontinued later in the year and was not taken up again until 9 years later (Raistrick, 1949; Dacre, 1950). By this time the yield of rugulosin obtainable from *P. rugulosum* strain H.Z. was very much smaller than that obtained previously, but two other strains of the same species, i.e. strains S.M. 1 and S.M. 14, which had not been kept in laboratory culture for so long a time, gave very considerable yields of the colouring matter. In this connexion it is of interest to note that, according to Raper & Thom (1949), while newly isolated strains of *P. rugulosum* usually sporulate freely and have a bright orange-red reverse, the same strains after being held in laboratory culture for a long time sporulate much less heavily and have an almost

white reverse. No rugulosin could be detected in three such strains.

The yields of crude crystalline rugulosin obtained by ether extraction of the dried and defatted mycelium from the three strains of *P. rugulosum* used in its preparation were considerable and amounted to 21 % of the dry weight of the mycelium of strain S.M. 1, 10–13 % from strain S.M. 14 and 7–8 % from strain H.Z. In addition, small amounts of crystalline rugulosin were isolated from strain P. 50 and from strain N.R.R.L. 1045 which is a direct descendant of Dr Charles Thom's original type strain.

Rugulosin has also been isolated from cultures of *P. wortmanni* grown on Czapek-Dox solution. Hence, this species, which has bright yellow perithecia embedded in yellowish orange mycelium, links the perithecia-producing *P. luteum* series with strains in the *P. rugulosum* series which do not produce perithecia. Although no rugulosin was detected by Howard & Raistrick (1949, 1950, 1954*a, b*) in *P. islandicum*, this species is clearly related genetically to both *P. rugulosum* and *P. wortmanni*, since skyrin, which is one of the crystalline colouring matters isolated in considerable amounts from *P. islandicum*, has now been obtained in crystalline form from *P. wortmanni* and has been detected with certainty in several strains of *P. rugulosum*. The amount of purified crystalline skyrin isolated from *P. wortmanni* was approximately 0.5 % of the dry weight of the mycelium and of purified rugulosin 0.7 % but, since exhaustive working up of the solvent extracts was not attempted, the amounts actually present in the mycelium are probably considerably greater.

RUGULOSIN

Considerable difficulty was experienced in establishing the empirical formula for rugulosin. Samples crystallized from ethanol or acetone, in yellow cubes or prisms, and dried to constant weight in high vacuum at 100° or below, are very hygroscopic. Provided suitable precautions are taken, however, they analyse well for $C_{30}H_{24}O_{11}$, a formula which is

in agreement with that for pentaacetylrugulosin, $C_{40}H_{34}O_{16}$. Analysis of samples dried to constant weight at 140–150° leads however to the formula $C_{30}H_{22}O_{10}$ for rugulosin, which is in agreement with that established for pentamethylrugulosin, i.e. $C_{35}H_{32}O_{10}$, and, as will be seen later, explains best most of its properties which are known at present.

Rugulosin melts with very vigorous decomposition at about 293°. It has a high dextro rotation in dioxan solution, $[\alpha]_{461}^{18} + 605^\circ$, which falls to an almost constant value of $+222^\circ$ after prolonged exposure to light. It contains two *C*-methyl groups but no methoxyl groups. It is practically insoluble in water but dissolves in aqueous alkalis, including sodium bicarbonate and ammonia, giving strong yellow to orange solutions. Its ethanolic solution gives an olive-brown ferric reaction. Rugulosin has a markedly specific antibacterial activity, being very highly active against certain bacilli, streptococci and corynebacteria, but is much less active against Gram-negative organisms. Therapeutic tests of rugulosin administered to mice infected with *Streptococcus pyogenes* showed good protection (five alive out of six), but failed completely with *Bacterium coli*. It is moderately antifungal, completely inhibiting the growth of *Pythium intermedium* de Bary at a concentration of 1:50000.

The presence of five hydroxyl groups in the molecule of rugulosin was established by (a) the Peterson & West (1927) method, involving acetylation at 37° with acetic anhydride and pyridine and estimation, by titration, of the amount of acetic anhydride used. (b) The isolation from the reaction mixture of pentaacetylrugulosin, bright yellow prisms, m.p. 210° (decomp.), analysing for the pentaacetate of $C_{30}H_{24}O_{11}$. (c) The preparation, by prolonged methylation with methyl iodide and silver oxide, of rugulosin pentamethyl ether, i.e. $C_{30}H_{17}O_5(OC_2H_5)_5$, lemon-yellow needles, m.p. 255–256°, insoluble in alkalis.

A number of decomposition products of rugulosin have been isolated which establish the relationship of this substance to skyrin, the second colouring matter of *P. rugulosum*, and also to some of the colouring matters of *P. islandicum*, e.g. skyrin, flavoskyrin, rubroskyrin and iridoskyrin. When rugulosin is heated with zinc dust in an atmosphere of hydrogen, 2-methylanthracene is formed. Hence, although it is not claimed that rugulosin is a polyhydroxyanthraquinone, it is clearly related to this class of colouring matters. This conclusion is confirmed by the following observations. (i) Chrysophanol (4:5-dihydroxy-2-methylanthraquinone) (structure I) was isolated in almost 50% yield from the decomposition products formed by prolonged heating of a solution of rugulosin in aqueous 2*N* sodium hydroxide. (ii) Thermal decomposition of rugulosin in an atmosphere of nitrogen at 300–305°

led to the formation of about 12% of 1 mole of carbon dioxide, about 24% by weight of chrysophanol and 20% of emodin (4:5:7-trihydroxy-2-methylanthraquinone) (structure II). (iii) Chrysophanol and emodin were also formed, together with 28.5% of 1 mole of carbon dioxide, by heating rugulosin with copper chromite and quinoline in an attempt to decarboxylate it (Adkins & Connor, 1931; Kinney & Langlois, 1931). (iv) When a solution of rugulosin in concentrated sulphuric acid was heated at 140° an obvious change took place since the colour, initially pure yellow, changed to a stable purplish crimson. The product of the reaction, isolated in 30% yield, has been named aurantio-rugulosin. It has the molecular formula $C_{30}H_{18}O_8$, crystallizes in orange-red needles or brick-red prisms, m.p. 325°, is insoluble in *N* sodium carbonate and gives solutions in sodium hydroxide and cold concentrated sulphuric acid which are almost indistinguishable in colour from those given by emodin in the same reagents. It forms a tetraacetate, bright yellow hexagonal plates, m.p. 294–295°, and a tetramethyl ether, yellow plates, m.p. 362°.

(I)

Chrysophanol

(II)

Emodin

(III)

Islandicin

(IV)

Catenarin

It is of interest to compare the properties of rugulosin, $C_{30}H_{22}O_{10}$, with those of rubroskyrin, $C_{30}H_{22}O_{12}$, from *P. islandicum* (Howard & Raistrick, 1954*b*), since these properties are so similar that they justify the belief that rugulosin and rubroskyrin belong to the same class of compounds. Any difference in their properties, particularly of colour reactions, are readily explained by postulating that rubroskyrin contains two more hydroxyl groups than does rugulosin. Thus the thermal decomposition of rugulosin, which may be represented by the equation

is analogous to that for the thermal decomposition of rubroskyrin advanced by Howard & Raistrick (1954*b*)

islandicin being 1:4:5-trihydroxy-2-methylanthraquinone (structure III), and catenarin being 1:4:5:7-tetrahydroxy-2-methylanthraquinone (structure IV).

It will be seen that islandicin is 1-hydroxy-chrysophanol and catenarin is 1-hydroxyemodin. Hence, if rubroskyrin does indeed prove to be a dihydroxyrugulosin, it follows that these two hydroxyl groups are both attached to the rubroskyrin molecule at positions which become position 1 in its 2-methylanthraquinone decomposition products, islandicin and catenarin.

The conversion by concentrated sulphuric acid of rugulosin into aurantio-rugulosin, which may be represented by the equation

is also analogous to the conversion of rubroskyrin into iridoskyrin, also by concentrated sulphuric acid (Howard & Raistrick, 1954b)

Comparison of the properties of aurantio-rugulosin, $\text{C}_{30}\text{H}_{18}\text{O}_8$, and iridoskyrin, $\text{C}_{30}\text{H}_{18}\text{O}_{10}$ shows equally striking similarities. Both substances have high melting points (325° and 358–360°), both are dimorphic and both have two *C*-methyl groups. While they are both soluble in aqueous sodium hydroxide, they are insoluble in aqueous sodium carbonate, although their parent substances dissolve readily in this reagent. Further, while aurantio-rugulosin gives a tetraacetate and a tetramethyl ether indicating that four of the oxygen atoms are present in the molecule as hydroxyl groups, iridoskyrin gives a hexaacetate and a hexamethyl ether indicating that iridoskyrin contains six hydroxyl groups. The four oxygen atoms remaining in both aurantio-rugulosin and iridoskyrin are believed to be present as two quinone groupings in each case. The differences in the colour reactions given by the two substances are readily explained by the presence of two more hydroxyl groups in iridoskyrin than in aurantio-rugulosin. Thus, while iridoskyrin gives an intensely blue solution in cold concentrated sulphuric acid and a purple-blue, rapidly fading, solution in *N* sodium hydroxide, the corresponding colours given by aurantio-rugulosin are crimson and a stable red with a purple shade.

SKYRIN

Skyrin was first described by Howard & Raistrick (1954*a*, *b*) and was isolated by them in pure crystalline form from two different strains of *P. islandicum*. We have isolated pure skyrin from *P. wortmanni* as the pyridine salt from which crystalline skyrin was recovered and converted into the hexaacetate, m.p.

293° (decomp.) alone or in admixture with hexa-acetylskyrin from *P. islandicum*. Skyrin from *P. wortmanni* also gave the same series of colour reactions as are given by skyrin from *P. islandicum*. On reductive cleavage with sodium dithionite in alkaline solution it likewise gave a good yield of emodin.

The isolation of pure skyrin from the dried mycelium of strains of *P. rugulosum* proved to be much more difficult. The ether extracted mycelium of *P. rugulosum* strains S.M. 1 and S.M. 14 was extracted with acetone, the precipitated mannitol was collected by filtration and the acetone filtrate was chromatographed on magnesium oxide columns giving two distinct bands. The lower yellow rugulosin band was separated with methanol and the upper purplish band containing skyrin, which could not be eluted with solvents, was separated mechanically and decomposed with 2*N* hydrochloric acid and ice. The resulting brownish red flocks were collected, washed and dried, giving a product which had all the reactions of skyrin but could not be crystallized. It was therefore acetylated, and the acetate was purified from boiling ethanol giving skyrin hexaacetate, m.p. 294° (decomp.).

Shibata, Tanaka, Chihara & Mitsuhashi (1953) investigated the colouring matters present in laboratory cultures of two species of *Endothia*, *E. parasitica* (Murr.) Anderson & Anderson, the chestnut-blight fungus and one of the most uniformly destructive fungous parasites known, and *E. radicalis* Fr. They isolated, by solvent extraction of the dried mycelium of each of these species, two crystalline colouring matters which they named endothianin, orange plates with no definite melting point, and radicalisin, yellow, dextro-rotatory rhombic crystals which have no definite melting point but decompose at approx. 282°. Professor Shoji Shibata, the senior of these four investigators, has shown, while on study leave in our laboratory, that endothianin is identical with skyrin and radicalisin is identical with rugulosin. It has therefore been arranged that all future work on the two colouring matters will be carried out in Professor Shibata's laboratory in Tokyo.

EXPERIMENTAL

THE COLOURING MATTERS OF *PENICILLIUM RUGULOSUM* THOM

Cultures

The following strains of *Penicillium rugulosum* Thom were used for the bulk preparation of rugulosin: *Strain H.Z.* Isolated in September 1937 by Mr G. Smith from hops from an oast house in Kent, England. *Strain S.M. 1.* Received in August 1946 from Mr Mandi, Imperial College of Science and Technology, London, as an antibiotic-producing Indian mould. *Strain S.M. 14.* Isolated here in December

1946 by Mrs S. Marcus from a rotten pear. These three strains were identified by Mr G. Smith.

Rugulosin was also isolated from the following strains, though in very much smaller yields: *Strain N.R.R.L. 1045*. Received in July 1948 from Dr Kenneth B. Raper, Northern Regional Research Laboratory (N.R.R.L.), U.S. Department of Agriculture, Peoria, Illinois, U.S.A., as Dr Charles Thom's original type strain. *Strain P. 50*. Received in December 1929 from Professor Ph. Biourge, University of Louvain, Belgium.

No rugulosin could be detected in the following strains: *Strain L.S.H.T.M. Ad. 27*. Purchased in 1925 from the British National Collection of Type Cultures, catalogue number 592. *Strain L.S.H.T.M. B.B. 14*. Isolated in 1929 by Mr G. Smith as a culture contaminant. *Strain L.S.H.T.M. P. 241*. Received in December 1943 from Dr K. B. Raper as *P. rugulosum* N.R.R.L. 1055.

Cultural conditions

Batches of 50 or 100 l. conical flasks, each containing 350 ml. Czapek-Dox solution (glucose, 50.0 g.; NaNO_3 , 2.0 g.; KH_2PO_4 , 1.0 g.; KCl, 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; distilled water, 1 l.), were plugged with cotton wool, sterilized and inoculated with a spore suspension of one of the chosen strains, i.e. H.Z. or S.M. 1 or S.M. 14, of *P. rugulosum* Thom. The flasks were incubated in the dark at 24° until the glucose content of the medium, determined polarimetrically, had decreased to 0.5% or less, when the cultures were harvested and worked up.

Isolation of the colouring matters of *Penicillium rugulosum*

At the end of the incubation period the orange-yellow mycelial mats were separated by filtration from the pale yellow culture solution which was discarded. The mycelial mats were washed with water, squeezed as dry as possible in a tincture press, dried in a vacuum oven at 40–45° and ground to a fine powder in a coffee mill. This powder was exhaustively extracted, first with light petroleum (b.p. 40–50°) in a large metal Soxhlet extractor. On removal of the solvent from the extract there remained 'fat' containing only a little colouring matter (Table 1, column 6).

The extracted mycelium was air dried and then re-extracted with ether until the extract, initially orange-yellow, became colourless. Considerable quantities of orange-yellow crystals separated during the extraction and were collected. The crystals, m.p. 275–280° (decomp.), consisted essentially of rugulosin together with small amounts of skyrin (Table 1, column 7).

The ether-extracted mycelium was again air dried and finally extracted completely with acetone. The dark orange-yellow extract contained further small amounts of rugulosin and most of the skyrin, which were separated by chromatographing on magnesium oxide columns (see below), and considerable quantities of almost colourless crystals, which were collected and identified as mannitol as follows. They were recrystallized several times from methanol (with charcoal) giving rosettes of colourless plates or prisms, m.p. and mixed m.p. with authentic mannitol, 168–170°. The identity of the mannitol was confirmed by conversion into its tribenzoylacetate derivative which was obtained as colourless needles ex ethanol, m.p. and mixed m.p. with an authentic specimen, 216–218° (lit. 215–217°).

The acetone extract, from which mannitol had been separated, was passed through chromatographic adsorption columns (2.5 × 15 cm.), packed with freshly ignited MgO (British Drug Houses Ltd., heavy quality). All the five rugulosin producing strains, i.e. H.Z., S.M. 1, S.M. 14, N.R.R.L. 1045 and P. 50, produced two distinct colour bands, an upper purplish band which could not be eluted with solvents and a lower yellow band. Washing with methanol resulted in almost complete separation of the two bands which were then drained free from solvents. The columns were extruded from the tubes and the two bands were separated mechanically, separately suspended in ice-water and decomposed with 2N-HCl. The lower band gave yellow flocks of pure rugulosin which were collected, washed and dried and crystallized from ethanol in the usual way. The upper purplish band gave brownish red flocks of skyrin which could not be crystallized. However, the substance gives the following reactions which are indistinguishable from those given by skyrin from *P. wortmannii*. It does not melt below 360° nor does it sublime; with conc. H_2SO_4 it gives a dark red colour changing very quickly to emerald green; it is insoluble in saturated NaHCO_3 but dissolves readily in 2N- Na_2CO_3 and 2N-NaOH giving deep purple solutions which are stable in air.

The crude skyrin from strain S.M. 1 was acetylated by heating it (0.5 g.) with anhydrous sodium acetate (2.5 g.) and acetic anhydride (5 ml.). Hexaacetylskyrin, isolated in the usual way, was repeatedly purified from boiling ethanol giving finally a product, m.p. 294° (decomp.). (Found: C, 63.5; H, 4.0. Calc. for $\text{C}_{42}\text{H}_{30}\text{O}_{18}$, i.e. $\text{C}_{30}\text{H}_{12}\text{O}_4$ (O.CO.CH_3)₆, C, 63.8; H, 3.8%.)

Purification of crude rugulosin

Since rugulosin has not a true melting point but decomposes on heating to about 290°, the criterion of purity adopted was constancy of optical rotation.

The crude rugulosin from *P. rugulosum* strain H.Z. (25.8 g.), m.p. 275–280° (decomp.), was ground with cold acetone (50 ml.), filtered and washed with acetone giving a reddish brown filtrate and a yellow cryst. product (21.6 g.), m.p. 285° (decomp.). A portion of this product (5 g.) was fractionally crystallized from acetone (200 ml.) giving three fractions, 2.60, 1.53 and 0.60 g., all melting with decomposition at 285°. These fractions were combined and a portion (3.8 g.) was dissolved in hot dioxan (10 ml.) and fractionally crystallized by the addition of light petroleum (b.p. 40–60°) giving two fractions, both melting with decomposition at 287–290°, i.e. 2.7 g. (fraction 1) and 0.8 g. Fraction 1 was finally crystallized fractionally from successive amounts of ethanol (75, 50 and 30 ml.) giving three fractions, 1.6 g. (fraction 2), 1.2 g. (fraction 3) and 0.57 g. (fraction 4), all melting with decomposition at 287–289°. Portions of the 2.7 g. fraction, m.p. 287–290° (decomp.), referred to above and of each of these three fractions were dried to constant weight in high vacuum at 60–65° and their optical rotations were determined in dioxan (c, 0.40 in all four cases) in a 2 dm. tube in the Hg green (5461 Å) light with the following results. Fraction 1, $[\alpha] +550^\circ$; fraction 2, $[\alpha] +604.7^\circ$; fraction 3, $[\alpha] +604.6^\circ$; fraction 4, $[\alpha] +604.5^\circ$.

In a second experiment 18.2 g. of acetone-washed crude rugulosin from strain H.Z. was crystallized from dioxan-light petroleum (b.p. 40–60°) and then from ethanol and gave 14.6 g. of cryst. rugulosin, $[\alpha]_{5461}^{18} +602.8^\circ$ (c, 0.40 in dioxan).

Table 1. *Cultural characteristics and yields of metabolic products of five strains of Penicillium rugulosum*

For details see text.

Strain of <i>P. rugulosum</i> , cat. no.	No of flasks	Incubation period (days)	Residual glucose (%)	Wt. of dried mycelium (g.)	Wt. of crude fat (g.)	Wt. of crystalline rugulosin (g.)
H.Z.	100	42	0.17	336	39.3	25.8
S.M. 14	50	21	0.15	220	2.3	27.5
S.M. 1	50	28	0.5	200	—	45.0
N.R.R.L. 1045	3	35	1.94	4.29	0.26	0.05
P. 50	2	32	0.42	4.78	0.03	0.02

The rugulosin obtained from strains S.M. 1 and S.M. 14, and prepared 9 years later, was identical in all its properties with that from strain H.Z. Details of representative batches of all three strains are given in Table 1.

General properties of rugulosin

Rugulosin contains C, H, O but not N, S or Cl. (Found, (a) on sample ex *P. rugulosum* strain H.Z. crystallized from ethanol and dried to constant wt. in high vacuum at 60–65°: C, 64.2, 64.4; H, 4.7, 4.65%; OCH₃, nil; mol.wt. cryoscopic in dioxan, 413. (b) On sample ex acetone, hermetically sealed after drying in high vacuum at room temp. and prior to sending to analyst: C, 64.3; H, 4.5%. (c) On sample ex *P. rugulosum* strain S.M. 14 crystallized from ethanol, dried in high vacuum at room temp. and hermetically sealed: C, 64.5; H, 4.1%; mol.wt. cryoscopic in dioxan, 420. C₃₀H₂₄O₁₁ requires C, 64.3; H, 4.3%; mol.wt. 560. (d) On sample ex *P. rugulosum* strain S.M. 14 crystallized from ethanol and dried to constant weight at 140° in high vacuum: C, 66.25; H, 4.05; C-CH₃, 4.8%. (e) On sample ex *P. rugulosum* strain S.M. 1 ex ethanol and dried at 150° in high vacuum: C, 65.8; H, 4.1%. C₃₀H₂₂O₁₀ requires C, 66.4; H, 4.1; 2 C-CH₃, 5.54%; mol.wt. 542.) Shibata *et al.* (1953) give the mol.wt. of radicalisin (=rugulosin), determined by X-ray analysis, as 562.

Rugulosin crystallizes from ethanol in large dull-yellow cubes which readily lose solvent of crystallization and crumble to a yellow powder on exposure to air or in an evacuated desiccator. It crystallizes from ether or acetone as bright yellow blunt-ended prisms and rods, and from methanol as yellow rosettes of elongated prisms. When freed from solvents of crystallization, rugulosin is markedly hygroscopic so that unusual precautions must be taken in preparing samples for analysis. It is also light-sensitive, hence a yellow sample exposed to bright sunlight soon becomes almost brick-red in colour and the optical rotation of dioxan solutions falls from an initial value of $[\alpha]_{461}^{25} + 605^\circ$ to an almost constant value of $+222^\circ$ after exposure to light for periods of 14 days or more.

Rugulosin does not sublime on heating in a high vacuum but decomposes very vigorously at its m.p. (approx. 293°) to a dark-red liquid. It is readily soluble in acetone, dioxan, pyridine, ethyl acetate and glacial acetic acid; moderately soluble in methanol, ethanol, ether, CHCl₃, butanol and benzene, and is almost insoluble in light petroleum and water. Its solutions in CHCl₃ and glacial acetic acid are bright yellow in colour with slight green fluorescence. It is fairly readily soluble in 2N-NaOH, 2N-Na₂CO₃, saturated NaHCO₃ and NH₄OH with the formation of strong yellow to orange-yellow solutions, but is only soluble with difficulty in aqueous potassium acetate to

give a light yellow solution. Rugulosin gives a very characteristic reaction with conc. H₂SO₄ in which it dissolves in the cold to give a pure yellow solution with a slight fluorescence which changes on heating through brown to a stable purplish crimson colour. It is, however, insoluble in conc. HCl either hot or cold. Its ethanolic solution gives an olive-brown colour with FeCl₃, but does not give a ppt. with Brady's reagent (0.32% 2,4-dinitrophenylhydrazine in 2N-HCl), nor does an acetone solution restore the colour to Schiff's reagent. Rugulosin does not decolorize bromine in CHCl₃, nor does its ethanolic solution decolorize acid KMnO₄ solution. It does not form a semicarbazone, methyl ester or *p*-bromophenacyl ester.

Derivatives of rugulosin

Pentaacetylrugulosin. A quantitative estimation of the number of acetyltable hydroxyl groups present in rugulosin was carried out as follows by the Peterson & West (1927) method. A solution of vacuum dried rugulosin (0.1873 g.) in 5.00 ml. of a mixture of acetic anhydride (1 vol.) and pyridine (2 vol.) was kept in a glass-stoppered flask for 3 days at 37°. A control, with the same amount of reagents but without rugulosin, was similarly kept. The two solutions were poured into ice-water (200 ml.) and titrated to phenolphthalein with 0.5N-NaOH. The respective titrations were 31.70 and 35.36 ml., i.e. acetic anhydride used = 3.66 ml. 0.5N-NaOH. This corresponds to an uptake of 5.48 acetyl groups by rugulosin (C₃₀H₂₄O₁₁) or 5.30 groups by C₃₀H₂₂O₁₀.

The precipitated acetate was collected by filtration, dried (0.24 g.) and crystallized 3 times from ethanol giving almost colourless plates, m.p. 206–210° (decomp.). The acetate was further treated by shaking out its solution in CHCl₃ with saturated aqueous NaHCO₃ when the initial very pale yellow colour of the CHCl₃ solution changed to a bright yellow. This solution was washed with water, the solvent was removed *in vacuo* and the dry residue was crystallized from ethanol giving *pentaacetylrugulosin* as long, bright yellow prisms, m.p. 210° (decomp.). (Found: C, 62.4, 62.5; H, 4.6, 4.6. C₄₀H₃₄O₁₆, i.e. pentaacetate of C₃₀H₂₄O₁₁, requires C, 62.3; H, 4.45; C₄₀H₃₂O₁₅, i.e. pentaacetate of C₃₀H₂₂O₁₀, requires C, 63.8; H, 4.3%.)

Pentamethylrugulosin. A solution of rugulosin (0.85 g.) in methyl iodide (50 ml.) was refluxed for 8 hr. with freshly prepared dry Ag₂O (20 g.). Ag₂O was then separated by filtration and extracted with boiling methyl iodide. The filtrate and washings were concentrated to about 5 ml. and allowed to evaporate in the atmosphere giving bright lemon-yellow needles (1.0 g.), m.p. 244°, from which *pentamethylrugulosin* was obtained, by four recrystallizations from ethanol, as characteristic sheaves of fine lemon-

yellow needles, m.p. 255–256°. The compound is insoluble in alkalis and gives no colour with ethanolic FeCl_3 . It gives colour reactions with conc. H_2SO_4 similar to those given by the parent compound. (Found: C, 68.4, 68.1; H, 5.7, 5.2; OCH_3 , 25.2, 25.2; C-CH_3 , 3.22; mol.wt. cryoscopic in camphor, 572. $\text{C}_{35}\text{H}_{32}\text{O}_{10}$, i.e. $\text{C}_{35}\text{H}_{17}\text{O}_5(\text{OCH}_3)_5$, requires C, 68.6; H, 5.3; 5OCH_3 , 25.3; 1 C-CH_3 , 2.45%; mol.wt. 612.) Optical rotation $[\alpha]_{5461}^{25} + 1027.5^\circ$ (c, 0.2 in dioxan).

Decomposition of rugulosin

(a) *By sulphuric acid.* A solution of rugulosin (0.334 g.) in conc. H_2SO_4 (15 ml.) was held at 140° for 30 min. The colour of the solution, initially pure yellow, changed quickly to a stable purplish crimson. The solution was cooled, poured into ice-water (150 ml.), and the resulting dark precipitate was extracted with CHCl_3 (200 ml.) giving a yellow solution which was shaken out with aqueous $\text{N-Na}_2\text{CO}_3$ (100 ml.). On removal of the solvent from the washed CHCl_3 solution there remained orange-red needles (0.10 g.), m.p. 316–318°, recrystallization of which from glacial acetic acid gave *aurantio-rugulosin*, m.p. 325°, $[\alpha]_{5461}^{25}$, nil (c, 0.1 in dioxan). (Found: C, 71.25, 71.1; H, 3.8, 4.0. $\text{C}_{30}\text{H}_{18}\text{O}_8$ requires C, 71.15; H, 3.6%.)

Aurantio-rugulosin is dimorphic and crystallizes from glacial acetic acid either as orange-red sheaves of needles or as brick-red prisms or plates with a constant m.p. of 325°. It is insoluble in $\text{N-Na}_2\text{CO}_3$ or saturated aqueous NaHCO_3 but gives colour reactions with aqueous 2N-NaOH or conc. H_2SO_4 which are almost indistinguishable from those given by chrysophanol and emodin in the same reagents, i.e. 2N-NaOH, a red solution with a purplish shade; cold conc. H_2SO_4 , a crimson solution with a purplish shade. Aurantio-rugulosin is also formed when pentamethylrugulosin is heated with conc. H_2SO_4 at 140°.

Tetraacetyl aurantio-rugulosin. A solution of aurantio-rugulosin (0.12 g.) and anhydrous sodium acetate (1.0 g.) in acetic anhydride (5 ml.) was refluxed for 2 hr. The crude acetate, recovered in the usual way, was crystallized thrice from glacial acetic acid giving *tetraacetyl aurantio-rugulosin* as small bright yellow hexagonal plates, m.p. 294–295°. (Found: C, 66.9; H, 4.25. $\text{C}_{35}\text{H}_{26}\text{O}_{12}$ requires C, 67.7; H, 3.9%.)

Tetramethyl aurantio-rugulosin. A solution of aurantio-rugulosin (0.35 g.) in anhydrous acetone (100 ml.) was boiled, and redistilled dimethyl sulphate (2 ml.) and anhydrous K_2CO_3 (2.0 g.) were added in 0.5 ml. and 0.5 g. portions at suitable intervals during 8 hr. boiling under reflux. The mineral salts were separated by filtration and the filtrate and washings were evaporated to dryness *in vacuo*. Crystallization of the residue from ethanol gave *tetramethyl aurantio-rugulosin* (0.27 g.) as small yellow prismatic plates, m.p. 362°, unchanged on further crystallization and sublimation at 280–300° in high vacuum. (Found: C, 72.6, 72.2; H, 4.8, 5.0; OCH_3 , 21.7, 21.9; C-CH_3 , 4.3. $\text{C}_{34}\text{H}_{26}\text{O}_8$ requires C, 72.6; H, 4.7; 4OCH_3 , 22.1; 2 C-CH_3 , 5.3%.)

(b) *Thermal decomposition.* Rugulosin (1.0 g.) was heated in a stream of N_2 freed from O_2 and CO_2 . The N_2 and volatile products were passed through a bubbler containing 0.2N-Ba(OH) $_2$. The tube containing the rugulosin was immersed in a metal bath maintained at 300–305° for about 5 min. CO_2 (9.68 mg.) was evolved $\equiv$ 12.3% of theory (78.5 mg.) for 1 mol. CO_2 /mol. rugulosin, $\text{C}_{30}\text{H}_{24}\text{O}_{11}$.

The solid products of the reaction consisted of a very slight orange sublimate and a blackish residue (0.87 g.).

The whole was extracted with CHCl_3 , separated by filtration from the black insoluble residue, and the orange-yellow solution was shaken repeatedly with $\text{N-Na}_2\text{CO}_3$. The purple-red carbonate-soluble fraction so obtained was separated, acidified with 2N-HCl and extracted with CHCl_3 . The CHCl_3 solution was evaporated to dryness *in vacuo*. The Na_2CO_3 -insoluble material remaining in the CHCl_3 after Na_2CO_3 extraction was also recovered by evaporation of the solvent.

The Na_2CO_3 -soluble material (0.21 g.) consisted of orange-yellow crystals, m.p. 250–252°, which were purified by two crystallizations from glacial acetic acid followed by sublimation in high vacuum, giving bright orange prisms, m.p. 254–256°. The substance was identified as emodin (4:5:7-trihydroxy-2-methylanthraquinone) by its colour reactions and by the fact that its m.p. was not depressed on admixture with authentic emodin, m.p. 258°. (Found: C, 66.9; H, 3.6. Calc. for $\text{C}_{11}\text{H}_{10}\text{O}_5$: C, 66.7; H, 3.7%.) The identity was confirmed by conversion into the acetate. The substance (0.09 g.) was refluxed for 2 hr. with acetic anhydride (2 ml.) and fused sodium acetate (0.20 g.). The yellow amorphous acetate (0.095 g.), recovered in the usual way, was crystallized thrice from ethanol giving large bright yellow needles of triacetylemodin, m.p. 198° alone or in admixture with an authentic specimen. (Found: C, 63.6; H, 4.2. Calc. for $\text{C}_{21}\text{H}_{16}\text{O}_5$: C, 63.6; H, 4.1%.)

The Na_2CO_3 -insoluble material (0.24 g.) consisted of orange-yellow plates, m.p. 176–180°, which were purified by three crystallizations from ethanol followed by sublimation in high vacuum, giving orange-yellow plates, m.p. 193–194°. The substance was identified as chrysophanol (4:5-dihydroxy-2-methylanthraquinone) by its colour reactions and by the fact that its m.p. was not depressed on admixture with authentic chrysophanol, m.p. 194°. (Found: C, 71.4; H, 4.5. Calc. for $\text{C}_{15}\text{H}_{10}\text{O}_4$: C, 70.9; H, 4.0%.) The substance was acetylated by the method described above for triacetylemodin giving diacetylchrysophanol as pale yellow plates from glacial acetic acid, m.p. 207–208° alone or in admixture with authentic diacetylchrysophanol. (Found: C, 67.5; H, 4.4. Calc. for $\text{C}_{15}\text{H}_{14}\text{O}_6$: C, 67.45; H, 4.2%.)

(c) *By hot aqueous sodium hydroxide.* A solution of rugulosin (0.20 g.) in 2N-NaOH (40 ml.) was heated on the boiling water bath for 8 hr. The initial intensely orange-yellow solution soon darkened to red. Next day 2N-NaOH (10 ml.) was added and heating was continued for a further 8 hr. The solution was cooled, acidified with 2N-HCl and extracted with CHCl_3 (250 ml.). After removal of the solvent *in vacuo*, the orange residue (0.10 g.) was sublimed in high vacuum giving an orange-yellow crystalline sublimate (0.09 g.), m.p. 186–188° raised to 190–192° on crystallization from ethanol. The substance was identified as chrysophanol by colour reactions, m.p. and mixed m.p. with an authentic specimen.

(d) *By heating with copper chromite and quinoline.* A mixture of rugulosin (0.5193 g.), copper chromite (0.2 g.; as prepared by Adkins & Connor, 1931) and pure quinoline (7 g.), contained in a small flask fitted with an air condenser, was heated in an oil bath. A slow stream of CO_2 -free N_2 was passed through the apparatus and the issuing gases were bubbled through aqueous 0.2N-Ba(OH) $_2$. The bath temperature was held at 215–220° until evolution of CO_2 ceased. 28.5% of the theoretical value for 1 g. mol. of CO_2 per g. mol. of rugulosin was evolved. After cooling, the contents of the flask were acidified and extracted with

CHCl_3 . On removal of the solvent a crude mixture of emodin and chrysophanol remained. These substances were isolated, purified and identified as described in (b) above.

(e) *By heating with zinc dust in hydrogen.* A mixture of rugulosin (1 g.) and Zn dust (20 g.) was heated in a combustion tube in a slow stream of H_2 . A sublimate of almost colourless plates (5 mg.), m.p. 196–198°, which appeared in the cool part of the tube, was collected and crystallized from ethanol giving 2-methylantracene as almost colourless leaflets, m.p. 206–208°, not depressed on admixture with an authentic specimen, m.p. 207–208°. A further quantity of 2-methylantracene (35 mg.), m.p. 196–198°, was obtained by ether extraction of the remainder of the tube.

Rugulosin was recovered unchanged after treatment of (i) its CHCl_3 solution with ozone; (ii) its solution in aqueous $\text{N-Na}_2\text{CO}_3$ with sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$); (iii) its solution in dilute aqueous NaOH with H_2O_2 .

THE COLOURING MATTERS OF *PENICILLIUM WORTMANNI* KLÖCKER

The strain of *P. wortmanni* used in this part of the investigation was received in July 1948 from Dr K. B. Raper of the Northern Regional Research Laboratory (N.R.R.L.), U.S. Department of Agriculture, Peoria, Illinois, U.S.A., under the number N.R.R.L. 1017. It was received originally by Thom from Biourge in 1924, who had it directly from Klöcker, and is therefore presumably type (Raper & Thom, 1949, p. 584).

The cultural conditions were the same as those followed in the cultivation of *P. rugulosum*. The cultures were harvested after 24 days' incubation, when the mycelium was thick, dull orange on the surface and with a dirty greenish reverse. The yield of vacuum-dried mycelium was 4.16 g. per flask containing 350 ml. of Czapek-Dox 5% glucose solution.

Isolation and purification of the colouring matters

The dried and ground mycelium was extracted, like *P. rugulosum*, first with light petroleum, then with ether and finally with methanol. Little colouring matter was present in either the light-petroleum extract, which contained much crude 'fat', or in the methanol extract which, however, contained large amounts of other solids.

The ether extracts deposited appreciable amounts of orange coloured solid and further amounts of material, similar in appearance, were obtained by working up the mother liquors. The different crops were sorted according to their reactions with cold conc. H_2SO_4 : fraction A giving a purple colour changing to green consisted of crude skyrin; fraction B giving a reddish colour contained both skyrin and rugulosin; fraction C with no distinctive colour reaction was still contaminated with fatty material but contained some rugulosin and a trace of skyrin. From two separate batches of 50 flasks each of Czapek-Dox solution the yields of the different fractions obtained were: fraction A, 0.7 + 1.0 g.; fraction B, 2.2 + 2.7 g.; fraction C, 4.06 + 4.1 g.

Purification and identification of skyrin

Fraction A (1.5 g.) was repeatedly crystallized from dioxan–water giving 0.7 g. of minute reddish orange prisms which were recrystallized from pyridine giving the pyridine salt of skyrin (0.8 g.) as short, deep red prisms which

blackened without melting when heated. (Found: C, 68.7; H, 4.1; N, 4.76. Calc. for $\text{C}_{30}\text{H}_{18}\text{O}_{10} \cdot 2\text{C}_5\text{H}_5\text{N}$: C, 69.0; H, 4.1; N, 4.0%.)

The pyridine salt was converted into the free colouring matter as follows. The substance (0.20 g.) was dissolved in the minimum amount of aqueous $2\text{N-Na}_2\text{CO}_3$, the solution was diluted with water (2 vol.) and acidified to Congo red with $2\text{N-H}_2\text{SO}_4$. The resulting micro-crystalline, bright orange product (0.14 g.) was recrystallized from acetone as orange-red rectangular plates (0.07 g.) which did not melt on heating to 380°. It gave reactions identical with those given by skyrin from *P. islandicum* with the following reagents. (a) A deep purple stable solution in N-NaOH or $\text{N-Na}_2\text{CO}_3$. (b) In cold conc. H_2SO_4 a solution which is initially deep cherry red changing in about 1 min. to a stable emerald green. For further identification this substance was acetylated in the usual way with acetic anhydride and sodium acetate. The crude acetate obtained was crystallized first from acetic acid and then twice from CHCl_3 –ethanol, giving fine yellow needles, m.p. 293°. On admixture with authentic hexaacetylskyrin from *P. islandicum*, m.p. 296°, the mixture also melted at 296°.

The crude pyridine salt (0.19 g.) was dissolved in $\text{N-Na}_2\text{CO}_3$ (30 ml.), and a solution of sodium dithionite $\text{Na}_2\text{S}_2\text{O}_4$ (3.5 g.) in water (16 ml.) was added and the mixture was shaken for 5 min. N-HCl (30 ml.) was then added and the resulting orange ppt. was collected on a kieselguhr pad, washed and dried. The crude product was extracted in a Soxhlet, first with ether (30 hr.) and then with CHCl_3 (16 hr.). Both extracts were evaporated to dryness and the combined residues (0.09 g.) were sublimed in high vacuum at 170–180°, giving a crystalline orange sublimate (0.05 g.), m.p. and mixed m.p. 256° with an authentic specimen of *Frangula* emodin (4:5:7-trihydroxy-2-methylantraquinone). The two specimens also gave identical reactions (i) with cold conc. H_2SO_4 , a dark red, stable, non-fluorescent solution with a slightly bluish shade; (ii) readily soluble in aqueous $2\text{N-Na}_2\text{CO}_3$ giving a dark red solution which is very similar in shade to that given in conc. H_2SO_4 .

Purification and identification of rugulosin

The crude fractions B from the two batches were combined (4.9 g.) and fractionated from acetone giving an undissolved residue (0.07 g.) of fairly pure skyrin and three crops from the solution, i.e. crude rugulosin (2.1 g.); very impure rugulosin (1.45 g.); and skyrin (0.08 g.).

The corresponding crude fractions C (8.16 g.) were also fractionated from acetone giving 0.11 g. of pure rugulosin, 1.01 g. of fairly pure rugulosin, 1.10 g. of crude skyrin and 1.7 g. of a mixture of the two substances. The two crops of impure rugulosin (2.1 + 1.01 g.) were combined and crystallized from ethanol giving rugulosin (2.27 g.) as yellow, rectangular tablets, m.p. 293° (decomp.) either alone or mixed with rugulosin from *P. rugulosum*. (Found, on sample dried to constant weight in high vacuum at 100°: C, 64.4; H, 4.2. $\text{C}_{30}\text{H}_{24}\text{O}_{11}$ requires C, 64.3; H, 4.3%.) $[\alpha]_{\text{D}}^{20} +604^\circ$ (c, 0.25 in dioxan) falling to $+590^\circ$ overnight. Like rugulosin from *P. rugulosum* it gave an intensely orange-yellow solution in aqueous NaOH and the characteristic reaction with conc. H_2SO_4 , i.e. a pure yellow solution in the cold changing, on heating at 140°, through brown to a stable purplish crimson. It also gave an olive-brown ferric colour in ethanol.

ANTIBACTERIAL ACTIVITY OF RUGULOSIN

Since rugulosin is almost insoluble in water or in nutrient broth it was necessary to modify the methods in common use for the determination of antibacterial activity. Preliminary investigations having shown that a saturated solution of rugulosin in broth had a marked bactericidal effect on *Staphylococcus aureus* the following three methods were used. In recording the results obtained the source of the organism used is indicated by either L.S.H. (from the Department of Bacteriology, London School of Hygiene and Tropical Medicine) or N.C. (from the National Collection of Type Cultures, Medical Research Council). The figures recorded indicate the minimum concentration of rugulosin giving complete inhibition of growth after 24 hr. incubation at 37°, except where stated otherwise.

A. Pure rugulosin (0.200 g.) was dissolved in 8.20 ml. 0.1N-NaOH, the amount which a separate titration had shown to be necessary for neutralization to phenolphthalein used as an external indicator. The bright yellow solution was diluted to 10 ml. with sterile water and added to 40 ml. of sterile 0.2% glucose-nutrient broth—which became very turbid—giving a concentration of 1:250 of rugulosin. Serial dilutions in glucose-broth, going up in steps of two, were prepared, held at 37° for 1 hr. and inoculated with one loopful of a 24 hr. culture of the organism under investigation. A control culture in glucose-broth containing no rugulosin was set up for each organism investigated, and good growth was obtained in all cases. The following results were obtained. 1:2000 *Pseudomonas fluorescens* (L.S.H. incubated at 24°). 1:8000 *Staph. aureus* (Oxford H). 1:16 000 *Staph. aureus* (L.S.H. no. 1, L.S.H. no. 2, N.C. 3095). <1:256 000 *Bacillus subtilis* (L.S.H.). <1:1 024 000 *Staph. aureus* (N.C. 3093); *B. anthracis* (L.S.H.); *Corynebacterium diphtheriae gravis* (L.S.H.); *C. diphtheriae mitis* (L.S.H.). The cultures of the last four organisms, showing total inhibition at 1:1 024 000, were reinoculated and then incubated for a further 24 hr. No growth was obtained in any case.

B. Pure rugulosin (0.200 g.) was dissolved in dioxan (20 ml.). 0.5 ml. of the resulting pale yellow clear solution was added to 4.5 ml. of 0.2% glucose-broth and well mixed giving a 1:1000 solution of rugulosin. Serial dilutions in glucose nutrient broth were made and inoculated as in method A with one of an extensive series of micro-organisms with the following results. 1:2000 *Staph. albus* (N.C. 5955); *Staph. citreus* (N.C. 7415); *Staph. aureus* (N.C. 7860); *Bacterium coli* (L.S.H. D15; N.C. 86); *Salmonella typhi* (N.C. 2128); *Salm. typhi-murium* (N.C. 5710); *Salm. paratyphi-B* (N.C. 5705); *Salm. enteritidis* (N.C. 5765); *Shigella shigae* (N.C. 4837); *Mycobacterium phlei* (N.C. 525). and *M. smegmatis* (N.C. 523), incubated 7 days at 37°. 1:4000 *Staph. aureus* (Oxford H; N.C. 7878); *Staph. pyogenes* (N.C. 3750); *Pseudomonas pyocyanea* (L.S.H.). 1:8000 *Staph. aureus* (L.S.H. no. 1). 1:16 000 *Vibrio cholerae* (L.S.H. M40). 1:32 000 *Staph. pyogenes* (N.C. 3761); *B. subtilis* (L.S.H.); *Actinomyces hortoni* (N.C. 600). 1:128 000 *Staph. pyogenes* (N.C. 4163). 1:512 000 *C. diphtheriae intermedius* (L.S.H. F8; 48 hr.). 1:1 024 000 *Staph. pyogenes* (N.C. 3095). 1:2 048 000 *Streptococcus viridans* (N.C. 3165; 48 hr.); *C. diphtheriae gravis* (N.C. 5985; 48 hr.); *B. anthracis* (N.C. 5444); *B. cereus* (N.C. 8035). 1:4 096 000 *Strep. pyogenes* (N.C. 2432; 48 hr.); *C. diphtheriae mitis* (L.S.H. F16; 48 hr.).

Control experiments showed that dioxan has little inhibitory effect on the growth of micro-organisms, hence its use as a solvent for rugulosin which dissolves readily in it. Both dioxan and ethanol permit full growth of *Staph. aureus* Oxford H strain at a concentration of 1:160 in nutrient broth.

We are indebted to Mrs S. Marcus of this Department for the anti-bacterial tests recorded in sections A and B.

C. A suspension of rugulosin (25 mg.) in sterile nutrient broth (12.5 ml.; pH 7.6) was held in a sterile flask at 60° for 1 hr. and was then serially twofold diluted in nutrient broth. In order to ensure as uniform distribution as possible, the rugulosin was kept suspended during the dilution by sucking twice into the pipette and vigorously ejecting back into the tube by blowing. Each of the broth dilutions was inoculated with 0.1 ml. of an 18 hr. broth culture of the test organism. The results, which were read at 18 hr. unless otherwise stated, follow. 1:500 *Salm. typhi*; *Bact. coli*; *Proteus vulgaris*; *Mycobacterium avium*; *M. tuberculosis* var. *bovis*; *M. tuberculosis* var. *hominis* (these three strains were tested in Dubos' medium and the results read after 10 days). 1:1000 *Vibrio cholerae*; *Salm. sonnei*. 1:2000 *Salm. dysenteriae*; *Salm. paratyphenteriae*; *Haemophilus pertussis* in Bordet-Gengou medium. 1:4000 *Streptococcus pyogenes* (in blood). 1:8000 *Staph. aureus*; *Ps. aeruginosa*. 1:32 000 *Strep. agalactiae*. 1:125 000 *Strep. pyogenes*, in broth+10% serum. 1:250 000 *C. pyogenes*. 1:500 000 *Strep. pyogenes* in broth.

Therapeutic tests were carried out against *Strep. pyogenes* and *Bact. coli* as follows. Groups of six mice were infected with 10 000 lethal doses of the infecting organism with appropriate controls. A suspension of rugulosin in sterile distilled water (0.5 mg. in 0.5 ml.) was administered intraperitoneally immediately after infection, 6 hr. later and on the following day. The results are summarized in Table 2.

We are indebted to Mr S. R. M. Bushby of the Wellcome Foundation Ltd. for the above tests, which were carried out in April 1949 under the aegis of the Therapeutic Research Corporation of Great Britain Ltd.

Table 2. *Therapeutic tests of rugulosin in bacterial infections in mice*

Infecting organism	No. of mice	No. of mice surviving on day		
		1	2	3
<i>Strep. pyogenes</i>	6	6	5	5
Controls	6	1	0	—
<i>Bact. coli</i>	6	0	—	—
Controls	6	0	—	—

ANTIFUNGAL ACTIVITY OF RUGULOSIN

An aqueous solution of rugulosin (0.03 g.) was made by dissolving it in hot phosphate buffer (100 ml.; pH 7.0). Five dilutions, going up in steps of two, were made in phosphate buffer of the same pH and were held for 5 min. at 100°. While still hot, 1 ml. of each dilution was mixed with 14 ml. of hot melted sterile Czapek-Dox sucrose agar and the mixture was poured into sterile Petri dishes, which, when cold, were inoculated with small squares cut from the agar of a Petri dish culture of *Pythium intermedium* de Bary. The cultures were then incubated at 24° for 3 days, when the control plates were completely overgrown. The

following results were obtained. Rugulosin concentrations, 1:50 000, no growth; 1:100 000, partial inhibition, growth retarded and very thin; 1:200 000, partial inhibition; 1:400 000, normal growth.

SUMMARY

1. Two crystalline colouring matters—one red and the other yellow—have been isolated from the mycelium of laboratory cultures of a number of strains of *Penicillium rugulosum* Thom and from one strain of *P. wortmanni* Klöcker.

2. The red colouring matter is skyrin, $C_{30}H_{18}O_{10}$, previously reported from *P. islandicum* Sopp.

3. The yellow colouring matter, which has not been described previously and has been named rugulosin, $C_{30}H_{22}O_{10}$, forms yellow prisms, m.p. about 293° (decomp.) which are strongly dextro-rotatory. It has a markedly specific antibacterial activity and is moderately antifungal.

4. Good yields of chrysophanol (4:5-dihydroxy-2-methylanthraquinone) and emodin (4:5:7-trihydroxy-2-methylanthraquinone) have been obtained by degradation of rugulosin.

5. Treatment of rugulosin with concentrated sulphuric acid at 140° gives aurantio-rugulosin, $C_{30}H_{18}O_8$, which is believed to be a substituted dianthraquinone.

6. The structural relationship of rugulosin and rubroskyrin, $C_{30}H_{22}O_{12}$ from *P. islandicum*, is discussed as well as that of aurantio-rugulosin and iridoskyrin, $C_{30}H_{18}O_{10}$, from *P. islandicum*.

7. It is believed that rugulosin and rubroskyrin belong to a new class of colouring matters which, while not anthraquinones themselves, readily give rise to well known polyhydroxyanthraquinones.

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The Reactivity of the Cystine Linkages in Wool towards Reducing Agents

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Of all the various reactive side linkages in wool, none has been studied more extensively than the cystine linkage. This is probably accounted for by the fact that the keratins contain a much higher proportion of this amino acid than other proteins. Possibly because of this there has been a tendency to explain any anomalous physical or chemical behaviour of wool in terms of cystine.

In a very comprehensive series of studies of the reactivity of the cystine linkages in wool, Phillips and his colleagues have examined the behaviour of untreated and modified wools towards sulphites, alkalis, formaldehyde and thioglycolic acid (Elsworth & Phillips, 1938, 1941; Middlebrook & Phillips, 1942a, b, 1947; Cuthbertson & Phillips, 1945; Lindley & Phillips, 1945, 1947; Carter,

Middlebrook & Phillips, 1946; Phillips, 1946.) As a result of this work, the combined cystine of wool has been divided into two fractions of differing reactivity, each fraction being further divided into two subfractions. For convenience, the more reactive fraction has been termed (*A* + *B*) and the less reactive portion (*C* + *D*). By definition, that portion of cystine which reacts with sodium bisulphite solutions to give thiol groups on subsequent hydrolysis is known as fraction (*A* + *B*). Most of this fraction reacts in the cold. When rinsing with water is interposed between bisulphiting and hydrolysis, the residual thiol-S determined in the hydrolysate gives a measure of subfraction *B*, subfraction *A* being obtained by difference.

Fraction (*C* + *D*) is defined as that portion of the cystine which does not give cysteine and S-cysteine

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