Studies in the Biochemistry of Micro-Organisms

114. VIRIDICATOL AND CYCLOPENOL, METABOLITES OF *PENICILLIUM VIRIDICATUM* WESTLING AND *PENICILLIUM CYCLOPIUM* WESTLING*

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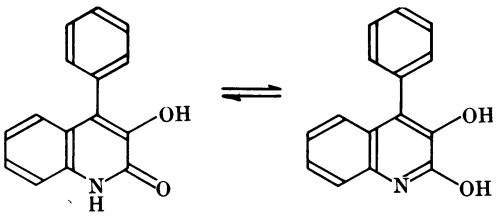
Bracken, Pocker & Raistrick (1954) described the isolation of two nitrogen-containing products from different cultures of *Penicillium cyclopium* Westling. One of these, previously isolated from *Penicillium viridicatum* Westling by Cunningham & Freeman (1953) and named viridicatin, $C_{15}H_{11}NO_2$, was shown to be 2,3-dihydroxy-4-phenylquinoline or its keto-tautomer (I), by degradative methods and unequivocal synthesis.



(I) Viridicatin

The second product, which was called cyclopenin, was isolated as a colourless crystalline solid, m.p. 207°, analysis of which led to the formula $C_{17}H_{14}N_2O_3,\frac{1}{2}H_2O$; or (from methanol) as needles, m.p. 110–120° with loss of water, and remelting at 207°, analysis in this case suggesting a dihydrate, $C_{17}H_{14}N_2O_3,2H_2O$. Though no derivatives could be obtained, the formula was supported by the results of acid hydrolysis, which gave over 90% yields of both carbon dioxide and methylamine, together with a solid product, m.p. 246–252°, which by sublimation and repeated crystallization yielded pure viridicatin, m.p. 268°. The yield of crude product, m.p. 246–252°, calculated as viridicatin, was also over 90%. The hydrolysis was therefore written:

$$C_{17}H_{14}N_2O_3 + H_2O \rightarrow CO_2 + CH_3 \cdot NH_2 + C_{15}H_{11}NO_2$$

and two possible formulae were put forward to account for this reaction and other properties of cyclopenin.

During a study of the biosynthesis of these metabolites, two of us (M. L. and K. M.) obtained paper-chromatographic evidence of two further products occurring alongside viridicatin and cyclopenin in a strain of *P. viridicatum* (cf. Luckner & Mothes, 1962, 1963). Examination of samples of the two forms of cyclopenin obtained by Bracken *et al.* (1954) also gave paper-chromatographic evidence that they were mixtures of two similar compounds. This paper presents some results of a joint study of these products.

Crude cyclopenin was obtained as described by Bracken *et al.* (1954) from culture filtrates of *P. cyclopium* strain LSHTM no. 72. Purification was effected by boiling the crude product with benzene, in which cyclopenin is moderately soluble. The evaporated benzene extract was extracted with carbon tetrachloride, and the soluble fraction was crystallized from ethyl acetate–light petroleum to give needles, m.p. 183–184°, which, however, were chromatographically not quite pure. By the use of paper chromatography on a preparative scale, chromatographically pure cyclopenin was obtained, m.p. 183–184°. Analysis agreed well with the formula $C_{17}H_{14}N_2O_3$, with no solvent of crystallization; it was optically active, $[\alpha]_{5461}^{20} - 291°$ in methanol.

The benzene-insoluble material was crystallized repeatedly from an ethyl acetate–benzene mixture to give prisms, m.p. 215°, which were chromatographically free from cyclopenin. Analysis of this substance agreed with the formula $C_{17}H_{14}N_2O_4$, which has one oxygen atom more than the molecular formula of cyclopenin. Its properties closely resemble those of cyclopenin (see below), the additional oxygen atom being almost certainly present as a hydroxyl group, and we therefore name it cyclopenol [Luckner & Mothes (1962, 1963) referred to cyclopenin and cyclopenol as 'cyclopenin A' and 'cyclopenin B', but these names are now abandoned]. Cyclopenol has $[\alpha]_{5461}^{20} - 309°$ in methanol.

Cyclopenin was methylated by diazomethane to a monomethyl derivative, $C_{13}H_{16}N_2O_3$. Cyclopenol was similarly converted into a dimethyl derivative, $C_{19}H_{18}N_2O_4$.

* Part 113: Birkinshaw, Kalyanpur & Stickings (1963).

Acid hydrolysis of cyclopenin yielded, as described by Bracken *et al.* (1954), 1 mol.prop. of carbon dioxide, methylamine and viridicatin. The crude hydrolysis product was fairly pure viridicatin, m.p. 267° (88% yield); crystallization from ethanol raised the m.p. to 269–270°. We believe the crude hydrolysis product, m.p. 246–252°, described by Bracken *et al.* (1954), was a mixture of viridicatin and viridicatol (see below), which on recrystallization yielded pure viridicatin.

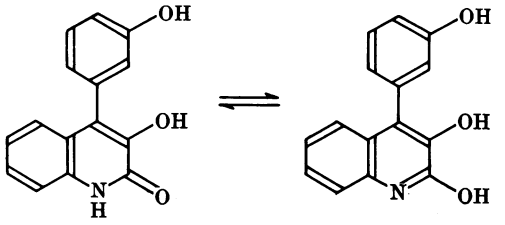
Acid hydrolysis of cyclopenol yielded 1 mol.prop. of carbon dioxide, 1 mol.prop. of methylamine, and a compound, m.p. 280°, whose molecular formula, $C_{15}H_{11}NO_3$, showed it to contain one oxygen atom more than the molecular formula of viridicatin. This compound we name viridicatol [it was called 'alkaloid X' by Luckner & Mothes (1962, 1963)]. It gives a green colour with ferric chloride, identical with that given by viridicatin. Its ultraviolet-absorption spectrum is similar to that of viridicatin.

Culture filtrates from *P. viridicatum* strain V 41 (Luckner & Mothes, 1963) were treated in a similar manner, but the crude methanol extract was purified by passage through alumina and then separated by thin-layer chromatography. After further purification, pure cyclopenin and cyclopenol were obtained, and were identified by mixed m.p. with the compounds obtained from *P. cyclopium*.

The mycelium from this strain of *P. viridicatum* was extracted with acetone, and the extract was adsorbed on an alumina column.

Material giving a green colour with ferric chloride was eluted by the use of aqueous methanolic hydrochloric acid, and was separated by preparative chromatography into viridicatin, m.p. 268°, identified with a sample prepared by Bracken *et al.* (1954), and viridicatol, m.p. 280°, identified by mixed m.p. with the compound obtained by hydrolysis of cyclopenol.

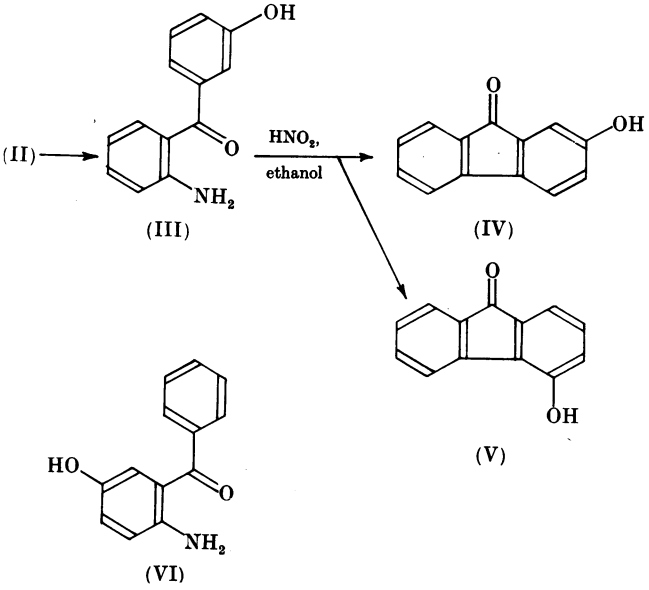
These facts show that the two new compounds contain an extra oxygen atom in a corresponding position in the molecule. A minor product of the acid hydrolysis of cyclopenol was shown to be *m*-hydroxybenzoic acid, obtained in about 6% yield. This suggests the formula (II) for viridicatol.



(II) Viridicatol

The presence of a phenolic group is supported by the fact that viridicatol (and cyclopenol) can be detected as pink spots on paper chromatograms by spraying with diazotized sulphanilic acid–sodium carbonate, whereas viridicatin and cyclopenin give no colour under these conditions. In addition, acetylation of viridicatin yielded a monoacetate, but viridicatol gives a diacetyl derivative.

Degradation of viridicatol by alkaline oxidation, with the method used for viridicatin by Bracken *et al.* (1954), yielded products corresponding to those obtained from viridicatin, namely a substance $C_{13}H_{11}NO_2$, as yellow needles, m.p. 102°, which was soluble in dilute acids and sodium hydroxide, but not in sodium hydrogen carbonate; and its oxalyl derivative, $C_{15}H_{11}NO_5$. By analogy with viridicatin, we expected the compound $C_{13}H_{11}NO_2$ to be a hydroxy derivative of 2-aminobenzophenone. It was therefore diazotized and treated with hot ethanol, to yield the known 2-hydroxyfluorenone (IV), m.p. 211°, identified by mixed m.p. with a synthetic specimen (Diels, 1901).



The isolation of 2-hydroxyfluorenone restricts the structure of the amine $C_{13}H_{11}NO_2$ to two possibilities, namely 2-amino-3′-hydroxybenzophenone (III) and 2-amino-5-hydroxybenzophenone (VI). We reject the latter possibility on the following grounds:

(1) A minor product was isolated from the diazo-coupling reaction, m.p. 245°. Insufficient material was available for complete identification, but its properties suggest that it is the known 4-hydroxyfluorenone (V), m.p. 250–251° (Huisgen & Rist, 1955). This would be expected from (III), but could

not be formed from (VI), which could give only one coupling product.

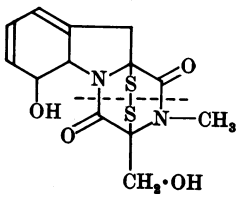
(2) Compound (VI) contains the *p*-aminophenol group, which would be expected to be sensitive to alkaline oxidation. The amine $C_{13}H_{11}NO_2$ is stable to boiling aq. 10% (w/v) sodium hydroxide in the presence of air.

(3) By treatment with aqueous zinc chloride at 200°, the benzophenone was cleaved (Königs & Nef, 1886). Paper chromatography of the product revealed the presence of aniline and the absence of *o*-, *m*- and *p*-aminophenol. 2-Aminobenzophenone and 2-amino-4′-hydroxybenzophenone also gave aniline under these conditions.

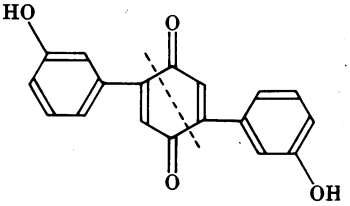
(4) The isolation of *m*-hydroxybenzoic acid from the acid hydrolysis of cyclopenol, of which viridicatol is a degradation product, strongly supports structure (II) for viridicatol and therefore structure (III) for the amine.

We therefore believe that the amine $C_{13}H_{11}NO_2$ has the structure 2-amino-3′-hydroxybenzophenone (III), and hence that viridicatol is 2,3-dihydroxy-4-(3-hydroxyphenyl)quinoline, or its keto-tautomer, 3-hydroxy-4-(3-hydroxyphenyl)-2-quinolone. Chemical evidence, and the presence of a strong infrared-absorption band at 1650 cm.$^{-1}$ in both viridicatol and viridicatin, suggest that the quinolone structure predominates in both cases.

The structure of viridicatol is of interest from the biosynthetic viewpoint. Luckner & Mothes (1962, 1963) have shown that in viridicatin C-2, C-3 and C-4 and the 4-phenyl group are derived from phenylalanine or a close relative. The presence of a phenolic group in viridicatol at first suggested a *p*-hydroxyl group, with a corresponding derivation from tyrosine; 2-amino-4′-hydroxybenzophenone was synthesized for comparison with the product from viridicatol, but found not to be identical. The introduction of a *m*-hydroxyl group into a grouping derived from phenylalanine is more unusual, but two examples are already known among fungal metabolites, namely gliotoxin (VII) from *Trichoderma viride* (Bell, Johnson, Wildi & Woodward, 1958), and volucrisporin (VIII) from *Volucrispora aurantiaca* (Divekar, Read & Vining, 1959; Divekar, Read, Vining & Haskins, 1959).



(VII) Gliotoxin



(VIII) Volucrisporin

In each of these cases, phenylalanine has been shown to act as a precursor for the *m*-tyrosine moiety, showing that *meta*-hydroxylation can take place in the aromatic ring (Suhadolnik & Chenoweth, 1958; Winstead & Suhadolnik, 1960; Read & Vining, 1959). Study of the biosynthesis of viridicatol is still in progress, but preliminary results indicate that phenylalanine can also act as a precursor in this case.

EXPERIMENTAL

The C, H and N determinations were by Dr A. Schoeller (Kronach) and Dr H. Jeschkeit (Organisch-chemisches Institut der Universität Halle). Ultraviolet absorption was measured on a Hilger spectrophotometer. Infrared absorption was measured on a Perkin–Elmer Infracord, and a VEB Zeiss Jena model UR10.

Isolation of metabolites from Penicillium cyclopium, *LSHTM no.* 72

Organism. The culture employed was the same as that used by Bracken *et al.* (1954). It was prepared from a sand culture preserved since 1948. A subculture is maintained by the Commonwealth Mycological Institute, Kew, Surrey (CMI no. 89374).

Cultural conditions and harvesting. The methods used by Bracken *et al.* (1954) were employed. The crude mixture of cyclopenin and cyclopenol which separated from the methanol concentrate melted at 200–205°, after sintering and resetting above 100°. Yields of 4–8 g./100 flasks were obtained.

Separation of the mixture. Separation methods were tested by the use of paper chromatography (Luckner & Mothes, 1963), with carbon tetrachloride–acetic acid (99:1, v/v) or more simply pure carbon tetrachloride. In addition to the ferric chloride spray, followed by heating at 100° to develop the green colour, a diazo spray was used [10 ml. of 0·3% sulphanilic acid in N-HCl plus 0·6 ml. of 5% (w/v) $NaNO_2$, then 20 ml. of 10% (w/v) Na_2CO_3, used immediately]. This gave a red colour with cyclopenol (and viridicatol), but no colour with cyclopenin (or viridicatin).

The crude mixture of cyclopenin and cyclopenol (0·668 g.) was boiled with benzene (300 ml.) for 30 min., and then allowed to stand overnight. The benzene solution was filtered and the insoluble residue again subjected to the same treatment. The insoluble residue, m.p. 207°, was purified by repeated crystallization from ethyl acetate–benzene (1:40, v/v), giving chromatographically pure cyclopenol as prisms, m.p. 215° (yield 0·24 g.).

The combined benzene filtrates were evaporated, and the residual solid was extracted with carbon tetrachloride and allowed to stand overnight. It was filtered from an insoluble residue (m.p. 207°), then concentrated to about 5 ml. On treatment with light petroleum (b.p. 40–60°), crude cyclopenin was obtained as a white amorphous precipitate, which crystallized from ethyl acetate–light petroleum (b.p. 40–60°) in needles, m.p. 183–184° (yield 0·14 g.). When a spot of a solution of this product was applied to a filter paper and then sprayed with the above diazo spray, a faint red colour was obtained. The product was purified as follows:

Small amounts (20–30 mg.) were dissolved in acetone (2 ml.) and the solution was evenly distributed between two base-lines, 0·5 cm. apart and 44 cm. long, drawn 4 cm. from the end of a Whatman no. 1 paper sheet (50 cm. × 24 cm.). The paper was bent and clipped to form a cylinder and introduced into a chromatography tank containing carbon tetrachloride at the bottom. The solvent front was allowed to ascend about 18 cm., requiring about 3 hr. After drying, two 5 cm. strips were cut from either side of the paper and sprayed with the diazo spray. The faint red colour revealed on the chromatogram due to cyclopenol was found never to travel beyond 10 cm. from the starting line, whereas cyclopenin went almost with the solvent front. The area on the main chromatogram containing cyclopenin was allocated by holding the paper against a light source, when it appeared as a white band about 3 cm. wide. The paper containing this band was cut into small pieces and extracted with cold acetone, giving a gum which crystallized on treatment with a few drops of ethyl acetate. In this way crude cyclopenin (0·15 g.) applied to six papers yielded, after crystallization from ethyl acetate–light petroleum (b.p. 40–60°), pure cyclopenin (yield 0·11 g.) separating in long fine needles, m.p. 183–184°. A solution applied to a filter paper gave no colour when sprayed with the diazo spray.

Isolation of metabolites from Penicillium viridicatum *no. V* 41

Organism. The strain used was a single-spore culture obtained from *P. viridicatum*, LSHTM no. BBA/8, by the procedure described by Luckner & Mothes (1963).

Cultural conditions. A Raulin–Thom medium of the following modified composition was used: glucose, 50·0 g.; ammonium tartrate, 6·0 g.; tartaric acid, 0·5 g.; $MgSO_4,7H_2O$, 0·8 g.; K_2CO_3, 0·4 g.; $(NH_4)H_2PO_4$, 0·3 g.; $FeSO_4,7H_2O$, 0·05 g.; $ZnSO_4,7H_2O$, 0·05 g.; water, 1000 ml. This was distributed in 500 ml. amounts in Fernbach flasks, sterilized by steaming on 2 successive days, and sown with a suspension in water of spores obtained by growing the mould on a special mannitol–agar medium (Luckner & Mothes, 1963). Batches of 25 flasks were cultured for 14 days in the dark at 24°.

Isolation of cyclopenin and cyclopenol from the culture filtrate. The charcoal-adsorption method was used, as described by Bracken *et al.* (1954) for *P. cyclopium*, but the methanol eluate, after concentration to 100 ml., was passed through a column of alumina, and the mixture washed out with more methanol. The eluate was concentrated to about 10 ml., then separated by thin-layer chromatography.

The plates (8 cm. × 16 cm.) were prepared by spreading a suspension of Kieselgel G (Merck) in water (35 g. suspended in 150 ml. of water, then allowed to stand for 45 min.) and then drying at 100°. The methanol concentrate was applied to the plates in a line 1 cm. from the end. After drying, the plates were introduced into small cylinders containing a chloroform–methanol mixture (24:1, v/v), and the solvent was allowed to run for about 11 cm. from the starting line. The plates were then dried. One plate was sprayed with dilute aq. ferric chloride, then heated: the green bands indicated the positions of cyclopenin and cyclopenol (mean R_F values 0·40 and 0·15 respectively). The areas on the other plates containing the two compounds were removed separately and collected in two glass columns, each containing a small layer of alumina at the bottom, and eluted with methanol.

The dried cyclopenin fraction was repeatedly crystallized from ethyl acetate–light petroleum (b.p. 40–60°) to give needles, m.p. 183–184°, alone or mixed with cyclopenin from *P. cyclopium*.

The dried cyclopenol fraction was extracted with hot benzene, and the insoluble part then crystallized from ethyl acetate–benzene in prisms, m.p. 215°, alone or mixed with cyclopenol obtained from *P. cyclopium*.

Isolation of viridicatin and viridicatol from the mycelium. The mycelium from 25 flasks was pressed and then extracted with acetone. The extract was cooled, filtered and then passed through an alumina column, which was washed with methanol until no more colour was eluted. The adsorbed viridicatin and viridicatol were eluted with a mixture of methanol and 1N-HCl (1:1, v/v) until the eluate no longer gave a green colour with ferric chloride. Ascorbic acid (0·3 g.) was added to the green eluate until the green colour just disappeared (destruction of a heavy-metal complex). The solution was then concentrated under reduced pressure to about 100 ml. and cooled. The precipitated solid was filtered and dried (2·5 g.).

The crude mixture was dissolved in methanol (500 ml.) and applied to paper chromatograms (1·25 mg./strip), and developed with carbon tetrachloride–acetic acid (99:1, v/v) in tanks saturated with 50% (v/v) acetic acid (Luckner & Mothes, 1963). After 6 hr., the papers were dried: the areas on the chromatograms containing viridicatin and viridicatol showed as faint violet bands. They were cut off and extracted separately with methanol.

The dried viridicatin fraction was crystallized from ethanol and sublimed in a high vacuum, giving viridicatin, m.p. 268°, not depressed when mixed with an authentic specimen prepared by Bracken *et al.* (1954).

The dried viridicatol fraction was treated similarly. The sublimate, pure viridicatol, melted at 280°, alone or mixed with a specimen obtained by hydrolysis of cyclopenol (see below).

Cyclopenin

Properties. Cyclopenin readily crystallizes from ethyl acetate–light petroleum (b.p. 40–60°) in colourless needles, m.p. 183–184°; $[\alpha]_{5461}^{20} - 291°$ (*c* 1·2 in methanol) (Found: C, 69·4, 69·1; H, 4·7, 4·7; N, 9·5, 9·7. $C_{17}H_{14}N_2O_3$ requires C, 69·4; H, 4·8; N, 9·5%). It is soluble in carbon tetrachloride, benzene, chloroform, ethyl acetate or ethanol, and insoluble in light petroleum and water. It is insoluble in aq. $NaHCO_3$, but dissolves in dilute NaOH. It is insoluble in dilute mineral acids, but is slowly decomposed. In ethanolic solution it gives no immediate colour with ferric chloride, but a green colour develops slowly on standing at room temperature, or rapidly on heating.

Methylcyclopenin. A solution of cyclopenin (0·14 g.) in methanol (10 ml.) was treated with excess of ethereal diazomethane. The reaction mixture was kept overnight at room temperature, and was then concentrated under reduced pressure. The resulting gummy residue crystallized on the addition of ether. It was recrystallized from aq. ethanol giving *methylcyclopenin* as prisms, m.p. 207°, sintering at 200°. Methylcyclopenin is soluble in ethanol, chloroform or ethyl acetate, and insoluble in water (Found: C, 70·1; H, 5·2; N, 9·2. $C_{18}H_{16}N_2O_3$ requires C, 70·1; H, 5·2; N, 9·1%).

Hydrolysis of cyclopenin with 2N-*hydrochloric acid.* A suspension of cyclopenin (0·27 g.) in 2N-HCl (50 ml.) was heated on a boiling-water bath in a stream of CO_2- and

oxygen-free nitrogen. The evolved gases were passed through two bubblers each containing 25 ml. of 0·0875N-$Ba(OH)_2$. Heating was stopped after 3 hr., and the contents of the bubblers were titrated against 1·00N-HCl. The observed titration difference was 1·8 ml. of 1·00N-HCl, equivalent to 1·00 mole of CO_2/mole of cyclopenin.

The hydrolysis solution was kept overnight at 0°, and then the solid was filtered, washed with water and dried, m.p. 267° (yield 0·19 g., corresponding to 0·88 mole of viridicatin/mole of cyclopenin). The product was purified by crystallization from ethanol, separating in needles, m.p. 269–270°, not depressed when mixed with a sample of viridicatin obtained by Bracken *et al.* (1954).

The acidic filtrate, after separation of viridicatin, was evaporated to dryness under reduced pressure, leaving a crystalline residue (20 mg.). It was recrystallized from ethanol and a few drops of ether giving fine colourless needles, m.p. 229°, not depressed on admixture with authentic methylamine hydrochloride.

Cyclopenol

Properties. Cyclopenol crystallizes from ethyl acetate–benzene (1:40, v/v) in prisms, m.p. 215° (decomp.); $[\alpha]_{5461}^{20}$ −309° (*c* 1·3 in methanol) (Found: C, 66·3; H, 4·6; N, 9·1. $C_{17}H_{14}N_2O_4$ requires C, 65·8; H, 4·5; N, 9·0%). It is soluble in ethanol or hot ethyl acetate, sparingly soluble in chloroform, and insoluble in carbon tetrachloride, benzene or ether.

Other properties are as described above for cyclopenin.

Dimethylcyclopenol. A solution of cyclopenol (0·68 g.) in methanol (30 ml.) was treated with 1% ethereal diazomethane solution (100 ml.) and the reaction mixture left overnight. It was evaporated under reduced pressure to give a gum which crystallized on treatment with ether (yield 0·66 g.). Dimethylcyclopenol was recrystallized from ethyl acetate–light petroleum (b.p. 40–60°) in shining needles, m.p. 167–169° (Found: C, 67·4; H, 5·3; N, 8·5; O·CH_3, 9·2. $C_{19}H_{18}N_2O_4$ requires C, 67·5; H, 5·3; N, 8·3; 1 O·CH_3, 9·2%). It is soluble in ethyl acetate, chloroform, ethanol or acetone, and insoluble in light petroleum.

Hydrolysis of cyclopenol with 2N-hydrochloric acid. A suspension of cyclopenol (0·697 g.) in 2N-HCl (40 ml.) was hydrolysed as described for cyclopenin. CO_2 was evolved equivalent to 4·35 ml. of 1·00N-HCl (0·96 mole/mole of cyclopenol).

The hydrolysis solution was kept overnight at 0°, and the solid was filtered, washed and dried, m.p. 274° (yield 0·47 g., corresponding to 0·83 mole of viridicatol/mole of cyclopenol). Crystallization from ethyl acetate yielded viridicatol as needles, m.p. 280°, which could be sublimed in a high vacuum to remove traces of solvent.

The acid filtrate, after the separation of viridicatol, was repeatedly extracted with ether. The combined ethereal extracts were washed with $NaHCO_3$ solution, and dried. The gummy residue after evaporation of this extract gave a precipitate when treated with 2,4-dinitrophenylhydrazine, but gave a negative Schiff's test for aldehydes; no pure substance has so far been obtained from this fraction. The $NaHCO_3$ layer was acidified and extracted with ether. The ethereal extract on evaporation left a residue (20 mg.) which could be purified by recrystallization from boiling chloroform or by sublimation. It melted at 205°, alone or mixed with an authentic specimen of *m*-hydroxybenzoic acid; the infrared-absorption spectra in Nujol were also identical (Found: C, 60·8; H, 4·4; Calc. for $C_7H_6O_3$: C, 60·9; H, 4·3%).

The original reaction solution was finally evaporated to dryness under reduced pressure, leaving a solid residue (yield 0·17 g., corresponding to 1·1 mole of methylamine hydrochloride/mole of cyclopenol). It was recrystallized from ethanol in shining plates, m.p. 229°, alone or mixed with authentic methylamine hydrochloride.

Viridicatol

Properties. Viridicatol, obtained either from the mycelium of *P. viridicatum* or by hydrolysis of cyclopenol, can be crystallized from ethyl acetate, m.p. 280°, but for analysis it was sublimed in high vacuum to remove traces of solvent (Found: on sample from *P. viridicatum*: C, 70·8; H, 4·7; N, 5·5; on sample from hydroysis of cyclopenol: C, 71·4; H, 4·5; N, 5·4. $C_{15}H_{11}NO_3$ requires C, 71·1; H, 4·4; N, 5·5%). The ultraviolet-absorption spectrum in methanol resembles that of viridicatin: $\lambda_{max.}$ 226, 284, 304 (shoulder), 316, 329 (shoulder) mμ; log ϵ: 4·45, 3·95, 3·96, 4·04, 3·88 respectively. Infrared-absorption maxima in Nujol include: 1650 cm.$^{-1}$ (medium), 1635 cm.$^{-1}$ (strong), 3200 cm.$^{-1}$ (strong).

The compound is optically inactive. It is soluble in ethanol and acetone, sparingly soluble in ethyl acetate, and insoluble in benzene, chloroform, carbon tetrachloride, ether and water. It dissolves in dilute alkalies and is precipitated again on acidification, but is insoluble in $NaHCO_3$ solution. Its ethanolic solution gives with ferric chloride an intense green colour indistinguishable from that given by viridicatin.

Diacetylviridicatol. A solution of viridicatol (0·133 g.) in pyridine (1 ml.) was treated with acetic anhydride (1 ml.) and the reaction mixture was kept overnight at room temperature. It was then poured into cold water and allowed to cool. The separated crystals were filtered and recrystallized from ethyl acetate–light petroleum (b.p. 40–60°). *Diacetylviridicatol* separated in needles, m.p. 195–196° (Found: C, 67·4; H, 4·6; N, 4·4. $C_{19}H_{15}NO_5$ requires C, 67·7; H, 4·5; N, 4·2%). It is soluble in ethanol and ethyl acetate, but insoluble in water and light petroleum.

Alkaline oxidation of viridicatol. Method (*a*). A solution of viridicatol (50 mg.) in aq. 10% (w/v) KOH (5 ml.) was treated with 30% (w/v) hydrogen peroxide (0·5 ml.), and the reaction mixture was refluxed for 2 hr. After cooling, it was adjusted to pH 7 with dilute HCl, then extracted with chloroform. After evaporation of the chloroform, the residue was crystallized from carbon tetrachloride, giving *2-amino-3′-hydroxybenzophenone* as yellow needles, m.p.102°.

Method (*b*). Carbon dioxide-free oxygen was passed at room temperature through a solution of viridicatol (0·445 g.) in ethanolic 10% (w/v) KOH (30 ml.). After a few minutes, the colourless solution turned yellow, and the colour deepened with time. The reaction was stopped after 10 hr., when a sample no longer gave the green colour with ferric chloride. The solution was then diluted with water, and ethanol was removed under reduced pressure. The residual solution was made slightly acid with dilute HCl, then neutralized with $NaHCO_3$ until a yellow precipitate appeared. The mixture was extracted with ether, and the ethereal extract was washed with a little water and dried. On evaporation of the solvent, a yellow gum was obtained (yield 0·298 g.) which crystallized on treatment with carbon tetrachloride. Recrystallization from carbon tetrachloride

and a few drops of chloroform yielded 2-amino-3′-hydroxybenzophenone as yellow needles, m.p. 102°.

The original reaction mixture, after removal of the amine, was again acidified, and extracted with ether. On evaporation of the extract, a crystalline residue (yield 0·12 g.) was obtained. It was purified by crystallization from ethyl acetate–light petroleum (b.p. 40–60°), from which *2-oxalylamino-3′-hydroxybenzophenone* separated in needles, m.p. 205° (decomp) (Found: C, 63·0; H, 4·1; N 5·5. $C_{15}H_{11}NO_5$ requires C, 63·2; H, 3·9; N, 4·9%). It is soluble in ethanol, ethyl acetate and ether, but insoluble in water. It dissolves in $NaHCO_3$ solution, but not in dilute acids. Its ethanolic solution gave no colour with ferric chloride. On paper, it gives a yellow spot with the diazo spray.

Properties of 2-amino-3′-hydroxybenzophenone. 2-Amino-3′-hydroxybenzophenone separates from carbon tetrachloride in yellow needles, m.p. 102° [Found: on sample obtained by method (*a*): C, 73·8; H, 5·5; N, 6·2; on sample obtained by method (*b*): C, 73·0; H, 5·6; N, 6·9. $C_{13}H_{11}NO_2$ requires C, 73·3; H, 5·2; N, 6·6%]. Infrared-absorption maxima in Nujol include: 1620 cm.$^{-1}$ (strong, bonded C=O), 1640 cm.$^{-1}$ (weak, NH), 3315 cm.$^{-1}$ (medium, NH_2), 3380 cm.$^{-1}$ (medium, NH_2), 3610 cm.$^{-1}$ (unbonded OH). The C=O absorption for 2-aminobenzophenone is at 1630 cm.$^{-1}$, that for 2-amino-4′-hydroxybenzophenone (Ullmann & Bleier, 1902; Stoermer & Gaus, 1912) at 1610 cm.$^{-1}$.

The compound is soluble in ethanol, ethyl acetate, benzene and chloroform, but sparingly soluble in carbon tetrachloride. It dissolves in dilute mineral acids and in dilute NaOH, but not in aqueous $NaHCO_3$. It can be extracted from its solution in acid slowly by ether. It gives no colour with ferric chloride, but the phenolic group is demonstrated on paper by coupling reactions with diazotized benzidine (brown) or sulphanilic acid (yellow), and by reduction of the Folin–Ciocalteu reagent. It also gives a yellow colour with the *p*-dimethylaminobenzaldehyde reagent (Luckner, 1963), typical of an aromatic amine.

Alkaline hydrolysis of 2-oxalylamino-3′-hydroxybenzophenone. A solution of 2-oxalylamino-3′-hydroxybenzophenone (0·1 g.) in aq. 10% (w/v) NaOH (20 ml.) was heated under reflux for 3 hr. After cooling, the alkaline solution was made slightly acid and then neutralized with $NaHCO_3$, and extracted with ether. The gum obtained (yield 70 mg.) was crystallized from carbon tetrachloride and a few drops of chloroform, giving yellow needles, m.p. 102°, alone or mixed with the previously isolated 2-amino-3′-hydroxybenzophenone.

Conversion of 2-amino-3′-hydroxybenzophenone into 2-hydroxyfluorenone. A solution of 2-amino-3′-hydroxybenzophenone (0·556 g.) in ethanol (15 ml.) was treated with conc. HCl (14 ml.) and water (50 ml.), and then the mixture was cooled to 0° in an ice bath. Sodium nitrite (1 g.) was added to the cold solution in small amounts, with stirring, during a period of 30 min. The diazonium salt solution was added in portions to boiling ethanol (100 ml.) and finally the mixture was refluxed for 2 hr. The ethanol was then removed under reduced pressure, giving an orange–red precipitate. After cooling, it was filtered (0·37 g., 73% yield). Crystallization from dilute acetic acid gave needles, which were repeatedly sublimed in a high vacuum, giving a dark-red powder, m.p. 211°, alone or mixed with a specimen of 2-hydroxyfluorenone synthesized from fluorenone (Diels, 1901) (Found: C, 79·5; H, 4·3. Calc. for $C_{13}H_8O_2$: C, 79·0; H, 4·2%).

The small residue left after sublimation had an orange colour and m.p. 245°. It gave a red colour on treatment with alkali, as does 2-hydroxyfluorenone. 4-Hydroxyfluorenone has m.p. 250–251° (Huisgen & Rist, 1955).

Cleavage of 2-amino-3′-hydroxybenzophenone. A solution of the amine (1–2 mg.) in aq. 50% (w/v) zinc chloride (0·1 ml.) was heated in a small sealed tube for 6 hr. at 200°. After cooling, the contents of the tube were made alkaline with NaOH, then extracted with chloroform. The chloroform extract was concentrated to about 1 ml. Then in 0·2 ml. amounts it was chromatographed, with the carbon tetrachloride system or the cyclohexane–methanol system (Luckner & Mothes, 1963). The spots revealed after spraying with *p*-dimethylaminobenzaldehyde (Luckner, 1963) had R_F 0·26 and R_F 0·23 respectively in the two systems, and were identical with those of aniline run under the same conditions. Controls of *o*-, *m*- and *p*-aminophenol applied on the chromatograms showed the absence of such compounds in the hydrolytic product.

Cleavage of 2-aminobenzophenone, and of 2-amino-4′-hydroxybenzophenone, also showed the presence of aniline.

SUMMARY

1. The product cyclopenin, isolated from a strain of *Penicillium cyclopium* Westling by Bracken, Pocker & Raistrick (1954), has been shown to be a mixture of two closely related compounds, and these have been separated and characterized.

2. For one of these, $C_{17}H_{14}N_2O_3$, m.p. 183–184°, $[\alpha]_{5461}^{20} - 291°$, the name cyclopenin is retained, since it has the empirical formula and hydrolysis products described by Bracken *et al.* (1954).

3. The second compound, $C_{17}H_{14}N_2O_4$, m.p. 215° (decomp.), $[\alpha]_{5461}^{20} - 309°$, is named cyclopenol.

4. Cyclopenin and cyclopenol are also produced by culture filtrates of a strain of *Penicillium viridicatum* Westling, from the mycelium of which are obtained two corresponding metabolites: viridicatin, $C_{15}H_{11}NO_2$ (3-hydroxy-4-phenyl-2-quinolone), a known metabolite of this species and of *P. cyclopium*; and viridicatol, $C_{15}H_{11}NO_3$, m.p. 280°.

5. Acid hydrolysis of cyclopenol yields nearly 1 mol.prop. each of carbon dioxide, methylamine and viridicatol, and a little *m*-hydroxybenzoic acid.

6. Viridicatol undergoes alkaline oxidation to an amine, $C_{13}H_{11}NO_2$, which is converted by diazotization and cyclization into 2-hydroxyfluorenone.

7. It is concluded that the amine is 2-amino-3′-hydroxybenzophenone, and that viridicatol is 3-hydroxy-4-(3-hydroxyphenyl)-2-quinolone.

8. The biogenetic significance of this structure is discussed.

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Insulin and Incorporation of Amino Acids into Protein of Muscle

2. ACCUMULATION AND INCORPORATION STUDIES WITH THE PERFUSED RAT HEART*

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Kipnis & Noall (1958) found that insulin *in vitro* stimulated the accumulation of the non-utilizable amino acid, α-aminoisobutyric acid, by isolated rat diaphragm. Their discovery suggested that an action of insulin to promote the accumulation of amino acids might be the means by which the hormone enhances the incorporation of amino acids into protein in this tissue (Sinex, MacMullen & Hastings, 1952; Krahl, 1953). However, the extent to which insulin does in fact influence the intracellular accumulation of amino acids is still uncertain. Manchester & Young (1960) found insulin to be without effect on the uptake of alanine, leucine, phenylalanine, lysine, ornithine, aspartic acid and glutamic acid under the conditions in which it increased that of aminoisobutyric acid, although it did stimulate the uptake of glycine. Guroff & Udenfriend (1961) were unable to find a stimulation by insulin of the uptake of tyrosine or of another non-utilizable α-methyl amino acid, α-methyltyrosine. Akedo & Christensen (1962) have demonstrated an enhancement by insulin of the accumulation of sarcosine, L- and D-isovaline, cycloleucine, methionine and proline, though not of serine, valine, histidine or norleucine. Thus there is little support for the view that insulin promotes amino acid incorporation, an effect observable for every amino acid studied (Manchester & Young 1958; Wool & Krahl, 1959), through the stimulation of accumulation. However, the usefulness of experiments of this sort has been questioned by Kipnis, Reiss & Helmreich (1961). Because they were unable to find an increase in the rate of incorporation of [^{14}C]proline into diaphragm protein as its specific radioactivity in the cell amino acid pool rose, they suggested that the pool from which amino acids are taken for protein synthesis is more limited and not congruous with the total measurable amino acid pool. The lack of an effect of insulin on the entry of amino acids into the total measurable pool would not preclude an effect of the hormone on entry into a more limited fraction. However, the significance of the observation of Kipnis *et al.* (1961) could be simply that the specific radioactivity of ^{14}C-labelled amino acids penetrating into the cell rises to its maximum value in the region of the cell membrane and the ribosomes of the sarcoplasmic reticulum much more rapidly than in the total amino acid pool of the tissue which includes amino acids dissolved in water associated with the myofibrils.

In each instance cited the tissue studied has been the isolated rat diaphragm, either the cut or intact preparation (Kipnis & Cori, 1957). To both prepara-

* Part 1: Manchester (1961).