## 147. *The Chemistry of Bacteria. Part VII.* The Structure of Violacein.*

By J. A. Ballantine, C. B. Barrett, R. J. S. Beer, Stephen Eardley, Alexander Robertson, B. L. Shaw, and T. H. Simpson.

Examination of a methylation product of violacein suggests two possible molecular formulæ for the pigment, namely $C_{21}H_{15}O_3N_3$ and $C_{20}H_{13}O_3N_3$. The latter formula offers the more satisfactory explanation of the behaviour of violacein with alkaline reagents and leads to structure (IV). Other structures, based on the $C_{21}$ formula, are briefly discussed.

Violacein, the violet pigment of *Chromobacterium violaceum*, is also produced by a marine bacterium, isolated from the skin slime of iced North Sea Cod and provisionally designated *Chromobacterium*, Strain B.C.1. This organism differs from *Chromobacterium violaceum* morphologically, in its behaviour towards antibiotics, and in being chitinivorous.† Recognition of the identity of the pigments from the two sources has been facilitated by the discovery that violacein can be methylated with methyl sulphate and potassium carbonate, giving a blue highly crystalline (and easily crystallised) compound, $C_{23}H_{18}O_2N_3{\cdot}OMe$ or $C_{22}H_{16}O_2N_3{\cdot}OMe$. The formation of this derivative must involve the introduction of at least three methyl groups, since on degradation with alkali and zinc dust it gave the trimethyl derivative (I; R = Me) of the $C_{20}$ acid, γ-(5-hydroxy-3-indolyl)-α-(3-oxindolyl)-γ-oxobutyric acid, previously reported,[1,2] and on being pyrolysed furnished

* Part VI, *J.*, 1957, 4810.

† We are grateful to Dr. J. H. Shewan of the Torry Research Station, Aberdeen, for supplying this information.

[1] Ballantine, Barrett, Beer, Boggiano, Clarke, Eardley, Jennings, and Robertson, *J.*, 1957, 2222.

[2] Barrett, Beer, Dodd, and Robertson, *J.*, 1957, 4810.

5-methoxy-1-methylindole and 1-methyloxindole. On the basis of this evidence there are two possible molecular formulæ for the parent pigment, namely, $C_{21}H_{15}O_3N_3$ and $C_{20}H_{13}O_3N_3$, neither of which is inconsistent with the rather erratic analytical data obtained with violacein samples. At present these data are of uncertain value because the insolubility of the pigment and its tendency to retain solvents, *e.g.*, pyridine, make absolute purification difficult. From the results so far obtained in the degradation of violacein it appears clear that a $C_{20}$ or $C_{21}$ empirical formula is to be preferred to the formula, $C_{42}H_{28}O_7N_6$, suggested by Wrede and Swane.[3] By the magnesium iodide method partial demethylation of the methylated pigment gave a product which, although it could not be satisfactorily purified, afforded crystalline acetyl and benzoyl derivatives. The analyses of these derivatives, however, do not serve to distinguish between the $C_{20}$ and the $C_{21}$ formula.

An important feature in the chemistry of violacein is the behaviour of the compound with alkaline reagents, a preliminary account of which has been given by Wrede and Swane.[3] According to these authors violacein dissolves in aqueous sodium hydroxide, giving an intense emerald-green solution which soon becomes red, a change much accelerated on warming. From this solution a red solid (A) is precipitated by saturation with carbon dioxide, and on acidification with mineral acid the filtrate gives an unstable yellow solid (B) which darkens rapidly. Wrede and Swane considered solids (A) and (B) to be very closely related to violacein, claiming that both regenerate violacein with hot pyridine and give the acetyl derivative of the pigment on acetylation.

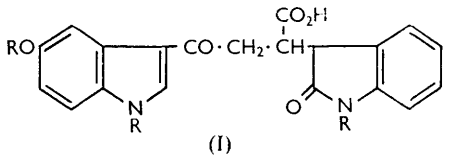
We have confirmed the production of compounds (A) and (B) from violacein but, whereas an examination of (A) supports Wrede and Swane's view that it is a modified form of violacein, a study of the yellow solid (B) indicates that it is a degradation product. Thus, on acetylation, the compound (B) gives a product which superficially resembles the acetyl derivative of violacein but has a different infrared absorption and a lower nitrogen content. This acetate was found to be identical with the " red lactone " [1] derived from the $C_{20}$ acid and therefore has structure (II; R = Ac). As the substance (B) is *not* precipitated from the alkaline reaction mixture with carbon dioxide it may reasonably be regarded as a carboxylic acid. The conditions under which (B) is formed differ from those which give rise to the $C_{20}$ acid only in the absence of zinc, a reducing agent. It seems probable that compound (B) is an oxidised form of the $C_{20}$ acid, *viz.*, (III; R = H), a structure which accounts for the colour of (B) and for its instability, since compounds of this type are very easily converted into unsaturated lactones.[2] The darkening of compound (B) may be attributed to the formation of the lactone (II; R = H). On this account it has not been possible to purify compound (B), and the almost black solid (C), presumably (II; R = H) derived from it by the action of dilute mineral acid, has yielded only minute amounts of crystalline material. Nevertheless, a number of transformations have been carried out which support the above views. Thus, as expected, reduction of compound (B) with zinc and alkali affords the $C_{20}$ acid. On acetylation, material (C) is converted into the " red lactone " (II; R = Ac) and on methylation into the " magenta lactone " [1] (II; R = Me) which was also obtained from the methyl derivative of violacein by degradation with alkali and treatment of the resulting unstable yellow product, presumably (III; R = Me), with dilute aqueous acid.

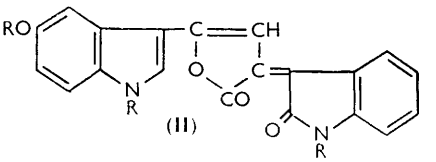
The formation of the yellow products (III; R = H and Me) by the relatively mild treatment of violacein and its methyl derivative respectively with alkali is explained on the assumption that violacein has the molecular formula, $C_{20}H_{13}O_3N_3$. The pigment must then have structure (IV) which would be expected to be alkali-sensitive. This structure also readily accounts for the formation of the $C_{20}$ acid and ammonia by reductive hydrolytic fission of violacein.

On the other hand structures based on a $C_{21}H_{15}O_3N_3$ formula, *e.g.*, (V) and (VI), do not appear to offer a satisfactory explanation of the degradation reactions discussed above.

[3] Wrede and Swane, *Arch. exp. Pathol. Pharmakol.*, 1937, **186**, 532.

Whilst a compound of formula (V) might conceivably yield a 3-acylindole on alkaline hydrolysis, the generation of the carboxyl group in compounds (III; R = H and Me) and in the $C_{20}$ acid presents difficulties. Stoicheiometric considerations indicate that the carbon atom which is lost would separate as methanol but attempts to isolate this unit


(I)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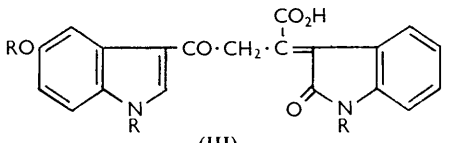
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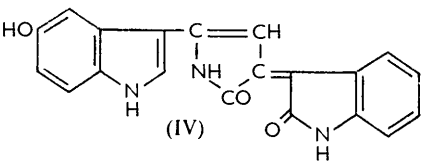
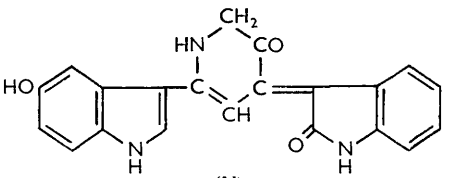
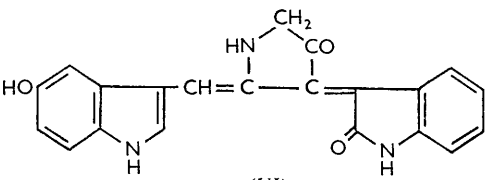

(IV)


(V)


(VI)

from the hydrolysate have so far been unsuccessful. The structure (VI), which was originally proposed to account for the formation of a supposed indolylpyrrylmethene [4] from acetylviolacein by the action of hydriodic acid, does not explain the formation of either the carbonyl or the carboxyl group in the alkaline degradation products, and structures of the trisubstituted methene type referred to in Part III [4] are similarly excluded. Clearly structure (IV), which is otherwise acceptable, could not give rise to an indolylpyrrylmethene, and it therefore seems necessary to re-examine this hydriodic acid degradation product.

In connection with the isolation of violacein from *Chromobacterium*, B.C.I. it may be noted that one of us (A. R.) in collaboration with Dr. T. S. Subramaniam (lately of this Department) isolated in 1939 a pigment from *Chromobacterium janthinum* which appeared to be closely related to, if not identical with, violacein (the compounds had identical ultraviolet absorption spectra), a result in agreement with the view that biologically *Chr. janthinum* is very similar to *Chr. violaceum*.[5]

## Experimental

(With B. G. Boggiano, K. Clarke, and T. S. Subramaniam.) *Violacein from* Chromobacterium violaceum.—The organism grows and pigments well at room temperature on a solid medium containing agar (1·8—2·0%), glycerol (2%), and proteose peptone (Difco; 1%) made up in tap-water. For large-scale cultivation, this medium was supported on enamel trays (20″ × 15″); inoculation was effected with sterile cotton-wool plugs from vigorous cultures growing on a similar medium contained in glass casseroles. After 12—14 days the bacterial mass was scraped from the surface of the medium, spread on filter-paper, and allowed to dry. The resulting brittle, almost black product was milled and then extracted (Soxhlet) with acetone, the pigment separating, usually highly crystalline. Impurities were removed from the crystalline solid by washing it with water and repeatedly extracting it with hot chloroform.

[4] Beer, Jennings, and Robertson, *J.*, 1954, 2679.
[5] Topley and Wilson, " Principles of Bacteriology and Immunity," Arnold, London, 1943, p. 497.

Purified for analysis by repetition of the acetone-extraction process, violacein formed almost black prisms with a green sheen (Found: C, 69·3, 69·0, 69·5; H, 3·8, 4·1, 4·3; N, 11·7, 11·1; O, 15·0. Calc. for $C_{21}H_{15}O_3N_3$: C, 70·6; H, 4·2; N, 11·8; O, 13·4. Calc. for $C_{20}H_{13}O_3N_3$: C, 70·0; H, 3·8; N, 12·2; O, 14·0. Calc. for $C_{20}H_{13}O_3N_3,\frac{1}{2}C_3H_6O$: C, 69·3; H, 4·3; N, 11·3; O, 15·1%). Repeated crystallisation from aqueous pyridine gave the *pyridine complex* in violet needles (Found: C, 70·7; H, 4·3; N, 13·0, 12·2. $C_{20}H_{13}O_3N_3,C_5H_5N$ requires C, 71·1; H, 4·3; N, 13·3%). 80 Well-pigmented trays gave approximately 8 g. of crystalline violacein and 3·5 g. of less pure amorphous material isolated from the acetone filtrate.

*Isolation of Pigment from* Chromobacterium B.C.1.—This marine *Chromobacterium*, strain B.C.1., was isolated from the skin slime of iced cod from the North Sea and although similar to *Chromobacterium violaceum* (NCTC 7917) it differed in the respects tabulated.

| Test | *Chromobacterium* B.C.1 | *Chromobacterium violaceum* |
|---|---|---|
| Chitin | Digested | No action |
| Milk | Made slightly alkaline; clotted | Completely peptonised |
| Flagella | Peritrichous | Single polar |
| Antibiotics | (*a*) Not inhibited by 10 m.c.g. erythromycin tablets | Inhibited by 1 and 10 m.c.g. erythromycin tablets |
| | (*b*) Pigmentation suppressed by terramycin | Pigmentation unaffected by terramycin |

Growth and pigmentation of the organism were good only under strongly aerobic conditions and consequently were poor in fluid culture. Originally it was grown on nutrient agar containing considerable quantities of chitin, but later satisfactory growth with good pigmentation was obtained on " Oxoid " nutrient agar containing 1% of glucose. This medium was supported on enamel trays (16″ × 13″) and was surface-inoculated from liquid cultures. Pigmentation was maximal after incubation at 25° for 10—14 days. The mass of bacterial cells was scraped from the agar surface and defatted by extraction (Soxhlet) with light petroleum (b. p. 60—80°) and the pigment then extracted with hot acetone. Evaporation of the extract left the compound as a blue-black microcrystalline powder which was purified by repeatedly washing it with hot water and hot light petroleum until the washings were colourless (yield, *ca.* 80 mg. per tray). This pigment had the same properties as violacein and by a comparison of visible, ultraviolet, and infrared absorption spectra the compound was shown to be identical with the pigment from *Chromobacterium violaceum*.

*Methylation of Violacein.*—(*a*) A mixture of violacein (5·0 g. from *Chromobacterium*, Strain B.C.1.), methyl sulphate (10 ml.), an excess of potassium carbonate, and acetone (500 ml.) was heated under reflux for 8 hr., then filtered (wash with acetone), and the combined filtrate and washings were concentrated in a vacuum to a small volume. The residue was agitated overnight with aqueous sodium hydrogen carbonate to decompose unchanged methyl sulphate, and the residual blue solid isolated, dissolved in benzene, and purified by chromatography on aluminium oxide. Evaporation of the benzene eluate left the *methylated pigment* which crystallised from alcohol in blue needles (4 g.), m. p. 218° (Found: C, 72·1; H, 5·3; N, 10·4; OMe, 8·0%; *M*, 380. $C_{23}H_{18}O_2N_3$·OMe requires C, 72·2; H, 5·3; N, 10·5; OMe, 7·8%; *M*, 399. $C_{22}H_{16}O_2N_3$·OMe requires C, 71·7; H, 5·0; N, 10·9; OMe, 8·1%; *M*, 385).

(*b*) Application of the same methylation procedure to violacein (200 mg.) from *Chromobacterium violacein* followed by recrystallisation of the product from benzene gave blue needles (150 mg.), m. p. 127°, containing solvent of crystallisation; the unsolvated *compound* separated from ethyl acetate in blue needles, m. p. 220°, $\lambda_{max.}$ (in EtOH) 270, 376, 580 mμ (log ε 4·31, 3·88, 4·29), $\lambda_{min.}$ 243, 333, 440 mμ (log ε 4·26, 3·65, 3·34) (Found: C, 71·8, 72·0; H, 5·1, 5·2; N, 10·4, 10·7; OMe, 8·2, 7·8%; *M*, 405), and was shown by mixed m. p. determination and by comparison of visible, ultraviolet, and infrared absorption spectra to be identical with the methylated pigment, m. p. 218°, from (*a*). Methylated violacein is readily soluble in chloroform or benzene, moderately soluble in alcohol, and insoluble in aqueous sodium hydroxide. It did not form an oxime and was recovered in good yield after being heated with acetic anhydride and sodium acetate under reflux for ½ hr.

A mixture of methylated violacein (100 mg.), in alcohol (15 ml.), 2N-aqueous sodium hydroxide (15 ml.), and zinc dust (200 mg.) was heated under reflux for 1 hr., filtered, and acidified, giving the trimethyl derivative [1] of the $C_{20}$ acid (60 mg.), m. p. 260°, which crystallised from acetone in prisms, m. p. and mixed m. p. 267°.

*Partial Demethylation of Methylated Violacein.*—A solution of methylated violacein (55 mg.)

in benzene was added to ethereal magnesium iodide (freshly prepared from 500 mg. of iodine and an excess of magnesium), and the mixture heated under reflux for ½ hr. After evaporation of the solvents in a vacuum the residual green-red complex was heated in a vacuum at 150° for 1 hr. and the residue decomposed with dilute acetic acid. The resulting blue solid did not crystallise satisfactorily and was converted by acetic anhydride–pyridine into the *acetyl derivative* which after purification by chromatography on aluminium oxide from chloroform–benzene crystallised from alcohol and then light petroleum (b. p. 100—120°) in dark purple needles (25 mg.), m. p. 219—221° (Found: C, 70·3; H, 5·0; N, 9·8. $C_{21}H_{12}O_3N_3Me_2$·COMe requires C, 70·2; H, 5·0; N, 9·8. $C_{20}H_{10}O_3N_3Me_2$·COMe requires C, 69·7; H, 4·6; N, 10·2%). Prepared by the benzoyl chloride–pyridine method, the *benzoyl derivative* of the partially demethylated product separated from acetone–light petroleum (b. p. 60—80°) in dense, dark blue needles, m. p. 241—243° (Found: C, 73·6; H, 4·7; N, 8·7. $C_{30}H_{23}O_4N_3$ requires C, 73·6; H, 4·7; N, 8·6. $C_{29}H_{21}O_4N_3$ requires C, 73·3; H, 4·5; N, 8·8%).

Methylation of the partially demethylated material by the standard method regenerated methylated violacein, m. p. and mixed m. p. 215—218°.

*Pyrolysis of Methylated Violacein.*—An intimate mixture of methylated violacein (1·5 g.) and zinc dust (15 g.) was heated to 300—310° under reduced pressure in a slow stream of carbon dioxide, and the oily distillate (180 mg.) thus obtained was purified by chromatography on aluminium oxide. From this 5-methoxy-1-methylindole (39 mg.), m. p. and mixed m. p. 104—105°, was eluted with ether–light petroleum (b. p. 40—60°), a trace of 5-methoxyindole with ether, and then 1-methyloxindole (109 mg.), m. p. and mixed m. p. 85—87°, with ether–chloroform (100 : 1).

*Degradation of Violacein with Alkali.*—Violacein (500 mg.) was heated with 0·1N-aqueous sodium hydroxide (70 ml.) in nitrogen under reflux for 35 min. The undissolved violacein (*ca.* 50 mg.) was removed by filtration and the solution then heated for a further 5 min., cooled, and saturated with carbon dioxide (by addition of " dry ice "). The precipitated " red solid " (A) (150 mg.) was collected and on acidification with 2N-hydrochloric acid the filtrate gave a bright yellow solid (B) (150 mg.) which was thoroughly washed with water (in contact with acid, this yellow solid rapidly became green and eventually almost black) and dried. Longer interaction of violacein with alkali gave relatively less " red solid " and more " yellow solid."

*Reactions of the Yellow Solid* (B).—When kept at room temperature the solution obtained by heating the yellow solid (B) (170 mg.) under reflux with acetic anhydride (15 ml.) and sodium acetate (200 mg.) for 10 min. slowly deposited a red crystalline product, m. p. *ca.* 280°, having an infrared absorption spectrum identical with that of the " red lactone " [1] derived from the $C_{20}$ acid. Treated with zinc dust and hot alkali, the yellow solid (B) was converted into the $C_{20}$ acid, m. p. and mixed m. p. *ca.* 245°, having the requisite infrared absorption spectrum.

With warm dilute hydrochloric acid the solid (B) was converted into an almost black solid (C) which was extracted (Soxhlet) with ethyl acetate and partially purified by chromatography from the same solvent on silica gel. The resulting dark amorphous product gave wine-red or purple solutions, differing from those given by violacein. A solution of the *substance* in acetic acid deposited a small quantity of very dark plates with a green sheen, m. p. *ca.* 330° (Found: N, 7·9. $C_{20}H_{12}O_4N_2$ requires N, 8·1%).

*Reactions of Solid* (*C*).—The partly purified solid (C) (200 mg.) was mixed intimately with potassium carbonate and heated under reflux with acetone (30 ml.) and methyl sulphate (1·0 ml.) for 17 hr. The filtered solution was concentrated, the sticky residue was triturated with water, and the crude product was isolated with benzene and chromatographed from this solvent on silica. Eluted with benzene containing a little ethyl acetate and then recrystallised from benzene, the product formed dark needles with a green reflux (50 mg.), m. p. 268°, identical with the " magenta lactone " [1] and having the requisite infrared absorption spectrum. Heated with acetic anhydride and sodium acetate, the solid (C) gave the " red lactone " [1] in dark red needles, m. p. and mixed m. p. 282°.

*Reactions of the Red Solid* (*A*).—On being heated with dilute aqueous sodium hydroxide under reflux for 1 hr., the red solid (A) (450 mg.) was partially converted into the yellow solid (B) (200 mg.) with some unchanged starting material (*ca.* 100 mg.). With methyl sulphate and potassium carbonate in boiling acetone, the red solid (A) (150 mg.) gave the methyl derivative of violacein (60 mg.), m. p. and mixed m. p. 220°; more methyl derivative (40 mg.) was obtained by further methylation of an acetone-insoluble salt-like by-product which had been digested with dilute aqueous acid.

*Degradation of Methylated Violacein with Alkali.*—The solution formed by heating the methyl derivative of violacein (500 mg.) with acetone (150 ml.) and N-aqueous sodium hydroxide (150 ml.) in nitrogen for 1 hr. was filtered, cooled, and acidified, giving a yellow solid. This mixture was warmed and the resulting dark solid (400 mg.) collected and dried. On purification by chromatography from benzene on silica and then by crystallisation from benzene this gave the " magenta lactone " [1] in dark needles with a green sheen (100 mg.), m. p. and mixed m. p. 266°, having the requisite infrared absorption spectrum (Found: C, 71·4; H, 4·8; N, 7·6. Calc. for $C_{23}H_{18}O_4N_2$: C, 71·5; H, 4·7; N, 7·3%).

UNIVERSITY OF LIVERPOOL.
TORRY RESEARCH STATION, ABERDEEN.

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