A NEW PRODIGININE (PRODIGIOSIN-LIKE) PIGMENT FROM STREPTOMYCES. ANTIMALARIAL ACTIVITY OF SEVERAL PRODIGININES

NANCY N. GERBER

Waksman Institute of Microbiology, Rutgers University,
The State University of New Jersey, New Brunswick, New Jersey 08903, U.S.A.

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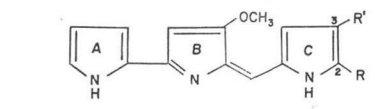
Two prodigiosin-like pigments from *Streptomyces* sp. were shown to be undecylprodiginine (**I**) and butylcycloheptylprodiginine (**V**). The antimalarial activity of five prodiginine pigments is given.

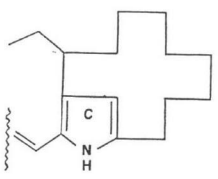
Several new prodiginine (prodigiosin-like) pigments have been isolated in recent years[1-3]. The antimicrobial activity of some prodiginines has been reported[1,4] as well as the antimalarial activity of prodigiosin itself.[5] In this report we describe the isolation and structure proof of another novel prodiginine molecule and summarize the results from the antimalarial testing of five prodiginines.

Streptomyces sp. Y-42 was isolated from leaf and grass compost, maintained on yeast-dextrose slants and, on routine transfer, seen to have one red colony. This strain was extremely erratic and variable in pigment production. Early work with *S.* sp. Y-42 was also complicated by the simultaneous production of a hydroxyquinone type of pigment. It was red in acid, blue in base, diffused in agar, and found in broth and cells. Prodiginine pigments are red in acid, yellow in base, do not diffuse in agar and occur in cells only. The two pigments could be separated by column chromatograpy but the similarity of the visible absorption maxima in acid solution (525 and 530 nm) made the usual spectrophotometric assay for prodiginine pigments impossible. Selection experiments, which involved plating out *S.* sp. Y-42 and picking the most desirable single colonies, furnished a substrain which reliably gave prodiginine pigments free of hydroxyquinone. Thin-layer chromatography (TLC) of solvent extracts of red cells of *S.* sp. Y-42 disclosed two prodiginine pigments: A, a faster moving, elongated, orange-pink spot and B, a slower moving, compact, true pink spot. The preparation and purification of these two pigments is given in the experimental section.

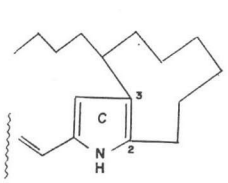
Fig. 1. Structures of some prodiginine pigments.



I R = $-(CH_2)_{10}CH_3$, R' = H
II R = $-(CH_2)_8CH_3$, R' = H
III R = CH_3; R' = $-(CH_2)_4CH_3$



IV



V

Pigment A was identical in its visible absorption maxima (525 nm in acid ethanol, 528 nm in acid chloroform) and TLC behavior with authentic samples of nonylprodiginine (**II**) and undecylprodiginine (**I**) which cannot be differentiated by these techniques. Pigment A was conclusively identified as undecylprodi-

ginine (**I**) by its mass spectrum ($M^+=393$) and by chromic acid oxidation to undecanoic acid.[8]

The TLC behavior of pigment B most nearly resembled that of authentic samples of prodigiosin (**III**) and metacycloprodigiosin (**IV**)[2] but the spot was slightly behind that of **IV** and ahead of **III**. The mass spectrum of B disclosed a strong molecular ion at 391 mass units, thus like **IV**, B had an eleven carbon aliphatic chain. The mass spectrum of B was, however, clearly different from that of **IV**. Since the visible absorption maxima of B (537 nm in acid ethanol, 542 in acid chloroform) were identical with those of **III** and different from **IV**, it was surmised that the aliphatic chain was attached to the aromatic nucleus at carbons 2 and 3 of ring C as for **III**. The nmr spectrum verified this feeling since a strong singlet at δ 6.38, characteristic of H at carbon C-4, was observed. The range for H at carbon C-3 is δ 5.6~5.9[6] in 3 examples. The nmr spectrum also showed $ArC\underline{H}_2$ at δ 2.3 (2H) and ArCH at δ 3.1 (1H) which indicated branching at one of the aliphatic carbons alpha to the pyrrole ring. The single terminal aliphatic methyl band at δ 0.9 was a very unsymmeterical triplet which indicated that the chemical shift of the hydrogens in the adjacent methylene group was close to δ 0.9. Thus the alkyl substituent on the aliphatic ring must be ethyl or larger.

In order to decide which of the aliphatic carbons (in addition to 1′) was linked to the pyrrole ring chromic acid oxidation was used. This degradation destroys all the pyrrole rings but leaves the aliphatic chain unaltered except where it has been bonded. The mono- and dibasic acids as well as ketoacids obtained from the oxidation of various prodiginine pigments have been reported.[8] The acids were examined in the form of their methyl esters by gas chromatography (GC). Pigment **IV**, of known structure, was selected as a model for investigation. From **IV** the two major aliphatic acids obtained were α-ethylundecanedioic acid and 10-oxododecanoic acid. A lesser amount of α-ethyldecanedioic acid was obtained. The structures were deduced by: (1) comparison of the retention times with those of authentic unbranched dibasic esters, with authentic 10-oxoundecanoic acid and with the products from the oxidation of other known prodiginine pigments. (2) The method of ester exchange which clearly differentiates between mono- and dibasic esters including ketoesters.[8] (3) Treatment with dilute alkaline permanganate for several days at 28°C followed by reesterification which destroys peaks due to ketoesters.

Pigment B gave major amounts of an α-branched 12 carbon diacid and an 11 carbon ketoacid with minor amounts of the α-branched 13 carbon diacid. When the ketoacid was removed by alkaline permanganate treatment it was possible to prepare by GC samples of the two branched diesters for mass spectroscopy. Because of their lesser volatility the dipentylesters were used. The mass spectra verified them as dipentyl esters of 12 and 13 carbon diacids. The small molecular ions at 370 and 384 nm were clearly visible. Both esters showed M-butyl, that is M-56, due to cleavage with rearrangement of a bond β to the carbonyl group. These peaks were 42% and 20% respectively of the base peaks which were from cleavage of the α linkage (M-115) in both cases. In comparison, the mass spectrum of the unbranched, dipentyl undecanedioate showed a small molecular ion at 356; the base peak was M-87 (M-Pent) and the β cleavage peak (M-129) was stronger than that from α cleavage. Thus the α-branched diacids were α-butyloctanedioic and α-butylnonanedioic acids and the alkyl substituent of pigment B was *n*-butyl; carbon 7′ was attached to the pyrrole ring.

In order to decide which α carbon was branched, the nmr spectrum of B was reexamined in acid salt and free base forms. A survey of the nmr spectra of alkylpyrroles[7] and of some synthetic prodiginines[8] suggested that the difference in chemical shift between the acid salt and free base form was greater for hydrogens of the methyl or methylene groups attached to carbon 2 than for those attached to carbon 3. This was verified experimentally for prodigiosin (**III**) and nonylprodiginine (**II**) as shown in Table 1. In addition, the large shift of the hydrogens of the α-methylene group in pigment B showed that this group was attached at carbon 2. Thus the structure of pigment B was established as **V**.

Prodiginine pigments **I**, **IV**, **V** as well as cyclononylprodiginine[8] and cyclomethyldecylprodiginine[1,8] in peanut oil were tested by one subcutaneous administration into mice, 3 days after infection with *Plasmodium berghei* KBG 173 malaria. The test methods have been described.[9] At each dose level 5 mice were used. Without treatment, death occurred at 6~8 days. Deaths before 6 days were regarded as due to toxicity of the test substance. The results are shown in Table 2.

Table 1. Chemical shifts of α-methyl and methylene hydrogens in some prodiginines.

Compound	Group	δ Free base	δ HCl salt	Δ
III	2-methyl	1.8 s	2.55 s	0.75
	3-methylene	2.25 br s	2.4 t	0.15
II	2-methylene	2.2 br s	2.9 t	0.7
B(=V)	methylene	2.2	2.9~3.6 mainly 3.3	1.0

Table 2. Antimalarial activity of some prodiginines.

Test No.[a]	Dose in mg/kg	Increase in mean survival time (days) for mice treated with: I·HCl salt undecylprodiginine	IV·HCl salt metacycloprodigiosin	V·HCl salt butylcycloheptylprodiginine	Cyclononylprodiginine free base	Methylcyclodecylprodiginine free base
1	10	0.3	0.5	0.7	2.1	1.9
	20	0.5	3.9	—	3.9	2.3
	40	0.7	4.9	4.5	5.9	5.3
	80	1.1	5.9	—	8.5	6.9
	160	1.5	8.5	7.5	9.9	12.9
	320	—	9.3	—	0.0[b]	0.0[b]
3	10		0.5	0.5	1.9	1.7
	20		4.1	0.7	3.7	2.3
	40		5.1	4.7	6.1	5.5
	80		5.9	6.7	8.7	7.1
	160		8.5	—	—[c]	12.4

[a]Different test No. means different groups of mice and different times.
[b]All toxic deaths.
[c]Three cured *i.e.* survived 60 days. Two toxic deaths.

Experimental

The yeast-dextrose, BENNETT's and soybean media have been described[3] as well as procedures for thin-layer chromatography, spectrophotometric assay of pigment solutions, mass spectra[3] and gas chromatography.[1] Nmr spectra were obtained in $CDCl_3$ with tetramethylsilane as an internal standard on a Varian T-60 instrument. Yeast-CZAPEK medium: 2 g Difco yeast extract,

2 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 30 g sucrose per liter of distilled water, pH adjusted to 7.3 before autoclaving.

Selection of pigment-producing substrain of *Streptomyces* sp. Y-42. *S.* sp. Y-42 was streaked out on plates of half strength BENNETT's agar and on yeast-CZAPEK agar. After several days at 28°C the reddest single colonies were selected and restreaked on yeast-CZAPEK plates. From these the reddest colonies were transferred to yeast-CZAPEK and BENNETT's agar slants. Blue pigment, diffusing into the agar was also observed. Somewhat later these cultures were streaked out on plates of yeast-CZAPEK agar made with Sigma Chemical Co. *i*-inositol (meso inositol) instead of sucrose. After 5~7 days at 28°C most colonies showed red intracellular (prodiginine) and blue diffusing (hydroxyquinone) pigment but a few colonies were observed with strong red pigmentation only. These were selected and used in all subsequent work.

Undecylprodiginine (I) and butylcycloheptylprodiginine (V) from *S.* sp. Y-42. The selected substrain was maintained on BENNETT's agar slants, incubated at 28°C and when well grown, stored at 5°C. For production, growth from a slant was used to inoculate 5 flasks of BENNETT's broth 50 ml/250 ml Erlenmeyer flask. The flasks were shaken at 28°C, 220 rpm, for 4 days, then the resulting mixture of cells and broth was used to inoculate 40 production flasks at 5 ml per flask. The production medium (250 ml per 2-liter Erlenmeyer flask) was 2 g Difco yeast extract, 4 g $NaNO_3$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO \cdot 7H_2O$, 10 g *i*-inositol per liter of distilled water, pH adjusted to 7.3 before sterilization. Calcium carbonate, about 500 mg per flask, was added before sterilization. Production flasks were incubated at 28°C on the reciprocal shaker, 68 strokes per minute, at 28°C for 2.5~3 days then the red cellular material separated by centrifugation. The wet sludge obtained was shaken overnight with acetone (1.5~2 liters) and after spectrophotometric assay (yields were 4~6 mg per flask), concentrated to 1/10~1/5 volume. This mixture was diluted to 4 liters with water and shaken with 500 ml of CH_2Cl_2. After separation of the dark lower layer 200 ml of concentrated HCl was added to the aqueous portion which was then shaken again with CH_2Cl_2. The combined CH_2Cl_2 extracts were washed with dilute acid, dilute base and evaporated to dryness. The residue in benzene was purified by column chromatography on alumina (250 g) as for the other prodiginine pigments then the two pigments are separated by column chromatography on silica (200 g).[3] Typical recoveries were 70 mg **I** and 130 mg **V** from a 40-flask batch. Air-dried pure pigments were each dissolved in CH_2Cl_2 and the CH_2Cl_2 solutions shaken with 10% HCl. The dried CH_2Cl_2 solutions were concentrated to about 50 ml and 50 ml of hexane added. Thereafter the mixture was reduced in volume by a gentle steam of air and hexane added until crystallization of the HCl salt was well started. The final volumes were about 20 ml and the mixtures were chilled several days before filtration. **I**·HCl, dark red needles, mp 104~106°C (lit. mp 106~107[4]). For comparison **II**·HCl prepared in the same way melted at 125~130°C. **V**·HCl, dark purple, irregular amorphous pieces which did not melt sharply, about 170~175°C. **V**·$HClO_4$, rods from ethanol-H_2O, became amorphous 120~160°C and melted with decomposition about 200°C. The mass spectrum of **V** showed peaks in order of decreasing intensity at 391 (M^+), 91, 175, 348, 334, 262, 195.5 (M^{++}), 376, 118 and 251 mass units. For comparison the peaks from **IV** were 391 (M^+), 307, 320, 175, 376, 360, 249, 262, 230, 195.5 and 91 mass units.

Undecylprodiginine (**I**) and metacycloprodigiosin (**IV**) from *S. longisporuber*. *S. longisporuber* IMRU 3762 was maintained on BENNETT's agar slants transferred every 16 weeks, incubated at 28°C and when well grown stored at 5°C. For production, growth from a slant at least 2 weeks old was used to inoculate one flask of yeast dextrose broth, 50 ml/250 ml Erlenmeyer flask. When well grown (usually 2~3 days) the growth was pipetted into 8 similar flasks of BENNETT's broth. After 3 days shaking at 28°C the pale pink growth was used to inoculate 24 production flasks (250 ml of soybean medium/2-liter Erlenmeyer flask) which were shaken at 28°C on the reciprocal shaker at 100 strokes per minute or the rotary shaker at 200 rpm. After 4 days, the uniformly red cells were filtered with suction and treated as described above

for *S.* sp. Y-42. Yields by spectrophotometric assay were 20~30 mg/liter. The recovery of pure pigments from a 10-liter batch was **I**, 7 mg, **IV**, 300 mg. The perchlorate salt of **IV** was prepared using 100 ml ethanol, 3 ml 7 % $HClO_4$ followed by gradual addition of 150 ml of 0.7 % $HClO_4$ to the hot solution. After several days at 5°C, 287 mg of dark shiny crystalline **IV**·$HClO_4$ was obtained identical in visible absorption spectra and TLC behavior with an authentic sample of **IV**·HCl supplied by WASSERMAN.[2] The melting point was not sharp, about 220~230°C. For antimalarial testing the HCl salt was prepared as described for **V** except that **IV**·$HClO_4$ (750 mg) dissolved in CH_2Cl_2 was shaken twice with 10 % NH_3 before shaking with 10 % HCl. The final volume for crystallization was about 50 ml. Filtration gave 552 mg of dark purple crystals mp 205~210°C (lit. mp 214~216)[2]

Alkaline permanganate oxidation of degradation products. One mg or less of ketoacid or ketoester was shaken at 28°C for 2 days with 25 mg of $KMnO_4$ in 5~10 ml of 1 % NaOH. The mixture was centrifuged to remove MnO_2, decolorized by warming with a few drops of ethanol and centrifuged again. The colorless supernatant was acidified with H_2SO_4, extracted 4 times with CH_2Cl_2 and the residue from the combined CH_2Cl_2 extracts esterified in the usual way with BCl_3·MeOH. By this procedure authentic, synthetic 10-oxoundecanoic acid[1] was converted to dimethylnonanedioate.

Microscale preparation of pentyl esters. To 1 mg or less of acid or acid mixture in a small test tube was added 400 μl benzene, 100 μl pentanol, 25 μl of concentrated sulfuric acid and 1 piece of "Drierite." After overnight at 50°C the mixture was pipetted from the "Drierite" into a second small test tube where it was shaken cautiously with enough 5 % aqueous sodium bicarbonate to neutralize the acid. The upper benzene layer was pipetted into a third test tube, clarified by standing some hours with 1 piece of "Drierite" then examined directly by gas chromatography.

Cyclononyl and cyclomethyldecylprodiginine free bases. (For structures see Ref. 1.). For antimalarial testing the free bases were prepared from the perchlorate salts. Perchlorate salt (720 mg) in 500 ml of 10 % NH_3 was shaken 3 or 4 times with 200~300 ml hexane. The residue from the combined hexane extracts was dissolved in 300 ml 95 % ethanol then 2 % NH_3 added until crystallization was well started. After overnight more 2 % NH_3 was added (total 250 ml) and after another night at 5°C the orange needles were filtered off and dried in vacuum, mp of cyclononylprodiginine 158~160°C, of cyclomethyldecylprodiginine, 61~65°C.

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