# Minor Prodiginine Pigments from *Actinomadura Madurae* and *Actinomadura Pelletieri*

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Five minor prodiginine pigments were isolated from *Actinomadura madurae* and *A. pelletieri* fermentations. Two of the pigments were shown to be methylcyclooctylprodiginine (**7**) and ethylcyclononylprodiginine (**8**). For the others, structures containing oxygen functions in the aliphatic chains (**11**, **12** and **13**) were suggested.

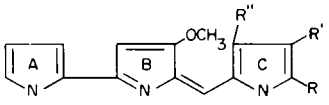
Recently we have reported the isolation and structure proof of nonylprodiginine, **1**, and cyclononylprodiginine, **2**, from red strains of *Actinomadura madurae* (1) as well as cyclomethyldecylprodiginine, **3**, from *A. pelletieri* (2). In order to obtain amounts of **2** and **3** sufficient for antimalarial screening we developed reliable procedures for their production by submerged fermentation in shaken flasks.

Previously we had noted that solvent extracts of *A. madurae* R39 or *A. pelletieri* 610 (hereafter called R39 or 610) cells examined by tlc showed, in addition to **2** or **3**, faint spots of similar color and fluorescence but different $R_F$ values. During the purification of **2** and **3** we obtained eluate fractions enriched in one or another of these minor pigments. By further column chromatography as for **2** and **3**, we obtained small amounts of pure R39FF, R39MF, R39SF, 610FF and 610MF.

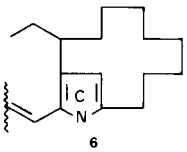
All of the minor pigments had the characteristic visible absorption spectra of prodiginine pigments (1b). Their maxima were consistent with dialkyl substitution of the chromophore, not mono (1a). The large solvent shifts (Table I) and intense yellow fluorescence on tlc plates in long wavelength uv light, both characteristic of **2** and **3** but not of **1**, **4** (undecylprodiginine), **5** (prodigiosin), or **6** (metacycloprodigiosin) (3), strongly suggested that the minor pigments were of the macrocyclic type. This was confirmed in the case of R39FF and 610FF by their nmr spectra which showed two singlets (6.7 and 5.9 $\delta$) and four sets of doublets (6.6, 6.5, 6.05 and 5.95 $\delta$ all $J \sim 3.5$) as had been previously observed for the aromatic and olefinic protons of **1** (1a).

Since all prodiginine pigments are red in the salt form and yellow in the free base form, the color of a tlc spot while the plate is running is a rough indication of the amount of pigment in each form. All spots are pink when dry. Under normal tlc conditions (1b) R39FF is yellow, **3** is pink, 610FF and **2** are orange. The $R_F$ values are nearly identical. We surmised that the observed different basicities were due to more or less steric hindrance caused by different ring sizes. Thus R39FF was the weakest base, had the most steric hindrance, thus the smallest ring; **3** was the strongest base, had least steric hindrance and the largest ring. The other two were intermediate and of the same ring size.

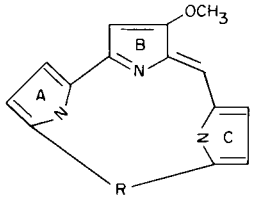
Previously we had found that **1** and **4**, identical in tlc behavior, could be distinguished by their partition coefficients, 1.3 and 2.1, in benzene-ethanol-water-acetic acid. Partition experiments on the macrocyclic pigments used hexane-ethanol-water-ammonia because in the acidic system the distribution between layers seemed to depend




**1** R $(CH_2)_8CH_3$; R′ R″ H
**4** R $(CH_2)_{10}CH_3$; R′ R″ H
**5** R $CH_3$; R′ $(CH_2)_4CH_3$; R″ H
**9** R R″ $CH_3$; R′ H
**10** R R″ $CH_3$; R′ $CH_2CH_3$




**6**




**2** R $(CH_2)_9$
**3** R $CH(CH_2)_9$ ($CH_3$)
**7** R39FF: R $CH(CH_2)_7$ ($CH_3$)
**8** 610FF: R $CH(CH_2)_8$ ($CH_2CH_3$)
**11** R39MF: R $(CH_2)_8CO$
**12** R39SF: R $CHOH(CH_2)_8$

**13** 610MF: R $CHC(X)(CH_2)_5C(Y)$ ($CH_3$)
X O; Y HOH
or
X HOH; Y O

TABLE I

Some Physical Properties of Macrocyclic Prodiginine Pigments

| Pigment | Maximum in acid EtOH | $CHCl_3$ | Solvent shift | Partition coefficient in hexane-EtOH-$H_2O$-$NH_3$ |
|---|---|---|---|---|
| **2** | 542 | 551 | 9 | 9 ± 1 |
| R39FF | 543 | 551 | 8 | 13 ± 1 |
| R39MF | 547 | 554 | 7 | 0.9 |
| R39SF | 538 | 547 | 9 | ——— |
| **3** | 539 | 549 | 10 | 28 ± 4 |
| 610FF | 541 | 550 | 9 | 30 ± 4 |
| 610MF | 538 | 547 | 9 | 1.0 |

TABLE II

Data from the Mass Spectra of Prodiginine Pigments

| Pigment | Peak height/sum of peak heights at 91, 104 and 118 | | | Inlet temperature and date |
|---|---|---|---|---|
| | 91 | 104 | 118 | |
| **2** (a) | 8 | 75 | 17 | 250°, 3/72 |
| **2** perchlorate salt | 6 | 61 | 33 | 250°, 5/70 |
| **2** perchlorate salt | 7 | 72 | 21 | 325°, –/67 |
| **3** Zn salt | 11 | 12 | 77 | 280°, –/67 |
| **3** Zn salt | 12 | 12 | 76 | ———, –/66 |
| **3** perchlorate salt | 8 | 10 | 82 | 250°, 5/70 |
| **3** perchlorate salt | 24 | 17 | 59 | 320°, 5/70 |
| **3** perchlorate salt | 23 | 14 | 63 | 350°, 5/70 |
| **6** HCl salt | 66 | 10 | 24 | 290°, 1/67 |
| **1** Zn salt | 70 | 5 | 25 | ———, –/66 |
| **4** lit. ref. (5) | 76 | 3 | 21 | ———, ——— |
| **4** | 66 | 6 | 28 | ———, –/69 |
| **5** | 60 | 10 | 30 | 250°, 4/72 |
| **9** lit. ref. (6) | 65 | 0 | 35 | ———, ——— |
| **10** lit. ref. (6) | 81 | 0 | 19 | ———, ——— |
| 4-MeO-2, 2′-bi pyrrole lit. ref. (7) | 73 | 16 | 11 | ———, ——— |
| | | | | Other significant ions in decreasing intensity order |
| R39FF | 17 | 14 | 69 | M = 363 |
| R39MF | 23 | 44 | 33 | M = 377 |
| R39SF | 12 | 67 | 21 | M-$H_2O$ = 361; M = 379 |
| 610FF | all 5 or 6% as intense as 362 | | | M = 391; M-Et = 362 |
| 610MF | 28 | 22 | 50 | M-$H_2O$ = 403; M = 421 |

(a) Pigments without a literature reference were prepared by us and spectra were obtained on the free base form on diatomaceous earth unless otherwise stated.

TABLE III

Products from the Chromic Acid Degradation of Prodiginine Pigments

| Pigment | Major Product (a) | Minor Products (a) |
|---|---|---|
| 1 | nonanoic acid | decanoic acid |
| 4 | undecanoic acid | dodecanoic acid |
| 2 | nonanedioic acid (azelic acid) | decanedioic acid (sebacic acid) |
| 3 | 10-oxoundecanoic acid 11-oxododecanoic acid (b) | several, not identified |
| R39FF | 8-oxononanoic acid (c) 9-oxodecanoic acid (c) | ------- ------- |
| R39MF | nonanoic acid (d) | decanoic acid |
| R39SF | nonanoic acid (d,e) | decanoic acid |
| 610MF | nonanoic acid (e) | ------ |

(a) Products were separated by gas chromatography of the methyl esters and identified by GC comparisons with authentic samples except as noted. (b) Identified as homologous with 10-oxoundecanoic acid by GC as described in reference (2). (c) Identified as homologous with 10-oxoundecanoic acid by GC by the method of reference (2) shown to be mono esters not diesters by ester exchange (9). (d) Negative micro carbonyl tests with 2,4-dinitrophenylhydrazine. (e) Shown to be diester not mono by ester exchange (9).

mainly on base strength. The results (Table I) suggested that each "FF" pigment was similar in aliphatic chain length to the corresponding major pigment from the same strain. Structures **7** and **8** for the "FF" pigments fulfill the above suggestions for chain length and ring size. These structures were confirmed by the nmr spectra which showed a doublet at 1.3 δ for R39FF and a triplet at 0.9 δ for 610FF. In both the methylene band was centered at 1.4 δ.

The single most useful method for structure elucidation of prodiginine pigments is mass spectroscopy. Using the direct inlet of a Hitachi RMU-6 instrument good spectra were routinely obtained on sub-milligram amounts of pure pigment in free base form which had been absorbed on a diatomaceous earth GC support by evaporation of a carbon tetrachloride solution. Earlier spectra had been obtained of various salt forms on different instruments. In spit eof experimental variations, the mass spectra of **3**, for example, were always similar and clearly recognizable. For all prodiginine pigments $M^+$ and $M^{++}$ were apparent. Considering the previously known pigments (upper part of Table II) in every case the predominant peak in the 80-150 range was 91, 104 or 118 mass units (m.u.). Frequently it was the second strongest peak in the spectrum; $M^+$ was usually strongest. Since the 91 m.u. peak was predominant in the spectrum of 4-methoxy-2,2′-bipyrrole as well as **6**, **1**, **4**, **5**, **9** and **10** it must represent a fragment of rings A and B, not C. Since 91 m.u. = $C_6H_5N$ the fragment must contain the atoms of ring A as well as two carbons of ring B because fragmentations which would leave ring B intact are not favored (4). It was already known that the predominant side chain cleavage in prodiginine pigments is β to a pyrrole ring: 266 m.u. in **5** (4), 255 m.u. in **1** (1b) or **4** (5). Thus **2** undergoes both types of cleavage to give the observed predominant ion at 104 m.u. For **3** the main ion at 118 m.u. furnished proof that the α-methyl branch is adjacent to ring A not ring C, a point which had been in some doubt (2).

Previously the degradation of **1**, **2** and **3** with potassium permanganate had resulted in very low yields of mixtures of aliphatic acids (2). Using chromic acid better yields and simpler mixtures were obtained so that useful results from 1-2 mg. of pigments were achieved (Table III).

For R39FF the $M^+$, the 118 m.u. peak and the chromic acid oxidation products all verify its structure as **7**. For 610FF the $M^+$ and M-Et peaks dwarfed all others in the mass spectrum, confirming formula **8**. The strong $M^+$ for R39MF and the formation of a new pigment of lower $R_f$ from R39MF and $NaBH_4$ under conditions where **2** and R39SF were recovered unchanged, suggested a carbonyl group on a nine carbon aliphatic chain. The weak $M^+$ and strong $M\text{-}H_2O$ of R39SF suggested an alcohol group on a nine carbon chain. For both, the 104 m.u. peaks indicated an unbranched chain and the isolation of nonanoic acid from chromic acid oxidation showed that the oxygen functions were present at carbons 1′ or 9′. Careful TLC comparisons of R39SF and the $NaBH_4$ reduction product of R39MF as well as mass spectra showed that they were not identical although the reduction product did show the expected peak at 361 m.u., $M\text{-}H_2O$. Thus, the most reasonable structures for R39MF and R39SF were **11** and **12** respectively since a carbonyl group next to ring A would not allow for the observed 104 m.u. peak. The weak $M^+$, strong $M\text{-}H_2O$ and 118 m.u. peak from 610MF suggested a carbonyl group and an alcohol function on a methyl-branched eleven carbon chain. 610MF, like R39MF, gave a new pigment of low $R_f$ after overnight treatment with sodium borohydride in ethanol. The isolation of nonanoic acid from the chromic acid oxidation implied that the oxygen functions were at carbons 1′ and 9′. See reference (10).

## EXPERIMENTAL (8)

Cyclononylprodiginine (**2**).

*A. madurae* R39 was maintained on yeast-dextrose or Bennett's agar slants, transferred every 16 weeks, incubated at 28° until well grown, then stored at 5°. For production a slant was used to inoculate one flask of yeast-dextrose broth, 50 ml./250 ml.

Erlenmeyer flask. The flask was shaken on a rotary action shaker Model V, New Brunswick Scientific Co., New Brunswick, New Jersey at 250 rpm and 28° until the organism was well grown (7-14 days) then the growth from this flask was pipetted into 8 similar flasks of yeast-dextrose broth. After 5 days shaking at 28° the growth from these flasks was used to inoculate 24 flasks of soybean medium; 250 ml./2 l. Erlenmeyer flask which were incubated at 28° on a reciprocal shaker 68 strokes/minute. After 3 days the growth was uniformly red; after 7 days in order to kill the organism, 100 ml. of chloroform was added to each flask and they were shaken overnight. The combined cells, broth and chloroform mixture was filtered by suction using filter aid or more rapidly in a pilot plant Sharples centrifuge. The chloroform layer contained about half of the pigments; the broth was discarded; the cells were shaken overnight with acetone, 100 ml. per original production flask which extracted most of the remaining pigment. Yields by spectrophotometric assay were 60-80 mg./l. of medium.

Cyclomethyldecylprodiginine (**3**).

The procedure was the same as for **2** except as noted. The microorganism was *A. pelletieri* 610. The 8 seed flasks were shaken for 7 days; the 24 production flasks contained Bennett's medium and were shaken 14 days. Growth usually was variously pink after 4-5 days. The combined cells, broth and chloroform mixture filtered rapidly with suction; only about one-tenth of the pigments were in the chloroform ext. Yields were 10-40 mg./l. of medium.

Purification Procedures.

A typical acetone extract, 3.1 l. with 400 ± 50 mg. pigments, was shaken with 20 parts water and 1 part hexane. After 18 hours the pigment-containing hexane layer was washed with dilute acid then dilute base. For **3** some color remained in the aqueous layer so that recoveries were lower. The concentrated hexane solution was applied to a 400 g. column of alumina in benzene. The alumina had been previously stirred several hours with distilled water, filtered and air dried for several days. Pigments **1**, **2** and **7** (from R39) or **4**, **3** and **8** (from 610) were readily eluted with benzene; chloroform was needed for **11**, **12** and **13**. The concentrated benzene eluate was stirred with dilute ammonia to convert all prodiginines to the free base form. It was then applied to a 200 g. column of Baker silica gel powder 60-200 mesh made up in chloroform which had been freed from traces of acids by shaking with aqueous bicarbonate. Elution with bicarbonate-washed chloroform furnished first **7** or **8** followed by **2** or **3**; elution with increasing concentrations of ethyl acetate in chloroform gave **1** or **4**. Often fractions containing **7** and **2** or **8** and **3** were obtained. They were resolved by repeated chromatography on silica. Chloroform eluates from alumina columns were cleaned up on silica using chloroform and ethyl acetate as described, but separations of **11** and **12** from each other and from **1** as well as the purification of **13** were carried out on alumina eluting with benzene and chloroform. Chloroform extract of cells were taken to dryness, dissolved in benzene and purified by column chromatography as described above for the hexane solution. Yields of **7**, **8**, **11** and **13** were typically 2-5% of **2** or **3**, **12** was noticeably less. Only **7** was obtained in amounts sufficient for the preparation of the perchlorate salt (1b). The others were reasonably stable in the free base form in benzene at 5°.

Partition Experiments.

Ten to twenty times the amount of pure pigment in solution necessary for a strong tlc spot was taken to dryness in a glass stoppered centrifuge tube. At 28° exactly 3 ml. each of the upper and lower layers from a mixture previously equilibrated overnight at 28° was added to each tube. For **1** and **4** the partition mixture was benzene-ethanol-water-acetic acid 15:10:5:1; for the macrocyclic pigments hexane-ethanol-water-28% ammonia 20:15:5:1 was used. The tubes were shaken vigorously then allowed to stand at 28° until both layers were clear. From the acidic mixture exactly 1 ml. of each layer was withdrawn and diluted to 4 ml. with ethanol for spectrophotometric assay. For the alkaline mixture 0.5 ml. of upper and 2 ml. of lower layer was used and both assay solutions were acidified with concentrated hydrochloric acid before assay. K = optical density at the maximum in the solution prepared from the upper layer/optical density at the maximum in the lower layer solution. For comparison of K values parallel experiments were always carried out at the same time.

Chromic Acid Oxidation.

Ten mg. of pigment, either free base or perchlorate salt was dissolved in 1 ml. of acetone. To this was added 15 ml. of 15% aqueous sulfuric acid then dropwise a solution of 1 g. of chromic oxide in 5 ml. of distilled water. The mixture became homogeneous in a few moments and after overnight at room temperature was extracted 4 times with methylene chloride. The residue from the methylene chloride extracts was esterified with boron trichloride-methanol. The resulting esters were examined by gas chromatography as previously described (2).

Ester Exchange (**9**).

To 30-100 μ l. of the usual toluene or methylene chloride solution of ester used for gas chromatography was added 500 μ l. of an ethanol-sodium hydroxide solution. This solution had been prepared by dissolving 1 piece of sodium hydroxide (about 0.1 g.) in 80 ml. of absolute ethanol. After overnight at 50° the reaction mixture was diluted with water and extracted 3 times with methylene chloride. The combined extracts were washed once with water and concentrated in a stream of air to a volume about equal to that taken originally.

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(8) Media. Yeast-Dextrose: 10 g. Difco yeast extract, 10 g. Cerelose (commercial grade glucose) made up to 1 l. with tap water, *p*H adjusted to 7.0-7.2 before sterilization. Bennett's:

1 g. Difco yeast extract, 1 g. Difco Beef extract, 2 g. Sheffield Farms N-Z amine-A (a casein hydrolysate), 10 g. Cerelose made up to 1 l. with tap water, *p*H adjusted to 7.3. Soybean: 10 g. Staley's soybean meal, 20 g. Cerelose, 10 g. Wilson Co. meat peptone 851C, 5 g. of sodium chloride made up to 1 l. with tap water, *p*H adjusted to 7.5. For solid media add 15 g. agar per l.

All stages of production and purification were monitored by TLC on Eastman chromagram #6061. Plates were normally 4 inches tall and run 10-15 minutes in chloroform or chloroform-ethyl acetate 9:1. The $R_F$ values vary with loading and with sample purity. All spots fade rapidly in light and air.

The amount of pigment in extracts and solutions were estimated spectrophotometrically. Mg. pigments = optical density at the maximum in acid ethanol times volume of solution in ml./300.

(9) The method of ester exchange is useful for distinguishing between mono and diesters on a small scale. Mixtures of known, homologous, aliphatic unbranched mono and diesters like those in Table III were tested. The difference in retention time of a homologous pair such as dimethylnonanedioate and dimethyl decanedioate was defined at Δ-homo and the change in retention time caused by ester exchange, such as dimethyl nonandioate to diethyl nonanedioate was defined as Δ-exchange. Under a given set of experimental condtiions for the diesters Δ-exchange $>>$Δ-homo and for the mono esters, including keto esters, Δ-exchange $<<$Δ-homo.

(10) The possibility that some portions of R39MF, R39SF and 610MF might be artifacts formed by air oxidation of 2 or 3 in chloroform solutions cannot be rigorously excluded. However, chloroform extracts of whole broths always showed, by tlc, these minor fluorescent spots when first examined and the amounts did not seem to increase with time or extent of manipulation. Pigments 2 and 3 did deteriorate in solution, especially in light, but the products were dark colored, non-fluorescent spots which did not move from the origin during tlc. A few mg. of chromatographically pure 2 in chloroform solution was shaken with sterile soybean medium which had been adjusted to the normal final *p*H of the fermentation (8.5). After 4 days shaking tlc disclosed no new fluorescent spots.