PRODIGIOSIN-LIKE PIGMENTS FROM *ACTINOMADURA* (*NOCARDIA*) *PELLETIERI*

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Two prodigiosin-like pigments from *Actinomadura pelletieri* were shown to be undecylprodiginine (III) and methylcyclodecylprodiginine (VI). The antimicrobial activity of four prodiginine pigments is given.

Prodigiosin[1] (I) is the bright red pigment of *Serratia marcescens* and was probably responsible for many medieval "miracles" involving the appearance of blood stains on the Holy Host[2]. Two red pigments with the methoxytripyrrole nucleus of **I** (prodiginine) have been isolated from *Streptomyces longisporus ruber*. They are undecylprodigiosin[3] (undecylprodiginine) (III) and metacycloprodigiosin (IV)[4]. Recently we reported nonylprodiginine (II)[5] and cyclononylprodiginine (V)[6] to be produced by red strains of *Actinomadura*[7] (*Nocardia*) *madurae*.

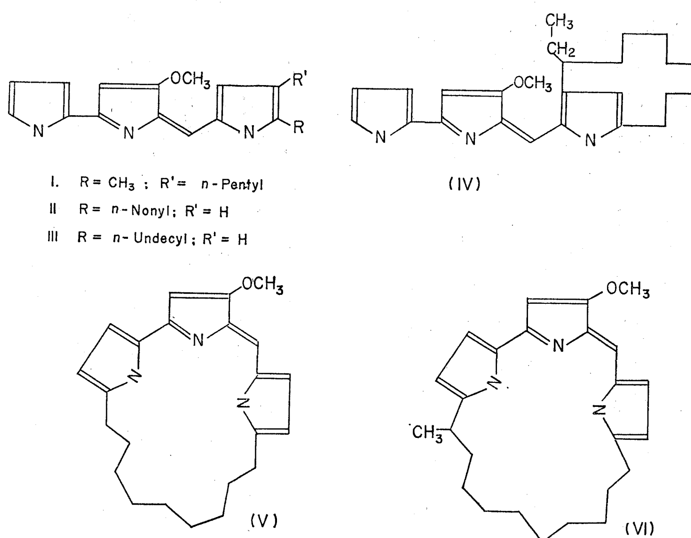
We have detected, by thin-layer chromatography (TLC) and visible absorption spectroscopy, similar pigments in 16 strains of the closely related organism, *A. pelletieri*, which is normally red. The slower moving TLC spot was shown, in the case of strain 687, to be undecylprodiginine (III) by comparison with an authentic sample. Although **II** and **III** have identical visible absorption spectra and similar TLC behavior, they can be readily distinguished on a 0.1 mg scale by their partition coefficients. The K's for **II** and **III** in C_6H_6 - EtOH - H_2O - HOAc (15 : 10 : 5 : 1) were 1.5 and 2.5 respectively.

The pigment corresponding to the faster moving TLC spot was obtained in quantity from strain 610. A comparison of it with **V** showed some similarities. The visible absorption maximum was 539 mμ in acidic EtOH, 549 in hexane. In the mass spectrum, the base peak was the molecular ion at 391, M^{++} at 195.5 was also strong. The nmr spectrum showed two singlets at 6.7 and 5.8 δ which were assigned to hydrogens at carbons 5 and 7 as well as bands characteristic of two AB systems each containing one "α" and one "β" type of pyrrole proton[8]. Thus (as has been previously explained in greater detail[6]) the alkyl chain must be attached at positions 2 and 10.

However, in contrast to **V**, the nmr spectrum of **VI** showed an area corresponding to 2 hydrogens, not 4, at the characteristic location for -C$\underline{H}_2$-Ar, 2.9 δ. Furthermore, the mass spectrum (see Fig. 2) of **V** shows that fragmentation ions with 1 and 3 -CH_2-units derived from the side chain predominated over those with 0, 2, 4, 5 or more -CH_2-units. In contrast, from **VI**, ions with 2 and 4 -CH_2-units predominated. This

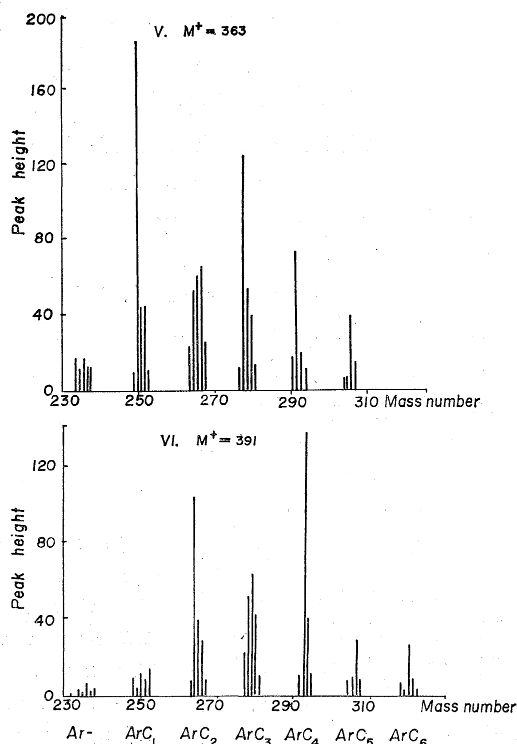
suggested that one carbon α to the aromatic portion of the molecule had a methyl branch on it. If this were the case then one might expect **VI** to be optically active. The circular dichroism of **V** and **VI** were measured in EtOH. The tracing for **V** was flat while that for **VI** showed a small but definite peak at 530 mμ indicating optical activity not due to impurities.

Fig. 1. Structures of the prodiginine pigments



Previously, we had investigated **II** and **V** by oxidation with dilute potassium permanganate in aqueous pyridine[6]. The acids obtained were methylated and analyzed by gas chromatography (GC). Pigment **II** gave a 1:4 mixture of methyl nonate and methyl decanoate; **V** furnished a 2:7:5 mixture of the dimethyl esters of azelaic, sebacic, and undecanedioic acids. All were identified by comparison with authentic samples and the structure of dimethyl sebacate was verified by its mass spectrum. Thus, in this oxidation, one obtains a mixture of acids derived from the aliphatic part of the molecule. Oxidative cleavage may occur α to the pyrrole ring (methyl nonate from **II**) or in such a way that the product includes one of the ring carbons (methyl decanoate from **II**). For **V**, one would expect 3 different dicarboxylic acids with sebacic acid predominating, since it could be formed in two different ways.

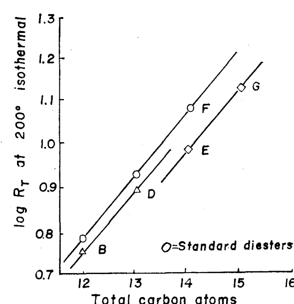
Fig. 2. Portions of the mass specta of V and VI



However, **VI** did not furnish the dimethyl esters of undecanedioic, dodecanedioic and tridecanedioic acids as would have been expected from an unbranched eleven carbon aliphatic chain. Instead, GC revealed 5 main peaks whose retention times are given in Fig. 3 compared to those for sebacic, undecanedioic and dodecanedioic dimethyl esters. For a homologous series run under isothermal conditions, the log of

the retention time versus carbon number is a straight line (except for the lowest members of the series). Series of esters having the same type of characteristic groupings give parallel straight lines[9]. Thus, Fig. 2 shows that B and D are homologs, as well as E and G, but they are not members of the same series. Material from peaks B and D gave a positive microtest for carbonyl with 2, 4-dinitrophenylhydrazine[10]. Furthermore, B was identical with an authentic sample of methyl 10-oxoundecanoate in GC retention time and TLC behavior of the dinitrophenylhydrazone derivatives. Thus B and D are methyl 10-oxoundecanoate and methyl 11-oxododecanoate where oxidative cleavage at the branched end has occurred α to the pyrrole ring. The other pair of products, in which at the branched end one carbon from the pyrrole ring is included, would be α-methylundecanedioic and α-methyldodecanedioic acids. A direct comparison with an authentic sample was not possible but the retention times of E and G are consistent with these structures since branched chain fatty esters have shorter retention times than the corresponding unbranched ones[9].

Fig. 3. The retention time of the methyl esters obtained from **VI** and of the 3 standard dimethyl esters



The retention time of peak F was identical with that of dimethyl dodecanedioate, however its distinct "mushroom" odor suggested that it was some other type of degradation product since the diesters are odorless under our GC conditions. One might imagine that **VI** was a mixture of pigments containing enough of one with an unbranched ten carben chain to furnish dimethyl dodecanedioate on oxidation. However, this was disproved by the complete absence of any M^{++} at 188.5 in the mass spectrum of **VI**.

Although most strains of *A. pelletieri* gave **VI**; strain 757 furnished **II** and **V**. Six red strains of *A. madurae* were available for study. The majority furnished **II** and **V**, 554 produced only **III**, shown by its partition coefficient compared to those of **II** and **III**. Strain 507 gave a pigment whose partition coefficient was intermediate between those of **II** and **III**. The mass spectrum demonstrated that it was a mixture of **II** and **III** (M^+ at 393 and 365) not a pigment with a 10 carbon side chain. In all cases strain identification had been made previously[11]. The presence of C-9 and C-11 side chains but not C-10; as well as coproduction of **II** and **III**

Table 1. Antimicrobial activity of proginine pigments

Organism	Minimum inhibitory concentration (μg/ml agar)			
	I	II	V	VI
Staphylococcus aureus 15	10, 5*	25	25	25
Mycobacterium smegmatis 3	10, 5*	50*, 25*	25	25
Nocardia asteroides 3318	25, 10*	50*, 25*	50*, 25*	25*
Actinomadura madurae R-28	10	50, 25*	25	25
Streptomyces somaliensis 395	10	25	25, 10*	10
M. rhodochrous 271	10, 5*	50, 25*	25*	25
Trichophyton mentagrophytes 171	10, 5*	50*	25*	25*
Ceratostomella ulmi 185	50, 25*	(−)**	(−)	(−)
Saccharomyces cerevisiae 216	25*	(−)	(−)	(−)
Prototheca zopfii 328	25, 10*	(−)	(−)	(−)
Pacilomyces varioti 3	25*	(−)	(−)	(−)
Aspergillus niger 13	25*	(−)	(−)	(−)

* Partial inhibition ** (−)=Not active

by strain 507 suggests that the side chains are biosynthesized from acetate units by chain extension as in fatty acids. Then, at some point before the extended chain is fully reduced (for example: at the R-CH=CH-COOH stage), cyclization may occur. The methyl branch in **VI** is considered to be α to ring A not ring C on the basis of these biogenetic suggestions which are for the actinomycetic prodiginine pigments only. The terminal stage in the biosynthesis of prodigiosin has been shown to involve coupling of 4-methoxy-2,2′-bipyrrole-5-carboxaldehyde and 2-methyl-3-amypyrrole[1].

The antimicrobial activity of pigments **I**, **II**, **V** and **VI** is shown in Table 1. Prodigiosin (**I**), showed modest activity against some gram positive bacteria, actinomycetes and fungi. The others had a similar but weaker activity.

Experimental

The production, isolation and purification of the prodiginine pigments has been described previously[5].

Authentic 10-oxoundecanoic acid was prepared from 10-undecynoic acid using the method of SISIDO[12] and esterified with BCl_3-MeOH.

Permanganate Oxidation of **VI**. To 18 mg of **VI** perchlorate salt in 4 ml of pyridine was added 5 ml of $NaIO_4$-$KMnO_4$ solution (1 g of $NaIO_4$+0.1 g $KMnO_4$/100 ml distilled water) and 25 mg of $KMnO_4$. The mixture was shaken at 28°C additional portions of $KMnO_4$ were added after 4 hours (10 mg), 4 hours (10 mg) and overnight (10 mg). Control flasks with pyridine only or with pyridine and 10-oxoundecanoic acid to which the $NaIO_4$-$KMnO_4$ solution had been added were nol decolorized during several days at 28°C. Four hours after the last addition of $KMnO_4$, the reaction mixture was centrifuged; the MnO_2 was stirred with very dilute aqueous Na_2CO_3, centrifuged again and the combined supernatants decolorized by warming with a few drops of EtOH. After centrifugation this supernatant was acidified with H_2SO_4 and extracted 5 times with $CHCl_3$. The residue from the $CHCl_3$ solution (8 mg) was esterified with BCl_3-MeOH and analyzed by gas chromatography on an F and M model 700 dual column machine equipped with a thermal conductivity detector and a 180×0.65 cm column of 10 % SE-30 on Diatoport 60～80 mesh using He flow at 50 ml/min. The 5 essentially equal-sized peaks had retention times between 29 and 36 minutes when the column temperature was started at 80°C and raised 4°C/min. Peak areas indicated a total yield of about 1.5 mg. There were no peaks in the methyl decanoate range (about 20 minutes under these conditions). Low-boiling esters such as dimethyl oxalate would have been lost during evaporation of solvent.

Antimicrobial Assays. Weighed samples of the pure perchlorate salts were dissolved in $CHCl_3$ and transformed into the free base form by shaking with aqueous NH_3. The $CHCl_3$ solutions were evaporated at room temperature under a stream of CO_2 and the vacuum-dried residues dissolved in distilled dimethylsulfoxide. Assays were carried out using the agar-streak dilution method[13] on nutrient agar+0.5 % NaCl and 1.0 % glucose. Each assay was run twice and compared to a solvent control. All were incubated at 28°C. Final readings were made at 24 hours for bacteria, 48 hours for mycobacteria and some filamentous fungi, 5 and 7 days for *Ceratostomella ulmi*, *Trichophyton mentagrophytes* and all actinomycetes.

None of the compounds tested had any activity against *Escherichia coli* 54, *Proteus vulgaris* 73, *Candida albicans* 204, *Trichoderma koningi* 55 or *Penicillium notatum* 40. Prodigiosin was inactive against *Pseudomonas aeruginosa* 77. The concentrations used for **I** were 50, 25, 10, 5 and 1 μg/ml of agar; the other three were tested at 50, 25 and 1 μg/ml. Dilutions were in dimethylsulfoxide.

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