IDENTIFICATION OF A RED PIGMENT FROM *STREPTOMYCES COELICOLOR* A3(2) AS A MIXTURE OF PRODIGIOSIN DERIVATIVES

Sir:

As part of a broad program on the genetics of *Streptomyces coelicolor* A3(2)[1], RUDD and HOPWOOD[2] carried out genetic studies on a non-diffusible red mycelial pigment produced by this organism. By UV irradiation of strain B18, a mutant blocked in the synthesis of the diffusible pigment actinorhodin[3], they obtained a series of red$^-$ mutants. These all mapped in a narrow region of the chromosome and were divided into 5 mutant classes based on cosynthesis studies. Class A served as a converter to B, C and E, classes B and C gave red pigment production in mixed culture and each served as secretor to A, as did class E, and class D showed no cosynthesis with any other class. Additional genetic work on this system has been reported by FEITELSON and HOPWOOD[4]. In the present note we report on the chemical nature of the red pigment.

Experimental

Materials

The following mutant strains of *S. coelicolor* A3(2), obtained from the laboratory of Prof. HOPWOOD, were used: *act* mutants B18[3] and 2827, a new prototrophic recombinant strain carrying the marker *act* 3; *red* mutants B707 (class A), B717 (class B), B701 (class C), B713 (class D) and B229 (class E). Other cultures were obtained from Prof. H. ZÄHNER, Tübingen (*S. collinus* Tü 105 and Tü 353) or from the culture collection of this laboratory (*S. griseus, S. aureofaciens, Bacillus subtilis*). *Streptomyces* strains were maintained as spore suspensions in 20% glycerol at −20°C. Reference samples of undecylprodigiosin were obtained from Profs. H. H. WASSERMAN at Yale University and N. N. GERBER at Rutgers University. 3-Methoxy-5-phenylpyrrole-2-carboxaldehyde was a gift from Prof. E. CAMPAIGNE, Indiana University.

Fermentations

The red pigment complex was produced in liquid culture with strains B18 or 2827 in tomato - oatmeal medium (2 g Heinz or Gerber baby oatmeal, 2 g Contadina fancy tomato paste, 100 ml boiling tap water). Fermentations were carried out either in 500-ml Erlenmeyer flasks containing 100 ml medium agitated at 300 rpm and 30°C on a New Brunswick rotary shaker for 7 days or in a 14-liter New Brunswick Microferm fermentor containing 10 liters medium and 10 ml 50% polypropylene glycol as antifoam incubated for 2 weeks at 30°C with aeration (1 liter/minute) and agitation (300 rpm).

The mutasynthetic red pigment was produced in 500-ml Erlenmeyer flasks containing 100 ml "*Streptomyces* Complete Medium" (CM)[3] incubated at 28°C and 300 rpm on a rotary shaker. 3 - Methoxy - 5 - phenylpyrrole- 2 -carboxaldehyde (10 mg) dissolved in a minimal amount of EtOH was added to 5 days old cultures of strain B229 and the cultures were harvested 48 hours later.

Mutasynthetic Experiments

Cultures were inoculated in separate patches of 1.3×1.3 cm on CM agar medium on Petri dishes (5 *red* mutant classes on one dish or one strain per dish) and grown up for 6 days at 30°C. Filter paper discs of 0.6 cm diameter were each impregnated with 1 mg, 5 mg (3-methoxy-5-phenylpyrrole-2-carboxaldehyde) or 20 mg (pyrrole-2-carboxaldehyde) of a potential mutasynthetic precursor. Each disc was placed on the edge of a culture lawn so that half of it covered the culture and half lay on top of the medium. The plates were further incubated at 30°C and changes in pigmentation were observed. Cultures of strains B18 and 2827 served as reference.

Pigment Isolation

At the end of the fermentation, cultures were filtered, and the washed mycelium was lyophilized and then extracted overnight with MeOH in a Soxhlet extractor. The MeOH extract was concentrated, diluted with H_2O and extracted several times with $CHCl_3$. The combined $CHCl_3$ solutions were washed with dilute acid, dilute base and H_2O, dried over sodium sulfate and evaporated to give the crude extract. The pigments were then isolated by medium pressure LC on Michel-Miller columns[5] packed with silica gel LP-1 (Whatman) impregnated with sodium bicarbonate, using benzene containing 0~10% EtOAc as solvent for step gradient separation. The development of the column was followed by analytical TLC (silica gel, $CHCl_3$ - MeOH, 19:1, containing a few drops of conc NH_4OH, orange pigment Rf 0.83, pink pigment 0.54, purple pigment 0.05) and fractions containing pure single

Table 1. Spectral data for the isolated orange and pink pigments and reported values for butylcycloheptylprodiginine.

	Orange pigment	Pink pigment	Reported for butylcycloheptylprodiginine
UV $\lambda_{max}(\varepsilon)$	530 (sh, 6,700), 462 nm (40,100) in 0.5% NaOH - MeOH HCl salt: 530 (100,500), 502 nm (sh, 40,500) in MeOH	541.5 nm (98,000) in acidic $CHCl_3$	542 nm in acidic $CHCl_3$
MS (m/z)	393 (M^+), 378, 364, 350, 336, 322, 308, 294, 280, 266, 253, 252, 238, 221, 127, 91	391 (M^+), 376, 348, 334, 175, 91	391 (M^+), 376, 348, 334, 175, 91
^1H NMR (δ, ppm)	1.24 (t, 3H), 1.33 (b, 18H), 2.85 (t, 2H), 4.01 (s, 3H), 6.20 (d, 1H), 6.34 (m, 1H), 6.55 (m, 1H), 6.76 (m, 1H), 6.90 (m, 1H), 6.97 (s, 1H), 7.23 (m, 1H)	0.9 (t, 3H), 1.2~1.3 (b, 16H), 2.30 (t, 2H), 3.1 (m, 1H), 3.92 (s, 3H), 6.09 (s, 1H), 6.34 (m, 1H), 6.38 (s, 1H), 6.68 (d, 1H), 6.91 (m, 1H), 7.06 (s, 1H)	0.9 (t, 3H), 1.2 (b, 16H), 2.3 (t, 2H), 3.1 (m, 1H), 3.9 (s, 3H), (low field signals not reported)

components were pooled and evaporated to dryness. Fractions containing mixtures were further resolved by preparative layer chromatography ($NaHCO_3$-impregnated silica gel, 1 mm thick, benzene - EtOAc, 9: 1, orange pigment Rf 0.48, pink pigment 0.21, purple pigment 0.09).

Spectroscopy

UV and IR spectra were recorded on Gilford 250 and Beckman IR-33 spectrophotometers, respectively. ^1H NMR spectra were obtained on Varian FT-80 and Nicolet NC-470 spectrometers. Low resolution mass spectra (EI or CI, isobutane) were recorded on a Dupont 21-492BR or a Finnegan 4000 mass spectrometer and high resolution and FD mass spectra on a Varian MAT 731 instrument.

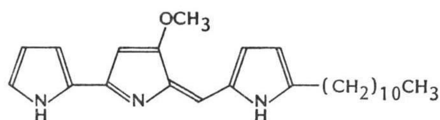
Results and Discussion

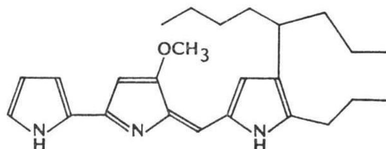
Extraction of the mycelium from shake cultures of *S. coelicolor* A3(2) mutants B18 and 2827 followed by TLC analysis of the extract showed that the red mycelial pigment represented a mixture of pigments. The major component was an orange pigment which had the highest Rf value, followed by a pink pigment and, only in mutant 2827 but not B18, traces of a low Rf purple pigment. Following optimization of culture conditions and isolation procedures, the pigments were obtained from larger scale cultures on tomato - oatmeal medium in amounts of 35~45 mg/liter for the orange pigment, 20~25 mg/liter for the pink pigment and less than 3 mg/liter for the purple pigment.

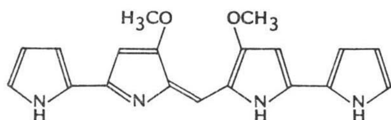
Spectral characterization of the orange pigment indicated a molecular formula $C_{25}H_{35}N_3O$ and gave data (Table 1) which closely matched those published for undecylprodigiosin (I)[6~8]. Direct comparison (IR, mixed mp) with an authentic reference sample obtained from Prof. WASSERMAN confirmed the identity of the orange pigment with undecylprodigiosin.

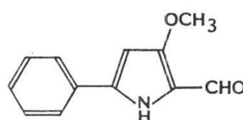
The pink pigment showed a molecular weight of 391 and spectral data (Table 1) closely matching those reported for butylcycloheptylprodiginine (II)[9]. The characteristic mass spectral fragmentation pattern distinguishes it from the isomeric metacycloprodigiosin[10], also a natural product[10,11]. Although no reference sample was available for a direct comparison, the spectral data strongly support the identity of the pink pigment with butylcycloheptylprodiginine. Both compounds I and II have been isolated from *Streptomyces* species before[6~9,12,13]. In fact, an unidentified prodigiosin-type pigment isolated in BROCKMANN's laboratory[14] from an Actinomycete later classified as *S. coelicolor*[15] shows spectral characteristics resembling those of the orange pigment undecylprodigiosin.

The amounts of the purple pigment obtained were insufficient for adequate spectral characterization. The acid-base indicator properties and the chromatographic behavior are similar to those reported for a pigment first isolated by GREEN *et al.*[16] and later identified by WASSERMAN *et al.*[17] as a dipyrrolyldipyrromethane analog (III) of prodigiosin. However, any relationship between these compounds is very tenuous,









because no reference material could be obtained for a direct comparison.

The biosynthesis of prodigiosins, as shown by studies in *Serratia marcescens* as well as in *Streptomyces,* proceeds by a bifurcated pathway[18~20]. One branch produces a bipyrrolaldehyde and the other a substituted monopyrrole; these two components then condense to form the prodigiosin pigments. RUDD and HOPWOOD[2] had shown that their five classes of *red* mutants all mapped in a narrow region of the *S. coelicolor* chromosome, suggesting either that all these mutants are in one branch of the pathway or that the genes for both branches are in one cluster. To prove this point we carried out some mutasynthetic experiments with these mutants.

Earlier work with *S. marcescens* had led to the incorporation of a variety of monopyrrole analogs[21,22], but no modifications in the bipyrrole moiety were achieved. When we tested pyrrole and 2-formylpyrrole with the five mutants, using the cosynthesis technique described by RUDD and HOPWOOD[2], no red pigment formation was observed. However, when 3 methoxy-5-phenyl-2-pyrrolecarboxaldehyde (**IV**), an analog of the bipyrrolealdehyde precursor, was tested, red pigment formation was seen with mutants of classes A, B, C and E. Class D, thought to be a regulatory mutant[2], gave a weak, ambiguous response. Compound **IV** also induced red pigment formation in three other *Streptomyces* strains, *S. collinus* Tü 105 and Tü 353 and *S. griseus* ATCC 12648, but not in *S. aureofaciens* B 1287 and in *B. subtilis*. A small sample of the induced red pigment was isolated from a liquid culture of a class E mutant supplemented with **IV**. The mass spectrum showed a molecular weight of 390 and the ^{1}H NMR spectrum indicated the presence of a phenyl, a methoxy, an aliphatic methyl and several methylene groups. These limited spectroscopic data clearly indicate that the compound is the product of mutasynthetic conversion of **IV** into a prodigiosin-like material.

The above results identify HOPWOOD's "red pigment" as a mixture of, predominantly, undecylprodigiosin and butylcycloheptylprodiginine. They also present what appears to be the first mutasynthetic modification of the bipyrrole moiety of prodigiosin. Finally, they demonstrate that the *red* mutants of classes A, B, C and E, isolated by RUDD and HOPWOOD[2], are probably all blocked in the bipyrrolealdehyde branch of the pathway and that the ability to synthesize monopyrrole moieties and to condense them with a suitable bipyrrolealdehyde analog is more widespread in Streptomycetes.

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