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PRODIGIOSENE

by

Michael Kahler Elson

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In Charge of Major Work

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Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

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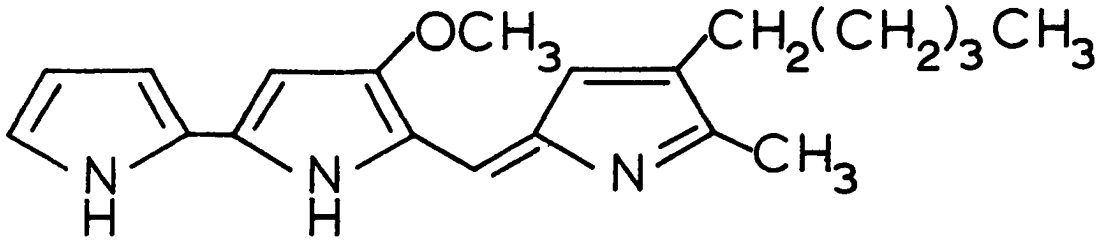
INTRODUCTION

Prodigiosin (I) is the red pyrrolyldipyrrolylmethene pigment produced by the wild type of the microorganism *Serratia marcescens*. Its biosynthesis and antimicrobial properties have been a source of continuing interest. Prodigiosin was the subject of a recent review (32).

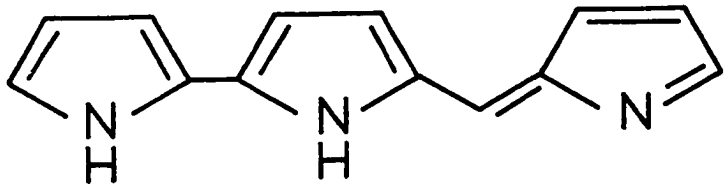


I

Prodigiosin-like pigments, differing only in the alkyl substituents on the monopyrrole portion of the pigment, have also been isolated from certain species of *Streptomyces* and *Actinomyces*. All crude extracts of prodigiosin and prodigiosin-like pigments have been noted to contain additional pigments, none of which has been fully characterized.

Several nomenclature systems are in use, one for the prodigiosin-like pigments from *Streptomyces* and the other for a series of substituted pyrrolyldipyrrolylmethenes with the same basic ring structure of prodigiosin but without the methoxyl group.

In this investigation the unsubstituted pyrrolyldipyrrolylmehtene II was synthesized.



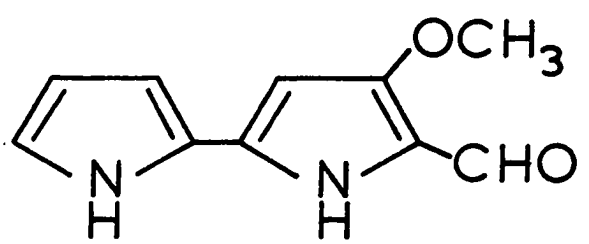
II

Prodigiosene is proposed as a name for compound II and as the basis for a uniform nomenclature system for prodigiosin and the prodigiosin-like pigments. A series of substituted prodigiosenes was also synthesized and their visible, ultraviolet, infrared, nuclear magnetic resonance, and mass spectra are reported.

REVIEW OF LITERATURE

Prodigiosin was first isolated by Wrede from *Serratia marcescens*. Wrede and co-workers (35, 33, 34) prepared several crystalline salts of the pigment. By oxidation of the pigment and its degradation products Wrede (36, 37, 38, 39) was able to identify correctly the three pyrrolic constituents of the pigment: pyrrole, 3-methoxypyrrole and 2-methyl-3--amylpyrrole (MAP). Wrede proposed two possible structures, one a pyrrolyldipyrrolylmethene, the other a tripyrrolylmethene. On the basis of available spectral data, Wrede incorrectly chose the tripyrrolylmethene structure without further work.

In 1965 Santer and Vogel (22) isolated a prodigiosin precursor from strain 9-3-3, a *Serratia* mutant that does not produce pigment. This precursor, which could condense with MAP to yield prodigiosin (26), was identified as 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde (MBC), structure III.



III

In 1962 Rapoport and Holden (20) unequivocally confirmed the structure of prodigiosin by synthesizing MBC and condensing it with synthetic MAP.

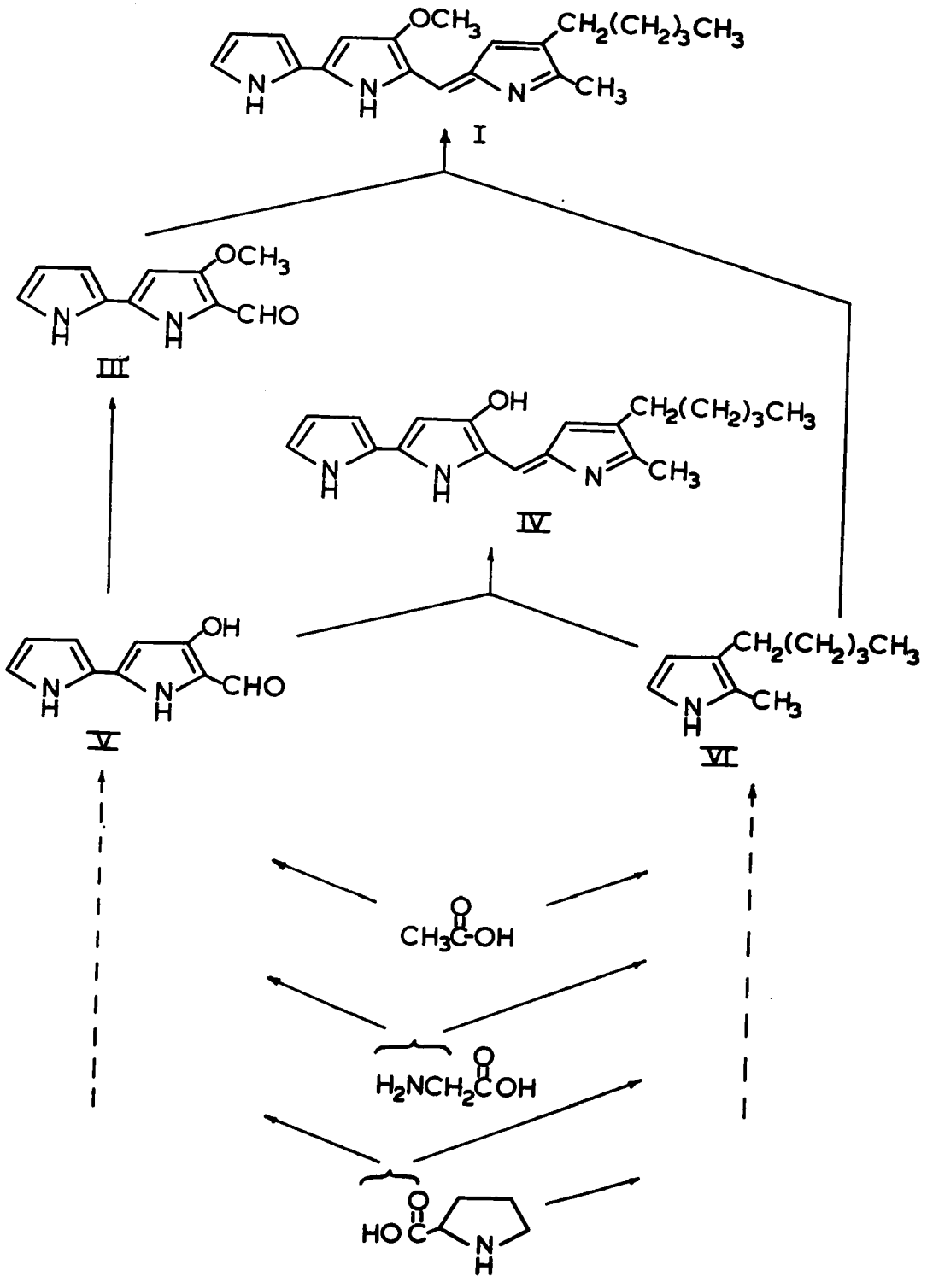
Establishment of the structure of prodigiosin has renewed interest

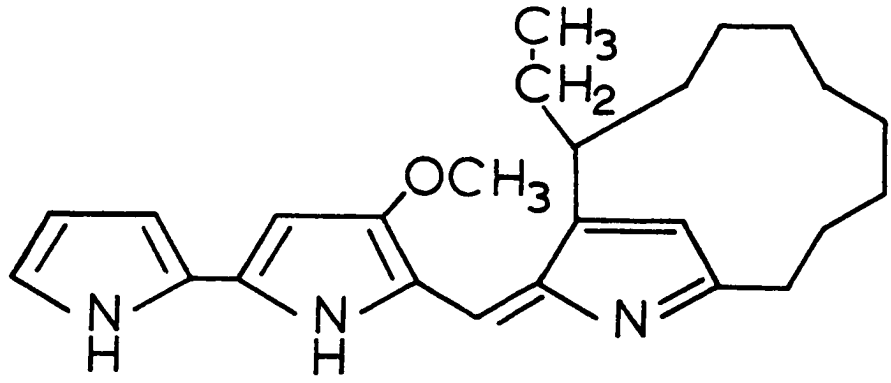
in biosynthetic investigations. The only firmly established step in the biosynthesis of prodigiosin is the coupling of MAP and MBC as shown in Figure 1. Mutants are available that have metabolic blocks at various points on the path to prodigiosin (17) but elucidation of the pathway is hampered by the fact that only small amounts of unstable precursors are produced. One such mutant, strain OF, produces an orange pigment norprodigiosin (IV) that may be converted to prodigiosin by methylation with diazomethane (13). From the broth of strain OF, Burgus (5) isolated a compound identified as 4-hydroxy-2,2'-bipyrrole-5-carboxaldehyde (HBC), structure V, because it yields MBC after treatment with diazomethane.

At the other end of the pathways to prodigiosin, radioisotope techniques have indicated that acetate and glycine (but not the glycine carboxyl) are incorporated into prodigiosin (14). Proline is a better pigment precursor than glycine. The carboxyl carbon of proline is incorporated into the MAP (VI) and MBC (III). The methylene carbon of glycine is incorporated equally into the bipyrrole and monopyrrole portions of the pigment (23). The pathway to prodigiosin appears to be bifurcated but seems to have a common or closely related starting point (17).

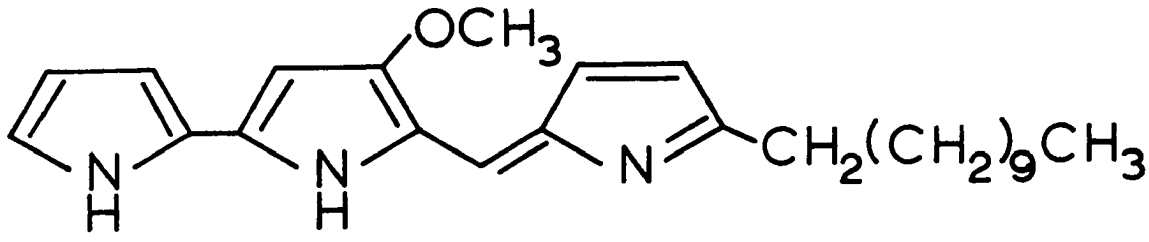
Prodigiosin itself is not limited to *Serratia*. It has been isolated (16) and identified (21) from a marine organism that is not *Serratia*. An *Actinomycete* has also been reported to produce prodigiosin (19). A pseudomonad bacterium is reported to produce a prodigiosin-like pigment (11). Prodigiosin-like pigments have been found in *Actinomyces* and *Streptomyces* strains. Pigments VII and VIII were isolated by Rodgers (21) and Wasserman (27). Pigment VIII is identical to the pigment characterized by Harashima (12).

Figure 1. Scheme for biosynthesis of prodigiosin by *Serratia marcescens*


OCH3
CH2(CH2)3CH3
N
H
N
H
N
CH3
I
OCH3
CHO
III
OH
CH2(CH2)3CH3
IV
OH
CHO
V
CH2(CH2)3CH3
CH3
VI
O
CH3C-OH
O
H2NCH2COH
O
HOC
N
H

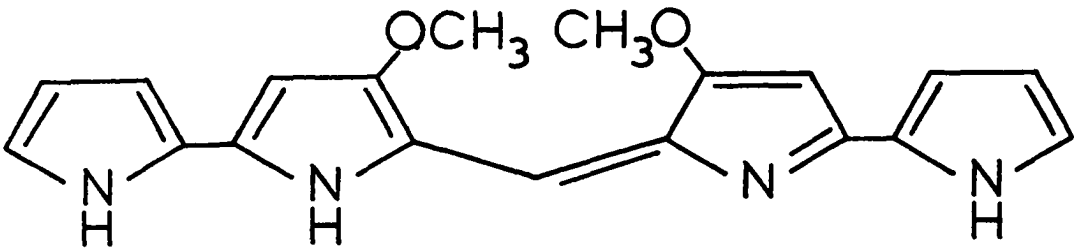


VII



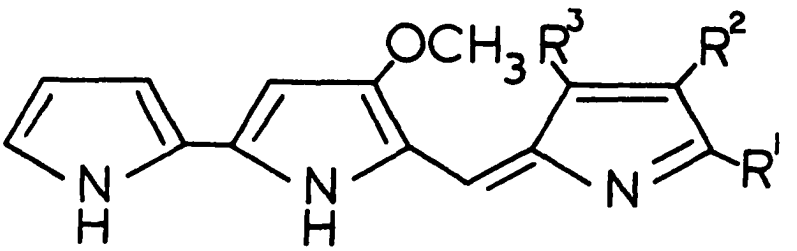
VIII

A pigment related to prodigiosin has been isolated from *Serratia marcescens* strain 9-3-3 by Feider (10) and from *S. marcescens* strain 114 by Wasserman (26) who showed IX to be its structure. Wasserman showed that the pigment was produced *in vitro* by condensation of MBC and 4-methoxy-2,2'-bipyrrole. He suggested that 4-methoxy-2,2'-bipyrrole might be a precursor to MBC in the prodigiosin biosynthesis pathway but noted that MBC will deformylate in acidic conditions. Castro (8) also noted this phenomenon with formylmonopyrroles.



IX

Other prodigiosin-like pigments have been produced by adding alkyl pyrroles to the growth media of strain 9-3-3, which condenses them with MBC (28,29). Pyrroles used were 2-methylpyrrole, 2,4-dimethylpyrrole, and 2,4'-dimethyl-3-ethylpyrrole, giving structures X, XI, and XII respectively. Strain 9-3-3 was not able to incorporate unsubstituted pyrrole.

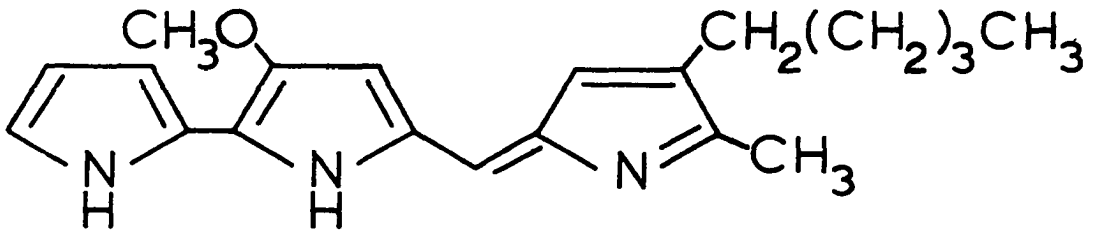


X $R^1=CH_3$ $R^2=H$ $R^3=H$

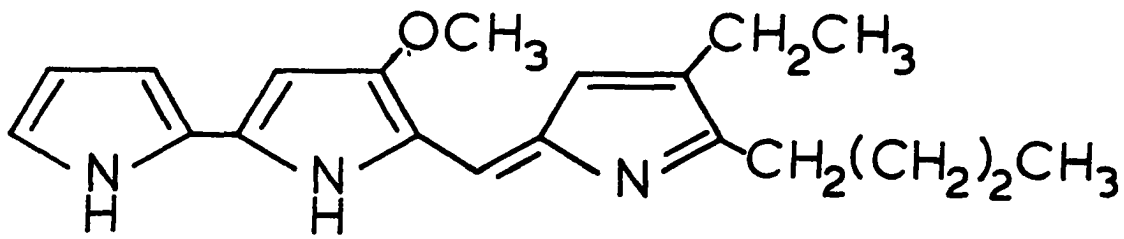
XI $R^1=CH_3$ $R^2=H$ $R^3=CH_3$

XII $R^1=CH_3$ $R^2=CH_2CH_3$ $R^3=CH_3$

Two isomeric prodigiosins were synthesized by Rapoport and co-workers in their synthesis of prodigiosin. The compound 3-methoxy-2,2'--bipyrrole-5-carboxaldehyde was condensed with MAP to give structure XIII. A byproduct of their MAP synthesis was 2-butyl-3-ethylpyrrole which was condensed with MBC to yield structure XIV.



XIII



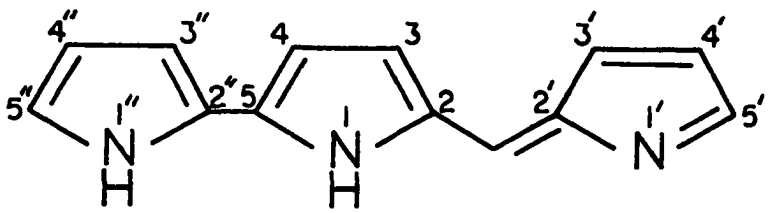
XIV

A constant theme running through all of the reports on prodigiosin and prodigiosin-like pigments is the occurrence of several pigment fractions, with the red prodigiosin type usually the main fraction. Burgus reported chromatographic separation of prodigiosin extracts into three to five fractions. Rechromatography of these fractions yielded similar elution patterns. The Williams group (31) demonstrated the presence of a blue pigment, isolated from old *Serratia marcescens* cultures, that has a Rast molecular weight of 775. The pigment was postulated to be a dimer of

prodigiosin (molecular weight 323). Several colored fractions were reported in the purification of the "syntrophic" pigments produced when one mutant supplied precursors to another (28).

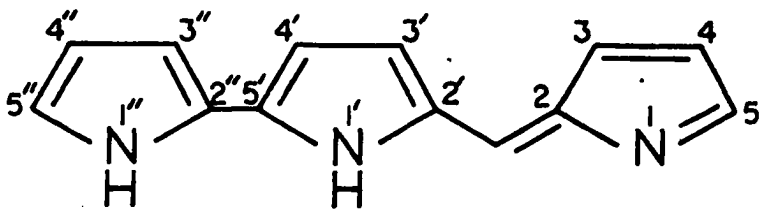
The mass spectrum of prodigiosin was reported to be quite simple by A. J. Jackson *et al.* (15) in the second paper of a series on the mass spectrometry of pyrroles. The parent ion was the base peak. A weak ion was observed for a loss of 15 from the parent ion. The only intense ion, other than the parent ion, correspond to a loss of 57, the cleavage of C_4H_9 from the amyl side chain. Harashima reported the mass spectrum of the prodigiosin-like pigment (12) isolated from a strain of *Streptomyces*. Again, the parent ion was the base peak. Then a weak ion for a loss of 15 was observed and the ion corresponding to M-141 was the second most intense peak. Loss of the alkyl side chain seems to be the most characteristic fragmentation in the mass spectrum of prodigiosin-type pigments.

There have been reports of synthesis of pyrrolyldipyrrolylmethene compounds similar to prodigisin, but without a methoxyl substituent. Bullock *et al.* (4) synthesized a number of pyrrolyldipyrrolylmethenes in their investigations of modified porphyrin ring systems. Their bipyrrole compounds were synthesized via the Ullmann reaction that affords only symmetrically substituted bipyrroles. Their highly substituted pyrrolyldipyrrolylmethenes were prepared by condensing a formylbipyrrole with a monopyrrole, or a bipyrrole with a formylmonopyrrole. These pyrrolyldipyrrolylmethenes were named as pyrrolyl derivatives of dipyrrolylmethenes with the numbering system shown in structure XV.



XV

This numbering system is in contrast with a nomenclature suggested by Harashima (12) for the prodigiosin-like pigment of their *Streptomyces* strain. The Japanese workers differentiated the prodigiosin-like pigments on the basis of number of carbons ("prodigiosin-C25") and order of isolation. Harashima's numbering system is illustrated in structure XVI.



XVI

In the paper on the synthesis of prodigiosin, Rapoport and Holden (20) did not use a special nomenclature system and referred to the prodigiosin isomers simply as isomers.

Ermili (9), who follows Bullock's nomenclature, synthesized two pyrrolyldipyrrolylmethenes from unsubstituted bipyrrole obtained by the method of Rapoport (19), and alkyl-substituted formylmonopyrroles. Elemental analysis and melting points but no spectral data were reported.

EXPERIMENTAL

Materials

Organism

Mutant strain WCF was supplied by Dr. Robert P. Williams of Baylor University College of Medicine. Strain WCF was obtained by ultraviolet irradiation of strain Nima, a wild-type strain of *Serratia marcescens* (30).

Medium

Culture medium was that of Williams and co-workers (31). It consisted of: yeast extract, 0.1 percent; enzymatic casein hydrolyzate, 0.2 percent; glycerol, 1.0 percent; ammonium citrate, 0.5 percent; magnesium sulfate, 0.05 percent; sodium chloride, 0.5 percent; ferric ammonium citrate, 0.005 percent; made up in deionized water. The final pH was adjusted to 7.1.

Instruments and reagents

All reagents used were either CP or Analytical grade, unless otherwise noted. Skelly B, chloroform, and acetone used for column chromatography were redistilled.

Pyrrole, 2-formylpyrrole, and 2,4-dimethyl-3-ethylpyrrole were obtained from Aldrich Chemical Company, 2371 North 30th Street, Milwaukee, Wisconsin 53210. The 2,4-dimethylpyrrole was obtained from K & K Laboratories, Inc., 1212 Express Street, Plainview, New York 11803.

Elemental analyses were performed by Ilse Beetz Midroanalytisches Laboratorium, 8640 Kronach, Postfach 460, West Germany.

Ultraviolet-visible spectra and absorbance data were obtained with

either a Cary Model 15 recording spectrophotometer, a Beckman DB recording spectrophotometer, or a Beckman DU spectrophotometer.

Infrared spectra were obtained with a Perkin-Elmer Model 21 double beam spectrophotometer.

Nuclear magnetic resonance spectra were obtained with a Varian Model A-60 spectrometer. Samples were dissolved in approximately 0.5 ml of deuterochloroform with tetramethylsilane as internal standard.

Mass spectra were obtained with an Atlas CH 4 mass spectrometer using a TO 4 ion source. All samples for mass spectral analysis were hydrobromide salts, unless otherwise noted.

The pH measurements were made with a Beckman Model 76 expanded scale pH meter.

Melting point determinations, uncorrected, were made with a Mel-Temp capillary melting point apparatus.

Concentration of solutions was accomplished on a Büchi Rotavapor with a bath temperature of not more than 35° unless otherwise noted.

General Methods

Cultures

Frozen stock and working cultures Frozen working cultures of strain WCF were prepared by inoculating 3 ml of sterile Williams' medium in 15-ml screw-cap tubes. Inoculum was from Williams' agar stock slants (Williams' medium with 2.0 percent agar). The inoculated tubes were allowed to grow for 25 hours at room temperature, then quick-frozen in a dry ice-acetone bath. The frozen working cultures were stored in a freezer until needed.

Inocula Cultures for inoculation of shake flasks were prepared by inoculating 10 ml of sterile Williams' medium in 15-ml screw-cap tubes with a loop of strain WCF from a quick-thawed working culture. The inoculum culture was grown in the dark at room temperature for 24 hours and then used to inoculate shake flasks.

Broth cultures Two-liter Erlenmeyer flasks each containing 400 ml of Williams' medium were stoppered with cotton plugs and autoclaved. Each flask was aseptically inoculated with 5 ml of a Williams' medium 24 hour inoculum tube of strain WCF. The flasks were shaken in the dark for 24 hours in a New Brunswick Scientific Company Model G-25 gyrotory incubator at room temperature.

Thin-layer chromatography

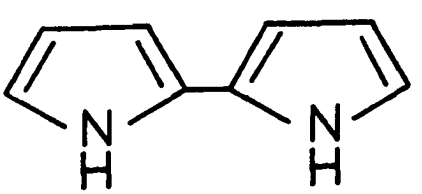
Analytical thin-layer chromatography Progress of synthetic reactions and of purification procedures was followed by using thin-layer chromatography. Plates were prepared using 3 × 1 inch glass microscope slides. The plates were coated with E. Merck Silica Gel G or Silica Gel GF 254. Five g of the silica gel was vigorously shaken with 10 ml of distilled water. The resulting slurry was spread evenly over 20 microscope slides with an aluminum spreader and slide-holding tray made in the chemistry shop of Iowa State University. The coated plates were air-dried and activated in an over at 110° for 30 minutes. Activated plates were stored in a desiccator over $CaCl_2$ until needed. Samples were applied to the plates with micropipettes made by drawing out capillary tubes. The plates were developed in small solvent-saturated chambers. For preservation, thin-layer chromatograms were sprayed with Neatan

(E. Merck). After drying of the Neatan, the thin-layer was removed from the plate with Scotch transparent tape.

Spray reagents Visualization of the monopyrroles and bipyrroles on the thin-layer plates was accomplished with an Ehrlich spray reagent. Acid catalyzed condensation of p-dimethylaminobenzaldehyde with the free alpha position of the monopyrrole or bipyrrole gives a colored dye (24). Color development was rapid at room temperature unless otherwise noted. The spray reagent consisted of one g of p-dimethylaminobenzaldehyde dissolved in 100 ml of 1 N methanolic HCl (8.4 ml of concentrated HCl diluted to 100 ml with absolute methanol).

Column chromatography

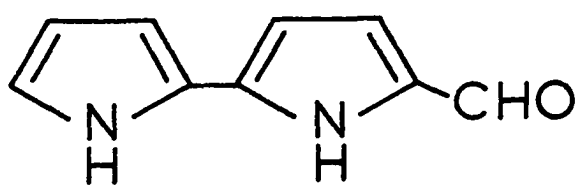
Kontes Chromaflex columns, fitted with sintered glass disks at the bottom and with teflon stopcocks were used. Columns were prepared by pouring a slurry of sorbent and solvent into the column and allowing it to pack by settling and solvent flow. A 2-mm pad of sand was added to the top of the sorbent to stabilize it. The prodigiosin and prodigiosin analogs prepared by R. H. Williams (28) were chromatographed on Hyflo Supercel (Johns Manville Co.) by his method. Some prodigiosene pigments were chromatographed on silicic acid: Celite (2:1), eluted with 10 percent acetone in chloroform.



2,2'-Bipyrrole

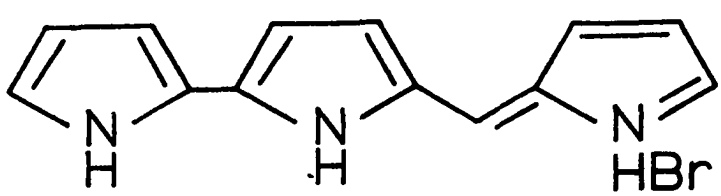
The method of Rapoport and Castagnoli (20) was followed with some modification. A mixture of 20 g of 2,2'-(pyrrolin-1'-yl)-pyrrole (20) and 10 g of 10 percent palladium-on-charcoal in 1000 ml of xylene was refluxed under nitrogen with vigorous stirring. After 48 hours the hot solution was filtered and the catalyst was digested twice with hot xylene. The combined solutions were cooled to room temperature and were extracted with an aqueous solution of KH_2PO_4 to recover any unreacted 2,2'--pyrrolinylpyrrole. Alkalization of the aqueous extract gave a flocculent precipitate that was extracted into methylene dichloride. The methylene dichloride was dried and evaporated on the Rotovapor to give a small amount of the 2,2'-pyrrolinylpyrrole.

The xylene solution, containing the bipyrrole, was dried and evaporated on the Rotavapor. The residue was digested with 50 ml of chloroform to remove any 2,2'-pyrrolinylpyrrole not previously extracted, 2,2'-bipyrrole being relatively insoluble in chloroform. Filtration of the digest and sublimation of the insoluble portion at 90° (0.2 mm Hg) yielded the bipyrrole. The 2,2'-bipyrrole was crystallized from benzene. Yield, 7.5 g; mp, 189-190°.



5-Formyl-2,2'-bipyrrole

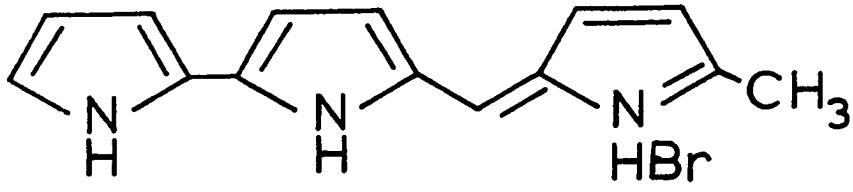
Preparation of 5-formyl-2,2'-bipyrrole was similar to the method used by Bullock *et al.* (4). One g of 2,2'-bipyrrole was dissolved in 10 ml of dimethylformamide in a three-necked flask fitted with a stirrer, nitrogen sweep, and a dropping funnel. The flask was cooled in an ice bath. A complex of 1.16 g of $POCl_3$ and one ml of dimethylformamide was added dropwise over a 15 minute period. The reaction mixture initially turned green on addition of the complex and then solidified. After addition was completed the ice bath was removed and the reaction mixture was then dissolved in water and 10 percent NaOH was added until the odor of dimethylamine was noticed. The mixture was then warmed slightly and the aldehyde was precipitated (occasionally it was necessary to add more NaOH). The aldehyde was obtained by filtration, dissolved in ethanol, decolorized with charcoal, and crystallized from an ethanol-water system. Yield, 0.74 g; decomp, 234-240°.



Prodigiosene

A solution of 132 mg of 2,2'-dipyrrole and 95 mg of 2-formylpyrrole in 5 ml of ethanol was warmed on a steam bath. Seven drops of 48 percent HBr were added. The solution immediately became deep purple. The hydrobromide salt crystallized in about 30 minutes. The solution was filtered.

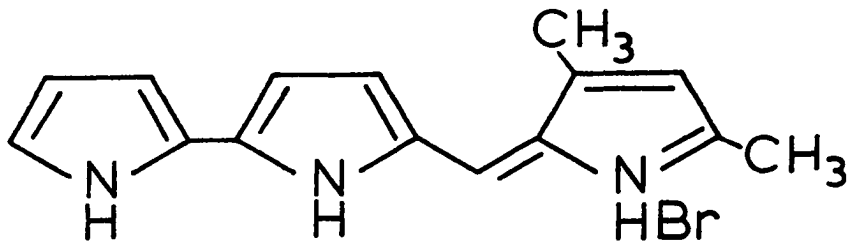
The insoluble portion was dissolved in a small amount of warm chloroform. The chloroform solution was filtered and an equal volume of Skelly B was added dropwise while the solution was warmed on the steam bath. The solution was filtered to remove amorphous precipitated material. More Skelly B was added dropwise to the warmed solution until crystallization began. The solution was cooled and the long metallic-blue needles were collected and vacuum-dried at 40°. Yield, 62 mg; mp, <360°. *Anal.* Calcd. for $C_{13}H_{12}N_3Br$: C, 53.8; H, 4.14; N, 14.5. Found: C, 53.71; H, 4.20; N, 14.44.



2-Methylprodigiosene

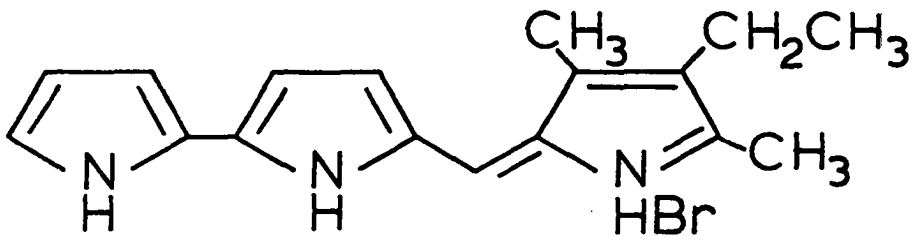
A mixture of 81 mg of 2-methylpyrrole and 160 mg of 5-formyl-2,2'--bipyrrole in 15 ml of ethanol was warmed on the steam bath until solution occurred. The solution was allowed to cool and then seven drops of 48 percent HBr were added. The solution immediately turned dark purple. After an hour the solvent was removed on the Rotovapor. The residue was dissolved in a small volume of warm chloroform, filtered and applied to a silicic acid:Celite column. Elution was accomplished with 10 percent acetone in chloroform. A small amount of blue pigment preceded the main fraction. The main fraction was collected, the solvent was evaporated, and the pigment was dissolved in a small amount of chloroform. An equal

volume of warm Skelly B was added and a precipitate formed. The solution was filtered and more Skelly B was added to the solution while scratching with a glass rod to promote crystallization. The solution was cooled and the purple crystals were collected. Yield, 42 mg. A second crop was also obtained. The crystals were vacuum-dried at 40°. Mp, 179-180°, decomp. *Anal.* Calcd. for $C_{14}H_{14}N_3Br$: C, 55.3; H, 4.61; N, 13.8. Found: C, 55.18; H, 4.60; N, 13.60.



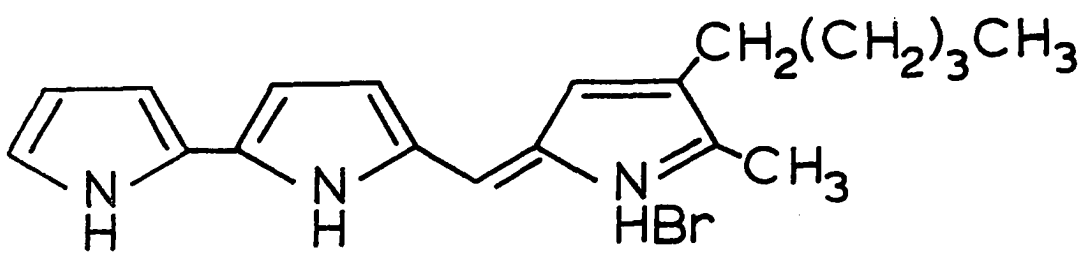
2,4-Dimethylprodigiosene

A mixture of 160 mg of 5-formyl-2,2'-bipyrrole and 95 mg of 2,4--dimethylpyrrole in 15 ml of ethanol was warmed on the steam bath until solution occurred. The solution was removed from the bath and allowed to cool. Seven drops of 48 percent HBr were added. The solution immediately turned dark purple. In 30 minutes the hydrobromide crystallized. The crystals were obtained by filtration and dissolved in a small amount of warm chloroform. The solution was filtered and an equal volume of warm Skelly B was added. The solution was filtered again to remove an amorphous precipitate. More Skelly B was added until crystallization started. The solution was allowed to cool, and after filtration the long blue needles were vacuum-dried at 40°. Yield, 116 mg; mp, 203-204.5°. *Anal.* Calcd. for $C_{14}H_{16}N_3Br$: C, 56.6; H, 5.04; N, 13.2. Found: C, 56.67; H, 5.10; N, 13.34.



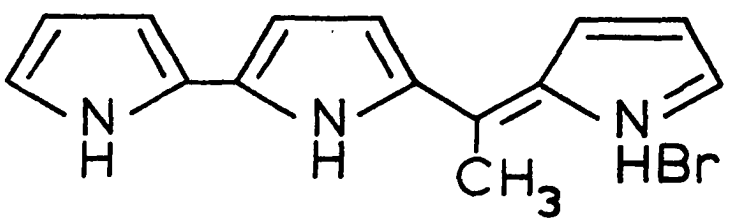
2,4-Dimethyl-3-ethylprodigiosene

A mixture of 160 mg of 5-formyl-2,2'-bipyrrole and 123 mg of 2,4-dimethyl-3-ethylpyrrole in 15 ml of ethanol was warmed on the steam bath until solution occurred. The solution was allowed to cool, and seven drops of 48 percent HBr were added. The solution immediately turned dark purple. In 20 minutes the hydrobromide crystallized. The crystals were obtained by filtration and dissolved in a small amount of warm chloroform. The chloroform solution was filtered and then an equal volume of Skelly B was added. The solution was filtered again to remove an amorphous precipitate. Warm Skelly B was added until crystallization started and then the solution was cooled. The green crystals were filtered and vacuum-dried at 40°. Yield, 164 mg; mp, 229.5-231°. *Anal.* Calcd. for $C_{17}H_{20}N_3Br$: C, 58.9; H, 5.78; N, 12.1. Found: C, 58.94; H, 5.84; N, 12.03.



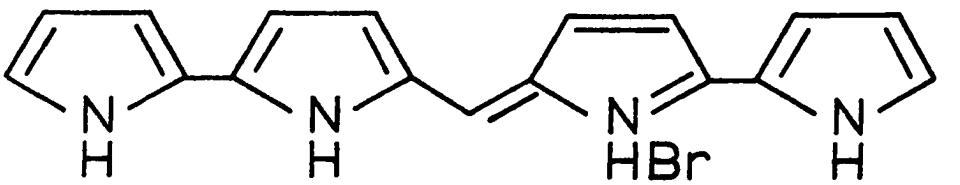
2-Methyl-3-amylprodigiosene

A sample of 2-methyl-3-amylpyrrole, derived from the reduction of 1 g of 2-methyl-3-valeroyl-5-ethoxycarbonylpyrrole according to Castro (7) but not distilled, was added to 160 mg of 5-formyl-2,2'-bipyrrole in 15 ml of ethanol. The mixture was warmed until solution occurred. The solution was cooled and placed under a nitrogen atmosphere, and then seven drops of 48 percent HNr were added. The solution immediately turned purple. After one hour the ethanol was removed on the Rotovapor. The residue was dissolved in a minimal volume of chloroform. The solution was filtered and placed on a silicic acid:Celite column. The 2-methyl-3--amylprodigiosene was eluted with 10 percent acetone in chloroform. The major band was followed by a fraction containing 2-methylprodigiosene. The 2-methyl-5-amylprodigiosene solution was evaporated on the Rotovapor. The residue was dissolved in a small volume of chloroform. Skelly B was added, while scratching with a glass rod, until crystallization started. The solution was cooled. The fine dark crystals were collected and vacuum-dried at 40°. Yield, 59 mg; mp, 123-123.5°. *Anal.* Calcd. for $C_{19}H_{24}N_3Br$: C, 60.7; H, 6.45; N, 11.4. Found: C, 60.86; H, 6.54; N, 11.24.



5-Methylprodigiosene

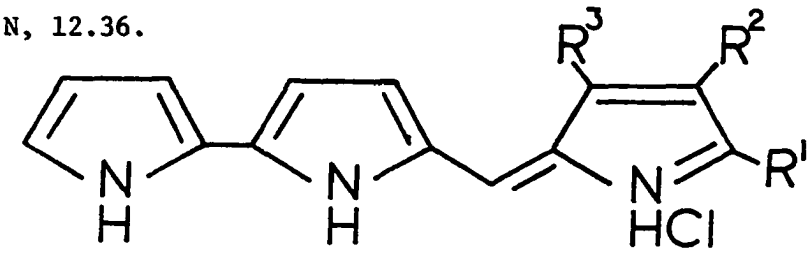
An ethanol solution of 132 mg of 2,2'-bipyrrole and 109 mg of 2-acetylpyrrole was brought to reflux under nitrogen. Seven drops of 48 percent HBr were added. The solution turned purple and slowly darkened. The solution was refluxed for 2 hours, then allowed to cool. Ethanol was removed on the Rotovapor. The residue was dissolved in a minimal volume of warm chloroform, filtered, and placed on a silicic acid: Celite column. The 5-methylprodigiosene was eluted with 15 percent acetone in chloroform. The eluted solution was evaporated and the residue was crystallized from chloroform-Skelly B. The small purple crystals were collected and vacuum-dried at 40°. Yield, 39 mg; mp, 208-209°. *Anal.* Calcd. for $C_{14}H_{14}N_3Br$: C, 55.3; H, 4.61; N, 13.8. Found: C, 55.44; H, 4.70; N, 13.76.



2-(pyrrol-2'-yl)-prodigiosene

A mixture of 160 mg of 2-formyl-2,2'-bipyrrole and 132 mg of 2,2'-bipyrrole in 15 ml of ethanol was warmed on the steam bath until solution occurred. The solution was allowed to cool and then seven drops of 48 percent HBr were added. The solution immediately turned blue. In 30 minutes the hydrobromide crystallized. Ethanol was removed on the Rotovapor. The residue was recrystallized from chloroform-Skelly B. The

crystals were vacuum-dried at 40°. Yield, 222 mg; decomp, 215°. *Anal.* Calcd. for $C_{17}H_{15}N_4Br$: C, 56.6; H, 4.25; N, 16.22. Found: C, 56.68; H, 4.23; N, 12.36.



R^1=CH R^2=$CH_2(CH_2)_3CH_3$ R^3=H

2-Methyl-3-amyl-6-methoxyprodigiosene

R^1=CH_3 R^2=H R^3=CH_3

2,4-Dimethyl-6-methoxyprodigiosene

R^1=CH_3 R^2=CH_2CH_3 R^3=CH_3

2,4-Dimethyl-3-ethyl-6-methoxyprodigiosene

For mass spectral analysis, samples of the three substituted prodigiosenes prepared by R. H. Williams (28) were dissolved in ethanol, converted to the free bases, and transferred to chloroform. The chloroform solutions were concentrated and chromatographed on Hyflo Supercel, eluted with 0.2 percent methanol in Skelly B. Pigment fractions were concentrated on the Rotovapor and transferred to chloroform. Anhydrous HCl was bubbled into the chloroform solutions. The amorphous hydrochlorides were precipitated by adding Skelly B to the solution while on the Rotovapor. The hydrochlorides were filtered, vacuum-dried at 40°, and submitted for analysis.

RESULTS AND DISCUSSION

Test of 5-Formyl-2,2'-Bipyrrole as a Prodigiosin Precursor

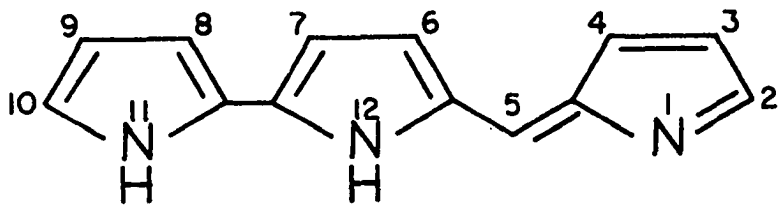
An ethanolic solution of 50 mg of 5-formyl-2,2'-bipyrrole was added to 24-hour cultures of strain WCF in Williams' broth. The flasks were restoppered and returned to the shaker. After 2 hours one of the flasks was removed for inspection. No pigment had been produced. A 0.5 ml portion of an ethanolic mother liquor from an MBC crystallization procedure was added. The flask was returned to the shaker. Within 15 minutes a pink color had formed indicating production of pigment. Another flask was removed after 6 hours. The culture appeared slightly green but no pigment was produced. Pigment was produced upon subsequent addition of MBC. The last flask was removed after 12 hours. Again, the culture appeared slightly green.

Evidently strain WCF is not able to condense 5-formyl-2,2'-bipyrrole with MAP. The presence of MAP and of the coupling enzyme was demonstrated by prodigiosin production on addition of MBC.

Since strain WCF excretes MAP into the medium, at the end of the experiment one ml of concentrated HCl was added to the culture filtrate. No prodigiosene pigment was produced on acidification, possibly indicating that the 5-formyl-2,2'-bipyrrole had been taken up or modified by the bacterium. That is, the bipyrrole was not available to condense with MAP non-enzymatically (after 36 hours). Very dilute aqueous ethanol solutions of 5-formyl-2,2'-bipyrrole and 2,4-dimethyl-3-ethylpyrrole give a purple color when acidified.

Nomenclature

Condensation of 2,2'-bipyrrole and 2-formylpyrrole gave the unsubstituted pyrrolyldipyrrolylmethene, structure II.



II

We propose the name prodigiosene for this compound because it contains the basic skeleton of prodigiosin. The name prodigiothene (indicating the methene structure) was rejected thince it ith hard to thay. The compound was numbered beginning with the monopyrrole because this is where the substituents vary in the naturally occurring prodigiosin-like pigments isolated so far. Thus, prodigiosin and the prodigiosin-like pigments form a series of 6-methoxyprodigiosenes. In our system, Bullock's (4) ethyl 5-(3",4"-dimethylpyrrol-2"-yl)-3,3'4,5'-tetramethylprodigiosene--3-carboxylate, which is easier to visualize.

Spectra

Visible-ultraviolet spectra

The visible-ultraviolet spectra of the synthetic prodigiosenes in acid and base, Figures 2 through 5, may be compared to those of the 6-methoxyprodigiosenes obtained by R. H. Williams (28). The data are summarized in Table I.

Figure 2. Ultraviolet-visible spectra

Top. Prodigiosene

——— Spectrum in ethanol 0.01N in HCl

---- Spectrum in ethanol 0.01N in NaOH

Bottom. 5-Methylprodigiosene

——— Spectrum in ethanol 0.01N in HCl

---- Spectrum in ethanol 0.01N in NaOH

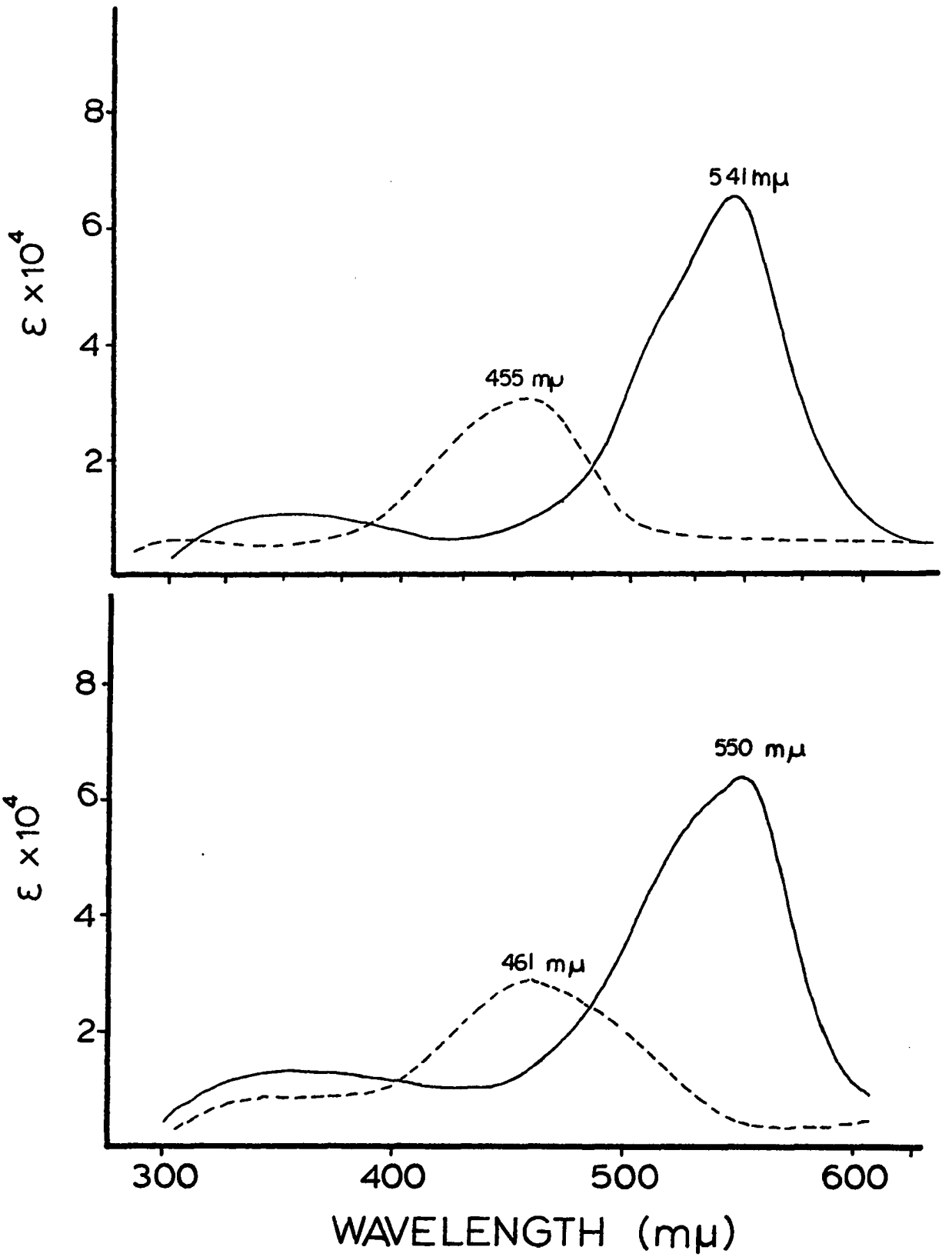

8
6
4
2
ε x10⁴
541mμ
455 mμ
8
6
4
2
ε x10⁴
550 mμ
461 mμ
300
400
500
600
WAVELENGTH (mμ)

Figure 3. Ultraviolet-visible spectra

Top. 2-Methylprodigiosene

——— Spectrum in ethanol 0.01N in HCl

---- Spectrum in ethanol 0.01N in NaOH

Bottom. 2,4-Dimethylprodigiosene

——— Spectrum in ethanol 0.01N in HCl

---- Spectrum in ethanol 0.01N in NaOH

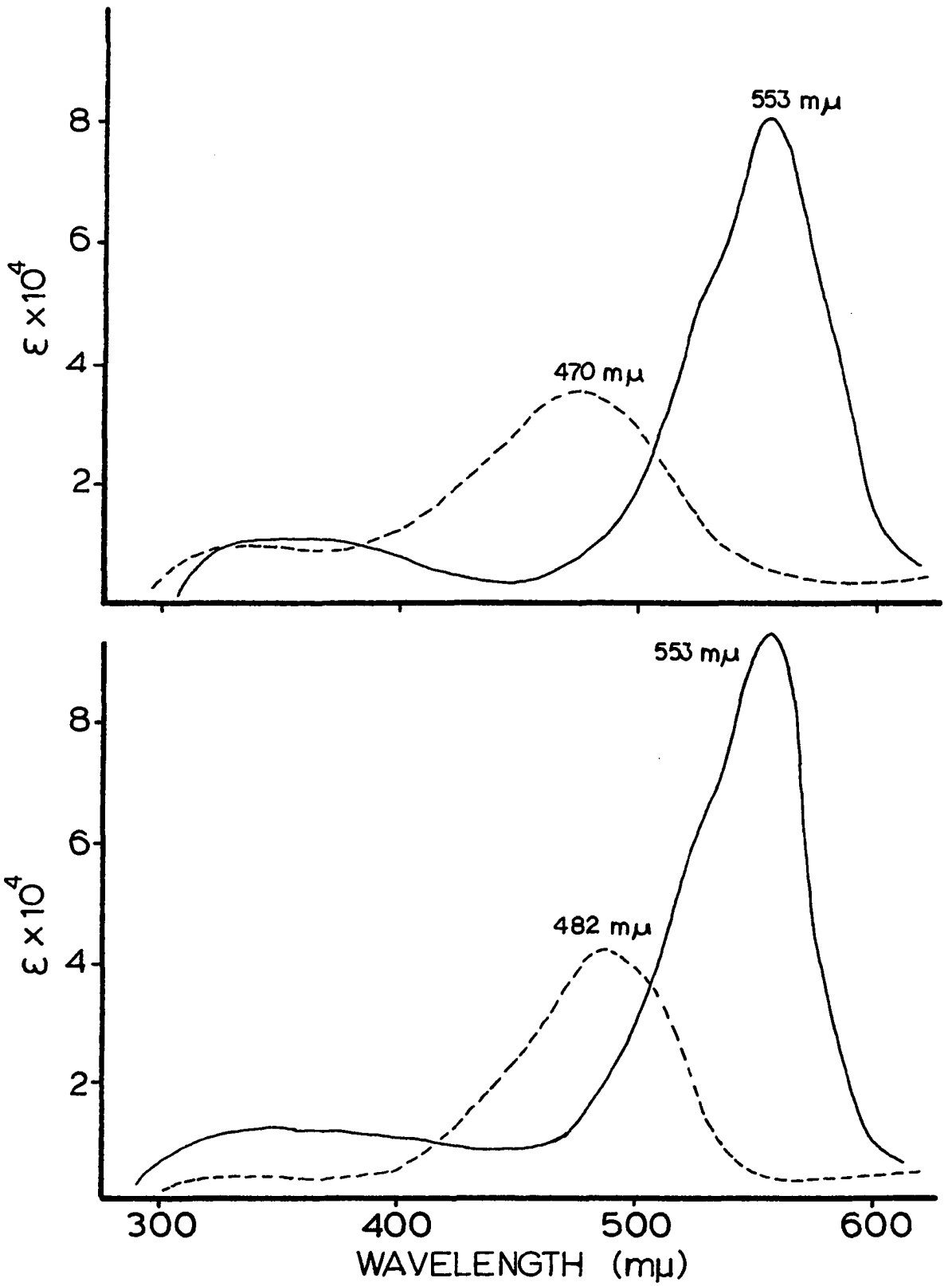

553 mμ
470 mμ
ε x 10^4
8
6
4
2
553 mμ
482 mμ
ε x 10^4
8
6
4
2
300
400
500
600
WAVELENGTH (mμ)

Figure 4. Ultraviolet-visible spectra

Top. 2,4-Dimethyl-3-ethylprodigiosene

——— Spectrum in ethanol 0.01N in HCl

---- Spectrum in ethanol 0.01N in NaOH

Bottom. 2-Methyl-3-amylprodigiosene

——— Spectrum in ethanol 0.01N in HCl

---- Spectrum in ethanol 0.01N in NaOH

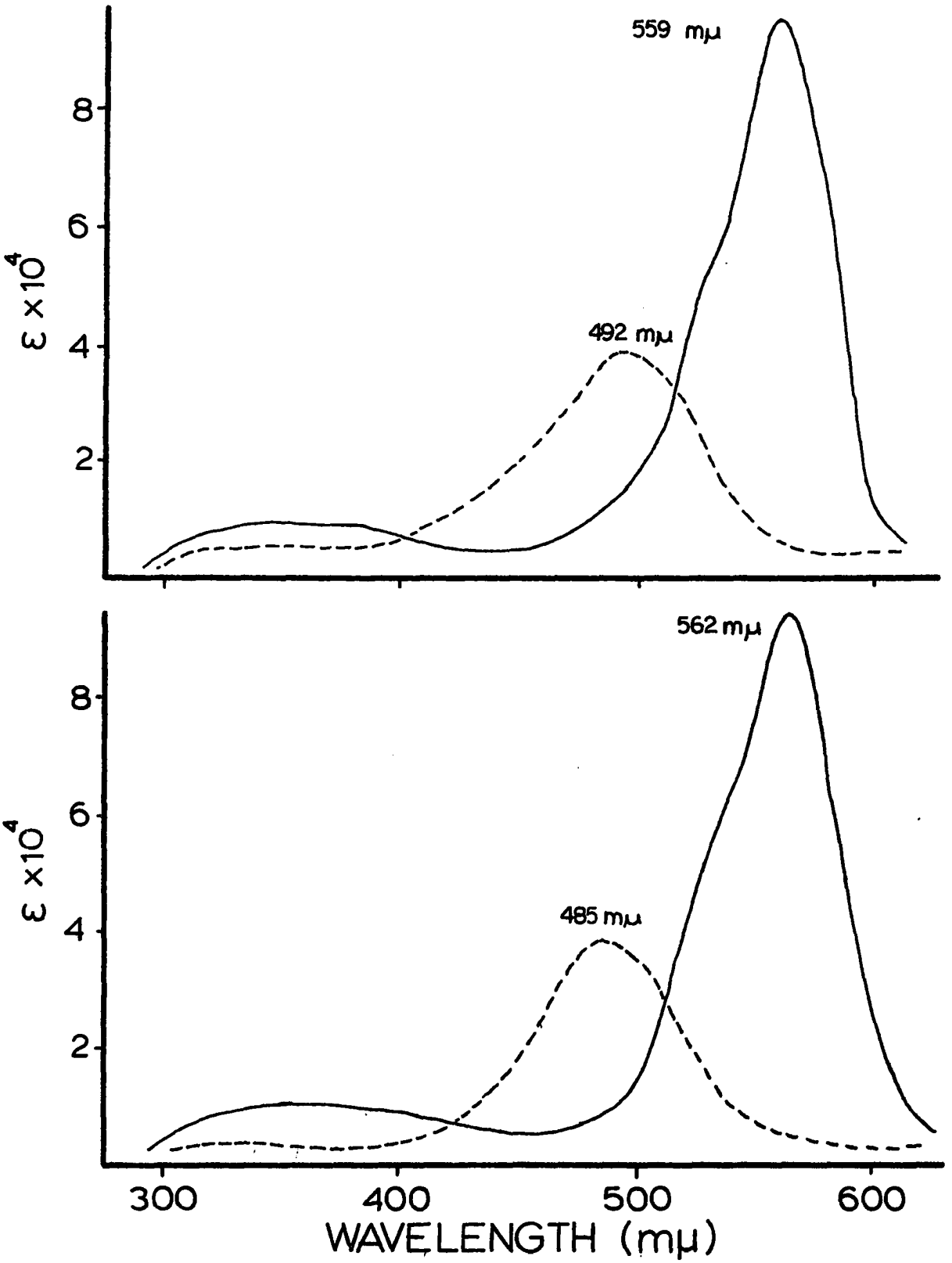

559 mμ
492 mμ
562 mμ
485 mμ
ε x10^4
8
6
4
2
300
400
500
600
WAVELENGTH (mμ)

Figure 5. Ultraviolet-visible spectra of 2-(pyrrol-2'-yl)-
-prodigiosene

——— Spectrum in ethanol 0.01N in HCl

---- Spectrum in ethanol 0.01N in NaOH

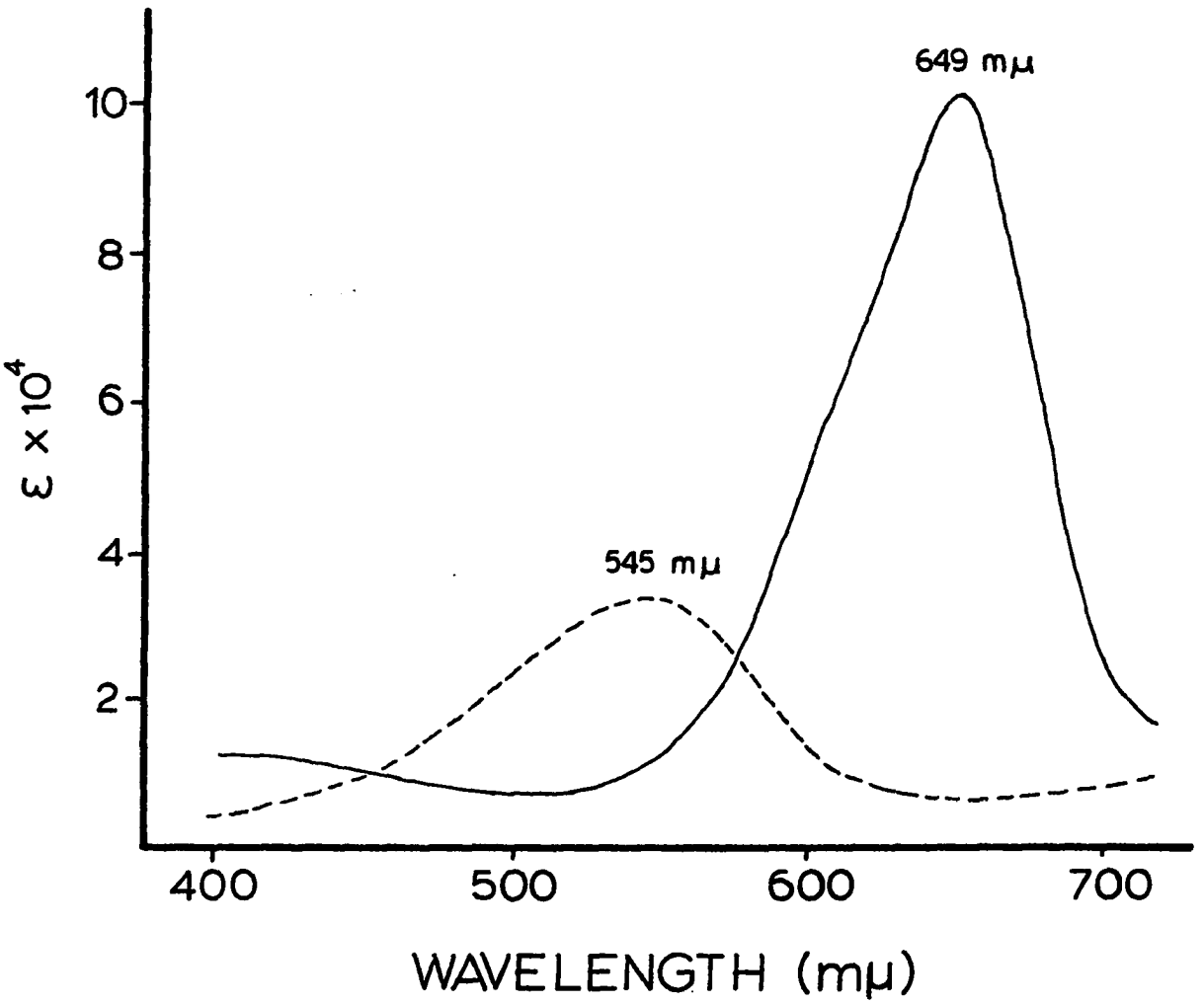

649 mμ
545 mμ
10
8
6
4
2
ε x 10⁴
400
500
600
700
WAVELENGTH (mμ)

Table 1. Molar absorptivities of some prodigiosene pigments

Sample	Acid Form		Basic Form	
	λ_{max}	$\varepsilon_{\lambda_{max}}$	λ_{max}	$\varepsilon_{\lambda_{max}}$
prodigiosin[a]	537 mμ	10.6×10^4	466 mμ	4.3×10^4
2,4-dimethyl-6-methoxyprodigiosene[b]	537 mμ	-----	465 mμ	-----
2,4-dimethyl-3-ethyl-6-methoxyprodigiosene[b]	537 mμ	12.2×10^4	465 mμ	5.12×10^4
2-undecyl-6-methoxyprodigiosene[c]	525 mμ	10.7×10^4	460 mμ	4.22×10^4
prodigiosene	541 mμ	6.40×10^4	455 mμ	3.27×10^4
5-methylprodigiosene	550 mμ	6.25×10^4	461 mμ	2.84×10^4
2-methylprodigiosene	553 mμ	8.02×10^4	470 mμ	3.61×10^4
2,4-dimethyl prodigiosene	553 mμ	9.66×10^4	482 mμ	4.31×10^4
2,4-dimethyl-3-ethyl prodigiosene	559 mμ	9.57×10^4	492 mμ	3.98×10^4
2-methyl-3-amyl prodigiosene	562 mμ	9.55×10^4	485 mμ	3.95×10^4
2-(pyrrol-2'-yl)-prodigiosene	649 mμ	10.6×10^4	545 mμ	3.52×10^4
2-(pyrrol-2'-yl)-4,6-dimethoxy prodigiosene[d]	595 mμ	-----	---	-----

Source:

[a]Castro *et al.* (6)

[b]Williams (28)

[c]Harashima (12)

[d]Feider (10)

The λ_{max} of the protonated form of three of the 6-methoxyprodigiosenes is 537 mμ and is 525 mμ for the naturally occurring 2-undecyl-6--methoxy compound. The λ_{max} of the synthetic prodigiosenes generally shifts to longer wavelength as the amount of alkyl substitution increases. The absorptivity also increases with alkyl substitution, possibly reflecting less tendency to associate in solution.

Infrared spectra

The infrared spectra of the synthetic prodigiosene hydrobromides in KBr pellets are shown in Figures 6 and 7.

Nuclear magnetic resonance spectra

The nmr spectrum of 5-formyl-2,2'-bipyrrole (Figure 8) is included to show that the bipyrrole was not diformylated, since Bullock (4) reported diformylbipyrrole as a byproduct of formylation of substituted bipyrroles. The spectrum was obtained with dimethylsulfoxide as solvent. Integration shows one aldehyde proton, two NH protons and five aromatic protons, indicating monoformylation.

Integration of the nmr spectrum of prodigiosene (Figure 8) accounts for two NH protons and nine protons in the aromatic region (eight aromatic protons and a single vinyl CH proton).

The nmr spectrum integration of 5-methylprodigiosene (Figure 8) shows the three methyl protons present as a doublet in the spectrum, and eight aromatic protons. A small amount of CH_2 impurity is also present.

The nmr spectrum of 2-methylprodigiosene (Figure 9) shows a sharp singlet due to the methyl group. Integration accounts for three methyl protons, seven aromatic protons and one vinyl CH proton. Downfield there

Figure 6. Infrared spectra in KB_r

Top. 5-Formyl-2,2'-bipyrrole

Center. Prodigiosene

Bottom. 2,4-Dimethylprodigiosene

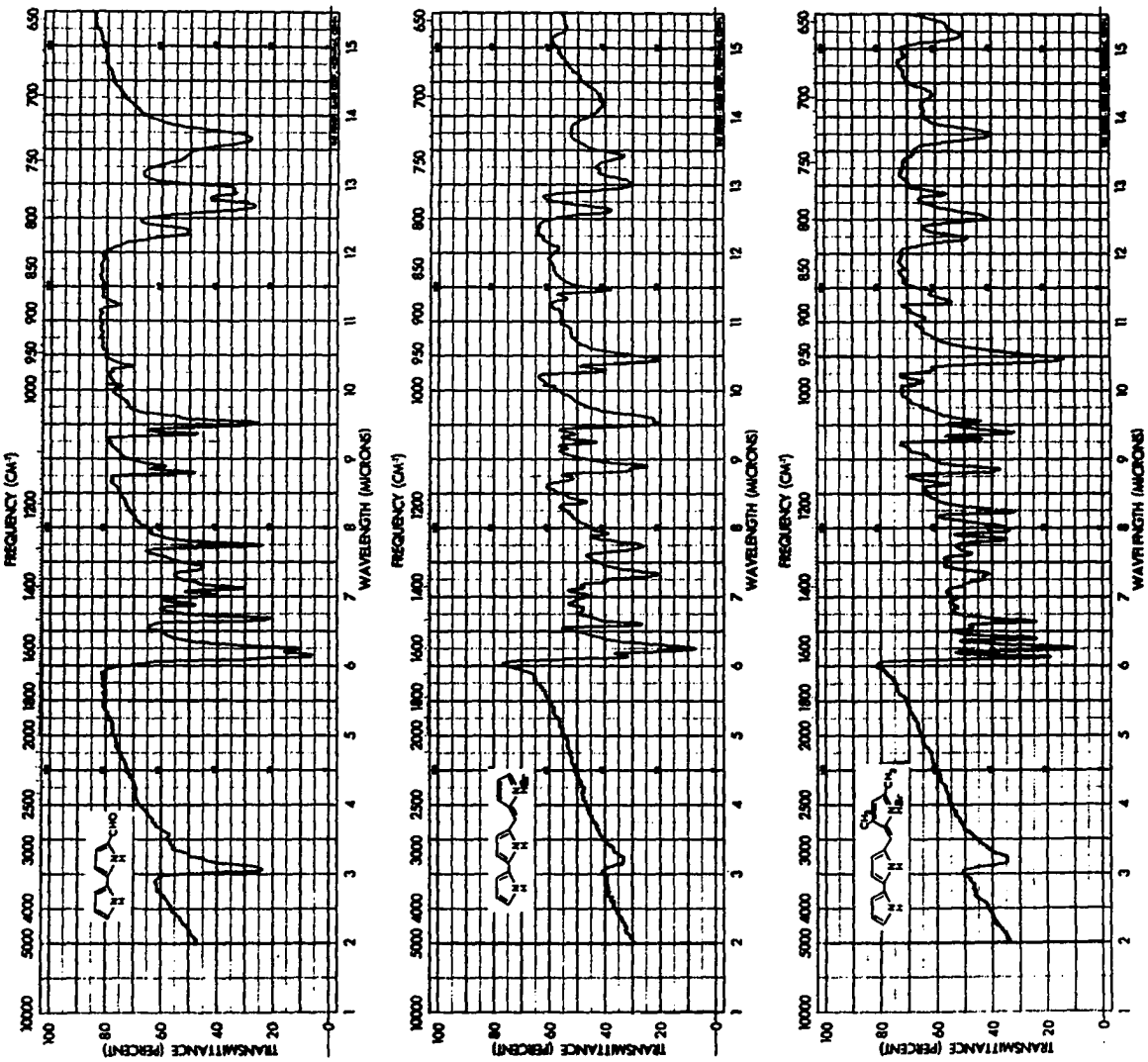
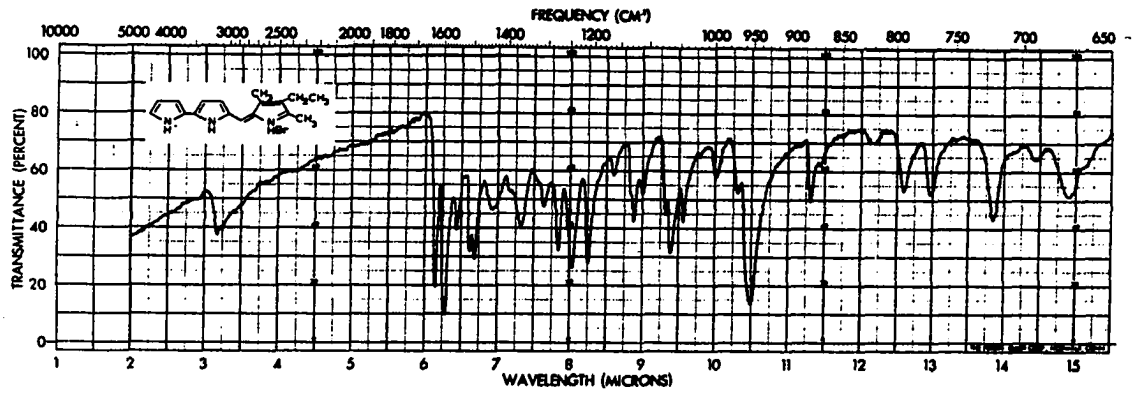

FREQUENCY (CM-1)
WAVELENGTH (MICRONS)
TRANSMITTANCE (PERCENT)

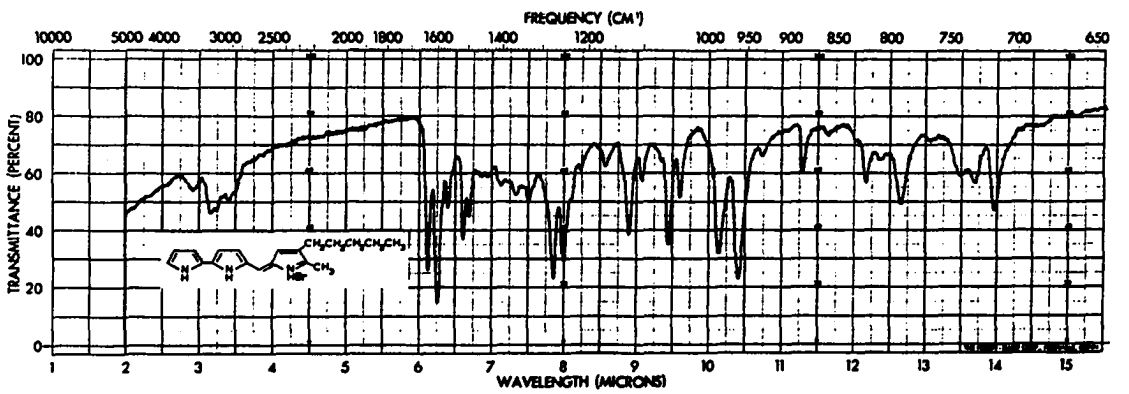
Figure 7. Infrared spectra in KB_r

Top. 2,4-Dimethyl-3-ethylprodigiosene

Center. 2-Methyl-3-amylprodigiosene

Bottom. 2-(Pyrrol-2'-yl)-prodigiosene


FREQUENCY (CM⁻¹)
10000 5000 4000 3000 2500 2000 1800 1600 1400 1200 1000 950 900 850 800 750 700 650
TRANSMITTANCE (PERCENT)
100 80 60 40 20 0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)


FREQUENCY (CM⁻¹)
10000 5000 4000 3000 2500 2000 1800 1600 1400 1200 1000 950 900 850 800 750 700 650
TRANSMITTANCE (PERCENT)
100 80 60 40 20 0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

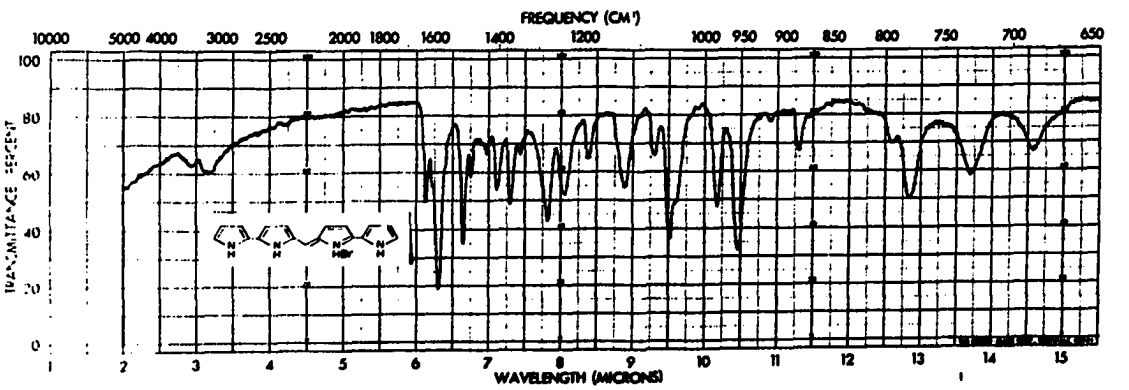

FREQUENCY (CM⁻¹)
10000 5000 4000 3000 2500 2000 1800 1600 1400 1200 1000 950 900 850 800 750 700 650
100 80 40 0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

Figure 8. Nuclear magnetic resonance spectra

Top. 5-Formyl-2,2'-bipyrrole (in dimethyl sulfoxide)

Center. Prodigiosene hydrobromide

Bottom. 2-Methylprodigiosene hydrobromide

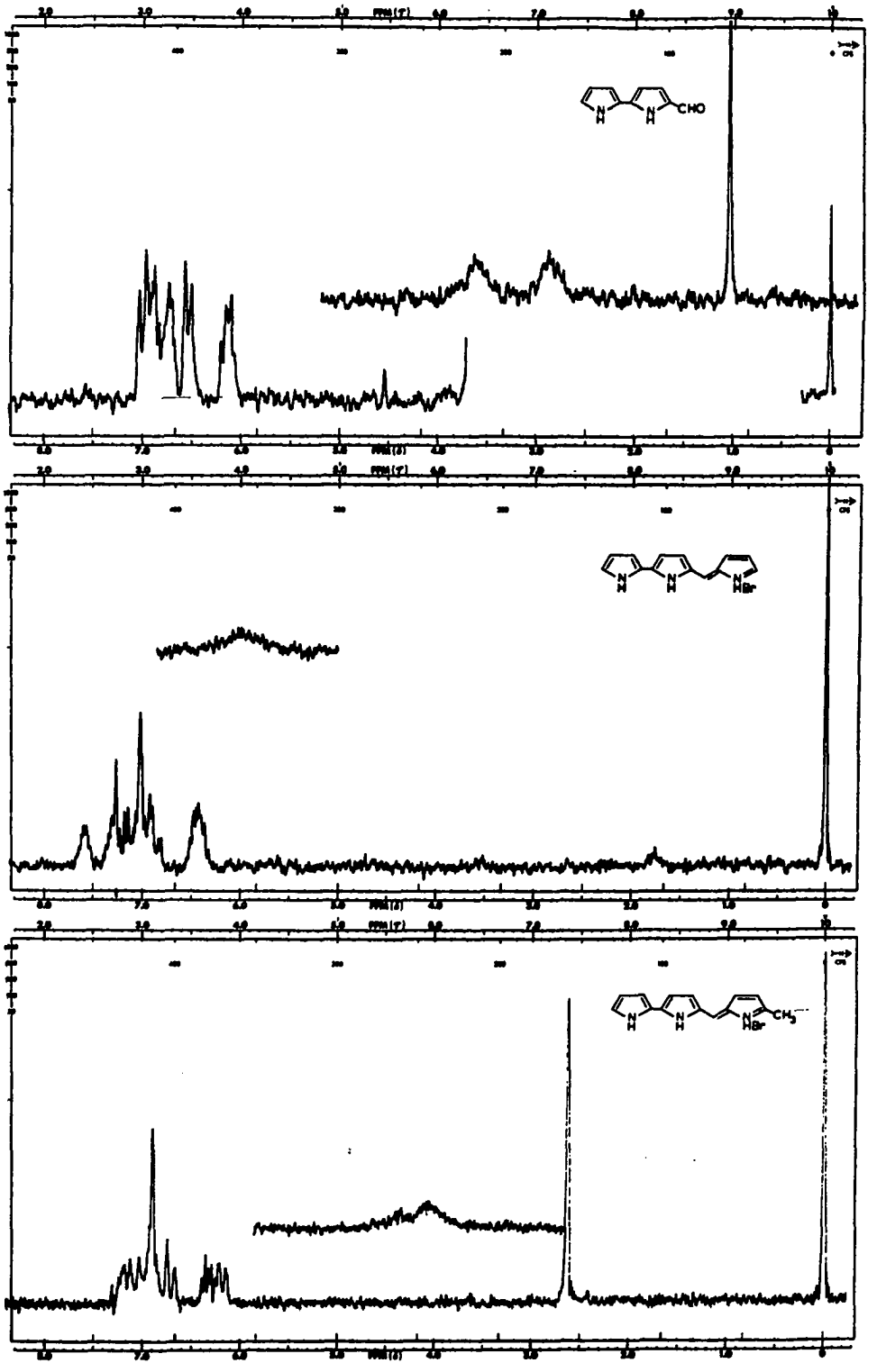
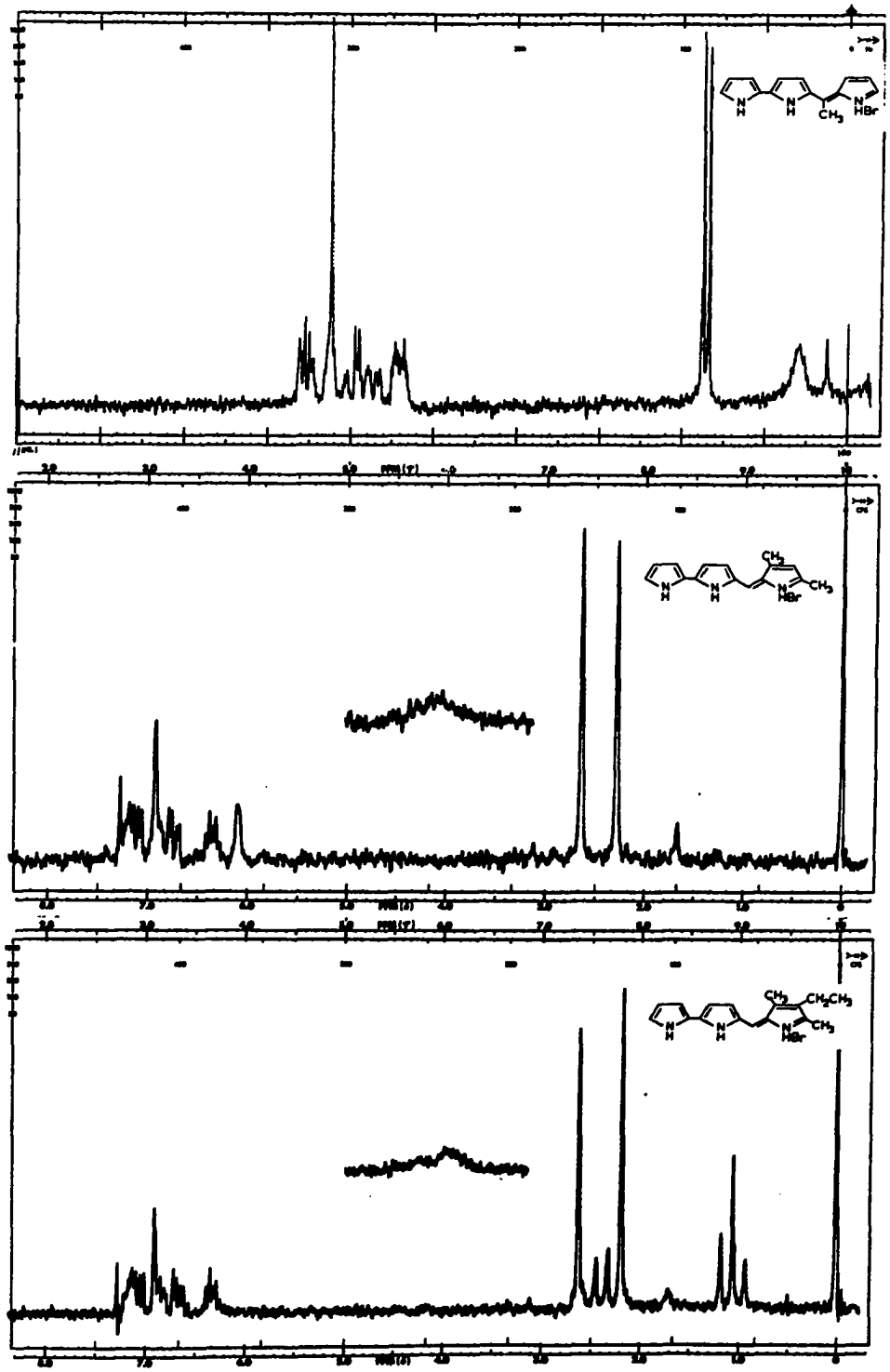

CHO
NH
Br
CH3

Figure 9. Nuclear magnetic resonance spectra

Top. 5-Methylprodigiosene hydrobromide

Center. 2,4-Dimethylprodigiosene hydrobromide

Bottom. 2,4-Dimethyl-3-ethylprodigiosene hydrobromide



are two NH protons.

The nmr spectrum of 2,4-dimethylprodigiosene (Figure 9) shows two sharp methyl proton peaks that integrate to three protons each. Integration also shows six aromatic protons and further downfield two NH protons. The single vinyl CH proton does not appear. The aromatic region, which should include the vinyl CH proton, integrates to about 6.2 protons, less than the expected seven.

The nmr spectrum of 2,4-dimethyl-3-ethylprodigiosene (Figure 9) shows a triplet for the terminal methyl protons of the ethyl group. The quartet for the methylene protons of the ethyl group is between the two singlets for the two ring methyl group protons. Integration shows three protons in the triplet, two protons in the quartet and three protons in each singlet. Integration also accounts for the five aromatic protons, the single vinyl CH proton and two NH protons downfield.

Integration of the nmr spectrum of 2-methyl-3-amylprodigiosene (Figure 10) accomodates the three protons in the terminal methyl of the amyl group, six aliphatic CH protons in a broad band, the two methylene protons of the amyl group next to the pyrrole ring and three protons of the 2-methyl group. Also there are seven protons in the aromatic region, six aromatic protons and one vinyl CH proton, and two NH protons downfield.

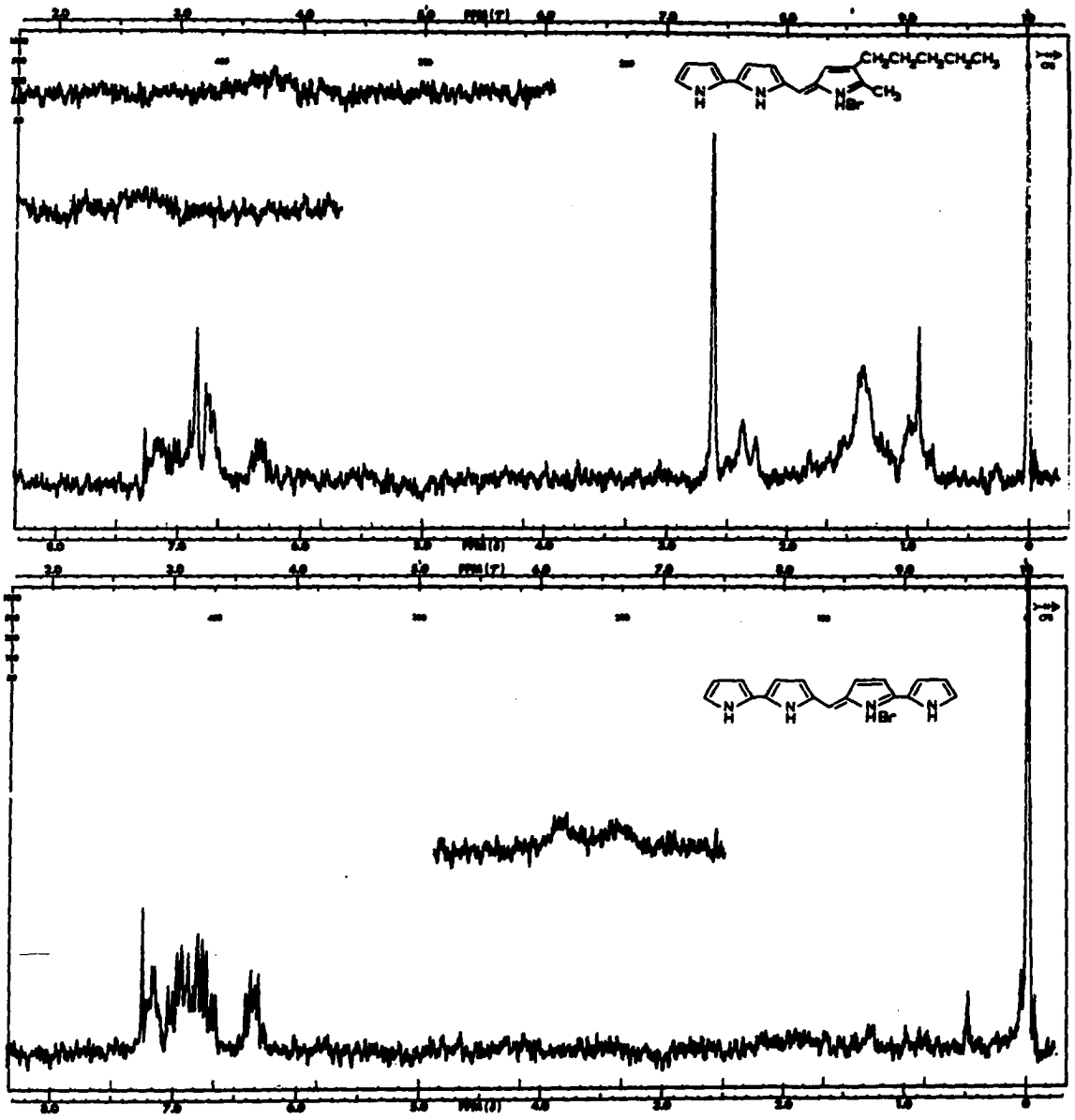
The compound 2-(pyrrol-2'-yl)-prodigiosene (Figure 10) was not soluble enough in deuterochloroform to obtain integration steps high enough to measure precisely. However, integration is consistent with three NH protons, ten aromatic protons, and one vinyl CH proton.

The nmr spectra reported here were obtained on hydrobromide salts. A disadvantage of determining the spectrum of the salt rather than the

Figure 10. Nuclear magnetic resonance spectra

Top. 2-Methyl-3-amylprodigiosene hydrobromide

Bottom. 2-(Pyrrol-2'-yl)-prodigiosene



free base is that the aromatic region is more complex and difficult to interpret. The hydrobromides were used in this case because only small amounts of the compounds were available and conversion to the free base is usually accomplished in only 20 to 30 percent yield. Also, the free bases are less stable. Williams reported the nmr spectra of prodigiosin, 2,4-dimethyl-6-methoxylprodigiosene and 2,4-dimethyl-3-ethyl-6-methoxy-prodigiosene in free base form (Figure 11). It can be seen that the aromatic portion of these spectra is better defined than in the spectra obtained in this investigation. It would be desirable to have nmr spectra of the free bases of the whole series of prodigiosenes.

Mass spectra

The mass spectrum of 2,2'-bipyrrole (Figure 12) is essentially identical to that reported by Rodgers (21). It is presented here to illustrate the behavior of the prodigiosenes in the mass spectrometer. The base peak of 2,2'-bipyrrole is the molecular ion M (m/e=132). The next intense peaks are at M-27 and M-28 (m/e=105 and m/e=104). These peaks may be accounted for on the basis of the fragmentation mechanism proposed by Budzikiewicz and co-workers for pyrrole (3).

The mass spectrum of 5-formyl-2,2'-bipyrrole (Figure 13) also has the molecular ion as its base peak. Loss of the formyl group gives the next intense peak, M-29 (m/e=131). The M-29 peak is followed by the characteristic m/e=105 and m/e=104 peaks.

The prodigiosenes were placed in the mass spectrophotometer as hydrobromide salts. Subsequent heating decomposed the salts (1) and spectra of the free bases were obtained. In each case, small peaks of

Figure 11. Nuclear magnetic resonance spectra[a]

Top. Prodigiosin

Center. 2,4-Dimethyl-3-ethyl-6-methoxyprodigiosene

Bottom. 2,4-Dimethyl-6-methoxyprodigiosene

[a]Source: Williams (30)

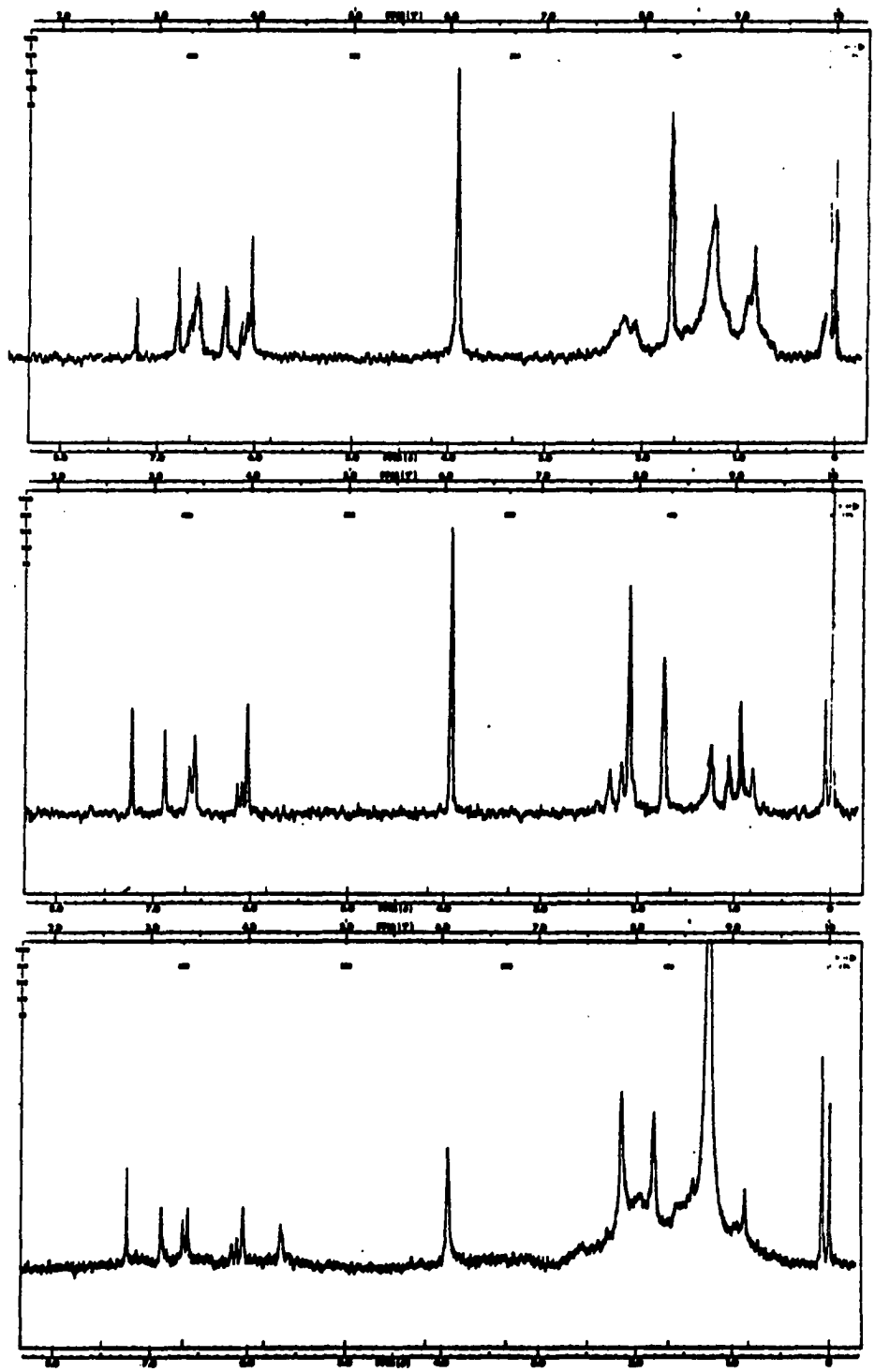


Figure 12. Mass spectrum of 2,2'-bipyrrole

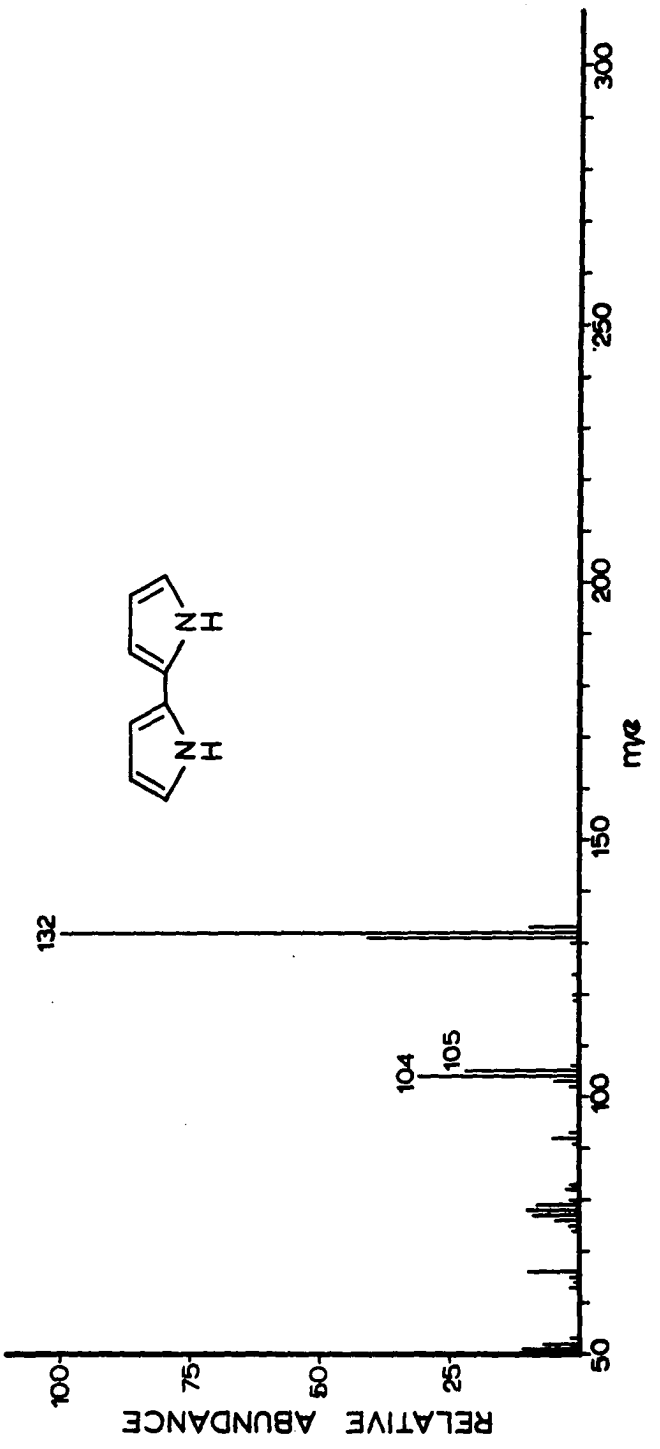
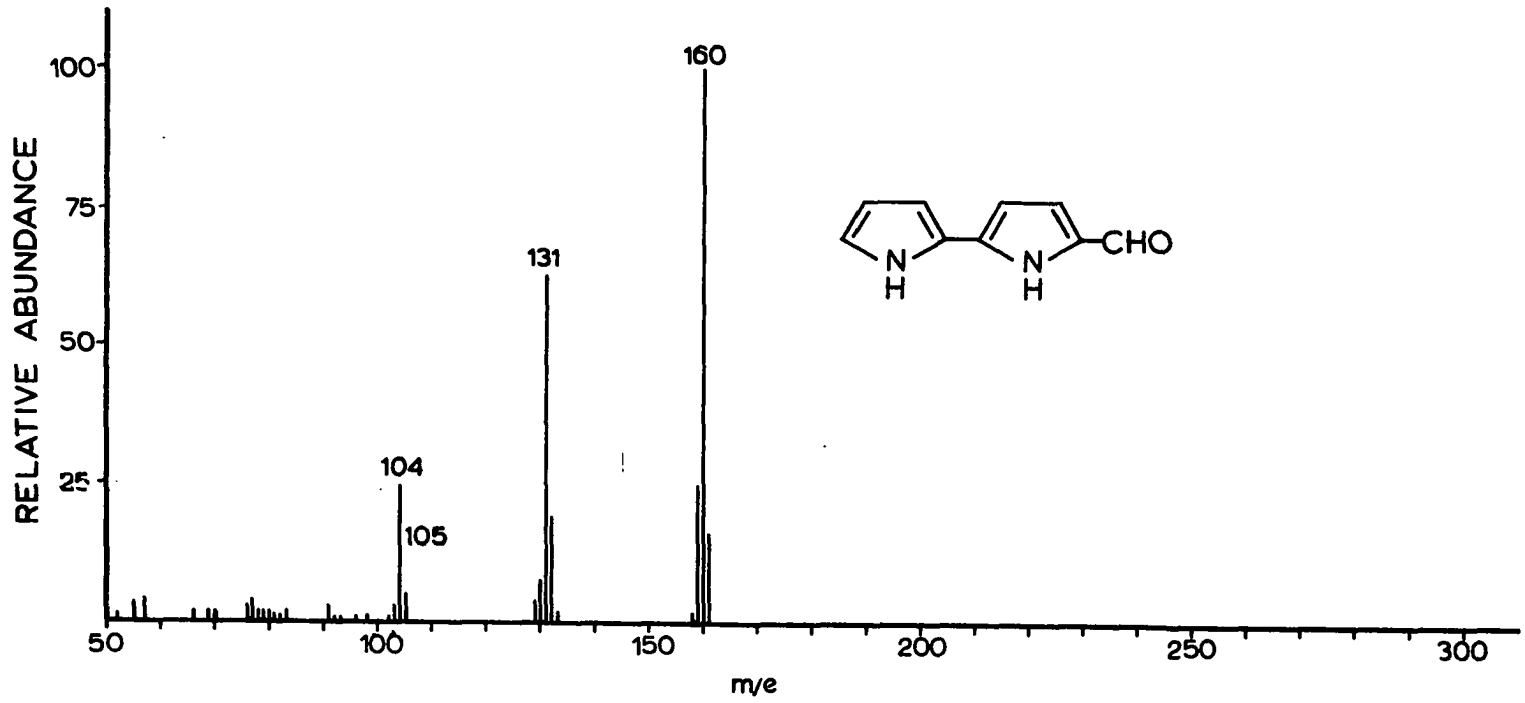

RELATIVE ABUNDANCE
100
75
50
25
50
100
150
200
250
300
m/e
132
104
105
NH
NH

Figure 13. Mass spectrum of 5-formyl-2,2'-bipyrrole

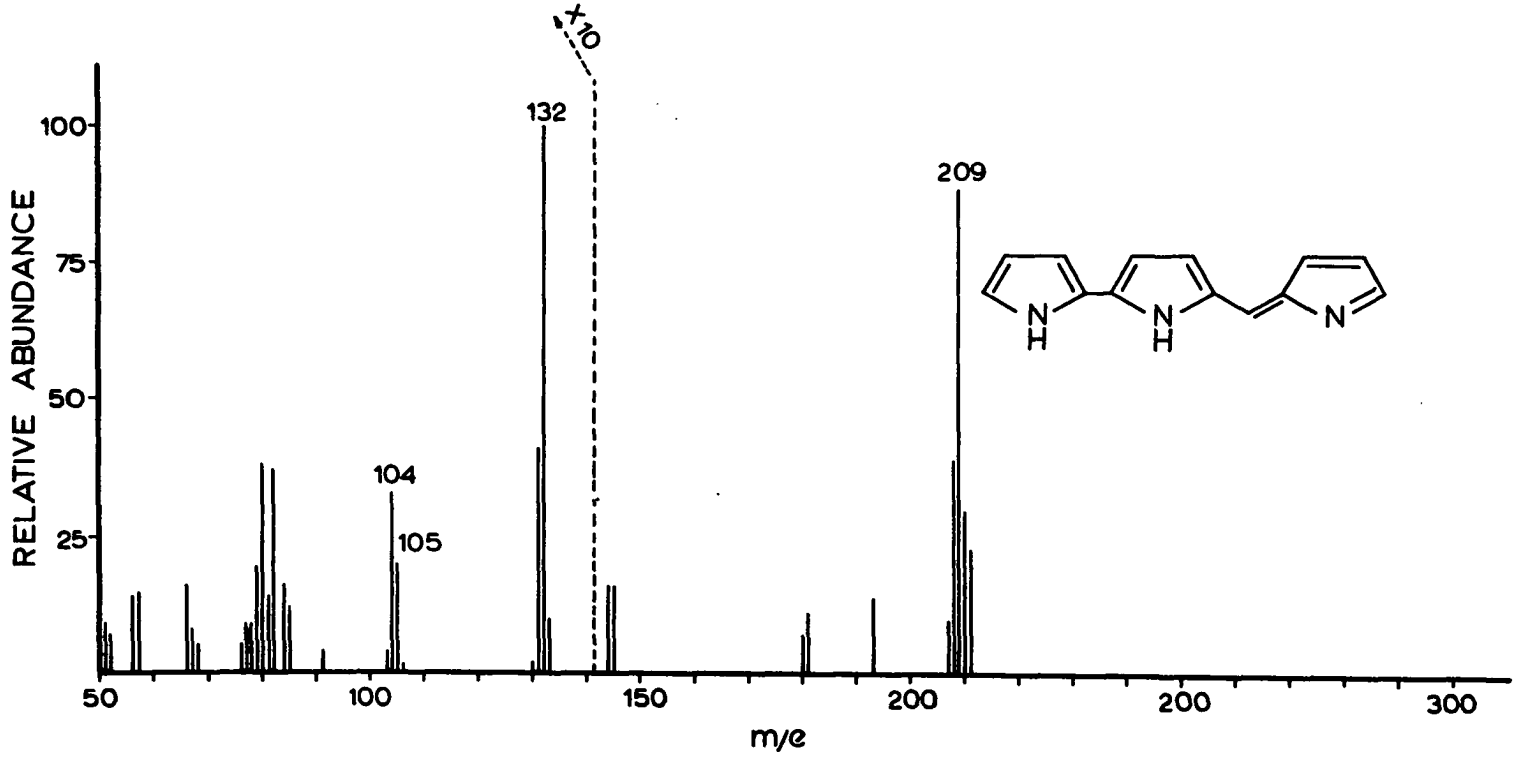

RELATIVE ABUNDANCE
100
75
50
25
160
131
104
105
50
100
150
200
250
300
m/e
N
H
N
H
CHO

equal intensity were observed at M+80 and M+82. These peaks correspond to the hydrobromide, Br having two isotopes of mass 79 and 81 in approximately equal abundance.

Prodigiosene (Figure 14) has a strong molecular ion in its mass spectrum. The spectrum is devoid of any other significant peaks until m/e=132, which is its base peak. Also present are the m/e=105 and m/e=104 peaks. A prominent peak is m/e=80, characteristic of methylpyrrole. Finally there is a peak, m/e=66, indicating cleavage of the 2,2'-bipyrrole bond or of the methylene carbon-monopyrrole bond.

It should be noted that the m/e=132 peak is not the m/e=131 peak expected from cleavage of the bond between the bipyrrole and the methylene carbon. It appears that cleavages of the methylene bonds are probably the result of pyrolysis from the heating necessary to decompose the hydrobromide salt and volatilize the molecule for ionization in the mass spectrometer. This possibility is supported by the fact that pyrolysis of the 6-methoxyprodigiosenes has been used as a fruitful degradative method for several studies (37, 21, 28). In our laboratory the monopyrrole fragment has been the only isolable product, but Wasserman has isolated bipyrroles as well as monopyrroles after pyrolysis. Cleavage of pyrrolylmethene bonds in the mass spectrometer has been reported by Jackson (15) who listed mass spectra of thirteen dipyrrolylmethene hydrobromides; ions representing cleavage of the methylene bonds never exceeded 17 percent of the base peak. In the case of prodigiosene and other in the present series, ions with an m/e ratio of 1 greater than those expected from ionization-induced cleavages of the methylene bonds were found. They were in much greater abundance than expected from the dipyrrolylmethene analogy.

Figure 14. Mass spectrum of prodigiosene


RELATIVE ABUNDANCE
100
75
50
25
X10
132
209
104
105
50
100
150
200
200
300
m/e
N
H
N
H
N

The mass spectrum of 5-methylprodigiosene (Figure 15) shows a strong molecular ion, m/e=223, followed by the next intense peak corresponding to M-15 (m/e=208). The next intense peak is the base peak, m/e=132, indicating pyrolysis has occurred.

The mass spectrum of 2-methylprodigiosene (Figure 16) has a strong molecular ion, m/e=223. The M-15 ion (m/e=208) is the next intense peak. In this case m/e=132 is not the base peak. The 2-methylpyrrole expected from cleavage or pyrolysis provides the base peak (m/e=80).

The mass spectrum of 2,4-dimethylprodigiosene (Figure 17) has a strong molecular ion (m/e=237), which appeared to lose only one methyl group to give M-15 (m/e=222). The peak corresponding to m/e=207, from loss of both methyl groups, is quite small. It was possible to obtain the spectrum with less heating than in the previous case. As expected, the m/e=132 peak is not the base peak. However, the base peak (m/e=94) is also the base peak of trimethylpyrrole (molecular weight, 95). The ratio of peak intensities of m/e=95 to m/e=94 is close to that reported for trimethylpyrrole (2).

The mass spectrum of 2,4-dimethyl-3-ethylprodigiosene (Figure 18) was also obtained with less heating. A strong molecular ion, m/e=265, is observed. Ions corresponding to the loss of one methyl, M-15 (m/e=250), and two methyls, M-30 (m/e=235), are observed.

The mass spectrum of 2-methyl-3-amylprodigiosene (Figure 19) has a strong molecular ion, m/e=293. The next intense peak is M-15 (m/e=278). Then a more intense peak corresponding to M-57 (m/e=236) is observed. This peak is explained by cleavage of C_4H_9 from the amyl group. At lower m/e ratios ions derived from pyrolysis were observed. Most notable are

Figure 15. Mass spectrum of 2-methylprodigiosene

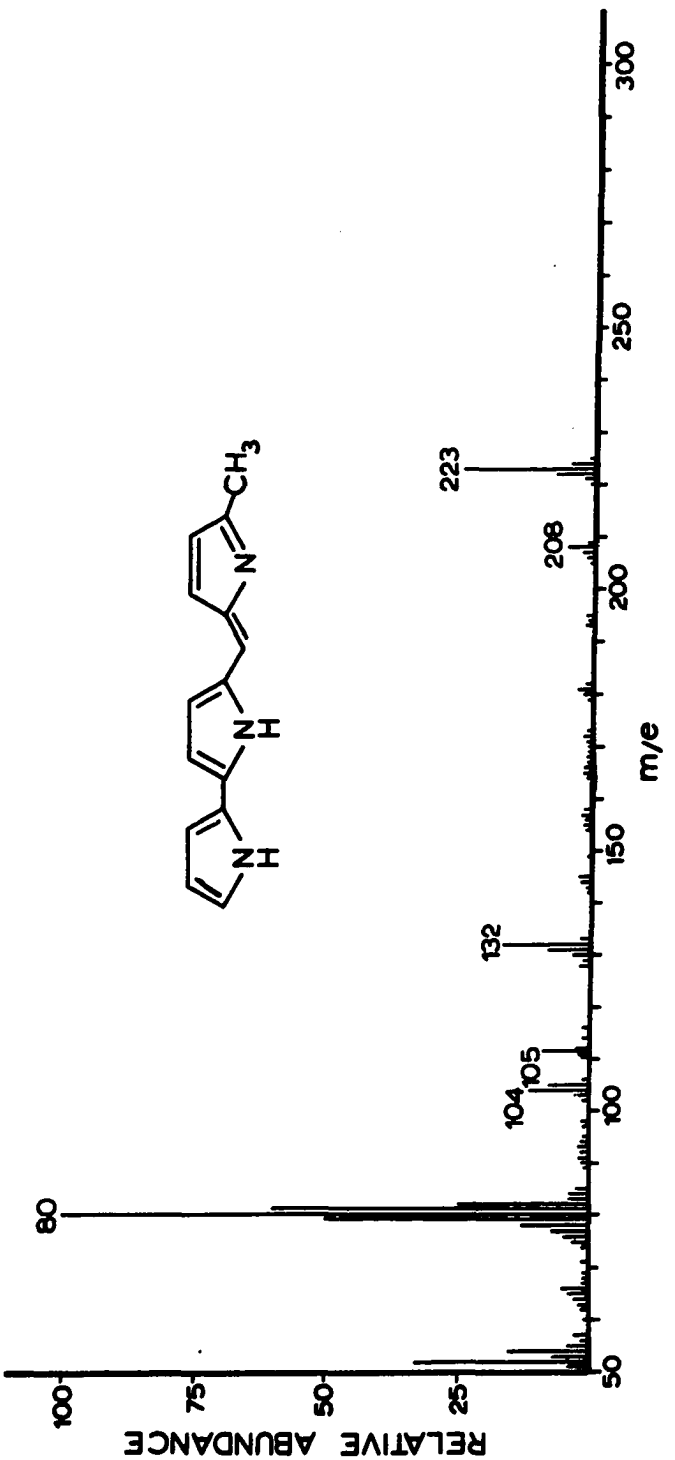

RELATIVE ABUNDANCE
100
75
50
25
80
104
105
132
223
208
50
100
150
200
250
300
m/e
NH
NH
N
CH3

Figure 16. Mass spectrum of 5-methylprodigiosene

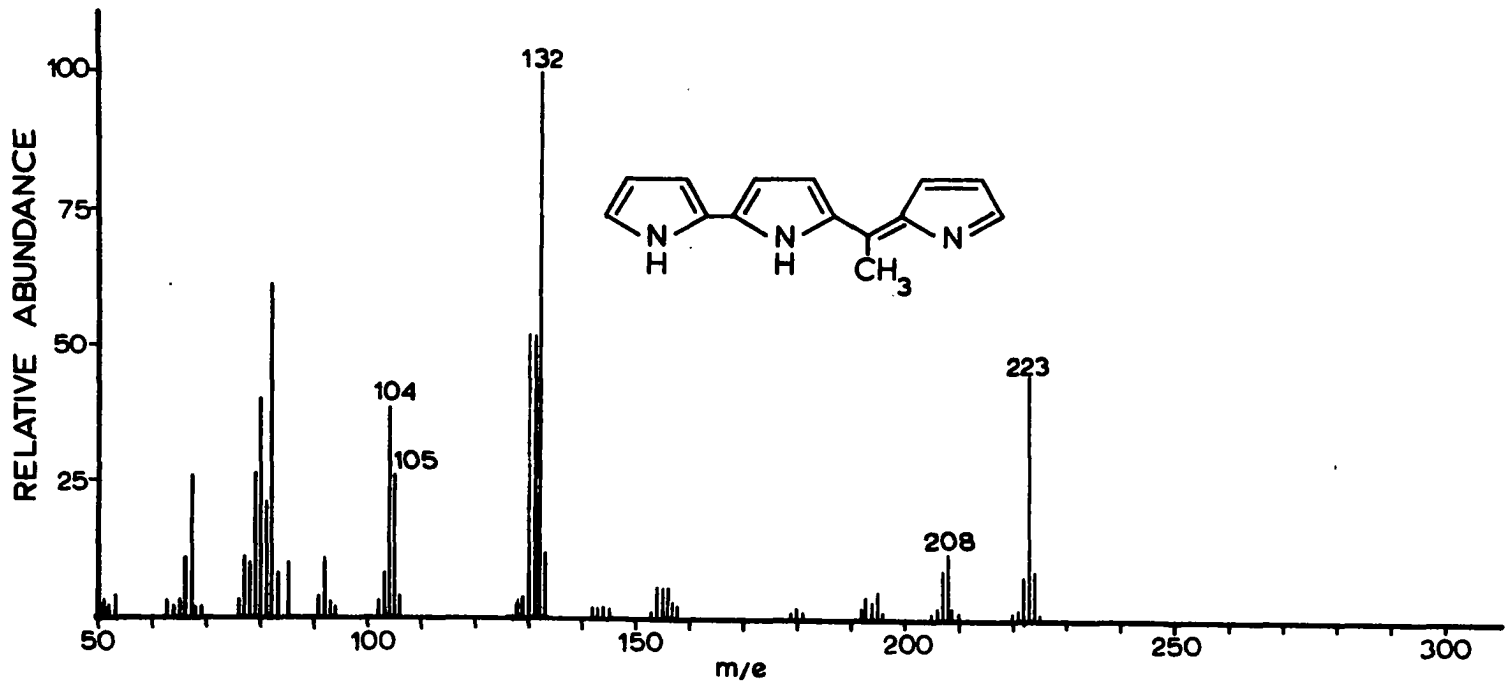

RELATIVE ABUNDANCE
100
75
50
25
132
104
105
223
208
N
H
N
H
CH3
N
50
100
150
200
250
300
m/e

Figure 17. Mass spectrum of 2,4-dimethylprodigiosene

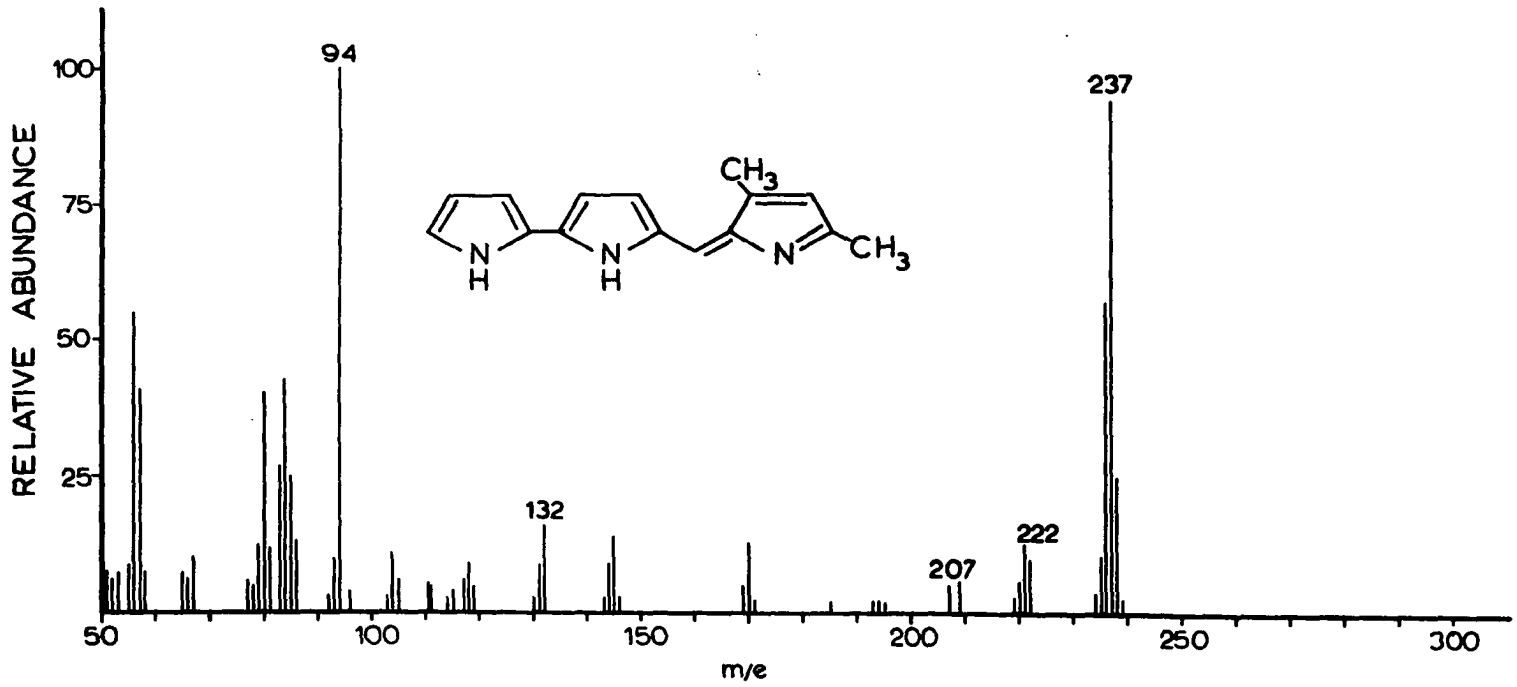

RELATIVE ABUNDANCE
100
75
50
25
94
237
132
222
207
CH3
N
H
N
H
N
CH3
50
100
150
200
250
300
m/e

Figure 18. Mass spectrum of 2,4-dimethyl-3-ethylprodigiosene

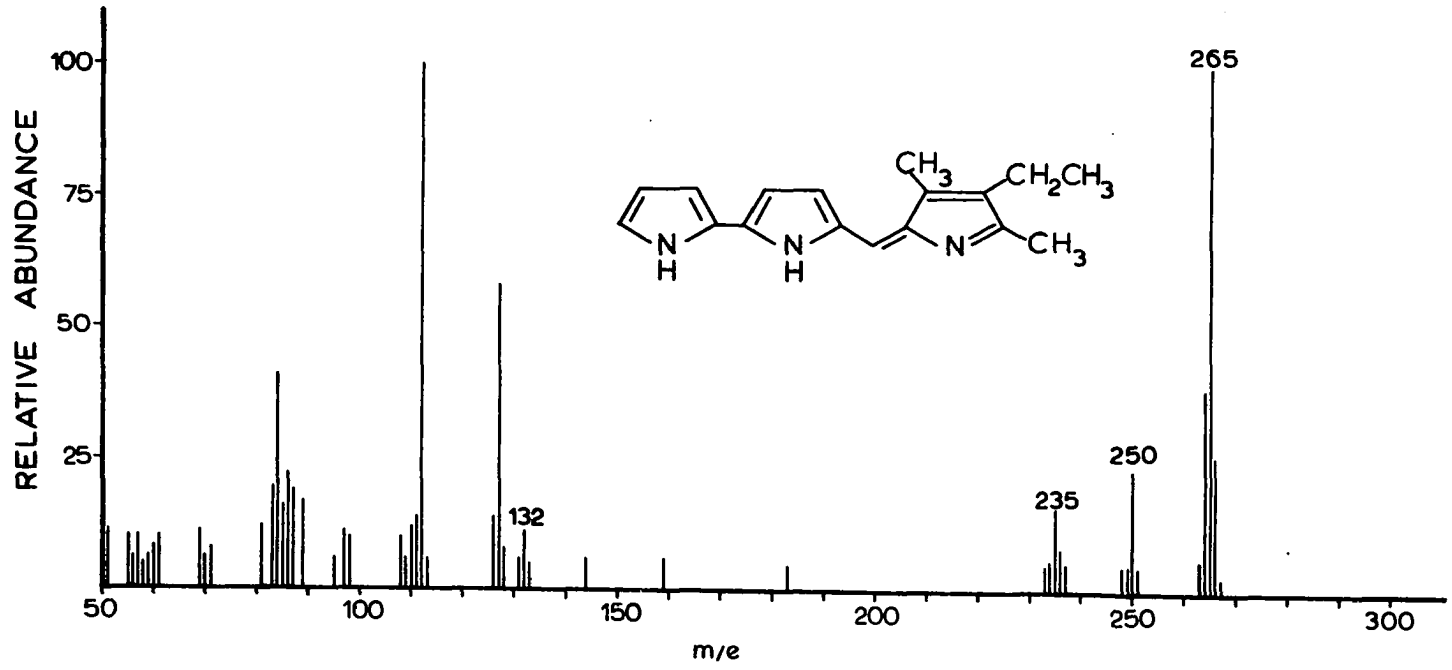
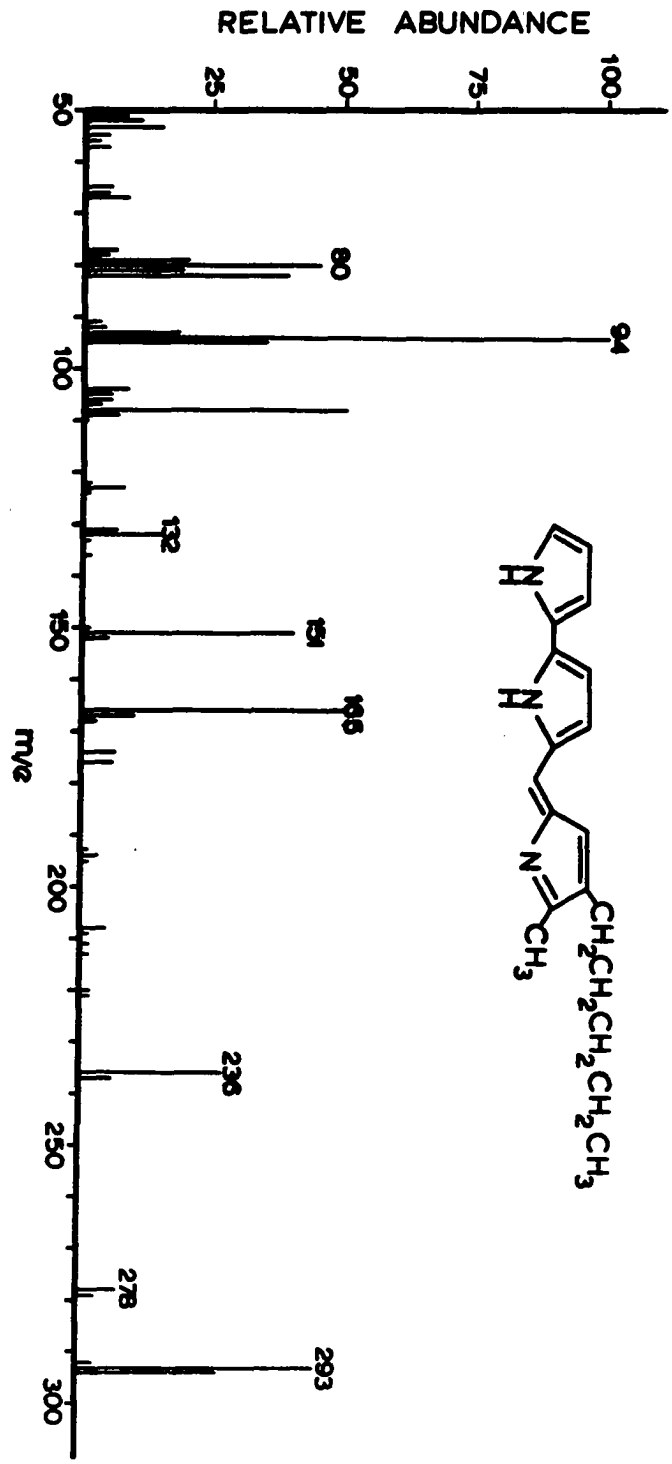

RELATIVE ABUNDANCE
100
75
50
25
50
100
150
200
250
300
m/e
265
250
235
132
CH3
CH2CH3
CH3
N
H
N
H
N

Figure 19. Mass spectrum of 2-methyl-3-amylprodigiosene


RELATIVE ABUNDANCE
25
50
75
100
50
80
94
100
132
150
151
166
m/z
200
236
250
278
293
300
N
H
N
H
N
CH3
CH2CH2CH2CH2CH2CH3

ions characteristic of a dimethylamylpyrrole (m/e=166), a methylamylpyrrole (m/e=151), a trimethylpyrrole (m/e=108), a dimethylpyrrole (m/e=94), and a monomethylpyrrole (m/e=80).

The mass spectrum of 2-(pyrrol-2'-yl)-prodigiosene (Figure 20) is quite simple. A strong molecular ion (m/e=274) is observed. There are no other significant peaks until m/e=132 where the spectrum is quite similar to that of 2,2'-bipyrrole.

The mass spectrum of 2,4-dimethyl-6-methoxyprodigiosene (Figure 21), a pigment prepared by R. H. Williams, is quite simple. The molecular ion (m/e=267) is the base peak. An M-15 ion (m/e=252) is present. However, an M-31 ion due to loss of the methoxyl (m/e=236) is more intense. Also present is an ion corresponding to a loss of both the methyl and methoxy groups (m/e=221).

The mass spectrum of 2,4-dimethyl-3-ethyl-6-methoxyprodigiosene (Figure 22), also prepared by R. H. Williams, is similarly quite simple. The molecular ion (m/e=295) is the base peak. An M-15 ion (m/e=280) is observed as is an M-31 ion (m/e=264).

In the mass spectrometer, these last two examples of 6-methoxyl-prodigiosenes show a fragmentation of the alkyl substituents in a manner similar to the fragmentation of prodigiosin. However, prodigiosin shows no significant peak corresponding to loss of the methoxyl group, although the syntrophic pigments of Williams and the 2-undecyl-6-methoxyprodigiosene of Harashima (12) exhibit peaks corresponding to loss of methoxyl, M-31.

Figure 20. Mass spectrum of 2-(pyrrol-2'-yl)-prodigiosene

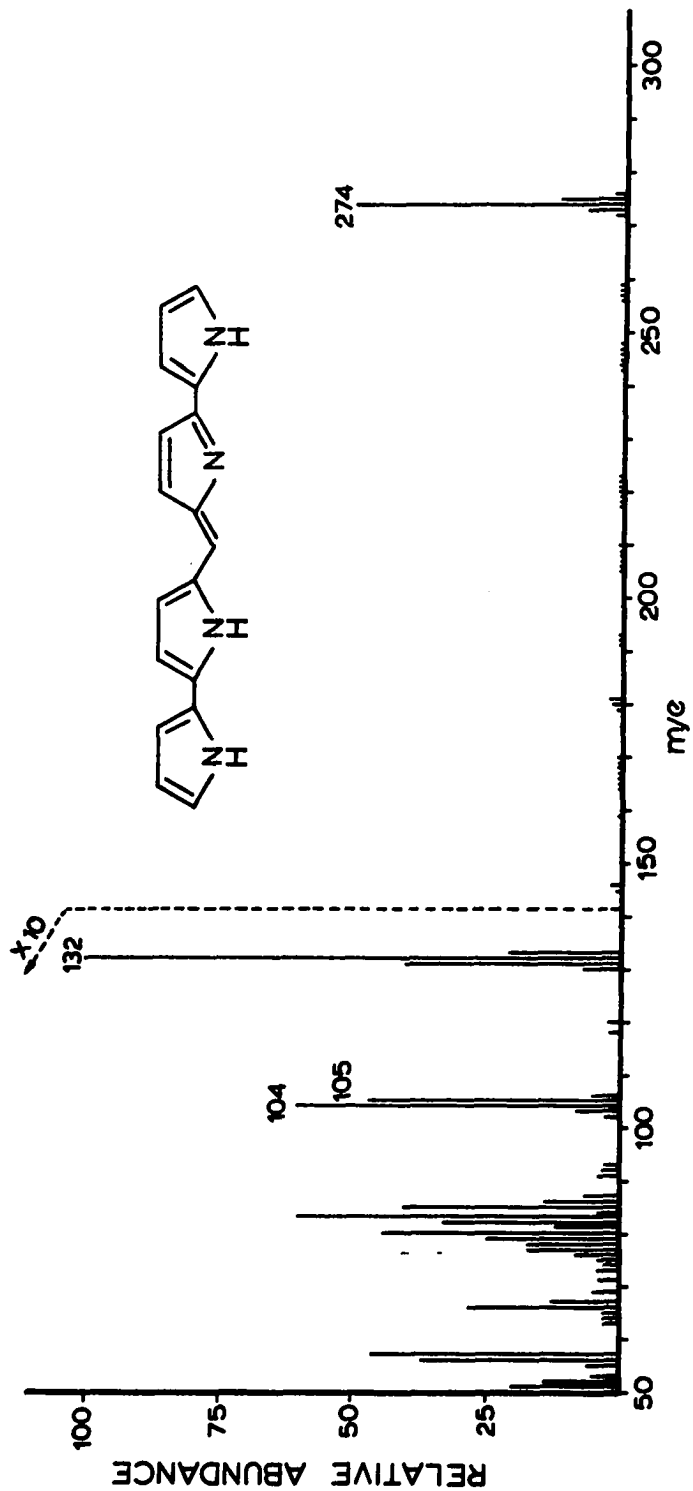

RELATIVE ABUNDANCE
100
75
50
25
50
100
150
200
250
300
m/e
274
132
x10
104
105
NH
N
NH
NH

Figure 21. Mass spectrum of 2,4-dimethyl-6-methoxyprodigiosene

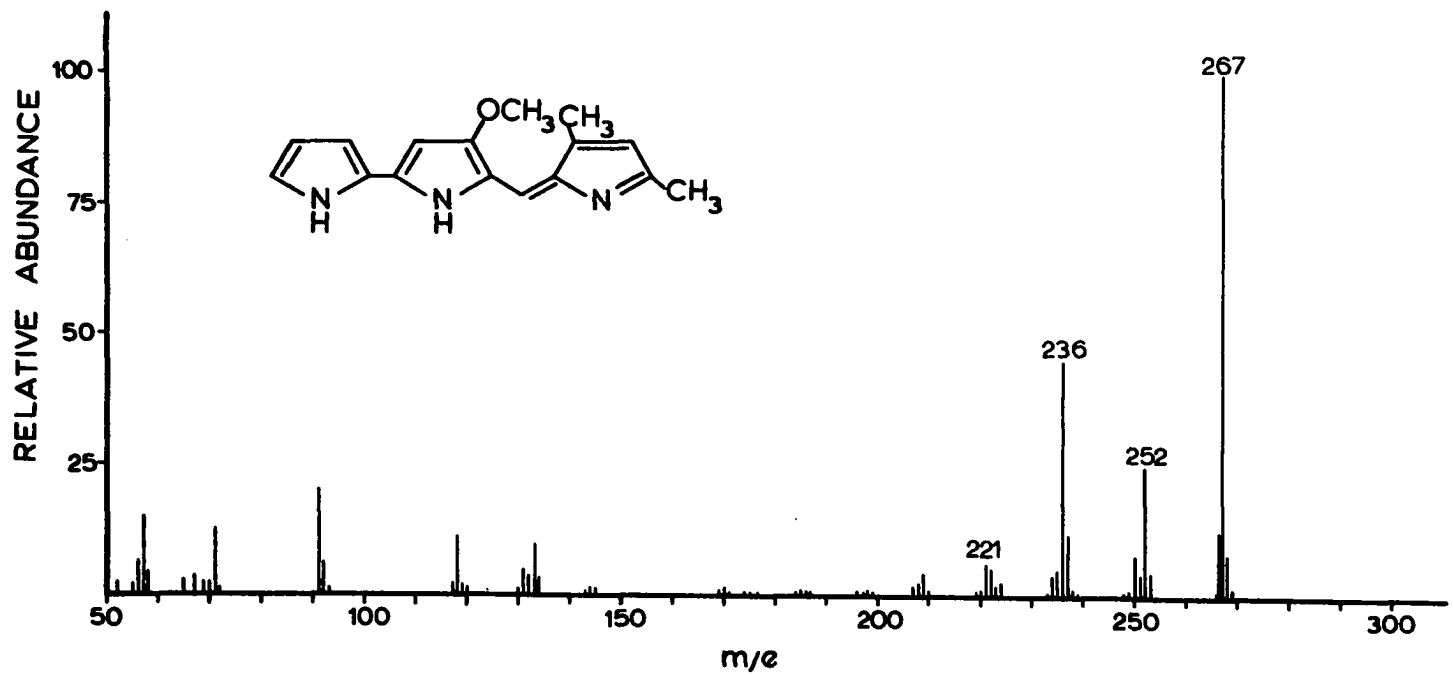
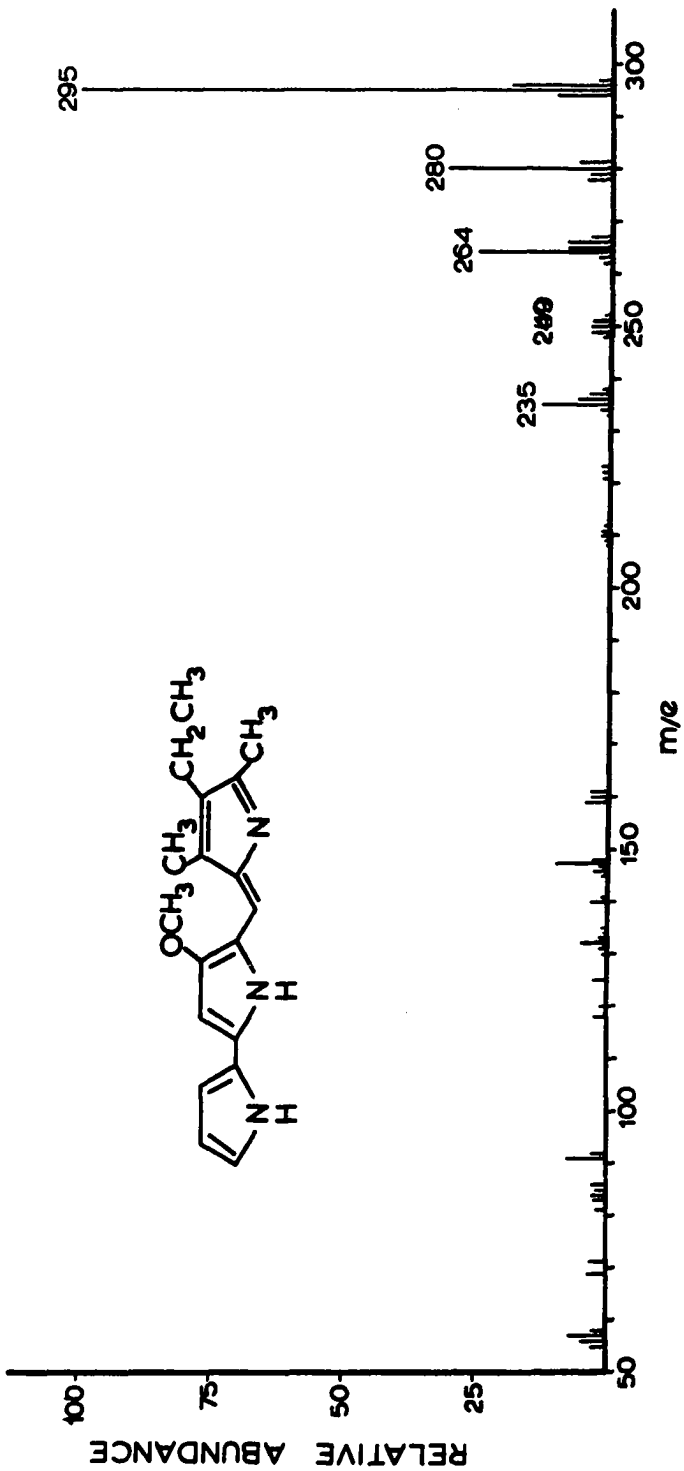

RELATIVE ABUNDANCE
100
75
50
25
50
100
150
200
250
300
m/e
267
236
252
221
OCH3
CH3
CH3
N
H
N
H
N

Figure 22. Mass spectrum of 2,4-dimethyl-3-ethyl-6-methoxyprodigiosene


RELATIVE ABUNDANCE
100
75
50
25
50
100
150
200
250
300
m/e
295
280
264
235
OCH3
CH3
CH2CH3
CH3
N
H
N
H

Chemistry of Prodigiosenes

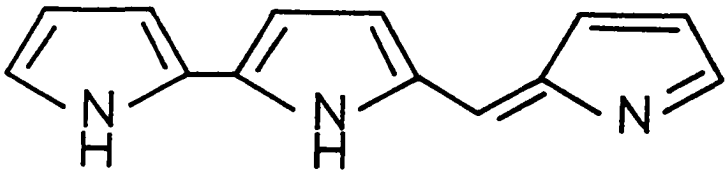
During the synthesis of the prodigiosenes, 2-(pyrrol-2'-yl)--prodigiosene was observed as a byproduct in each case. Its identity was shown by comparison of Rf's on thin-layer chromatography and by the appearance of a small peak (m/e=274) in the mass spectrum. The 2-(pyrrol--2'-yl)-prodigiosene could have been produced by deformylation and condensation of the resulting bipyrrole with formylbipyrrole. This mechanism has been reported for formylpyrroles (8). Deformylation may account for its presence in the original reaction mixtures but 2-(pyrrol--2'-yl)-prodigiosene was also produced in purified solutions of other prodigiosenes. The rate of formation in ethanol was much greater than in chloroform. In about two hours an ethanol solution of 2-methylprodigiosene was 80 percent converted to 2-(pyrrol-2'-yl)-prodigiosene. Chromatography of old samples of prodigiosene hydrobromides showed the presence of 2--(pyrrol-2'-yl)-prodigiosene. In the case of prodigiosenes with more highly substituted monopyrrole rings, a similar behavior of the monopyrrole portion was observed. In the mass spectrum of 2,4-dimethyl-6-methoxyprodigiosene, ions with m/e ratios expected for a tetramthyldipyrrolylmethene were observed. Ot a lesser extent similar ions were observed with 2,4-dimethyl-prodigiosene, 2,4-dimethyl-3-ethylprodigiosene, and 2-methyl-3-amyl--prodigiosene. These ions increased with temperature and time of vaporization of the sample in the mass spectrometer.

This type of pyrrole-bipyrrole exchange behavior may offer an explanation for some of the many unidentified pigments that have been observed in purification of the 6-methoxyprodigiosenes isolated from natural sources.

SUMMARY

1. 5-Formyl-2,2'-bipyrrole was synthesized and was found not to be utilized for pigment production by *Serratia marcescene* strain WCF, which utilizes either the 4-methoxy or 4-hydroxy derivative for prodigiosin synthesis.

2. 5-Formyl-2,2'-bipyrrole was condensed *in vitro* with pyrroles to give a series of pigments with the same basic ring system as that of prodigiosin.



3. The unsubstituted ring system (illustrated above) was named prodigiosene and a convenient systematic nomenclature was proposed for prodigiosin-like compounds.

4. The visible, infrared, nmr, and mass spectra of the prodigiosenes synthesized were shown to be consistent with proposed structures and clearly analagous to spectra of the presently identified natural prodigiosenes (prodigiosin and the "C25-prodigiosins").

5. Pyrrole-bipyrrole exchange behavior observed with synthetic prodigiosenes was postulated to account for some of the heterogeneity characteristic of 6-methoxyprodigiosenes isolated from natural sources.

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