Prodigiosin-25 C
Isolation and the Chemical Structure

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A water-insoluble red antibiotic pigment was isolated from mycelia of a strain of Streptomyces. It was found that the pigment is a new C25-prodigiosin-analogue and the authors propose to designate it prodigiosin-25 C. The chemical structure (XI) has been deduced from visible absorption spectra, NMR spectra, mass spectra and analysis of degradation products of the pigment.

Many investigators have reported isolation of prodigiosin-like pigments from various strains of Streptomyces.1-4 Presence of prodigiosin was also demonstrated in a strain of Streptomyces.5 Recently the authors isolated a new C25-pigment from red mycelia of a strain of Streptomyces and found that the isolated pigment was also an analogue of prodigiosin. The authors called it prodigiosin-25 C* and reported preliminarily the physicochemical properties of the isolated pigment, whose partial structure was also proposed.6

This communication describes details of the isolation and physicochemical characterization of prodigiosin-25 C. Also the chemical structure of this pigment has been presented on the basis of visible absorption spectra, NMR spectra, a mass spectrum and analysis of degradation products.

The pigment was isolated as a hydrochloride, C25H36N3OCl, showing dimorphism (m.p. 76-78°C and 105.5-106°C), the free base, C25H35N3O (m.p. 91-91.5°C) or a perchlorate (m.p. 167°C). It was soluble in chloroform, benzene, acetone, ethyl ether, ethanol, methanol and hot petroleum benzine, sparingly soluble in cold petroleum benzine, and completely insoluble in water at all pH-values. The solubilities mentioned above and absorption spectra in acid and alkaline methanol, \( \lambda_{\text{max}}^{\text{HCl-MeOH}} = 525 \text{ m} \mu \) (\( \varepsilon = 107000 \)), \( \lambda_{\text{max}}^{\text{NaOH-MeOH}} = 460 \text{ m} \mu \) (\( \varepsilon = 42200 \)), as illustrated in Fig. 1, are characteristic of prodigiosin-25 B.

digiosin. As in the case of prodigiosin and prodigiosin-25 A, the isolated pigment forms a zinc complex (m.p. 102-103°C). This is an indication, as Wrede already stated as an evidence for the partial structure of prodigiosin, that the pigment is a dipyrryl methene derivative. Reflux of the hydrochloride with hydroiodic acid in acetic acid

afforded degradation products. Careful fractionation of them gave at least three components, two of which developed red-purple-red colors and the one blue color when mixed Ehrlich's reagent (Fig. 2). This coloration suggests that the products may be pyrrole and bi-pyrrole derivatives, because it is well known that many pyrrole derivatives develop red-purple-red colors and that bipyrrole derivatives give blue-green colors with Ehrlich's reagent.

All the properties mentioned above indicate that the pigment has evidently the pyrryl dipyrryl methene system in the molecule just as prodigiosin and prodigiosin-25 A have.

On the other hand the physical properties, particularly the IR spectra illustrated in Fig. 3 and 4, are distinctly different from those of the known pigments. Therefore it is concluded that the pigment, prodigiosin-25 C, is a new compound.

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The NMR spectrum of the free base, illustrated in Fig. 5, shows a methoxy (6.11, 3H), an aromatic methylene (7.89, 2H) signal and signals assigned to seven olefinic protons (3.1–4.3, 7H). Neither N-methyl nor aromatic methyl signal was detected. A broad NH signal (~1.75, 2H, 60°C, room temperature) was also observed. All these signals can be reasonably explained when prodigiosin-25 C has a pyrryl dipyrrol methene structure carrying a methoxy group as in the case of prodigiosin\textsuperscript{11,13,14} and prodigiosin-25 A,\textsuperscript{3,15–17} while only a single aliphatic side chain, an undecyl group, may attach to one of the three pyrolye rings. The triplet signal with the highest \(\tau\)-value (9.13, 3H) can be assigned to an aliphatic terminal methyl and any additional 3-H signal was not detected in the aliphatic methyl region. Therefore the side chain may not be branched but a straight one (n undecyl chain). The authors wish to tentatively number the pyrrole rings, carbon and nitrogen atoms for convenience as illustrated in the formula I.

The presence of a AB-quartet (3.59 and 4.23, \(J_{AB}=3.5\) c.p.s.) in the aromatic proton region of the NMR spectrum indicates the presence of two vicinal unsubstituted sites in one of the rings, and the presence of a triplet (3.94, 1H) indicates that either the ring I or III carries no substituent. The 2H-signal at ~3.4 can be assigned to ring protons located at 3" and 5" or at 3 and 5. One of the two singlets (3.19 and 4.01, each 1H) is assigned to an isolated ring proton and the other to the methene proton.

The mass spectrum, as illustrated in the Fig. 6 also supports the idea that the undecyl chain is the straight one. The base peak (m/e=393) represents a molecular ion of the free base and the second intense peak, M-141 (m/e=252), is most likely attributable to a fragment represented by the formula II. Though considerably less in its intensity than the M-141 peak, a peak, M-155 (m/e=238), also appeared corresponding to the fragment III produced by removal of the undecyl residue from the molecule. This is a character of mass spectra of alkyl pyrroles, as reported, by Budzikiewicz et al.\textsuperscript{18}

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Presence of metastable peaks at m/e~162 and at a m/e=224~225 suggests that the fragment II was produced from the molecular ion and the fragment III from the fragment II respectively by one step degradation. Between the base peak and the second M-141 peak there are observed 9 peaks, M-15, M-29, M-43, M-57, M-71, M-85, M-99, M-113 and M-127, differing in m/e-values by 14×n (n=1, 2, 3...8) from each other. Fragments corresponding to these peaks may be produced by stepwise removal of C₇-units from the straight side chain of the molecule. Removal of a methoxy residue from the fragment II may afford a fragment IV corresponding to the peak, M-172 (m/e=221). On the other hand, the presence of two peaks, M-231 (m/e=162) and (M-262) (m/e=131), differing from each other by 31 corresponding to a methoxy residue, suggests that the former peak was converted to the latter by losing a methoxy radical. The peak, m/e=162, most likely corresponds to a
methoxybipyrrole fragment and the peak, m/e=131, to a bipyrrole fragment as represented by the formulas V and VI. Therefore

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\text{V} \quad \begin{array}{c}
\text{N} \\
\text{H} \\
\text{H} \\
\text{N} \\
\text{OCH}_3
\end{array} + \quad \text{VI} \quad \begin{array}{c}
\text{N} \\
\text{H} \\
\text{H} \\
\text{N} \\
\text{OCH}_3
\end{array}
\]

it is considered that the methoxy group attaches not to the ring I but to the ring II or III. The occurrence of the bipyrrole and methoxybipyrrole fragments also suggests that the undecyl chain may attach to the ring I but not to the bipyrrole moiety. If the undecyl chain would attach to the ring II or III, the peaks corresponding to the fragments represented by the formulas VII and VIII should be more intense than the peaks corresponding to the fragments V and VI according to the mode of fragmentation of alkyl pyrroles. However the intensities of the peaks corresponding to the fragments VII and VIII are considerably lower than the peaks, M-231 and M-262, corresponding to the fragments V and VI. Thus the ring carrying the undecyl chain is most likely the ring I. On the other hand, as already described, the presence of the triplet in the aromatic proton region indicates that either the rings I or III carries no substituent. Therefore it seems reasonable to conclude that the methoxy group attaches to the ring II and not to the ring III. The visible absorption spectra, as shown in Fig. 1, showed a resemblance to those of prodigiosin (IX) rather than to those of the isomeric compound X synthesized by Rapoport and Holden, in which the methoxy group is located at the position 4'. Hence the methoxy group seems to be located at the position 3' but not at 4'.

As to the position of the \(\pi\)-undecyl chain the position 4 must be excluded, since the structure in which the undecyl chain is located at the position 4 is not compatible with the NMR spectrum showing a AB quartet and only two singlets in the aromatic proton region. Therefore, the structure of prodigiosin-25 C is most likely to be XI or XII. Furthermore, the NMR spectrum (60 MC) of this pigment differs from that of prodigiosin in the aromatic proton region as follows. In place of the singlet (3.65\(\tau\), III) in the spectrum of prodigiosin the AB quartet (3.55 and 4.19\(\tau\)) appeared in the spectrum of prodigiosin-25 C. The \(\tau\)-value (3.55) of the A part of the AB-quartet in the spectrum of prodigiosin-25 C is essentially identical with that (3.65) of the singlet in the spectrum of prodigiosin. The

19) H. H. Wasserman, personal communication.
difference mentioned above suggests that the singlet in the spectrum of prodigiosin can be assigned to the proton located at 3-position, and the AB quartet in the spectrum of prodigiosin-25 C to the protons at the positions 3 and 4 of the ring I. Therefore it seems more reasonable to consider that the structure of prodigiosin-25 C is represented by the formul a formula XI.

Meanwhile, Wasserman et al.20 isolated two prodigiosin-like pigments, one of which had been shown to be identical with the compound XI prepared by condensation of 4-methoxy 2, 2'-bipyrrrole 5-aldehyde and 2-n-undecyl pyrrrole, and suggested that the compound XI may be identical with prodigiosin-25 C.* Therefore a direct comparison was made between prodigiosin-25 C and the compound XI prepared by Wasserman et al. Mass spectral cracking patterns of both compounds were essentially identical, as illustrated in Fig. 6. No depression in melting point was observed when the two samples were mixed. The comparison undertaken by Wasserman et al.20 also showed that the two compounds were identical in all respects including the IR spectra in solution. Therefore the structure of prodigiosin-25 C has been decided to be the formula XI.

Like prodigiosin and prodigiosin-25 A,21 prodigiosin-25 C also showed considerable antibi otic activity, though being somewhat toxic to mice (LD_{50} 26.7 mg/kg body weight). It was bactericidal to Micrococcus pyogenus var. aureus 209 and M. flavus at 100 µg/ml, and to Bacillus agri, M. luteus and M. lysodeikticus at 20 µg/ml, and bacteriostatic to B. subtilis (PCI 219) at 20 µg/ml. It showed no activity against Candida albicans Penicillium crysogenum, Saccharomyces cerevisiae, E. coli, Mycobacterium 607, M. phlei and Pseudomonas fluorescens. While pro-
digiosin-25 A was reportedly active against Mycobacterium 607,21 prodigiosin-25 C was not active against this Mycobacterium and M. phlei. It should be also mentioned that prodigiosin-25 C showed no detectable insecticidal activity against house-flys.*

** EXPERIMENTAL **

** Materials.**

Aluminum Oxide: Aluminum oxide (Brockmann, Merck) was washed with water and ethanol successively, dried overnight at 120~150°C, and ground in a mortar.

Magnesium Oxide: Magnesium oxide was prepared from magnesium hydroxide (Kanto Kagaku Co., Ltd., G. R.) by ignition.

Hyflosuper Cel: Hyflosuper Cel (Wako Pure Chemical Industries, Inc.) was used after drying overnight at 105°C.

Florisil: Florisil (Wako Pure Chemical Industries, Inc., 60~100 mesh) was activated according to Kiribuchi et al.21)

Pigment Production: The constitution of the medium used for the pigment production was as follows: it contained, per liter, 20 g of glucose, 10 g of soluble starch, 1 g of meat extract, 4 g of dried yeast, 2 g of sodium chloride, 25 g of soybean meal and 0.05 g of dipotassium phosphate. The medium was autoclaved for 15 minutes after being brought to pH 9. Strep tomyces, strain 28-24, isolated by one of the authors (J. N.) and considered to resemble S. ruber,** was inoculated into 15 ml of the medium in a 100 ml-Erlenmeyer flask. After being shaken for 3~4 days at 30°C, the culture was poured into 1.5 l of the same medium in a 5 l-Erlenmeyer flask. After further shaking for 4 days, the mycelia of the organism were harvested by centrifugation. About 3 kg of red mycelia were obtained from 9 cultures.

Pigment Extraction: Three kilograms of wet mycelia were extracted repeatedly with 31 each portion of acetone. The combined extract (about 12 l) was extracted twice with each 51 portion of chloroform after being mixed with 91 of distilled water.

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20) H. H. Wasserman, G. C. Rodgers, Jr. and D. D. Keith, personal communication.

* After the manuscript had been subjected to publication, a preliminary report, of their works appeared in Chemical communications 1966, 826.


** Identification of this strain is now in progress.

* The insecticidal activity of prodigiosin-25 C was examined by a courtesy of Prof. Y. Ohshima, Kyushu University, to whom the authors wish to express their cordial thanks.

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and the chloroform solution was washed with distilled water to remove acetone, dried over anhydrous sodium sulfate and evaporated in vacuo. The resulting residue was dissolved in 50 ml of 1:4 (v/v) mixture of chloroform and petroleum benzine.

Chromatography and Crystallization of the Hydrochloride: The pigment solution thus obtained was passed through a column of aluminum oxide (3.5x15 cm) and the column was further washed with the same solvent mixture. The main pigment was adsorbed on the column forming a bright red band. Below the main band a yellow and a pink band appeared. The yellow band was eluted from the column with the same solvent mixture (Fr. 1) and the pink band with 1:2 (v/v) mixture of chloroform and petroleum benzine (Fr. 2). The main red band was eluted with 1:1 (v/v) mixture of chloroform and petroleum benzine and evaporated in vacuo under a stream of nitrogen. The residue was dissolved in 50 ml of 1:4 (v/v) mixture of acetone and petroleum benzine and washed through a column of magnesium oxide (magnesium oxide-Hyflorsuper Cel 2:1, w/w, 5x15 cm). The main pigment was adsorbed on the column forming a yellow band. Below the main band a faint yellow and a pink band were observed. When the column was washed further with 1:4 (v/v) mixture of acetone and petroleum benzine, the faint yellow band first (Fr. 3) and then the pink band (Fr. 4) were eluted from the column. After the pink band was eluted completely, the main yellow band was developed with 1:4 (v/v) mixture of ethanol and petroleum benzine. Then the solution was kept overnight at 0-4°C and then afterward for several hours at -20°C. The fine red needle-like crystals precipitated were collected by filtration followed by washing with a small volume of cold petroleum benzine. About 150 mg of the crude crystals were obtained, which were further purified by repeated crystallization followed by drying in vacuo for 4 hours at 60°C. The purified material melted once at 76-78°C, then solidified and melted again at 105.5-106°C (crystal I). Anal. Found: C, 69.92; H, 8.46; N, 10.01; Cl, 8.56; O, 3.52. Calcld. for C25H36OCl: C, 69.83; H, 8.44; N, 9.77; Cl, 8.24; O, 3.72%. Molecular weight of the hydrochloride was determined to be 440 by Signer's method using acetone as the solvent. Analysis by mass spectrometry (Fig. 6) gave the molecular weight of the free base, 393. Both of the determined values are exactly consistent with the theoretical values. Prolonged heating (30-60 minutes) of the crystal I at 75°C gave crystal II, which melted at 105-106°C but did not melt at 76-78°C. During the heating loss of weight was less than 1%. Recrystallization of crystal II at lower temperature yielded again crystal I. Although the IR spectra of the two crystals were somewhat different when measured in KBr-disks, as illustrated in Fig. 3, the IR spectra of both crystals in solution were exactly identical, as illustrated in Fig. 4-B.

The Free Base

One hundred milligrams of the hydrochloride were dissolved in 200 ml of benzene, washed with 200 ml of distilled water, shaken twice with 200 ml each portion of 1.5 N-aqueous ammonia, filtered through a thin layer of anhydrous potassium carbonate and evaporated in vacuo under a stream of nitrogen so that the volume of the solution was reduced to about 500 ml, washed twice with water to remove ethanol, shaken twice with 300 ml each portion of 0.1 N-hydrochloric acid, filtered through dried filter paper to remove a minute amount of moisture and a purple insoluble matter and evaporated in vacuo under a stream of nitrogen. The residue thus obtained was dissolved in about 30 ml of warm petroleum benzine and cooled to room temperature. At this stage crystallization of the pigment had better be avoided. The solution was kept overnight at 0-4°C and then afterward for several hours at -20°C. The fine red needle-like crystals precipitated were collected by filtration followed by washing with a small volume of cold petroleum benzine. About 150 mg of the crude crystals were obtained, which were further purified by repeated crystallization followed by drying in vacuo for 4 hours at 60°C. The purified material melted once at 76-78°C,* then solidified and melted again at 105.5-106°C (crystal I). Anal. Found: C, 69.92; H, 8.46; N, 10.01; Cl, 8.56; O, 3.52. Calcld. for C25H36OCl: C, 69.83; H, 8.44; N, 9.77; Cl, 8.24; O, 3.72%. Molecular weight of the hydrochloride was determined to be 440 by Signer's method using acetone as the solvent. Analysis by mass spectrometry (Fig. 6) gave the molecular weight of the free base, 393. Both of the determined values are exactly consistent with the theoretical values. Prolonged heating (30-60 minutes) of the crystal I at 75°C gave crystal II, which melted at 105-106°C but did not melt at 76-78°C. During the heating loss of weight was less than 1%. Recrystallization of crystal II at lower temperature yielded again crystal I. Although the IR spectra of the two crystals were somewhat different when measured in KBr-disks, as illustrated in Fig. 3, the IR spectra of both crystals in solution were exactly identical, as illustrated in Fig. 4-B.

* All the melting points described in this communication are the uncorrected data.  
The residue was dissolved in a small volume of hot ethanol containing several drops of concentrated aqueous ammonia. To the clear orange-red solution thus obtained a solution of 1.5 N-aqueous ammonia was added dropwise until the solution became slightly turbid. After cooling at -20°C, orange crystals of the free base precipitated, which were collected by filtration and washed with a small volume of cold ethanol containing about 10% by volume of 1.5 N-aqueous ammonia (yield 75 mg). After repeated crystallization and drying over phosphorus pentoxide for 4 hours at 60°C, the crystals melted at 91-91.5°C. Anal. Found: C, 76.05; H, 8.88; N, 11.10. Calcd. for C_{25}H_{35}N_{3}O; C, 76.29; H, 8.95; N, 10.68%. The IR spectrum in solution and the NMR spectrum of the free base are illustrated in Fig. 4-A and Fig. 5 respectively.

Other Derivatives
A perchlorate, m.p. 167°C, and a zinc complex, m.p. 102-103°C were obtained by the methods described by Wrede and Hettec7) and Wrede.10) Analysis of the zinc complex. Found: C, 70.29; H, 7.05; N, 10.06. Calcd. for (C_{25}H_{34}ON_{3})_{2}Zn: C, 70.02; H, 8.01; N, 9.88°c.

Spectroscopies
Visible and ultraviolet absorption spectra, as illustrated in Fig. 1, were recorded with a Cary automatic recording spectrophotometer Model 14 PM-50. The hydrochloride and the free base gave essentially the same absorption spectra. It should be mentioned that the absorption spectrum in alkaline methanol was recorded immediately after the preparation of the solution, since on standing a while at room temperature in alkaline methanol the spectrum of the pigment somewhat varied. An isobestic point lies at 482 mp. When a methanolic solution of the pigment was mixed with 1/10 its volume of buffer, pH 6-9, and the light absorbancy at 527 mp* was plotted against the pH of the added buffer, a titration curve was obtained and the apparent pKa-value** was determined to be 7.62.

Infra-red absorption spectra were recorded with a Kentron IR spectrometer, Model-D 301 equipped with NaCl optics.

NMR spectrum was recorded with Nihondenhahi NMR spectrometer, JNM C-60 (60 MC) or with a Varian NMR spectrometer HA-100 (100 MC) in deuteriochloroform containing tetramethyl silane as an internal standard.

Mass spectra were measured by the direct insertion method with a Hitachi mass spectrometer Model RMU-6D; chamber voltage 70 eV, target current 52 μA, total current 80 μA, sample heater 240°C, chamber heater 225°C.

Degradation of the Hydrochloride with Hydroiodic Acid
Five milligrams of the hydrochloride were refluxed with 1.5 ml of a mixture of hydroiodic acid (d= 1.654), acetic anhydride and glacial acetic acid (2:4:3) for 6-7 minutes under a stream of nitrogen in an oil bath at 100-105°C. After cooling the reaction mixture was poured into a mixture of 2 ml of concentrated aqueous ammonia and 20 ml of ethanol chilled below -10°C. The mixture was extracted twice with 30 ml each of peroxide-free ethyl ether after being mixed with 40 ml of 0.5 N-sodium thiosulfate. The combined extract was washed 3 times with 30 ml each portion of distilled water, dried over anhydrous sodium sulfate and evaporated under a stream of nitrogen. The residue was dissolved in 5 ml of petroleum benzine and passed through a column of florisil (1 x 5 cm). The column was washed with 35 ml total of petroleum benzine and then eluted with 10% acetone in petroleum benzine. The filtrate and the eluate were fractionated into 5 ml portions. Fractions 2 to 6 (component I) and fractions 11 to 19 (component II) were Ehrlich-positive and combined separately. Each combined fraction was evaporated in vacuo under a stream of nitrogen and dissolved in 5 ml of ethanol. When the reaction mixture, neutralized with ethanolic ammonia, was extracted with petroleum benzine, instead of ethyl ether, a part of a third component of the products remained unextracted in aqueous ethanol-layer. This component was extracted twice with 30 ml each portion of ethyl ether and the combined extract was evaporated, without a prior washing with water, and the residue was dissolved in 15 ml of ethanol (component III).
Fig. 2 shows absorption spectra of the three components after being mixed with Ehrlich’s reagent, which was prepared by dissolving 2 g of p-dimethylamino benzaldehyde in 50 ml of ethanol and mixed with 2 ml of concentrated hydrochloric acid. One ml of the test solution was mixed with 2 ml of the reagent. After standing 30 minutes at 40~45°C, spectra of the reaction mixture were recorded with a Beckman spectrophotometer Model DB against references prepared by omitting p-dimethylamino benzaldehyde from the reaction mixtures.

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