A New Red Dipyrromethene Pigment from *Candida boidinii*

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Candida boidinii, Growth on Ethanol, Presence Monofluoracetate, Red Dipyrromethene Pigment, Structure Elucidation

The yeast *Candida boidinii* ATCC 32195 produces a cell-bound red pigment during growth on 1% ethanol in the presence of 0.05% monofluoracetate. Its isolation and purification are described. The structure was elucidated by chemical degradation and physical methods, mainly mass and nuclear magnetic resonance spectroscopy.

Introduction

Several microorganisms show conspicuous colouring caused by excretion of colouring matter into the media or pigmentation of the cell. Often carotenoides, phenazine and pyrrole compounds, as well as azaquinones and anthocyanes, are responsible for these findings. Among the red compounds produced by bacteria prodigiosin from *Serratia marcescens* [1] and some analogous substances such as undecylprodigiosin from *Streptomyces longisporusruber* [2, 3] and a cyclic pigment from *Alteromonas rubra* [4] are well known examples.

In the case of yeasts red pigments are formed by *Candida pulcherrima* [5] and *Candida lipolytica* [6]. The first yeast produces pulcherrimin, a red pyrazine derivative, the latter the antibiotic tryptanthrin, derived from L-tryptophan. Frequently the pigments are secondary metabolites and are produced mostly under growth limiting conditions. The red pigment of the yeast *Candida boidinii*, first investigated because of utilization of methanol as sole carbon source [7], was observed during studies on citrate accumulation in the presence of inhibitors [8]. The presence of monofluoracetate, besides ethanol as the carbon source, seemed to be a prerequiste for pigment formation.

Methods

Medium and cultivation conditions

The yeast *Candida boidinii* ATCC 32195 was grown in a synthetic medium described by Sahm and Wagner [7]. Only methanol was entirely replaced by ethanol and 0.05% sodium monofluoracetate was added to the synthetic medium. The organism was cultivated batchwise in a 80 l bioreactor (type b 50, Giovanola Frères SA, Monthey, Switzerland) fitted with an intensor system. 69.5 l synthetic medium was inoculated with 0.5 l of a 24 h-culture of *Candida boidinii* in the same medium. Incubation was performed at 30 °C with a stirring rate of 1000 min^{-1} and an aeration rate of 0.3 vvm without adjustment of the pH. After 72 h the cell suspension was harvested by centrifugation at 6000 rpm for 20 min and washed twice with 0.9% sodium chloride solution.

Isolation and purification of the red pigment **(1)**

The wet biomass, 840 g from 70 l-fermentation, was extracted three times with 1.2 l acetone until the red colour was removed from the yeast. The water-containing acetone solution was evaporated at 30 °C under reduced pressure, the water-pigment-suspension was acidified to pH 3 and extracted three times with 200 ml CCl_4. The extracts were dried with sodium sulfate and evaporated to a viscous residue which was applied to a short silica gel column (Kieselgel Woelm, ICN-Pharmaceuticals GmbH, Eschwege). Elution with CCl_4, CCl_4/acetone (25:1) and CCl_4/acetone (9:1) led to separation of lipophilic products. Further elution with CCl_4/methanol (4:1) yielded the crude pigment. After evaporation of the solvent under reduced pressure the crude pigment was chromatographed on plates with silica gel 60 PF 254/366 (Merck, Darmstadt) using toluene/dioxane/acetic acid (45:15:1.5)

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as solvent system. The R_f-values of three red components were as follows: 0.59 (main component), 0.49, 0.39. The pigment bands were eluted immediately from plates with CCl_4/CH_3OH (4:1), the resulting solutions were washed with water and evaporated to dryness.

The purified pigment from the main component was obtained by dissolving the residue in CCl_4/CH_3OH (9:1) and precipitating the red pigment by dropwise addition of *n*-hexane. The yield was 30 mg of a red amorphous substance with a melting region of 197–203 °C.

Spectral methods

Ultraviolett and visible absorption spectra were obtained with a Beckman model 25 spectrophotometer.

^1H and ^{13}C nuclear magnetic resonance spectra were recorded at ambient temperature, at 400 and 100 MHz respectively, on a Bruker WM-400 NMR spectrometer operating in the Fourier transform mode and locked to the deuterium resonance of the solvent, $CDCl_3$. Shifts are reported in ppm relative to TMS. ^1H nuclear Overhauser enhancement (nOe) difference spectra were recorded using the standard software package. The mass spectra were recorded on the A.E.I. MS-9 (high resolution) and MS-30 (field desorption, gc/ms) instruments. For the infrared spectra an Perkin-Elmer IR 521 spectrophotometer was used.

Methylester of red pigment **(2)**

44 mg of the red amorphous pigment (**1**) obtained as described above were dissolved in 130 ml hot methanol. After adding 2 mg of *p*-toluene sulfonic acid the solution was stirred at room temperature for 4 h. Methanol was removed and replaced by CH_2Cl_2. The organic solution was washed with 20 ml water and dried over Na_2SO_4. The solvent was reduced under vacuum and the pigment purified by thin layer chromatography on silica gel with *n*-hexane/acetone (3:2). R_f-values: 0.59 (main component), 0.53, 0.47. The main red nonpolar band of the methylester was scraped from the plate (36 mg).

p-Bromphenacylester of red pigment **(3)**

30 mg of the red pigment (**1**), 20 mg triethylamine and 60 mg *p*-bromphenacylbromide in 35 ml acetone were stirred for 4 h. After filtration of the precipitated triethylammonium bromide the solution was reduced in vacuum and chromatographed on a silica gel plate with *n*-hexane/acetone (3:2). Elution of the red band at the R_f-value of 0.56 with $CHCl_3/CH_3OH$ (9:1) from the plate led to 24 mg (59%) of *p*-bromphenacylester.

Chromic acid and chromate degradation of the red pigments **(1)** *and* **(2)**

Oxidative degradation of the original red pigment (**1**) and of its methylester derivative (**2**) was carried out on silica gel plates a) with chromic acid (1%) in 2N H_2SO_4 and b) with sodium dichromate in the presence of potassium hydrogen sulfate according to the method of Rüdiger [9].

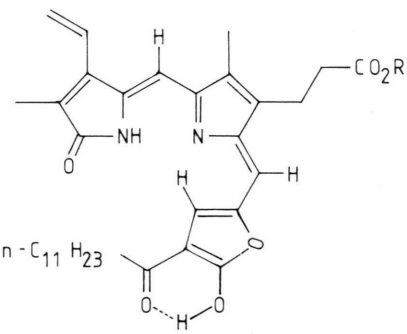
Bayer-Villiger oxidation of p-bromphenacylester **(3)**

The Bayer-Villiger oxidation on (**3**) was performed using *m*-chloroperbenzoic acid and trifluoracetic acid. After saponification with 1N NaOH/dioxane (1:1) and esterification with diazomethane the reaction mixture was analyzed by coupled gas chromatography and mass spectrometry.

Results and Discussion

Formation of the red pigment **(1)**

The formation of the red pigment (**1**) (see Fig. 1) is not growth associated which is characteristic for a secondary metabolite. In the exponential growth



1 R = H
2 R = CH_3
3 R = CH_2-CO-C_6H_4-Br

Fig. 1. Structure of the red pigment (**1**) and its synthetic methylester (**2**) and *p*-bromphenacylester (**3**) derivatives.

phase with a specific growth rate of 0.18 h^{-1} no pigment is produced but is formed during the subsequent stationary growth phase and reached the highest value after 72 h. The facultative methylotrophic *Candida boidinii* produces the pigment only in the presence of fluoracetate, the precursor of the aconitase inhibitor, fluorcitrate. The unique molecular structure of the red pigment, which is so far unknown, combines precursors of bile pigments with products of fatty acid biosynthesis.

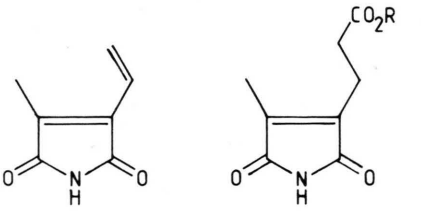
The red pigment (**1**) displayed no antimicrobial activity against *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Candida albicans, Schizosaccharomyces pombe,* and *Mucor hiemalis.*

Isolation and purification of the red pigment (**1**)

The red pigment was extracted from the wet biomass with acetone and purified by silica gel chromatography, leading to 30 mg of a red amorphous substance. By new chromatography of the two minor red components (yield: 1 mg) the original three omponents of the extract were observed again, indicating an isomeric state of a single compound. The main component, which had given a single band, also formed several bands after repeated chromatography on silica gel using the same solvent system. For bile pigments it is well known [10] that esterification improves their stability without alteration of their structure. However, for **1** no single methyl derivative could be prepared, even after treatment with methanol and *p*-toluene sulfonic acid.

Identification of the red pigment

Structural studies were carried out with the original red pigment (**1**), with the mono-methyl (**2**) and the *p*-bromphenacylester (**3**) derivatives (see Fig. 1). The absorption maxima of the original red pigment and its methyl derivative were at 555 nm and 325 nm indicating a conjugated system of double bonds. No optical activity could be detected, neither by ORD nor by CD spectroscopy. The infrared spectrum of **1** showed the following absorption characteristics (cm^{-1}): 3430 (NH, OH); 2920, 2850: aliphatic – CH_2 –; 1715, 1690: unsaturated ring ketone C=O (strong); 1640, 1590: C=N; 1520: pyrrole C=C (strong). By field desorption mass spectrometry the molecular ion of **1** was found at *m/e* 562. On high resolution mass-spectrometry the sufficiently volatile methylester **2** gave a molecular ion at *m/e* 576, 3197 which corresponds to the empirical formula $C_{34}H_{44}O_6N_2$ (calculated: 576, 3199). From these data as well as from the absorption characteristics of the visible and infrared spectra a conjugated system of two pyrrole rings and three further double bonds were suggested. The high intensity of the molecular ion at *m/e* 576 reflects the stability of the fully conjugated system. The accompanying lower mass peaks account for the successive fragmentation of a propionic ester chain: *m/e* 561 (M^+-CH_3), 545 (M^+-OCH_3), 517 (M^+-CO_2CH_3), 503 (M^+-$CH_2CO_2CH_3$), 489 (M^+-$CH_2CH_2CO_2CH_3$). The stability of the fully conjugated pigment was analogous to that of alkylated dipyrromethenes in which the fragmentation occurs primarily at the side-chain position rather than at the methine bridge. As the molecular ion was even-numbered and, in addition, the elemental analysis of this compound ($C_{34}H_{44}O_6N_2$; calculated: C 70.83, H 7.63, O 16.66, N 4.86; found: C 70.60, H 8.51, O 16.67, N 4.29) did not allow three nitrogen atoms, two conjugated pyrrole rings as well as one other conjugated ring was indicated. In order to prove these structural characteristics the original acidic pigment (**1**) and its methylester derivative (**2**) were degraded oxidatively with sodium dichromate as well as with chromic acid. The procedures are reported as mild techniques in structural studies of bile pigments [9]. In the presence of potassium hydrogen sulfate (pH 1.7) bile pigments are degraded to imides by sodium dichromate without hydrolysis of the ester bonds. On such oxidations the rings B and C of biliverdine led to pyrrole dialdehydes as intermediates in imide formation. Both red compounds **1** and **2** were oxidized according to this procedure for 2 h with sodium dichromate. After



4 **5** R=H **5a** R=CH_3

Fig. 2. Oxidative degradation products of the red pigment (**1**) and its synthetic methylester derivative (**2**).

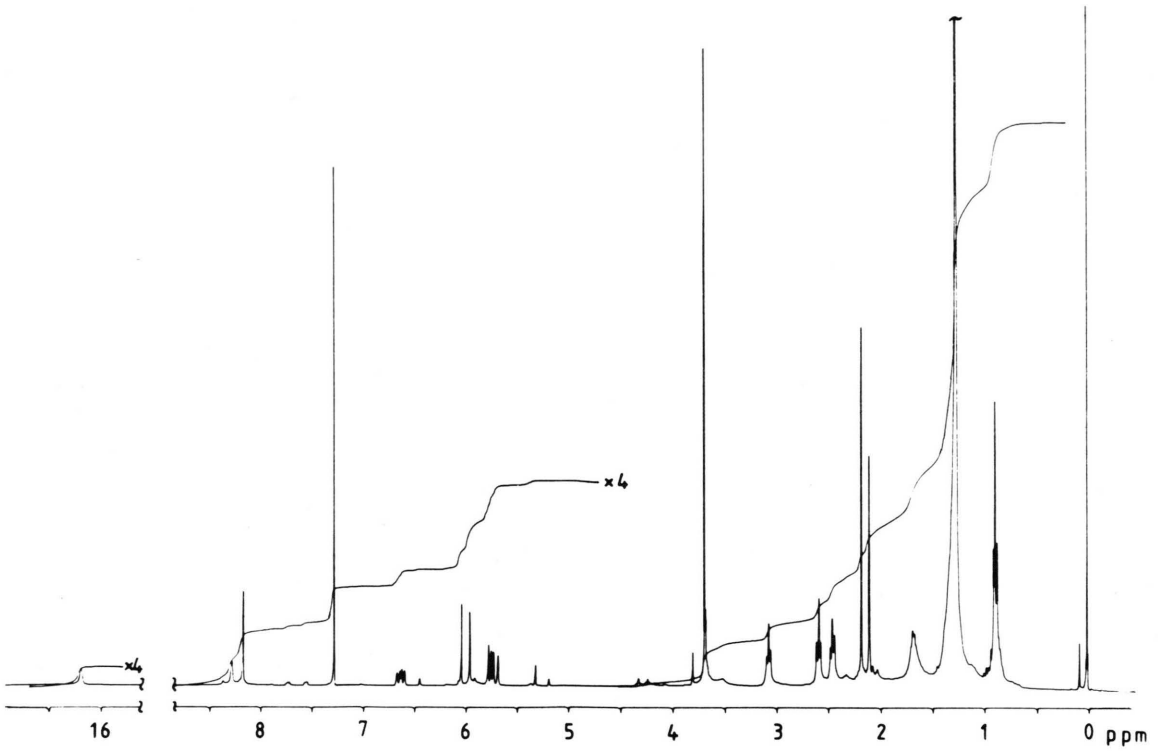


Fig. 3. 400 MHz ^{1}H-NMR spectrum of **2** in $CDCl_3$.

development of the thin layer plates in the solvent system CCl_4/acetic acid ethylester/cyclohexane (5:3:1) methylvinylmaleimide (**4**) and hematic acid imide (**5**) (see Fig. 2) could be detected with chlorine/toluidine in the case of the original red pigment (**1**). The polar imide (**5**) could be esterified by methanol/*p*-toluenesulfonic acid to the methylester (**5a**). The R_f-values of 0.26 of this ester and 0.43 were in accordance with authentic imide samples, derived from oxidation of biliverdine. Oxidation of the methylester derivative (**2**) of the red pigment led to hematinic acid imide methylester (**5a**) as well as methylvinylmaleimide.

The analogous degradation of **1** and **2** with chromic acid only gave hematimic acid imide or hematimic acid imide methylester, the latter was hydrolyzed within 4 h to the acid form. The three reaction products were separated on silica gel plates and subjected to mass spectral analysis. The relative intensities of the molecular and other ions, characteristic fragmentation pattern as well as the metastable ions are identical with the literature data for these known imides. Preliminary NMR measurements indicated a long alkyl chain attached to the chromophore system. Thus to study the remainder of the molecule, the *p*-bromphenacylester (**3**) was subjected to a Bayer-Villiger oxidation. After saponification and esterification dodecanoic acid methylester as the solely fatty acid derivative was identified by gc/ms spectroscopy. This result showed that a 11-membered alkyl chain is bound to the conjugated system via a keto group.

Further structural elucidation required the analysis of the ^{1}H and ^{13}C NMR spectra of **1** to **3**. The 400 MHz ^{1}H NMR spectrum of the methyl ester (**2**) is shown in Fig. 3. Comparison of this with the known spectra of the bile pigments allows immediate identification of two pyrrolic methyl groups at 2.09 and 2.17 ppm, a methyl propionic ester group with chemical shifts at 2.56 (t), 3.06 (t) and 3.67 (s) ppm and the ABX spin system of a vinyl group with chemical shifts at 5.70, 5.76 and 6.62 ppm, respectively. Three sharp methine singlet signals, each corresponding to one proton, are found at 5.94, 6.03

and 8.15 ppm, and two broad singlet peaks at 8.26 and 16.17 ppm corresponding to NH and a hydrogen-bonded enolic OH, respectively.

As the assembly of the various units in the molecule could not be reliably deduced from the shift data ^{1}H nOe difference spectra were recorded. Irradiation of the methyl group at 2.14 ppm gave pronounced nOe effects for the singlet at 6.03 ppm and the methylene groups at 2.56 and 3.06 ppm of the propionic ester group. Irradiation of the methyl group at 2.09 ppm caused a significant effect for the signal of the vinyl group at 5.70 ppm and a smaller effect on the signal at 6.62 ppm of the same group; no effect upon any of the methine peaks was observed. Conversly irradiation of the methine signal at 6.03 ppm caused effects upon the vinyl group signal at 6.62 ppm and the methyl group at 2.14 ppm. This data indicates the pyrrole rings are substituted as shown in Fig. 4 and have a methine bridge with a *Z* configuration.

An nOe effect on the signal at 2.44 ppm was observed upon irradiation of the methine at 5.94 ppm. This was confirmed by irradiation at 2.44 ppm which caused effects at 5.94 ppm and 1.66 ppm, and indicates that this methine proton is in close proximity to the α-methylene of the long alkyl side chain. Finally irradiation of the lowest field methine signal at 8.15 ppm caused a small effect upon the low field methylene signal of the propionic ester side chain.

Hence the ^{1}H data and other physical and chemical data allow the following structures shown in Fig. 5 to be proposed.

The ^{13}C data are given in Table I. The assignments of the hydrogen-bearing carbons follows from the single frequency off-resonance proton decoupled spectrum and comparison with literature data. Although the quaternary carbons can not be unambiguously assigned, their general shift position

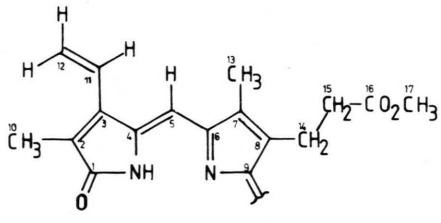


Fig. 4. Partial structure of dipyrromethene part of **2** from ^{1}H nOe data.

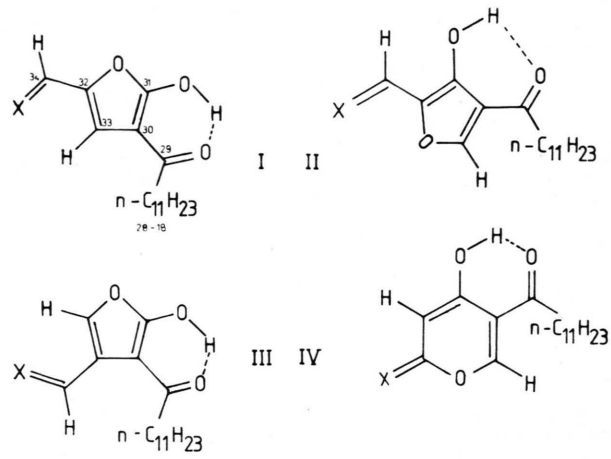


Fig. 5. Possible structures of **2** from ^{1}H-NMR data and other physical and chemical data. X corresponds to the partial structure shown in Fig. 4.

are compatible with the proposed structures. Of particular interest are the two methine carbon signals at 107.73 and 136.63 ppm which correlate with the proton signals at 5.94 and 8.15 ppm, respectively, and have ^{1}J(CH) values of 172.4 and 154.1 Hz, respectively (determined from a proton coupled ^{13}C spectrum). Inspection of the literature

Table I. ^{13}C NMR data for **2** in $CDCl_3$.

(i) Hydrogen-bearing carbons

Shift	Multiplicity [a]	Assignment [b]	^{1}J(CH)
9.50	q	10, 13	
9.87	q		
14.15	q	18	
19.97	t	14	
22.73	t	19	
26.34	t	27	
29.08–29.75		21–26	
31.95	t	20	
33.89	t	28	
35.35	t	15	
51.86	q	17	
94.72	d	5	160.2
107.73	d	33 [c]	172.4
123.88	t	12	160.2
125.99	d	11	155.6
136.63	d	34 [c]	154.1

(ii) Quaternary carbons at:
128.26, 129.96, 133.57, 140.38, 140.50, 140.80, 141.32, 165.87, 167.74, 168.39, 171.54, 172.29(16), 182.30(29)

[a] From SFORD ^{13}C spectrum.
[b] For numbering see Figs. 4 and 5.
[c] See text.

data for furan [11] and 4-pyrone [12] indicates that for the above four systems $^1J(CH)$ values for carbons α to the ring oxygen should be approx. 200 Hz and approx. 170–175 Hz for β-carbons. Thus the data for **2** are only compatible with the signal at 107.73 ppm arising from a β-carbon and hence structure I of Fig. 5 is the most likely structure for **2**. This is also compatible with the nOe data for the attached proton which interacts with the α-methylene of the long alkyl side chain. The second methine carbon, with a $^1J(CH)$ value of 154.1 Hz, has to be assigned to the carbon adjacent to the second pyrrole ring and this value is in keeping with $^1J(CH)$ values of approx. 154 Hz found for the bridge methine carbons in the bile pigments [13].

Lactam-lactim and keto-enol tautomerism would then account for the problems encountered in the preparation and separation of these compounds.

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