Pseudoverdin, a Compound Related to the Pyoverdin Chromophore from a *Pseudomonas aeruginosa* Strain Incapable to Produce Pyoverdins [1]

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Dedicated to Professor Carl Djerassi on the occasion of his 70th birthday

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Pseudomonas aeruginosa Mutant Strain, Biogenesis, Coumarin Derivative, Pyoverdins, Siderophores

From a genetically manipulated strain of *Pseudomonas aeruginosa* ATCC 15692 (PAO1 strain) a compound named pseudoverdin, 3-formylamino-6,7-dihydroxycoumarin, was obtained which is related to the typical pyoverdin chromophore and thus allows to shed some light on the biogenesis of the latter.

Introduction

Members of the fluorescent group of the genus *Pseudomonas* are characterized by the production of siderophores ("pyoverdins") when grown under iron deficiency [2, 3]. Their common structural unit is the chromophore (**1**), (1*S*)-5-amino-2,3-dihydro-8,9-dihydroxy-1*H*-pyrimido-[1,2a]quinoline-1-carboxylic acid [4]. Its 5-amino group is bound amidically to a small dicarboxylic acid from the citric acid cycle and its 1-carboxyl group is linked to a peptidic residue. From *Pseudomonas aeruginosa* ATCC 15692 (PAO1 strain) the pyoverdins C-E were isolated and their structures could be elucidated [5].

During complementation studies of *Pseudomonas aeruginosa* PAO pyoverdin-deficient mutants by conjugational mobilization of a *Pseudomonas aeruginosa* PAO1 cosmid bank one bacterial clone with a restored yellow-green fluorescent wild type phenotype could be isolated [6]. This clone was shown to contain a cosmid derivative, pPYP17, containing a 15.1 kb PAO1 chromosomal DNA insert. Strains harboring this cosmid were characterized by the production of a compound named pseudoverdin very similar to the *Pseudomonas aeruginosa* PAO pyoverdins as far as its fluorescence, its UV/VIS spectral properties and its capacity to chelate iron(III) are concerned. However, it differs from the pyoverdin in its biological properties as it is unable to transport iron into the cells. This feature correlates well with the lack of the peptide portion of pyoverdins since it is known that their peptide part plays a determinant role in receptor-ligand interactions [7]. Moreover, pseudoverdin is constitutively produced by the pPYP17-containing bacteria irrespective of their growth conditions, whereas the pyoverdin biosynthesis is strictly controlled by the supply of Fe(III) [8]. These observations suggest that the 15.1 kb DNA fragment corresponds to a deregulated pyoverdin biosynthesis gene cluster containing genes involved in the biosynthesis of the chromophore part of the pyoverdin molecule [6].

In this communication we wish to describe the structure elucidation of pseudoverdin (**2**) isolated from the culture medium of the genetically manipulated *Pseudomonas aeruginosa* strain described above. As will be shown it sheds some light on the biogenesis of the pyoverdin chromophore.

Reprint requests to Prof. Budzikiewicz.

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Experimental

Strain and growth conditions

The pseudoverdin producing strain used in this study was *Pseudomonas aeruginosa* PAO6624/pPYP17. This strain was obtained by conjugational transfer of the pMMB33-cosmid derivative pPYP17 [6] into the pyoverdin-deficient mutant PAO6624 [9]. Due to intrinsic instability of the construct, growth experiments were systematically started from a stock suspension strain PAO6624/pPYP17 maintained under glycerol at −80 °C. A loop of the stock suspension was streaked at the surface of an agar-solidified King's B medium [10] supplemented with 500 μg/ml kanamycin. Among the colonies which developed after 72 h of growth at 37 °C two different phenotypes were observed. Some colonies, usually smaller than the others, were characterized by a strong yellow-green pigmentation and by the exhibition of a strong fluorescence when illuminated under UV-light (365 nm) developed around the colonies in a diffused halo. These colonies with such a restored wild type phenotype were typical for PAO6624/pPYP17. The second phenotype was characterized by non-pigmented colonies, without any fluorescent halo and corresponded to the phenotype previously observed for the pyoverdin-deficient mutant PAO6624. However, their growth in the presence of kanamycin indicated that these colonies should correspond to PAO6624 cells still harboring a defective pPYP17 cosmid. Five to ten single colonies developing a yellow-green fluorescent halo were selected, homogenized in sterile distilled water and used directly as the culture inoculum. Liquid cultures in the presence of 300 μg/ml kanamycin were grown either in succinate media [2] for the production of pseudoverdin or in iron supplemented ($FeCl_3$, 200 μM) succinate media for the production of ferri-pseudoverdin at 37 °C with shaking (200 rpm) in 1-liter Erlenmayer flasks containing 500 ml medium.

Isolation and purification of 3-formylamino-6,7-dihydroxycoumarin (**2**)

In order to show that no structural changes occurred by addition of Fe(III) to the culture medium both iron-free cultures and culture media obtained after addition of $FeCl_3$ were worked up. (a) Iron-free cultures (5 l) were brought to pH 6.5 by addition of HCl and the cell material was removed by centrifugation. The liquid phase was passed through a XAD-column (1 × 0.1 m) with H_2O containing increasing amounts of CH_3OH; thus non-fluorescent material was removed. Finally the fluorescent fraction was desorbed with pure CH_3OH (66 mg raw material from 1 l culture medium). (b) The Fe(III) complex is not stable enough for a work-up by chromatography as described. Therefore, the brown suspension obtained after addition of $FeCl_3$ to the culture medium (5 l) was freed from cell material by centrifugation. The remaining liquid was concentrated i.v. to about one tenth of its volume. In this way a dark precipitate formed which was removed by centrifugation. Evaporation i.v. to dryness yielded 310 mg raw material from 1 l culture medium. An aqueous solution of the Fe(III) complex was treated with ethylendiamine tetraacetic acid (EDTA) at pH 4.5 and subjected to repeated chromatography on Sephadex LH-20 with CH_3OH/H_2O 1:1 (column: 50 × 5 cm; detection at 254 and 310 nm). The material obtained according to procedure (a) was purified in the same way. The amount of **2** obtained by either procedure was *ca.* 2 mg/l culture medium.

Equipment used

HPLC: HPLC-pump 64, Knauer (Bad Homburg); UV/VIS-filterphotometer 734 (Bad Homburg); column: Polygosil 60-C_{18}, 7 μ (Macherey u. Nagel, 4.6 × 250 mm). – Preparative HPLC: HPLC-pump Gynkotek, model 200; UV-spectrophotometer DuPont; column: Polygosil 60-C_{18}, 7 μ (Macherey u. Nagel, 8 × 240 mm). – NMR: Bruker AM 300 (^1H, ^{13}C NMR). – UV/VIS: Perkin-ELMER Lambda 7. – IR: Perkin ELMER Infrared Spectrophotometer 283. – EI-MS: Varian MAT 212 (70 eV).

Chemical preparations

3-Formylamino-6,7-dimethoxycoumarin (**5**) *from* (**2**)

Through a solution of 8 mg **2** in 10 ml CH_3OH, CH_2N_2 was bubbled for 1 h. The reaction mixture was kept at room temperature for 1 h, then all volatile material was removed i.v. Purification of **5** was achieved by preparative HPLC (Polygosil 60-C_{18}, 7 μ) with CH_3OH/H_2O 2:3 (v/v).

3-Acetylamino-6,7-dimethoxycoumarin (**7**)

In analogy to a published procedure [11], 2 g 2-hydroxy-4,5-dimethoxy benzaldehyde (**9**) were heated under stirring with 0.75 g glycine, 1.25 g dry sodium acetate and 2 g acetic anhydride for 6 h. The raw product was recrystallized from 60% C_2H_5OH. M.p. 248 °C, yield 0.3 g (11%), M^+ *m/z* 263. ^1H NMR (DMSO-d_6): δ = 9.59, 8.56, 7.26, 7.04, 3.83, 3.79, 2.13. ^{13}C NMR see Table I.

3-Amino-6,7-dimethoxycoumarin (**8**)

In analogy to a published procedure [12], 0.3 g **7** in 15 ml C_2H_5OH/HCl conc. (2:1 v/v) were refluxed for 15 min. The C_2H_5OH was distilled off on a steam bath, the residue was diluted with 50 ml H_2O and the mixture was made alkaline with $NaHCO_3$. The precipitate formed was collected, washed with H_2O and recrystallized from 60% C_2H_5OH. M.p. 170 °C, yield 0.2 g (79%), M^+ *m/z* 221. ^1H NMR ($CDCl_3$): δ = 6.7, 6.68, 6.64, 4.10, 3.87, 3.86 (all s). ^{13}C NMR see Table I.

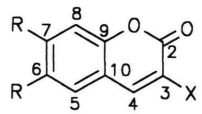
3-Formylamino-6,7-dimethoxycoumarin (**5**)

0.2 g **8** together with 7 mg HCOOH and 5 ml toluene were heated to 110–120 °C until the formation of H_2O ceased. The reaction mixture was evaporated i.v. to dryness, 50 mg of the raw material (yield 160 mg) were purified on a silica gel column (40 × 2.5 cm) with $CHCl_3/CH_3COCH_3$ (increasing the CH_3COCH_3 content by 1% v/v for every 500 ml $CHCl_3$). **5** was eluted with 4% CH_3COCH_3 and subjected to HPLC as described above. M.p. 246 °C, M^+ *m/z* 249. ^1H NMR (DMSO-d_6): δ = 10.06, 8.61, 8.37, 7.3, 7.06, 3.83, 3.79. ^{13}C NMR see Table I.

Results

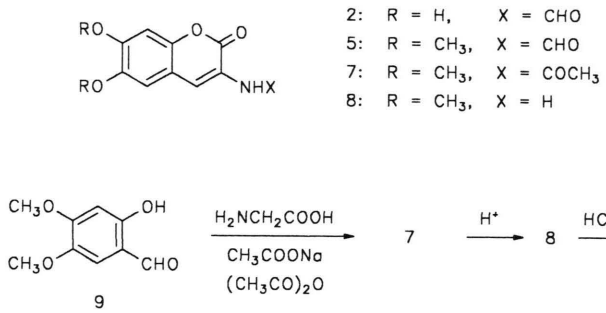
The structure of **2** was deduced from its spectral data and confirmed by an independent synthesis of its dimethyl ether. **2** has an absorption maximum at 356 nm (ε 8003; CH_3OH). Its EI mass spectrum shows a molecular ion at *m/z* 221 and successive losses of CO (*m/z* 193), CO (*m/z* 165), HCN (*m/z* 138) and again twice CO (*m/z* 110 and *m/z* 82) typical for the degradation of lactones and phenolic compounds. In the IR-spectrum (KBr) two carbonyl bands are seen at 1698 and 1652 cm^{-1}, which belong to the coumarin system (coumarin has 1705 cm^{-1}) and the formyl group, resp. The ^1H NMR spectrum (DMSO-d_6) shows three aromatic

Table I. ^{13}C NMR data (chemical shifts in ppm).



Compound	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10	X (CO; CH_3)	R
R: H[a]* X: H	160.4	116.4	143.6	128.1	124.4	131.8	116.4	153.9	118.8		
R: OH[a]* X: H	161.4	112.0	144.5	112.9	143.3	150.6	103.2	149.1	111.4		
R: OMe[a] X: H	160.7	113.5	142.8	108.0	146.2	152.8	99.9	150.0	111.2		
R: H[b] X: NH_2	158.8	133.3	125.4	108.0	124.5	124.8	115.5	148.0	121.9		
8 R: OMe[c] X: NH_2	159.7	130.2	112.0	106.2	143.9	148.8	99.9	146.5	113.5		56.3; 56.4
R: H[c]* X: NHCHO	157.3	123.8	123.4	127.9	125.0	129.7	115.9	149.7	119.4	161.4	
2 R: OH[c] X: NHCHO	157.8	120.5	125.3	111.9	143.4	148.7	102.6	144.5	110.8	161.0	
5 R: OMe[c] X: NHCHO	157.6	121.3	124.8	108.8	146.4	150.9	99.9	145.1	111.5	161.1	55.9; 56.1
R: H[c]* X: $NHCOCH_3$	158.8	123.9	123.3	127.8	125.2	129.6	116.3	149.9	119.8	169.4; 24.7	
7 R: OMe[c] X: $NHCOCH_3$	157.9	122.1	125.0	108.7	146.3	150.7	99.8	145.0	111.6	169.9; 23.8	55.9; 56.1

Spectra determined in DMSO-d_6 or * in $CDCl_3$ relative to tetramethylsilane (TMS). Data taken from literatur, [a] [13] and [b] [14]; [c] data acquired with a Bruker AM 300.



Scheme 1. Synthesis of pseudoverdin dimethyl ether.

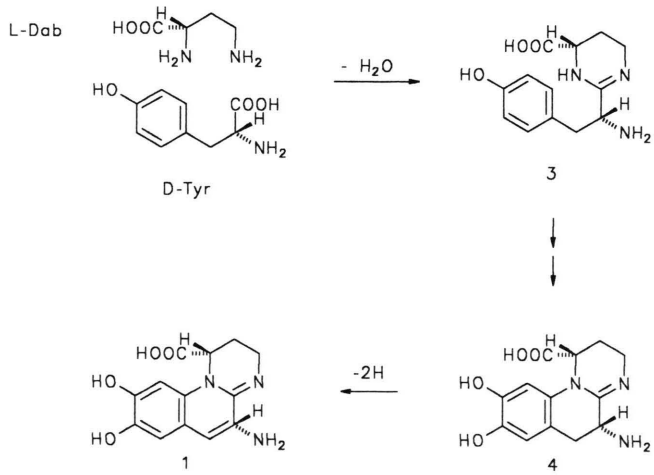
singlets at 6.75 (H-8), 6.95 (H-5) and 8.45 ppm (H-4; between H-4 and H-5 an NOE is observed) and two additional ones at 9.99 (NH) and 8.33 ppm (CHO). The ^{13}C NMR data of **2** and of related compounds can be found in Table I. **2** was transformed by treatment with CH_2N_2 in CH_3OH into its dimethyl ether **5** which proved to be identical (HPLC retention time, IR, ^1H and ^{13}C NMR, mass spectrum) with a synthetic compound prepared according to Scheme 1.

Discussion

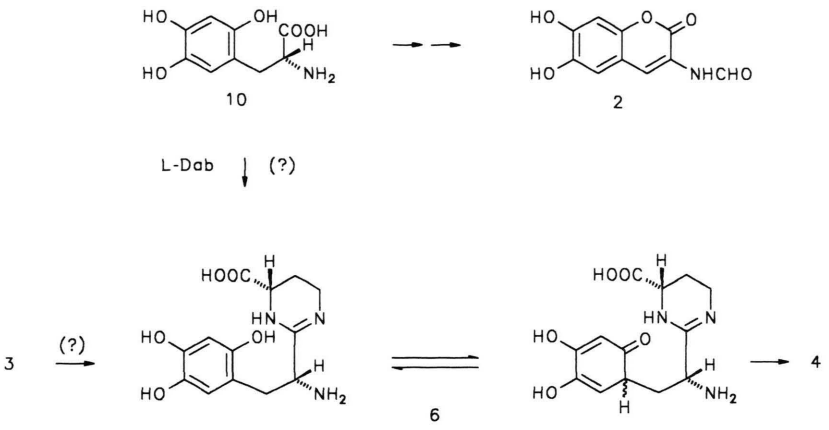
Until recently hardly anything was known about the biosynthesis of the pyoverdins. The first clue came from the observed co-occurrence of pyoverdins, dihydro-pyoverdins and ferribactins which possess identical peptide chains but differ in the nature of their chromophores. The ferribactin chromophore (**3**) was shown to be a condensation product of D-Tyr and L-2,4-diaminobutyric acid (L-Dab) which comprises all structural elements necessary for the formation of **4** and subsequently of **1** (Scheme 2) [15]. However, the cyclization step **3** → **4** and the moment of the introduction of the second (catecholic) hydroxyl group remained moot points. It may be surmised that these transformations occur at an early stage of the biosynthetic steps since only pyoverdins with both hydroxyl groups have been detected.

The isolation of pseudoverdin (**2**) suggests that Tyr can be oxidized to 2,4,5-trihydroxy-Phe (**10**) [16] which by condensation with Dab leads to **6.** If, however, **3** is an intermediate on the way to **1** introduction of the additional hydroxylgroup should occur on the stage of **3**. In either case, **6** possesses the necessary structural features for cyclization (an intramolecular Bucherer reaction) to furnish the dihydropyoverdin chromophore (**4**) (Scheme 3).

If the dihydropyoverdins are the immediate precursors of the pyoverdins pseudoverdin (**2**) does not fit into the biogenetic chain. Its formation



Scheme 2. Proposed biogenetic correlation of the chromophores of ferribactins and pyoverdins.



Scheme 3. Biogenetic correlation of pseudoverdin (**2**) and the pyoverdin chromophore (**1**).

could rather be the result of an aberrant pathway owing to the genetic manipulation. The introduction of the double bond could be due to a dehydrogenation step taking place en lieu of that leading from dihydropyoverdins to pyoverdins in those strains which are not capable to attach the peptide chain to the C-1 carboxyl group and the formylation of the amino group would correspond to the attachment of the dicarboxylic acids in pyoverdins.

Acknowledgements

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