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STRUCTURE OF THE YELLOW-GREEN FLUORESCENT PEPTIDE PRODUCED BY IRON-DEFICIENT *AZOTOBACTER VINELANDII* STRAIN O

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Abstract—The yellow-green fluorescent peptide (hereafter called Compound A-1) has been isolated from iron-deficient *Azotobacter vinelandii* strain O, and the total structure of compound A-1 was determined by X-ray analysis (chromophore moiety) and the subtractive Edman degradation (peptide moiety).

INTRODUCTION

THE presence of yellow–green pigments in cultures of Azotobacter was first reported by Beijerinck (1907).[1] Johnstone observed a pigment which fluoresced under UV light (3600 A).[2] Previous experiments[3,4] had shown that the pigment was produced under a low level of iron in the culture medium. Johnstone *et al.*[5] established that the fluorescence was not due to riboflavin, but produced by water-soluble, thermostable pigments. The pigment was shown to be a peptide with an attached chromophore (Bulen, LeComte 1962).[6]

Since iron is involved in nitrogen fixation and other electron-transfer systems, there is a correlation between the occurrence of the pigment and the availability of iron in the medium.

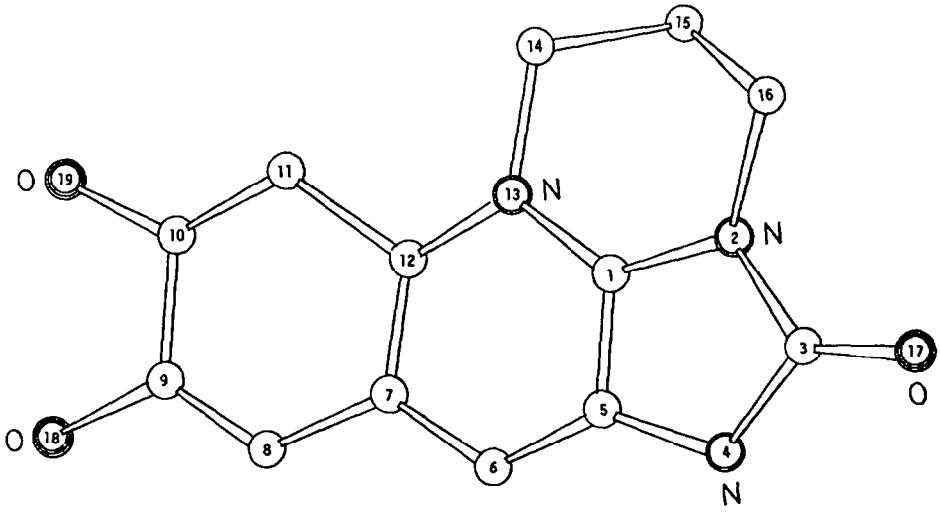
This report describes a new method of isolation and purification of this pigment, the X-ray analysis of the chromophore decarboxylated (Scheme 1), and the amino acid sequence analysis of the peptide moiety with the subtractive Edman degradation of partial acid hydrolysates of compound A–I (Scheme 2).

RESULTS AND DISCUSSION

(I) *The cultivation and isolation of compound A–I*

The pigment does not appear in the culture medium with an iron content of over 1 μg/ml, although the growth curve increases to 10^9 cells/ml in the stationary phase. In an iron-deficient medium (below 0·725 μg/ml), this pigment is produced, while the growth curve reaches only $0{\cdot}87 \times 10^6$ cells/ml in the stationary phase. The isolation of compound A–I is shown in Table 1.

SCHEME 1



SCHEME 2

Chromophore–(Asx)–(Homoser)–(Ser)–(Homoser)–(Citrulline)–(Ser)–(Gly)–(β-Hydroxy Asx)

TABLE 1. ISOLATION OF COMPOUND A–I

Culture 80 l

Sup. continue flow centrifugation at 0° — Ppt.
↓ 1% acetic acid soln.
Florisil column (7·0 × 5·0 cm)
washed with water
eluted with 20% acetone–0·1% ammonia
concentrated *in vacuo* below 40°
DEAE-cellulose column (5·0 × 25 cm) Cl^- type
eluted with 0·004N HCl by stepwise elution
concentrated *in vacuo* below 40° after neutralization
Paper electrophoresis (refrigeration: isopar H)
200 V/cm 1 hr pyridine-acetate buffer pH 3·7
eluted with 0·2 mole acetic acid.
Sephadex G–25 column (5·0 × 25 cm) fine
eluted with water
concentrated to a small bulk *in vacuo* at below 40°
precipitated with ethanol.
Yellow powder Compound A–I, 620 mg. dec 208°

The method of Bulen *et al.* was not suitable for the isolation of this pigment. That is, using the charcoal-celite column did not make for a better recovery (elution: 30% acetone), and separation by means of a Dowex 50 column decomposed the pigment. Compound A–I obtained by our method was found pure by paper chromatography and paper electrophoresis, and the mole ratio of the amino acid was constant, and it was identical with the original culture.

(II) *The determination of the molecular formula of the chromophore* (hereafter called Compound A–II)

The systematic disintegration of compound A–II is shown in Scheme 3. The paper chromatography of compound A series is shown in Table 2.

The titrated and elemental analyses of compound A–II presumed the molecular formula to be $C_{14}H_{11}N_3O_5$. The mass spectrum of compound A–II (parent peak, *m/e* 301), however, showed a loss of CO_2 $(M\text{-}44)^+$. The IR spectrum exhibited bands at 1740 cm^{-1}, 1550 cm^{-1}, 1400 cm^{-1} attributable to a carbonyl (b). This result prompted us to investigate the thermolysis of compound A–II in solution at higher temperatures (c). The UV absorption spectra of compounds A–II–M and A–II–D–P were the same; they did not change with the pH and usually showed an acidic type of spectrum throughout the pH range, suggesting a keto-enol tautomerism. The mass spectra of compounds A–II–M, A–II–D–P and of A–II–D–G were all the same (d-1, d-2). The mass spectral analysis of compound A–II–Ac established this molecular formula (e). M^+ $C_{16}H_{13}N_3O_6$, $M—C_2H_2O$ $C_{14}H_{11}N_3O_5$, $M—C_2H_2O—CO_2$ $C_{13}H_{11}N_3O_3$.

Structure of compound A–II–D as determined by X-ray analysis

A single-crystal X-ray analysis was executed to verify the structure of compound A–II–D. Large clear needles were mounted in capillaries. Preliminary photographs showed a 2/m Laue symmetry, with systematic absences for h0*l* ($l = 2n + 1$) and 0*k*0 ($k = 2n + 1$). The space group is, therefore, $P2_1/c$. The measured density of 1·56 $g.cm^{-3}$ indicated four molecules of compound A–II–D and eight molecules of water per unit cell ($\rho_{calc} = 1{\cdot}56\ g.cm^{-3}$). The diffractometer-measured cell constants are a = 10·342, b = 6·986, c = 21·525 Å, and $\beta = 115{\cdot}78°$. Intensities with $\theta \leqslant 70°$ were recorded with Cu-Kα radiation. After correction for background, Lorentz, and polarization effects, 1381 reflections greater than 3σ were used for the analysis. The structure was solved by the symbolic addition method.[7] Block-diagonal least-squares refinements with anisotropic thermal parameters for all the nonhydrogen atoms are currently at 8·0%. The molecular shape of compound A–II–D is shown in Scheme 1.

The NMR spectra of compounds A–II and A–II–D

(a) *Compound A–II–D.* (CF_3COOH): three aromatic protons (at $\delta = 7{\cdot}52$ C-8H, C-11H singlet, at $\delta = 8{\cdot}18$ C-6H singlet); three methylene protons (at $\delta = 2{\cdot}70$ C-15H_2 quintet, at $\delta = 4{\cdot}18$ C-16H_2 triplet, at $\delta = 4{\cdot}72$ C-14H_2 triplet); one imino proton (at $\delta = 9{\cdot}90$ broad singlet).

(DMSO-d_6): three aromatic protons (at $\delta = 7{\cdot}40$ C-8H, C-11H singlet, at $\delta = 7{\cdot}98$ C-6H singlet); three methylene protons (at $\delta = 3{\cdot}82$ C-16H_2 triplet, at $\delta = 4{\cdot}50$ C-14H_2 triplet, C-15H_2 overlapped solvent).

SCHEME 3

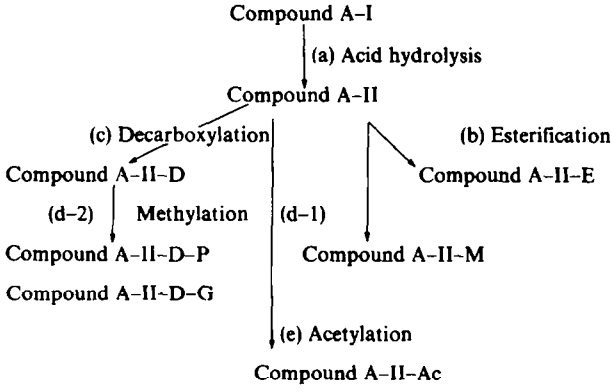


TABLE 2. PAPER CHROMATOGRAPHY OF COMPOUND A SERIES

Substance	Solvents[a] I	II
Compound A-I	0·05	0·90
Compound A-II	0·20	0·35
Compound A-II-E	0·63	0·55
Compound A-II-D	0·30	0·35
Compound A-II-Ac	0·72	—
Compound A-II-M	0·43	0·75
Compound A-II-D-G	0·52	0·63
Compound A-II-D-P	0·46	0·52

[a] Solvents: I. 1-butanol:acetic acid:water (4:1:2)
II. 3% aqueous ammonium chloride

TABLE 3. AMINO ACID COMPOSITION AND MOBILITY AND SEQUENCES OF THE PEPTIDE FRACTIONS

Peptide Compound	Asp	Ser	Homo	Gly	Cit	Y	W	Mobility
A-I	1	2	1	1	1	1	1	+0·53
peptide I	0	2	2	1	1	0		−0·77
Edman (1)		2	1	1	1			
(2)		1	1	1	1			
peptide II	0	2	1	1	1	1		+0·70
Edman (1)		1	1	1	1	1		
(2)		1	0	1	1	1		
peptide III	0	2	1	1	1	0		−0·58
Edman(1)		1	1	1	1			
(2)		1	0	1	1			
peptide IV	0	1	1	0	1	0		−1·14
Edman (1)		0	1		1			
(2)			0		1			
peptide V	0	0	0	1	0	1		+0·80
Edman (1)				0		1		

(b) *Compound A–II.* (CF_3COOH): three aromatic protons (at $\delta = 8{\cdot}26$ C-6H singlet, at $\delta = 7{\cdot}52$ C-11H singlet, at $\delta = 7{\cdot}47$ C-8H singlet); two methylene protons (at $\delta = 2{\cdot}50 \backsim 4{\cdot}70$ multiplet); one methine proton (at $\delta = 6{\cdot}00$); one imino proton (at $\delta = 9{\cdot}96$ broad singlet);

(DMSO-d_6): three aromatic protons (at $\delta = 7{\cdot}80$ C-6H singlet, at $\delta = 7{\cdot}52$ C-11H singlet, at $\delta = 7{\cdot}14$ C-8H singlet); three exchangeable protons (at $= 4{\cdot}50 \backsim 6{\cdot}50$ broad); one methine proton (at $\delta = 5{\cdot}35$); two methylene protons (at $\delta = 2{\cdot}50 \backsim 4{\cdot}30$ multiplet).

The NMR spectrum of compound A–II showed only 11 protons; when compound A–II was decarboxylated, the methine proton was changed to a methylene proton, and the combined position of $—CO_2$ was undoubtedly any one of C-14, -15, and -16. Because of the chemical shift of the methine proton was to a lower field, it was expected that the methine proton was combined with the C atom neighboring the N atom of a higher electron negativity (C-14 or C-16). It was suggested that the three aromatic protons which were affected the $—CO_2$ had to be nearly the same as it, since the structure of the chromophore was rigid (C-14). This is in agreement with the results of the X-ray analysis of the methylated chromophore by Corbin *et al.*[8,9]

(III) *Amino acid sequence*

(a) *The isolation of fluorescent peptide by partial hydrolysis with dilute acid.*[10,11] Compound A–I (7 mg) was hydrolysed with 0·03 N HCl (7 ml) in a sealed tube at 105° for 4 hr. After neutralization, the hydrolysate was concentrated, put on a DEAE-cellulose column (3·0 × 23 cm; $H_2O \backsim 0{\cdot}01$ N HCl gradient elution), and separated into two fractions, A and B. Fraction A was identified as compound A–II. Fraction B was further purified by paper chromatography (R_f values; Solvent I, 0·38; II, 0·60). It will be called Compound A–I″. Compound A–I″, involved with chromophore (0·0339 μ mole), was further hydrolysed with 6 N HCl at 110° for 24 hr in a sealed tube. Aspartic acid (0·0342 μ mole) was obtained in the hydrolysate. Therefore, compound A–I″ can be said to consist of 1 mole of compound A–II and 1 mole of aspartic acid. Both compounds, A–I and A–I″, were negative to ninhydrin.

(b) *Isolation of peptides.* Compound A–I (14 mg) was hydrolysed with 0·03 N HCl (5 ml) in a sealed tube at 105° for 4 hr. After neutralization, the hydrolysate was chromatographed at 53° on a column (0·8 × 50 cm) of the amino acid analyser, JEOL 6AH. The eluate was continuously analysed with the apparatus and then fractionated. Each ninhydrin-positive fraction was desalted using a Biorad AG 50 W × 2 column (1·0 × 15 cm), NH_4^+ type, by Dreze's method,[12] and then purified further by paper electrophoresis. Ninhydrin- and tolidine-positive fractions were eluted with 0·2 mole acetic acid, hydrolysed with 6N HCl, and analysed. The subtractive Edman degradation was carried out as has been described by Konigsberg and Hill.[13] The amino acid composition and the mobility and sequences of the peptide fractions are shown in Table 3.

Y was identified as a β-hydroxy Asp by comparing it with a synthetic material by Dakin's method.[14,15] W was identified with a homoserine lactone, which appeared during the hydrolysis of homoserine. The mobility of the paper electrophoresis was demonstrated with a relative value, as that of Asp was + 1. The enzymatic degradation of compound A–I was all negative—pepsin, bacterial proteinase, and carboxypeptidase. The recovered amino acids amounted to 10%. As a result of many considera-

tions, the best conditions of dilute acid hydrolysis were found to be at 105° for 24 hr. Under these conditions, the yield of amino acid was approximately 6 fold.

We may conclude from the experiment described above that the combination point of the chromophore and peptide is the C-terminal of chromophore (C—14—COOH) and the N-terminal of Asp.

EXPERIMENTAL

(I) *The Cultivation and isolation of compound A–I*

The *Azotobacter vinelandii* strain O was obtained from the general culture collection of the Department of Bacteriology, University of Wisconsin, Madison, Wisconsin. Cells were cultured in Bulk's nitrogen-free, iron-deficient medium[16] at 30° for 60 hr in a fermentation tank (N.B.S. microfarm fermenter, Model 107), with aeration (1·5 l/min). The iron content of the medium was determined by the method of Shinohara.[17] The sucrose (Fe: 0·00001 %) used in the medium was obtained from Nakarai Chemicals Co. Ltd., while the water used was a deionized water distilled by the use of an all-glass apparatus. The isolation of compound A–I is demonstrated in Table 1. (Found (70°/10^{-4} mmHg): C, 44·51; H, 5·76; 15·32. Calc. $C_{44}H_{59}N_{13}O_{23}\cdot 3H_2O$: C, 44·33: H, 5·46: N, 15·28%). S. P. Cl was zero: UV absorption spectrum: $\lambda_{max}^{0.1NHCl}$ nm (ε): 224 (4·06 × 10^4) 380 (1·94 × 10^4), $\lambda_{max}^{0.1NNaOH}$ nm (ε): 240 (3·63 × 10^4), 434 (1·86 × 10^4).

(II) *Purification of compound A–II and derivatives, and X-ray analysis*

The NMR spectra were recorded on a Japan Electron Optics Lab. C-60 HL-type apparatus, the UV spectra, with a Hitachi EPS-3 recording spectrophotometer, and the mass spectra, with a C.E.C. 21–101 B-type double -focus mass spectrometer. Whatman No. 1 or Toyo Roshi No. 51A filter papers were used for paper chromatography and electrophoresis. Ascending paper chromatography was carried out at room temp, and paper electrophoresis was performed at pH 3·7 or 6·5 (pyridine–acetate buffer)[18] at a potential gradient of 200 V/cm for 30 min.

X-ray analysis

The intensities were measured on a Hilger and Watts four-circle automatic diffractometer, Y-290. All the computations were performed on a Facom 230-60 at the Nagoya University Computation Center, using our programs.

(a) *Compound A–II.* Compound A–I (480 mg) was hydrolysed with 6N HCl (90 ml) in a sealed tube at 110° for 48 hr. After the HCl had then been removed, the hydrolysates were applied on a DEAE-cellulose column (5·0 × 25 cm), Cl^-type, and the yellow-green fluorescent eluted with 0·001N HCl by step-wise elution. The desired fraction was recrystallized with hot water, yellow needles; compound A–II, 60 mg (yield, 47·6%): dec 216°.

Compound A–II (11·118 mg) was dissolved in water (100 ml) and titrated with 0·11125N KOH on a pH-Stat. The end point was observed at 0·338 ml of the base, giving a neutralization equivalent of ~300. The *pKa* was 6·25 ± 0·02. (Found (70°/10^{-4} mmHg): C, 45·27; H, 4·97; N, 11·56. Calc. $C_{14}H_{11}N_3O_5\cdot 4H_2O$: C, 45·04: H, 5·13: N, 11·26%): Found (100°/10^{-4} mmHg): C, 52·65: H, 3·86: N, 12·87. Calc. $C_{14}H_{11}N_3O_5\cdot H_2O$: C, 52·66: H, 4·10: N, 13·16%): UV absorption spectrum $\lambda_{max}^{0.1NHCl}$ nm (ε): 224 (5·92 × 10^4), 378 (2·78 × 10^4), $\lambda_{max}^{0.1NNaOH}$ nm (ε): 240 (4·56 × 10^4), 432 (2·49 × 10^4): mass spectrum: $(M-CO_2)^+$ $C_{13}H_{11}N_3O_3$, Detm. 257·0755, Calc. 257·0799.

(b) *Esterification.* Compound A–II (34 mg) was suspended in dry EtOH (10 ml): the suspension was saturated with dry HCl gas in an ice–water bath for 1 hr, and then refluxed for 1 hr to dissolve compound A–II completely. The mixture was then concentrated *in vacuo* below 40° and purified by passing it through a DEAE-cellulose column (5·0 × 25 cm). The desired fraction was evaporated to dryness *in vacuo*: a yellow ppt was then obtained by recrystallization from EtOH-ether: compound A–II–E, 33 mg (yield, 77·2%). (Found (70°/10^{-4} mmHg): C, 43·97: H, 4·98: N, 9·73: Cl, 15·91. Calc. $C_{16}H_{15}N_3O_5\cdot 2H_2O\cdot 2HCl$: C, 43·83: H, 4·79: N, 9·58: Cl, 16·21%): UV absorption spectrum: $\lambda_{max}^{0.1NHCl}$ nm (ε): 224 (4·74 × 10^4), 378 (2·51 × 10^4), $\lambda_{max}^{0.1NNaOH}$ nm (ε): 240 (4·72 × 10^4), 430 (2·52 × 10^4), mass spectrum: $(M-CO_2)^+$ $C_{15}H_{15}N_3O_5$, Detm. 285·1093, Calc. 285·1109.

(c) *Decarboxylation.*[19] To compound A–II (96 mg) we added nitrobenzene (1 ml): the mixture was then heated for 30 min at 160 ~ 190°. After the subsequent addition of 2N HCl (10 ml), the soln was extracted with ether to remove the nitrobenzene. The water phase was concentrated and purified by passing it

through a DEAE-cellulose column (5·0 × 25 cm). The desired fraction was then recrystallized with hot water and a few drops of conc HCl, yellow needles: compound A–II–D: 63 mg (yield, 59·1%). (Found (100°/10^{-4} mmHg); C, 51·83; H, 4·46; N, 13·84; Cl, 12·09. Calc. $C_{13}H_{11}N_3O_3 \cdot 1/2\ H_2O \cdot HCl$: C, 51·57; H, 4·29; N, 13·88; Cl, 11·73%): UV absorption spectrum: $\lambda_{max}^{0.1N\,HCl}$ nm (ε): 224 (5·29 × 10^4), 374 (2·43 × 10^4), $\lambda_{max}^{0.1N\,NaOH}$ nm (ε): 239 (5·39 × 10^4), 428 (2·75 × 10^4).

(d) *Methylation.* (i) A mixture containing compound A–II (20 mg), Ag_2O (30 mg), and MeI (20 ml) was agitated for 15 hr at room temp. After filtration, the filtrate was extracted with water. The water phase was concentrated and purified by gel filtration using a Sephadex G-25 (fine) column (3·0 × 25 cm: elution: water). Purple fluorescent, compound A–II–M: mass spectrum: $(M-CO_2)^+$ $C_{16}H_{17}N_3O_3$ Detm. 299·1225, Calc. 299·1268.

(ii) Compound A–II–D (47 mg) was added to MeOH (40 ml) and diazomethane, and the mixture was agitated overnight at room temp. The mixture was then concentrated and purified by chromatography using a cellulose powder column (3·0 × 15 cm: developer: H_2O). Green fluorescent, compound A–II–D–G: mass spectrum: M^+ $C_{16}H_{17}N_3O_3$ Detm. 299·1249, Calc. 299·1268.

(e) *Acetylation.* To a mixture of 1 ml dry pyridine and 1 ml Ac_2O, a 50 mg portion of compound A–II was added: the whole was then left standing for 40 hr at room temp. The mixture was concentrated to dryness *in vacuo* below 40°, and then purified by chromatography using a silica-gel column (1·0 × 15 cm, developer: $CHCl_3$:MeOH, 9:1): the blue fluorescent was subsequently concentrated and further purified on a cellulose powder column (3·0 × 20 cm: developer: BuOH:MeOH:H_2O, 3:1:1). Blue fluorescent, compound A–II–Ac: mass spectrum: M^+ $C_{16}H_{13}N_3O_6$ Detm. 343·0899, Calc. 343·0881.

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