PRODUCTION OF A NOVEL RED PIGMENT, RUBROLONE, BY *STREPTOMYCES ECHINORUBER* SP. NOV.

II. CHEMISTRY AND STRUCTURE ELUCIDATION

W. SCHÜEP, J. F. BLOUNT, T. H. WILLIAMS and A. STEMPEL

Chemical Research Department, Hoffmann-La Roche Inc., Nutley, New Jersey 07110, U.S.A.

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Streptomyces echinoruber sp. nov. produces several red pigments. The major component, rubrolone, has been identified as 8(R),9(R),10(S),10a(R)-tetrahydro-9,10,10a,11-tetrahydroxy-3,8-dimethyl-1-propyl-6aH(S)-pyrano[2″,3″: 5′,4]furo[2′,3′: 5,6]azuleno[2,3-c]pyridine-5,13-dione (**1**) by single crystal X-ray analysis of a suitable derivative. A second pigment, B, is probably structurally closely related.

Streptomyces echinoruber (X-14077, NRRL 8144) isolated from a sample of soil collected in eastern Argentina was found to produce a group of five or more red pigments in submerged culture under suitable conditions[1,2]. The water-soluble pigments were readily distinguished from each other by thin-layer chromatography as described below.

The culture produced one major pigment, A (rubrolone), a structurally related minor component B, a third pigment C which was not obtained pure, as well as traces of several other red compounds. The three pigments A, B and C were separated by thin-layer chromatography on silica gel F254 plates using methanol-chloroform (1: 3) as a solvent system. The Rf values were as follows: pigment B, 0.28; pigment A, 0.13; and pigment C, 0.03.

Assays on the fermentation were carried out by separating the crude pigment mixture on thin-layer plates and estimation of the compounds by means of UV spectroscopy at 520 nm[1].

The broth filtrate obtained after a fermentation of culture X-14077[1] was extracted with *n*-butanol and the solvent removed at reduced pressure and low temperature. The residue contained the major pigment A in a concentration of about 20% together with a minor pigment B and other pigments. Separation of the pigments A, B, and C by chromatography on silica gel using chloroform with increasing amounts of methanol as eluant afforded the compounds in a purity of 70~80%. Further purification was obtained by adsorption on XAD_2 resin followed by elution with water containing increasing amounts of methanol.

Rubrolone (A) is a nitrogen-containing amorphous solid, very soluble in water and methanol, less soluble in ethanol and almost insoluble in anhydrous butanol. It exhibited an optical rotation of $[\alpha]_D^{25}$ $-937.5°$ (*c* 0.2, H_2O) and characteristic UV maxima at 216, 275, 421 and 523 nm. The ORD/CD curves showed a multiple COTTON effect; the first was negative at 550 nm. A broad hydroxyl band at 3400 cm^{-1} as well as bands at 1704, 1635 and 1577 cm^{-1} was observed in the IR(KBr) spectrum. The ^1H-NMR spectrum showed the presence of 19 unexchangeable protons and the highest mass observed in the MS was *m/e* 407. The ^{13}C NMR spectrum taken in DMSO-d_6 revealed the presence of 23 carbons. The dissociation constants were measured to be $pKa_1=1.37$, $pKa_2=6.36$ and $pKa_3=12.8$.

It was soon recognized from these physical-chemical data that the empirical formula of the red pig-

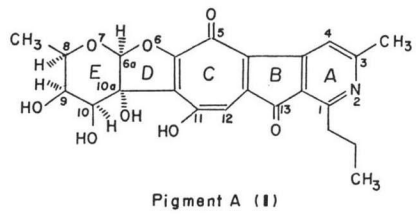
ment A was $C_{23}H_{23}NO_8$. Since the ^{1}H-NMR spectrum showed 6 methine protons of which at least 4 were attached to oxygen, it was apparent that a sugar type side chain or perhaps a glucoside might be present in the molecule. Several attempts to hydrolyze pigment A under various conditions resulted in inseparable mixtures of degradation products. On the other hand, acid catalyzed reaction with acetone led to a less polar compound (**2**) (Scheme 1), containing an isopropylidene group. The UV spectrum indicated no change in the chromophore whereas the ^{1}H-NMR spectrum now clearly revealed the presence of a propyl, an aromatic methyl and a sugar-like CH_3–CH–CH–CH-group as well as 3 other protons in the anomeric, 3.5~3.8 ppm region.

Reaction of **2** with benzoyl chloride in chloroform solution, with or without potassium carbonate as catalyst, resulted in a red monobenzoate (**3**). Under carefully controlled conditions **2** reacted with diazomethane to give, in moderate yield, a crystalline yellow methyl ether (**4**), which was also obtained by methylation with methyl iodide in the presence of silver oxide. The latter reaction also gave access to a minor crystalline orange compound, **5** which like **4** contained an aromatic methoxyl group as well as a hydroxyl group.

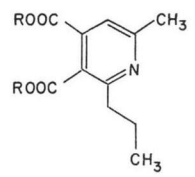
Oxidation of the red pigment A (**1**) with nitric acid followed by esterification of the corresponding sodium salt of the resulting acid with *p*-bromophenacyl bromide gave a crystalline diester (**6**). The structure of this oxidation product was elucidated from the NMR spectrum.

At this point it was apparent, that the quickest way to determine the structure of pigment A was probably a single crystal X-ray analysis. For this purpose, considering the molecular weight, it was deemed necessary to introduce a heavy atom. The remaining hydroxyl in the methyl ethers, **4** and **5**, seemed to be a suitable reaction group. However, the reactivity towards acylating agents was so poor that another approach had to be found. The isopropylidene derivative (**2**) was much more reactive and afforded monoesters in moderate yields upon reaction with substituted benzoyl chlorides in chloroform solution (*e. g.* **7**). These compounds were usually amorphous solids which reacted with methyl iodide/

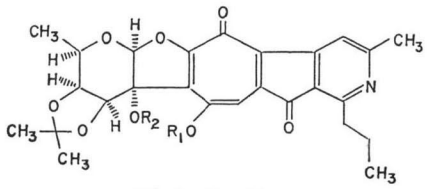
Scheme 1.



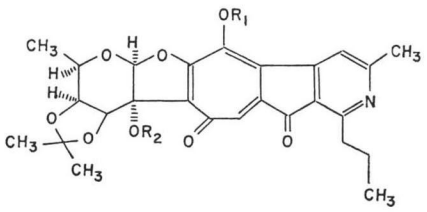
Pigment A (**1**)
Rubrolone



(**6**) R = $BrC_6H_4COCH_2$

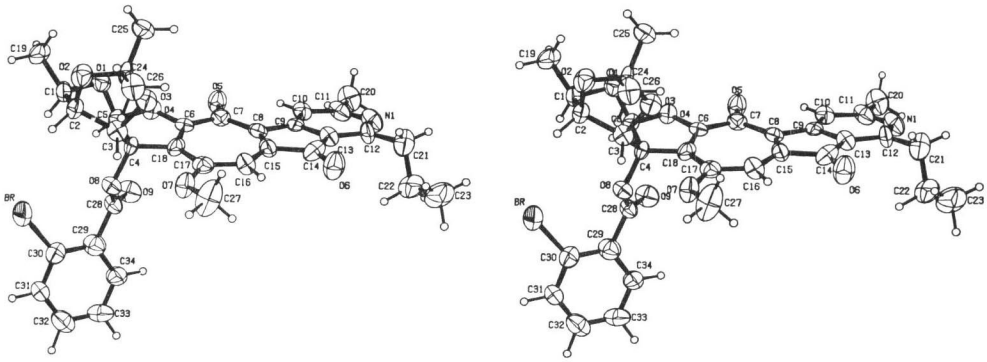


(**2**) $R_1 = R_2 = H$
(**3**) $R_1 = H$ $R_2 = C_6H_5CO$
(**5**) $R_1 = CH_3$ $R_2 = H$
(**7**) $R_1 = H$ $R_2 = o\text{-}BrC_6H_4CO$
(**9**) $R_1 = CH_3$ $R_2 = o\text{-}BrC_6H_4CO$

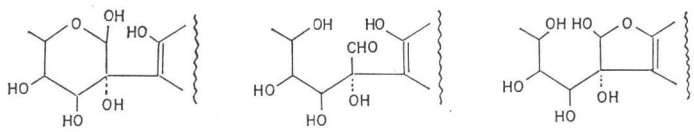


(**4**) $R_1 = CH_3$ $R_2 = H$
(**8**) $R_1 = CH_3$ $R_2 = o\text{-}BrC_6H_4CO$

Fig. 1. A stereoscopic drawing of a molecule of **9** showing its absolute configuration



Scheme 2.



(10) (11) (12)

silver oxide in chloroform solution to give two isomeric yellow and orange ethers, **8** and **9**. Some of these methyl ethers crystallized and the *o*-bromobenzoyl derivative (**9**) proved to be the most suitable for X-ray analysis. The structure and absolute stereochemistry of this derivative (Fig. 1) was determined to be 10a-(2-bromobenzoyloxy)-8(R), 9(R), 10(S), 10a(R)-tetrahydro-9, 10-(isopropylidenedioxy)-11-methoxy-3, 8-dimethyl-1-propyl-6aH(S)-pyrano[2″, 3″: 5′, 4′] furo [2′, 3′: 5, 6] azuleno [2, 3-c] pyridine-5, 13-dione (**9**).

From the structure of **9** it could be inferred that the red pigment A had structure **1**. However the first intermediate in the synthesis of **9** was the isopropylidene derivative (**2**) which was prepared under acidic conditions. Under these conditions, it would also be possible that the cyclic acetal was formed if the red pigment A existed in any of the open forms **10**, **11** or **12**. Quantitative periodate oxidation of **1** excluded the open forms **10**~**12** and was in agreement with structure **1**. Further there was no evidence of the formation of acetaldehyde which would be expected from forms **11** and **12**. In addition, removal of the isopropylidene group in **2** with aqueous trifluoracetic acid gave a product whose NMR spectrum was identical to that of pigment A (**1**) untreated with acid.

With the structure of **1** known, the reasons for some of the anomalous reactions became apparent. The isomeric methyl ethers, **4** and **5**, did not react with acylating agents because the free hydroxyl group at the D/E bridge is tertiary. However if the enolic group at position 11 was free, it acylated readily and the acyl group migrated to the tertiary alcohol. The phenolic group was then free for etherification or further esterification.

Pigments A (**1**) and B (**13**) are probably closely related structurally based on the following evidence. The ultra-violet spectra of A and B and their derivatives are almost identical indicating a similar chromophore. Mass spectra of the isopropylidene derivative of B (**14**) and the methyl ether of **14** (compound **15**) gave a molecular ion consistent with the molecular weight of the corresponding derivatives of pigment A.

A solution of an equimolar mixture of pigments A and B showed differences in the NMR spectrum

in three areas. The largest difference (0.30 ppm) was observed in the chemical shift of the anomeric protons, then in the methyl doublets (0.13 ppm) and least in the tropolone ring protons (0.07 ppm). The similarity in the remainder of the spectrum suggests the probability that the pigments are isomeric.

Experimental

General

Melting points were determined on a Thomas Hoover capillary melting point apparatus and are not corrected. Spectral measurements were performed by members of the Physical Chemistry Department of Hoffmann-La Roche Inc. using the following instruments: NMR, Varian HA-100 spectrometer with tetramethylsilane as internal standard (chemical shifts in δ (ppm) and coupling constants in Hz); IR, Beckmann IR 9 spectrometer (absorptions in cm^{-1}); UV, Cary Model 14 spectrometer (λ_{max} in nm, ε in parentheses): MS, Joelco OISG or CEC 21-110 spectrometers with a direct inlet system (70 eV). Chromatography was carried out on E. Merck Silica Gel 60 (0.063~0.200 mm). Thin-layer chromatograms (TLC) were run on E. Merck pre-coated Silica Gel 60 F-254 plates in tanks saturated with the indicated solvent mixtures. The spots were detected by (a) observation under a 254 nm source or visible light, (b) spraying with a 5% solution of anisaldehyde in ethanol containing 5% sulfuric acid, and (c) heating with a hot air gun.

Fermentation and isolation of red pigments A (**1**) and B (**13**)

Fermentation broths and concentrated solvent extracts of pigments from such broths were prepared as described in ref. 1. In a typical experiment, the residue (236 g) from a butanol extract of 236 liters of fermentation broth (**1**) which contained ~40 g of pigment A by UV assay, was extracted with methanol (3 liters) and filtered. To the filtrate 890 g of silica gel (0.2~0.5 mm) were added and the solvent removed at reduced pressure. The resulting solid was charged on a column containing 3 kg of silica gel. Chloroform containing increasing amounts of methanol (0~25%) was used as eluant. Fractions of 1 liter were taken and three main pools were made. The first (8.6 g) was a mixture of pigment A and pigment B. The second (31.6 g) and third (18.7 g) contained **1** in purities of 84% and 79%, respectively determined by UV assay.

The first pool was rechromatographed on XAD_2 resin. Using water with increasing amounts of methanol (0~100%) as eluant, 945 mg of pigment B (**13**) was isolated as an amorphous red solid; UV (H_2O): 218 (18,000), 273 (21,300), 333 (8,600), 412 (6,700), 520 (5,200); IR (KBr): 3400 (broad), 1720, 1650, 1590, 1580, NMR (DMSO): 0.92 (t, 3, J=7 Hz, $\underline{CH_3}$–CH_2); 1.21 (d, 3, J=7 Hz, $\underline{CH_3}$–CH); 1.63 (m, 2, CH_3–$\underline{CH_2}$); 2.43 (s, 3, $\underline{CH_3}$–Ar); 2.93 (t, 2, J=7 Hz, $\underline{CH_2}$–Ar); 3.47 (s, 1, $\underline{CH}$); 3.79 (q, 1, J=7 Hz, CH_3–$\underline{CH}$); 3.80 (s, 1, $\underline{CH}$); 5.41 (s, 1, $\underline{CH}$); 6.36 (s, 1, $\underline{CH}$); 8.11 (s, 1, $\underline{CH}$).

In the same way a 10-g portion of crude pigment A was further purified to yield 3.7 g of pure material and several other slightly contaminated fractions with a purity above 90%. **1** exhibited the following physical-chemical properties; UV (H_2O): 216 (18,800), 275 (31,500), 300 (infl. 9,600), 325~328 (sh. 800), 400 (sh. 7,300), 421~422 (9,500), 523 (8,600); IR (KBr): 3400 (broad), 1704, 1635, 1577, 1560; NMR (DMSO): 0.92 (t, 3, J=7 Hz, $\underline{CH_3}$–CH_2); 1.08 (d, 3, J=7 Hz, $\underline{CH_3}$–CH); 1.65 (m, 2, CH_3–$\underline{CH_2}$); 2.49 (s, 3, $\underline{CH_3}$–Ar); 2.97 (t, 2, J=7 Hz, $\underline{CH_2}$–Ar); 3.46 (s, 1, $\underline{CH}$); 3.75 (q, 1, J=7 Hz, CH_3–$\underline{CH}$); 3.80 (s, 1, $\underline{CH}$); 5.18 (s, 1, $\underline{CH}$); 6.43 (s, 1, $\underline{CH}$); 8.15 (s, 1, $\underline{CH}$); $[\alpha]_D^{25}$–937.5° (*c* 0.2, H_2O).

An equimolecular mixture of pigments A and B in DMSO had the following nmr spectrum: 0.92 (t, 3, J=7 Hz, $\underline{CH_3}$–CH_2); 1.08, 1.21 (d, 3, J=7 Hz, $\underline{CH_3}$–CH); 1.64 (m, 2, CH_3–$\underline{CH_2}$); 2.46 (s, 3 $\underline{CH_3}$–Ar); 2.95 (t, 2, J=7 Hz, $\underline{CH_2}$–Ar); 3.47 (s, 1, $\underline{CH}$); 3.76 (q, 1, J=7 Hz, CH_3–$\underline{CH}$); 3.80 (s, 1, $\underline{CH}$); 5.11, 5.42 (s, 1, $\underline{CH}$); 6.43, 6.36 (s, 1, $\underline{CH}$); 8.13, 8.12 (s, 1, $\underline{CH}$).

Isopropylidene derivative (**2**)

To a solution of 4.0 g of crude **1** in 200 ml of absolute methanol and 400 ml of acetone, 10 g of molecular sieves and 2 ml of concentrated sulfuric acid were added and the reaction mixture heated to reflux for 2 hours. The molecular sieves were removed by filtration and 8 g of anhydrous sodium acetate were added to the filtrate. After removing the solvents, the residue was dissolved in chloroform, washed with water, dried (Na_2SO_4), filtered and concentrated under reduced pressure giving 3.6 g of crude **2**.

Further purification of the isopropylidene derivative was carried out by chromatography on silica gel using $CHCl_3$ - EtOH (9: 1) to give the compound* as a red amorphous solid; IR (KBr): 1720, 1650, 1600; UV (EtOH): 223 (17,300), 278 (28,600), 305 (infl. 11,600), 328~329 (8,900), 343 (infl. 8,200), 393 (infl. 7,200), 416 (10,200), 433 (infl 8,700), 540 (8,200); MS (*m/e*): 481 (Calcd. for $C_{26}H_{27}NO_8$ 481).

Monobenzoate of isopropylidene derivative (**3**)

To a solution of 1.5 g of crude **2** in 120 ml of chloroform, 1.5 ml of benzoyl chloride was added. After stirring at room temperature for 16 hours a second portion of 0.5 ml of benzoyl chloride was added and stirring continued for 24 hours. After the addition of 1 g of potassium carbonate and stirring for an additional 4 hours, the reaction mixture was filtered, the filtrate concentrated under reduced pressure and the residue chromatographed on silica gel. Chloroform - ethanol (9: 1) eluted 1.12 g of **3** as an amorphous red solid; IR (KBr): 1730, 1650, 1600; UV (EtOH): 228 (27,700), 277 (22,000), 420 (7,300), 528 (5,800).

Methyl ethers **4** and **5** of isopropylidene derivative (**2**)

(a) By reaction with diazomethane: To 100 mg of the isopropylidene derivative (**2**) dissolved in 2 ml of methanol, an ethereal solution of diazomethane (1.2 eq.) was added at room temperature. After stirring for 2.5 hours a second portion of diazomethane (1 eq.) was added and the reaction mixture stirred overnight. The solvents were then removed at reduced pressure and the residue chromatographed on silica gel using $CHCl_3$ - ether (1: 1) to give 75 mg of a yellow compound. Upon addition of ethanol 15 mg of **4** crystallized, mp. 185~187°C, $[\alpha]_D^{25}$ +150.2° (*c* 0.78, $CHCl_3$), Calcd. for $C_{27}H_{29}NO_8$ (495.50): C 65.45, H 5.90, N 2.83%; Found C 65.21, H 6.03, N 2.78%: MS (*m/e*): 495 (M^+); IR (KBr): 1710, 1575; UV (EtOH): 213 (19,300), 253 (35,300), 296 (17,570), 305~306 (17,400), 372~373 (9,100), 400 (infl. 5,200).

(b) By reaction with silver oxide/methyl iodide: To a solution of 1.5 g of **2** in 600 ml of chloroform, 1.5 g of silver oxide and 15 ml of methyl iodide were added and the reaction mixture heated under anhydrous conditions to 40°C. After 1.5 hours a second portion of 6 ml of methyl iodide and 1 g of silver oxide were added. After a total reaction time of 3 hours, the reaction mixture was filtered through Hyflo, the filtrate concentrated and then the methyl ethers formed separated by chromatography on silica gel. Chloroform containing up to 50% ether eluted 1.0 g of **4**, recrystallized from EtOH; yellow crystals mp. 185~187°C, no depression of mixed melting point with the compound obtained by the diazomethane reaction; and 280 mg of a second methyl ether (**5**), recrystallized from methylene chloride-*n*-hexane, orange crystals mp. 150~152°, $[\alpha]_D^{25}$ −122° (*c* 0.261, $CHCl_3$); IR (KBr): 1722, 1590; UV (EtOH): 218 (25,000), 255 (23,780), 334 (19,200), 486~488 (6,650); MS (*m/e*): 495 (M^+); Calcd. for $C_{27}H_{29}NO_8$ (495.50); C 65.45, H 5.90, N 2.83%; Found C 65.00, H 5.84, N 2.68%.

6, Product of nitric acid oxidation of **1**

A solution of 1 g of crude **1** (in 7 ml of concentrated nitric acid and 1 ml of water) was heated for one hour on a steam bath, then 3 ml of concentrated nitric acid was added and the mixture kept at room temperature overnight. The reaction mixture was concentrated under reduced pressure; 20 ml of water was added and again concentrated. This was repeated 5 times. The residue was dissolved in 5 ml of water, neutralized with 2 N sodium hydroxide solution (towards phenolphthalein), 1 drop of 2 N hydrogen chloride solution and 1 g of *p*-bromophenacyl bromide as well as a small amount of ethanol were added to give a clear solution upon warming. The reaction mixture was heated on a steam bath for 4 hours, stripped down and the residue extracted with chloroform. The chloroform extract was concentrated and charged on a silica gel column eluting with ether - cyclohexane (3: 1). The main fraction, 467 mg, could be recrystallized from ethanol giving **6**, mp. 93~96°C, IR (KBr): 1730, 1700, 1590; Calcd. for $C_{27}H_{23}Br_2NO_6$ (617.29): C 52.53, H 3.76, N 2.27, Br 25.89%; Found C 52.18, H 3.90, N 2.19, Br 25.70%.

o-Bromobenzoate (**7**)

To a solution of 500 mg of **2** in 150 ml of chloroform, 400 mg of *o*-bromobenzoyl chloride was added and stirred for 72 hours at room temperature. An additional 200 mg of *o*-bromobenzoyl chloride was added and stirring continued for 40 hours. Solvent was removed under reduced pressure and the residue

* NMR spectral data for all compounds were in agreement with the structures assigned.

redissolved in chloroform and charged on a column containing 120 g of silica gel in chloroform. After elution with 300 ml of chloroform, the column was eluted with chloroform - methanol (9: 1) and the fractions containing the product (TLC) were combined and concentrated to dryness under reduced pressure.

To remove some *o*-bromobenzoic acid that was present, the residue was redissolved in chloroform and washed with 50 ml of saturated sodium bicarbonate solution, water and dried (Na_2SO_4). The organic layer was concentrated to dryness and the residue crystallized from ethanol. Recrystallization from ethanol gave red crystals; mp 192°C dec.; IR (KBr): 1720, 1645, 1595; UV (EtOH): 230 (infl. 21,000), 279 (28, 400), 305 (infl. 10,900), 378 (infl. 7,300), 420 (sh. 9,080), 434 (9,680), 540~542 (7,640).

Methyl ethers (**8**) and (**9**) of the *o*-bromobenzoate (**7**)

To a solution of 310 mg of **7** in 120 ml of chloroform, 3 ml of methyl iodide and 400 mg of silver oxide were added and the mixture stirred overnight at room temperature. An additional 2 ml of methyl iodide and 200 mg of silver oxide were added and stirring continued for 4 hours. Filtration separated the solid material and the filtrate was concentrated to dryness under reduced pressure. The residue was dissolved in a small amount of chloroform, charged on a silica gel column and eluted with a mixture of chloroform - ether (9: 1). Fractions were collected and pooled based on TLC. The first pooled material gave 98 mg of a yellow solid (**8**) which had IR (KBr): 1735, 1715, 1580; UV (EtOH): 254 (36,250), 274 (infl. 23,000), 294 (infl. 18,350), 319 (infl. 14,400), 357~358 (sh. 9,300), 375 (9,500), 400 (infl. 6,000); MS (*m/e*): 677 (M^+ calcd. for $C_{34}H_{32}BrNO_9$ 677). The second pooled fraction gave 24 mg of **9** which crystallized from ethanol as orange crystals, mp 210~214°C dec; IR (KBr): 1735, 1715, 1620, 1600; UV (EtOH): 207 (37,100), 239 (21,200), 245 (21,200), 257 (21,500), 267 (21,300), 334 (15,000), 483~485 (5,550); MS (*m/e*): 677 (M^+). These crystals were used for single crystal X-ray analysis.

Isopropylidene derivative of pigment B (**14**)

To a solution of 300 mg of pigment B (**13**) in 10 ml of methanol and 40 ml of acetone, 0.1 ml of concentrated sulfuric acid and 2 ml of 2,2-dimethoxypropane were added. The reaction mixture was refluxed for 1.5 hours and another 2 ml portion of 2,2-dimethoxypropane was added. After 30 minutes, 500 mg of anhydrous sodium acetate was added to the cooled reaction mixture and the solvent was removed at reduced pressure. The residue was dissolved in chloroform and washed with water, dried (Na_2SO_4), filtered and concentrated. The reaction product was purified by chromatography on silica gel. Chloroform - ethanol (9: 1) eluted 210 mg of **14** isolated as an amorphous red solid; IR (KBr) 1710, 1630, 1580 cm^{-1}; UV EtOH 217~218 (19,000), 277 (26,900), 300 (sh. 10,700), 328 (8,600), 342~343 (sh. 8,200), 395 (sh. 6,900), 413 (9,000), 537~538 (7,400) MS: M^+ *m/e* 481 (calcd. for $C_{26}H_{27}NO_8$ 481).

Methyl ether of isopropylidene derivative of pigment B (**15**)

To a solution of 165 mg of **14** in 30 ml of chloroform, 5 ml of methyl iodide and 330 mg of silver oxide were added. After stirring for 4 hours at ambient temperature, the reaction mixture was filtered through diatomaceous earth and the filtrate concentrated. The residue was chromatographed on magnesium silicate and eluted with chloroform - ether (19: 1). After concentration of the eluate and crystallization from ether - hexane, 56 mg of **15** was obtained as yellow crystals; mp 182~184°C; IR (KBr) 1720, 1650, 1590; UV (EtOH) 216 (16,640), 253 (32,000), 295~297 (17,600), 355 (8,100), 372 (8,500), MS M^+ *m/e* 495 (Calcd. for $C_{27}H_{29}NO_8$ 495). The corresponding orange compound was observed but was not eluted from the column.

Crystallography

Crystals of **9** are tetragonal, space group $I4_1$, with a=20.312(7), c=14.507(7) A, and d_{calcd}=1.505 g cm^{-3} for Z=8. The intensity data were measured on a Hilger-Watts automated four-circle diffractometer (Ni-filtered Cu Kα radiation, θ-2θ scans, pulse height discrimination). The size of the crystal used for data collection was approximately 0.08×0.08×0.4 mm; the data were corrected for absorption (μ= 25.8 cm^{-1}). Of the 2.333 accessible reflections for $2\theta<127°$, 1,610 were considered to be observed.

The structure was solved by a multiple solution procedure[3]. After preliminary anisotropic refinement of the non-hydrogen atoms, the positions of the hydrogen atoms were calculated. The hydrogen atoms were included in the structure factor calculations but their parameters were not refined. The final refinement was carried out by block diagonal least squares in which the matrix was partitioned into three

blocks. Two parallel refinements were carried out. In the second refinement the antipode of **9** was refined as the molecule located at 0.5+x, y, −z relative to the molecule in the first refinement. The final weighted R values for the two refinements were 0.0830 and 0.0864. Thus, according to the test described by HAMILTON[4], the absolute configuration can be taken as the one corresponding to the lower R value at a significance level better than 0.005.

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