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Two novel yellow pigments natronochrome and chloronatronochrome from the natrono(alkali)philic sulfur-oxidizing bacterium *Thialkalivibrio versutus* strain ALJ 15

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Abstract—Two novel membrane-bound yellow pigments natronochrome (**1**) and chloronatronochrome (**2**) were isolated from the obligately chemolithoautotrophic sulfur-oxidizing natrono(alkali)philic bacterium *Thialkalivibrio versutus* strain ALJ 15. They were derivatives of fully unsaturated fatty acids with a phenyl group, and their structures were determined by spectral data.

Soda lakes represent unique saline habitat with extremely high pH and alkalinity due to the presence of high concentration of sodium carbonate. Despite this, fully structured and diverse prokaryotic communities develop even in hypersaline soda brines.[1,2] Recently, a new branch of obligately chemolithoautotrophic sulfur-oxidizing bacteria has been discovered in soda lakes, which includes three new genera in the Gammaproteobacteria: *Thialkalimicrobium*, *Thialkalivibrio*, and *Thioalkalispira*.[3,4] All of them belong to obligate haloalkaliphiles, which mean absence of growth at neutral pH and optimum growth in saline media with pH around 10. Since most of the isolates preferred the sodium carbonate salts over the sodium chloride, they should be called natrono(alkali)philes in contrast to (halo)alkaliphiles. The aerobic chemolithoautotrophic sulfur bacteria usually referred to as 'colorless' in contrast to the anaerobic purple sulfur bacteria. However, all the extremely salt-tolerant *Thialkalibibrio* strains (about 50) isolated from the hypersaline soda lakes produce yellow pigments. The low-salt-tolerant *Thialkalivibrio* and *Thioalkalispira* strains do not produce any pigments.[3,4] The pigments were found to be associated with the membrane fraction of the cells in these novel bacteria. This letter reports the isolation and structural elucidation of two novel yellow pigments from *Thialkalimicrobium versutus* strain ALJ 15.

High-density cultivation of *T. versutus* strain ALJ 15 was performed in 15 L fermentor with pH and dissolved oxygen control at air overpressure of 0.5–0.8 atm and 10–12 L of culture. The medium contained ($g\,L^{-1}$): Na_2CO_3–95; $NaHCO_3$–15; NaCl–16; K_2HPO_4–1; KNO_3–1, pH 10.1. After sterilization, 0.5 mM $MgCl_2 \cdot 6H_2O$, 1 mL/L of trace metal solution and 200 mM sodium thiosulfate were added to the mineral base. The cells were harvested by centrifugation and lyophilized.

The lyophilized cells (ca. 300 g) were air shipped to Japan, and then rehydrated. Pigments were extracted with MeOH. The extract was partitioned with $CHCl_3$ and water, and then $CHCl_3$ layer was evaporated. The pigments were subjected to column chromatography on DEAE-Toyopearl 650M, and eluted with hexane/acetone (1:1). The fraction was further purified by

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preparative TLC on silica gel G developed with CH_2Cl_2/acetone (19:1) followed by HPLC on μBondapak C18 column with MeOH/H_2O (8:2) to yield compound **1** (2 mg) and **2** (2 mg). A minor compound with the same absorption spectrum as **1** was eluted just before **1** on HPLC. All the procedures were performed under dim light.

Compound **1** was obtained as a yellow amorphous solid and showed absorption maxima at 262, 333, (408), 428, (445) nm in MeOH/H_2O (8:2), indicating the presence of conjugated polyene system. The molecular formula of **1** was determined to be $C_{23}H_{24}O_3$ by HR-EIMS.[5] The characteristic IR absorptions showed the presence of a hydroxy group (2925 cm^{-1}), an ester carbonyl group (1714 cm^{-1}), and a conjugated olefin (1008 cm^{-1}). The ^{13}C NMR spectrum (Table 1) including DEPT experiment revealed the presence of 2 methyls, 17 sp^2 methins, and 4 quaternary carbons (δ_C 121.9, 137.9, 153.8, and 167.6). Taking into account the IR absorptions, the quaternary carbons at δ_C 153.8 and 167.6 were assigned to a carbon bearing a hydroxy group and an ester carboxyl carbon, respectively. ^1H NMR (Table 1) showed the presence of a methyl group (δ_H 2.27), a methoxy group (δ_H 3.75), 17 sp^2 methins, and a hydroxy group (δ_H 7.47). Of the 17 methins, COSY, TOCSY, and HSQC experiment revealed the three aromatic protons attributed to 1,2,3-trisubstituted benzene ring [δ_H 6.68 (d, J = 8 Hz; H-19), 7.04 (dd, J = 8, 8 Hz; H-20), and 7.13 (d, J = 8 Hz; H-21)] and 14 olefinic protons located on heptaene chain. These spectral data suggested that **1** included a phenyl group bearing a methyl and a hydroxy groups, heptaene chain, and carboxy methyl moieties. The HMBC correlations CH_3 (δ_H 2.27)/C-16, C-17, C-18, OH (δ_H 7.47)/C-18, H-20 (δ_H 7.04)/C-18, H-21 (δ_H 7.13)/C-16, C-15, and H-15 (δ_H 6.38)/C-16, revealed that a heptaene chain, a methyl group, and a hydroxy group were attached to a benzene ring at C-16, C-17, and C-18, respectively as shown in Figure 1. NOESY correlations CH_3 (δ_H 2.27)/H-15 and H-14/H-21 were also in agreement with this structure (Fig. 2). On the other hand, the HMBC correlations OCH_3 (δ_H 3.75)/C-1, H-2/C-1, H-3/C-1, C-2, C-5, and H-4/C-2 revealed the partial structure of the carboxy methyl terminal group, as shown in Figure 1. The 6 sp^2 methins at δ_C 132.8, 133.2, 133.4, 134.6, 134.8, and 135.8 attributed to C-7 to C-12 in heptaene chain could not be completely assigned because of overlapping of attached ^1H NMR signal (δ_H 6.40–6.50). The geometry of heptaene chain was assumed as all E from NOESY correlations (Fig. 2) and the similarities of the ^1H and ^{13}C NMR spectral data of related compounds.[6–8] Therefore, the structure of the compound **1** was determined to be Formula **1** and it was named natronochrome.

Compound **2** was also obtained as a yellow amorphous solid and it showed absorption maxima at 260, 332,

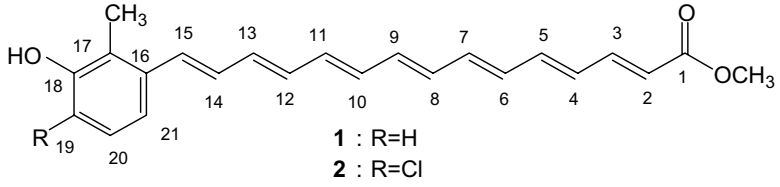

Table 1. ^{13}C (125 MHz) and ^1H (500 MHz) NMR data for natronochrome (**1**) and chloronatronochrome (**2**) in $CDCl_3$

Position	1			2		
	δ_C	δ_H mult. (J, Hz)	HMBC (^{13}C)	δ_C	δ_H mult. (J, Hz)	HMBC (^{13}C)
1	167.6	—		167.6	—	
2	120.1	5.88 d (15.5)	C-1	120.1	5.88 d (15.5)	C-1
3	144.6	7.34 dd (15.5, 11.5)	C-1, C-2, C-5	144.6	7.34 dd (15.5, 11.5)	C-1, C-2, C-5
4	130.3	6.35 dd (15.5, 11.5)	C-2	130.1	6.35 dd (15.5, 11.5)	C-2
5	140.9	6.63 dd (15.5, 11.5)		140.8	6.63 dd (15.5, 11.5)	
6	132.1	6.34 dd (15.5, 11.5)		132.1	6.34 dd (15.5, 11.5)	
13	137.4	6.48 dd (15.5, 11.5)		137.4	6.48 dd (15.5, 11.5)	
14	130.8	6.76 dd (15.5, 11.5)	C-13, C-15	129.9	6.74 m	C-13, C-15
15	130.9	6.38 d (15.5)	C-14, C-16	131.1	6.76 m	C-14, C-16
16	121.9	—		123.5	—	
17	137.9	—		136.6	—	
18	153.8	—		149.4	—	
19	114.1	6.68 d (8)	C-21	118.5	—	
20	126.3	7.04 dd (8,8)	C-18	125.7	7.12 d (8)	C-18
21	118.1	7.13 d (8)	C-15, C-16	117.8	7.05 d (8)	C-15, C-16
1-OCH_3	51.5	3.75 s	C-1	51.5	3.75 s	C-1
17-CH_3	11.3	2.27 s	C-16, C-17, C-18	12.1	2.29 s	C-16, C-17, C-18
18-OH	—	7.47 s	C-18	—	5.61 s	C-17, C-18, C-19

The ^{13}C and ^1H data of C-7 to C-12 in **1** are as follows: δ_C 132.8, 133.2, 133.4, 134.6, 134.8, 135.8, and δ_H 6.40–6.50 (overlapped).
The ^{13}C and ^1H data of C-7 to C-12 in **2** are as follows: δ_C 133.0, 133.5, 133.8, 134.3, 134.7, 135.7, and δ_H 6.40–6.50 (overlapped).

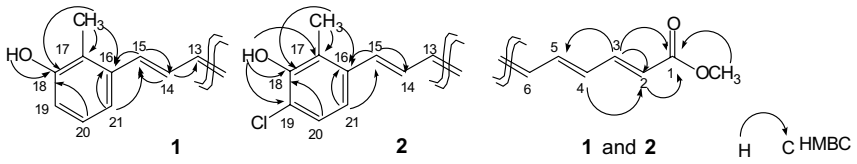

Figure 1. HMBC correlations of *natronochrome* (**1**) and *chloronatronochrome* (**2**).

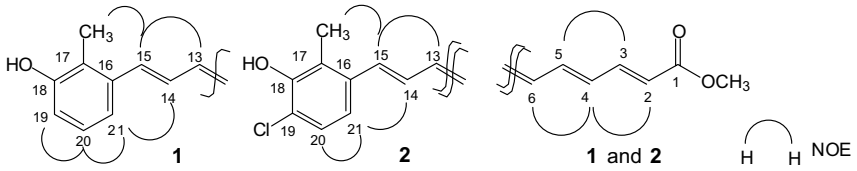

Figure 2. NOESY correlations of *natronochrome* (**1**) and *chloronatronochrome* (**2**).

(407), 426, (442) nm in MeOH/H_2O (8:2). The molecular formula of **2** was determined to be $C_{23}H_{23}O_3Cl$ by HR-EIMS.[9] ^{1}H NMR (Table 1) showed the presence of a methyl group (δ_H 2.29), a methoxy group (δ_H 3.75), 16 sp^2 methins, and a hydroxy group (δ_H 5.61). The COSY, TOCSY, and HSQC revealed the presence of 2 aromatic protons showing AB spin system [δ_H 7.05 (d, $J = 8$ Hz; H-21) and 7.12 (d, $J = 8$ Hz; H-20)] and 14 olefinic protons located on heptaene chain. The ^{13}C NMR spectrum (Table 1) including DEPT experiment revealed the presence of 2 methyl, 16 sp^2 methins, and 5 quaternary carbons (δ_C 118.5, 123.5, 136.6, 149.4, and 167.6). These spectral data indicated that the compound **2** is a chloride of **1**. Comparing ^{13}C NMR data with **1** and HMBC correlations, the quaternary carbon at δ_C 118.5 was assigned to a carbon bearing a chlorine atom. The chloride substituted position was deduced to be C-19 by following HMBC correlations: OH/C-19, H-20/C19, and H-21/C-19. Other parts of the structure were also confirmed by COSY, TOCSY, NOESY, HSQC, and HMBC experiments as shown in Figure 1. Therefore, the structure of the compound **2** was elucidated to be Formula **2** and it was named chloronatronochrome.

The basic structure of these two novel pigments is a fully unsaturated fatty acid with a phenyl group, and a few related structures have been reported. However, the substituted group(s) on the phenyl group and the esterified group are different in each compound. Compound **1** and **2** have a methyl and a hydroxy groups on the phenyl group, and a methyl ester. Physarigins A–C from a myxomycete *Physarum rigidum*[6] xanthomonadins from a bacterium *Xanthomonas juglandis*[7] and flexirubin from a bacterium *Cytophaga johnsonae*[8] have such as a hydroxy, a methoxy, and a ammonium groups, and complex esterified groups. Furthermore, compound **2** is notable in possessing a chlorine atom in the molecule. It is reported that halobacteria have carotenoids to protect from high light and oxygen radicals, and that pea increases superoxide dismutase when expressed to NaCl stress.[10] *T. versutus*, which grows under high salt and is exposed to high light, does not produce carotenoids but these yellow pigments. Since the pigments were found in the membrane fraction not the soluble one from these alkaliphilic sulfur-oxidizing bacteria, their functions might be protection from oxygen radicals and high-light stress, and an additional ion barrier at high salt as membrane components. Further functional experiments are in progress.

Acknowledgements

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