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Characterization of a novel unconjugated pteridine glycoside, cyanopterin, in *Synechocystis* sp. PCC 6803

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Abstract

A new pteridine glycoside, called cyanopterin, was isolated from *Synechocystis* sp. PCC 6803 and its structure was elucidated as 6-[1-(4-*O*-methyl-(α-D-glucuronyl)-(1,6)-(β-D-galactosyloxy]methylpterin by chemical degradation and ^{1}H- and ^{13}C-NMR spectroscopic means. Cyanopterin is constitutively synthesized at a relatively high intracellular concentration that is comparable to that of chlorophyll *a* in a molar ratio of approximately 1 to 1.6. The in vivo oxidation state of cyanopterin is primarily the fully reduced 5,6,7,8-tetrahydro form. The cellular function is unknown at present. The findings have established a model system, using *Synechocystis* sp. PCC 6803, for studies of the physiological functions of unconjugated pteridine glycosides found mostly in cyanobacteria.

Keywords: Cyanopterin; 6-Hydroxymethylpterin glycoside; *Synechocystis* sp. PCC 6803

1. Introduction

Pteridine glycosides that have various kinds of sugars attached to the side chains at C-6 of the pterin ring are unique in their abundance in some prokaryotes [1]. So far, they have been found in large amounts mostly in cyanobacteria [2–5] and, to a lesser extent, in anaerobic photosynthetic bacteria, *Chlorobium limicola* and *Chlorobium tepidum* [6,7], and a chemoautotrophic archaebacterium *Sulfolobus solfataricus* [8]. Much smaller amounts were in N_2-fixing chemoheterotrophs *Azotobacter agilis* [9] and *Azotomonas insolita* [10], a purple sulfur bacterium *Chromatium* strain D, and several purple non-sulfur photosynthetic bacteria [11]. Most of their chemically identified structures were biopterin glycosides except a few 6-hydroxymethylpterin or neopterin glycosides.

Generally, pteridine glycosides share some common characteristics: their occurrence is confined to a few classes of prokaryotes, they are produced in relatively large quantities, and some structural varia-

Abbreviations: COSY, correlation spectroscopy; DMSO, dimethylsulfoxide; DQF-COSY, double quantum filtered correlation spectroscopy; HMQC, heteronuclear multiple quantum coherence correlation spectroscopy; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy; UV-VIS, ultraviolet-visible light absorption

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tions occur in the pterin and sugar moieties. We believe that all of these characteristics will be related to the physiological function of the pteridine glycosides in various types of microorganisms. Their function, however, is obscure in contrast to some unconjugated pteridines that are well established for their cofactor functions with many enzymes as well as for their abundant presence as pigments [12]. Tetrahydrobiopterin in higher animals is involved in aromatic amino acid hydroxylation, glyceryl esterification, and nitric oxide synthesis. Molybdopterin is widely distributed in all organisms and essential in enzymes catalyzing a diverse array of oxidation–reduction reactions including xanthine dehydrogenase, sulfite oxidase, aldehyde oxidase, nitrate reductase, and others.

Earlier findings of pteridine glycosides in photosynthetic bacteria prompted functional investigation of pteridines in photosynthesis using broken spinach chloroplasts. For example, tetrahydropteridines were shown to stimulate photosynthetic phosphorylation and cytochrome *c* photo-oxidation, and it was postulated that pteridines stimulate electron transport in photosynthesis [13,14]. This function was further supported by the chemical properties of pteridines. They have low redox potential of ~ -0.7 V [15,16] and also the potential for free radical formation [17,18], which can be important in electron transfer and light-mediated reactions. However, hitherto no further studies have been reported demonstrating their involvement in vivo.

Despite the ubiquitous presence of pteridine glycosides in cyanobacteria, there has been no systematic approach to investigate their physiological functions. Even the most recent reports of new pteridine glycosides in cyanobacteria resulted from spontaneous findings [4,5]. Although unconjugated pteridine glycosides seem to be restricted to a few classes of prokaryotes, the fact that cyanobacteria contain relatively high levels of the pteridine glycosides and contribute much to photosynthesis in aquatic ecosystems justifies more intensive investigation of these compounds in a well-defined model organism.

We investigated a new pteridine glycoside in a well-known photosynthetic cyanobacterium, *Synechocystis* sp. PCC 6803, since it retains several advantages for our future studies of pteridine glycosides. The organism is one of the most intensively studied cyanobacteria regarding photosynthesis and the sequence of its genome has recently been decoded [19]. At the same time, the organism is readily transformable. In this paper, we report the isolation and the methods leading to the structural elucidation of the new pteridine glycoside, called cyanopterin, and present some characteristics, possibly related to its physiological function.

2. Materials and methods

2.1. Culture conditions

Synechocystis sp. PCC 6803 from Pasteur Culture Collection was donated by Dr. Y.M. Park in Korea Basic Science Institute, Korea. The strain was grown at 30°C in Medium C [20]. Liquid cultures of 1.6 l were grown under continuous white light (100–120 μmol m^{-2} s^{-1}) while stirring in 2-l flasks and bubbling with air. Smaller cultures in 100 ml flasks were maintained on rotary shakers at 150 rpm without air bubbling.

2.2. Chemicals

All chemicals used were of reagent grade. Neopterin, pterin, biopterin, 6-hydroxymethylpterin, and reduced forms of 6-hydroxymethylpterin were purchased from Dr. B. Schircks Laboratories, Jona (Switzerland). Dowex 50WX4-400 was purchased from Sigma. DeltaPak C18 prep-column (15 μm, 30 mm×30 cm) was purchased from Waters and Inertsil ODS-3 C18 analytical column (4.6×150 mm) was from GL Sci. (Japan).

2.3. Isolation and purification of cyanopterin

Cyanopterin was purified by the method of Cha et al. [6] with some modifications. Frozen cells (38.3 g in wet weight) suspended in 4 vols. of TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) were mixed with an equal volume of acidic KI/I_2 solution (2/1% in 1 N HCl). The mixture was incubated for 10 min at room temperature and centrifuged for 15 min at 10 000×*g*. Solid ascorbic acid was added to the supernatant until the dark brown color disappeared. The solution was loaded on the Dowex 50WX4-400 cation ex-

changer column (70 ml) pre-equilibrated with water. The column was washed with water and eluted with 2% NH_4OH at a flow rate of 60 ml/h. Fractions containing blue fluorescent material under UV light were combined and concentrated using a rotary evaporator. The resulting dry yellow powder was dissolved in water and filtered through a 0.2-μm membrane. The concentrated sample was subjected to HPLC (Kontron) equipped with DeltaPak C18 prep-column and UV detector (Kontron 430). The column was eluted with a isocratic 0.05% acetic acid and 8% aq. MeOH at a flow rate of 8 ml/min. The UV-absorbing peak at 276 nm with the retention time of 15.5 min was collected and evaporated to dryness (23 mg) using a rotary evaporator and speed-vac. concentrator. The purity was confirmed by analytical HPLC.

In order to cleave the attached sugars from the pterin moiety, the purified cyanopterin was dissolved in 1 N HCl and incubated at 100°C for 3 h. The sample was chromatographed on the same C18 prep-HPLC column with 8% aq. MeOH at a flow rate of 8 ml/min. The UV-absorbing peak eluting at 15 min was collected and evaporated to dryness as described above.

2.4. Quantitative analysis of cyanopterin

Cells were harvested in a 1.5-ml tube by centrifugation at 14 000 rpm for 10 min. To determine intracellular cyanopterin, the precipitated cells were suspended in 100 μl of TE buffer plus 20 μl of 10% SDS and mixed with an equal volume of acidic KI/I_2 solution. Extracellular cyanopterin was determined from the culture supernatant that had been oxidized with iodine. The mixtures were incubated at room temperature in the dark for an hour and centrifuged to discard precipitates. Aliquots of the supernatants were mixed with 5% ascorbic acid to reduce excess iodine and subjected to HPLC. The oxidized cyanopterin was separated on an Inertsil ODS-3 C18 column and measured using fluorescence detector (HP 1046A) at 350/450 nm (Ex/Em). Cyanopterin was eluted at 7.8 min in 10 mM K_2HPO_4, pH 6.0 at a flow rate of 1.2 ml/min. With 5% methanol in the buffer, the peak was shifted to 3.2 min at the same flow rate.

The molar concentration of cyanopterin was determined by measuring its acid hydrolyzed pterin moiety, 6-hydroxymethylpterin.

2.5. Assay of in vivo oxidation state of cyanopterin

To determine whether the cyanopterin in cells ex-

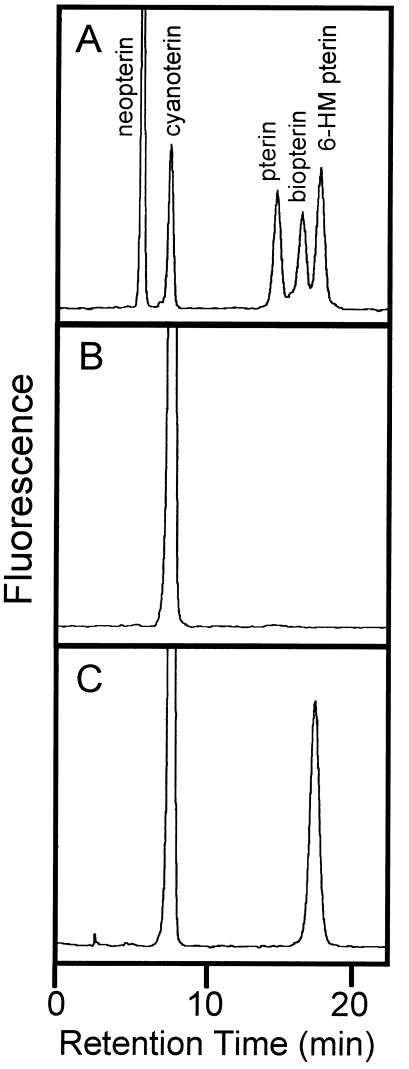


Fig. 1. HPLC profile of cyanopterin and its acid hydrolyzed product. (A) Purified cyanopterin co-injected with standard pteridines. (B) Cyanopterin in cellular extract of *Synechocystis* sp. PCC 6803 oxidized with acidic iodine solution. (C) Acid hydrolyzed cyanopterin in cellular extract. Cell extract was prepared as described in Section 2 and chromatographed on a column of Inertsil ODS-3 C18 column (4.6×150 mm). Acid hydrolysis of cellular extract was performed in the acidic iodine solution itself for 30 min at 80°C. Pteridines were eluted isocratically with 10 mM K_2HPO_4, pH 6.0, at a flow rate of 1.2 ml/min and detected with a fluorescence detector (350/450 nm).

ists as the dihydro or tetrahydro form, the method of Fukushima and Nixon [21] was adopted. Cells were harvested fresh from exponential phase, suspended in 0.02% ascorbic acid solution, and mixed with an equal volume of 2 N HCl to disrupt the cells. After 10 min at room temperature, the supernatant obtained by centrifugation was oxidized by equal volume of KI/I_2 solution (2/1% in water). After standing for different time intervals, 5% ascorbic acid was added and oxidized amount of cyanopterin was measured by HPLC. Reduced forms of 6-hydroxymethylpterin prepared fresh in 40 μM concentrations were oxidized and quantified in the same manner.

2.6. Determination of chlorophyll a

Chlorophyll *a* concentration was determined according to the method of Mackinney [22]. Harvested cells were suspended in 1 ml of 80% acetone and incubated at room temperature in the dark for 20 min. After centrifugation, absorption of the supernatant was measured at 663 nm.

3. Results

3.1. Chemical characterization of cyanopterin

Cyanopterin was the major fluorescent peak observed in the HPLC chromatogram of the iodine oxidized cell extract of *Synechocystis* sp. PCC 6803 (Fig. 1B). The retention time of the peak was clearly distinguishable from the other commercially available pteridines in the applied solvent system (Fig. 1A). When the iodine oxidized cell extract was heated at 100°C for 30 min, and then subjected to HPLC, a new peak appeared with the concomitant reduction of the cyanopterin peak (Fig. 1C), indicating that cyanopterin is a glycoside. The new peak was identical with 6-hydroxymethylpterin in its retention time, as confirmed by co-injection with an authentic sample. The loss of the attached hydrophilic sugar moiety would allow 6-hydroxymethylpterin to be eluted later than cyanopterin. Cyanopterin was purified in high yield (23 mg/38.3 g of cells in wet weight) by consecutive chromatographies on Dowex resin and a C18 prep-column. The UV-VIS spectrum of purified cyanopterin in water generated four apparent peaks at 192, 236, 276 and 345 nm, respectively (Fig. 2). The molar extinction coefficient at 345 nm of cyanopterin in water was calculated as $4.626 \times 10^3 \pm 117$. The spectra observed at different

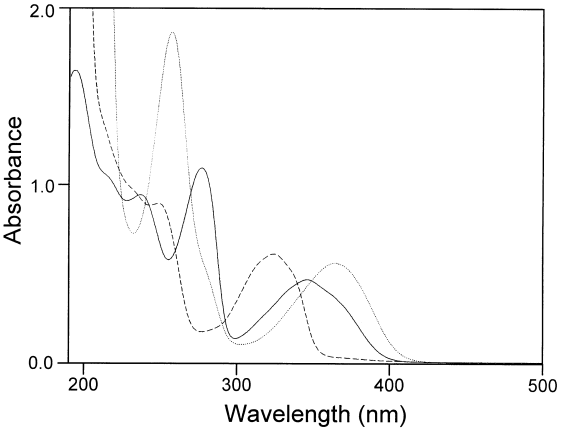


Fig. 2. Absorption spectra of purified cyanopterin. Spectrum was obtained with oxidized cyanopterin (0.1 mM) in H_2O (———), in 0.1 N HCl (- - -), and in 0.1 N NaOH (····), respectively.

Table 1
^1H- and ^{13}C-NMR chemical shifts (ppm) and coupling constants (Hz) of cyanopterin[a]

Sugar	a (^1H)		a (^{13}C)	b (^1H)		b(^{13}C)	p	(^1H)	(^{13}C)
1-H	4.62	$J_{1,2} = 7.6$	104.47	4.96	$J_{1,2} = 3.6$	99.90	2	–	153.10
2-H	3.63	$J_{2,3} = 9.8$	72.40	3.65	$J_{2,3} = 9.5$	72.29	4	–	161.35
3-H	3.70	$J_{3,4} = 3.16$	74.20	3.77	$J_{3,4} = 9.5$	74.02	4a	–	128.21
4-H	3.97	$J_{4,5} < 2.0$	70.48	3.32	$J_{4,5} = 10.0$	83.09	6	–	153.58
5-H	3.92		75.14	4.19	–	71.02	7	8.99	151.37
6-H	3.74	$J_{5,6'} = 7.6$	69.20	–	–	174.58	8a	–	148.23
	3.88	$J_{5,6''} = 10.4$							
OCH_3				3.47		61.91	6-CH_2	5.05, 5.20	70.79

[a]a = galactose, b = glucuronic acid, p = pterin; D_2O, 300K, 600 MHz.

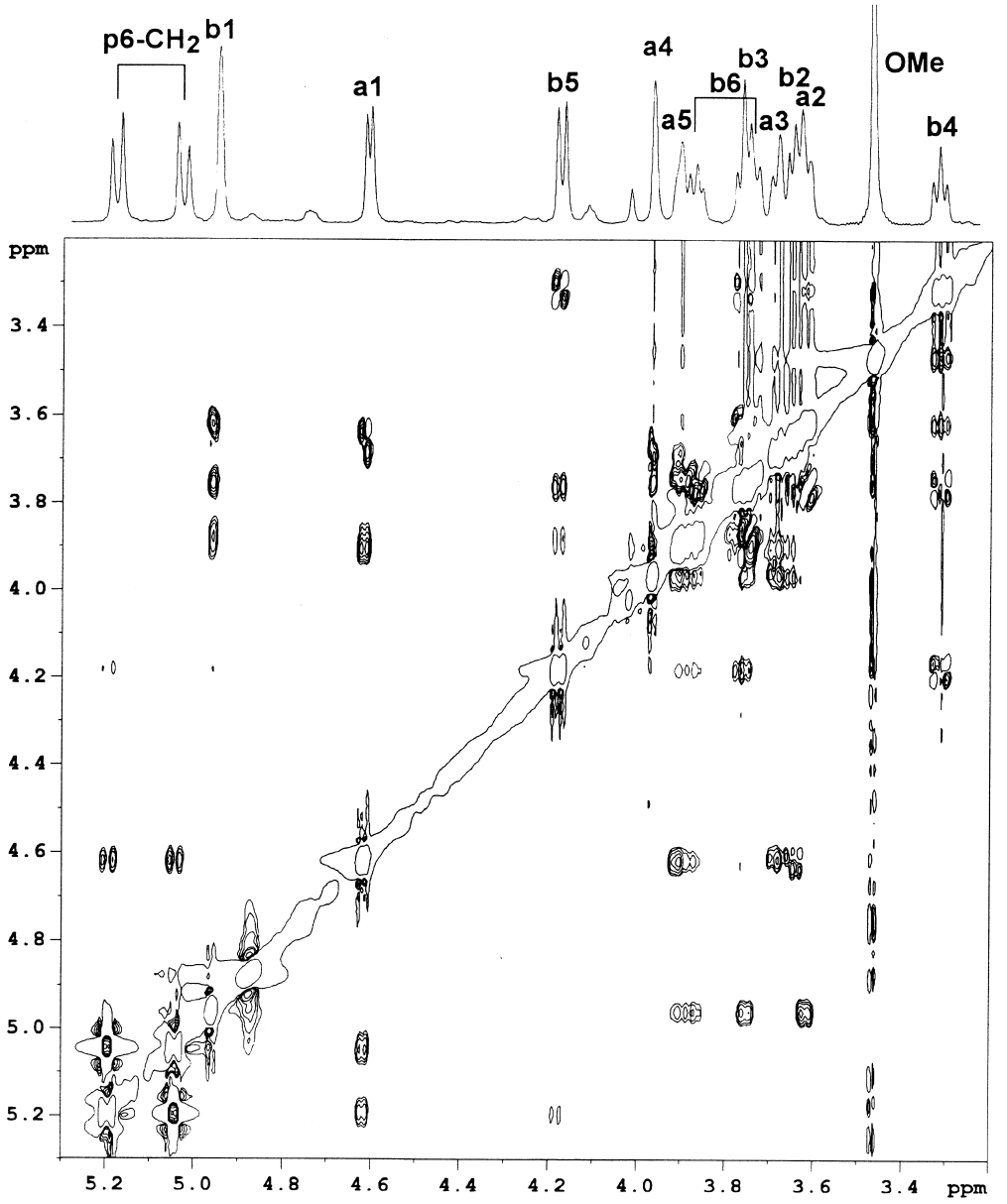


Fig. 3. The two-dimensional rotating frame nuclear Overhauser spectrum (ROESY) shows through-space connectivities via dipolar couplings in cyanopterin. A transglycosidic ROE is detected between b1-H and a6-H (a = galactose, b = glucuronic acid, p = pterin). Two inter-residue ROEs between a1-H and p6-CH_2 prove the attachment of the pterin aglycon (all NMR spectra: D_2O, 300 K, ^1H = 600 MHz).

pHs were not only typical of those of unconjugated pterins, but also very similar to those of another pteridine glycoside, limipterin [6]. These results strongly suggested that *Synechocystis* sp. PCC 6803 cells contain high levels of the new compound (cyanopterin) which, when oxidized by iodine, becomes 6-hydroxymethylpterin glycoside.

3.2. NMR and mass spectroscopy

The purified cyanopterin was analyzed by mass spectrometry and NMR spectroscopy. From acid hydrolysis of the substance, the pterin moiety was cleaved to form 6-hydroxymethylpterin identified by spectroscopic comparison with an authentic sample.

The mass spectrum of cyanopterin showed *m/e* 193 for M^+. The 300 MHz ^1H NMR spectrum in DMSO-d_6 showed resonances for seven protons. The proton resonance at singlet 8.74 ppm was assigned to the C-7 proton of the pterin; the resonance at singlet 4.63 ppm to the CH_2 of the hydroxymethyl group and the broad resonance at 7.07 ppm to the remaining four protons of NH, NH_2, and OH.

The structure of the sugar moiety of cyanopterin could be derived from the ^1H- and ^{13}C-NMR spectra recorded at a 600 MHz NMR spectrometer, see Table 1. The ^{13}C-NMR spectrum clearly showed the presence of 20 carbons in cyanopterin, seven of which corresponded unambiguously to the hydroxymethylpterin. The other 13 peaks corresponded to the sugar moiety, as expected from a disaccharide unit. The spin systems were determined by a DQF-COSY spectrum and the connectivity of the two sugar moieties and the aglycon was identified via dipolar couplings (rotating frame NOEs) in a ROESY spectrum (Fig. 3). Proton bearing carbons were assigned in a HMQC spectrum (Fig. 4). The connectivity was

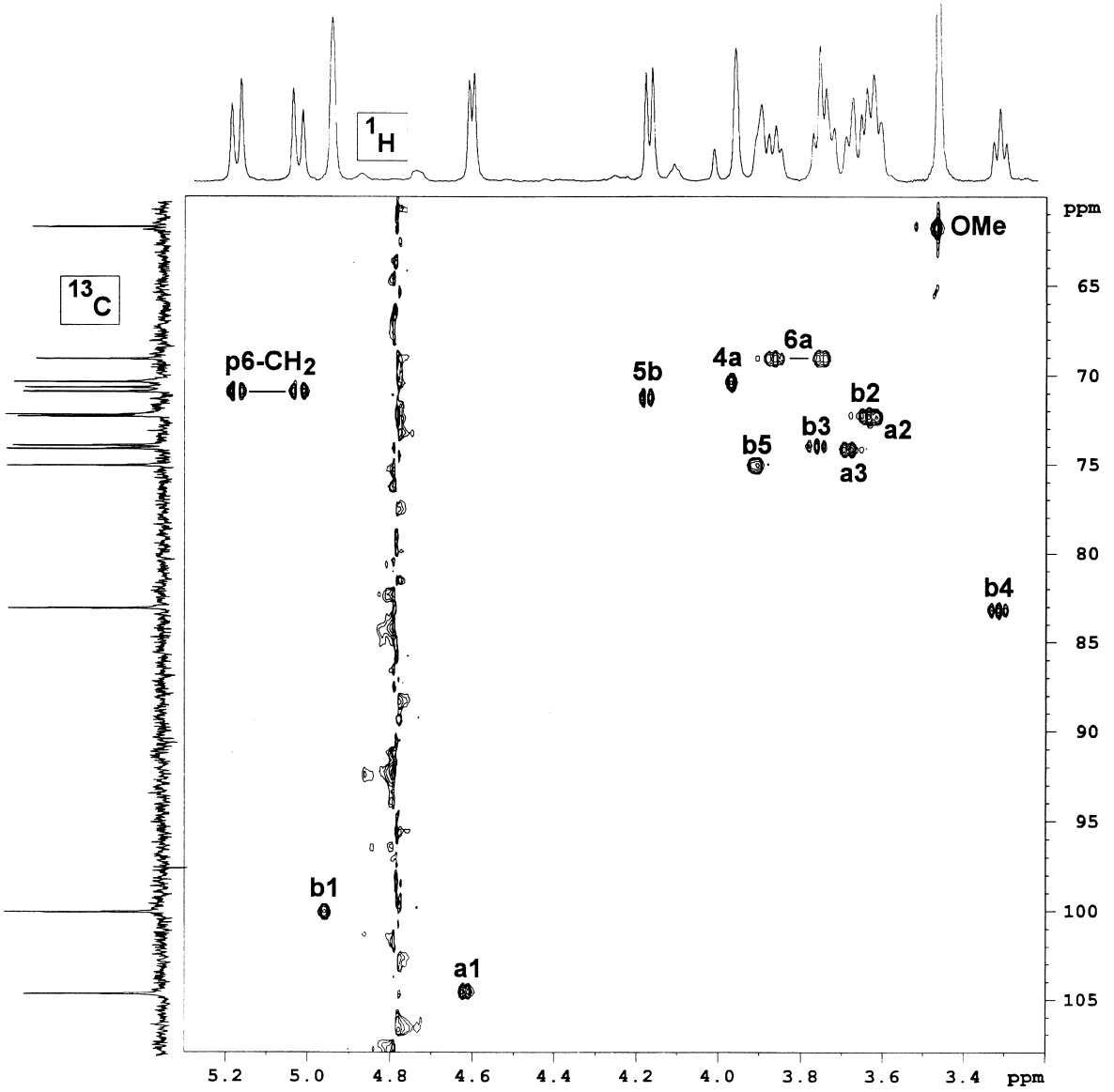


Fig. 4. $^1J_{C,H}$ connectivities are identified from this heteronuclear multiple-quantum coherence (HMQC). The two anomeric CH-groups are well separated at lowest field, the *O*-methylation of ring b leads to a deshielding of b4-C.

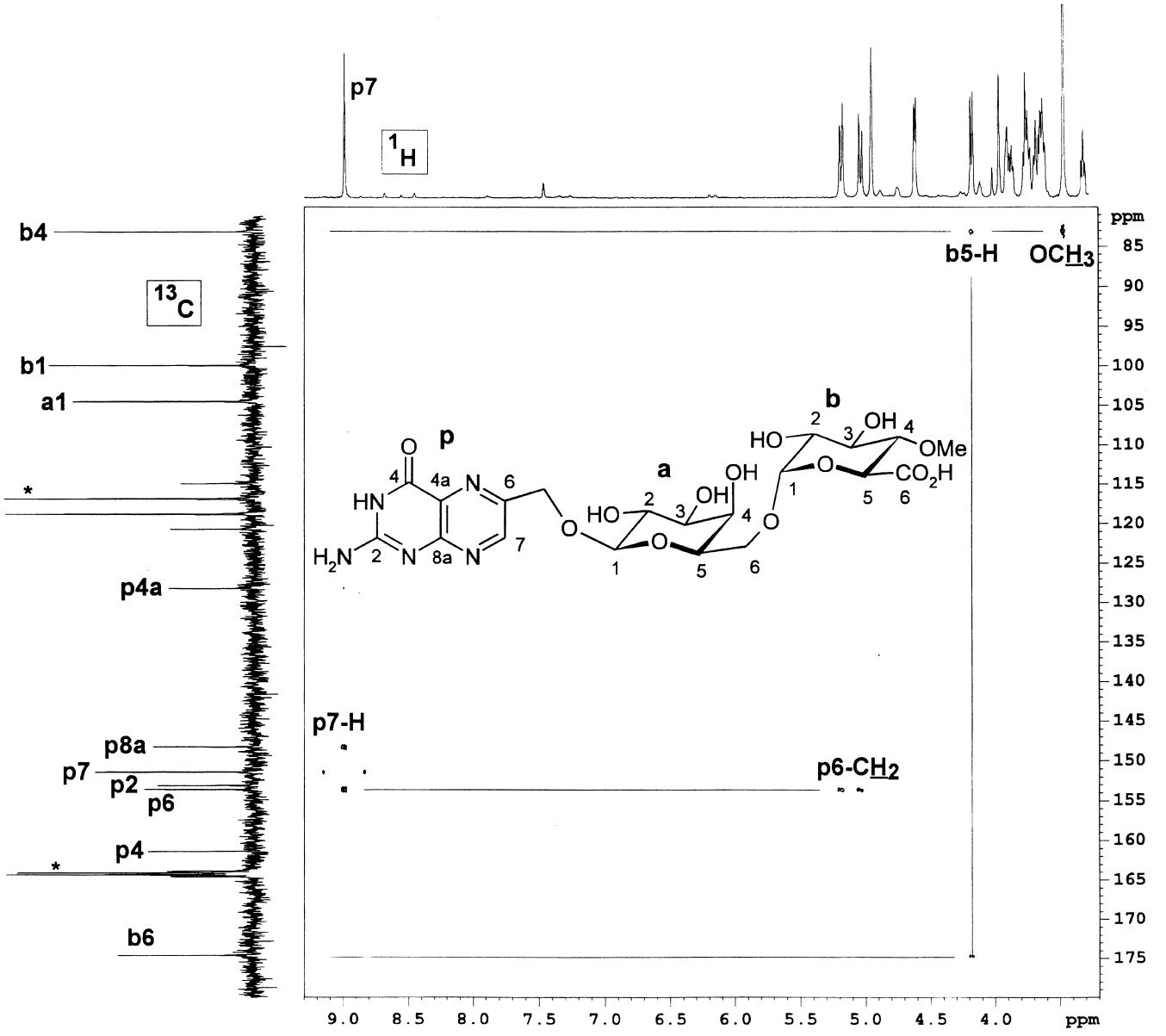
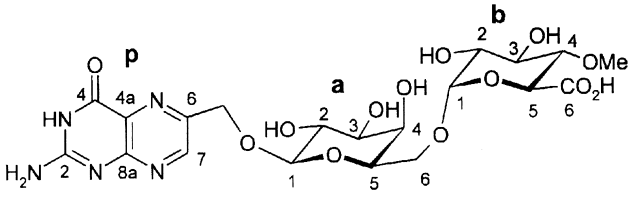


Fig. 5. Gradient selected heteronuclear multi-bond correlation (HMBC) of cyanopterin. b4-C shows a $^{3}J_{C,H}$ coupling to the methyl group and to b5-H. The carboxylic acid group b6-C shows a $^{3}J_{C,H}$ coupling to b5-H. Both p6-C and p8a-C show heteronuclear coupling to p7-H, only p8a-C is also correlated to p6-CH_2. (*Trifluoroacetic acid to increase the solubility of cyanopterin.)

independently proven via long range $^{3}J_{C,H}$ couplings in a gradient selected HMBC spectrum (Fig. 5). The CH_2-group of the aglyconic pteridine shows intense ROE contacts to the glycosidic proton at 4.62 ppm (Fig. 3). The large anomeric coupling of 7.6 Hz and the small $J_{3-H,4-H}$ and $J_{4-H,5-H}$ couplings could confirm this sugar as the β-configuration of galactose (ring a). The low-field 6-H shows a transglycosidic ROE contact to the anomeric proton of the second carbohydrate moiety resonating at 4.96 ppm and also shows a small $J_{1-H,2-H}$ coupling of 3.6 Hz. The ring couplings are large (>9 Hz) and the spin system ends with 5-H at 4.19 ppm. The 4-C is low-field shifted to 83.1 ppm (Fig. 4), and the 4-H shows a ROE contact to the methyl group at 3.47 ppm. We identified this sugar as the α-configuration of 4-*O*-methylated glucuronic acid (ring b). The *O*-methylation was independently proven via the heteronuclear

three-bond coupling between Gal 4-C and the *O*-methyl protons (Fig. 5). In the same spectrum the carbonyl of the carboxylic acid was found at 174.6 ppm by its $^3J_{C,H}$ coupling to Gal 5-H.

Consideration of all these data leads to the cyanopterin structure of 6-[1-(4-*O*-methyl-(α-D-glucuronyl)-(1,6)-(β-D-galactosyloxy]methylpterin:



3.3. Intracellular concentration of cyanopterin

Intracellular cyanopterin was determined from aliquots of the culture during cell growth in the photoautotrophic condition. Its intracellular content was highly dependent on the growth of cells. In order to show the linear correlation between the cell growth and cyanopterin content, chlorophyll *a* was determined and its molar concentration was plotted against that of cyanopterin (Fig. 6). They increased at the same rate and maintained a molar ratio of approximately 1 to 1.6. The extremely high intracellular concentration of cyanopterin suggests that it may have some essential housekeeping role in the cell that requires higher levels than would be required for a cofactor function.

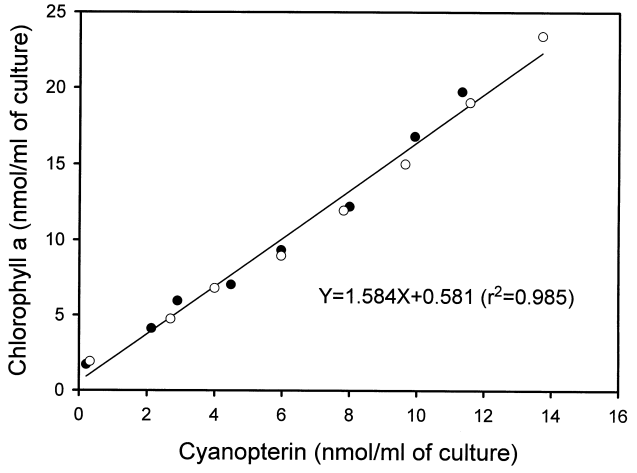


Fig. 6. Linear regression plot of intracellular cyanopterin and chlorophyll *a* in *Synechocystis* sp. PCC 6803. Cells were grown in Medium C under photoautotrophic conditions with air bubbling. Aliquots of cultures were harvested every 12 or 24 h and the precipitated cells were analyzed for intracellular cyanopterin and chlorophyll *a*. They were determined from two independent cultures (closed and open circles, respectively) and plotted against each other. Linear regression showed a molar ratio of approximately 1.6 between cyanopterin and chlorophyll *a* (r^2 = 0.985).

3.4. In vivo oxidation state of cyanopterin

To extract the native form of intracellular cyanopterin without affecting its reduced state, fresh cells were suspended in 1 N HCl plus 0.01% ascorbic acid. Oxidized cyanopterin in the cell extract was equivalent to 0.19% of the total amount obtained after iodine oxidation. The level increased to 3.2% when ascorbic acid was omitted in the cell extraction. These indicate that most of intracellular cyanopterin exists in its reduced form that is amenable to oxidation. In order to differentiate the dihydro and tetrahydro forms of intracellular cyanopterin, the oxidized amount of cyanopterin was measured during iodine oxidation and compared with those of the reduced forms of 6-hydroxymethylpterin. The method was adopted from Fukushima and Nixon [21], who showed that H_4-biopterin was more slowly oxidized to biopterin than H_2-biopterin. The time course of oxidation in Fig. 7 shows that the oxidation of intracellular cyanopterin was slow and generally followed the time course of 5,6,7,8-tetrahydro-6-hydroxymethylpterin during a period of 1 h. On the other hand, 7,8-dihydro-6-hydroxymethylpterin was almost completely oxidized within 1 min, which is congruent with the results obtained with 7,8-dihydrobiopterin [21]. The reducing effect of ascorbic acid in the cell extract was negligible on the rate of iodine oxidation of intracellular cyanopterin. When an excess amount of ascorbic acid was added to the 7,8-dihydro-6-hydroxymethylpterin solution, still more than 80% of dihydro form was oxidized within 1 min. Even with 5-fold higher amount of iodine in the cell extract, oxidation of intracellular cyanopterin was never completed within 1 min (data not shown). Therefore, these results strongly suggest that the majority of cyanopterin exists in the 5,6,7,8-tetrahydro form in vivo, analogously to other pteridine glycosides, such as limipterin and tepidopterin [6,7].

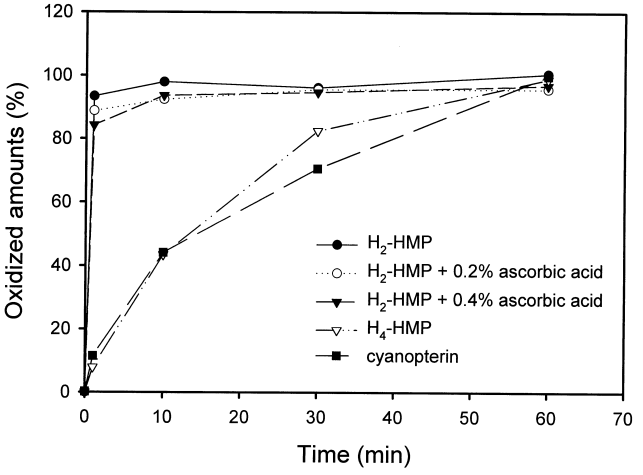


Fig. 7. Time course of oxidation of intracellular cyanopterin by iodine. Cellular extract was prepared in 1 N HCl plus 0.01% ascorbic acid as described in Section 2. Solutions (40 μM) of reduced forms of 6-hydroxymethylpterins were prepared fresh in 1 N HCl. Oxidation was performed in equal volume of KI/I_2 solution (2/1% in water) and stopped with 5% ascorbic acid. Oxidized pteridines were measured by HPLC. Data are expressed as percentage of the oxidized amount: dihydro form of 6-hydroxymethylpterin (●), plus 0.2% ascorbic acid (○), plus 0.4% ascorbic acid (▼), tetrahydro form of 6-hydroxymethylpterin (▽), and cyanopterin (■).

4. Discussion

We describe the complete procedure for the isolation and structural elucidation of cyanopterin from *Synechocystis* sp. PCC 6803 and the characteristics of the compound that may be possibly related to the physiological function. We demonstrated that the structure of cyanopterin is 6-[1-(4-*O*-methyl-(α-D-glucuronyl)-(1,6)-(β-D-galactosyloxy]methylpterin and its native intracellular form is primarily in the 5,6,7,8-tetrahydro state. We also could show that cyanopterin is synthesized constitutively in high amounts comparable to the amount of chlorophyll *a*.

Cyanopterin is unique in its structure since an unusual disaccharide is attached to 6-hydroxymethylpterin to produce a new type of pteridine glycoside which has not previously been found in nature. Glycosylation of the relatively insoluble 6-hydroxymethylpterin results in a marked increase of water solubility which apparently allows the high intramolecular concentration of the pteridine. In this context, cyanopterin may have, as a disaccharide derivative, an even greater advantage in its solubility than corresponding monoglycosides. The increased hydrophilicity also resulted in the faster elution of cyanopterin, as compared to 6-hydroxymethylpterin, from a C18 reverse-phase column (Fig. 1). It is also interesting to note that cyanopterin is derived from 6-hydroxymethylpterin, which has previously been found in another *Synechocystis* sp. [3].

It was clearly shown that intracellular cyanopterin content was constitutive with cell growth and maintained a molar ratio of approximately 1 to 1.6 with chlorophyll *a* (Fig. 6). We also demonstrated, in data not shown, that extracellular cyanopterin content in the medium was very low until the exponential phase and slightly increased to a level of 5.3% of the intracellular content at the stationary stage, possibly resulting from cell lysis. In contrast to *Chlorobium tepidium* [7], no other types of pteridines were detected in the medium, while 6-hydroxymethylpterin and higher amount of cyanopterin were present extracellularly in unhealthy cultures (data not shown). These results may indicate that cyanopterin is essential for cellular function.

The intracellular oxidation state of cyanopterin was determined to be primarily the 5,6,7,8-tetrahydro form. The time course of iodine oxidation (Fig. 7) suggested that the majority of intracellular cyanopterin exists in its tetrahydro form. Other pteridine glycosides, limipterin and tepidopterin found in anaerobic photosynthetic sulfur bacteria, also exist as tetrahydro forms in vivo [6,7]. Tetrahydrobiopterin, the best studied unconjugated pteridine, functions in its tetrahydro form as a cofactor of monooxygenases [12]. In addition, tetrahydro forms of pteridines were shown to be active in stimulating photosynthetic electron transport [13–16].

Until now, pteridine glycosides have been found in various kinds of prokaryotes, such as cyanobacteria, photosynthetic green sulfur or non-sulfur bacteria, and chemoheterotrophic and chemoautotrophic bacteria. Except for the last two, all others share the common characteristics of phototrophism. Thus, it is of interest to consider what role pteridine glycosides may have that is related to photosynthesis. Earlier works suggested that tetrahydropteridines stimulate photophosphorylation and play a primary role in photosynthetic electron transport [13–16]. Pteridines also have been postulated as the putative candidate for the chromophore related to blue light photoreception [23–25]. However, since chemotrophs

also possess pteridine glycosides [8–10] a role directly related to light may not be the case.

In conclusion, our results suggest that the tetrahydro form of cyanopterin has some essential physiological function in *Synechocystis* sp. PCC 6803, which may be revealed through genetic manipulations of the decoded genome of *Synechocystis* [19]. Based on the elucidated structure of cyanopterin and the genome sequence of *Synechocystis* sp. PCC 6803, we have already identified several genes coding for enzymes putatively involved in the biosynthesis of cyanopterin (in preparation).

Acknowledgements

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