

PTERINS OF THE CYANOBACTERIUM *APHANIZOMENON FLOS-AQUAE*

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**Abstract**—The cyanobacterium *Aphanizomenon flos-aquae* contains various glycosides of a pterin that had previously been tentatively identified as 6-(threo-1,2-dihydroxypropyl)-pterin. The pterin is now identified as 6-(L-threo-1,2-dihydroxypropyl)pterin (6-L-threo-biopterin) by NMR and mass spectrometry, thin layer chromatographic comparisons with authentic samples and by optical rotation. The 6-L-threo-biopterin glycoside-1 from toxic strain NH-1 gave rise to xylose and glucose on hydrolysis, whereas its glycoside-2 from Cambridge Collection and nontoxic NH-1 strains gave mannose and glucose. This pterin may be a useful marker for certain species of cyanobacteria.

## INTRODUCTION

The cyanobacteria are prokaryotic organisms, formerly, and still often referred to as blue-green algae. The freshwater species *Aphanizomenon flos-aquae* (L.) Ralfs, has been widely investigated because it can occur in highly toxic forms. During the course of our studies on some toxic strains [1], attention was turned to the highly fluorescent substances which accompanied the toxins in the early stages of isolation. These fluorescent substances were identified as pterin glycosides and were also found in non-toxic strains. The parent pterin, which had not previously been reported in a cyanobacterium, was tentatively identified as 6-threo-1,2-dihydroxypropyl)pterin (6-threo-biopterin) [2].

High concentrations of pterins (2-amino-4-hydroxy-pteridines) in the cyanobacterium *Anacystis nidulans* were first reported by Forrest *et al.* [3]. Hatfield *et al.* [4] surveyed the cyanobacteria and found that biopterin was the principal pterin in species of *Anabaena*, *Nostoc*, *Anacystis* and *Synechococcus*, and that it occurred in the form of various glycosides. *Synechocystis* was unusual in that its principal pterin was 6-hydroxymethyl-pterin (ranachrome-3), which also occurred in glycosidic form.

The present paper describes the parent pterin and its derivatives found in strains of *Aph. flos-aquae*.

## RESULTS AND DISCUSSIONS

It was previously shown that the highly fluorescent substances in various strain of *Aph. flos-aquae* (F-2, F-6, F-Camb, F-7, F-8) all gave rise to the same compound, called F-1, on acid hydrolysis [2]. Oxidation with potassium permanganate gave rise to pterin-6-carboxylic acid, showing it to be a 6-substituted pterin, but TLC

indicated that F-1 was not identical with any of the usual naturally occurring pterins nor with oxidized sepiapterin or isosepiapterin.

The mass spectrum of the trimethylsilylated (TMSi)-derivative of F-1 was compared with those of the TMSi-derivatives of biopterin, 6-(threo-1,2-dihydroxypropyl)-pterin (6-threo-biopterin), neopterin and monapterin (Table 1). Although no molecular ions were observed, peaks at  $m/z$  541 ( $M - 72$ ) and 469 ( $M - 2 \times 72$ ) were noted for neopterin and monapterin but not for biopterin, threo-biopterin and F-1. On the other hand, the latter three TMSi-derivatives showed small fragments at  $m/z$  453 ( $M - 72$ ), 438 ( $M - 72 - 15$ ) and 381 ( $M - 2 \times 72$ ). The latter compounds also had small fragments at  $m/z$  276, whereas those at  $m/z$  205, 379 and 424 were less intense than those for neopterin or monapterin. We did not observe a strong peak at  $m/z$  409 resulting from the cleavage of the penta-TMSi-derivatives of neopterin and monapterin and the tetra-TMSi-derivatives of biopterin and threo-biopterin between the  $\alpha$  and  $\beta$  carbon atoms of the 6-side chain [5, 6]. However, all derivatives gave characteristic intense fragments at  $m/z$  265 ( $409 - 2 \times 72$ ) and 337 ( $409 - 72$ ) [7]. Thus the mass spectral data of the TMSi-derivative of F-1 was more closely consistent with biopterin/6-threo-biopterin than with neopterin/monapterin.

The high resolution proton NMR data for F-1 were almost identical to those reported for biopterin in DMSO [8], the major difference being that the 7-H resonance was at 0.34 ppm lower field than for biopterin. The identity of F-1 with 6-threo-biopterin rather than biopterin was shown by direct TLC comparison with samples of synthetic 6-(threo-1,2-dihydroxypropyl)pterin (6-threo-biopterin) [8, 9]. That F-1 is 6-L-threo-biopterin was deduced from its  $[\alpha]_{25}^D + 72^\circ$  (lit.  $[\alpha]_{25}^D + 95^\circ$  (9)).

Table 1. Mass spectral fragmentation of the trimethylsilyl-derivative of F-1 with those of neopterin, monapterin, biopterin and *threo*-biopterin

<i>m/z</i>	TMSi-Neopt.	TMSi-Monap.	TMSi-Biopt.	TMSi- <i>threo</i> - Biopt.	TMSi-Deriv. of F-1		
	(613) A	(613) B	(525) C	(525) D	Prep I	II	Fragment
73	100	96	100	100	90	100	Si <sup>+</sup> Me <sub>3</sub>
202	0.6	0.4	1.4	2	5	0.7	
205	5	5	0.3	0.5	0.7	0.4	
207	3	1.0	0.4	0.6	0.8	—	
234	7	5	6	9	11	6	
249	10	8	8	12	14	7	
265	94	100	61	92	100	40	409 - 2 × 72
276	0.8	0.3	4	7	4	4	
290	2	2	0.6	0.7	—	1.0	
292	3	0.5	0.4	0.5	0.8	0.8	
337	22	19	16	15	19	30	409 - 72
366	0.5	0.3	0.8	1.1	1.1	0.6	M - 2 × 72 - 15 (C/D)
379	0.7	1.2	0.07	0.05	—	0.2	
381	0.2	0.2	0.5	0.8	0.7	0.6	M - 2 × 72 (C/D)
409	0.7	0.4	0.4	0.12	—	1.2	(see text)
424	0.4	0.5	—	0.02	—	—	
438	—	0.04	0.3	0.2	—	1.2	M - 72 - 15 (C/D)
453	—	0.05	0.2	0.06	—	0.6	M - 72 (C/D)
454	0.6	0.5	—	—	—	0.2	M - 2 × 72 - 15 (A/B)
469	0.4	0.6	—	—	—	—	M - 2 × 72 (A/B)
526	—	0.09	—	—	—	—	M - 72 - 15 (A/B)
541	0.13	0.11	—	—	—	—	M - 72 (A/B)

This is the first reported occurrence of 6-*threo*-biopterin in cyanobacteria. Kidder and Dewey [10], however, have reported ciliapterin in the ciliated protozoan *Tetrahymena pyriformis*. The structure of 6-*threo*-biopterin was proposed for ciliapterin, and an L-configuration proposed on the basis of its biological activity. The L-configuration for F-1 is consistent with the fact that the known pterins, biopterin, monapterin and sepiapterin, derived from the biosynthetic intermediate dihydro-neopterin triphosphate [11], except for neopterin itself, possess a L-configuration. The *Aphanizomenon* pterins are glycosides of 6-L-*threo*-biopterin. Its glycoside-1 from toxic NH-1 strain gave rise to xylose and glucose on hydrolysis, and its glycoside-2 from non-toxic NH-1 and Cambridge strains gave rise to mannose and glucose. Of the other cyanobacteria examined, no 6-L-*threo*-biopterin was detected in three strains of *Microcystis aeruginosa*, and one out of two strains of *Anabaena flos-aquae* showed its presence (unpublished results). Because of its apparent limited occurrence, 6-L-*threo*-biopterin may be a useful marker for certain cyanobacteria.

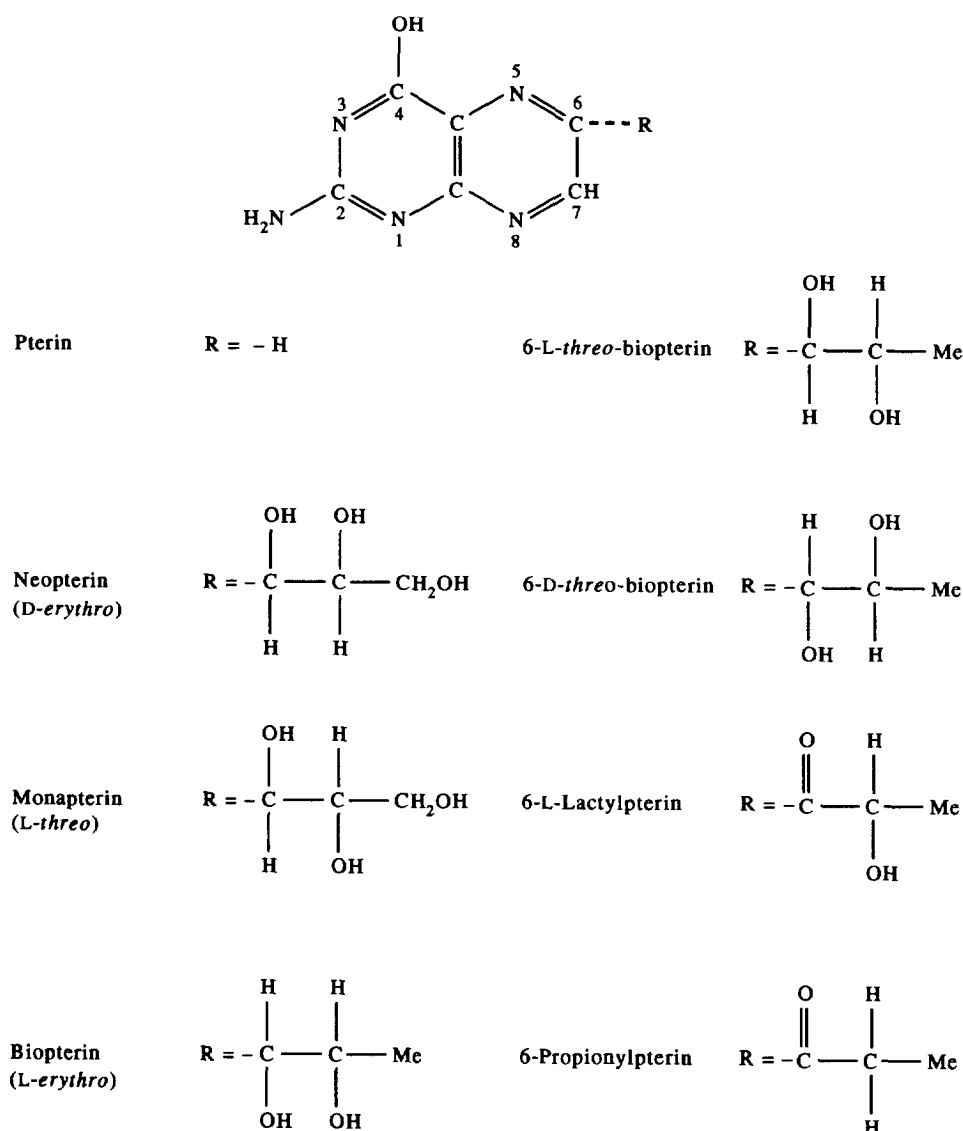
#### EXPERIMENTAL

*Algal strains and culture conditions.* The strains of *A. flos-aquae* used in this study were the following: (1) *NH-1 toxic strain*—culture of cells isolated by Dr W. W.

Carmichael, Wright State University, Dayton, Ohio, from a toxic bloom in a farm pond near Durham, New Hampshire; (2) *NH-1 non-toxic strain*—culture of cells of previously toxic NH-1 which had lost its toxicity to mice; (3) *Cambridge strain (non-toxic)*—culture of cells from the Algae Collection, Cambridge University, England. All laboratory cultures were grown in ASM 1 medium [12] at 20–22° under constant fluorescent lighting (cool white) at approximately 50  $\mu$ E.

*Pterins.* For sepiapterin and isosepiapterin, ca 25 frozen heads from *sepia* mutant *Drosophila* (Connecticut Valley Biological Supply Co., Southamptn, MA) were placed in a small test tube with 0.25 ml of 95% EtOH and crushed with a glass rod. H<sub>2</sub>O (0.25 ml) was added and the crushing continued. The mixt. was filtered and the extract frozen. Paper chromatography of the extract in solvent system E (below) showed the presence of a very strong yellow fluorescent zone corresponding to sepiapterin [13, 14] and a faster weaker yellow fluorescent zone corresponding to isosepiapterin [13, 15]. MnO<sub>2</sub> or Br<sub>2</sub> oxidation of the extract led to the formation of a blue fluorescent oxidation product (6-L-lactyl-pterin) from sepiapterin [16] and blue fluorescent 6-propionyl-pterin from isosepiapterin [17] (Fig. 1).

*Chromatographic systems.* For TLC, E. Merck analytical Silica Gel 60 plates without fluorescent indicator were used. Solvent systems; (A) *n*-BuOH-Me<sub>2</sub>CO-0.1 M



NaH<sub>2</sub>PO<sub>4</sub> (4:5:1) for pterins and sugars; (B) *iso*-PrOH-2% NH<sub>4</sub>-OAc (2:1) for pterins; (C) *n*-BuOH-HOAc-H<sub>2</sub>O (2:1:1) for pterins. For PC, Whatman No. 1 paper was used. Solvent systems; (D) EtOAc-pyridine-H<sub>2</sub>O (45:18.75:15) for sugars; (E) *n*-PrOH-1% NH<sub>3</sub> (2:1) for pterins. Pterins were detected by fluorescence under long wavelength UV. Sugars were detected by spraying with aniline hydrogen oxalate and heating or by periodate-benzidine spray [18].

**Mass spectrometry.** Mass spectra were obtained with a Hewlett-Packard Model 5988A quadrupole mass spectrometer at an ion source temp. of 200° and an ionization energy of 70 eV. For prepn of the TMSi-derivatives, 0.5 mg of pterin was treated with 100  $\mu$ l of TRI-SIL/BSA (Pierce Chemical Co., Rockford, IL) for 1 hr at 70–80° in a capped small reaction vial, and the reaction mixt. taken to dryness in a vacuum desiccator.

**NMR spectrometry.** A high resolution proton NMR spectrum in DMSO- $d_6$  was acquired at 360 MHz on a Bruker AM360 NMR spectrometer.

**Pterin glycoside isolation procedure.** The extraction procedure was similar to that described by Hatfield *et al.* [4]. Lyophilized cells (2.5 g) were suspended in 250 ml of 0.5 M HOAc, with 125 mg of powdered MnO<sub>2</sub> added, and the mixt. stirred for 24 hr. Although the pterins probably occur partially or totally as dihydro- and tetrahydro-derivatives, they are oxidized to the pterins by the MnO<sub>2</sub> treatment. The mixt. was then filtered through Whatman No. 1 paper and the filtrate passed through a Buchner funnel containing a bed of previously washed decolorizing wood carbon (1.5 g of Nuchar S-N, Fisher Scientific Co., Boston, MA). The bed was then washed with 100 ml of H<sub>2</sub>O and the fluorescent substances eluted with 50% EtOH containing 1% conc. NH<sub>3</sub>. The eluate

was concentrated *in vacuo* to ca 2 ml and the concentrate streaked along the origins of 4 sheets of Whatman 3 MM paper (18.5 × 28 cm, origin along the 28 cm edge). The sheets were developed ascendingly in *n*-BuOH–HOAc–H<sub>2</sub>O (2:1:1) and the intense fluorescent zone was cut out, shredded and the fluorescent substance eluted with water. The eluate was concd *in vacuo* to ca 2 ml, the concentrate streaked along the origin of an E. Merck Silica Gel 60 prep. TLC plate and the plate developed in *iso*-PrOH–2% NH<sub>4</sub>OAc (2:1). The intensely fluorescent zone was scraped out and the fluorescent component eluted with 85% EtOH. The eluate was again concd and the chromatography on Whatman 3MM repeated using 2 sheets. The final eluate was concd to dryness *in vacuo* to give the pterin fr. (6-*L*-threo-biopterin glycoside-1 from toxic NH-1 strain, its glycoside-2 from non-toxic NH-1 and Cambridge strains). This fr. was obtained in a yield of ca 3–5 mg g<sup>-1</sup> dry cells. The material contained NaOAc, which could be removed by extraction with EtOH.

*Hydrolysis for the identification of parent pterin and sugars.* Approximately 5 mg of pterin glycoside were dissolved in 1.5 ml of 2 N H<sub>2</sub>SO<sub>4</sub> and heated in a boiling water bath for 1 hr. The hydrolysate was cooled and neutralized to pH 5–6 with a soln of Ba(OH)<sub>2</sub>. The pptd BaSO<sub>4</sub> was removed by centrifugation and the supernatant solution concd *in vacuo* to ca 0.5 ml for TLC and PC.

*Isolation of 6-*L*-threo-biopterin.* About 30 mg of glycoside 1 or 2 were hydrolysed as described above and 200 mg of activated charcoal (Nuchar S-N) added to the supernatant soln remaining after removing the BaSO<sub>4</sub>. The charcoal was removed by centrifugation, washed with water and the pterin eluted from the charcoal with 50% EtOH containing 1% conc. NH<sub>3</sub>. The eluate was concd to dryness, the residue extracted with a small amount of absolute EtOH and the insoluble 6-*L*-threo-biopterin recrystallized from 10% HOAc [8] as a pale yellow crystalline powder.  $[\alpha]_{25}^D + 72^\circ$  (c 1.6 mg in 2 ml 0.1 N HCl); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ 1.04 (3H, *d*, *J* = 6.4 Hz, 3'-H<sub>3</sub>), 3.85 (1H, *m*, 2'-H), 4.44 (1H, *dd*, *J*<sub>1</sub> = 4.6 Hz, *J*<sub>2</sub> = 5.0 Hz, 1'-H), 4.61 (1H, *d*, *J* = 5.7 Hz, 2'-OH), 5.51 (1H, *d*, *J* = 5.3 Hz, 1'-OH), 6.84 (2H, *br s*, 2-NH<sub>2</sub>), 8.71 (1H, *s*, 7-H), 11.39 (*br s*, 3-NHCO).

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