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The occurrence of the *cis/trans* geometric isomerism of myxoxanthophyll and 4-ketomyxoxanthophyll in the cyanobacterium *Anabaena* sp. PCC7120*

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The cyanobacteria of the genus *Anabaena* have recently been recognized as a potential source of secondary metabolites of pharmacological and biotechnological importance. In particular, myxoxanthophylls – specific carotenoid glycosides that accumulate in cyanobacterial cells, are attracting increasing interest. *Anabaena* (*Nostoc*) sp. PCC7120, a filamentous, mesophilic, nitrogen-fixing cyanobacterium, is a model organism used in biochemical and genetic studies. The carotenoid pool of *Anabaena* sp. PCC7120 consists of five main species of pigments, namely β-carotene, echinenone, canthaxanthin and two derivatives of myxoxanthophyll: myxoxanthophyll ((3R,2'S)-myxol 2'-fucoside) and 4-ketomyxoxanthophyll ((3S,2'S)-4-ketomyxol 2'-fucoside). Recent findings show that the carotenoid biosynthesis pathway functions in *Anabaena* sp. PCC7120 cells are affected by environmental factors. Specifically, the balance between β-carotene and ketocarotenoids alters according to the temperature conditions. In this study, a new method, based on single-step liquid adsorption chromatography was developed and applied to separate a fraction containing myxoxanthophyll and 4-ketomyxoxanthophyll from *Anabaena* sp. PCC7120 cells. It was found that this method allowed a high purity fraction of carotenoid glycosides to be obtained from pigment pools as extracted from cyanobacterial cells. The subsequent analysis using the methods HPLC and LC/MS demonstrated that this fraction consists of a mixture of compounds with different retention times. On the basis of their fragmentation spectra and optical properties, these compounds were identified as geometrical isomers of myxoxanthophyll and 4-ketomyxoxanthophyll, including the dominant all-*trans* forms and less abundant *cis* forms. Proposals regarding the structures of myxoxanthophyll isomers are made.

Keywords: carotenoid glycosides, geometric isomers, HPLC, LC/MS, cyanobacteria, *Anabaena*

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Abbreviations: HPLC/DAD, high-performance liquid chromatography with diode-array detection; LC/MS, liquid chromatography–mass spectrometry; RT, retention time

INTRODUCTION

Myxoxanthophylls (2'-myxol glycosides) are a group of carotenoids found almost exclusively in cyanobacteria, where they are common constituents of the carotenoid pool. They are glycosides in which the single sugar residue (predominantly a pentose) is linked by an ether bond either to the carotenoid core, myxol ((3S,4E,6E,8E,10E,12E,14E,16E,18E,20E,22E,24E)-25-[(4R)-4-hydroxy-2,6,6-trimethylcyclohexen-1-yl]-2,6,10,14,19,23-hexamethylpentacosa-4,6,8,10,12,14,16,18,20,22,24-undecaene-2,3-diol) or keto- and/or hydroxy- myxol derivatives (Takaichi *et al.*, 2001; Takaichi *et al.*, 2005; Takaichi *et al.*, 2006; Iwai *et al.*, 2008).

Anabaena sp. PCC7120 (henceforth *Anabaena* 7120), originally described as *Nostoc muscorum* (Adolph & Haselkorn, 1971) is a filamentous, mesophilic, nitrogen-fixing cyanobacterium (Rippka *et al.* 1979). It is a model organism widely used in biochemical and genetic studies on heterocyst-forming cyanobacteria (Koksharova & Wolk, 2002; Banerjee *et al.*, 2013). As previously mentioned, the carotenoid pool of *Anabaena* 7120 consists of five main species of pigments: β-carotene, echinenone, canthaxanthin and two myxoxanthophyll derivatives: myxoxanthophyll ((3R,2'S)-myxol 2'-fucoside) and 4-ketomyxoxanthophyll ((3S,2'S)-4-ketomyxol 2'-fucoside) (Takaichi *et al.*, 2005; Kłodawska *et al.*, 2019).

The carotenoid biosynthesis pathway in *Anabaena* 7120 has been extensively studied (Takaichi *et al.*, 2005; Mochimaru *et al.*, 2008). In particular, genes encoding key enzymes involved in the biosynthesis of carotenoids in *Anabaena* have been identified (see: Takaichi & Mochimaru, 2007; Takaichi, 2011; Sugiyama & Takaichi, 2020 for reviews). The initial stages of carotenogenesis are common to cyanobacteria and plants (see: Sandmann, 2021, for an extensive review). The first step in carotenoid biosynthesis in *Anabaena* is the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP, C20) into phytoene (C40). This process is catalyzed by phytoene synthase, an enzyme encoded by the *crtB* gene (Chamovitz *et al.*, 1992, Sandmann & Misawa 1992, Martínez-Férez *et al.*, 1994). The subsequent desaturation and cyclization catalyzed by phytoene desaturase (CrtP), ζ-carotene desaturase (CrtQ) and two lycopene cyclases (CruA , CruP) result in the synthesis of carotenes. Among oxygenic phototrophs, *Anabaena* 7120 exclusively contains a ζ-carotene desaturase (CrtQa) that can utilize both all-*trans* and 9-9'-di-*cis*-ζ-carotene as substrates and produce all-*trans*-lycopene (Linden *et al.*, 1994). Lycopene cyclization leads to the differentiation of hydrocarbons with cyclic ionone rings at one (γ-carotene) and at two ends

(β-carotene). Subsequently, β-carotene hydroxylase (CrtR) catalyzes the synthesis of myxol, which is a precursor of carotenoid glycosides – myxoxanthophylls formed after fucose binding by 2'-O-glycosyltransferase (Takaichi & Mochimaru, 2007). Similarly to other filamentous cyanobacteria, *Anabaena* has two distinct β-carotene ketolases: CrtW and CrtO with different substrate specificities (Mochimaru *et al.*, 2005). CrtW hydroxylase is considered to be the key enzyme that catalyzes the synthesis of keto-myxoxanthophyll from myxoxanthophyll (Mochimaru *et al.*, 2005; Makino *et al.*, 2008).

The biological role of myxoxanthophylls in cyanobacterial cells has not been fully elucidated. Studies performed on the *Synechocystis* PCC6803 loss-of-function mutant with impaired fucose synthetase have demonstrated the role of myxoxanthophyll in the formation of both the cell wall and thylakoid membranes (Mohamed *et al.*, 2005). An increased accumulation of myxoxanthophylls in various cyanobacterial species has also been documented under a range of different stress conditions. In particular, biotic stress conditions accompanied by phosphatidylglycerol depletion resulted in an increase in myxoxanthophyll concentration in the thylakoid and plasma membranes of *Synechocystis* PCC6803 (Domonkos *et al.*, 2009). In addition, the monomerization of photosystem I in *Synechocystis* led to the overaccumulation of myxoxanthophyll formed at the expense of β-carotene (Kłodawska *et al.*, 2015). Similar overaccumulation of carotenoid glycosides in cyanobacterial cells has also been observed under abiotic stress conditions, including exposure to the herbicide fluridone, which disrupts carotenoid biosynthesis (Millie *et al.*, 1989), high light intensities (Steiger *et al.*, 1999), suboptimal growth temperature (Várkonyi *et al.*, 2002) and elevated growth temperatures (Kłodawska *et al.*, 2019).

All structures of myxoxanthophyll compounds documented and described so far concern geometric all-*trans* isomers of these carotenoids, but studies on *cis* isomers are lacking. An explanation for this could be the analogy with other carotenoids common in nature, which exist and perform their physiological functions mainly in the all-*trans* form (Zechmeister, 1962). Examples of these are β-carotene, lutein or zeaxanthin, where *cis* isomerization is often one of the first steps in the degradation of these compounds (Xiao *et al.*, 2018). However, this is not the rule, and there are natural, stable *cis* carotenoids of physiological importance (Zechmeister, 1962). An example of this is 9-*cis*-neoxanthin, which is a precursor to the synthesis of abscisic acid, an important plant hormone (Parry *et al.*, 1990; Takaichi & Mimuro, 1998). Another example is the pool of polycis carotenoids found in 'Tangerine' tomato fruits (Clough & Pattenden, 1979). 15-*cis* carotenoids have been identified in photosynthetic reaction centers of plants, cyanobacteria, and photosynthetic bacteria (Bialek-Bylka *et al.*, 1995, 1996, 1998). More recently, 9-*cis*-neoxanthin and 13-*cis*-violaxanthin have been shown to participate in the structural stabilization of plant photosynthetic membranes (Grudziński *et al.*, 2001; Zubik *et al.*, 2011). Furthermore, an increased accumulation of 9-*cis*-β-carotene by the alga *Dunaliella bardawil* in plastidic oil bodies through the action of the specialized enzymes 9-*cis*/all-*trans* β-carotene isomerases has been observed under stress conditions (Davidi & Pick, 2017). In a recent report, the occurrence of 9 or 9'-*cis*-myxoxanthophyll and 13 or 13'-*cis*-myxoxanthophyll has been suggested in the cyanobacterium *Synechocystis salina* (Nováková *et al.*, 2020).

Cis compounds may also appear in cyanobacteria as intermediates in the carotenoid biosynthesis pathway. This takes place at the stage of the transformation of phytoene to lycopene. This process can follow one of two paths. The first, called plant-type, involves CrtP (phytoene desaturase) and CrtQ (ζ-carotene desaturase). During this process, initially formed *cis* compounds (15-*cis*-phytoene; 9,9',15-tri-*cis*-ζ-carotene; 9,9'-di-*cis*-ζ-carotene; 7,7',9,9'-tetra-*cis*-lycopene) are subsequently isomerized to all-*trans* compounds via the action of the CrtH enzyme (*cis*-carotene isomerase) or via direct photoisomerization (Giuliano *et al.*, 2002; Takaichi & Mochimaru, 2007). The other one is the so-called bacterial type path, in which only one enzyme CrtI (phytoene desaturase) is involved. In cyanobacteria, this type has been observed exclusively in *Gleobacter violaceus* (Takaichi & Mochimaru, 2007).

Although the occurrence of myxol 2'-fucoside and 4-ketomyxol 2'-fucoside has been confirmed among *Anabaena* 7120 xanthophylls, and all-*trans* isomers of these compounds were structurally identified using NMR spectroscopy, the existence of the *cis* forms of these carotenoids has only been demonstrated in pigment extracts from the cells of these cyanobacteria (Takaichi *et al.*, 2005). In this study, the fraction containing a pool of carotenoid glycosides was purified on the semi-preparative scale from pigment extracts of *Anabaena* 7120. We report the occurrence and semiquantitative distribution of the geometric isomers of myxoxanthophylls in this cyanobacterium. The structure of the isomeric forms of carotenoid glycosides is proposed.

MATERIALS AND METHODS

Organism and culture conditions

Anabaena PCC7120 (UTEX 2576 strain), was cultured photoautotrophically in liquid medium BG11 (-N) under diazotrophic conditions, according to Rippka and others (Rippka *et al.,* 1979), at a temperature of 23°C and constant illumination with a photon flux density of 60 μmol photons m^{-2} s^{-1}, using fluorescent lamps (Philips TL-D 18W/33-640). The cultures of cyanobacteria with a total volume of 3 L were aerated on a rotary shaker at 150 rpm in volumes of 200 mL in 500 mL flasks. The culture growth was assessed by measuring the optical density at 750 nm (OD_{750}) using a V650 spectrophotometer (Jasco Co., Tokyo, Japan).

Isolation of the carotenoid glycoside fraction

To keep the carotenoid pigments intact, all steps of the isolation procedure were performed under dim light or in darkness, shielding the vessels using aluminum foil where possible. Cyanobacterial cells from 3 L cultures in a stationary phase, typically 21 days after inoculation (OD_{750}=0.5-0.8), were harvested by centrifugation (9000×*g*, 5 min). The supernantant was discarded and the cell pellet was sonicated on ice for 10 min, with the power set at 70% using a Vibracell VCX 750 sonicator (Sonics & Materials Inc., Newtown, CT), in approximately 200 mL of an extraction mixture of acetone:methanol 7:2 (v:v) (Takaichi *et al.*, 2005) added in 3 aliquots of equal volume. The resulting pigment extract was evaporated to dryness on a rotary vacuum evaporator (Rotavapor, Büchi Labortechnik AG, Flawil, Switzerland) in the dark, at 30° C. The dry extract was redissolved in a mixture of petroleum ether (boiling point 40–60°C) and acetone in an 8:2 ratio (v:v). This solution was then poured into an adsorption chromatography column, filled with (meta)silicic acid (80 μm mesh,

Mallinckrodt Pharmaceuticals, St. Louis, MO) in a volume of 300 mL, equilibrated with the same solvent mixture and isocratically eluted using a solvent of the same composition. The fraction containing carotenoid glycosides was separated from other pigments (chlorophyll, other carotenoids), collected, reconcentrated under vacuum to dryness and stored in darkness, under nitrogen, at –30°C until use.

Liquid chromatography and LC/MS

The composition of the myxoxanthophyll fraction was examined by HPLC/DAD using a Jasco PU-4180 chromatography system equipped with a Jasco MD-410 diode array detector (Jasco Co., Tokyo, Japan) and a Zorbax SB-C18 column (dimensions 4.6×150 mm; porosity 3.5 μm; Agilent Technologies, Santa Clara, CA). A test sample (100 μL) of the myxoxanthophyll fraction was dissolved directly in an eluent composed of acetonitrile, methanol and water in ratios 72:8:1 (v:v:v). Chromatographic separation was performed according to Myśliwa-Kurdziel and others (Myśliwa-Kurdziel *et al.,* 2012) under the following conditions: flow rate – 0.7 mL/min increasing to 1.4 ml/min in the first 16.5 min. The absorption spectra of the eluate (300–600nm) were recorded every 0.2 s. LC/MS was performed with a 4000 Q-TRAP mass spectrometer (Applied Biosystems Inc., Waltham, MA), accompanied by an ESI ionization unit and a liquid chromatograph (Shimadzu Co., Kyoto, Japan), equipped with a 100 mm C-18 column.

Spectrophotometry

All spectral measurements were made with a V650 spectrophotometer (Jasco Co., Tokyo, Japan) using Hellma Analytics (Müllheim, Germany) quartz cuvettes (700 μl, 1 cm optical path). The scan speed was set to 100 nm/min with a resolution of 1 nm and a 1 nm UV/Vis band width setting.

Chemical structures and models

The structures of the compounds were drawn using ACD/ChemSketch (Freeware) 2019.2.0 by Advanced Chemistry Development, Inc. (Toronto, Canada). The 3D molecular models were generated using Avogadro, an open source molecular builder and visualization tool, version 1.2.0. http://avogadro.cc (Hanwell *et al.*, 2012).

RESULTS AND DISCUSSION

The acetone:methanol extract from *Anabaena* 7120 cells contained all the pigments previously identified in this cyanobacterium (Takaichi *et al.,* 2005; Kłodawska *et al.*, 2019). The separation method based on adsorption chromatography with (meta)silicic acid as a stationary phase efficiently separated the fraction of most polar carotenoids from other pigments, including chlorophyll *a* and other xanthophylls (echinenone, canthaxanthin), which were not observed on the HPLC/DAD chromatogram of this fraction (Fig. 1A). The HPLC/DAD chromatogram at 470 nm (Fig. 1A) shows one major peak (retention time 5.05 min; 62.43% of the total chromatogram area) and four minor peaks (retention times 1.58, 5.71, 6.22, and 7.12 min; 2.67%, 13.24%, 12.75% and 3.50% of the total peak area, respectively). The absorption spectra of the separated compounds are shown in Fig. 1B. They are very similar to each other in the visual range. In particular, substance 1, with a retention time of RT=5.05 min, has an absorption maximum $(\lambda)_{max}$=480 nm; substance 2, with retention time RT=1.58 min, $(\lambda)_{max}$=450 nm; substance 3, with retention time RT=5.71 min, $(\lambda)_{max}$=476 nm; substance 4, with retention time RT=6.22 min; $(\lambda)_{max}$=475 nm; substance 5, with retention time RT=7.12 min; $(\lambda)_{max}$=470 nm. These absorption spectra correspond to the absorption spectra of 4-keto-myxoxanthophyll (479 nm) and myxoxanthophyll (473 nm), known from the literature (Mochimaru *et al.,* 2005; Takaichi *et al.,* 2005). The observed differences of 1 nm could be attributed either to instrumental disparity or to the effect of the solvent used. Thus, for all detected peaks, the optical properties in the visible region are consistent with the absorption spectra of 4-ketomyxoxanthophyll and myxoxanthophyll.

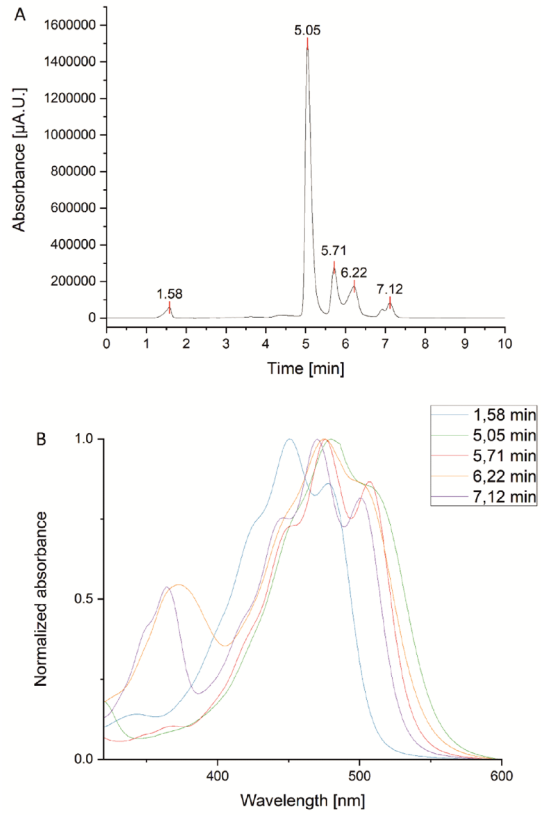

Figure 1. Analysis of cartotenoid glycosides fraction of *Anabaena* 7120
(**A**) HPLC/DAD chromatogram at 470 nm, retention times of individual substances are marked; (**B**) Absorption spectra corresponding to the separated substances; more detailed description in the text.

Further analysis by LC/MS allowed us to confirm the presence of a total of 9 substances in the fraction studied, including 6 substances with an ion mass spectrum corresponding to 4-ketomyxoxanthophyll and 3 substances corresponding to myxoxanthophyll (Fig. 2). In fact, the observed mass-to-charge ratios for the parent ion of m/z=744 and 730, corresponded to the predicted molecular weights of 4-ketomyxoxanthophyll and myxoxanthophyll, respectively. A summary of the retention times of the carotenoid glycosides from *Anabaena* 7120 based on the LC/MS chromatogram at 450 nm (Fig. 2A) along with the corresponding m/z of selected ions determined by mass spectrometry and compound identification, is presented in Table 1. On the basis of the predicted molecular mass, the compounds 1,3,4,5,6,7 could be assigned as 4-ketomyxoxanthophyll and com-

Table 1. Summary of retention times of compounds present in the myxoxanthophyll fraction from *Anabaena* 7120 based on LC chromatogram along with corresponding selected ions determined by mass spectrometry with the identification of the compound.

Compound No.	Retention time [min]	Identifed compound	Ions [*m/z*]						% of content
1	10.32	4-keto myxoxanthophyll	744.46	581.4	563.39	523.35			54.8
2	11.42	Myxoxanthophyll	730.48	583.41	567.42	549.41			14.56
3	13.03	4-keto myxoxanthophyll	744.46	730.48	581.4	563.29	539.39	313.27	0.78
4	14.59	4-keto myxoxanthophyll	744.46	730.48	581.4	563.29	539.39	313.29	0.94
5	14.85	4-keto myxoxanthophyll	744.46	581.4	563.39	523.35			1.68
6	15.20	4-keto myxoxanthophyll	744.46	581.4	563.39	523.35			9.25
7	15.35	4-keto myxoxanthophyll	744.46	581.4	563.39	523.35			9.42
8	16.06	Myxoxanthophyll	730.48	-	567.42	539.29		313.27	1.55
9	16.61	Myxoxanthophyll	730.48	-	567.42	549.41			7.01

pounds 2,8,9 as myxoxanthophyll. The observed mass spectra of 4-ketomyxoxanthophyll and myxoxanthophyll are presented in Figs. 2B and C, respectively. Thus, both HPLC/DAD and LC/MS analysis shows the presence of only myxoxanthophylls and their derivatives in the fraction of polar carotenoids as isolated by adsorption on (meta)silicic acid. The absence of any other substances indicates the high purity of the myxoxanthophyll fraction isolated from *Anabaena* 7120 biomass *via* the method used in this study. Taking into account the fact that compounds 3,4,5 and 8 are present in the fraction at low concentrations, i.e., below 2% of total peak area based on a chromatogram at 450 nm, one can conclude that compounds 1,2, 6,7 and 9 correspond to the chemical individuals initially identified using the HPLC/DAD method.

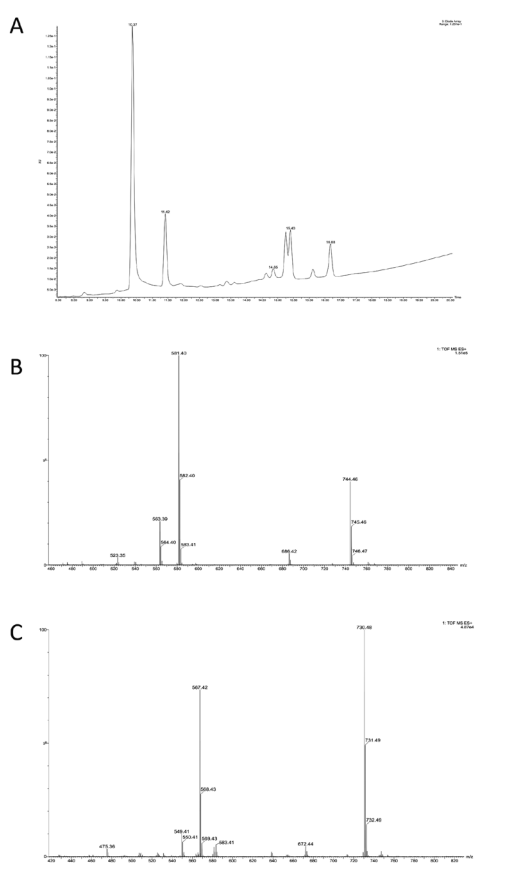

Figure 2. LC/MS analysis of *Anabaena* 7120 myxoxanthophyll fraction; chromatogram at 450 nm (A) and mass spectra (ESI, positive ions) of peaks with retention times of 10.32 min – ketomyxanthophyll (B) and 11.42 min – myxanthophyll (C)

In addition to the occurrence of several compounds of the same molecular weight, the existence of *cis* isomers in the fraction of polar carotenoids from *Anabaena* 7120 is suggested by the presence of the so-called *cis* band on the absorption spectra (Melendez-Martinez *et al.,* 2013). In particular, peaks with retention times of 6.22 min and 7.12 min are accompanied by absorption spectra with bands of $(\lambda)_{max}$=364 nm and $(\lambda)_{max}$=372 nm respectively. Unlike carotenoid *trans* isomers, *cis* isomers have an additional band in the near ultraviolet spectral region (near 360 nm), making it specific for them (Zechmeister, 1962; Xiao *et al.*, 2018). Therefore, the presence of an additional absorption band with a maximum in near UV suggests that the compounds eluted in peaks with retention times of 6.22 min and 7.12 min may correspond to *cis*-isomeric forms of myxoxanthophylls. A summary of the main absorption maxima of the compounds present in the myxoxanthophyll fraction of *Anabaena* 7120, as detected using the HPLC/DAD method, is shown in Table 2.

Further evidence supporting the notion that some of compounds identified are, in fact, *cis* forms of myxoxanthopylls, is the shift of the absorption maxima of *cis* isomers towards ultraviolet by 4–6 nm for mono-*cis* isomers and 8–12 nm for di-*cis* isomers (Xiao *et al.*, 2018). The substance with an RT of 1.58 min is probably an oxidized product of decomposition because it is characterized by the large spectral shift of 20–30 nm of $(\lambda)_{max}$ and it has a much shorter retention time, unlike the other substances. The four remaining peaks can be divided into two pairs based on their Vis maxima: peaks with RT=5.05 min and RT=6.22 min show $(\lambda)_{max}$=480 nm and 475 nm, respectively. On the contrary, the peaks with RT=5.71 min and RT=7.12 min show $(\lambda)_{max}$=476 nm and 470 nm respectively. Similar spectral shifts of absorption maxima are observed in both of these pairs: 5 nm for the first one and 6 nm for the other pair of peaks. The later peaks in each pair show the absorption band in the near-UV region. This band is not observed in peaks with shorter RT. These results suggest that the pool of

Table 2. Summary of retention times and main absorption maxima of compounds present in the myxoxanthophyll fraction from *Anabaena* 7120 as detected by HPLC/DAD method.

Compound No.	Retention time [min]	Absorption maximum (Vis) [nm]	Absorption maximum (UV) [nm]
1	1.58	450	342
2	5.05	480	n.d.
3	5.71	476	n.d.
4	6.22	475	364
5	7.12	470	372

myxoxanthopylls consists of several forms that differ in terms of their optical properties. In particular, on the basis of the differences in the retention times detected, which correspond to those previously described by other authors (Takaichi *et al.*, 2005), the first pair can be deduced to be all-*trans* 4-ketomyxoxanthophyll and its *cis* isomers and the second one to be all-*trans* myxoxanthophyll and its *cis* isomers. The fact that LC/MS analysis showed the presence of more compounds can be attributed to the circumstance that these presumed *cis* isomers were not well separated in the HPLC/DAD analysis and appeared as one peak. In addition, it is difficult to determine which bonds can be isomerized, especially for compounds with a structure distinct from that of classical carotenoids. Given shift values of 5 nm, and 6 nm, it can be assumed that mono-*cis* isomers are present (Xiao *et al.*, 2018). The exact identity of individual isomers requires further investigation using X-ray crystallography and/or nuclear magnetic resonance spectrometry.

To analyze the effect of isomerization on the spatial structure of carotenoid glycosides, 3-D models of all-*trans* myxoxanthophyll and several monocis myxoxanthophylls were generated using molecular modeling algorithms as implemented in the chemical editor Avogadro (Hanwell, 2012). A single isomerization on bonds 9, 13 and 15 – localized in the center of the myxoxantophyll molecule was adopted in order to visualize the putative structural differences between all-*trans* and *cis* isomers better. The structures of 15-*cis* carotenoids occur in nature, an example of which is 15-*cis*-phytoene, an intermediate product in carotenoid biosynthesis (Giuliano *et al.*, 2002). Another example is the presence of 15-*cis*-γ-carotene and 15-*cis*-chlorobactene in the reaction centers of the green sulfur bacterium *Chlorobium tepidum* (Bialek-Bylka *et al.*, 1998). Additionally, 15-*cis*-β-carotene can be found in the reaction centers of the photosystem I of the cyanobacterium *Synechococcus vulcanus* (Bialek-Bylka *et al.*, 1998) and in both photosystem I and II of spinach (Bialek-Bylka *et al.*, 1995; Bialek-Bylka *et al.*, 1996). The results of 3-D modeling are presented in Fig. 3. As can be seen, the isomerization on the bond at positions 9,13 and 15 generates remarkable alterations in the overall shape of the myxoxanthophyll molecule. In particular, the all-*trans* form has a linear structure, resembling a rigid rod with polar poles at its ends. On the contrary, *cis* forms have a more pincer-like shape, where the polar and hydrophilic regions located at the poles are closer together, while the hydrophobic, hydrocarbon center of the terpenoid chain is exposed in the direction opposite to the myxol and sugar moieties. The position of the *cis* bond affects both the length of the polyene chain and the angle between its polar regions. The values of distance and the angle measured between carbons C7 and C2' of the main polyene chain, as predicted for geometrical isomers, are presented in Table 3. These structural modifications result in changes in the size and shape of myxoxanthophyll molecules. In particular, the predicted total length of the all-*trans* isomer molecule is about 3.3 nm, whereas the 15-*cis* isomer is about 2.2 nm long as measured along the long axis. As a consequence, this mutual orientation of the molecule fragments may cause stronger adsorption of the *cis* isomers to the stationary phase during chromatographic separation due to the higher polarization in the perpendicular plane. This effect may explain the longer

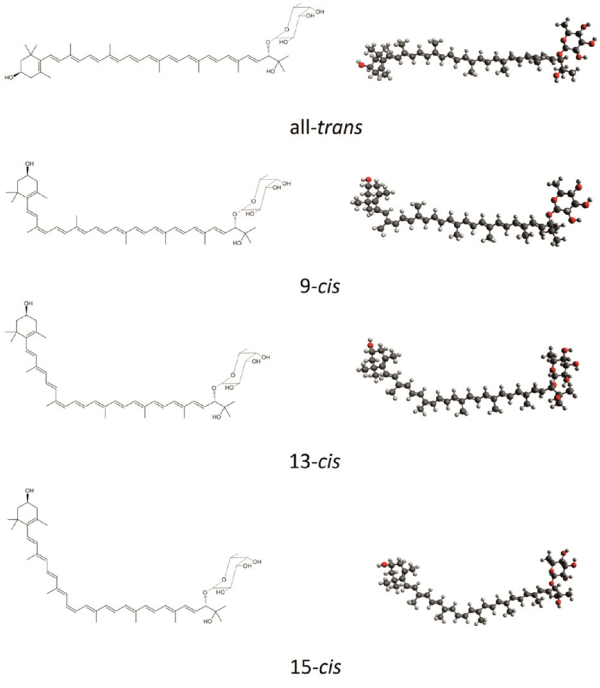

Figure 3. Structural formulas (left) and Avogadro model predictions (right) of all-*trans*-myxoxanthophyll and 9, 13,15-*cis*-myxoxanthophylls.
Note the change in spatial shape from the "rod" of molecule (all-*trans* isomer) to the "pincers" of molecule (15-*cis* isomer).

Table 3. Values of distance and angle measured between carbons C7 and C2' of the main polyene chain of computer-generated 3D model of myxoxanthophyll.
For angle measurement C15' was used as a third point of measurement. Structure generation and measurements were made in Avogadro.

Isomer	Distance [nm]	Angle [°]
all-*trans*	2.5846	3.3
9-*cis*	2.4931	8.5
13-*cis*	2.4280	13.0
15-*cis*	2.3988	19.0

retention times observed in HPLC for molecules identified as *cis* isomers.

The isomerization process may occur prior to cyclization, especially since the biosynthesis of myxoxanthophyll from lycopene, which is the base molecule for the synthesis of all cyanobacterial xanthophylls, is not well understood (Takaichi & Mochimaru, 2007; Sugiyama & Takaichi, 2020). Then the *cis* forms will be precursors of the ultimate all-*trans* form. Otherwise, it may also occur by conversion of the final all-*trans* form of myxoxanthophyll and its derivatives. Then the *cis* forms will be the initial products of the *trans* form degradation (Xiao *et al.*, 2018). Degradation processes can occur either naturally in cells or artificially when compounds are isolated from biological material. These processes can be enhanced by various abiotic factors, including high temperature, intensive light, and contact with oxygen and/or acidic environment (Zechmeister, 1962; Xiao *et al.*, 2018). It is worth noting that the weak acidic conditions that may have been created by contact with the stationary phase during preparative LC chromatography ((meta)silicic acid) have previously been shown to be unfavorable for the purification of carotenoid epoxides (Britton and Young, 1993). The lack of epoxy groups in myxoxanthophyll structures and the short contact time of the carotenoid glycoside fraction with the LC stationary phase make its chemical modification unlikely during purification under our experimental conditions.

In cyanobacteria subjected to a low temperature or phosphatidylglycerol depletion, increased myxoxanthophyll content was detected in the fraction of thylakoid membranes (Varkonyi *et al.*, 2002; Domonkos *et al.*, 2009). In higher plants, the violaxanthin cycle coordinates the protection of photosynthetic apparatus through zeaxanthin (Demmig-Adams 1990; Latowski *et al.*, 2004). As this mechanism is not present in cyanobacteria, and carotenoids are known to affect the structure of photosynthetic membranes (Gruszecki & Strzałka, 2005), we propose that specific myxoxanthophyll forms, including their *cis* isomers, might be involved in the stabilization of photosynthetic membranes and in this way contribute to the acclimation of cyanobacteria to environmental conditions.

CONCLUSIONS

In this study, based on adsorption chromatography on (meta)silicic acid, we isolated a fraction containing polar carotenoids from the model cyanobacteria *Anabaena* 7120. Subsequent analysis by HPLC/DAD and LC/MS revealed that this fraction represents a mixture of compounds that could be identified as myxoxanthophylls and 4-ketomyxanthophylls on the basis of their optical properties and predicted molecular weights. The differences in their absorption spectra, namely the presence of remarkable bands in the near-UV region, leads us to the conclusion that at least two of the identified compounds may represent *cis* isomers of myxoxanthophylls. Putative structures of these *cis* forms are proposed. Thus, the cellular pool of carotenoid glycosides in *Anabaena* 7120 contains both *trans* and *cis* derivatives. The biological significance of the *cis* isomers of carotenoid glycosides in cyanobacteria requires further research.

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Declarations

The authors have no conflicts of interest to declare.

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