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CAROTENOIDS OF *ANACYSTIS NIDULANS*, STRUCTURES OF CALOXANTHIN AND NOSTOXANTHIN*

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Key Word Index—*Anacystis nidulans*; Cyanophyta; blue–green algae; carotenoids; caloxanthin, nostoxanthin; 2*R*,3*R*,3′*R*-β,β-carotene-2,3,3′-triol; 2*R*,3*R*,2′*R*,3′*R*-β,β-carotene-2,3,2′,3′tetrol; absolute configuration; biosynthetic consideration

Abstract—Reinvestigation of the carotenoids of *Anacystis nidulans* has confirmed the occurrence of β,β-carotene (β-carotene), β,β-caroten-3-ol (cryptoxanthin), β,β-carotene-3,3′-diol (zeaxanthin) and 2*R*,3*R*,3′*R*-β,β-carotene-2,3,3′-triol (absolute configuration assigned in the present work). In addition the previously unknown 2*R*,3*R*,2′*R*,3′*R*-β,β-carotene-2,3,2′,3′-tetrol has been isolated. The triol and the tetrol are considered identical with caloxanthin and nostoxanthin, respectively, for which allenic structures have been suggested by others. The chirality of these compounds followed from CD and ^{1}H NMR considerations.

INTRODUCTION

Early examinations of the carotenoids of *Anacystis nidulans*, reviewed elsewhere [1], revealed the presence of β-carotene (**1**), zeaxanthin (**3**) and unidentified xanthophylls. Later Stransky and Hager [2, 3] designated the two most polar xanthophylls, also encountered in *Synechococcus elongatus*, *Nostoc commune* and *Calothrix parietina*, caloxanthin and nostoxanthin. Allenic structures for caloxanthin (**6**) and nostoxanthin (**7**) were assigned on the basis of higher relative polarity than zeaxanthin (**3**), formation of diacetates upon acetylation, allene absorption in IR and formation of zeaxanthin (**3**) on treatment with $LiAlH_4$. However, as already pointed out [4] the structures **6** and **7** are not compatible with the same visible light absorption as for zeaxanthin (**3**).

Pinewitsch and Wasiljeva [1] reported the presence in *A. nidulans* of β-carotene (**1**) and six xanthophylls identified as β-cryptoxanthin (**2**), zeaxanthin (**3**), an ester of zeaxanthin, an unknown carotenoid, caloxanthin and nostoxanthin. However, no allene absorption around 1930 cm^{-1} could be confirmed for caloxanthin and nostoxanthin. From strong absorption at 1130 cm^{-1} they claimed the presence of ether groups. Recently, Smallidge and Quackenbush [5] identified a polar xanthophyll in *A. nidulans* as β,β-carotene-2,3,3′-triol (**4**) without stereochemical assignments.

In light of previous conflicting evidence a reinvestigation of the carotenoids of *A. nidulans* was undertaken. The chiroptical properties of the triol **4** were of particular interest for stereochemical assignment.

RESULTS AND DISCUSSION

In the present work the following distribution pattern was established after saponification of the crude pigment extract: β-Carotene (**1**), 28% of total; β-Cryptoxanthin (**2**), 2%; Zeaxanthin (**3**), 37%; β,β-Carotene-2,3,3′-triol (**4**, caloxanthin) 22%; and β,β-Carotene-2,2′,3,3′-tetrol (**5**, nostoxanthin) 11%. This analysis includes all carotenoids present in amounts higher than 1% of total. Identical chromatographic properties (TLC) of the individual carotenoids before and after saponification revealed the absence of esterified xanthophylls.

The chromatographic pattern observed and the relative quantities found are almost identical to those reported earlier [1, 2]. Identity of caloxanthin and nostoxanthin with our two most polar carotenoids follows from relative polarity and abundance as well as electronic spectra.

β-Carotene (**1**), β-cryptoxanthin (**2**) and zeaxanthin (**3**) were identified from chromatographic and spectral properties in direct comparison with authentic compounds. CD spectra of **2** and **3** *ex A. nidulans* corresponded to that of zeaxanthin (**3**) [6], confirming 3*R* and 3*R*,3′*R*-configuration, respectively [7, 8].

Structure **5** for the most polar xanthophyll (nostoxanthin) is assigned from the following data. β-Carotene-type chromophore is supported by the visible spectrum. The MS revealed a molecular weight of 600, compatible with $C_{40}H_{56}O_4$. The presence of four primary or secondary hydroxy groups is consistent with the formation of a tetraacetate. Only common 92 and 106 mass unit losses from the polyene chain on electron impact disfavour the presence of hydroxymethyl substituents on the chain [9], and general absence of primary hydroxy functions follows from the ^{1}H NMR spectrum. Location of the four hydroxy groups in secondary positions in two β-rings by a (2 + 2) rather than a (3 + 1) distribution pattern follows from the ^{1}H NMR evidence available for 2-, 3- and 4-monohydroxylated β-ring carotenoids (Scheme 1, including β,β-carotene-2,2′-diol (**8**), β,β-carotene-3,3′-diol (**3**) and β,β-carotene-4,4′-diol (**9**) with ^{1}H NMR methyl

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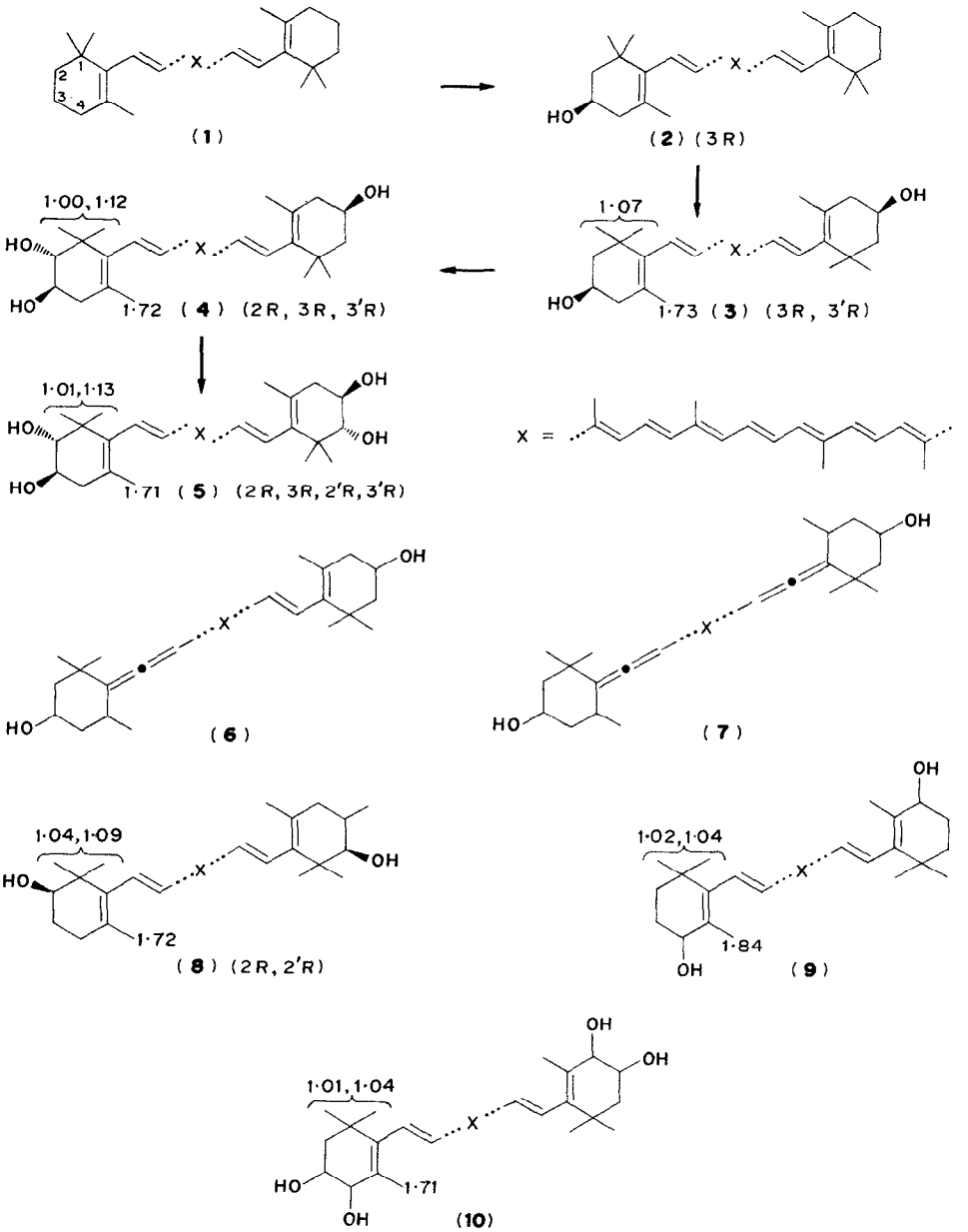
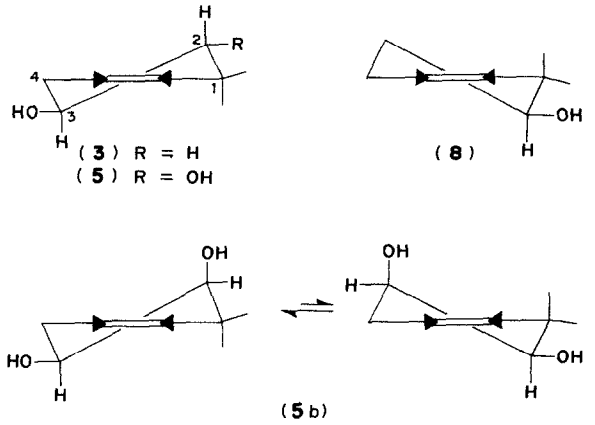


Scheme 1. Interrelations of hydroxycarotenoids.



Scheme 2. Conformations of hydroxycarotenoids.

assignments) [10, 11]. *Gem* dimethyl signals at δ 1.13 (6H) and δ 1.01 (6H) for nostoxanthin can only be accommodated with two hydroxy groups in each of two identical β-rings. Either 2,3- or 3,4-α-glycol arrangement follows from a two proton doublet at δ 3.32 (J 10.5 Hz), revealing a *trans* diaxial coupling for the C-2(2') or C-4(4') protons.

Crustaxanthin (**10**) is claimed to be β,β-carotene-3,4,3',4'-tetrol [12] with the adjacent hydroxy groups *trans* [13]. The corresponding *cis* tetrol is reported to undergo rapid dehydration with HCl–$CHCl_3$ [14] in contrast to the 3,4,3',4'-di-*trans*-tetrol [13]. Nostoxanthin suffered no elimination under conditions where β,β-caroten-4-ol (isocryptoxanthin) was smoothly dehydrated. However, the non-identity of nostoxanthin with crustaxanthin follows from the *gem* dimethyl signals in the ^{1}H NMR spectrum which in the case of 3,4-diol substitution occur at δ 1.01 and

1.04 [15] compared with δ 1.01 and 1.13 for nostoxanthin with consequent 2,3-diol substitution. Significant differences in IR are also noted; crustaxanthin exhibiting strong absorption at 1030 cm^{-1} for allylic hydroxyl [12], which is absent for nostoxanthin (**5**).

The chiroptical properties of nostoxanthin allowed assignment of absolute configuration by the conformational rule [11, 6]. The CD of nostoxanthin (**5**) and its tetraacetate were almost identical in shape, sign and magnitude with that of 3*R*,3′*R*-β,β-carotene-diol (**3**, zeaxanthin). Conformational analysis [6], confirmed by ^{13}C NMR data [16], reveals that in zeaxanthin (**3**) the hydroxy groups prefer equatorial positions. Hence the chirality at C-3(3′) determines the helicity of the cyclohexene half-chair, responsible for the strong Cotton effect [11,6]. It is known that 3*R*,3′*R*-β,β-carotene-3,3′-diol (**3**) and 2*R*,2′*R*-β,β-carotene-2,2′-diol (**8**) [11, 17], with opposite preferred helicity [11] indeed give opposite Cotton effects (Scheme 2). Introduction of a *trans* substituent in 2-position in zeaxanthin (**3**) to give **5** would stabilize its preferred conformation. Also *cis* substitution providing **5b** would result in a preference of the same conformation in order to avoid serious diaxial 1:3 hydroxy:methyl interaction. The CD consequently provides no conclusive information about the relative configuration. However, *trans* diol substitution follows from the ^1H NMR data already discussed and 2*R*,3*R*,2′*R*,3′*R*-chirality for **5** is concluded. This is the first report of a 2,3,2′,3′-β,β-carotene-tetrol.

β,β-Carotene-2,3,3′-triol (**4**), also previously isolated and identified (including IR and MS) from *A. nidulans* [5], is considered identical with caloxanthin. Previous valid arguments in favour of the hydroxylation pattern rested on acetonide formation, typical of an α-glycol, and no allylic dehydration with HCl-$CHCl_3$. Additional evidence is presented here including ^1H NMR (see Experimental part and Scheme 1) and formation of a triacetate.

^1H NMR revealed the 2,3-*trans* relationship of the diol ring by *trans* diaxial coupling of the 2,3 protons (*J* 10.5 Hz). The CD spectrum, closely similar to that of zeaxanthin (**3**) supports by the same arguments used for nostoxanthin (**5**) that the absolute configuration for caloxanthin (**4**) is 2*R*,3*R*,3′*R*.

Cis glycols are known to undergo facile acetonide formation [18]. The formation of an acetonide in unreported yield [5] from the *trans* glycol **4** is, however, not without precedence [19]. The trihydroxy-β-carotene previously reported from *Phormidium* spp. [20, 21] may be identical with **4**.

The opposite configuration at C-2 for caloxanthin (**4**) and at C-2,2′ for nostoxanthin (**5**) relative to β,β-carotene-2,2′-diol (**8**) from the green algae *Trentepohlia iolithus* may reflect different biosynthetic pathways. In *A. nidulans* it appears likely that the introduction of the hydroxy groups in 2,2′ succeeds that of the hydroxy groups in 3,3′ since the 3,3′-diol and no 2,2′-diol was isolated, see Scheme 1. The existing C-3 hydroxy group may direct the introduction of the neighbouring hydroxy group in *trans* dieq. position. The possibility of 2*R*,2′*R*-β,β-carotene-2,2′-diol (**8**) being formed by cyclization of an epoxidic precursor has been discussed previously [11].

EXPERIMENTAL

Biological material. Anacystis nidulans (Dept. Biology Brookhaven National Laboratory) were grown at 25° in 180 l. polyethylene drums as previously described [4]. *Materials and methods* were as usually employed in the Norwegian laboratory and are summarized elsewhere [22, 23]. CD spectra were measured in EPA (Et_2O-isopentane-EtOH 5:5:2) on a Roussel Jouane dichrograph.

Isolation. 14.73 g freeze dried algae in 50 ml Me_2CO–MeOH (1:1) were ground in a mortar, the solvent removed by decantation and the procedure repeated until no further pigment could be extracted. After filtration and evaporation of the solvent, saponification of the combined extracts using Et_2O and 10% MeOH–KOH (1:1) for 2 hr at room temp. gave 54.8 mg residue containing 13.7 mg carotenoids (E°_{1cm} = 2500). Separation of pigments was carried out on TLC (Si gel) with 35% Me_2CO in hexane (AHE).

β,β-Carotene (**1**), *3R-β,β-caroten-3-ol* (**2**) *and 3R,3′R-β,β-carotene-3,3′-diol* (**3**). β-Carotene (**1**, 3.46 mg), β-cryptoxanthin (**2**, 0.25 mg) and zeaxanthin (**3**, 4.56 mg) were identified by their R_f values on Whatman (SG 81) circular kieselguhr—and Schleicher & Schüll (No. 288) alumina-filled papers, visible light and MS. Identity was confirmed by co-chromatography with authentic samples. On co-chromatography with authentic β,β-caroten-4-ol, β,β-carotene-2,2′-diol (**9**) and β,β-carotene-4,4′-diol the absence of these structural isomers were demonstrated. CD spectra of **2** and **3** were consistent with that of authentic zeaxanthin (**3**) [6].

2R,3R,3′R-β,β-Carotene-2,3,3′-triol (**4**). **4**, 2.72 mg had R_f (SG 81, 30% AHE) 0.54, λ_{max} (Me_2CO) 480.5, 453.5, (430) nm, ν_{max} (KBr) 3400 *m*, 3030 *w*, 2960 *s*, 2930 *s*, 2860 *s*, 1465 *m*, 1440 *m*, 1380 *w*, 1180 *w*, 1120 *m*, 1075 *w*, 1055 *m*, 970 *m* cm^{-1}; ^1H NMR δ (100 MHz, $CDCl_3$) 1.00 *s* and 1.12 *s* (Me-16,17), 1.07 *s* (Me-16′,17′), 1.72 *s* (Me-18,18′), 1.97 *s* (Me-19,19′,20,20′), 3.31 *d J* 10.5 Hz (H-2), *ca* 4.0 *m* (H-3,3′), 6.10 *s* and 6.12 *s* (H-7,7′,8,8′), 6.15–6.9 (H olefinic); MS, *m/e* 584 (100%, M), 568 (10%, M-16), 492 (13%, M-92), 478 (3%, M-106), 133 (24%), 91 (43%), 83 (22%), 69 (42%); CD (EPA) λ ($\Delta\epsilon$) 337 (+4.6), 312 (0), 283 (−16), 260 (0), 244 (+8.8), 231 (0), 222 (−10.4), 212 (0) nm. Acetylation gave a triacetate (*m/e* 710 (M), M-42, M-60, M-92, M-106) with R_f (SG 81; 10% AHE) 0.62 and the same CD properties as the triol. After treatment of **4** with 0.02 N HCl in $CHCl_3$ no change of chromatographical behaviour or vis. spectrum was observed in contrast to parallel reaction with β,β-caroten-4-ol.

*2R,3R,2′R,3′R-β,β-*Carotene-2,3,2′,3′-tetrol (**5**). **5**, available 1.34 mg, had R_f (SG 81, 30% AHE) 0.36; λ_{max} (acetone) 480.5, 453, (431) nm; ν_{max} (KBr) 3390 *m*, 3030 *w*, 2960 *s*, 2930 *s*, 2860 *s*, 1465 *m*, 1445 *m*, 1400 *w*, 1380 *m*, 1365 *m*, 1265 *w*, 1175 *w*, 1125 *w*, 1075 *w*, 1050 *m*, 970 *s* cm^{-1}; ^1H NMR δ (100 MHz, $CDCl_3$) 1.01 *s* and 1.13 *s* (Me-16,16′,17,17′), 1.71 *s* (Me-18,18′), 1.97 *s* (Me-19,19′,20,20′), 3.32 *d J* 10.5 Hz (H-2,2′), *ca* 3.8 (H-3,3′), 6.10 *s* (H-7,7′,8,8′), 6.15–6.9 (H olefinic); MS, *m/e* 600 (100%, M), 584 (31%, M-16), 568 (2%, M-16-16), 508 (11%, M-92), 494 (2%, M-106), 492 (2%, M-16-92), 133 (22%), 91 (33%), 69 (25%); CD (EPA) λ ($\Delta\epsilon$) 340 (+3.5), 313 (0), 282 (−16.7), 261 (0), 245 (+8.8), 232 (0), 220 (−11.9), 208 (0) nm. Acetylation gave a tetraacetate (*m/e* 768 (M), M-42, M-60, M-92, M-106) with R_f (SG 81; 10% AHE) 0.51 and the same CD properties as the tetrol. Treatment of **5** with 0.02 N HCl had no influence on the chromatographic behavior or the vis. spectrum.

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