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STEROLS AND TRITERPENOIDS FROM THE CYANOBACTERIUM *ANABAENA HALLENSIS*

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Key Word Index—*Anabaena hallensis*; cyanobacterium; sterols; triterpenoids.

Abstract—The sterol pattern of the cyanobacterium *Anabaena hallensis* has been investigated. Several sterols and triterpenes were found for the first time in the genus *Anabaena*.

INTRODUCTION

Blue-green algae (cyanobacteria) constitute a large group of oxygenic photoautotrophic procaryotes [1]. Due to their ability to survive and emerge in changing and stressful habitats, they are dominant autotrophs in many ecosystems. Up to today it was known that sterols occur in almost all algae. The discovery of sterols in cyanobacteria was made in 1968 for *Anacystis nidulans* and *Fremyella diplosiphon* where only sitosterol and cholesterol were found [2], but the occurrence of other sterols in *Formidium* has been demonstrated [3]. More than 17 sterols were characterized for three nitrogen fixing cyanobacterial genera *Anabaena*, *Nostoc* and *Nodularia* [4]. Thirteen sterols were identified recently in *Spirulina maxima* [5]. Evidently, sterols occur in cyanobacteria only in minute amounts (below 0.03% of cell dry weight). Therefore, identification was achieved by GC–mass spectrometry and GLC. However, triterpenoids of the hopane family, mainly C_{35} hopanepolyols, are present in several cyanobacteria strains at a level of up to 2 mg g^{-1} dry weight, allowing structural elucidation by ^1H and ^{13}C NMR [6, 7]. Consequently, to isolate hopane derivatives, amounts of biomass were used for extraction, which were insufficient for the detection of sterols [6, 7]. The cyanobacteria contain both saturated and unsaturated sterols [4]. The typical unsaturated sterols were as follows: cholesterol, isofucosterol, chondrilasterol, stigmasterol, brassicasterol, campesterol, 22-dehydrocholesterol, 24-methylcholest-7-en-3β-ol, 24-ethylcholesta-2,5-dien-3β-ol, 24-ethylcholest-7-en-3β-ol and 24-ethylcholesta-5,7 22-trien-3β-ol. Within several cyanobacteria up to 10 sterols of these types were found. In some strains such as *Anabaena cylindrica*, *A. viguieri*, *A. solitaria*, *Nostoc carneum*, *Nodularia harveyana* and *Microcystis aeruginosa* [4, 8, 9] the following saturated sterols were identified: 5α-cholestan-3β-ol, 24-methyl-5α-cholestan-3β-ol and 24-ethyl-5α-cholestan-3β-ol. Despite the fact that the blue-green algae are used as a source for nitrogen and phytohormones in agricultural crop production [10, 11], the nature of the algal promoting substances has not been studied. We expected that cyanobacteria may contain some sterols related to the precursors of brassinosteroids, a new class of plant hormones, as in the case of green algae [12].

We report here the sterol constituents of a nitrogen-fixing cyanobacterium *A. hallensis* isolated from paddy soils of northern Vietnam.

RESULTS AND DISCUSSION

The cyanobacterium *Anabaena hallensis* (family Anabaenaceae) used here was isolated from an algal matter of *Aphanothece*, an abundant association in paddy soils of North Vietnam [13]. From this material at least six other cyanobacterial strains were recently purified (Rippka, R., personal communication). Although the unicellular strain *Aphanothece pallida* is the major constituent of this algal matter, in our hands only *A. hallensis* grew in suspension state under batch culture conditions with a continuous stream of air–CO_2 (19:1) and possessed a large growth index. Bacteriologically pure culture was obtained by discrete UV radiation plus antibiotic treatment and a selection procedure as described before [13, 14]. This procedure was repeated weekly to prevent any contamination.

The cells of *A. hallensis* were extracted as described below. The 80% methanol fraction, probably containing polyhydroxyhopanes, was not further studied. The *n*-hexane fraction (2.5 g) was subjected to silica gel chromatography. The elution was carried out sequentially using mixtures of increasing polarity, first *n*-hexane–methylene chloride and then methylene chloride–methanol. From the fractions eluting with *n*-hexane–methylene chloride (1:3) and methylene

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chloride, two sterol zones were obtained with R_f 0.22 and 0.32 by silica gel TLC in chloroform–methanol (99:1). These sterol fractions were analysed by GC–mass spectrometry of free and acetylated samples. This method does not permit the configuration at C-24 of the 24-alkylsterols to be determined. The commonly occurring sterols in the cells of *A. hallensis* were identified by comparison with authentic samples and cited data [15, 16] as 5α-cholestan-3β-ol, cholest-5-en-3β-ol, 24ξ-methylcholest-5-en-3β-ol, 24ξ-methyl-5α-cholestan-3β-ol, 24ξ-ethylcholesta-5,22-dien-3β-ol, 24ξ-ethylcholest-5-en-3β-ol and 24ξ-ethyl-5α-cholestan-3β-ol. These sterols were identified previously in other nitrogen-fixing cyanobacteria [4, 5]. In addition to these compounds the following were also identified: 3β-hydroxy-24ξ-ethylcholest-5-en-7-one, 3β-hydroxy-24ξ-ethylcholest-5-en-7-one, 3β-hydroxycholest-5-en-7-one, β-amyrin, α-amyrin and 24-methylenecycloartanol. The occurrence of 7-oxosterols and the above triterpenes in cyanobacteria is reported here for the first time. 24ξ-Ethylcholest-5-en-3β-ol, 24ξ-methylcholest-5-en-3β-ol, 24ξ-ethylcholestan-3β-ol, 24-methylenecycloartanol, β-amyrin and α-amyrin occurred in relatively high concentration in *A. hallensis*.

β-Carotene (R_f 0.68, $[M]^+$ at m/z 536) and echinone (R_f 0.38, $[M]^+$ at m/z 550) were eluted with *n*-hexane–methylene chloride (1:1). Palmitic acid was eluted from this column by methylene chloride–methanol (19:1) and further separated by silica gel TLC (R_f 0.12, $[M]^+$ at m/z 256).

EXPERIMENTAL

Culture conditions. Cyanobacterium *A. hallensis* was isolated as described above and stored at the Algal Collection of the State University of Hanoi. The axenic culture was obtained by antibiotic treatment and UV discrete radiation as described before [13, 14]. For biomass production the cyanobacterium was fermented in 6 flasks of 5 l vol. containing 3 l BG-11 medium [17] under continuous illumination (white light, 3000 lux) and gassing with air–CO_2 (19:1) at 100 ml min^{-1} and room temp.

Extraction and purification of sterols. The cells of *A. hallensis* were harvested by centrifugation. The cells (1000 g fr. wt.) were washed once with bidest and disrupted in 4 l MeOH by an Ultra-Turrax (3 × 5 min). The MeOH extract was obtained by centrifugation. The extraction procedure was repeated 3 ×. The aq. phase remaining after concn of the combined filtrates was partitioned between $CHCl_3$–H_2O (1:1), $CHCl_3$ was evapd to dryness, and the residue was partitioned between *n*-hexane and 80% MeOH (1:1). Sepn of the *n*-hexane fr. was performed using a Silica gel 40 column. The elution was performed stepwise starting with *n*-hexane–CH_2Cl_2 (1:1) and followed by (1:3). Then CH_2Cl_2 and CH_2Cl_2–MeOH (19:1) were used as eluents. Sterol isolation was achieved by further prep. TLC with Silica gel 60 using $CHCl_3$–MeOH (99:1) as solvent. The TLC zones which co-chromatographed with the standard compounds campesterol (R_f 0.22) and β-amyrin (R_f 0.32), after CH_2Cl_2 elution, were re-chromatographed using identical conditions. The purified frs were subjected to GC-MS with 5α-cholestane as int. standard.

Acetylation of sterols. This was performed with pyridine–Ac_2O (1:1) containing 1% 4-(dimethylamino)-pyridine at room temp.

GC-MS. GC-MS of the sterols and triterpenoids was performed on an MD 800 (Fisons); EI (70 eV); source temp. 200°; column DB-5 (15 m × 0.32 mm, 0.25 μm film thickness, injector temp. 250°, interface temp. 300°, carrier gas He, flow rate 0.8 ml min^{-1}, splitless injection. Temp. programme (column): 170° for 1 min then elevated to 270° within 25 grd min^{-1}, then raised to 290° at 2 grd min^{-1}.

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