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BIOSYNTHESIS OF THE HETEROCYST GLYCOLIPIDS IN THE CYANOBACTERIUM *ANABAENA CYLINDRICA**

AGATA GAMBACORTA, ANNUNZIATA SORIENTE,† ANTONIO TRINCONE and GUIDO SODANO†‡

Istituto per la Chimica M.I.B., CNR, Via Toiano 6, 80072 Arco Felice (NA), Italy; †Dipartimento di Chimica, Università di Salerno, 84081 Baronissi (SA), Italy

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Key Word Index—*Anabaena cylindrica*; Cyanobacteria; heterocyst glycolipids; biosynthesis.

Abstract—Incorporation of [1-^{13}C] acetate into the heterocyst glycolipids of the cyanobacterium *Anabaena cylindrica* indicated that (i) the long chain of the aglycones originates from a *de novo* biosynthesis and not by elongation of a preformed shorter molecule and that (ii) there is no apparent interconversion between the hydroxyketone and alcohol glucosides.

INTRODUCTION

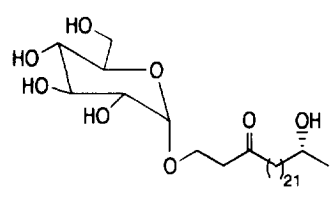
The site of nitrogen fixation in certain filamentous cyanobacteria resides in specialized cells called heterocysts which originate by differentiation of vegetative cells under aerobic conditions and in the absence of nitrogen salts. The heterocysts have a thick envelope containing a laminated layer of glycolipids and an outer homogenous layer of polysaccharide [1]. According to several authors [2, 3], the thick envelope plays a role in the protection of the enzyme nitrogenase from oxygen, restricting air diffusion to a level which allows sufficient nitrogen penetration while maintaining oxygen diffusion to a level which can be removed by respiratory scavenging.

The glycolipids constituting the laminated layer are unique to nitrogen-fixing cyanobacteria [4, 5] and their structures have only recently been fully elucidated in certain cyanobacteria [6, 7], including *Anabaena cylindrica* [8]. From the latter, **1**–**4** were isolated, the major being **3** and **1**. In particular, **3** seemed the most widespread heterocyst glycolipid in cyanobacteria.

The biosynthesis of the heterocyst glycolipids in *A. cylindrica* has also been previously investigated [9–11]. In the previous studies, sodium [1-^{14}C] acetate was used as precursor and the incorporation in the whole compounds was considered, no attempts having been made at localizing the label on the individual carbon atoms. The authors concluded that the activity of key enzymes involved in the biosynthesis of the heterocyst glycolipid fraction is stimulated during heterocyst differentiation and that in mature heterocysts the formation of the characteristic very long chain essentially ceases. Some

*Part 4 in the series 'The Heterocyst Glycolipids of Cyanobacteria'. For previous papers in the series see refs [6–8].

‡Author to whom correspondence should be addressed.



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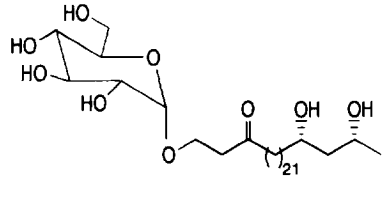

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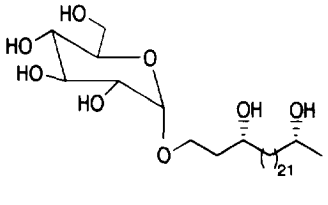

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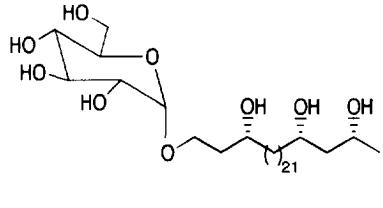

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additional conjectures were heavily affected by the scarce chemical characterization of presumptive intermediates and by the fact that **1** and **2** were incorrectly believed to be glucose esters of hydroxy fatty acids, while actually they have been shown to be glucosides of β-hydroxyketones [8].

We report here the results of our studies on the biosynthesis of the major heterocyst glycolipids in *A. cylindrica* (**1** and **3**) by using [1-^{13}C] acetate as precursor.

RESULTS AND DISCUSSION

Our biosynthetic studies were aimed at clarifying the following aspects. The unusually long alkyl chain (C_{26}–C_{28}) of the aglycones could in principle originate, through the acetate pathway, (i) by elongation of a preformed shorter chain, or (ii) by a *de novo* biosynthesis. The first mechanism, which is operative in animals and higher plants for the synthesis of very long chain fatty acids [12], would imply that the fatty acids of *A. cylindrica*, which have a normal length (C_{16}–C_{18}) [11], could be elongated and further modified and glucosylated to give rise to **1**–**4** during the heterocyst differentiation. The second hypothesis means that the aglycones of **1**–**4** are biosynthesized independently from the fatty acid biosynthesis.

In addition, an interesting aspect to be considered is the possible precursor product relationship between the pairs **1**, **3** and **2**, **4**, since in each pair one compound could be formed by oxidation (reduction) of the other.

In a first experiment, [1-^{13}C]acetate (25 mg l^{-1}) was administered to a culture of *A. cylindrica* (70 1) 9 days after inoculation and the cyanobacterium was harvested 4 days later. The results of the incorporation were monitored by ^{13}C NMR on the major glycolipids **1** and **3** after their isolation. The height increment of the signals of the carbons arising from the C-1 of the labelled acetate is shown in Fig. 1. No increments were found for the carbons arising from the C-2 of the acetate, nor for the glucose carbons. It is apparent from the data that there is about the same incorporation at both ends of the aglycones, excluding the possibility that the biosynthesis of these molecules could take place by an elongation process of a shorter chain compound. In fact, in that case, since the elongation proceeds in the direction going from C-26 to C-1, the incorporation at C-1, C-3 and C-5 should be greater than that at C-23 and C-25.

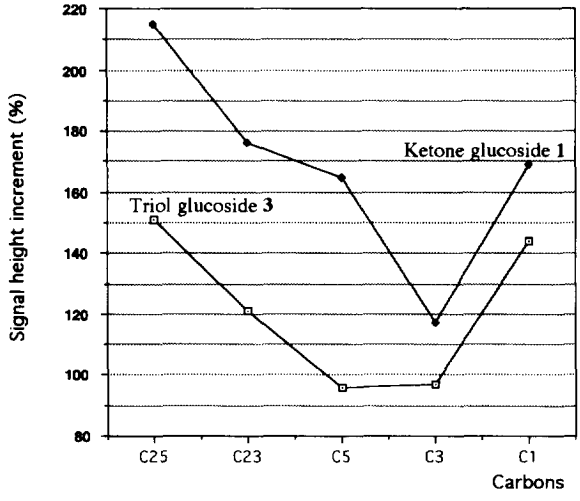

Fig. 1. ^{13}C NMR monitoring of the incorporation results after 4 days in **1** and **3**, following administration to *A. cylindrica* of 25 mg^{-1} of Me$^{13}CO_2H$.

It is also evident that the ketone glucoside **1** is more consistently labelled than the triol glucoside **3**, suggesting a possible role of the ketone as precursor of the triol. To clarify this point we decided to follow the incorporation of acetate during time in both compounds.

At first, in parallel experiments with unlabelled acetate it was found that supplying the fermenter with 18 mg l^{-1} of acetate, about half of the precursor was consumed after 24 hr and the remaining part after 48 hr. Therefore, in order to ascertain any precursor–product relationship between the ketone and the alcohol glucosides, we performed incorporation experiments by administration of 18 mg l^{-1} of labelled acetate and monitored the incorporation in both compounds by withdrawing aliquots of the cyanobacterium after 1, 2 and 4 days (Fig 2).

The incorporation data in the triol glucoside (Fig. 2) show that the biosynthesis proceeds at a good rate, since from 24 to 48 hr, when the labelled acetate is still present in the growth medium, there is a fairly good incorporation, while after 4 days the incorporation decreases, as a result of a dilution with molecules biosynthesized from the endogenous unlabelled acetate.

On the other hand, the biosynthesis of the ketone (Fig. 2) proceeds at a lower rate, after 4 days reaching incorporation values which are slightly greater than those of the triol. These data exclude that the ketone could be the precursor of the triol or vice versa, since in that case in the early stages of the biosynthesis one of the two compounds should be found much more labelled than the other. However, while on the basis of these data the interconversion between the glucosides **1** and **3** could be excluded, it is likely that it could take place at the aglycone stage before glucosylation.

In the previous biosynthetic studies with *A. cylindrica*, heterocyst differentiation was induced by transfer of cells grown in medium containing combined nitrogen to a medium lacking a combined nitrogen source [11], or by addition to the culture of tryptophan analogues[10]. Because of the difficulty of reproducing in a large scale the previous conditions, in our experiments cells were grown in the absence of combined nitrogen and the precursor was added in the late logarithmic growth phase. In these conditions, the heterocyst frequency is about 4% of the vegetative cells. However, when cells grown in medium containing combined nitrogen are transferred to a small volume of a medium lacking a combined nitrogen source [11], a quick heterocyst differentiation takes place and nitrogen fixation starts. Since in these conditions there is no growth and only differentiation takes place, in principle there could also be a difference in the metabolic route conducting to the glycolipids, possibly by utilization of

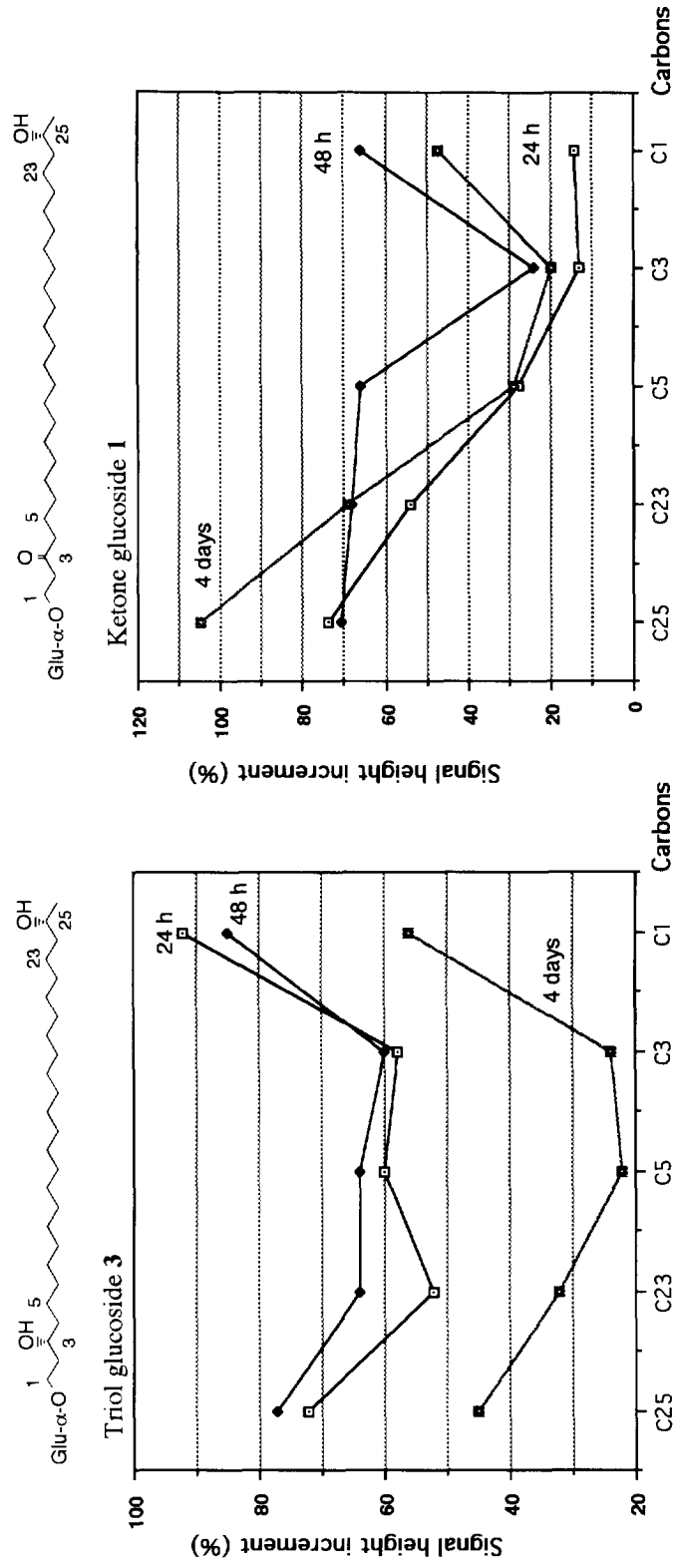

Fig. 2. ^{13}C NMR monitoring of the incorporation results at various times in **1** and **3** after administration to *A. cylindrica* of 18 mg^{-1} of $Me^{13}CO_2H$.

shorter preformed molecules. For these reasons we designed an experiment on a small scale (1l) in which the labelled acetate was supplied when the cells, grown in the presence of combined nitrogen, were transferred to a medium without combined nitrogen. The cells were harvested after 48 hr and the heterocyst frequency was found to be 12%. Because of the small total amount, from these cells only the most abundant glycolipid **3** was isolated and monitored by ^{13}C NMR spectrometry: the spectrum showed the same general trend of the spectra obtained from the other experiments, a similar incorporation at both ends of the aglycone of **3** having been found. The experiment fully confirms that the aglycones of the heterocyst glycolipids are the result of a *de novo* biosynthesis

EXPERIMENTAL

Administration of [1-^{13}C]*acetate and isolation of the glycolipids.* The labelled precursor (Sigma) was administered to *A. cylindrica*, grown in a 70-l 1 fermentor as previously described [8], after 9 days of growth. The concn of the residual acetate in the fermentor, supplied with labelled or unlabelled acetate, was determined enzymatically by using a commercial kit (Boehringer, Mannheim). The glycolipids were isolated as previously described [8].

In a small scale experiment, a batch culture in a 1l fermentor was grown for 2 days in BG-11_0 medium and then the medium was made 5 mM in NH_4Cl and the growth was continued for 10 days. The cells were collected by centrifugation under aseptic conditions at 3 800 *g*, were resuspended in BG-11_0 (1l) and the labelled precursor (18 mg) was added. The cells were harvested after 48 hr and the glycolipid **3** was isolated in the usual way.

^{13}C *NMR spectra.* ^{13}C NMR spectra were recorded in pyridine-d_5 on a Bruker AM 250 machine operating at 62.89 MHz. The height increments of enriched signals were calculated from $(a - b)/b$ where a = height of the enriched carbon and b = height of the same carbon in the unenriched compound. The value of b was calculated from $b = b' \cdot C_{24}/C_{24'}$ where b' is the height of the carbon in the spectrum of the unenriched sample and C_{24} and $C_{24'}$ are the heights of C-24, which was used as the internal standard, in the spectrum of the enriched and unenriched sample, respectively.

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