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Composite hopanoid biosynthesis in *Zymomonas mobilis*: *N*-acetyl-D-glucosamine as precursor for the cyclopentane ring linked to bacteriohopanetetrol

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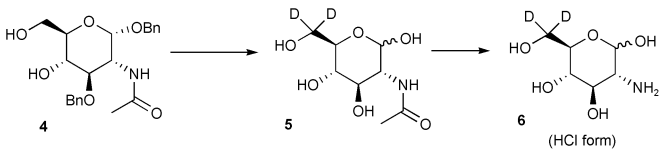
A selective labelling of the two major composite hopanoids of *Z. mobilis* with deuterated *N*-acetyl-D-glucosamine showed that this carbohydrate is a common precursor of the glucosamine or the cyclopentitol moieties respectively linked to bacteriohopanetetrol by a glycosidic or an ether bond.

Triterpenoids of the hopane series are widespread amongst eubacteria.[1] The bacterium *Zymomonas mobilis* is one of the best-known hopanoid producers. Bacteriohopanetetrol derivatives **1** and **2** linked either to glucosamine *via* a glycosidic bond or to a cyclopentane ring *via* an ether bond (Fig. 1) are the major constituents of the lipids.[2] Incorporation experiments performed with ^{13}C labelled acetate or glucose have been used to elucidate the biosynthetic pathways through which bacterial hopanoids are constructed. (i) They allowed the discovery of the long overlooked methylerythritol phosphate pathway for isoprenoid biosynthesis.[3–5] (ii) The polyhydroxylated C_5 side chain is derived from a D-pentose derivative linked *via* its C5 to the triterpene moiety.[3,6] (iii) First insights were obtained on the origin of the carbon skeleton of the cyclopentane ring of hopanoid **2**. It results from a hexose derivative by formation of a carbon–carbon bond between C1 and C5.[6] Calditol (Fig. 1, **3**), a similar cyclopentanic ether has been found in the archaean *Sulfolobus solfataricus*.[7]

Nothing is known on the nature of the direct precursor of such natural cyclopentanic ethers and on the enzyme reaction leading to the formation of the five-membered ring. Incorporation experiments that would lead to a selective labelling of the glucosamine and cyclopentane moieties of hopanoids **1** and **2** would be of great interest, since they may provide interesting information regarding a possible common precursor. The stereochemical pattern of both moieties is the same, suggesting that they are derived from the same biosynthetic intermediate, or that the glucosamine residue (before coupling to bacteriohopanetetrol or already attached to the tetrol) is the precursor of the cyclopentane. Incorporation experiments with labelled glucosamine ($GlcNH_2$) or *N*-acetylglucosamine (GlcNAc) may provide a way to selectively label the hexose derived substructures found in hopanoids **1** and **2**.

The synthesis of **5** and **6** (Scheme 1) starts with a one-pot oxidation/esterification of the known benzyl 2-acetamido-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside[8] **4** into its benzyl ester,[9] which is then reduced by $LiAlD_4$ to give the bisdeuterated isotopomer of starting glycoside **4**. Catalytic hydrogenolysis of the benzyl groups furnished the targeted [6,6-$^2H_2$]GlcNAc **5** which was hydrolysed in acidic conditions to give [6,6-$^2H_2$]$GlcNH_2$ **6**. Mass spectrometry of derivatives **5** and **6** confirmed the total bisdeuteration of the molecules. ^1H and ^{13}C spectra were compared with those of non-deuterated molecules and displayed the characteristic pattern of the CD_2OH group.

For the incorporation experiments, *Z. mobilis* was grown on glucose (10 g L^{-1}) with deuterium labelled $GlcNH_2$ **6** or GlcNAc **5** (1 g L^{-1}).[10] Standard procedures were employed for hopanoid isolation.[2,4] Mass spectrometry (by fast atom bombardment, FAB^+, and chemical ionisation, using methane as reactant gas, IC-CH_4) of the hepta-acetates **7** and **8** revealed interesting features. The total deuterium incorporation of the whole composite hopanoids **7** and **8** were therefore obtained from the isotopic profiles of the molecular peaks of the labelled hopanoids compared to those of hopanoids of natural isotope abundance. The incorporation was much more efficient when the incubation was performed in presence of [6,6-$^2H_2$]GlcNAc



Scheme 1 Synthesis of deuterated analogues **5** and **6**.

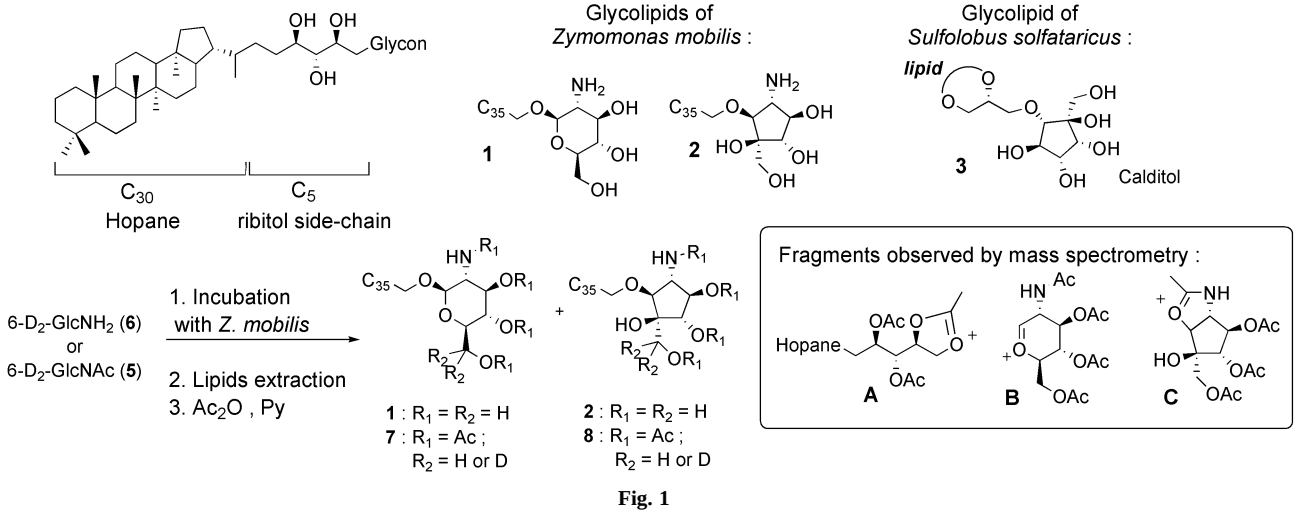


Fig. 1

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5 (around 20% incorporation for both the glycoside **7** and the cyclitol ether **8**) than of [6,6-$^2H_2$]$GlcNH_2$ **6** (around 8% incorporation for the glycoside and less than 5% for the cyclitol).

Isotope dilution occurred *via de novo* biosynthesis of glucosamine and cyclopentane ring from the non-labelled glucose utilised as carbon and energy source.

The incubation experiment with [6,6-$^2H_2$]$GlcNH_2$ **6** did not result in a very selective deuteration of the aglycon fragments: significant increase of the *m*/*z*+2 peaks was observed for the fragments corresponding to the bacteriohopanetetrol moiety (fragment **A**, Fig. 1) as well as in the fragments **B** or **C** corresponding to the glycon moeities of hopanoids **7** and **8** (Table 1). This suggests a catabolism of the deuterated free glucosamine and recycling of the resulting deuterated metabolites into anabolic pathways leading to the isoprene units and the pentose derivatives.

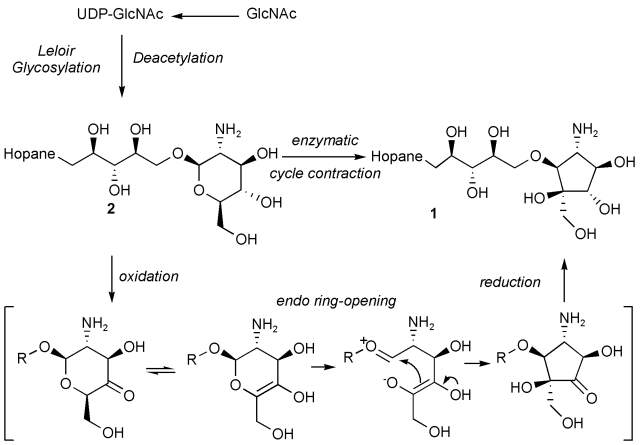
Interestingly, the analysis of the isotope distribution in the aglycon fragments **A** and the glycon fragments **B** and **C** of the same spectra revealed that the deuterium incorporation was mainly found in the glycon part of hopanoids **7** and **8** when [6,6-$^2H_2$]-*N*-acetylglucosamine **5** was used for the incubation experiments. Moreover, the deuterium incorporation for the glycon fragment **B** or **C** of respectively **7** or **8** was very similar and roughly corresponded to the total deuterium incorporation into the whole hopanoids. In addition, the similar isotope abundance found in the glycon moieties of hopanoids **7** and **8** suggests a precursor to product relationship between GlcNAc and the cyclopentitol. Intensity increases of the mass-spectra peaks were only observed for the (*m*/*z*+2) peaks, never for the (*m*/*z*+1) ones. This implies that only bisdeuterations occurred, a result consistent with an incorporation of the intact CD_2OH group of **5**. These observations indicated that no or little catabolism of the deuterated GlcNAc occurred and that this precursor was mostly directly utilised for further metabolic reactions. From this set of results, two conclusions can be reasonably drawn. (i) A GlcNAc derivative is a likely precursor of the cyclopentane ring of hopanoid **2**. (ii) The glycons of the glycoside **1** and the ether **2** share the same biogenetic pathway derived from GlcNAc, which is independent of the construction of the hopane and the C_5 side-chain substructures, both derived from glucose.

The incubation experiment with the deuterated GlcNAc analogue **5** addresses the general question of how GlcNAc is transformed within the cell to lead to composite hopanoids **1** and **2**. To date, there are no direct enzymatic pathways described to answer this question. Free glucosamine and GlcNAc do not represent usual substrates for enzyme reactions. However, GlcNAc has been shown to be the substrate of a kinase of *Escherichia coli* that converts this sugar into the corresponding 6-phosphate.[11] Moreover, a recycling of GlcNAc has been evidenced within the cells of *E. coli*, probably through

Table 1 Incorporation of ^2H into acetylated glycoside **7** and acetylated cyclitol **8**, analysed by mass spectrometry.[a]

		Incorporation into **8** (%)		
Precursor	Ionisation mode	Molecular ion	Fragment **A**	Fragment **B** or **C**
[6-$^2H_2$]GlcNAc	FAB^+	21	1	23
(**5**)	CI-CH_4	16	3	—[b]
[6-$^2H_2$]$GlcNH_2$	FAB^+	2	2	1
(**6**)	CI-CH_4	2	3	—[b]
		Incorporation into **7** (%)		
[6-$^2H_2$]GlcNAc	FAB^+	19	2	24
(**5**)	CI-CH_4	20	2	33
[6-$^2H_2$]$GlcNH_2$	FAB^+	6	7	10
(**6**)	CI-CH_4	8	9	13

[a] Incorporation percentages were calculated from the isotopic distribution of the labelled ions compared to that of the corresponding ions of natural isotope abundance. [b] Higher signal/noise ratio.



Scheme 2 Hypothetical biogenetic scheme for the biosynthesis of bacteriohopanetetrol-cyclitol ether **2** from *N*-acetylglucosamine.

a multistep enzymatic pathway that would regenerate UDP-GlcNAc from free GlcNAc.[11] Such a natural recycling pathway could be involved in the selective incorporation of deuterium labelled GlcNAc into the glycons of **1** and **2**. If verified, this would signify that UDP-GlcNAc is a common precursor of glycoside **1** and ether **2** (Scheme 2).

A Leloir-type glycosylation of bacteriohopanetetrol would lead to glycoside **1**, which would be further converted into the cyclopentanic ether **2**. At first sight, this process appears analogous to the well-established inositol-synthase mechanism,[12] with, however, a very significant difference: the putative mechanism implied in the biosynthesis of cyclitol **2** from glycoside **1** (Scheme 2) would involve an *endo* opening of the sugar ring giving rise to an oxonium intermediate whereas the inositol synthase pathway involves the formation of an intermediate aldehyde leading to an intramolecular aldol process.[12] Although such a mechanism has been described *chemically*,[13] there are no precedents in the literature of an *enzymatic* process involving this new reaction.

The putative mechanism depicted in Scheme 2 implies an oxidation that can occur at the C4 (the one selected in the scheme), C6 or even C5 by an oxidative *endo*-opening process. Because of the retention of two deuteriums, our experiments seem to rule out the oxidation at C6, and work is in progress to confirm this hypothesis. However, other mechanisms such as flavoenzymes catalysed reactions[14] might also be considered to explain this unprecedented ring contraction. The same hypothesis may be proposed as well for the biosynthesis of calditol.

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