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Carbohydrates

Identification of Living *Legionella pneumophila* Using Species-Specific Metabolic Lipopolysaccharide Labeling**

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Dedicated to Professor Jean-Marie Beau on the occasion of his 65th birthday

Abstract: *Legionella pneumophila is a pathogenic bacterium involved in regular outbreaks characterized by a relatively high fatality rate and an important societal impact. Frequent monitoring of the presence of this bacterium in environmental water samples is necessary to prevent these epidemic events, but the traditional culture-based detection and identification method requires up to 10 days. Reported herein is a method allowing identification of Legionella pneumophila by metabolic lipopolysaccharide labeling which targets, for the first time, a precursor to monosaccharides that are specifically present within the O-antigen of the bacterium. This new approach allows easy detection of living Legionella pneumophila, while other Legionella species are not labeled.*

In the summer of 1976 an outbreak of an unknown disease occurred at the annual convention of the American Legion in Philadelphia.[1] A total number of 221 people were infected and 34 died from the consequences of this infection. The causative agent was later identified as a bacterium,[2] which was given the name *Legionella pneumophila*.[3] Since then, outbreaks of legionellosis have occurred on many occasions, and numerous strains of pathogenic *L. pneumophila* have been identified. A characteristic aspect of infection by *L. pneumophila* is its high fatality rate, thus resulting in an important impact of each individual epidemic event. Monitoring of the presence of this bacterium in potential reservoirs, including cooling towers and other water-containing systems, is the major strategy used to limit the occurrence of outbreaks, and methods allowing fast and easy detection of living *L. pneumophila* could have a major impact since standard, culture-based methods can require up to 10 days.[4]

We have previously shown that metabolic glycan labeling,[5] a strategy in which a modified monosaccharide bearing a reporter function is metabolically incorporated into surface glycans, could be efficiently used to target bacterial lipopolysaccharides (LPS) without species specificity. In this first study, an azido derivative of Kdo, a bacterial monosaccharide, was incorporated into the LPS inner core of various Gram-negative bacteria, and detected by copper-catalyzed click-chemistry with an alkyne-modified fluorophore.[6] This strategy can be efficiently used to detect the overall presence of living Gram-negative bacteria. A much more impactful challenge would be to use metabolic lipopolysaccharide labeling not only to detect, but also to identify a living pathogenic bacterium of interest in the same operation by using an analogue of a monosaccharide which would be specifically present within the O-antigen of this bacterium. To the best of our knowledge, metabolic glycan labeling has not yet been used for direct species identification. The work presented here successfully addresses this challenge in the case of *L. pneumophila.*

The O-antigen of *L. pneumophila* serogroup 1, which is prevalent among infected cases,[7,8] is composed of an α(2→4) homopolysaccharidic repeat of 5-*N*-acetimidoyl-7-*N*-acetyllegionaminic acid (Leg5Am7Ac)[9] (Figure 1a). The biosynthesis of Leg (Figure 1b) starts from UDP-*N*,*N*′-diacetylbacillosamine, which is transformed into 2,4-diacetamido-2,4,6-trideoxy-D-mannopyranose (**1**) by the dual action of a hydrolyzing 2-epimerase. In the next step, the precursor **1** is directly transformed into *N*,*N*′-diacetyllegionaminic acid (Leg5Ac7Ac) by the action of an aldolase, in the presence of phosphoenolpyruvate (PEP).[10] This event controls the stereochemistry of the newly generated stereogenic center at C4. Legionaminic acid is then activated in the form of a cytidine monophosphate donor (CMP)-Leg5Ac7Ac. Further transformations are believed to occur at a later stage.

To target the Leg pathway for metabolic glycan labeling, we embarked upon the synthesis of an azido derivative of **1**,[11] namely 6-azido-2,4-diacetamido-2,4,6-trideoxy-D-mannopyranose (**2**, Figure 1c), as well as its less polar, monoacetylated derivative **3**, which we believed might enter more easily into

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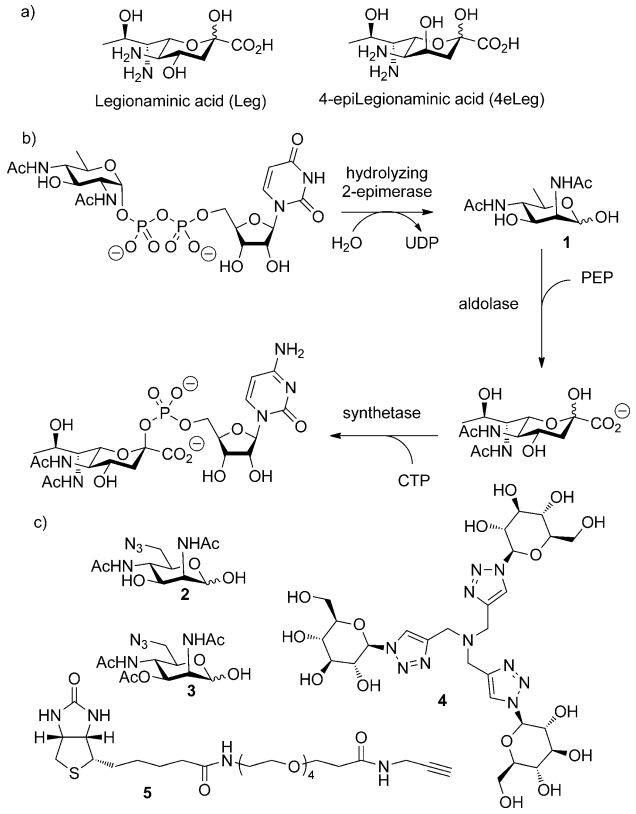
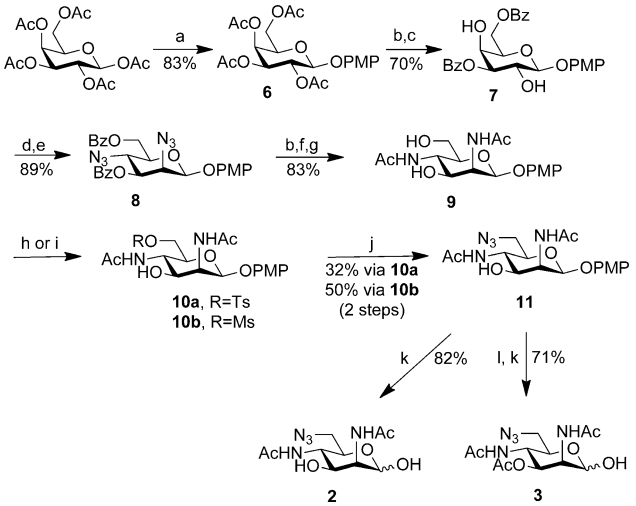


Figure 1. Structures of Leg and 4eLeg, the Leg pathway and molecules used in this study. a) Structure of Legionaminic (Leg) and 4epiLegionaminic (4eLeg) acids. b) Leg pathway in *L. pneumophila*, and the structure of intermediate **1**. c) Structure of analogues **2** and **3**, as well as ligand **4** and biotine-alkyne **5.**



Scheme 1. Reagents and conditions: a) *para*-methoxyphenol, $BF_3.Et_2O$, CH_2Cl_2;[12] b) CH_3ONa, CH_3OH;[13] c) 2-aminoethyl diphenylborinate, DIPEA, benzoyl chloride, CH_3CN;[14] d) Tf_2O, pyridine, CH_2Cl_2;[15] e) Bu_4NN_3, toluene;[15] f) $Pd(OH)_2/C$, H_2, CH_3OH;[15] g) Ac_2O, CH_3OH;[15] h) TsCl, pyridine;[6] i) MsCl, pyridine; j) NaN_3, DMF;[6] k) CAN, CH_3CN/H_2O (3:1);[16] l) Ac_2O, pyridine, CH_2Cl_2. CAN = ceric ammonium nitrate, DIPEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, Ms = methanesulfonyl, PMP = *para*-methoxyphenyl, Tf = trifluoromethanesulfonyl, Ts = 4-toluenesulfonyl.

the cell by passive transport, and be further transformed into **2** by the action of intracellular, nonspecific esterases. Once in the cell, **2** might act as a precursor of an azido-labeled analogue of legionaminic acid, and be further incorporated within the O-antigen of the bacteria. A synthetic strategy starting from D-galactose has therefore been developed to access **2** and **3**, and these products have been tested for specific labeling of the LPS of living *L. pneumophila* serogroup 1.

The compounds **2** and **3** were synthesized (Scheme 1) using a method inspired by the synthesis of **1** by Tsvetkov and co-workers.[15] The target compound, 6-azido-2,4-diacetamido-2,4,6-trideoxy-D-mannose (**2**), was prepared in 11 steps from the commercially available β-D-galactose pentaacetate with an overall yield of 17%, while 3-*O*-acetyl-6-azido-2,4-diacetamido-2,4,6-trideoxy-D-mannose (**3**) was obtained from the same starting material in 12 steps and 15% overall yield.

Glycosylation of *p*-methoxyphenol with β-D-galactose pentaacetate in the presence of boron trifluoride etherate gave **6** in a good 83% yield.[12] Zemplén deacetylation using sodium methoxide[13] and selective benzoylation using the method developed by Lee and Taylor led to **7** (70% yield over 2 steps).[14] Conversion of **7** into the bis(triflate) derivative, and its subsequent reaction with tetrabutylammonium azide in toluene resulted into the bis(azido) compound **8** (89%; 2 steps),[15] the *manno* configuration of which was confirmed by ^{1}H NMR spectroscopy ($J_{1,2} = 1.2$ Hz; $J_{2,3} = 3.6$ Hz; $J_{3,4} = 10.0$ Hz; $J_{4,5} = 10.2$ Hz). Conventional debenzoylation of **8** and reduction of the azido groups with H_2 and $Pd(OH)_2/C$ was followed by N-acetylation to give **9** in a high yield (82% over 3 steps).[15] The azido derivative **11** was obtained by selective tosylation or mesylation in pyridine, with subsequent nucleophilic substitution using sodium azide in dimethylformamide.[6]

In this strategy, mesylation and substitution gave better results (50%) than the tosylation route (32%). The final product **2** was obtained in a good yield (82%) from **11** by deprotection of the anomeric position using cerium ammonium nitrate.[16] Alternatively, the product **3** was prepared in two steps from **11** in a respectable 71% yield by acetylation and subsequent deprotection of the anomeric position using the same reaction conditions as before.[16]

We then selected four different strains of *L. pneumophila*, belonging to serogroup 1, including 1) a strain isolated from one of the victims of the historical Philadelphia outbreak,[17] 2) the Paris strain, which was responsible for nosocomial epidemics at the newly constructed, modern, and freshly opened Georges Pompidou Hospital in Paris in 2000, and has become endemic throughout Europe,[18] 3) the Lens strain, which infected 86 people and killed 17 in the north of France during the 2003–2004 winter and was therefore accountable for the most important epidemics in this country. These strains were grown in the presence of **2**, and the incorporation of the azido chemical reporter into LPS was monitored in a subsequent step by using copper-catalyzed azide–alkyne cycloaddition,[3] with copper sulfate, sodium ascorbate, TGTA [**4**; a water-soluble tris(triazolyl) ligand for copper (I)],[19] and

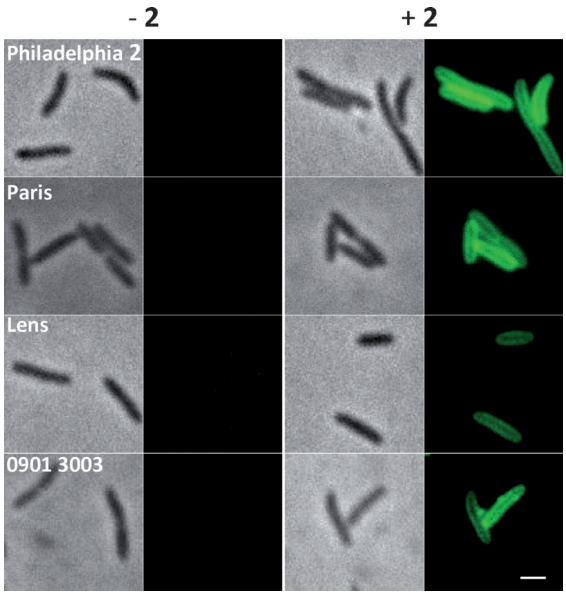


Figure 2. Detection of metabolically incorporated **2** by various *L. pneumophila* serogroup 1 strains as shown by Cu^{I}-catalyzed click reaction with **5**, and further visualization using an Alexa Fluor 488-IgG anti-biotin antibody. Phase contrast and fluorescence images in the presence (right panel) or absence of **2** (left panel). Scale bar = 1 μm.

a biotine-alkyne probe (**5**) for 30 minutes (Figure 1c). Biotin labeling was then visualized with a fluorescently labeled anti-biotin antibody. All strains showed highly distinctive fluorescence on their membrane, indicative of an effective metabolic incorporation of the chemical reporter (Figure 2). Those results are in contrast with other bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*, which both failed to show any labeling under the same conditions (see Figure S1 in the Supporting Information).

This highly encouraging result prompted us to test the specificity of our labeling strategy towards other strains of *Legionella*, which did not belong to the *pneumophila* species, and have therefore not been described to contain legionaminic acid within their LPS. A representative set of such strains, containing *L. gormanii*, *L. maceachernii*, *L. micdadei*, *L. anisa*, *L. feeli*, *L. jordanis*, *L. tucsonensis*, and *L. bozemanii* were therefore subjected to the same labeling conditions, and no membrane fluorescence was observed (see Figure S2), a result consistent with the absence of Leg within their lipopolysaccharides. The method is therefore able to efficiently discriminate *L. pneumophila* serogroup 1 from other *Legionella* not belonging to the *pneumophila* species.

We then evaluated the capacity of our method to label *L. pneumophila* strains belonging to other serogroups. This is an interesting point, since although serogroup 1 is found in most infected cases, other serogroups are abundant in the environment. The possibility to label these other serogroups would therefore be an important result allowing a better evaluation of the presence of *L. pneumophila* in a given sample. Most of these serogroups have been shown to contain another isomer of a 5-acetamidino-7-acetamido-3,5,7,9-tetradeoxynon-2-ulosonic acid, namely 5-*N*-acetimidoyl-7-*N*-acetyl-4-epi-legionaminic acid (4eLeg5Am7Ac),[20] with various degrees of 8-O-acetylation depending on the serogroup (Figure 1a). Contrary to the Leg pathway, the 4eLeg biosynthetic pathway has not been identified yet, but one could speculate that it might involve similar intermediates. Strains belonging to serogroups 3, 4, 5, 6, which represent together with serogroup 1 between 68 and 85% of the *L. pneumophila* present in the environment,[8] showed very bright membrane labeling (Figure 3). This result tends to indicate that Leg and 4eLeg biosyntheses apparently share **1** as a common precursor, and our method allows clear and specific detection of *L. pneumophila* strains that do not belong to serogroup 1.

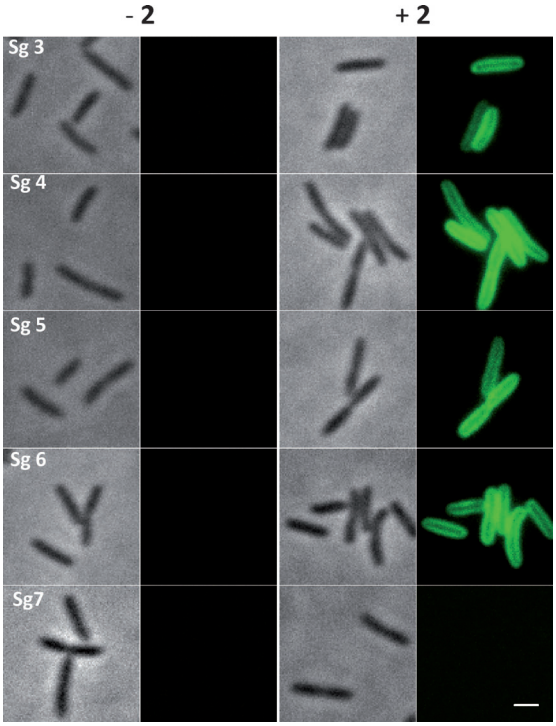


Figure 3. Detection of metabolically incorporated **2** by various *L. pneumophila* strains belonging to serogroups other than serogroup 1 (same conditions as used for Figure 2).

Interestingly, the only exception observed concerned a *L. pneumophila* strain belonging to serogroup 7 (Figure 3), a serogroup which is very poorly represented both in infection cases and in the environment.[7,8] Serogroup 7 has been described to present a still unidentified isomer of 5-acetamidino-7-acetamido-3,5,7,9-tetradeoxynon-2-ulosonic acid within its O-polysaccharide.[20] This observation is consistent with the absence of labeling, and tends to indicate that **1** is not an intermediate in the corresponding biosynthetic pathway. An isomer of **1** is most certainly involved as a substrate for the aldolase.

Another set of experiments was performed using **3**, a monoacetylated derivative of **2**. It was expected that this less-polar esterified compound might enter the bacterial cell more efficiently by passive transport, and could be further

incorporated into the LPS after deacetylation to **2** by nonspecific esterase activity within the cell. The availability and extent of such an activity inside bacterial cells has been the subject of recent debate within the scientific community.[21] No labeling was observed under our conditions, even in the case of the *L. pneumophila* strains that were efficiently labeled by **2** (see Figure S3), suggesting that such an esterase activity is not present at a sufficient level within the cell to allow efficient production of **2** from **3** and further metabolization and incorporation of **2** to a detectable proportion.

This work therefore leads to several interesting results: 1) The approach we have described appears as an efficient strategy to specifically detect and identify living *L. pneumophila*, a pathogenic bacterium of high sanitary and economic impact, and requires about one day on laboratory samples. To the best of our knowledge, this is the first time that metabolic labeling has been applied to species identification, through the use of a specific monosaccharide. 2) Interestingly, the absence of labeling under our laboratory conditions with an acetylated precursor strengthens the claim that nonspecific esterase activity within such bacteria might not be sufficient for the efficient liberation inside the cell of a previously acetylated carbohydrate precursor,[21] an aspect that should be evaluated when considering the use of this strategy for metabolic labeling experiments, prodrug approaches, or other biosynthetic applications. 3) The use of an analogue of a Leg precursor allows labeling of relevant serogroups using the same conditions. The overall success of this strategy offers some mechanistic insight into the biosynthesis of Leg and 4eLeg as **1** appears to be a common intermediate in these two metabolic pathways.

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