DOI: 10.1002/ange.201108127

Bioimaging

Click-Mediated Labeling of Bacterial Membranes through Metabolic Modification of the Lipopolysaccharide Inner Core

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Metabolic glycan labeling[1] has recently emerged as a very powerful method for studying cell-surface glycans, which has applications that range from imaging glycans in living multicellular organisms, such as zebrafish or mice, to the identification of metastasis-associated cell-surface sialoglycoproteins.[2] This strategy relies on the cellular biosynthetic machinery assimilating a modified monosaccharide that contains a bioorthogonal chemical reporter. The metabolic incorporation of this reporter into glycans can be further visualized by chemical ligation with a label, such as a fluorescent probe. Somewhat surprisingly, previous studies have mainly focused on the labeling of vertebrate glycans[3] by using derivatives of common monosaccharides, such as *N*-acetyl neuraminic acid (or its *N*-acetylmannosamine precursor), *N*-acetylglucosamine, *N*-acetylgalactosamine, and fucose.[1]

In spite of a much higher degree of diversity in their monosaccharide building blocks as well as an essential role in bacterium–host interactions and bacterial virulence, bacterial polysaccharides have been poorly explored in terms of in vivo structural modifications. Bacteria are divided into Gram-positive and Gram-negative bacteria. Whereas Gram-positive bacteria are surrounded by a peptidoglycan cell wall, Gram-negative bacteria are covered by a dense layer of lipopolysaccharides that are embedded in their outer membrane. These lipopolysaccharides are involved in the structural integrity of the cell and are often considered as determinants of pathogenicity. Although lipopolysaccharides appear to be an interesting target for specific and well-defined glycan metabolic labeling in Gram-negative bacteria, attempts to achieve this goal have been limited to the introduction of modified L-fucose derivatives into a customized, genetically engineered strain of *Escherichia coli*.[4] Although it is a very interesting proof of concept, this L-fucose-based approach has some limitations as L-fucose is not generally present within the lipopolysaccharides of all Gram-negative bacteria, but is found in the O-antigens of specific strains.[5] Secondly, free L-fucose is not an intermediate in the normal *E. coli* "de novo" pathway and, therefore, should not be directly activable into a nucleotide-sugar donor[6] without the introduction of an alternative pathway, known as the "salvage pathway", into the organism of interest by genetic engineering (metabolic pathway engineering). Furthermore, once activated in the form of a modified guanosine-5′-diphosphate–fucose (GDP–Fuc), the L-fucose analogue might be transformed into a correspondingly modified GDP–mannose (GDP–Man) by the reverse de novo pathway, and potentially further metabolized into various other compounds, a process which could result in the chemical reporter being spread through other pathways of sugar metabolism or beyond.

As a result of all of these limitations, and as our goal was labeling the lipopolysaccharides of bacteria with no genetic modification, we investigated whether another sugar could be used as a target for the metabolic modification of glycans.

From all of the potential targets, 3-deoxy-D-manno-octulosonic acid (KDO) appears to be a very attractive candidate. Indeed, KDO is a specific and essential component of the inner core of lipopolysaccharides,[7,8] and has long been considered as being present in the lipopolysaccharides of almost all Gram-negative species (as well as higher plants and algae), in which at least one residue is directly connected to lipid A (Scheme 1a).[9] Because of its vital importance, KDO has been considered as a determinant for the characterization of Gram-negative bacteria, and the KDO pathway as a potential target for the development of new antibacterial compounds.[10] In the KDO pathway (Scheme 1b), arabinose-5-phosphate (arabinose-5-P) is condensed with phosphoenolpyruvate (PEP) to give KDO-8-phosphate (KDO-8-P), which is then transformed into free KDO, and further activated to form the cytidine monophosphate (CMP)–KDO donor prior to lipopolysaccharide elaboration. For all of these reasons, we hypothesized that the KDO pathway, as a lipopolysaccharide-specific pathway, may be tolerant enough to incorporate a modified analogue of KDO, such as 8-azido-8-deoxy-KDO (**1**, Scheme 2), into the core of *E. coli* lipopolysaccharides, and potentially other Gram-negative bacteria. Given the presence of free KDO as an intermediate in the pathway, we postulated that if the cell penetration of this analogue of KDO was sufficient,[11] it could then be directly activated, partially replace endogenous KDO in lipopolysaccharides, and be detected on the cell surface by azide–alkyne click chemistry (Figure S1 in the Supporting Information).[12] Moreover, modification of the C8-position of KDO with a bioorthogonal azido group should prevent reverse metabolism by KDO-8-P

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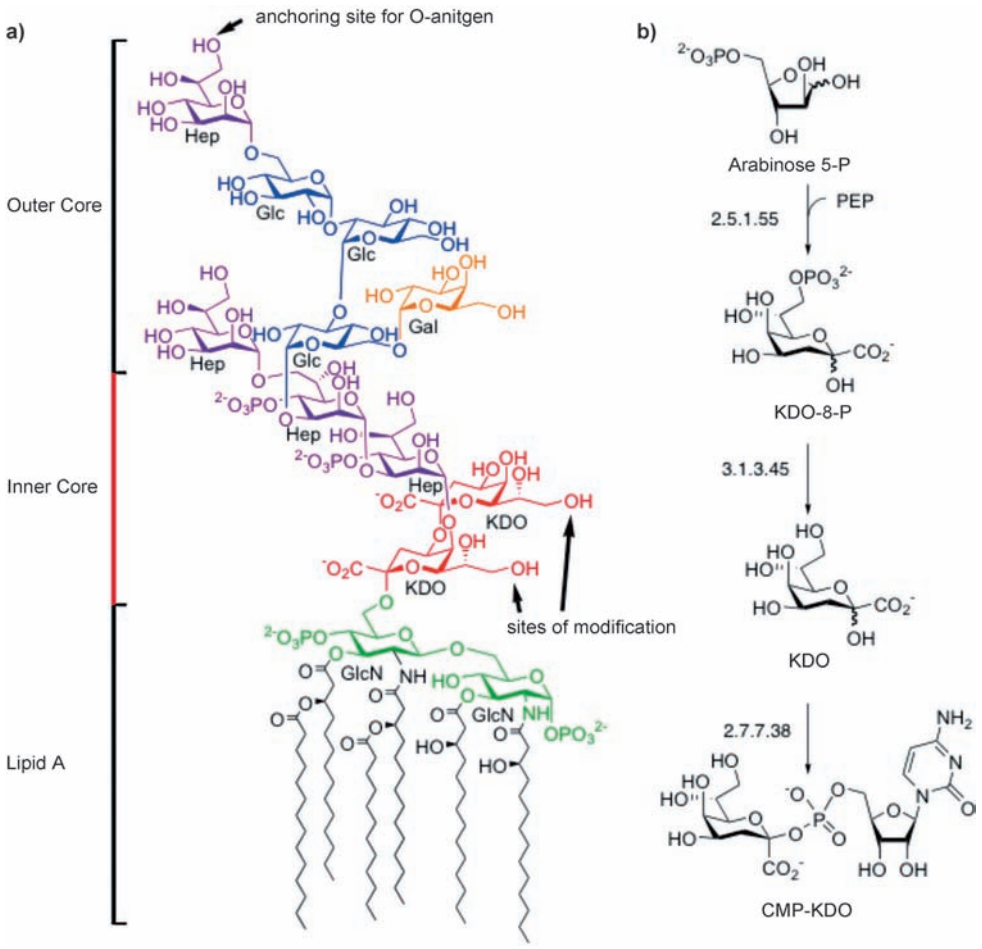
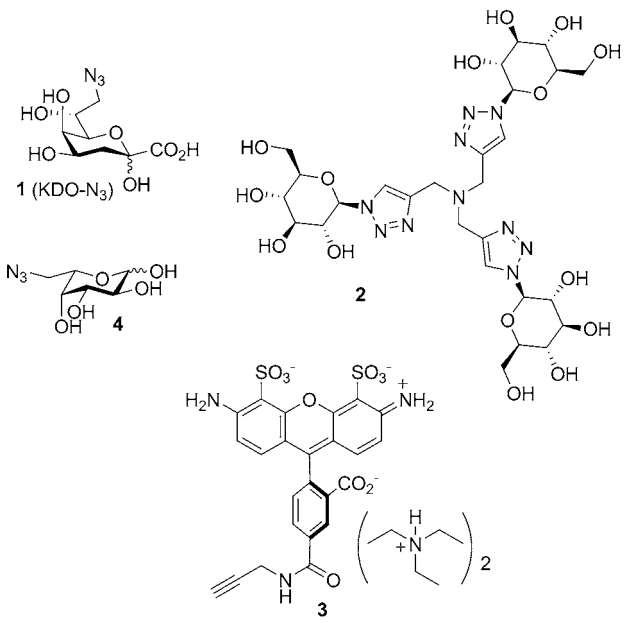
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201108127.



Scheme 1. a) Structure of the major component of *E. coli* K12 lipopolysaccharides. b) Summary of CMP–KDO biosynthetic pathway (KDO pathway). Gal: D-galactose; Glc: D-glucose; GlcN: 2-amino-2-deoxy-D-glucose; Hep: L-glycero-D-manno-heptose.

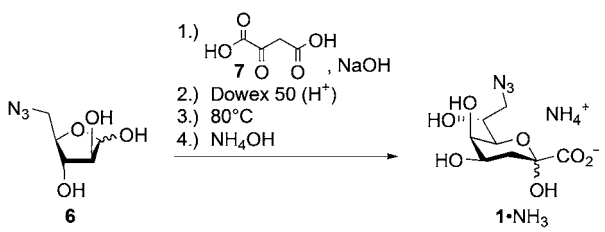


Scheme 2. Molecules **1**–**4**.

phosphatase (3.1.3.45), which should limit the potential dissemination of the chemical reporter into other carbohydrates and metabolites.

Among the many potential multistep synthetic strategies available to access **1**,[13] we prepared this compound in a straightforward manner (Scheme 3)[14] by using a method that was adapted from the approach described in 1963 by Ghalambor and Heath for the direct synthesis of KDO.[15] 5-Azido-5-deoxy-D-arabinofuranose[16] (**6**) was condensed with sodium oxaloacetate (**7**), which gave **1**, isolated as its ammonium salt in 57% yield (86% based on recovered **6**) after decarboxylation under slightly acidic conditions. Precursor **6** could be obtained by a very direct, simple, and time-saving strategy from commercial D-arabinose, as described in Supporting Information (Scheme S1 in the Supporting Information).

Nonpathogenic *E. coli* K12, which lacks an O-antigen,[17] was cultured overnight in the presence of **1**, and further treated with the water-soluble, glucose-derived tris(triazolyl) ligand **2**[19,20] and an Alexa Fluor 488 fluorophore that contains a terminal alkyne group (**3**) under optimized copper-catalyzed click reaction conditions[18] during a time course experiment. After 5 min of incubation under these click reaction conditions, the bacteria were labeled very brightly, whereas control experiments in the absence of **1** did not generate any significant fluorescence (Figures 1a and b). The fluorescence was mostly evident around the cell periphery, which suggests that the membranes were preferentially labeled as expected (Figure 1c). Interestingly, as the signal-to-noise ratio decreases with increasing time of incubation, only five minutes is



Scheme 3. Synthesis of ammonium 8-azido-3,8-dideoxy-D-manno-octulosonate.

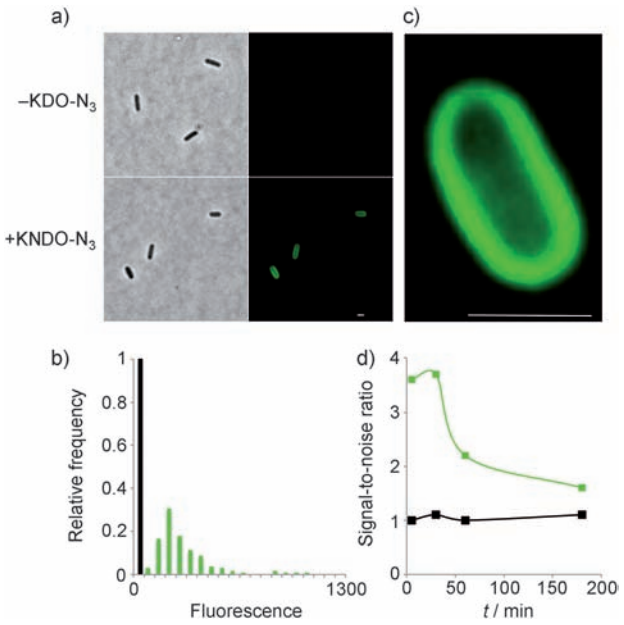


Figure 1. KDO-N_3 (**1**) metabolically labels *E. coli* lipopolysaccharides. a) Metabolically incorporated **1** in *E. coli* K12 was revealed by a Cu^I-catalyzed click reaction with **3** after 5 min. b) Frequency distribution of the bacterial fluorescence values in the presence (green bars) or absence of **1** (black bars). c) Deconvolution analysis indicates that fluorescence was concentrated at the cellular surface. The image was deconvoluted by using the Richardson–Lucy algorithm with an experimental point spread fusion. Scale bar = 1 μm. d) Ratio of fluorescence signal/background fluorescence plotted against the duration of the click reaction in the presence (green bars) or absence of **1** (black bars).

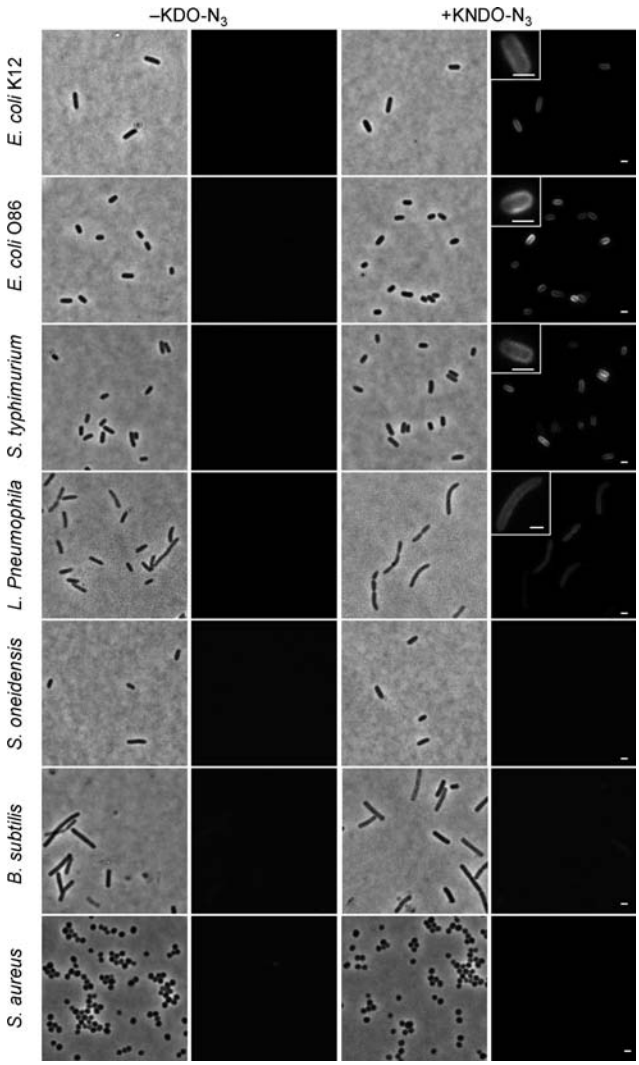


Figure 2. Detection of metabolically incorporated **1** by various bacterial strains as shown by the Cu^I-catalyzed click reaction with **3** after 60 min. Phase contrast and fluorescence images in the presence (right panel) or absence of **1** (left panel). Scale bar = 1 μm.

sufficient to obtain optimal labeling (Figure 1d, see also Figure S2 in the Supporting Information). We also tested the metabolic incorporation and labeling of an azido-modified fucose (6-azido-6-deoxy-L-galactose **4**)[21] in two *E. coli* strains by using our procedure: *E. coli* K12, which lacks L-fucose in its lipopolysaccharides, and O86 *E. coli*, which has L-fucose in the O-antigen portion of its lipopolysaccharides. No labeling of the cell surface was observed in these experiments, which confirms that **4** is not directly incorporated in the absence of an active L-fucose salvage pathway (Figure S3 in the Supporting Information).

To further consolidate our approach, we tested its efficiency and specificity on three other Gram-negative bacteria that use KDO (O86 *E. coli*, *Salmonella typhimurium*, and *Legionella pneumophila* strain Paris) and three negative controls, (*Shewanella oneidensis*, which has recently been shown to use 8-amino-8-deoxy-KDO instead of KDO in its lipopolysaccharides[22] and two Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*), which do not produce KDO.[23] As expected, the two *E. coli* strains, *S. typhimurium* and *L. pneumophila* Paris showed efficient and well-defined cell-surface labeling, whereas no labeling was observed with *S. oneidensis* or the Gram-positive bacteria (Figure 2, see also Figure S4 in the Supporting Information).

In conclusion, we have demonstrated that KDO analogue **1** can be metabolically assimilated and incorporated into bacterial lipopolysaccharides without the use of genetically modified bacteria. This labeling technique has potential applications in bacterial cell imaging, prodrug conjugation, and direct drug delivery, as well as in the extraction and characterization of lipopolysaccharides, a process which still needs some improvements.[24] More interestingly, the fact that **1** needs to be metabolically assimilated first, led us to use this method for the fast detection of metabolically active/viable Gram-negative bacteria (the overall process takes less than one day). This last application might be very powerful, as the detection of viable bacteria normally needs between two days and one month or more, depending on the bacterial strain. In the near future, the assimilation of KDO-N_3 will be coupled to the fluorescence in situ hybridization (FISH)[25] procedure, which will allow the specific detection of viable bacteria of interest.

Received: November 18, 2011
Revised: December 23, 2011
Published online: February 9, 2012

Keywords: carbohydrates · click chemistry · fluorescent probes · glycans · lipopolysaccharides

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