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ARTICLE

Synthesis of chemical tools to label the mycomembrane of corynebacteria using a modified Iron (III) chloride-mediated protection of trehalose

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Abstract. Trehalose-based probes are useful tools allowing the detection of the mycomembrane of Mycobacteria through the metabolic labeling approach. Some of them are trehalose analogues conjugated to fluorescent probes and others are functionalized with a bioorthogonal chemical reporter for a two-step labeling approach. The synthesis of such trehalose-based probes relies mainly on desymmetrization of natural trehalose using regioselective protection-deprotection steps to differentiate the eight hydroxyl groups, and involve a large number of steps. Here, in order to avoid these time-consuming steps, we reinvestigated our previously reported tandem protocol mediated by $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ with the aim of modifying the ratio of the products allowing the challenging desymmetrization of the C2 symmetrical disaccharide trehalose. We demonstrate the usefulness of this method in providing easy access to trehalose analogues with a bioorthogonal moiety or a fluorophore in C2 and an application for the study of the mycomembrane in live mycobacteria through either the one-step or the two-step labeling approach.

Introduction

Corynebacteriales are an order of actinomycetes including corynebacteria, and mycobacteria. Among them *Corynebacterium diphtheriae* is the causative agent of diphtheria, and *Mycobacterium tuberculosis* of tuberculosis. The latter is causing around 10 million of new infections yearly and one of the leading cause of mortality as a result of an infectious agent.¹ Besides, the nonpathogenic *Corynebacterium glutamicum* is of considerable economic benefit as glutamate and lysine world's largest producing strain.² All bacteria of this group have a complex and unusual diderm cell envelope that possesses features of both Gram-positive and Gram-negative bacteria.^{3,4} Beyond the classical plasma membrane, the cell wall core of these bacteria is made up of a specific matrix composed

of cross-linked peptidoglycan (PG) bound to arabinogalactan (AG) through a phosphodiester linkage between *N*-acetylglucosamine and a rhamnose units. In turn, AG is covalently bound through an ester link with the non-reducing ends of AG to long fatty acids named mycolic acids. The esterified AG are the major components of the inner part of the outer membrane referred to as the mycomembrane (MM) and whose outer leaflet is composed of non-covalently attached glycolipids the most abundant being trehalose monomycolate (TMM) and dimycolate (TDM).^{5,6} As its name suggests, the mycomembrane is mainly composed of mycolic acids which represent up to 40% of cell wall dry mass. Mycolic acids are very long α -branched β -hydroxylated fatty acids (C30-90) that are crucial for the survival of mycobacteria.^{7,8} They play a role in the virulence and the persistence of pathogenic mycobacteria within infected organisms and in the host immune response.⁹ Mycolic acids biosynthesis occurs in the cytoplasm where they are assembled and transferred by the polyketide synthase Pks13, onto the disaccharide trehalose leading to trehalose monomycolate (TMM)^{10,11} which is further exported across the inner membrane by an RND transporter of the MmpL3 family.^{12,13} In the cell envelope TMM is the mycolate donor for

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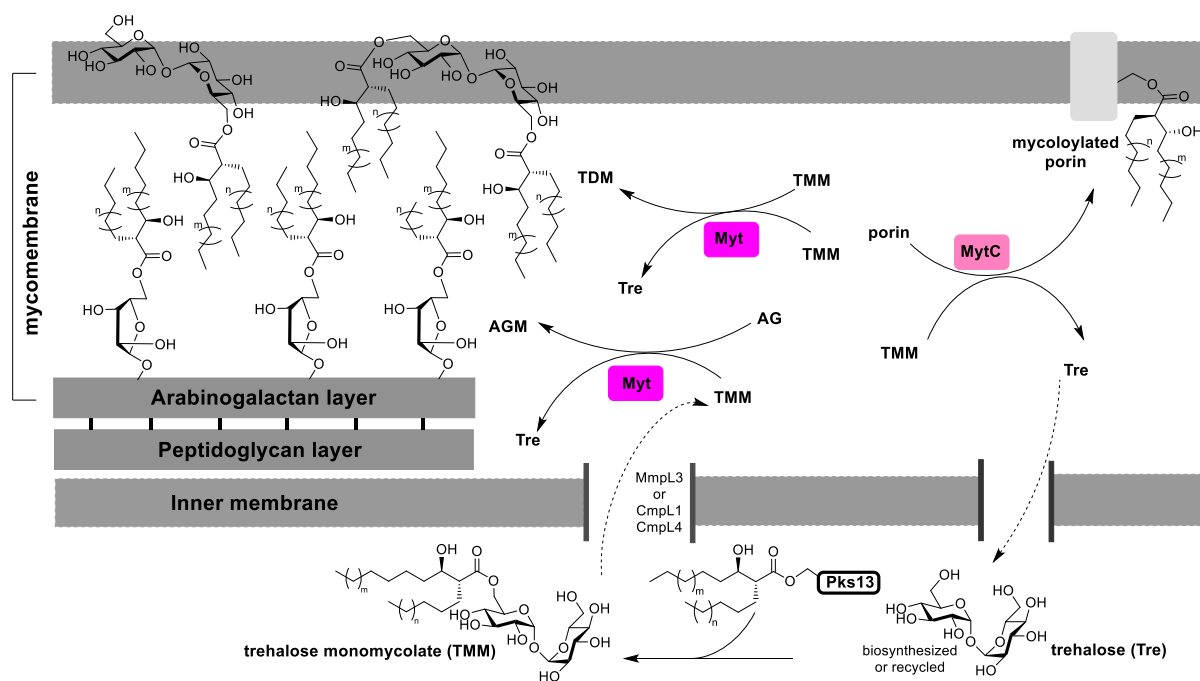


Figure 1. Schematic mycobacterial cell wall structure and overview of the mycoloylation of various acceptors: arabinogalactan, TMM and porins by several mycoloyltransferases. AG = arabinogalactan; TDM = trehalose dimycolate; Myt = mycoloyltransferase.

several acceptor molecules: (i) the AG layer, (ii) another TMM to form TDM or even (iii) porins, *ie* channel-forming proteins (Figure 1). These transfers are catalysed by multiple mycoloyltransferases that are key enzymes in the biogenesis of the envelope of *Corynebacteriales*. Four of them have been described in *M. tuberculosis* (Ag85), 6 in *M. smegmatis* (Fbp) and 6 in *C. glutamicum* (Myt).¹⁴

The mycomembrane is essential for the viability of mycobacteria and is responsible for low permeability of their envelope thus contributing to the intrinsic resistance to various antibiotics and is involved in persistence in host environments.^{15,5} Given their importance, mycomembrane components and their biosynthetic pathways have been extensively studied and several front-line drugs used for treating tuberculosis inhibit enzymes involved in MA synthesis.¹⁶

Several structural studies have been performed to elucidate the complex organisation of the cell envelope^{17,18} and over the last decade, chemists have developed bioorthogonal chemical reporters to study the dynamics of MM components.¹⁹ Since a few years, several trehalose-based probes targeting mycoloyltransferases have been reported allowing the visualization of the MM in live cells through the metabolic labeling approach (Figure 2). These new tools emerged after the pioneering work of Davis, Barry and coworkers.²⁰ Notably, they reported for the first time a fluorescent analogue of trehalose, FITC-Tre (Figure 2), in which one glucose unit of trehalose is methylated on the anomeric position. Interestingly, these fluorescent probes are processed by Ag85 allowing sensitive detection of *M. tuberculosis* under the one step labeling approach. A series of azide-modified trehaloses 2-, 3-, 4- and 6-TreAz (Figure 2), based on the natural trehalose core, were then described by the group of Bertozzi.²¹ In this two-step labeling

approach using the bioorthogonal chemical reporter strategy, the alkyne-functionalized fluorescent probe is delivered in a second step and is conjugated using CuAAC (Cu(I)-catalyzed Azide-Alkyne Cycloaddition) or SPAAC (Strain-Promoted Azide-Alkyne Cycloaddition). The same team has recently described several fluorophore-functionalized trehalose probes starting from their corresponding previously reported TreAz. The conjugated fluorophore was either fluorescein (2-, 3-, 4-, 6-FITre^{22a}), tetramethylrhodamine (6-TMRTre^{22b}), or the solvatochromic dye 4-*N,N*-dimethylamino-1,8-naphthalimide (DMN-Tre²³). Other 6-azido-modified trehaloses were later reported by Fullam.²⁴ These derivatives were also modified on the glucose unit bearing the azide *i.e.* the glucose was either formally replaced by a mannose (azido-mannotrehalose) or a galactose (azido-galactotrehalose). Additional probes, mimicking the mycoloyl donor TMM, were reported by the group of Swarts.²⁵ They consisted in acylated trehaloses in which the fluorescent or bioorthogonal moieties are positioned at the end of a short linear alkyl chain in place of the native mycolic acid (Figure 2, *N*- and *O*-AlkTMM). These probes were then implemented with derivatives bearing an extra photoactivatable diazirine (*O*- and *N*-x-AlkTMMC15) in order to identify mycolate-protein interactions.²⁶ In another approach, the same group developed deoxy-^[18F]fluoro-trehalose FDTre for positron emission tomography imaging.²⁷ Recently, we described the first alkyne-modified trehalose monomycolate probes closely mimicking the native TMM *Alkm*₁₃ α ₀TMM and *Alkm*₁₃ α ₁₀TMM including either a simple β -hydroxylated fatty acid chain which is a hallmark of the natural meromycolate chain or an extra α -branched chain similar to those of mycolic acids allowing a better processing by mycoloyltransferases.²⁸ In a recent work, Kiessling's group developed a quencher-trehalose-fluorophore probe QTF with a an alkyl chain bearing

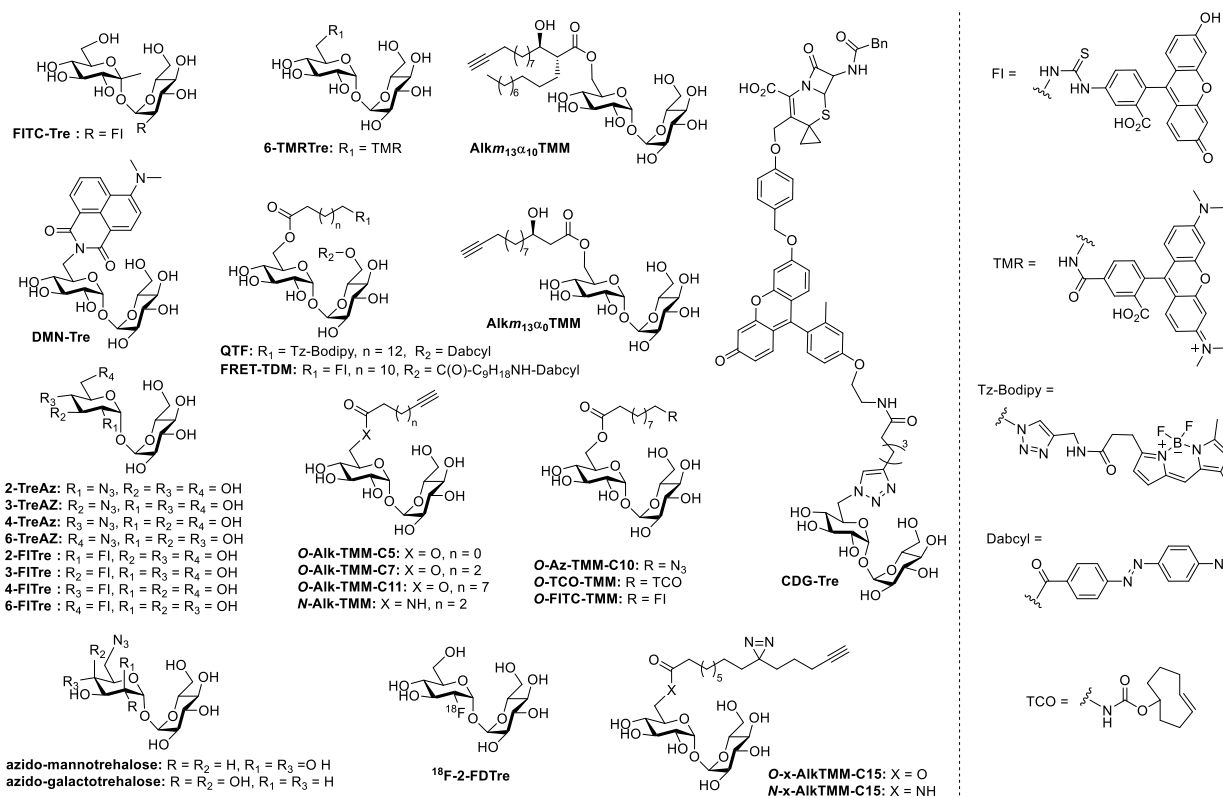


Figure 2. Overview of the previously reported trehalose-based probes bearing fluorescent or bioorthogonal moieties for mycobacteria labeling (see text for references).

the fluorescent dye BODIPY-FL fluorophore and the fluorescence quencher DABCYL linked to the 6'-hydroxyl of trehalose.²⁹ This elegant strategy was then also used by Swarts with another di-*O*-acylated trehalose FRET-TDM.³⁰ Finally, the new derivative CDG-Tre, was described by the Rao's group, targeting two *Mycobacterium tuberculosis* enzymes (*i.e.* Ag85 and BlaC).³¹

The synthesis of complex glycoconjugates, including fluorescent or bioorthogonal trehalose-based probes, usually requires multi-stage routes for the modification of the carbohydrate moieties. In the case of trehalose derivatives, regioselective protection and deprotection steps in order to differentiate the eight hydroxyl groups are required, as clearly exposed in recent

reviews.³² The desymmetrization of trehalose has long time been studied and several methods were reported in the literature.^{32,33} Briefly, the synthesis of unsymmetrical trehaloses can be achieved either starting from two different glucose units by creating the 1,1- α,α -linkage,³⁴ or directly from natural trehalose as inexpensive starting material, the latter being the most used approach. Indeed, native trehalose can be desymmetrized using the different reactivity between primary and secondary alcohols of the glucose units. For example, unsymmetrical trehaloses can be obtained by monoreaction at one of the two primary positions or after full functionalization of the two 6-OH and subsequent modifications of the secondary alcohols.³⁵ In this field, selective modifications in order to leave

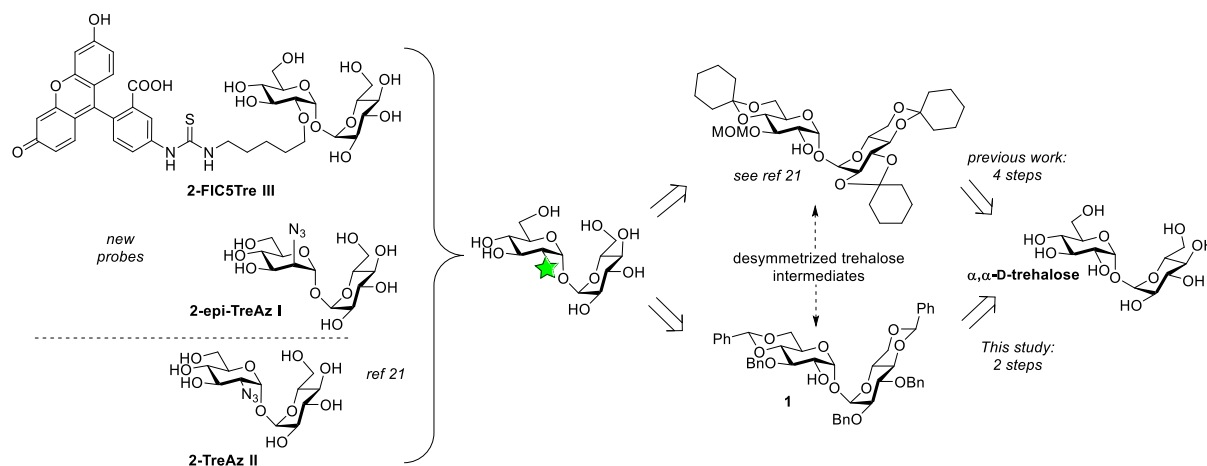


Figure 3. Our approach using tandem desymmetrization of trehalose, and structures of the trehalose-based probes prepared in this study.

one free secondary OH is still challenging and often requires multisteps processes.

In order to avoid time-consuming steps for the regioselective protection of carbohydrates, we^{36,37,38,39,40} and other^{41,42,43,44,45,46,47} groups have developed one-pot tandem protocols⁴⁸ mediated by acids for regioselective protection of per-*O*-silylated carbohydrates involving acetalation and reductive etherification. Among the various promoters used, we found FeCl₃•6H₂O to be the most efficient promoter for the protection of tri- and disaccharides.^{37,39} We have also used this cheap, easy to handle and environment friendly catalyst for the fast access to regioselectively protected trehalose derivatives in the synthesis of sulfolipid analogues from *M. tuberculosis*.^{49,50,51} We therefore envisioned that this method could provide easy access to various trehalose analogues useful for the study of the mycomembrane in live mycobacteria through either the one-step or the two-step labeling approach.

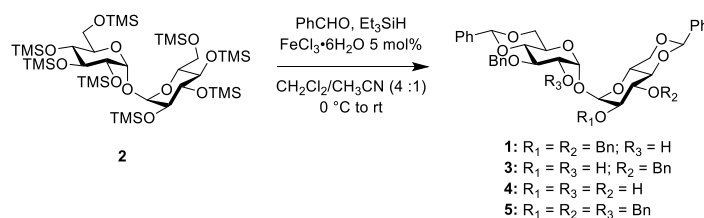
In a previous study, 2-TreAz II was synthesized starting from a desymmetrized trehalose intermediate obtained in four steps starting from trehalose (Figure 3, top).^{21,52,53} We thought that modifying the experimental conditions of our previously reported tandem protection of trehalose³⁷ would be useful for the rapid and one-pot access to a desymmetrized key intermediate allowing the fast introduction of a bioorthogonal moiety on C2 of trehalose (Figure 3, bottom). In this study, we report the synthesis of two new trehalose-based probes: 2-epi-TreAz I, an epimer of the known 2-TreAz II, and a conjugated fluorescein trehalose 2-FIC5Tre III starting from tri-*O*-benzylated trehalose 1, obtained in one-pot using modified conditions of our iron(III) chloride-mediated tandem catalysis, as well as a new access to 2-TreAz II (Figure 3). We also demonstrate the utility of the two new probes for the labeling of the mycomembrane of Corynebacteria by flow cytometry and fluorescence microscopy experiments.

Results and discussion

Chemical synthesis of trehalose-based probes

In our preliminary account with FeCl₃•6H₂O as promoter, we reported the one-pot protection of per-*O*-silylated trehalose 2 in the presence of 6 equiv of benzaldehyde, 2.2 equiv of triethylsilane and 5 mol% of FeCl₃•6H₂O leading to the symmetrical di-*O*-benzylated compound 3 in 61% yield (Table 1, entry 1).³⁷ In the course of this previous study, the desymmetrized tri-*O*-benzylated 1 and its mono-*O*-benzylated counterpart 4 were also identified as by-products in 15% and 20% yields respectively. We envisioned that the experimental conditions could be adjusted to obtain predominantly the desymmetrized key derivatives 1. Lowering benzaldehyde and triethylsilane amounts and the reaction concentration resulted in a decrease in yields for compounds 3 and 1 and the formation of the mono-*O*-benzylated derivative 4 as major compound in moderate yields (entry 2 and 3). In contrast, increasing amounts of benzaldehyde and triethylsilane, as well as reaction time, resulted in the formation of derivatives 1 and 3 in 38% and 36% yields respectively (entry 4). Under these conditions, we have

also isolated the tetra-*O*-benzylated derivative 5 as by-product. The proportion of expected compound 1 next fall to 31% yield when 10 mol% of catalyst were used (entry 5). Finally, the use of 10 equivalents of benzaldehyde and 4 equivalents of triethylsilane gave the expected desymmetrized derivative 1 in 40% yield (entry 6). We also tried to improve this yield by performing the reaction at higher concentration, but in this case compound 1 was only obtained in 31% yield (entry 7). When scaling up such reactions, yields can notoriously be limited by challenging purifications. As anticipated, we therefore observed a decreased but decent yield (i.e. 30-36%) when the synthesis of the tri-*O*-benzylated derivative 1 was performed on larger scales (i.e. 0.5-2.0 g of starting material). Thereby, we have access to the protected and desymmetrized trehalose 1 in one step starting from per-*O*-silylated trehalose 2 with a very satisfactory yield taking into account the five transformations catalyzed in one-pot (i.e. approximately more than 80% yield for each individual reaction).



Entry ^a	PhCHO (equiv)	Et ₃ SiH (equiv)	time	4 (%)	3 (%)	1 (%)	5 (%)
1	6	2.2	2 h	20	61	15	-
2 ^b	3.2	1.1	2 h	35	19	-	-
3 ^c	3.2	1.1	2 h	26	6	-	-
4	8	4	18 h	-	36	38	12
5 ^d	8	4	18 h	-	41	31	7
6	10	4	18 h	-	27	40	16
7 ^e	10	4	18 h	-	26	31	10

Table 1. modification of initial conditions in order to optimize the yield of compound 1: ^areactions were typically performed at 0.5-1 M; ^bthe bis-4,6-*O*-benzylidene derivative was also obtained in 30% yield; ^cin this case the reaction was performed at 0.1 M, the bis-4,6-*O*-benzylidene derivative was also obtained in 50% yield; ^d10 mol% of catalyst were used instead of 5 mol%; ^ein this case the reaction was performed at 2 M.

We took advantage of key precursor 1 for the synthesis of trehalose-based probes modified at C-2 for mycobacteria imaging. Indeed, trehaloses functionalized at the C-2 position were already described as efficiently processed by mycoloyltransferases, thus allowing the labeling of trehalose glycolipids in live mycobacteria. We chose to synthesize *i*) two azido-trehalose-based probes: the already described 2-TreAz II with native stereochemistry and the axial 2-epi-TreAz I in order to evaluate its processing by mycobacteria since its access is easier and shorter than the native 2-TreAz and *ii*) a new fluorescent trehalose analogue 2-FIC5Tre III with the native trehalose core alkylated at C-2 with a small linker (C5).

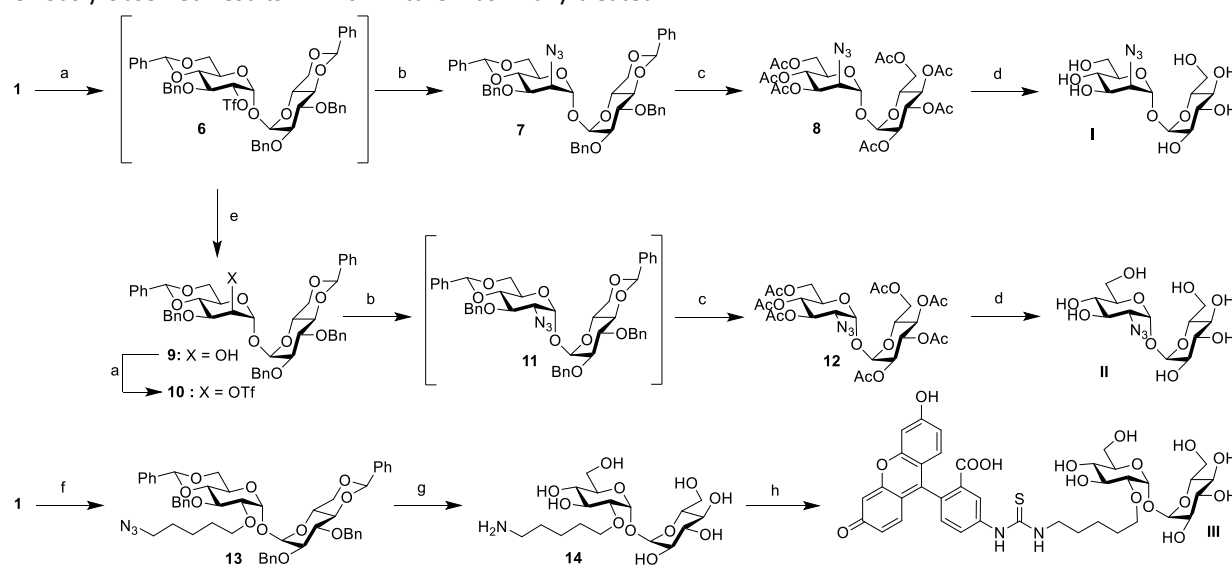
For the synthesis of probe **I**, derivative **1** was first converted quantitatively into triflate **6** using Tf_2O in pyridine, as a key intermediate. The azido moiety was next introduced by nucleophilic substitution of the triflate group in the presence of TBAN_3 to give compound **7** in 98% yield (Scheme 1). This two steps sequence resulted in the rapid introduction of the azido group as chemical reporter at a C-2 axial position starting from derivative **1**. For the deprotection of alcohol groups, we chose to use an excess of anhydrous FeCl_3 in dichloromethane, a known procedure for benzyl removal that preserves conjugated carbon-carbon double bonds^{50,54,55} and should also retain the azido moiety. Treatment of compound **7** with a suspension of an excess of anhydrous FeCl_3 in dry dichloromethane unfortunately resulted in a complex mixture of fully or partially unprotected derivatives and degradation products. The purification of the products, even after per-*O*-acetylation of the crude mixture (Ac_2O -pyridine), failed as well as attempted optimizations of this deprotection sequence. Finally, for the deprotection of **7**, we used the one-pot FeCl_3 - Ac_2O ⁵⁶ combination that has been previously reported to be very efficient for de-*O*-benzylation-acetylation of benzylated alcohols. Thus, compound **7** was treated with excess of anhydrous FeCl_3 and Ac_2O in dichloromethane leading to the per-*O*-acetylated derivative **8** in 61% yield. 2-epi-TreAz **I** was then obtained in high yield after de-*O*-acetylation in mild conditions (Scheme 1). For the synthesis of known 2-TreAz **II**, we performed a double-inversion process for the introduction of the azido group on a 2-equatorial position using the Lattrell-Dax nitrite-mediated inversion.^{57,58,59,60} Triflate **6** was first treated with NaNO_2 leading, after hydrolysis, to **9** in 46% yield. Derivative **9** was converted into **10** with Tf_2O and subsequent substitution of the triflate group was achieved using TBAN_3 . Under these optimized conditions, the expected compound **11** was obtained in a 2/1 mixture with an inseparable compound, supposed to be an elimination by-product, in agreement with previously observed results.²¹ This mixture was finally treated

with FeCl_3 - Ac_2O leading to per-*O*-acetylated 2-TreAz **12** in 25% overall yield starting from **9** (3 steps). Final de-*O*-acetylation of **12** led to 2-TreAz **II** in high yield. For the synthesis of the fluorescent analogue **III**, derivative **1** was first alkylated with 1-azido-5-iodo-pentane leading to alkylated derivative **13** in 87% yield. Complete hydrogenolysis performed in a mixture of 1,4-dioxane and water led to amino derivative **14** in high yield, which was then converted into the expected probe **III** after treatment with FITC (Scheme 1).

Mycomembrane labeling experiments

We first evaluated the intrinsic effect of the new probes 2-epi-TreAz **I** and 2-FIC5Tre **III** on *C. glutamicum* growth using strain ATCC13032. No growth inhibition with these analogues was observed upon treatment of *C. glutamicum* with up to 1 mM of probe, which indicates that neither the introduction of a bulky fluorophore nor the change of stereochemistry in position C2 affects bacterial growth (Figure S1, ESI[†]).

Next, we evaluated 2-epi-TreAz **I** and 2-TreAz **II** for MM labeling in *C. glutamicum* by quantitative flow cytometry analysis and qualitative fluorescence microscopy analysis. Wild-type *C. glutamicum* was cultured for 8 h with 2-epi-TreAz **I** and the previously reported 2-TreAz **II** as a control at 250 μM , then reacted with alkyne functionalized carboxyrhodamine 110 (CR110-CCH) using CuAAC. Interestingly, analysis by flow cytometry showed that the two epimers **I** and **II** have the same efficiency to label *C. glutamicum*, thus showing that stereochemical modification of the native C-2 position doesn't alter the processing by enzymes (Figure 4A) and illustrating the substrate tolerance of the mycoloyltransferases. Such tolerance on inversion of stereochemistry was also observed on an asymmetric trehalose analogue (i.e. mannotrehalose) which was shown to be resistant to degradation by mycobacterial enzymes.²⁴



Scheme 1. Synthesis of probes **I**, **II** and **III**. Reagents and conditions: a) Tf_2O (2.2 equiv), pyridine, CH_2Cl_2 , 0 °C to rt, 3h; b) TBAN_3 (4 equiv), toluene, 50 °C, overnight, (**7**, 98%, (two steps)); c) FeCl_3 (21 equiv), Ac_2O (40 equiv), CH_2Cl_2 , 0 °C to rt, 48h, (**8**, 61%; **12**, 25% (three steps)); d) K_2CO_3 (0.5 equiv), MeOH, rt, overnight, (**I**, **II**, 99%); e) NaNO_2 (18 equiv), DMF, 46% (two steps); f) NaH (3 equiv), N_3 -(CH_2)₅-I (3 equiv), DMF, 0 °C to rt, overnight, 87%; g) H_2 (1 atm.), $\text{Pd}(\text{OH})_2$, 1,4-dioxane/ H_2O (2:1), 40 °C, 3h30, quantitative; h) FITC (1.2 equiv), $\text{CH}_3\text{CN}/\text{NaHCO}_3$ buffer, 60 °C, 4 h, 66%.

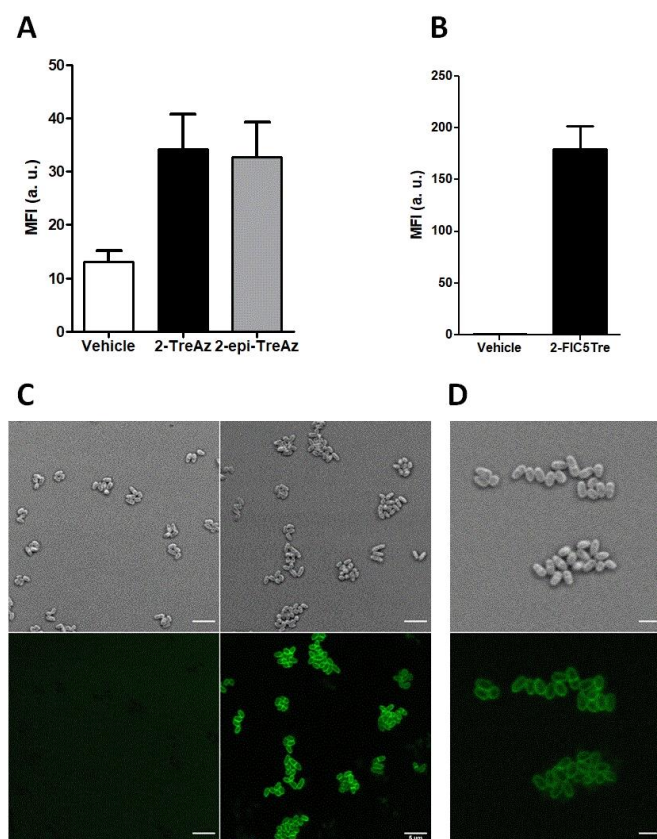


Figure 4. A. *C. glutamicum* strain ATCC1302 incubated with 250 μM of 2-epi-TreAz or 2-TreAz for 8 h, then reacted with CR110-CCH and analyzed by flow cytometry. B. *C. glutamicum* incubated with 100 μM of 2-FIC5Tre for 4 h and analyzed by flow cytometry. C. *C. glutamicum* incubated with 100 μM of 2-FIC5Tre (or untreated) for 4 h, then analyzed by fluorescence microscopy with bright field images on top. Scale bars = 5 μm . D. Zoomed-in view of *C. glutamicum* labeled with 100 μM of 2-FIC5Tre (different field). Scale bars = 2 μm . Data represent the mean of at least three replicate experiments.

We also evaluated the new 2-FIC5Tre **III** using a one-step labeling protocol. Wild-type *C. glutamicum* was cultured with 2-FIC5Tre **III** at 100 μM for 4 h. Analysis by flow cytometry showed a strong labeling of *C. glutamicum* (Figure 4B), which proved better than with the chemical reporter strategy. It was indeed reported that the secondary reagents used to click the fluorescent probes in the two-step labeling approach could have limited access to the MM and be engaged in nonspecific interactions.^{22a} Subsequently, fluorescence microscopy was used to visualize cell-surface incorporation of 2-epi-TreAz **I**, 2-TreAz **II** and 2-FIC5Tre **III**. In these experiments, 2-epi-TreAz **I** and the known 2-TreAz **II** showed similar behaviors (Figure S2 ES1†). 2-FIC5Tre **III** displayed strong labeling efficiency (Figure 4C, 4D). In all cases, consistent with previous observations with such probes, a membrane-specific labeling was clearly observed all around bacteria and more specifically at cell poles and septum, the major sites of cell membrane growth in *C. glutamicum*.

Conclusions

In conclusion the $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ -modified protocol appears to be a valuable method for the one-pot functionalization and desymmetrization of the C2-symmetrical trehalose, and we reported here a fast access to the two new MM chemical probes 2-epi-TreAz, and 2-FIC5Tre, as well as a new access to 2-TreAz. We showed that the desymmetrized key intermediate **1** is a valuable starting building block for the fast synthesis of trehalose-based probes. The two new probes were efficient to label *C. glutamicum* cell envelope as indicated by flow cytometry and fluorescence microscopy experiments. We found that the easily accessible 2-epi-TreAz probe is as effective as 2-TreAz, indicating a great tolerance of the mycoloyltransferases. We also observed a high labeling of the *C. glutamicum* envelope with fluorescent 2-FIC5Tre highlighting the efficiency of this one-step approach compared to the two-step bioorthogonal chemical reporter strategy in the case of TreAz analogues as previously observed. The synthesis of new tools for the study of the mycomembrane starting from the key intermediate **1** is underway in our laboratory.

Author Contributions

MC performed the syntheses of probes I and II. EL performed the synthesis of probe III and the biological evaluation of the probes. AB performed microscopy experiments and the fluorescence data experiments analysis. AL and LR were involved in the preparation of compound 1. KG was involved in probe III purification. CD carried out bacteria cultures. GD and DU brought their expertise in synthetic approach. NB brought his expertise in corynebacterial study. JMB was involved in tandem catalysis. BV brought his expertise on metabolic labeling and carefully proofread the manuscript. YB designed the study. DG and YB drafted the manuscript.

Conflicts of interest

There are no conflicts to declare.

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