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# First access to a mycolic acid-based bioorthogonal reporter for the study of the mycomembrane and mycoloyltransferases in *Corynebacteria*†

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**In this study, we report the first synthesis of an alkyne-based trehalose monomycolate probe containing a β-hydroxylated fatty acid and an α-branched chain similar to those of the natural mycolic acid. We demonstrate its utility for the labeling of the mycomembrane of *Corynebacteria* as well as for the study of mycoloyltransferases.**

*Corynebacteriales* are an order of *Actinobacteria* that include numerous human pathogens, such as the etiologic agents of tuberculosis, leprosy and diphtheria. They are characterized by an atypical envelope containing unique high molecular weight α-branched β-hydroxylated fatty acids (C30–90) called mycolic acids.[1] They have been shown to play a critical role in the structure and function of the cell wall.[2–4] At the end of their biosynthesis, mycolic acids are transferred onto the disaccharide trehalose leading to trehalose monomycolate (TMM). Once transported into the periplasm, TMM will play the role of a mycolate donor for mycolate acceptors (Fig. 1A): (i) the arabinogalactan (AG) layer, covalently linked to the peptidoglycan layer, (ii) another TMM to form trehalose dimycolate (TDM) or even (iii) proteins. Both TMM and TDM are part of a layer which associates non-covalently to a mycoloyl–arabinogalactan–peptidoglycan complex to form an outer membrane usually referred to as the mycomembrane (MM) which is unique to *Corynebacteriales* in term of structure and composition.[5,6] Some proteins that localize in the mycomembrane of *Corynebacterium glutamicum*, forming heterooligomeric complexes with channel-forming activity *in vitro*[7] have recently been shown to be covalently

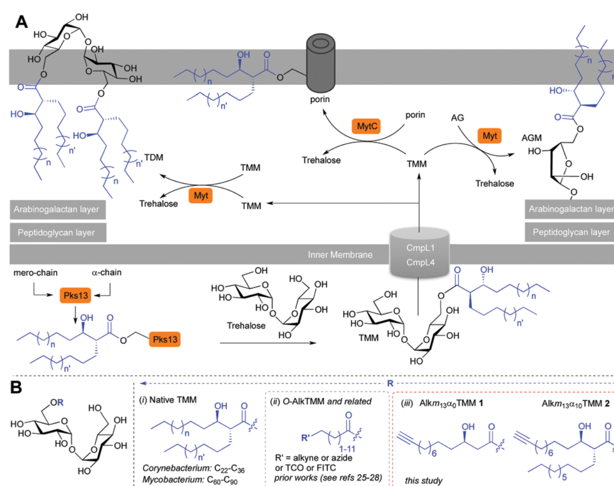


Fig. 1 (A) Schematic overview of the mycoloylation of various acceptors, arabinogalactan, TMM and porins by several mycoloyltransferases (Myt) in *C. glutamicum*. (B) Structure of (i) natural TMM, (ii) previously described trehalose chemical reporters esterified with unbranched and non-hydroxylated fatty acids (*O*-AlkTMM and related), and (iii) the two novel alkyne-based TMM-mimicking reporters, Alkm$_{13}$α$_{0}$TMM **1** and Alkm$_{13}$α$_{10}$TMM **2** described in this study.

modified at serine residues with mycolic acids.[8–10] This post-translational modification is essential for their activity.[11] The transfer of mycolic acids on their various acceptors is catalyzed by multiple mycoloyltransferases that are key enzymes in the biogenesis of the envelope of *Corynebacteriales*. Four Ag85 have been described in *M. tuberculosis*, 6 Fbp in *M. smegmatis* and 6 Myt in *C. glutamicum* which are the most studied *Corynebacteriales* so far.[12] They are collectively involved in the transfer of mycoloyl groups on trehalose and on the arabinose of AG but their individual *in vivo* specificity remains elusive.[13,14] Only MytC in *C. glutamicum* has been shown to be mainly involved in the mycoloyltransfer on proteins rather than on sugar acceptors.[15]

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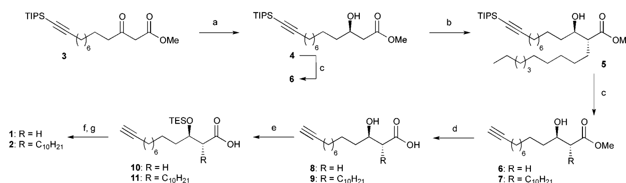
The unusual mycomembrane structure and composition of *Corynebacteriales* is responsible for low permeability of the envelope thus contributing to a natural resistance to various antimicrobial compounds and is involved in persistence in host environments. Given the essential role of the MM to mycobacterial species viability, MM components and their biosynthetic pathways are extensively studied because they provide opportunities for antibiotic development. Over the last decade, bioorthogonal chemical reporters have emerged as valuable tools for detecting bacterial cell surface components *in situ*.[16–19] Such developments include the study of MM components and several trehalose-based probes[20–23] have been developed allowing the visualization of the MM in live cells through the metabolic labeling approach.[24] TMM-based reporters have also been developed in recent years by the Swarts group for detecting MM components in mycobacteria (Fig. 1B). In these TMM mimics, the native mycolate is replaced by a shortened ester-linked alkyl chain with a terminal chemical reporter (*i.e.*, alkyne, azide, *trans*-cyclooctene or fluorescent tags).[25–28] After feeding of mycobacteria with these TMM surrogates, alkyne-tagged acceptors were labeled with a functionalized fluorophore using click chemistry. In a recent work, Kiessling's group developed an ingenious quencher–trehalose–fluorophore probe with an unbranched lipid bearing the fluorophore moiety enabling real-time imaging in a native cellular environment.[29] Until now, the synthesis of a mycolic acid-based reporter that tolerates a bioorthogonal group has never been described even though probes including the characteristic pattern of mycolic acids should be important for substrate recognition and for mycoloyltransferase catalyzed transesterification.

To further extend the set of chemical reporters for mycomembrane labeling and also for monitoring mycoloyltransferase activity *in vivo*, we sought to synthesize and demonstrate the utility of alkyne-based TMM probes 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.

To this aim, we designed two new probes with a terminal alkyne group: $Alkm_{13}\alpha_0$TMM **1** (Alkyne-mero$_{C13}$-$\alpha_{C0}$-trehalose monomycolate) which is a β-hydroxylated unbranched 6-*O*-acylated trehalose with a truncated alkyl chain (C13) and $Alkm_{13}\alpha_{10}$TMM **2** (Alkyne-mero$_{C13}$-$\alpha_{C10}$-trehalose monomycolate) which is a β-hydroxylated α-branched 6-*O*-acylated trehalose with two lipid chains mimicking the native meromycolic chain (C13) and the α-chain (C10) (Fig. 1B). The chain lengths were chosen to obtain soluble probes for the labeling experiments *in vivo* while closely mimicking the natural compounds, the predominant saturated form of TMM in *C. glutamicum* being the trehalose C32:0 corynomycolate [(2*R*,3*R*)-3-hydroxy-2-tetradecyloctadecanoate, see Fig. 1(B-i), with $n$ = 11 and $n'$ = 9]. The main challenges in the synthesis of native TMM and mycolic patterns are (i) to set up the anti-relationship at $C_2$–$C_3$ between the β-hydroxyl group and α-chain of mycolic acid and (ii) to perform a selective mono-6-*O*-esterification onto the $C_2$-symmetric trehalose. Procedures for the synthesis of native corynomycolic acid[30] have already been reported. However, these procedures required adaptations to introduce a terminal alkyne functionality or other bioorthogonal function onto the mycolic acid moiety making the synthesis of such chemical reporters far from trivial. Our approach is based on Noyori enantioselective reduction of β-ketoester and diastereoselective alkylation of the resulting β-hydroxyester, as reported by Genêt.[30h] Both probes **1** and **2** were obtained starting from the well-known 2,3,4,2′,3′,4′-hexakis-*O*-(trimethylsilyl)-α,α-D-trehalose[31] and the β-ketoester **3** bearing a protected alkyne moiety. Enantioselective reduction of β-ketoester **3**, obtained after a two-atom homologation of the corresponding carboxylic acid using Masamune's procedure[32] (see ESI†), was performed under atmospheric pressure of $H_2$ at 50 °C in the presence 2 mol% of the (*R*)-BinapRuBr$_2$ complex, leading to β-hydroxyester **4** in high yield (Scheme 1) and an excellent enantioselectivity (ee > 95%). The enantioselectivity and the 3-(*R*) absolute configuration of **4** were established using Mosher's method (see ESI†).[33] Interestingly, this reaction was performed with an excellent chemoselectivity as the alkyne group was not affected under these conditions. The presence of the bulky TIPS group was crucial to achieve this result since the use of the less hindered TES or TMS protecting groups led to complex reaction mixtures with concomitant reduction of alkyne to alkene. To the best of our knowledge, this is the first example of a chemoselective reduction of a β-ketoester featuring an unconjugated alkyne moiety using Noyori reduction. Diastereoselective alkylation of β-hydroxyester **4** at low temperature gave derivative **5** in a moderate 35% yield but with an excellent diastereoselectivity (dr > 95%).[34] The anti-relationship was assigned by NMR measurement of the H2–H3 coupling constant of a cyclic acetonide derivative of **9** (see ESI†). After removal of the TIPS groups of both derivatives **4** and **5** using TBAF, saponification of compounds **6** and **7** afforded β-hydroxy acids **8** and **9** in 98 and 93% yields, respectively. The secondary hydroxyl groups of **8** and **9** were then silylated with TESCl leading to **10** and **11**. Controlled monoesterification of **10** and **11** with 2,3,4,2′,3′,4′-hexakis-*O*-(trimethylsilyl)-α,α-D-trehalose followed by full deprotection under acidic conditions[31b] afforded the new TMM reporters **1** and **2** in 51% and 36% yields, respectively (2 steps).




Scheme 1 Syntheses of TMM reporters **1** and **2**. *Reagents and conditions*: (a) (*R*)-BinapRuBr$_2$ (2 mol%), $H_2$ (1 atm), MeOH, 50 °C, overnight, 98%; (b-i) LDA, THF, −78 °C, 1.5 h; (ii) 1-iododecane, HMPA, THF, −78 °C to −10 °C, overnight, 35%; (c) TBAF, THF, 0 °C to r.t., overnight, **6** 63%, **7** 85%; (d) NaOH, $H_2O$/MeOH, 45 °C, overnight, **8** 98%, **9**, 93%; (e) TESCl, pyridine, r.t., (on **8**), 60 °C, (on **9**), overnight, **10**, 55%, **11**, 62%; (f) 2,3,4,2′,3′,4′-hexakis-*O*-(trimethylsilyl)-α,α-D-trehalose, EDCI, DMAP, DCM, r.t., 58% (starting from **10**), 37 °C, 42% (starting from **11**); (g) Dowex 50WX8 ($H^+$ form), MeOH/DCM, **1** 87%, **2** 85%.

We first evaluated the intrinsic effect of probes **1** and **2** on *C. glutamicum* strain growth. As with the previously reported *O*-Alk-TMM, **1** does not show a growth inhibition effect upon treatment of *C. glutamicum* up to 500 μM. Probe **2** has a different

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behavior since a complete growth inhibition was observed at 100 μM (Fig. S3, ESI†) however without bactericidal activity as evidenced by growth recovery when the probe is diluted. Under the same conditions, **1** and **2** have no effect on Gram-negative *Escherichia coli* and Gram-positive *Bacillus subtilis* growths up to 1 mM (Fig. S4 and S5, ESI†), thus suggesting an effect relying on a specific pathway in *C. glutamicum*. The two probes therefore have different behaviors suggesting a different processing by bacteria depending on the presence of the α-chain. To further understand if the growth inhibition with probe **2** was dependent on a mycoloyltransferase activity, we undertook a competition experiment with the authentic predominant form of TMM substrate (C32:0), that we synthesized using the same approach as described above.[35] Bacterial cells were co-incubated with **2** (100 μM) and TMM (500 μM). A restoration of bacterial growth was then observed thus suggesting that **2** could be processed by the same enzymes as the natural TMM (Fig. S3, ESI†).

To further investigate the role of mycoloyltransferases in processing the probes, we performed a colorimetric *in vitro* assay using *p*-nitrophenyl palmitate (*p*-NPP) as a substrate surrogate to follow esterase activity of the most abundant mycoloyltransferase MytA. Interestingly, an inhibitory effect of the *p*-NPP hydrolysis activity was observed when the synthetic TMM or probe **2** was added, indicating a competitive effect of these species (Fig. S6, ESI†). Under the same conditions, probe **1** showed no effect on enzymatic activity. Hence, probe **1** with the β-hydroxylated unbranched chain seem to behave differently from the natural TMM while probe **2** appears to be a better TMM analogue. This experiment clearly demonstrates a specific interaction between probe **2** and MytA suggesting its processing by mycoloyltransferases in a cellular context. Additional experiments will be required to discriminate between the different mechanisms that could explain the observed competition in the presence of probe **2**, *i.e.*, normal acyl transfer to another TMM analogue, hydrolytic activity, or formation of a stable complex with MytA.

Next, we evaluated probes **1** and **2** for MM labeling in *C. glutamicum.* Since mycoloyltransferases are highly abundant and active during the log-phase growth, we postulated that the addition of the probes at the mid-log phase culture would make it possible to carry out labeling with a short time treatment and with low doses of the probe, thus limiting the inhibition effect for probe **2**. Wild-type *C. glutamicum* was cultured until mid-log phase (7 h), and then treated with probes **1** or **2** for 1 h at 5 and 50 μM, then reacted with azide functionalized carboxyrhodamine 110 (CR110-Az) using Cu(I)-catalyzed azide alkyne cycloaddition (CuAAC). Analysis by flow cytometry showed that both probes are efficient to label *C. glutamicum* and allowed a specific labeling even at a concentration as low as 5 μM, and that the highest levels of bacterial labeling was obtained with probe **2** even if the difference was less important at 50 μM (Fig. 2A).

Subsequently, fluorescence microscopy was used to visualize cell-surface incorporation of probe **2**. Alkyne specific fluorescence was observed and clearly, the signal localizes at the surface of bacteria with the fluorescence concentrated at the bacteria pole and septum. This is consistent with the assumed mycoloyltransferase localization during cell elongation to provide new trehalose

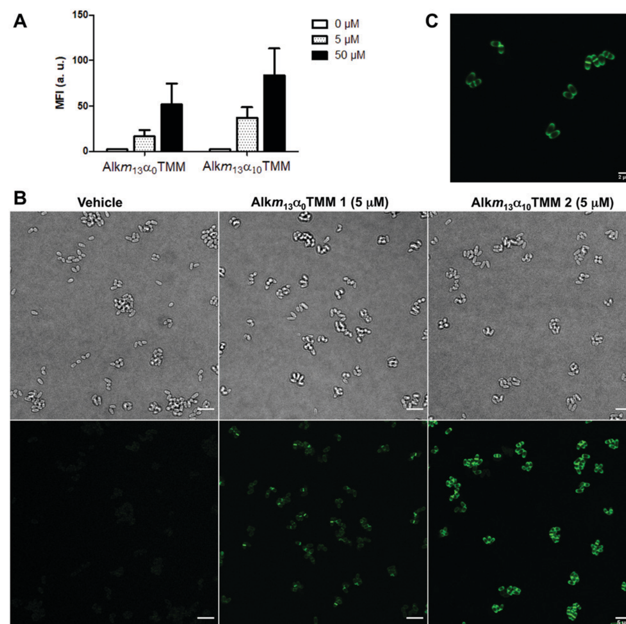


Fig. 2 *C. glutamicum* incubated with 5 or 50 μM of **1** or **2** (or untreated) for 1 h, then reacted with CR110-Az and analyzed by (A) flow cytometry (B) fluorescence microscopy with bright field images on top. Scale bars = 5 μm. (C) *C. glutamicum* labeled with 5 μM of probe **2**. Scale bars = 2 μm. Data represent the mean of three replicate experiments.

mycolates to be inserted into the MM (Fig. 2B, 5 μM and Fig. S7, 50 μM, ESI†). Similar results were observed with probe **1** but with less efficiency, further supporting a different behavior between these two compounds. The treatment of *E. coli* and *B. subtilis* that lack mycoloyltransferases afforded no detectable labeling (Fig. S8 and S9, ESI†) thus confirming specific incorporation of probes **1** and **2** into the mycomembrane.

In conclusion, we have developed a synthesis providing access to a trehalose monomycolate alkyne-based chemical reporter including, for the first time, a fatty acid chain closely mimicking the complex structure of hydroxylated and branched mycolic acids. We demonstrated that these probes are useful for MM labeling with a short labeling time and very low doses of the reporter in comparison with the protocols described so far. Initial experiments showed that the chemical reporter **2**, most closely mimicking TMM, revealed different biological behavior in comparison with the non-branched probe **1** both in the enzymatic assay *in vitro* with MytA and in the context of growth experiments. This could be explained by (i) a different binding mode either within the active site or in the non-catalytic trehalose binding site of the mycoloyltransferases (ii) a difference in selectivity among the 6 mycoloyltransferases for the two probes. The mycoloyltransferase catalyzed reactions proceed through two transesterification steps involving the serine of a catalytic triad. Several previous structural studies afforded mechanistic models underlying the role of the mycolate α-chain in the binding of TMM and the crucial conformational changes involved to block the hydrolysis of the acyl–enzyme intermediate.[36] When interacting with unbranched lipid or shorter trehalose ester, the enzymes could be either slowed down or

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disturbed from their acyltransferase activity. Indeed, a substantial acylhydrolase activity was already reported with Ag85s when acting on a simple lipid linkage.[36a] Additional studies are underway to determine the precise process by which the lipid chain surrogate of the different probes is transferred onto the acceptor in cells and to characterize the nature of the final clickable lipid chain deposited onto these mycoloyl acceptors. Finally, this work also paves the way to further studies of the structural determinants essential for the processing of such probes by the mycoloyl-transferases, which could help to better characterize the role and the mechanism of these enzymes.

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## Conflicts of interest

There are no conflicts to declare.

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