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Mara K. O'Neill^a, Brent F. Piligian^a, Claire D. Olson, Peter J. Woodruff
and Benjamin M. Swarts*

Tailoring trehalose for biomedical and biotechnological applications

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Abstract: Trehalose is a non-reducing sugar whose ability to stabilize biomolecules has brought about its widespread use in biological preservation applications. Trehalose is also an essential metabolite in a number of pathogens, most significantly the global pathogen *Mycobacterium tuberculosis*, though it is absent in humans and other mammals. Recently, there has been a surge of interest in modifying the structure of trehalose to generate analogs that have applications in biomedical research and biotechnology. Non-degradable trehalose analogs could have a number of advantages as bioprotectants and food additives. Trehalose-based imaging probes and inhibitors are already useful as research tools and may have future value in the diagnosis and treatment of tuberculosis, among other uses. Underlying the advancements made in these areas are novel synthetic methods that facilitate access to and evaluation of trehalose analogs. In this review, we focus on both aspects of the development of this class of molecules. First, we consider the chemical and chemoenzymatic methods that have been used to prepare trehalose analogs and discuss their prospects for synthesis on commercially relevant scales. Second, we describe ongoing efforts to develop and deploy detectable trehalose analogs, trehalose-based inhibitors, and non-digestible trehalose analogs. The current and potential future uses of these compounds are discussed, with an emphasis on their roles in understanding and combatting mycobacterial infection.

Keywords: analog; biocatalysis; biopreservation; chemical synthesis; chemoenzymatic synthesis; ICS-28; imaging; inhibitors; mycobacteria; trehalose.

Introduction

Trehalose (**1**) is a C_2 -symmetric, non-reducing disaccharide consisting of two glucose residues connected by a 1,1- α , α -glycosidic linkage (Fig. 1). It is abundant and widely distributed in nature, as it occurs in bacteria, yeast, fungi, plants, and invertebrates [1]. The distinctive structure and exceptional stability of trehalose give rise to its main biological function, which is stress protection [1]. The ability of trehalose to stabilize a wide range of biomaterials – including proteins, DNA, cells, and tissues – has been exploited for myriad preservation applications in the pharmaceutical, food, and cosmetic industries [2]. Beyond bioprotection, trehalose and its naturally occurring derivatives have various other functions, depending on the organism. For example, insects store high levels of blood trehalose, which can be rapidly utilized to permit flight [3]. In

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^aThese authors contributed equally to this work.

*Corresponding author: Benjamin M. Swarts, Department of Chemistry and Biochemistry, Central Michigan University, Mount Pleasant, MI 48859, USA, e-mail: ben.swarts@cmich.edu

Mara K. O'Neill, Brent F. Piligian and Claire D. Olson: Department of Chemistry and Biochemistry, Central Michigan University, Mount Pleasant, MI 48859, USA

Peter J. Woodruff: Department of Chemistry, University of Southern Maine, Portland, ME, USA

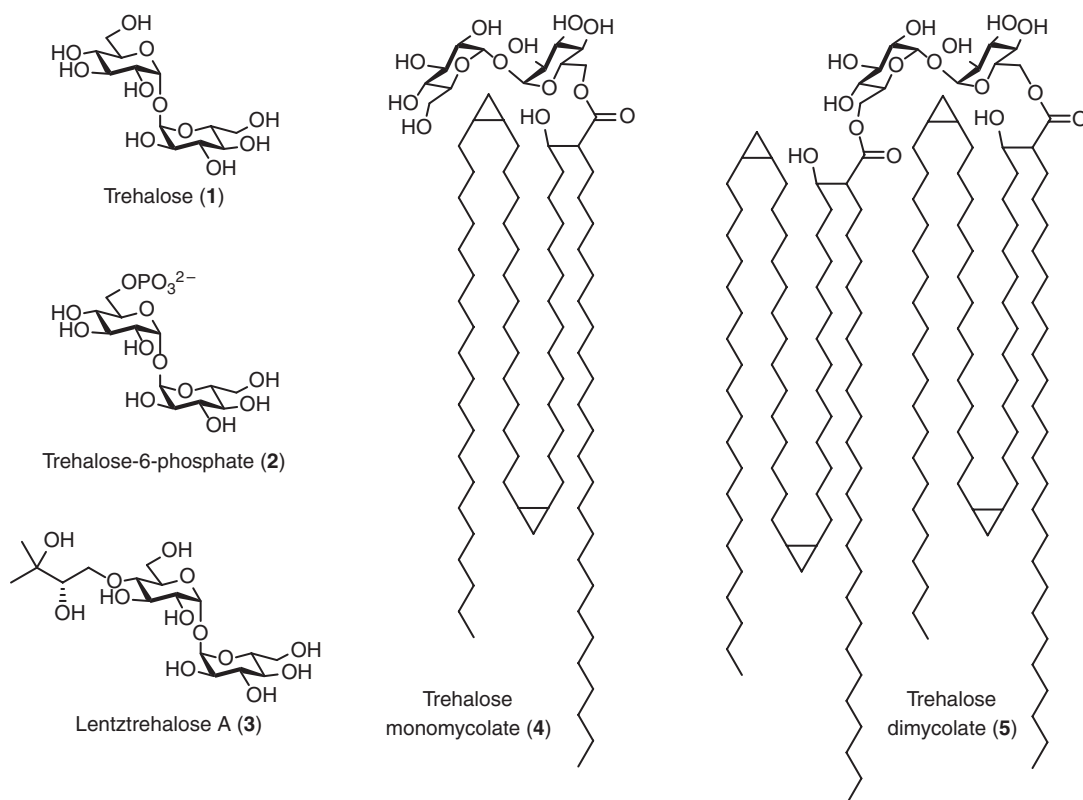


Fig. 1: Structures of trehalose and a few naturally occurring trehalose derivatives.

plants, trehalose's biosynthetic precursor, trehalose-6-phosphate (2), has been implicated in plant growth regulation and development [4]. Some actinobacteria express lentztrehalose A (3), which has a 2,3-dihydroxy-3-methylbutoxy group that renders the molecule resistant to degradation [5]. Trehalose is also essential for growth and virulence of globally significant pathogens such as *Mycobacterium tuberculosis*, which not only uses trehalose for energy storage and stress resistance, but also incorporates trehalose into virulence-associated cell envelope glycolipids (e.g. 4 and 5) [6].

Synthetic trehalose analogs, which contain structural modifications designed to impart particular properties or functions to the native disaccharide, are of interest for numerous applications. In relation to the expansive use of trehalose as a bioprotectant and food additive, there is considerable interest in developing metabolically stable versions of trehalose that, upon ingestion by a human, would not be broken down into glucose by the intestinal trehalase enzyme [7]. Indeed, trehalase-resistant trehalose analogs, including lentztrehalose A (3), may retain desirable properties (e.g. biomolecular stabilization, sweetness) while increasing residence time in the body and reducing caloric intake [5, 7]. Due to the importance of trehalose to various pathogens (e.g. *M. tuberculosis*) and insect vectors of pathogens (e.g. mosquitoes), as well as the lack of trehalose in mammals, recent research has focused on targeting trehalose metabolism in these organisms with analogs designed to facilitate disease diagnosis, treatment, or prevention of transmission [8, 9]. Unfortunately, the difficulties associated with trehalose analog synthesis have impeded progress in the development and applications of these compounds. Despite steady advances in the stereoselective construction of carbohydrates and carbohydrate analogs over the past few decades, the unique 1,1- α -glycosidic linkage of trehalose has rendered analog synthesis very challenging. In turn, it has been challenging to access trehalose analogs on a laboratory scale for research purposes, let alone on an industrial scale for commercial applications.

In this review, we discuss progress in the synthesis and applications of trehalose analogs. After a primer on trehalose metabolism to orient readers to this review's content, we provide an overview of the synthetic approaches used to produce trehalose analogs. Although chemical synthesis methods are briefly discussed

in this review, we point readers to two excellent reviews by the Kulkarni group focusing on chemical methods for 1,1- α,α -glycosylation and trehalose desymmetrization [10, 11]. It is noted that since 2013, a number of new reports describing chemical syntheses of various mycobacterial glycolipids have been published as well [12–18]. Here, we emphasize recent advances in trehalose analog synthesis using chemoenzymatic methods, which have been inspired by naturally occurring trehalose biosynthetic pathways. The bulk of this review is devoted to the various emerging applications of trehalose analogs, which range from the detection and treatment of pathogenic organisms to the development of non-digestible food additives. For readers interested in the latter topic, we highly recommend Desmet and co-workers' recent review, which mainly focuses on the biocatalytic production of hydrolytically stable trehalose analogs and their potential for use in the food industry [7]. We attempt to touch on most applications currently being pursued for trehalose analogs, paying particular attention to their current use and future potential in tuberculosis research and related areas. Overall, we seek to illustrate how rationally manipulating the structure of trehalose can engender this unique disaccharide with properties or functions that can benefit a wide range of fields.

Trehalose metabolism in nature

We begin this review with an overview of trehalose biosynthesis and utilization pathways that exist in nature. The purpose of this section is two-fold. First, it illustrates the biosynthetic pathways that have inspired chemoenzymatic methods to synthesize trehalose and trehalose analogs on laboratory and industrial scales. Second, it provides background for understanding many of the present and possible future biomedical and biotechnological applications of trehalose analogs.

Five pathways for de novo biosynthesis of trehalose have been reported (Fig. 2). In some organisms, multiple pathways may coexist. The most common route of trehalose biosynthesis in nature is the

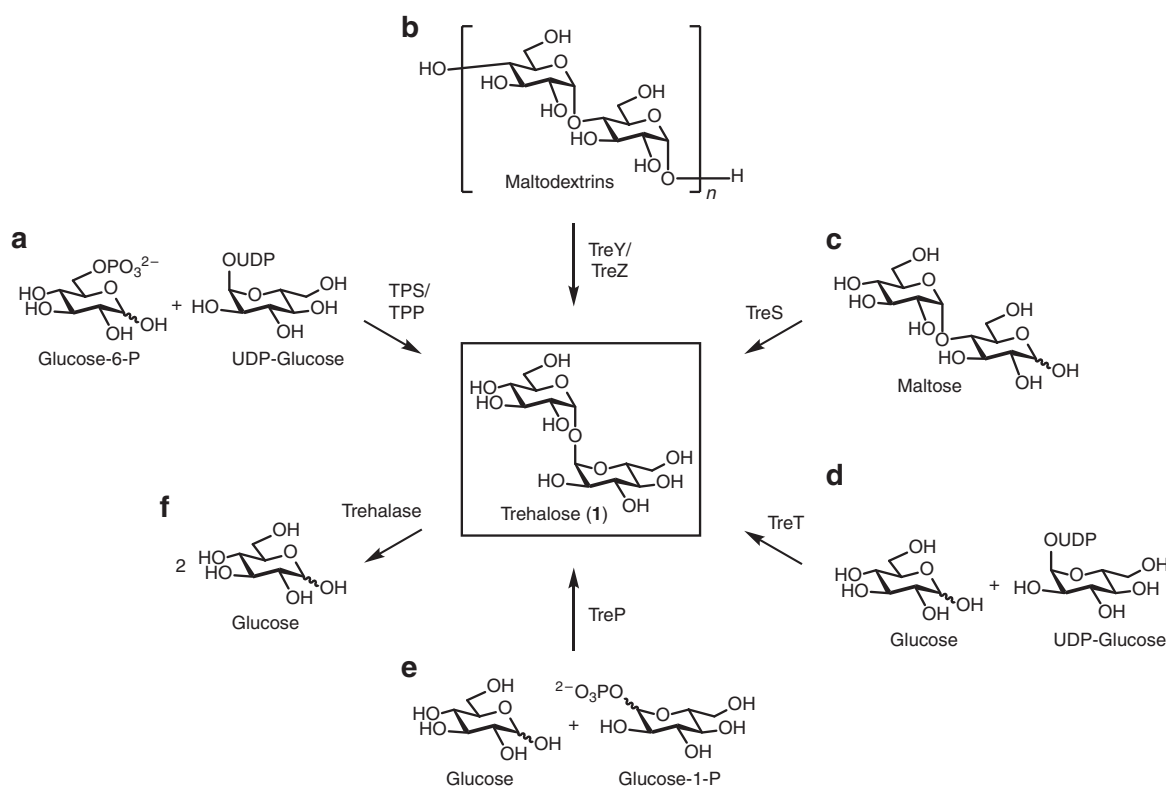


Fig. 2: Known trehalose biosynthesis (a–e) and degradation (f) pathways existing in nature. Note: TreP may catalyze both trehalose biosynthesis and trehalose degradation.

trehalose-6-phosphate synthase/trehalose phosphatase (TPS/TPP) pathway, which was first identified in yeast [19] and subsequently identified in many other eukaryotic and prokaryotic organisms, including bacteria, archaea, insects, and plants [1]. Commonly referred to as the OtsAB pathway in *Escherichia coli* [20], the two-step TPS/TPP pathway converts glucose-6-phosphate and uridine diphosphate (UDP)-glucose into trehalose-6-phosphate, which is subsequently dephosphorylated to yield trehalose (Fig. 2b). In some organisms, including species in the *Sulfolobus* and *Mycobacterium* genera, trehalose can be biosynthesized via the two-step TreY/TreZ pathway, which converts maltooligosaccharides into trehalose [21, 22]. In this pathway, TreY isomerizes the reducing end of the maltooligosaccharide substrate to generate maltooligosyltrehalose, which is then hydrolyzed by TreZ to give trehalose (Fig. 2b). Another trehalose biosynthesis pathway relies on trehalose synthase (TreS), which isomerizes the disaccharide maltose to trehalose (Fig. 2c) [23]. While the TreS pathway is involved in trehalose biosynthesis in vivo in some organisms, it appears to work in the opposite direction in *Mycobacterium* [24], instead converting trehalose to maltose, which is ultimately used to produce α -glucans [25, 26]. A fourth pathway, found in archaea, utilizes the trehalose-synthesizing glycosyltransferase TreT, which converts glucose and UDP-glucose into trehalose in one step (Fig. 2d) [27]. Finally, trehalose phosphorylase (TreP) is thought to break down trehalose to glucose and either α - or β -glucose-1-phosphate, depending on whether the TreP is retaining or inverting, respectively [7]. However, it is possible that TreP might also catalyze the reverse reaction, trehalose biosynthesis, in some organisms in vivo (Fig. 2e). The exploitation of some of these pathways for the laboratory synthesis of trehalose and trehalose analogs is covered later in this review.

The degradation of trehalose to glucose, which is catalyzed by the enzyme trehalase, is an important process in a multitude of organisms. In most scenarios, trehalose degradation occurs for the purposes of either (i) restoring cellular homeostasis after stress-induced trehalose overproduction; or (ii) producing glucose to meet energy and/or carbon demands [1]. Interestingly, although mammals do not have pathways for trehalose biosynthesis, they do possess intestinal and renal trehalase [28, 29], the former of which is known to be involved in the hydrolysis of ingested trehalose to glucose [30]. The presence of trehalase in humans has possible ramifications for many applications of trehalose and trehalose analogs, as discussed later.

In addition to biosynthetic and degradative pathways for trehalose, some organisms possess other pathways involving trehalose. Most notably, trehalose is central to the construction of the complex cell envelope of the *Corynebacterineae*, which include mycobacterial pathogens such as *M. tuberculosis*. Here, we summarize trehalose metabolic pathways that are conserved in mycobacteria (Fig. 3) because they are valuable

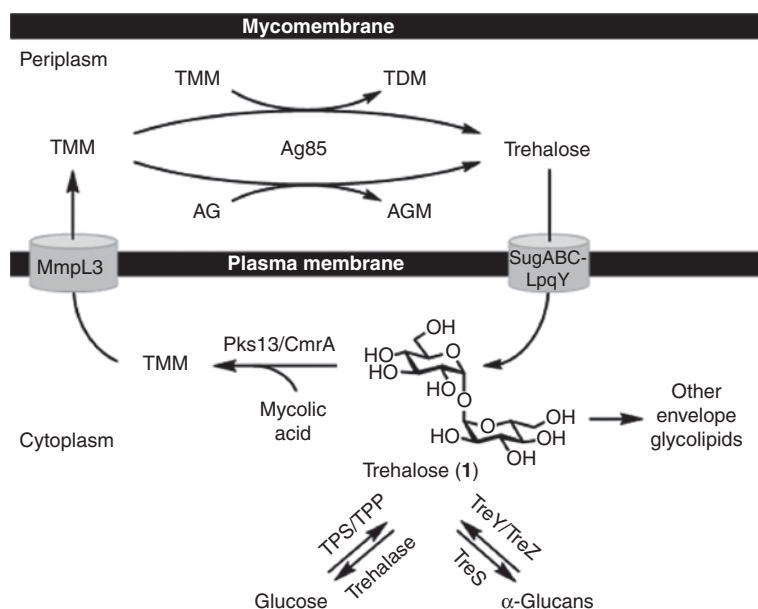


Fig. 3: Mycobacterial trehalose metabolism.

targets for the development of probes and inhibitors, as covered in detail later. For trehalose biosynthesis, mycobacteria use either the TPS/TPP or TreY/TreZ pathways [6, 22, 24, 31]. In an important pathway, trehalose is also lipidated, undergoing 6-*O*-mycoloylation by Pks13 to generate trehalose monomycolate [TMM (4), structure shown in Fig. 1] [32]. After crossing the plasma membrane via MmpL3 [33, 34], TMM donates its 6-*O*-mycoloyl group to two major acceptor molecules in service of constructing the unique mycobacterial outer membrane, or “mycomembrane.” These processes, which are catalyzed by the Ag85 complex, involve mycoloyl group transfer to either (i) terminal residues of the arabinogalactan polymer, generating arabinogalactan-linked mycolates (AGM); or (ii) a second molecule of TMM, generating trehalose dimycolate [TDM (5), shown in Fig. 1] [35–37]. AGM is the foundational inner leaflet component of the mycomembrane, while TDM resides in the mycomembrane’s outer leaflet and is an important contributor to mycobacterial pathogenesis [38–40]. When AGM and TDM are formed, free trehalose is released and reclaimed by the cell through the trehalose-specific transporter SugABC-LpqY [41]. This trehalose recycling step is essential for *M. tuberculosis* virulence and underscores the significance of trehalose uptake pathways, which appear to exist in many organisms. In addition to serving as the essential mediator of mycomembrane biosynthesis, trehalose is also incorporated into numerous other mycobacterial glycolipids, such as sulfolipids [42], polyacyltrehaloses [43], lipooligosaccharides [44], and the recently identified trehalose polyphosphates [45]. For a more extensive discussion of trehalose metabolism in mycobacteria, we refer readers to a recent review by Kalscheuer and Koliwer-Brandl [6].

The pathways described above, which can be categorized into trehalose biosynthesis, degradation, uptake, or elaboration to glycolipids, have important implications for the development and applications of trehalose analogs. Even the limited overview of trehalose metabolism provided above hints at various possible uses of trehalose analogs. Trehalose-based probes are critical tools for unraveling the complex functional and regulatory aspects of trehalose metabolism across all domains of life. Trehalose analogs containing detectable moieties – the archetypal example being ^{14}C -labeled trehalose – have applications ranging from basic biological inquiry to the detection of pathogens. Likewise, trehalose-based inhibitors can be useful as probes of trehalose-processing enzymes, and perhaps as therapeutic compounds in the future. The presence of trehalose uptake pathways in pathogens suggests the possibility of delivering trehalose-linked diagnostic or therapeutic cargo using Trojan horse-type strategies, which are of particular interest because trehalose metabolism is largely absent from mammals. On the other hand, mammals do have the ability to degrade trehalose into glucose. This must be considered in the contexts of pathogen targeting and consumption of native trehalose from food and pharmaceutical products. In this respect, non-degradable trehalose analogs may be of value for numerous applications. Lastly, trehalose biosynthetic pathways have the potential to be adapted for the chemoenzymatic synthesis of analogs, which is of high interest due to the drawbacks of chemical synthesis. Recent efforts in this area are highlighted in the next section of this review.

Approaches to trehalose analog synthesis

The development of synthetic methods to access trehalose derivatives has primarily been motivated by interest in the bioactive glycolipids of *M. tuberculosis*, such as TMM (4), TDM (5), and sulfolipid-1. Because these molecules have important immunomodulatory properties, it is necessary to have access to pure and structurally well-defined samples to elucidate their structure–activity relationships (SARs). Earlier reviews by Asselineau in 1978 [46] and Timmer and Stocker in 2012 [47] provided excellent accounts of the structures, activities, and chemical syntheses of naturally occurring trehalose-containing glycolipids. The decades-long work in this field has led to the development of numerous useful synthetic methods, while simultaneously calling attention to the significant challenges associated with synthesizing trehalose derivatives. Here, we discuss these challenges and provide an overview of the different approaches that have been used to synthesize modified trehaloses, including traditional chemical approaches and more recent chemoenzymatic approaches.

Chemical synthesis of trehalose analogs

The chemical synthesis of carbohydrates and their analogs has been driven forward by the development of reliable and general methods for the regioselective functionalization of hydroxyl groups and the stereoselective construction of glycosidic bonds. However, trehalose has an atypical 1,1- α -glycosidic linkage that joins two glucose units at their anomeric positions, making the molecule C_2 -symmetric. Many established carbohydrate synthesis methods are not well-suited to these structural features, so trehalose-specific synthetic methods have been developed. Generally, there are two chemical synthesis strategies that can be used to access trehalose analogs, including (i) stereoselective 1,1- α -glycosylation reaction between two appropriately functionalized glucoses (Fig. 4a); (ii) desymmetrization of natural trehalose using regioselective protection/deprotection steps (Fig. 4b).

With respect to the chemical glycosylation approach (Fig. 4a), 1,1- α -glycosidic bonds remain among the most difficult to forge with good stereoselectivity. First, the 1,1- α -glycosidic linkage is of the 1,2-*cis* variety, which is incompatible with neighboring-group participation strategies that are employed to stereoselectively form 1,2-*trans* linkages. Second, this type of glycosylation reaction involves the simultaneous generation of two glycosidic bonds, so three stereoisomers (α,α ; α,β ; β,β) are possible for symmetrical products and four stereoisomers (α,α ; α,β ; β,α ; β,β) are possible for unsymmetrical products. Not only are these reactions generally inefficient, but the isomeric products are very difficult to separate. Reflecting these challenges, early efforts to chemically synthesize native 1,1- α -trehalose through reactions between protected glucose hemiacetals and various protected glucosyl donors (e.g. 1,2-epoxy sugars, glycosyl halides, glycosyl trichloroacetimidates) usually resulted in complex reaction mixtures and low to moderate yields of the desired 1,1- α -product [48–50]. In fact, trehalose is now produced on an industrial scale by an enzymatic process due to low yields from chemical synthesis methods (discussed in further detail in the following section). Not surprisingly, classical chemical glycosylation methods have only been sparingly applied to the preparation of trehalose analogs.

A few custom chemical 1,1- α -glycosylation methods have been developed to overcome the problems discussed above. Two particularly inventive approaches that afforded excellent 1,1- α -stereoselectivity are discussed here. Ikegami and co-workers developed a method for the synthesis of ketoside trehalose analogs,

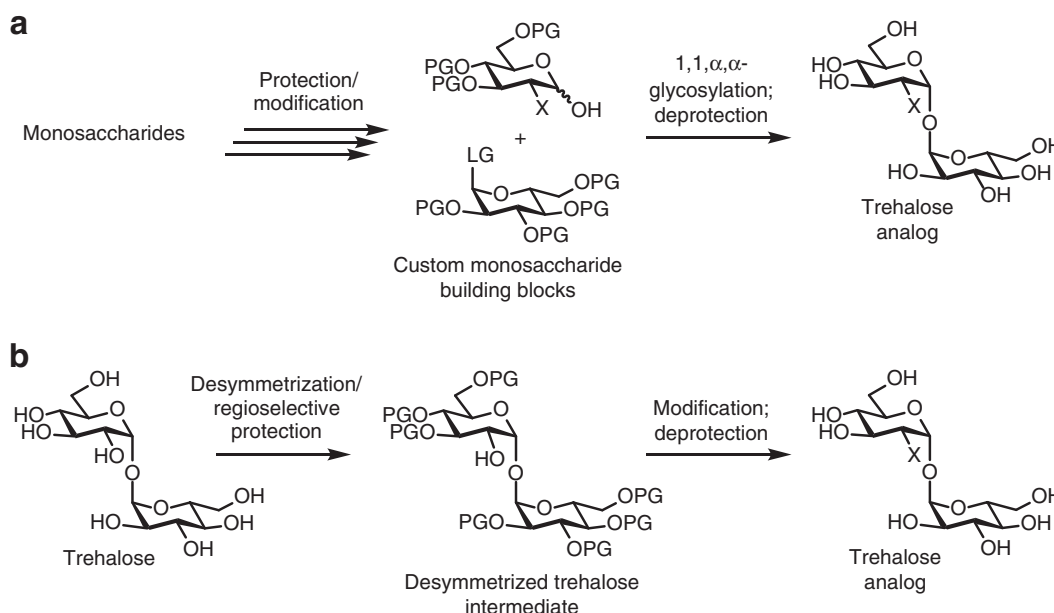


Fig. 4: Chemical synthesis of trehalose analogs via (a) 1,1- α -glycosylation between monosaccharide derivatives and (b) regioselective functionalization of trehalose. LG, leaving group; PG, protecting group; X, structural modification.

which were obtained by reacting an α -configured trimethylsilyl glycoside acceptor with either an *exo*-glycal or a ketose donor in the presence of a Lewis acid catalyst (Fig. 5a) [51]. Although custom sugar donors are required for this reaction, its complete 1,1- α,α -stereoselectivity and excellent yields (88–96 %) make it a powerful method. As discussed later, Ikegami's method was later applied to the synthesis of a fluorescent-tagged ketoside trehalose analog used for the detection of *M. tuberculosis* [52]. The Bertozzi group developed another method for chemical 1,1- α,α -glycosylation [53], which beautifully exploited intramolecular aglycon delivery [54] (Fig. 5b). In this case, the glycosyl acceptor and thioglycoside donor were first tethered to each other via a mixed acetal linkage, placing them in the proper orientation to undergo exclusive 1,1- α,α -glycosylation upon subsequent activation of the thioglycoside. This method, which also required specialized sugar building blocks, was used to synthesize complex sulfolipid-1 analogs from *M. tuberculosis* for immunological SAR studies [55, 56]. For a comprehensive review of chemical 1,1- α,α -glycosylation methods, readers are referred to a recent review article by Chaube and Kulkarni [10].

A more common approach to the chemical synthesis of trehalose analogs involves regioselective functionalization of native trehalose, which is abundant, inexpensive, and of course it already contains a 1,1- α,α -glycosidic bond (Fig. 4b). In this case, the main challenges include differentiation of similarly reactive hydroxyl groups (six secondary and two primary), as well as desymmetrization if unsymmetrical analogs are ultimately desired. Generally speaking, regioselective hydroxyl functionalization methods that work for simpler α -D-glucosides can also be applied to trehalose, with the obvious caveat that trehalose is a symmetrical disaccharide of glucose. A detailed review of these various methods is outside the scope of this review, but a few routinely used strategies are mentioned here. Differentiation of the primary hydroxyls at the 6-*O*-position(s) from the remaining hydroxyls via selective protection with bulky protecting groups (e.g. trityl [57], tosylate [58], *tert*-butyldimethylsilyl [59] [early examples referenced]) is commonly done, often en route to bioactive glycolipids bearing 6-*O*-acyl groups, such as TMM and TDM analogs. Differentiation of the secondary hydroxyl groups is more challenging, but a variety of chemistries – many invoking benzylidene

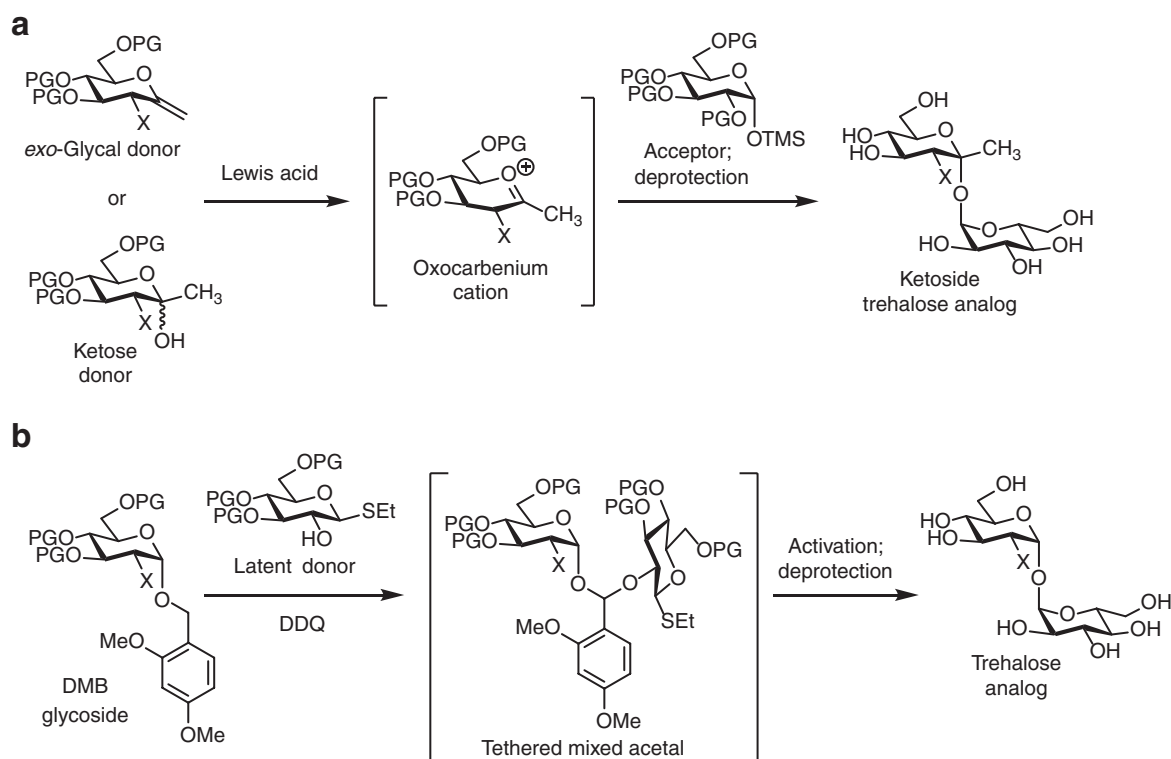


Fig. 5: 1,1- α,α -Glycosylation strategies using (a) ketoside formation and (b) intramolecular aglycon delivery to control stereochemical outcome. DMB, dimethoxybenzyl; TMS, trimethylsilyl.

[60] and cyclohexylidene [61, 62] acetal protecting groups for the 1,2- and 1,3-diols present in trehalose (early examples referenced) – have been developed for this purpose. The complete repertoire of regioselective functionalization methods for trehalose has enabled the preparation of numerous trehalose analogs, ranging from complex glycolipids to analogs bearing deoxy, fluoro, azido, and phosphate modifications. The synthesis and applications of select examples of such analogs are covered later in this review. For an in-depth discussion of regioselective trehalose functionalization methods, readers are pointed to a recent review article by Sarpe and Kulkarni [11].

The two chemical synthesis approaches described above offer the flexibility to access virtually any type of trehalose analog. However, it is clear that the unique structure of trehalose presents formidable challenges to synthetic chemists. While numerous methods have been developed to address these challenges, the chemical synthesis of trehalose analogs remains a daunting task due to lengthy multi-step syntheses, low overall yields, the need for synthetic expertise, and the generation of hazardous waste streams.

Chemoenzymatic synthesis of trehalose analogs

The existence of at least five *de novo* trehalose biosynthetic pathways in nature (Fig. 2a–e) provides ample opportunity for the development of enzymatic methods to produce trehalose and trehalose analogs. Trehalose-synthesizing enzymes are capable of forming 1,1- α , α -glycosidic bonds with exquisite stereoselectivity from simple, unprotected substrates in aqueous conditions. Together, these features address many of the drawbacks to chemical synthesis mentioned above. Therefore, enzymatic methods have gained increasing attention due to their potential to accelerate the development and applications of trehalose and trehalose analogs.

A breakthrough in the worldwide usage of native trehalose was the invention of an industrial-scale enzymatic synthesis of trehalose beginning from starch, which is inexpensive and abundant. Developed by Hayashibara Biochemical Laboratories, this process harnesses the TreY/TreZ pathway from the soil bacterium *Arthrobacter ramosus* [63]. While a number of steps and components are used to maximize the yield of this process, the two main steps are (i) TreY-catalyzed isomerization of starch maltooligosaccharides to maltooligosyltrehaloses; and (ii) TreZ-catalyzed hydrolysis to release free trehalose from maltooligosyltrehaloses (Fig. 2b) [63]. According to a 2011 review on the properties and applications of native trehalose, the Hayashibara process generates at least 30 000 tons/year of trehalose, which is then used in over 8000 commercial products in various industries [2]. This is a striking example of how biocatalysis can improve the overall efficiency and commercial viability of a synthetic process.

Despite the importance of the Hayashibara process for generating trehalose on an industrial scale, it is not easily adaptable to the production of trehalose analogs. Indeed, this would require a homogeneously modified starch substrate as well as sufficient tolerance of all enzymatic components (e.g. TreY and TreZ) for the unnatural substrate. Fortunately, other *de novo* pathways for trehalose biosynthesis have better characteristics for analog synthesis, namely the utilization of simple monosaccharide-based substrates.

The TPS/TPP pathway converts glucose-6-phosphate and UDP-glucose to trehalose over two steps. In principle, this pathway could be exploited for the chemoenzymatic synthesis of trehalose analogs by using a modified glucose-6-phosphate or UDP-glucose substrate. To our knowledge, the only example of the TPS/TPP pathway being used for in-flask analog synthesis was reported by the Barry and Davis groups in 2011 (Fig. 6) [52]. With the target molecule being 2-deoxy-2-fluoro-D-trehalose (^{19}F -2-FDTre, **9**), 2-deoxy-2-fluoro-D-glucose (^{19}F -2-FDG, **6**) was first phosphorylated at the 6-*O*-position using yeast hexokinase to generate intermediate **7**, which was then reacted with UDP-glucose in the presence of *E. coli* TPS to form 2-deoxy-2-fluoro-D-trehalose-6-phosphate (**8**). Removal of the phosphate group of **8** by alkaline phosphatase, rather than TPP, afforded the desired product **9**. This synthesis was quite efficient (62% yield over the final two steps), indicating that the TPS/TPP pathway has potential for trehalose analog synthesis. However, it requires the use of three separate enzymes to generate trehalose analogs from glucose analogs, which limits the overall efficiency and increases the probability that some substrate analogs will not work. Further studies are necessary

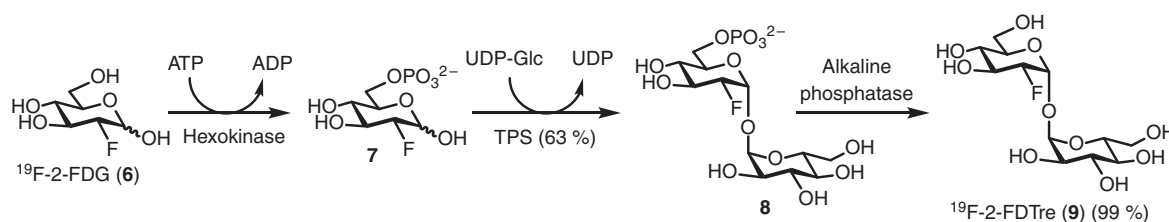


Fig. 6: Chemoenzymatic synthesis of fluorine-modified trehalose analog ^{19}F -2-FDTre (**9**) from the corresponding glucose analog ^{19}F -2-FDG (**6**) using a three-step method based on the TPS/TPP pathway.

to establish the scope of this method. Additionally, the requirement for 6-*O*-phosphorylation of the TPS substrate means that 6-position modifications are not possible using this strategy. Finally, UDP-glucose is a moderately expensive sugar donor (\$250/g), which could limit scale-up efforts.

Both the TreP (Fig. 2e) and TreT (Fig. 2d) pathways are attractive for trehalose analog synthesis from the standpoint that they directly convert glucose into trehalose in one step. A plethora of modified glucoses are commercially available, including those containing radiolabels, stable-isotope labels, click chemistry labels, and other structural modifications. In addition, the chemical synthesis of custom glucose building blocks is typically much more facile than the chemical synthesis of custom trehalose analogs. Thus, in principle, the TreP and TreT pathways are capable of converting these readily available glucose analogs into the corresponding trehalose analogs.

TreP is thought to mainly catalyze trehalose degradation to glucose and glucose-1-phosphate *in vivo*, but the reaction is reversible (Fig. 2e) [64]. Both inverting and retaining TreP enzymes exist in nature, and both have been evaluated for their tolerance for unnatural monosaccharide substrates [65–71]. In a promising example, the Desmet group showed that the thermostable inverting TreP enzyme from *Caldanaerobacter subterraneus* exhibited good activity on a variety of naturally occurring monosaccharides (D-galactose, D-xylose, L-arabinose, D-mannose, D-fucose, and L-fucose all had >10% relative activity compared to the natural substrate D-glucose) [70]. Despite exhibiting promising substrate promiscuity in wild type and engineered variants, inverting TrePs suffer from the use of sugar donor β -D-glucose-1-phosphate, which is extremely expensive (\$60/mg) and difficult to obtain in sufficient quantities for synthetic applications. Thus, the reaction scale is currently quite limited, which may explain the absence of product yields and characterization data in this report. Interestingly, the Desmet group is also developing enzymatic approaches to synthesize glycosyl phosphates such as β -D-glucose-1-phosphate, which, if efficient, could alleviate this problem [72, 73]. On the other hand, retaining TreP enzymes, which can produce trehalose from glucose and much cheaper α -D-glucose-1-phosphate (\$25/g), may provide a more efficient route to trehalose analogs. Unfortunately, to date all retaining TrePs that have been evaluated have shown poor stability and expression yields, and furthermore exhibited low substrate promiscuity [74–77]. Therefore, improvement of retaining TrePs through enzyme engineering will be needed for this approach to be fruitful. Additional details on TreP-catalyzed trehalose analog synthesis can be found in Desmet's 2015 review article [7].

Like TreP, the TreT pathway is attractive due to its one-step conversion of glucose to trehalose (Fig. 2d) [27]. In this case, however, the donor used is UDP-glucose, which as noted above is moderately expensive but much less so than β -D-glucose-1-phosphate. Early studies by the Lee group on the thermostable TreTs from *Thermotoga maritima* and *Pyrococcus horikoshii* indicated moderate to good activity on a few naturally occurring monosaccharides, such as galactose and mannose [78–80]. Furthermore, the trehalose stereoisomer galactotrehalose – discussed later due to its trehalase-resistance property – was synthesized on a semi-preparative scale in 10% isolated yield (based on the acceptor galactose) and characterized by ^{13}C NMR [80]. These preliminary studies encouraged further research on the TreT pathway for trehalose analog synthesis.

Recently, our group became interested in exploiting the thermostable TreT from *Thermoproteus tenax* for trehalose analog synthesis. Intriguingly, *T. tenax* TreT, which was first reported by Zaparty and co-workers in 2008, is unique among TreTs because it is unidirectional, i.e. it is incapable of degrading trehalose [81]. In combination with excellent thermostability and simple substrates, we predicted that the unidirectional

nature of *T. tenax* TreT would make it ideal for synthetic applications. In our initial report in 2014, we showed that this enzyme could indeed convert a wide variety of glucose analogs to trehalose analogs in high yield (up to 99 % yield as determined by HPLC, Fig. 7) [82]. The method has now been applied to the semi-preparative synthesis of numerous analogs bearing a variety of modifications (e.g. fluoro-, deoxy-, azido-, thio-, stereochemical, and isotopic modifications), with typical isolated yields of 60–80 % [82–84]. The rapid and facile procedures we have developed for TreT-catalyzed synthesis and product purification have facilitated access to novel trehalose analogs for mycobacteria research, as discussed in more detail later. As mentioned above, the cost of UDP-glucose is a limitation, plus *T. tenax* TreT showed poor activity on glucose analogs bearing 4-position modifications. Additional research will be necessary to address these issues.

In sum, chemoenzymatic synthesis using de novo trehalose synthesis enzymes is emerging as a powerful approach to the production of trehalose analogs. In particular, the TreP and TreT pathways, which allow one-step synthesis of trehalose analogs from glucose analogs, are of interest for further development and scale-up. The Hayashibara process for trehalose synthesis provides a model for how thermostable

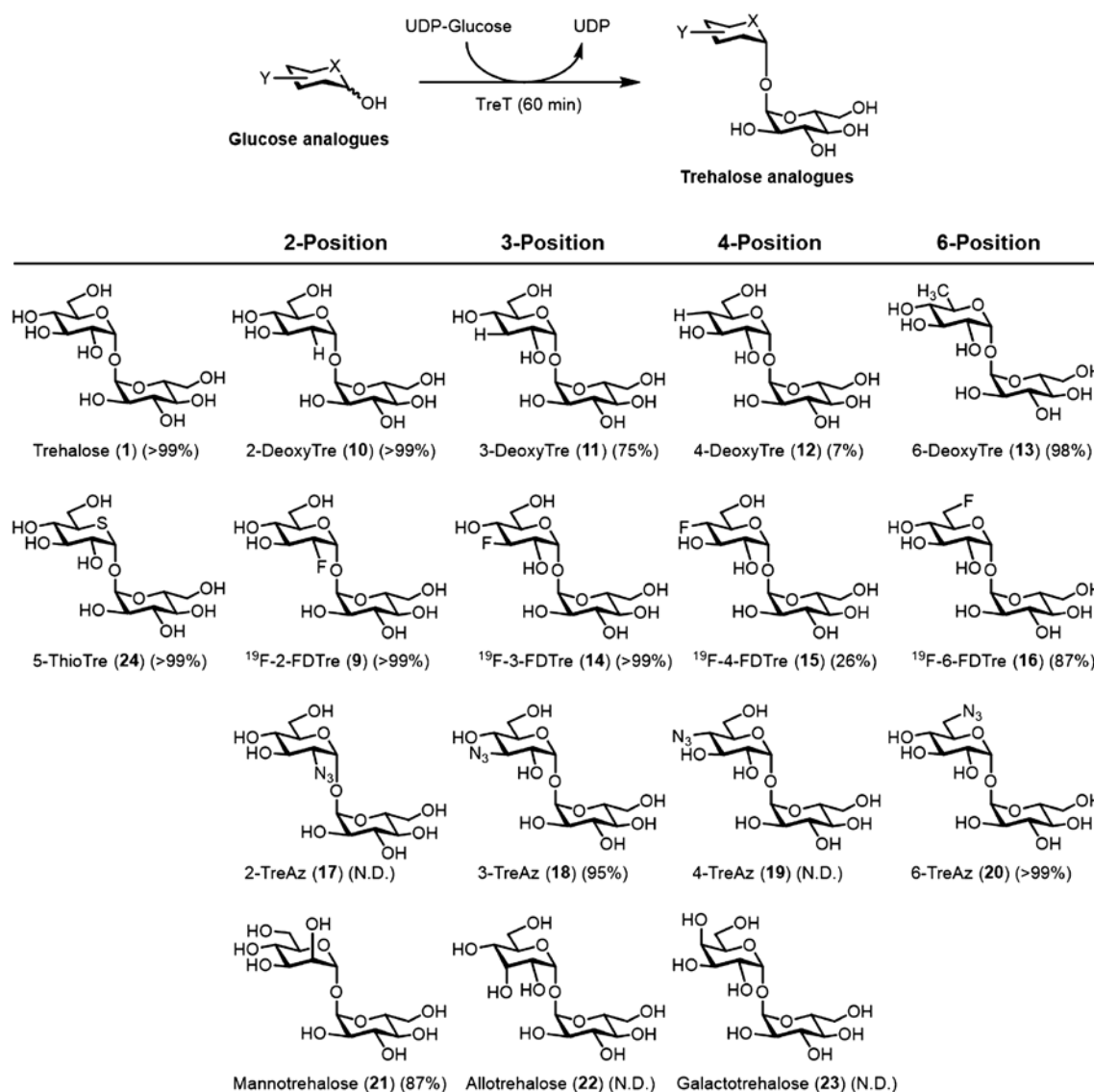


Fig. 7: One-step TreT-catalyzed synthesis of trehalose analogs (9–24). Yields were determined by HPLC. X and Y represent structural modifications. N.D., not detected.

trehalose-synthesizing enzymes might be adapted to large-scale analog production in the future. Compared to chemical synthesis, chemoenzymatic methods are straightforward, convenient, environmentally friendly, and very high-yielding for relatively simple analogs (e.g. monofunctionalized or isotope-labeled analogs). However, biocatalytic approaches are dependent on enzyme substrate promiscuity, which cannot be easily controlled. Thus, for more complex trehalose analogs, such as glycolipids, chemical synthesis remains the best option due to its versatility.

Applications of trehalose analogs

With an understanding of how trehalose analogs are produced, we now shift our focus to their applications in biomedical research and biotechnology. Given the increasing focus on using trehalose analogs in the study and targeting of pathogens, we first discuss compounds that have possible applications relating to the investigation, diagnosis, or treatment of diseases, namely those caused by mycobacteria. Then, we consider non-digestible trehalose analogs, which have several possible applications, including biopreservation. Due to the recent comprehensive review on the chemical synthesis and biological activities of mycobacterial trehalose glycolipids [47], we do not discuss this topic. For each type of trehalose analog covered herein, we discuss current use(s), method(s) of synthesis, and future directions for development and use.

Detectable trehalose analogs

^{14}C -labeled trehalose

Trehalose analogs modified with isotopic labels and other types of detectable chemical handles are invaluable research tools that may also have applications in medical imaging and diagnostics. The characterization of many trehalose metabolic pathways (e.g. those shown in Figs. 2 and 3) has been facilitated by the classical reagent in this category, ^{14}C -trehalose (**25**, Fig. 8), whose stable ^{12}C atoms have been exchanged for radioactive ^{14}C atoms. This modification enables sensitive tracking of trehalose in biological samples using techniques such as liquid scintillation counting and chromatography–autoradiography. Thus, ^{14}C -trehalose serves as a metabolic radiotracer whose abundance in a sample can be quantified and any apparent metabolic alterations to the molecule (e.g. elaboration to glycolipids or degradation to glucose) can be observed.

Mapping trehalose metabolism using ^{14}C -trehalose has been reported in a variety of organisms, including *Escherichia coli* [85], *Thermococcus litoralis* [86], and *Saccharomyces cerevisiae* [87], to highlight a few examples that encompass all three domains of life. Of particular interest to the biomedical research community, ^{14}C -trehalose has been used to elucidate trehalose metabolic pathways in pathogenic organisms, such as *M. tuberculosis*, thus presenting new opportunities for drug and diagnostic development. For instance, ^{14}C -trehalose has been used to investigate the role of the Ag85 complex in *M. tuberculosis* cell envelope biosynthesis [36], a pathway that is essential for mycobacterial growth and virulence and is therefore an

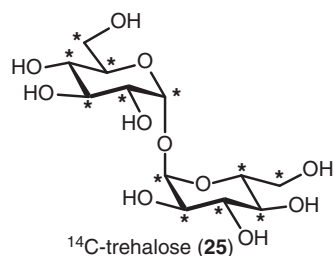


Fig. 8: Uniformly labeled ^{14}C -trehalose. Asterisk (*) indicates a ^{14}C -labeled atom.

attractive drug target. In another example, Kalscheuer and colleagues used ^{14}C -trehalose to characterize a novel virulence-associated trehalose transporter in *M. tuberculosis*, SugABC-LpqY [41]. In addition to *M. tuberculosis*, ^{14}C -trehalose has been used to study trehalose metabolism in related species, including the non-pathogenic model organism *M. smegmatis* and the industrial fermentation species *Corynebacterium glutamicum* [35, 88]. Although studying metabolism is the most common application of ^{14}C -trehalose, there have been other pursuits using the molecule as well. For instance, Hayek and co-workers used ^{14}C -trehalose to investigate the disaccharide for the long-term preservation of human pancreatic endocrine cells, which are used in tissue transplantation in diabetic patients [89]. In this case, ^{14}C -trehalose was used to track the intracellular accumulation of trehalose in pancreatic islet cells, which confirmed the successful translocation of exogenous trehalose into the cells [89].

^{14}C -trehalose can be acquired through either commercial purchase or in-house synthesis, both having advantages and drawbacks. Purchasing ^{14}C -trehalose is labor-free but presents a tremendous monetary cost (\$2500–5000/50 μCi). Of the reported methods for synthesizing ^{14}C -trehalose [90–92], the most effective method to date employs a mutant strain of *E. coli* that is unable to grow on glucose and is capable of overexpressing the trehalose-synthesizing TPS/TPP enzymes from an isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible plasmid [92]. Thus, this method calls for the incubation of the *E. coli* strain with exogenous ^{14}C -glucose, IPTG-induced gene expression, and synthesis and purification of ^{14}C -trehalose from the cells. This method is low-cost and generates pure ^{14}C -trehalose product with a reported overall yield of approximately 80 % [92]. However, ^{14}C -trehalose must be synthesized and purified from cells, which can be a time consuming process and may be inaccessible to some labs. Furthermore, the ^{14}C -glucose starting material or ^{14}C -trehalose product within cells can be funneled to other pathways, decreasing yield and increasing radioactive waste. Additional research aimed at improving the efficiency of ^{14}C -trehalose synthesis could bring down reagent costs and simplify in-house synthesis. Certainly, the chemoenzymatic approaches to trehalose analog synthesis discussed above could be applied to the production of ^{14}C -trehalose. For example, the unidirectional TreT enzyme from *T. tenax* [81, 82] could provide a rapid and high-yielding synthesis of ^{14}C -trehalose from relatively inexpensive ^{14}C -glucose, without the need for cell culture or laborious purification steps.

Stable isotope-labeled trehalose

Related to ^{14}C -trehalose, stable isotope-labeled versions of trehalose can also be beneficial for research purposes. For example, symmetrically ^{13}C - and ^2H -labeled trehalose are commercially available and can be used in combination with spectroscopic and spectrometric methods, e.g. NMR and mass spectrometry, to study the structure and metabolism of trehalose and its derivatives. In addition, custom stable isotope-labeled trehaloses have also been chemically synthesized for specific applications. For example, asymmetrical [$1\text{-}^{13}\text{C}$]-trehalose was synthesized using a 1,1- α,α -stereoselective glycosylation method and used to elucidate the conformation and dynamics of trehalose in aqueous solution by NMR [93]. Recently, ^2H -labeled trehalose was chemically synthesized through a one-step ruthenium-catalyzed deuteration of trehalose, and the product was used in conjunction with nuclear reaction analysis to evaluate trehalose uptake by *M. smegmatis* [94].

Fluorescent-labeled trehalose

^{14}C -trehalose is a powerful tool for studying trehalose metabolism, but radiotracers have a number of drawbacks. Radioactive facilities must be available and users must go through extensive training to work with radioisotopes. Such work also has safety concerns and generates radioactive waste streams that are difficult and expensive to dispose of. Stable isotope-labeled trehalose analogs are safer and easier to use, but their detection in samples can be difficult and of low sensitivity. From an experimental standpoint, both ^{14}C -containing trehalose radiotracers and stable isotope-labeled trehalose analogs cannot be adapted to the study

of intact, single cells. Therefore, alternative means to track trehalose metabolism are of interest for many experiments.

In 2011, the Barry and Davis groups reported the first trehalose-based fluorescent probe, named FITC-Tre (**26**, Fig. 9a) [52]. FITC-Tre is a ketoside trehalose analog containing a thiourea-linked fluorescein group at its 2'-position. The probe is conveniently deployed in a one-step metabolic labeling approach and it is capable of reporting on trehalose metabolism in living, single cells through fluorescence microscopy. The initial publication on FITC-Tre demonstrated its ability to label live *M. tuberculosis* cells through Ag85-mediated incorporation into surface glycolipids such as TDM (refer to Fig. 3 for a depiction of this metabolic pathway). This work had two important implications. First, it revealed that extracellular Ag85 had extraordinarily high substrate tolerance, since it accepted a substrate analog bearing a large fluorophore. Second, because the Ag85 pathway is absent from humans and other mammals, probes such as FITC-Tre could potentially be used to track mycobacterial trehalose metabolism in vivo. As a demonstration of this capability, FITC-Tre was used for the specific imaging of intracellular *M. tuberculosis* within macrophages (Fig. 9b). These findings suggest that trehalose-based probes could be useful for the detection and investigation of *M. tuberculosis* in complex environments, perhaps within a human patient or patient sputum sample. As discussed below, this concept is now being furthered through the development of trehalose-based tuberculosis diagnostic tools.

The synthesis of FITC-Tre was accomplished via chemical synthesis (Fig. 9c). The key step in the synthesis employed Ikegami's 1,1- α -glycosylation method [51] to couple two building blocks, ketose donor **27** and protected glucosamine hemiacetal acceptor **28**. Upon treatment with Lewis acid catalyst TMSOTf, monosaccharide derivatives **27** and **28** combined with good stereoselectivity to form α,α -ketoside **29** in 45 % yield, while the undesired α,β -isomer was generated in less than 10 % yield. Disaccharide **29**, which contained a Cbz-protected amine at the 2'-position, then underwent deacetylation and hydrogenolysis to remove the hydroxyl and amine protecting groups. The free amine of the intermediate was then chemoselectively conjugated to FITC, generating FITC-Tre (**26**) in 67 % yield over the final three steps. While this synthesis benefited from a glycosylation method with good stereoselectivity (6 : 1 α,α : α,β), it did require the use of custom monosaccharide building blocks, each of which required several steps to prepare from commercially available materials. Nonetheless, FITC-Tre represented a major advance in the development of trehalose analogs for studying mycobacteria, as exemplified by the additional work in this area discussed below. Furthermore, FITC-Tre has inspired next-generation fluorogenic trehalose analogs that produce fluorescence only upon binding to mycobacteria, which are currently under development.

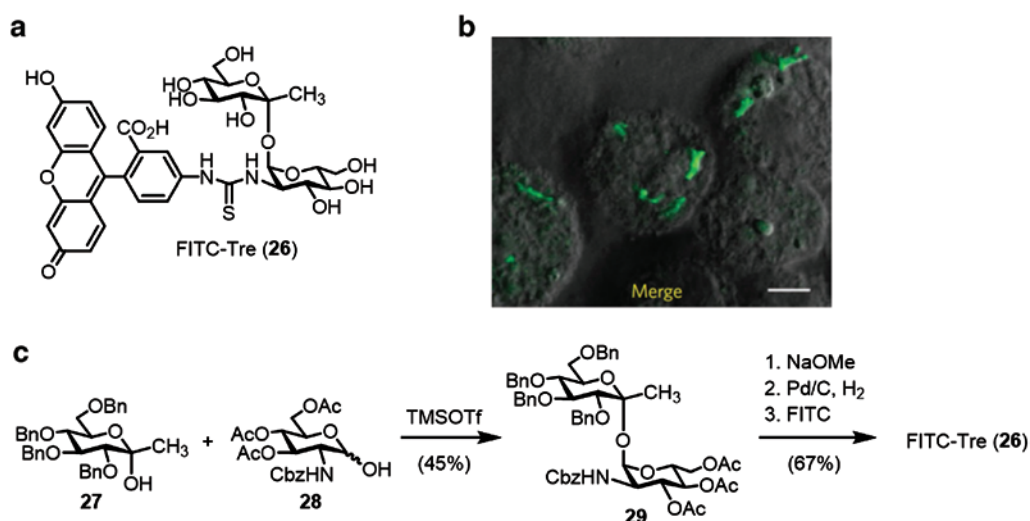


Fig. 9: (a) Structure of FITC-Tre (**26**). (b) Metabolic labeling of *M. tuberculosis* within host macrophages using FITC-Tre (image reprinted from Ref. [52] with permission). Scale bar, 5 μm . (c) Final steps in the chemical synthesis of FITC-Tre. Cbz, carboxybenzyl; FITC, fluorescein isothiocyanate; TMSOTf, trimethylsilyl trifluoromethanesulfonate.

Azide-labeled trehalose analogs

Fluorescent-labeled trehalose analogs such as FITC-Tre operate through one-step metabolic labeling, which is advantageous for some applications but has limitations for others. First, the fluorophore is a large modification that can impact the route or efficiency of metabolic incorporation and potentially affect the function of labeled biomolecules. Second, because in this approach trehalose is pre-labeled with a fluorophore, the probe has limited versatility. If a different functionality (e.g. a different fluorophore or an affinity tag) is desired, then a new probe must be synthesized and evaluated.

The bioorthogonal chemical reporter strategy, pioneered by the Bertozzi group, is a two-step labeling approach that is complementary to one-step approaches [95, 96]. The chemical reporter is an unnatural metabolite containing a small, abiotic functional group that does not interact or react with endogenous biomolecules. After metabolic incorporation of the chemical reporter into the cell, it can be detected via selective bioorthogonal reaction with a secondary reagent containing a fluorophore. The most commonly used reporter group is the azide, which is small, inert, and can selectively react with either (i) terminal alkyne-containing reagents through Cu-catalyzed azide-alkyne cycloaddition (CuAAC; “click” reaction) [97, 98]; or (ii) cyclooctyne-containing reagents through strain-promoted azide-alkyne cycloaddition (SPAAC; “Cu-free click” reaction) [99]. Compared to one-step labeling, the chemical reporter strategy allows for more efficient metabolic incorporation and lower structural/functional perturbation due to the small size and inertness of the azide. In addition, the modular nature of the bioorthogonal reaction confers high experimental flexibility, allowing virtually any type of cargo to be delivered to the labeled metabolite in its native environment.

In 2012, the Bertozzi group developed a series of detectable trehalose analogs bearing azides at all positions of the disaccharide (**17–20**, Fig. 10a) [100]. Referred to as TreAz analogs, these chemical reporters were shown to be metabolically incorporated into surface-bound trehalose glycolipids in various mycobacterial species, including *M. smegmatis*, *M. bovis*, and *M. tuberculosis*. Both CuAAC and SPAAC were used to deliver fluorophores to TreAz-labeled cells and permit subsequent detection by flow cytometry and fluorescence microscopy (Fig. 10b). Interestingly, it was shown through biochemical analysis that different TreAz analogs were incorporated by distinctive metabolic pathways. 3-TreAz (**18**) behaved similarly to FITC-Tre, undergoing extracellular incorporation into trehalose glycolipids via the Ag85 complex. However, 2-, 4-, and 6-TreAz (**17**, **19**, and **20**, respectively) were first imported into the cell via the trehalose transporter SugABC-LpqY, then incorporated into trehalose glycolipids from the inside-out. This finding, which indicates that TreAz analogs accurately mimic native trehalose in the cellular environment, reflects the subtleness of the azide modification to the disaccharide's structure and function. In addition to facilitating basic biological inquiries into trehalose metabolism, the TreAz two-step labeling strategy allows any type of chemical payload to be delivered to specifically to mycobacteria – potentially within a host – opening up new possibilities for tuberculosis diagnostic and therapeutic applications.

In Bertozzi's work, the synthesis of TreAz analogs **17–20** was accomplished using previously established methods for regioselective functionalization of trehalose. The most difficult of these syntheses were 2- and 3-TreAz. These target compounds both originated from a common synthetic intermediate (**30**), which was obtained from trehalose via Wallace and Minnikin's desymmetrizing cyclohexylidenation reaction [101] followed by regioselective 2-*O*-benzoylation [59] (Fig. 10c). Intermediate **30** could be elaborated to either 2- or 3-TreAz by carrying out multi-step double displacement sequences to replace the hydroxyl group with an azido group, followed by deprotection. The syntheses of 2- and 3-TreAz required 7–9 steps and proceeded in very low overall yield from trehalose. 4- and 6-TreAz were also accessed via reported trehalose desymmetrization methods, with the former taking six steps [100, 102] from trehalose and the latter taking four steps [103]. Although these syntheses were successful and provided enough material for evaluation, they were inefficient in terms of step count, time, and yield.

Our group's chemoenzymatic method for synthesizing trehalose analogs, reported in 2014, proved to be beneficial for accessing some TreAz analogs [82]. The TreT enzyme was capable of generating 3- and 6-TreAz from the corresponding commercially available azido glucoses in a single step in 95 and 99 % yield (HPLC),

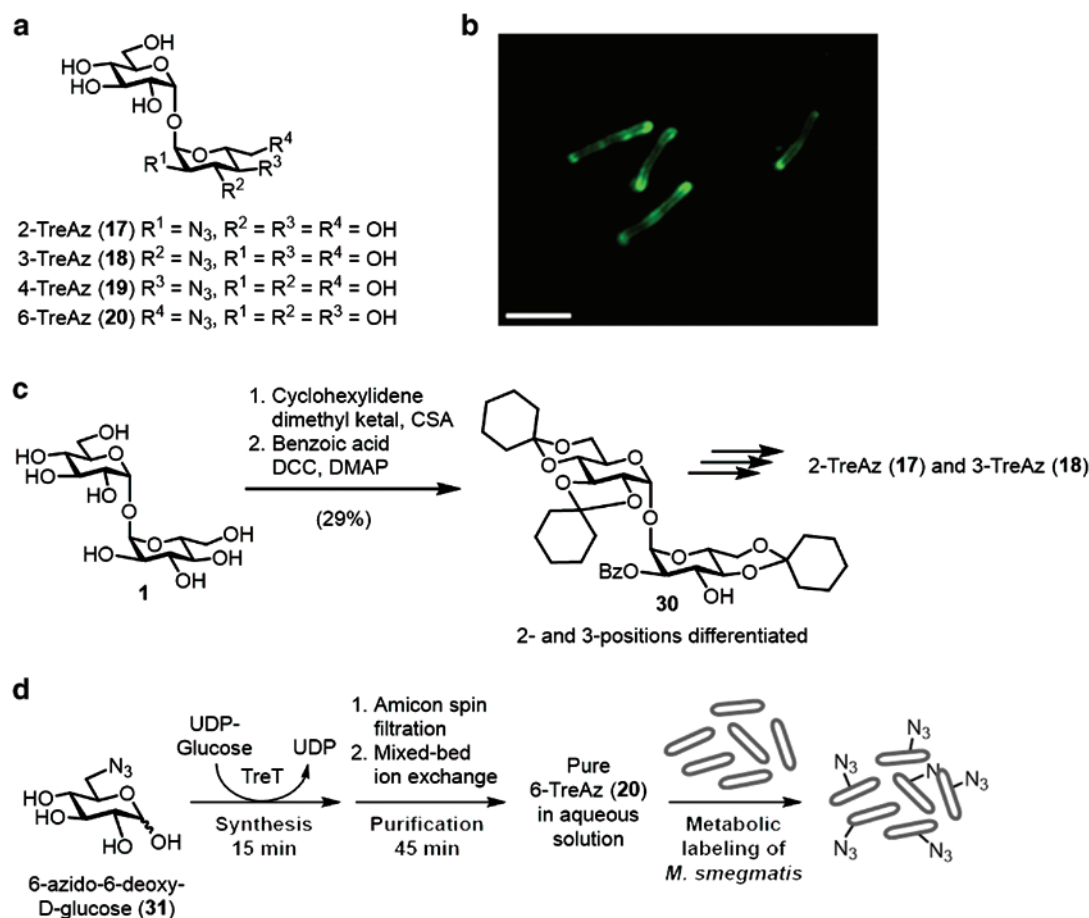


Fig. 10: (a) Structures of 2-, 3-, 4-, and 6-TreAz (**17–20**). (b) Metabolic labeling of *M. smegmatis* with TreAz followed by CuAAC with an alkyne-modified fluorophore (image reprinted with permission from Ref. [100]). Scale bar, 5 μ m. (c) An example of the approach used to synthesize TreAz analogs. Differentiation of the 2- and 3-*O*-positions of trehalose (**1**) was accomplished in two steps, giving an intermediate (**30**), which was elaborated to 2- and 3-TreAz (**17** and **18**). (d) Rapid TreT-catalyzed synthesis, purification, and administration of 6-TreAz (**20**) to *M. smegmatis* for a metabolic labeling experiment. CSA, camphor sulfonic acid; DCC, *N,N'*-dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine.

respectively. In addition, a purification protocol was developed that utilizes spin dialysis and ion exchange to deliver pure products quickly and in aqueous solution. Using this method, 6-TreAz was synthesized and purified in 60 min starting from 6-azido-6-deoxy-D-glucose (**31**), and it could be rapidly deployed in metabolic labeling experiments (Fig. 10d) [84]. Thus, chemoenzymatic synthesis employing TreT is preferable to chemical synthesis for accessing 3- and 6-TreAz on a semi-preparative scale. However, the wild-type TreT enzyme has not been able to generate 2- or 4-TreAz from the corresponding azido glucoses. At present, efforts are being made to address these limitations in reaction scope.

Alkyne-labeled trehalose monomycolate (TMM) analogs

FITC-Tre and TreAz analogs are valuable probes for tracking trehalose metabolism that have complementary attributes. Both probes operate by metabolic labeling of trehalose glycolipids. Another major envelope component of mycobacteria is AGM, which is generated through the Ag85-catalyzed transfer of mycoloyl lipid chains from TMM to terminal residues of the arabinogalactan polymer (refer to Fig. 3) [35–37]. This step in trehalose metabolism is essential for mycobacterial growth and virulence. Our group posited that a TMM-based chemical reporter would serve as a cellular probe of this important biochemical process and – because

only mycobacteria are known to possess TMM-processing enzymes – potentially have enhanced specificity for detecting/delivering cargo to the cell surface of mycobacteria as compared to FITC-Tre or TreAz.

In 2016, we reported the development of a TMM-mimicking reporter called O-AlkTMM (**32**), which contained a truncated, linear acyl chain with a terminal alkyne connected to the 6-*O*-position of trehalose (Fig. 11a) [104]. As hypothesized, metabolic labeling experiments confirmed that O-AlkTMM predominantly incorporated into the AGM layer via the action of Ag85. O-AlkTMM also exhibited high specificity for mycobacteria, as it robustly labeled *M. smegmatis* and the related species *C. glutamicum*, but it did not label Gram-negative *E. coli* or Gram-positive *Bacillus subtilis*.

Because O-AlkTMM has a chemical reporter group (terminal alkyne) that is orthogonal to the chemical reporter of 6-TreAz (azide), these two probes were used to simultaneously detect trehalose glycolipids and AGM in whole cells of *M. smegmatis* using two-color fluorescence microscopy (Fig. 10b) [104]. Such experimental flexibility will facilitate the study of cell envelope dynamics in intact mycobacterial cells. Recently, we also observed that O-AlkTMM can label and facilitate the identification of proteins modified by mycoloyl groups in *C. glutamicum* [105]. This form of protein post-translational modification (PTM) – termed protein *O*-mycoloylation – was recently discovered in *Corynebacterineae* [106, 107] but its scope and functional consequences remain unknown. O-AlkTMM will be a useful tool for identifying and characterizing proteins that contain this PTM and for probing the link between protein lipidation and trehalose metabolism in *C. glutamicum* and related organisms.

O-AlkTMM, along with several related TMM analogs with varying chain lengths and chemical reporter groups, were synthesized chemically by employing a trehalose desymmetrization approach originally developed by the Kulkarni group (Fig. 11c) [108]. After per-trimethylsilylation of trehalose, the intermediate was selectively desilylated at the 6-*O*-positions under basic conditions to give diol **33**. Mono-6-*O*-acylation of **33** was carried out with 6-heptynoic acid (or other azido or alkynyl fatty acids) in the presence of DCC and DMAP, followed by acid-mediated desilylation to give O-AlkTMM (or related chemical reporters). This procedure is straightforward, relatively fast, and provides high purity product. Other methods have been developed to directly mono-6-*O*-acylate unprotected trehalose either chemically or enzymatically, although removing all side products from the target compound is challenging [109, 110]. If these purification issues can be addressed, such one-step methods offer a powerful approach to the synthesis of TMM-mimicking probes.

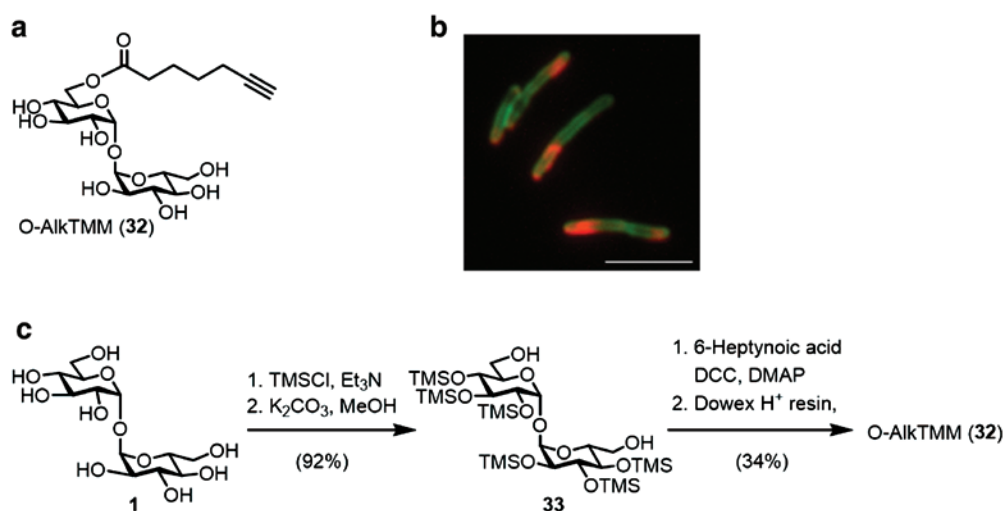


Fig. 11: (a) Structure of *O*-mycoloylation probe O-AlkTMM (**32**). (b) Simultaneous metabolic labeling of *M. smegmatis* with 6-TreAz and O-AlkTMM followed by sequential SPAAC and CuAAC reactions to deliver different fluorophores to trehalose glycolipids and AGM (image reprinted with permission from Ref. [104]). Scale bar, 5 μ m. (c) Chemical synthesis of O-AlkTMM using a 4-step trehalose desymmetrization approach.

^{18}F -labeled trehalose analogs

Tracking trehalose metabolism in large animals, including humans, could be desirable for numerous reasons. For example, trehalose is used in thousands of food products and is often taken as a dietary supplement [2]. In addition, it is being explored as a potential therapeutic compound for neurodegenerative diseases due to its ability to prevent protein aggregation [111]. Elucidating the biodistribution and pharmacokinetics of orally or intravenously administered trehalose could be highly informative in these pursuits. In addition, microbes that populate humans – whether it be commensal gut bacteria or pathogenic organisms such as *M. tuberculosis* – could potentially be imaged by monitoring trehalose uptake processes that are absent from the host [112, 113]. Unfortunately, fluorophore- and bioorthogonal tag-labeled trehalose analogs (e.g. FITC-Tre, TreAz, and O-AlkTMM) depend on optical imaging modalities and are therefore not well-suited to in vivo imaging in animals. Furthermore, bioorthogonal chemical reporters require two steps to permit detection, which is a challenge in complex organisms.

In their 2011 paper on FITC-Tre, Barry and Davis proposed the idea of ^{18}F -modified trehalose as a radiotracer to image *M. tuberculosis* infection in vivo [52]. ^{18}F -modified metabolites can be visualized by positron emission tomography (PET) imaging, which enables rapid, real-time monitoring of metabolic processes in living organisms in three dimensions. Indeed, 2-deoxy-2- ^{18}F fluoro-D-glucose (^{18}F -2-FDG) in conjunction with PET imaging has been used for decades to facilitate the detection and treatment of cancer [114]. Currently, bacteria-specific PET radiotracers that can image wild-type *M. tuberculosis* are in high demand but are not yet available [112]. An ^{18}F -modified trehalose analog capable of specifically detecting *M. tuberculosis* infection in animal models and humans would be an important tool in tuberculosis drug development and clinical diagnostics.

The synthesis of ^{18}F -modified compounds is very challenging due to the short half-life of ^{18}F ($t_{1/2} = 110$ min). To be viable for PET imaging applications, synthetic methods involving ^{18}F must be rapid and convenient. Toward this goal, in 2011 Barry and Davis developed a chemoenzymatic synthesis of the non-radioactive molecule ^{19}F -2-FDTre (**9**) modeled after the TPS/TPP pathway, as described earlier in this review (Fig. 6). Since this initial report, the method has been adapted by the same groups to synthesize radioactive ^{18}F -2-FDTre, which in preliminary studies has shown promise for imaging tuberculosis lesions in animal models [115]. A very attractive feature of this synthesis is that it utilizes commercially available ^{18}F -FDG as a starting material. A drawback of the method is that it requires three enzymes to convert ^{18}F -2-FDG into ^{18}F -2-FDTre, which limits the overall efficiency of the process.

Our group has also made progress toward the development of ^{18}F -modified trehalose analogs. In 2016, we reported the use of TreT catalysis to quantitatively convert non-radioactive ^{19}F -2-FDG (**6**) into ^{19}F -2-FDTre (**9**) in a single step in only 15 min (Fig. 12) [83]. The purification steps took 45 min, resulting in the production of a concentrated aqueous solution of pure ^{19}F -2-FDTre in a total of 60 min. This timeframe is compatible with the short half-life of ^{18}F , so the method should be adaptable to the synthesis of ^{18}F -2-FDTre. In addition to ^{19}F -2-FDTre, we synthesized ^{19}F -modified trehalose analogs containing the fluorine atom at the 3'-, 4'-, and 6'-positions (^{19}F -3-, ^{19}F -4-, and ^{19}F -6-FDTre, compounds **14–16**, shown in Fig. 7), mainly through chemoenzymatic synthesis. This panel of analogs was subjected to conformational analysis by NMR and molecular modeling, which showed that there was negligible difference in structure between native trehalose and

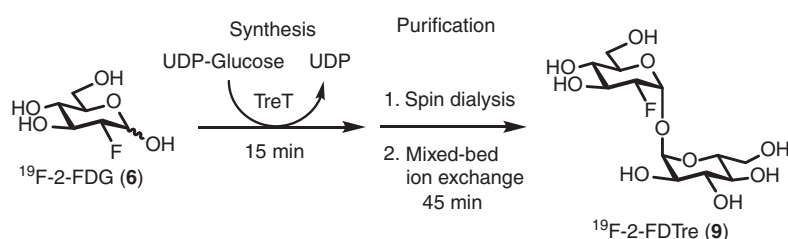


Fig. 12: Rapid TreT-catalyzed synthesis of ^{19}F -2-FDTre (**9**).

the fluorine-modified versions. In addition, three of the four analogs, including ^{19}F -2-FDTre, were shown to be taken up through the trehalose-specific transporter SugABC-LpqY in whole cells of *M. smegmatis*. Thus, this work established a robust method for the rapid and convenient synthesis of fluorine-modified trehalose analogs, confirmed that the analogs mimic the structure of native trehalose, and demonstrated that they could bind to mycobacteria through a non-mammalian trehalose recycling pathway. Together, the pioneering work of the Barry and Davis groups [52, 115], combined with our contributions [82, 83], should help to pave the way for continued development of trehalose-based PET imaging probes for tuberculosis diagnostics and other applications.

Trehalose-based inhibitors

The essentiality of trehalose metabolism in various pathogens, coupled with the lack of trehalose metabolism in humans (excepting trehalose degradation), makes it an attractive target for drug development. For example, *M. tuberculosis* has several trehalose-processing proteins that are required for virulence and are under intensive investigation as drug targets, including Pks13, MmpL3, Ag85, and SugABC-LpqY. Much of the recent progress in this area was covered in a 2016 review by Thanna and Sucheck [116]. Other prokaryotic pathogens also have trehalose metabolic pathways involved in physiology and pathogenesis [8, 9]. The importance of trehalose metabolism to insects [3] may provide opportunities for the development of new insecticides. In a similar vein, insects that act as disease vectors, such as mosquitoes for malaria, could also be targeted with trehalose metabolism inhibitors to prevent disease transmission. Common drug discovery approaches, such as natural products and high-throughput screening (HTS), will continue to be the main drivers of identifying inhibitors of trehalose metabolism. Indeed, HTS-based searches for anti-mycobacterial compounds have yielded numerous drug-like small molecules that act on trehalose metabolism, some of which are presently being pursued as possible treatments for tuberculosis [116]. Another approach that has shown promise is the rational design of inhibitors based on the structure of trehalose or trehalose derivatives. In keeping with the theme of this review, here we discuss the synthesis and evaluation of trehalose-based inhibitors in mycobacteria and other systems.

6-TreAz (**20**, Fig. 10) was the first trehalose analog shown to have anti-mycobacterial activity. In 1997, Belisle and co-workers found that 6-TreAz inhibited growth of *M. aurum* (MIC 200 $\mu\text{g/mL}$) [36]. 6-TreAz-treated bacteria showed marked reductions in TMM, TDM, and AGM, suggesting that the compound affected trehalose-mediated mycomembrane biosynthesis, perhaps via inhibition of Ag85 [36]. Following on Belisle's discovery of 6-TreAz activity, Barry and Davis chemically synthesized a panel of over 20 trehalose and keto-side trehalose analogs and assayed them against purified Ag85 enzyme and whole cells of *M. tuberculosis* (compounds **2**, **9**, **16**, **20**, **34–49**, Fig. 13) [52]. The chemical synthesis of these compounds involved a combination of stereoselective 1,1- α -glycosylation (Fig. 4a) and regioselective functionalization of trehalose (Fig. 4b). These analogs, which had a variety of modifications on the sugar rings (e.g. stereochemical inversion, deoxygenation, halogenation), were largely ineffective at inhibiting growth of *M. tuberculosis*. However, consistent with the earlier report from Belisle, it was found that analogs containing modifications at the 6-position, including 6-TreAz (**20**) and ^{19}F -6-FDTre (**16**), exhibited 50 % growth inhibition (MIC_{50}) of *M. tuberculosis* at concentrations in the 100–200 $\mu\text{g/mL}$ range. In a subsequent report, the same groups developed a trehalose analog bearing an electrophilic fluorophosphonate at the trehalose 6-position (**50**, Fig. 13) and demonstrated its ability to selectively and covalently inhibit Ag85 in vitro, although bacterial growth inhibition was not evaluated [117].

Modified versions of TDM have also been explored as anti-mycobacterial agents due to their potential ability to inhibit Ag85. Reynolds and co-workers synthesized a panel of symmetrical 6,6'-diamino-6,6'-dideoxytrehalose derivatives bearing *N,N'*-dialkylamino groups and 6,6'-bis(sulfonamido) groups and evaluated growth inhibition in an attenuated strain of *M. tuberculosis* and clinical isolates of *M. avium* [118]. While several compounds exhibited activity, *N,N'*-dioctylamino- and *N,N'*-didodecylamino-substituted TDM analogs (**52** and **53**) were the most potent (MIC 2–8 $\mu\text{g/mL}$) and acted on both *M. tuberculosis* and *M. avium*

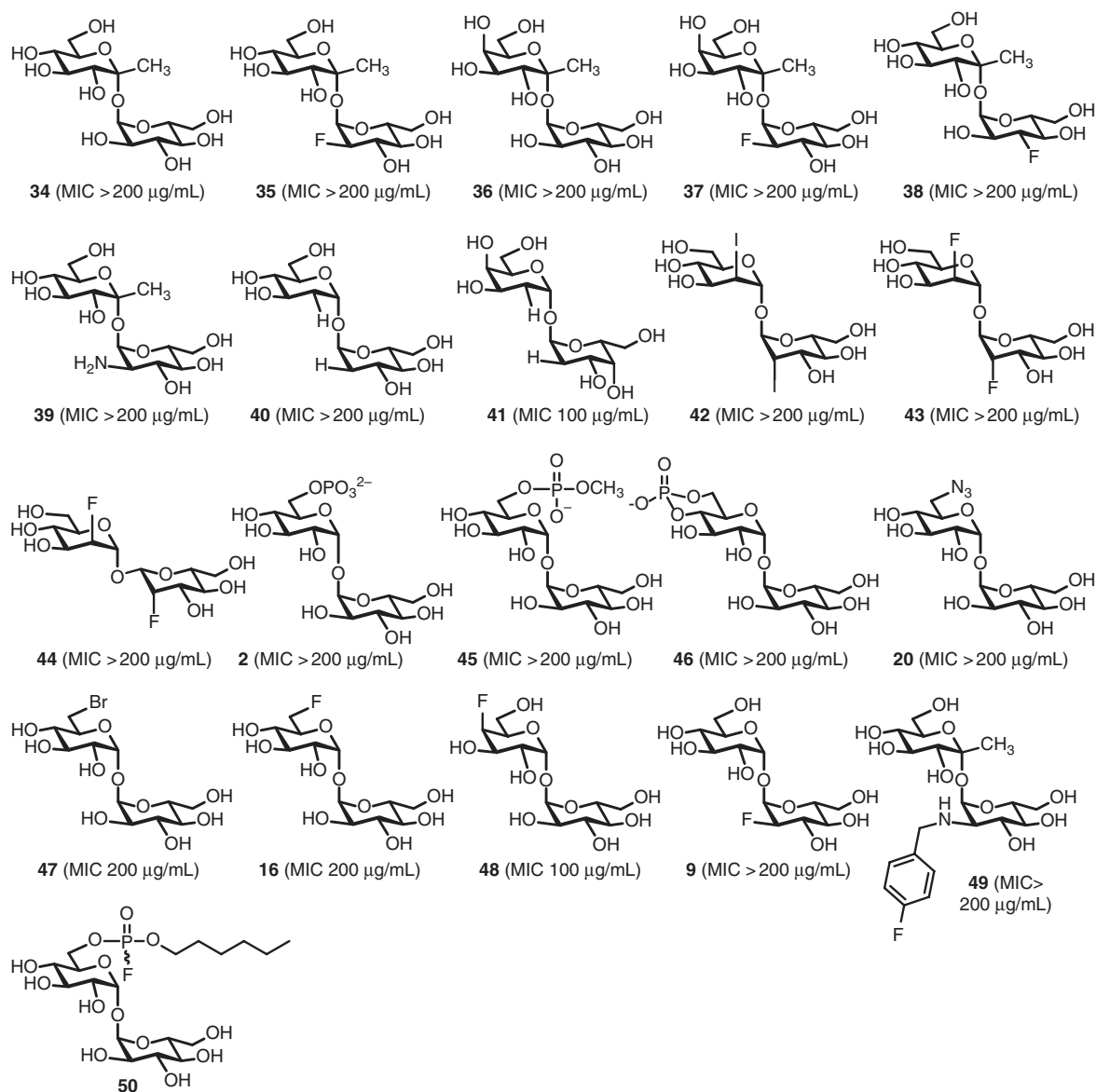


Fig. 13: Panel of trehalose analogs evaluated for inhibition of *M. tuberculosis* growth in Ref. [52]. The MIC value for each compound is given in parentheses.

(Fig. 14a). These structures were chemically synthesized through reaction of benzoyl-protected 6,6'-dibromo-6,6'-dideoxytrehalose derivative **51** with excess alkylamine to effect simultaneous *N*-substitution and debenzoylation, although the two-step yields were quite low (Fig. 14a). The Chang group also chemically synthesized a series of TDM analogs and screened them against non-pathogenic *M. smegmatis* [119]. The most active of these compounds contained “reversed” amide linkages stemming from each 6-position of trehalose. For example, compound **56** was obtained in four steps from diol **54** – first sequential Swern oxidation and Horner–Wadsworth–Emmons reaction to give **55**, then aminolysis and reduction – but the MICs of these derivatives were quite high (Fig. 14b) [119]. The Ronning and Sucheck groups chemically synthesized and evaluated a few related TDM-inspired analogs (**57–59**), also obtained from intermediate **55**, but they lacked activity against purified Ag85 and whole cells of *M. smegmatis* (Fig. 14c) [120].

Overall, trehalose analogs have had mixed success as inhibitors of mycobacteria, with some of the trehalose- and TDM-based structures discussed above showing activity against whole cells. Additional research is needed to determine whether these compounds can be optimized to attain higher potency in growth

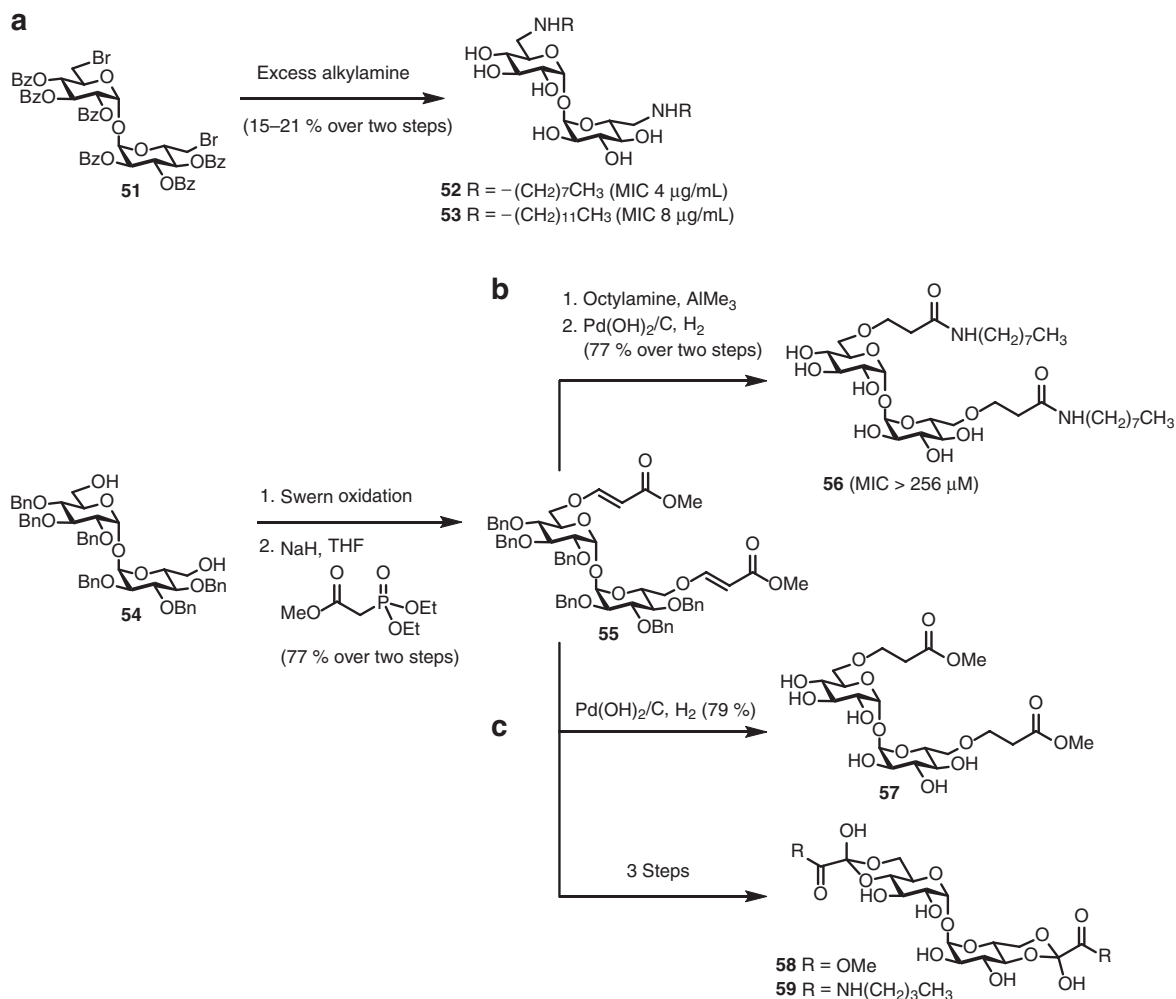


Fig. 14: TDM analogs developed as potential inhibitors of mycobacterial growth. (a) *N,N'*-dialkylated 6,6'-diamino-6,6'-dideoxytrehalose derivatives **52** and **53** from Ref. [118] (accessed through *N*-substitution of dibromide **51**) exhibited good activity against an attenuated strain of *M. tuberculosis*. (b) TDM analog **56** bearing a “reversed” acyl linkage from Ref. [119] showed moderate activity against *M. smegmatis*. (c) TDM analogs **57–59** from Ref. [120] displayed no activity against *M. smegmatis*.

inhibition assays. All of the trehalose-based inhibitors discussed were synthesized chemically. Chemoenzymatic approaches could increase synthetic efficiency for some simple inhibitors, e.g. compounds **16** and **20**, which can be made in high yield in one step using TreT catalysis [82]. Trehalose analogs may also have anti-biofilm activity, because trehalose-mediated export of mycolic acids is essential for biofilm formation in mycobacteria [121, 122]. Biofilm-like clusters of mycobacteria observed in TB animal models have been shown to correlate with bacterial persistence and drug tolerance, so anti-biofilm agents are of interest [123]. Preliminary work from our lab indicates that some trehalose analogs inhibit biofilm formation at lower concentrations than they inhibit growth. For both growth and biofilm inhibitors, efforts will be required to elucidate the mechanism(s) of action of trehalose analogs in cells, which may involve Ag85 inhibition as previously suspected, but could conceivably involve a number of other essential steps in cell envelope biosynthesis (Fig. 3).

Beyond anti-mycobacterial activity, trehalose-based inhibitors are of possible value in several additional areas. Other pathogens (or vectors that harbor them) have pathways for trehalose biosynthesis, degradation, and uptake that could be targeted for inhibition with trehalose analogs. Trehalose metabolism is critical to a number of bacterial pathogens, including *E. coli*, *Pseudomonas aeruginosa*, and *Burkholderia pseudomallei*, to name a few [8, 9]. Recently, the trehalose transporter AgTreT1 in the mosquito *Anopheles gambiae* was shown to be important to malaria infection caused by *Plasmodium falciparum*, suggesting that AgTreT1-mediated

trehalose uptake may be a target for preventing malaria transmission [124]. Trehalose breakdown into glucose by trehalase is critical for insect flight, making it a possible target for the development of novel insecticides. For example, trehazolin (**60**) and validoxylamine A (**61**) are naturally occurring insecticidal trehalose-like trehalase inhibitors (Fig. 15a) [125–127]. Trehalase-resistant trehalose analogs are of interest for other biotechnological applications too, as discussed in the next section of the review. Naturally occurring trehalose derivatives (and their synthetic analogs) have also shown interesting bioactivity in mammalian systems. For instance, brartemicin (**62**), a trehalose 6,6'-bisbenzoyl derivative, was isolated from *Nonomuraea* and shown to prevent tumor cell invasion (Fig. 15b) [128]. Based on this discovery, the Liu group chemically synthesized brartemicin analogs bearing modified aryl rings that were either ester- or amide-linked to the trehalose core [129, 130]. Of these compounds, **63** and **64** matched or slightly improved upon the inhibitory activity of the parent compound, while exhibiting no cytotoxicity.

Non-digestible trehalose analogs

Native trehalose belongs to a class of molecules called compatible solutes, which function to protect biomolecules from chemical stresses [131]. Trehalose can protect biomolecules from osmotic stress [132], thermal stress [133, 134], oxidative damage [135], and even radioactive stress [136]. These abilities have made trehalose a sought-after additive for applications that require long-term stabilization of reagents and medicines. For instance, trehalose is frequently an additive to vaccine formulations [137]. While trehalose is a powerful protein stabilizer, sweetening agent, and vaccine preservative, its use is somewhat limited by the fact that it can be consumed as a carbon source by most microorganisms. As noted earlier, trehalase splits trehalose into two glucose monomers for facile entry into traditional metabolic pathways [138]. While mammals do not synthesize trehalose themselves, they are able to break down trehalose present in the food they ingest. Trehalase is found across broad taxa, but in mammals the enzyme is only known to be expressed in the brush border of the intestine and the kidneys [139].

Several trehalose analogs are trehalase-resistant, and thus have limited ability to contribute to caloric intake by humans or be used as a carbon source by potential microbial contaminants. Thus, trehalase-resistant trehalose analogs have potential as low calorie food additives and stabilization agents. The analogs have varying degrees of resistance to trehalase, depending on the structural modification (Fig. 16). Galactotrehalose (α -D-glucopyranosyl- α -D galactopyranoside, **23**), a trehalose analog where one glucose is replaced

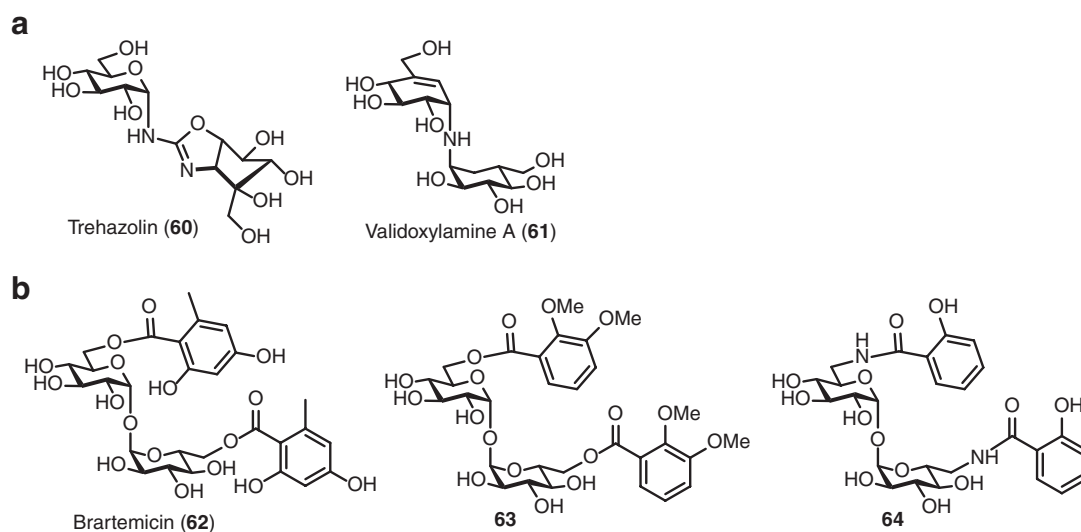


Fig. 15: (a) Naturally occurring trehalase inhibitors trehazolin (**60**) and validoxylamine A (**61**). (b) Brartemicin (**62**) and synthetic analogs (**63** and **64**) are being investigated as anti-invasive compounds with possible cancer chemotherapy applications.

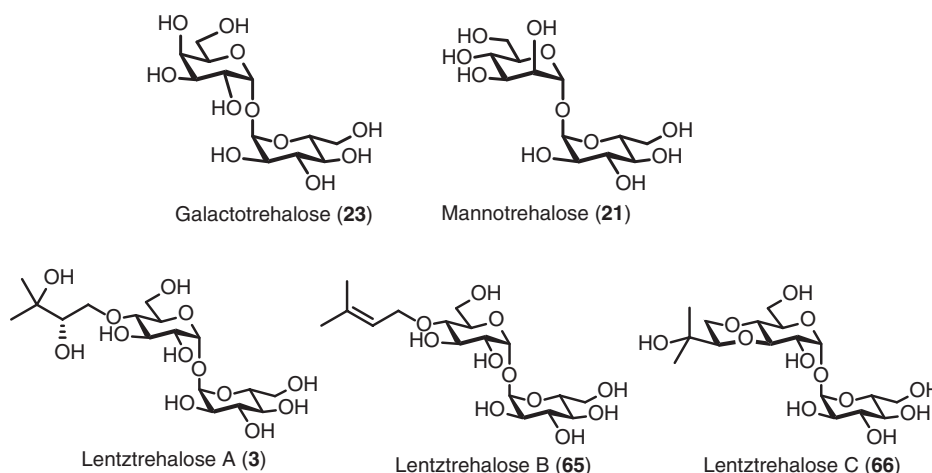


Fig. 16: Trehalose analogs that are resistant to degradation by trehalase, including trehalose epimers **21** and **23** and naturally occurring lentztrehaloses a–c (**3**, **65**, **66**).

by galactose, acts as a competitive inhibitor of trehalase [80]. It inhibits trehalase in the high micromolar/low millimolar range. More importantly, mammalian trehalase is unable to degrade galactotrehalose, making it a prime candidate for applications that benefit from resistance to degradation. For an excellent discussion of galactotrehalose, see Desmet's review [7].

Similarly, mannotrehalose (α -D-glucopyranosyl- α -D-mannopyranoside, **21**), has one of the glucose monomers replaced by the 2-position epimer, mannose. Mannotrehalose had no detectable activity against rat intestinal trehalase, indicating it could be used in comparable applications as galactotrehalose [80]. Additionally, mannotrehalose has been shown to inhibit other intestinal disaccharidases such as sucrase, maltase, and isomaltase [140]. Ryu et al. have suggested that by inhibiting intestinal disaccharidases, non-digestible trehalose analogs such as mannotrehalose (and by extension, galactotrehalose) would limit caloric intake, ultimately leading to a reduction in obesity and diabetes [140]. Additionally, non-digestible trehalose analogs could act as calorie-free food substitutes in low-calorie diet foods, simply by their inability to enter glycolysis. However, calorie-free food substitutes have a checkered history in both implementation and public acceptance, and the relative sweetness of these analogs has not been studied in great detail. Both galacto- and mannotrehalose have been synthesized on small scales enzymatically [71, 78, 80, 82], and are excellent candidates for production via TreT or TreP catalysis assuming these methods can be adapted to permit large-scale synthesis.

Recently, a new class of trehalose analogs was discovered in the actinomycete genus *Lentzea*. Unlike the unnatural galactotrehalose and mannotrehalose analogs discussed above, lentztrehalose analogs are found naturally in *Lentzea* sp. The lentztrehalose class is modified at the 4-position with a 2,3'-dihydroxy-3-methylbutoxy moiety (lentztrehalose A, **3**), which can be di-dehydroxylated (lentztrehalose B, **65**) or cyclized (lentztrehalose C, **66**) [5]. Lentztrehaloses do not inhibit trehalase like galactotrehalose or mannotrehalose. However, they are resistant to trehalase degradation, up to a point. Unlike galactotrehalose and mannotrehalose, trehalase has limited activity on the lentztrehalose class, and can break them apart given sufficient concentration and time [141]. Lentztrehalose A was tested against several mammalian cell lines and had low cytotoxicity, and was nontoxic when administered both orally and intravenously in a mouse model [141]. It has also been shown to induce autophagy, which has implications for the treatment of neurodegenerative diseases, as discussed below [142]. A recent report disclosed an efficient 6-step chemical synthesis of lentztrehalose A starting from trehalose, which allowed determination of the natural compound's absolute configuration and should facilitate future SAR studies [143]. Overproduction of lentztrehaloses in culture may provide an efficient route for large-scale production, although further optimization will be needed [142].

Aside from dietary applications, non-digestible trehalose analogs may be useful in protein stabilization applications. There is a great deal of precedent for using trehalose to stabilize biomolecules from osmotic,

thermal, and oxidative stress, as mentioned above. These properties have allowed trehalose to be employed as a food preservative, vaccine preservative, and general protein stabilizer [2]. However, the problem with adding any carbon source to food packaging or pharmaceutical is that it makes the item more prone to colonization by unwanted microorganisms. The non-digestible analogs galactotrehalose, mannotrehalose, and the lentztrehaloses should provide similar levels of protection as trehalose. However, these analogs' reduced reactivity with microbial trehalases may have the added benefit of limiting unwanted microbial contamination.

Finally, there may be applications for non-digestible trehalose analogs in diseases of protein aggregation. Trehalose moderates symptoms in disease models of Huntington's and Alzheimer's diseases by reducing protein aggregation [144–146]. However, trehalose has also recently been shown to activate autophagy in both Huntington's and Alzheimer's models. Trehalose easily crosses the blood brain barrier, and inhibits solute carrier 2A (SLC2A) class transmembrane saccharide transporters. This inhibition leads to cyclic AMP activation and ultimately autophagy [147]. Much like the caloric examples cited above, it is likely this process could be increased by using a trehalose analog that cannot be degraded by trehalase before it reaches its target. In this regard, there may be value in molecules such as lentztrehalose, which is resistant to degradation and exhibits autophagic induction activity. However, whether non-digestible trehalose analogs cross the blood brain barrier and inhibit SLC2A transporters has yet to be demonstrated.

Other applications

Applications of trehalose analogs are by no means limited to the areas that we focused on in this review. For example, immunostimulatory TDM from mycobacteria is a key component of Freund's adjuvant, which has been used for decades in vaccines. Simplified synthetic analogs of TDM, such as trehalose dibehenate, exhibit beneficial immunological characteristics and possess better toxicity profiles [148]. Recently, there have been reports of acetylated versions of trehalose with increased membrane permeability, which allow for enhanced uptake by mammalian cells and subsequent deacetylation by intracellular esterases [149]. This strategy for intracellular delivery of trehalose could prove invaluable for stabilizing cell lines and other biological products. The importance of trehalose metabolism in plant growth and development [4] may provide opportunities for the development of trehalose analogs that manipulate plant signaling for agricultural applications. Excitingly, during the proof stage of preparing this manuscript, the Davis group reported photo-activatable, plant-permeable analogs of trehalose-6-phosphate [150]. When administered to plants, these compounds underwent sunlight-mediated release of trehalose-6-phosphate, providing increased grain yield and enhanced recovery from stress [150]. In addition to biomedical and biotechnological uses, trehalose analogs will continue to play an important role in various areas of fundamental research, including SAR studies on bioactive trehalose derivatives, characterization of trehalose-processing enzymes, and determination of the *in vivo* functions of trehalose metabolic pathways in numerous living systems.

Conclusions and future directions

Trehalose analogs have a breadth of emerging applications in biomedicine and biotechnology, as well as basic research. The difficulty of trehalose analog synthesis has slowed the development and utilization of these compounds. Encouragingly, though, just like the applications of native trehalose escalated dramatically after the Hayashibara trehalose synthesis process was introduced, progress in the development of trehalose analogs has also correlated with the introduction of improved methods for their synthesis. Trehalose-specific chemical synthesis strategies involving stereoselective α,α -glycosylation or regioselective functionalization of trehalose offer high flexibility with respect to the target molecule structure, but they are generally lengthy, tedious, and inefficient. On the other hand, chemoenzymatic approaches to analog synthesis can be exceptionally fast, simple, and efficient, but they have limitations with respect to structural flexibility and

scale-up. Thus, although it is now possible to generate and evaluate trehalose analogs in a research setting, their commercial adoption will require further synthetic improvements in most cases. In our view, the most impactful improvement will involve scaling up chemoenzymatic syntheses of trehalose analogs.

In the meantime, there are several key areas where trehalose analogs have shown promise, warranting further research. Detectable trehalose analogs bearing stable isotopes, radionuclides, fluorophores, and click chemistry labels enable the study of trehalose metabolism in various contexts and may prove useful as diagnostic tools in the future. Toward this end, a better understanding of the specificity of trehalose-based probes for various microbial species is needed, as is a clearer picture of how these compounds are (or are not) metabolized by humans. Of particular interest in this area are ^{18}F -modified trehalose analogs, which could provide an unprecedented ability to visualize trehalose metabolism in vivo in animal models and humans. Fortunately, the past few years have witnessed the creation of novel synthetic methods that allow the rapid synthesis and evaluation of trehalose analogs containing fluorine and other detectable tags. The development of trehalose-based inhibitors has yielded some compounds with anti-mycobacterial activity, but their generally low potency suggests limited potential as drugs for treating tuberculosis. More likely, trehalose-based inhibitors will be useful as probes of trehalose-processing enzymes in vitro or in cells. Future efforts in this area should seek to better understand inhibitors' mechanism of action, improve their potency, and explore their uses outside of mycobacteria. Finally, significant progress has been made in identifying trehalose analogs that maintain native trehalose's beneficial features for food and pharmaceutical applications, but are resistant to enzymatic degradation. In these regards, molecules such as galacto- and mannotrehalose exhibit attractive properties, but they remain challenging to synthesize on a large scale.

Trehalose analogs are an exciting class of molecules with important applications on the horizon. Chemists' ability to tailor the structure of trehalose for specific applications will continue to be of paramount importance in these pursuits.

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