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Edaxadiene: A New Bioactive Diterpene from *Mycobacterium tuberculosis*

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Mycobacterium tuberculosis has infected up to one-third of the global population and remains one of the leading causes of fatal infections, as the resulting disease is responsible for close to two million deaths annually.[1] The bacterium enters and resides in its host cell macrophage by subverting phagosomal processing, such that following engulfment the resulting phagosome compartment remains at an early endosomal stage rather than maturing into a bactericidal phagolysosome. Several mechanisms have been proposed for this critical aspect of pathogenesis, and it seems that there are multiple means by which *M. tuberculosis* prevents phagosomal maturation.[1]

While there is clear evidence that mycobacterial specific cell surface lipids play a role in the arrest of phagosome maturation,[2] that for other effectors remains less definitive, with different genetic screens indicating roles for nonoverlapping sets of genes. One such screen, focused on primary effects early in the infection process, highlighted a role for the product of a five gene operon nominally involved in isoprenoid biosynthesis, with mutations in the two unique, presumably nonredundant genes (Rv3377c and Rv3378c) leading to a significantly decreased ability to prevent phagosomal maturation.[3] Specifically, while phagosomes containing wild type *M. tuberculosis* fail to acidify below pH 6.2, those containing the corresponding mutant mycobacteria acidify to pH 5.7. This more than 3-fold increase in proton concentration results in a significant reduction in bacterial proliferation in macrophage cell culture, and these mutants were among those with the most extreme phenotype,[3] indicating that the product of the operon plays a role in the initial stages of *M. tuberculosis* entry into macrophages. Later work demonstrated that the enzyme encoded by the first of these genes, Rv3377c, acts as a diterpene cyclase that produces bicyclic halimadienyl diphosphate (**1**) from the acyclic primary metabolite (*E,E,E*)-geranygeranyl diphosphate (GGPP) via a protonation-initiated (i.e., class II) cyclization mechanism.[4]

Given the similar phenotypic consequences for mutations in both Rv3377c, encoding the known class II diterpene cyclase, and the neighboring Rv3378c, we hypothesized that Rv3378c encodes an enzyme acting on halimadienyl diphosphate to produce a further elaborated product. In particular, while Rv3378c is annotated as encoding a hypothetical protein of unknown function, the translated sequence contains an aspartate-rich DDXXD divalent metal binding motif in common with enzymes catalyzing isoprenyl diphosphate ester cleavage and subsequent carbon–carbon bond formation in isoprenoid biosynthesis (i.e., isoprenyl diphosphate and class I terpene synthases, as well as prenyltransferases).[5] The purified recombinant enzymatic gene product of Rv3378c catalyzes just such a reaction, removing the pyrophosphate group of halimadienyl

Scheme 1. Cyclization of **1** to **3** Catalyzed by Rv3378c/MtEDS and the Corresponding Biomimetic Cyclization of **2** to **3**
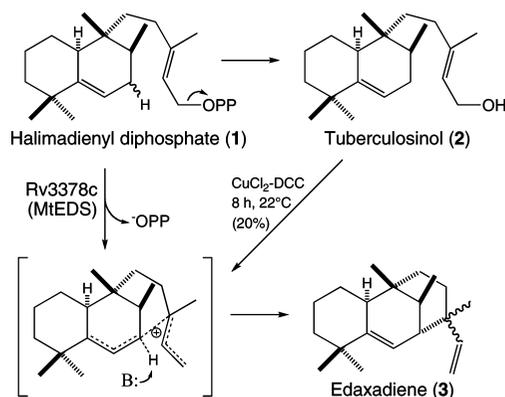
diphosphate to specifically form a single diterpene olefin (Figure S1).[6] Further characterization of this enzymatic reaction demonstrated the expected requirement for the divalent metal ion cofactor that is typical of isoprenyl diphosphate and class I terpene synthases (Figure S2).[5]

Although it was possible to produce and purify ∼250 μg of this enzymatically generated diterpene through an intensive effort, the Rv3378c encoded enzyme was found to be unstable in extended large-scale incubations. Thus, we turned to a biomimetic synthetic chemical reaction to generate sufficient product for structural characterization. Specifically, pseudourea-mediated dehydration[7] of the primary alcohol corresponding to hydrolytic dephosphorylation of **1**, which has been termed tuberculosinol (**2**),[4] and is readily produced by enzymatic dephosphorylation of **1** produced from GGPP by the Rv3377c encoded enzyme. The chemical reaction generated several diterpene olefins, with the desired product found in ∼20% yield (by GC-MS analysis), providing a convenient semisynthetic route for production of ∼1 mg of pure compound. Extensive comparison of NMR spectra verified the equivalence of this semisynthetically produced compound with the enzymatically generated diterpene.

Upon structural analysis by NMR (Figures S3−5 and Table S1), this diterpene was found to have a further cyclized (i.e., tricyclic) hydrocarbon backbone. However, rapid conformational dynamics of the newly formed ring (relative to **1/2**) prevented the acquisition of NOE data, and thus, it was not possible to definitively assign the stereochemistry of the geminal methyl–vinyl pair. Formation of the proposed additional six-carbon ring presumably occurs via concerted ring closure and deprotonation (Scheme 1). Notably, this represents a rather unusual terpene cyclization reaction, as these generally proceed via direct attack of a π bond on the delocalized allylic carbocation initially formed upon diphosphate ester cleavage.[5] The resulting diterpene further appears to have a previously

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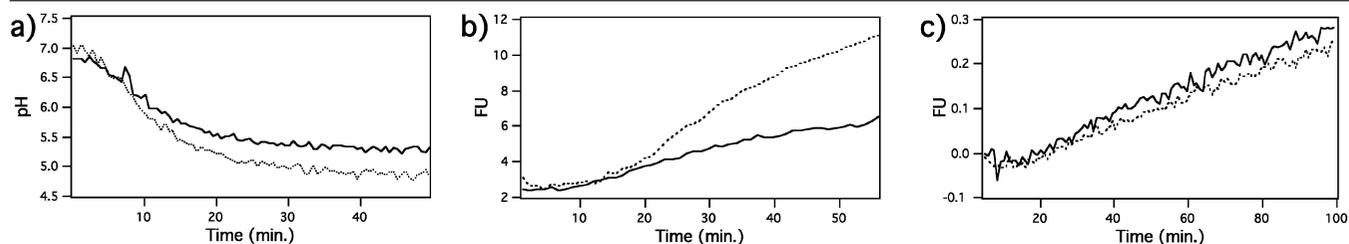§ Present address: Wenzhou Medical School, PRC.

Figure 1. Effect of edaxadiene (**3**) on phagosomal maturation. Analysis of the kinetics of phagosome maturation determined by ratio fluorometric measurements of (a) intraphagosomal pH (carboxyfluorescein), (b) intraphagosomal proteolytic activity (DQ Green protease substrate), and (c) intraphagosomal β-galactosidase activity (C12-fluorescein galactopyranoside), following bead uptake (solid lines = edaxadiene; dashed lines = control).

unidentified skeletal structure (**3**), which we propose to name edaxadiene, from the Latin edax—destructive or consuming.

Our results indicate that Rv3378c encodes a halimadienyl diphosphate (**1**) specific class I diterpene synthase that produces edaxadiene (**3**), and we propose to name this enzyme MtEDS. However, while this study was in progress, another report was found suggesting that Rv3378c removed the pyrophosphate group of **1** to form an equal molar mix of tuberculosinol (**2**) and the related tertiary alcohol isotuberculosinol,[8] although no biological activity was reported for these compounds, and we found no evidence for production of either compound with our enzymatic preparations.

To determine if edaxadiene (**3**) is able to directly affect phagosomal maturation, the pure compound was tested in bead based phagocytosis assays (Figure 1).[9] While phagosomes formed with control beads exhibited normal endosomal progression, including full acidification to pH 4.8, as well as the acquisition of proteolytic and β-galactosidase activity, beads coated with edaxadiene exhibited only partial maturation of their phagosome, with acidification arrested at pH 5.3 and a significant decrease in proteolytic activity. However, this clearly was a selective rather than generally toxic effect, as there was very little change in β-galactoside activity. This is consistent with continued accessibility of *M. tuberculosis*-containing phagosomes to components of the early recycling endosomal system.[1] Notably, edaxadiene's effect on acidification, an increased pH of ~0.5 units, is comparable to the effect of mutations in the Rv3377c and Rv3378c/MtEDS genes on the pH of *M. tuberculosis*-containing phagosomes, which were ~0.5 pH units more acidic than those containing wild type bacteria.[3]

Intriguingly, while MtEDS does contain the previously noted DDXXD motif, it does not exhibit any other homology to typical class I terpene synthases (i.e., MtEDS exhibits <10% overall amino acid sequence identity with any previously identified class I terpene synthase). Divergence in bacterial class I diterpene synthases has been previously noted,[10] although these are more similar to each other than MtEDS (≥15% identity). Furthermore, despite the lack of homology between the previously reported class I diterpene synthases from bacteria, and of those with the better known eukaryotic terpene synthases, all these contain a conserved NDX$_2$SX$_3$E motif in addition to the previously noted DDXXD motif. Indeed, in sequence comparisons of plant, fungal, and bacterial class I diterpene synthases,[11] it is the NDX$_2$SX$_3$E, rather than DDXXD, motif whose position seems to be better conserved. Every structurally characterized class I terpene synthase has been found to utilize both of these motifs in binding a catalytically requisite trinuclear divalent metal ion cluster, and mutational analysis has demonstrated that both motifs are critical for class I terpene synthase activity,[5] including seemingly less well conserved examples in some plant terpene synthases.[12] However, in addition to the distinct lack of sequence identity/similarity to any other

terpene synthase, bacterial or otherwise, MtEDS also does not contain this second NDX$_2$SX$_3$E divalent metal binding motif (Figure S6). Nevertheless, MtEDS exhibits a catalytic efficiency equivalent to that of the other characterized bacterial class I diterpene synthases ($k_{cat}/K_M = 1.1 \times 10^4$ M^{-1} s^{-1}),[10,13] including a reasonable affinity for its substrate (**1**; $K_M = 12$ μM; Figure S7). In addition, mutational analysis demonstrates a catalytic role for the originally noted DDXXD motif, as alanine substitution for these aspartates reduces catalytic activity 10^2–10^4-fold (Table S2). Thus, MtEDS may provide an example of extreme divergence from other class I terpene synthases, although a convergent evolutionary origin cannot be ruled out.

Regardless of evolutionary origin, the ability of edaxadiene (**3**) to directly inhibit endosomal progression presumably contributes to at least early suppression of phagosome maturation in the infection process of *M. tuberculosis*. Thus, it will be of considerable interest to identify the molecular target of **3** and further characterize the enzymatic activities involved in its biosynthesis, in particular, the class II diterpene cyclase encoded by Rv3377c and unusual class I diterpene synthase encoded by Rv3378c (MtEDS) dedicated to the production of **3**.

Supporting Information Available: Methods, NMR, MS and kinetic data, and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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