Drug Targeting *Mycobacterium tuberculosis* Cell Wall Synthesis: Genetics of dTDP-Rhamnose Synthetic Enzymes and Development of a Microtiter Plate-Based Screen for Inhibitors of Conversion of dTDP-Glucose to dTDP-Rhamnose

YUFANG MA,1 RICHARD J. STERN,1 MICHAEL S. SCHERMAN,1 VARALAKSHMI D. VISSA,1 WENXIN YAN,1 VICTORIA COX JONES,1 FANGQIU ZHANG,2 SCOTT G. FRANZBLAU,2 WALTER H. LEWIS,3 AND MICHAEL R. McNEIL1*

Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523; College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois 60612; and Department of Biology, Washington University, St. Louis, Missouri 63130

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An l-rhamnosyl residue plays an essential structural role in the cell wall of *Mycobacterium tuberculosis*. Therefore, the four enzymes (RmlA to RmlD) that form dTDP-rhamnose from dTTP and glucose-1-phosphate are important targets for the development of new tuberculostatics. *M. tuberculosis* genes encoding RmlA, RmlC, and RmlD have been identified and expressed in *Escherichia coli*. It is shown here that genes for only one isotype each of RmlA to RmlD are present in the *M. tuberculosis* genome. The gene for RmlB is Rv3464. Rv3264c was shown to encode ManB, not a second isotype of RmlA. Using recombinant RmlB, -C, and -D enzymes, a microtiter plate assay was developed to screen for inhibitors of the formation of dTDP-rhamnose. The three enzymes were incubated with dTDP-glucose and NADPH to form dTDP-rhamnose and NADP+ with a concomitant decrease in optical density at 340 nm (OD340). Inhibitor candidates were monitored for their ability to lower the rate of OD340 change. To test the robustness and practicality of the assay, a chemical library of 8,000 compounds was screened. Eleven inhibitors active at 10 μM were identified; four of these showed activities against whole *M. tuberculosis* cells, with MICs from 128 to 16 μg/ml. A rhodanine structural motif was present in three of the enzyme inhibitors, and two of these showed activity against whole *M. tuberculosis* cells. The enzyme assay was used to screen 60 Peruvian plant extracts known to inhibit the growth of *M. tuberculosis* in culture; two extracts were active inhibitors in the enzyme assay at concentrations of less than 2 μg/ml.

The necessity for new drugs against *Mycobacterium tuberculosis* due to increasing resistance to the present chemotherapeutic agents is well documented (6, 12, 21, 33, 41, 44). An attractive target for such new agents is the mycobacterial cell wall (2, 3, 29), since the wall is necessary for viability and several known drugs such as isoniazid (52) and ethambutol (11, 49) inhibit cell wall synthesis. The mycobacterial cell wall core consists of three interconnected macromolecules (Fig. 1). The outermost, the mycolic acids, are 70- to 90-carbon-containing, branched fatty acids which form an outer lipid layer in some ways similar to the classical outer membrane of gram-negative bacteria (5). The mycolic acids are esterified to the middle component, arabinogalactan (AG), a polymer composed primarily of D-galactofuranosyl and D-arabinofuranosyl residues. AG is connected via a linker disaccharide, α-L-rhamnosyl-(1→3)-α-D-N-acetyl-glucosaminosyl-1-phosphate, to the 6 position of a muramic acid residue in the peptidoglycan. The peptidoglycan is the innermost of the three cell wall core macromolecules.

This structural arrangement shows why AG is necessary for mycobacterial viability, as it tethers the lipid layer to the peptidoglycan layer. Moreover, a rhamnosyl residue, a sugar not found in humans, plays a crucial structural role in the attachment of AG to peptidoglycan (Fig. 1). l-Rhamnosyl residues are found in other bacteria as components of O antigens or extracellular polysaccharides but not as an essential cell wall component. l-Rhamnosyl residues are synthesized in nature by a single pathway requiring four enzymes (RmlA to RmlD) and beginning with TTP and α-D-glucose-1-phosphate (13, 19, 32, 36), as shown in Fig. 1. There is no salvage pathway for the formation of dTDP-l-rhamnose (dTDP-Rha) as with GDP-l-fucose (38, 39), and when l-rhamnose is utilized by bacteria as a carbon source, it is isomerized and broken down into smaller metabolites (26). Thus, the only way for *M. tuberculosis* to form the cell wall rhamnosyl residue is as shown in Fig. 1. In another study (J. A. Mills, K. Motichka, M. Jucker, H. P. Wu, B. C. Uhlic, R. J. Stern, M. S. Scherman, V. Vissa, W. Yan, M. Kundu, M. Kundu, and M. R. McNeil, unpublished data), it has been shown that the rhamnosyl transferase (encoded by the gene *wbbL*) that utilizes dTDP-Rha as a substrate to put the rhamnosyl residue into cell wall AG is essential for bacterial growth. Although the rhamnosyl transferase is a good drug target in itself, there are many advantages in targeting the four enzymes required to make its required substrate, dTDP-Rha. These include the facts that the enzymes are soluble (22, 28,
46), that crystal structures of these enzymes from bacteria are forthcoming (1, 15–17), and that for one of these enzymes, RmlB, detailed mechanistic studies have been performed (18, 34, 42, 45).

Although the completion of the entire genome sequence of \textit{M. tuberculosis} (8) greatly aids in the identification of the enzymes involved in dTDP-L-rhamnose synthesis, unambiguous identification from just the sequence data is problematical. In addition, it is important to determine whether additional genes encoding isoenzymes for any of the key conversions exist, because inhibition of multiple enzymes catalyzing the same reaction might be difficult. Here, we report experiments to determine which of the genes encoding proteins with homology to RmlA to RmlD actually encode dTDP-Rha formation enzymes. Following this, a microtiter plate assay to identify inhibitors of the conversion of dTDP-glucose to dTDP-rhamnose by \textit{M. tuberculosis} RmlB, -C, and -D was developed. The assay was used to screen both pure compounds and crude plant extracts.

**MATERIALS AND METHODS**

\textbf{Preparation of plasmids to express} \textit{M. tuberculosis} genes. Rv3264c, Rv3464, Rv3784, and Rv3468c were cloned into pET29b (Novagen, Madison, Wis.). PCR
was conducted using the following primers (coding sequence to the right of the hyphen): Rv3264c, 5′ GTAATTCCTAT-ATGCCAATC/CACAAAGTCTGAT3′ (sense) and 5′ CCGCTCAGTG-CCAACTCGTGAAGAATCCAGCC3′ (antisense); Rv3464, 5′ TTAATCAT-ATGGTGCTAGCTAACC3′ (sense) and 5′ TTTACAT-ATGGCGGTTGCTAGTC3′ (antisense); Rv3784, 5′ CGTGA GGCTATAGTAAGAATCTGCGC3′ (sense) and 5′ CCTGCAGG- TTAGGAAGCGGTGAAGCCTA3′ (antisense); and Rv3468c, 5′ GCCTAG GCATTGTAAGAACCATGATGCACCC3′ (sense) and 5′ ATCCCTGAG-TCAGGCGCTGCGGAGGCAAA3′ (antisense). The PCR products were subsequently cloned into pET29b using the NdeI and XhoI sites present in pET29b (the PCR products of Rv3784 and Rv3468c were cloned with AseI, which makes an overhang that can go into an NdeI site). Rv3464 was also cloned with an N-terminal His tag using 5′ TTAATCAT-ATGCCGTGCTAGTC3′ (sense) and 5′ TTTACAT-ATGGCGGTTGCTAGTC3′ (antisense) using the NdeI and XhoI sites to clone into pET11b (Novagen).

**Escherichia coli strains.** E. coli DH5α (Life Technologies, Inc., Grand Island, N.Y.) was used for cloning purposes. For expression, potential manB-bearing plasmids were electroporated into E. coli BL21(DE3) (Novagen); the potential rmlA- or manB-bearing plasmid (Rv3264c) was electroporated into E. coli sbl874(DE3) (22, 48).

**Assay for α-D-glucose-1-phosphate thymidylyltransferase (RmlA) and α-D-mannose-1-phosphate guanylyltransferase (ManB) activities.** To prepare the enzyme extract, E. coli sbl874(DE3) containing open reading frame (ORF) Rv3264c cloned into pET29b (and, separately, an empty pET29b control) was grown to an optical density (OD) of 0.6 to 0.7 with agitation at 37°C. The culture was induced (at 37°C) with isopropyl-β-D-thiogalactoside (IPTG) at 1 mM for 3 to 5 h, and harvested by centrifugation. The cells were then broken by sonication, and 8 ml of 100 mM Tris-HCl, pH 7.6, 1 mM EDTA, 25 μM pepstatin A, 50 μM leupeptin, 10 μg of pepstatin A, 25 μM leupeptin, 10 μg of pepstatin A, 25 μM leupeptin, and M pepstatin A, were added, including a control inhibitor containing 300 mM of dTDP and a control of DMSO only. A cocktail (75 μl) of HEPS buffer (50 mM, pH 7.6, with 1 mM MgCl₂ and 10% glycerol) containing 0.5 mM of NAD⁺, 20 mM of NADPH (prepared fresh daily), 1 μg of RmlB, 1 μg of RmlC, and 0.4 μg of RmlD was added to each well. (These enzyme amounts were found empirically to be in the range where the decrease of any of the three enzymes resulted in a decrease in overall activity.) The reactions were started by adding 20 mM of dTDP-Glc in 25 μl of the HEPS buffer to each well, and the plate was incubated at 30°C. At different time points, samples were examined on an enzyme-linked immunosorbent assay plate reader, typically at 0, 10, 20, 30, 60, 90, and 120 min at 340 nm, and the data were analyzed by comparing the slopes of the potential inhibitors with the slopes of the controls using the computer program Excel. To assay RmlA, a 1-h preincubation using the above cocktail, modified to contain 2 μg of Rml and no RmlB or RmlD, was performed, to prepare dTDP-6-deoxy-α-xylo-4-hexulose in situ. Then, 0.25 μg of RmlC and 4 μg of RmlD and NADPH were added, and the reaction was monitored as described above. To assay RmlD, dTDP-6-deoxy-α-xylo-4-hexulose in situ in the same fashion and then 10 μg of RmlC and 0.4 μg of RmlD were added and the reaction was monitored as described above.

**Assays against M. tuberculosis in culture.** Compounds were assayed against M. tuberculosis H37Rv in culture by the Alamar blue assay as described previously (9).

**RESULTS**

**Genes encoding RmlA.** We have reported the cloning and expression of an M. tuberculosis gene encoding RmlA (28). This gene was designated Rv0334 and identified as mlaI in the genome sequence paper (8). However, the genome was found to contain another ORF, Rv3264c, encoding a protein with strong homology to RmlA (8). In addition, this ORF was in an operon with wbbL and rmlD (see discussion of rmlD below), strongly suggesting a second mlaI gene, and Rv3264c was therefore designated mlaI2 (8). However, the protein encoded by this ORF also showed homology to ManB, the analogous enzyme in GDP-mannose synthesis that forms GDP-mannose from α-D-mannose-1-phosphate and GTP (α-D-mannose-1-phosphate guanylyltransferase).

To resolve this issue, ORF Rv3264c was cloned into pET29b and expressed in E. coli sbl874(DE3), a strain (48) which was modified to contain DE3 in the chromosome of either manB (as shown in Fig. 2) or mlaI (also shown in Fig. 2; also, the rml genes, which are part of the rfb cluster, are known to be deleted [35, 48]). Extracts from the bacterium transformed with pET29b-Rv3264c and pET29b (vector-only control) were assayed for both α-D-glucose-1-phosphate thymidylyltransferase (RmlA) and α-D-mannose-1-phosphate guanylyltransferase (ManB) activities in the reverse-direction reaction by monitoring the PPi-dependent loss of dTDP-Glc and/or GDP-Man as shown in Fig. 2. The result was that the enzyme expressed from the Rv3264c gene was active only against GDP-Man (Fig. 2), demonstrating that Rv3264c encodes ManB. These results were consistent with a recent report of the purification of ManB from Mycobacterium smegmatis (37), where the N-terminal sequence of the M. smeg-
Rv3634c has an N-terminal sequence identical, except for one amino acid, to that of UDP-galactose epimerase (GalE) purified from *M. smegmatis* (51). We thus conclude that this ORF encodes GalE rather than RmlB. GalE and RmlB enzymes catalyze similar oxidation at C-4 of a glucosyl residue and in general show strong homology to each other.

Rv3464 was cloned into pET29b and expressed in *E. coli* BL21(DE3), and the enzyme was partially purified. The band of the putative RmlB protein was blotted to nitrocellulose and trypsinized, and the resulting peptides was analyzed by liquid chromatography-mass spectrometry, which confirmed the identity of the expressed polypeptide. The two remaining candidates, Rv3784 and Rv3468c, were also cloned into pET29b and expressed in soluble form in *E. coli* BL21(DE3). Extracts containing the expressed protein were then assayed for dTDP-glucose dehydrogenase (RmlB) activity. Activity (OD at 318 nm [OD$_{318}$] = 0.425) was readily observed for the strain expressing Rv3464, but no activity was seen for the strains expressing Rv3784 (OD$_{318}$ = 0.046) or Rv3468c (OD$_{318}$ = 0.068) or a control strain containing only the empty pET29b vector (OD$_{318}$ = 0.074). These results are consistent with only one isotype of RmlB, the one that shows a very high homology with RmlB from other organisms, although the limitations of drawing conclusions from the lack of enzymatic activity are recognized.

**Genes encoding RmlC and for RmlD.** The *M. tuberculosis* genome sequence (8) shows only a single ORF encoding a protein with homology for RmlC (Rv3465) and also only a single ORF encoding a protein with homology for RmlD (Rv3626c). Both Rv3465 (46) and Rv3626c (22) have previously been expressed, and these genes do express the dTDP-6-deoxy-D-xylo-4-hexulose epimerase (RmlC) and dTDP-6-deoxy-L-lyxo-4-hexulose reductase (RmlD) enzymes, respectively. Thus, only single polypeptides with sequences corresponding to RmlC and RmlD are present in the *M. tuberculosis* genome.

**Development of a microtiter-based assay for RmlB, -C, and -D.** The fact that RmlD converts dTDP-6-deoxy-L-lyxo-4-hexulose to dTDP-Rha with the concomitant oxidation of NADPH to NADP$^+$ makes possible a facile microtiter plate assay. Thus, a mixture of RmlB (this study), RmlC (46), and RmlD (22) was incubated with dTDP-Glc (0.2 mM) and NADPH (0.2 mM) in microtiter plates at 37°C, and the OD$_{530}$ was monitored. Controls with no dTDP-Glc and with an inhibitor, dTDP, were also performed. The OD$_{530}$ drops in a linear fashion in the early part of the time course (Fig. 3B [control reaction]). It was found necessary to purify all enzymes to avoid a non-dTDP-Glc-dependent oxidation of NADPH. NAD$^+$ was also included in the reaction mixtures, since it was found that after purification of RmlB it was necessary to keep the enzyme active. It was also found that individual proteins had to be sufficiently pure not to reduce NAD$^+$ to NADH (which absorbs at OD$_{530}$), presumably via oxidation of glyceral present to help with the storage of the enzymes.

**Use of the microtiter plate-based assay to search for inhibitors.** The microtiter plate assay can be used to screen for inhibitors among drug candidates obtained in microtiter plate format. The slope of the oxidation of NADPH of wells with compounds is compared to that of the no-compound control and converted to percent inhibition by the formula.

![Diagram](image-url)
Thus, a perfect inhibitor would have a slope of 0 and 100% inhibition; a compound that showed no inhibition would have the same slope as the no-compound control and have an inhibition of 0%. The compound dTDP is run as a positive inhibitor control with typically 80% inhibition at 3 mM.

To test the robustness and practicality of the assay, 8,000 compounds supplied in microtiter plate format (Nanosyn, Tucson, Ariz.) were assayed. In terms of solubility and permeability, the Nanosyn compounds were selected based on the Lipinski “rule of 5,” specifically, for molecular weight, log P values, hydrogen bond donors, and hydrogen bond acceptors (27). With respect to these four criteria, 89% of the compounds of the library were in the “drug” category for all four criteria, and 9% of the compounds met three of the four criteria. The compounds were also selected for the presence of “drug-like” functional groups (14) and the lack of reactive groups such as aldehydes. The library was screened at a concentration of 10 μM.

Crude extracts made from Peruvian medicinal plants identified by an indigenous Aguaruna tribe were extracted in 95% ethanol, detannified, and tested in DMSO in a whole-cell assay of M. tuberculosis using the virulent human strain H37Rv (9, 50). Of the 492 extracts tested, 26% inhibited the growth of whole M. tuberculosis cells at 100 μg/ml. The ability of the enzyme assay to analyze such extracts was then tested. Sixty of the extracts active against whole M. tuberculosis cells were screened for their ability to inhibit the conversion of dTDP-Glc to dTDP-Rha. Two were quite active, and the inhibition activity of one, from a liana whose resin was applied by indigenous...
TABLE 1. Inhibitors of Rml enzymes and their activities against *M. tuberculosis* in culture

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<th>RmlC</th>
<th>RmlD</th>
<th>Enzyme(s) inhibited</th>
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people to heal wounds due to cuts, is shown at different concentrations in Fig. 4.

**DISCUSSION**

**Identification of dTDP-Rha-synthesizing genes.** Only one copy each of *rmlC* and *rmlD* is present in the *M. tuberculosis* genome (8), and we have shown previously that the genes do in fact encode the expected enzymes (22, 46). The situation for *rmlA* is now also seen to be unambiguous, as only two candidate genes are present and we have clearly shown the function of Rv0334 to be encoding RmlA (26) and that of Rv3264c to be encoding ManB (this study). The situation is not as clear-cut for the *rmlB* gene. Rv3464 clearly encodes RmlB, as active enzyme can be expressed from it. Rv3634c encodes a protein with homology to RmlB, but the fact that its N-terminal sequence is nearly identical to that of UDP-galactose epimerase (GalE) from *M. smegmatis* (51) indicates that this gene encodes the GalE protein. Two other possible candidates, Rv3784 and Rv3468c, have been identified in the genome sequence (8); our data suggest that these genes do not encode RmlB, because they can be expressed as soluble proteins that do not show dTDP-glucose dehydratase (RmlB) activity. However, we cannot rule out the possibility that the soluble proteins were merely inactive RmlB. Therefore, it is most likely that only one isoform of RmlB is present in *M. tuberculosis*, but this has not yet been established unequivocally.

**Genome organization of dTDP-Rha-synthesizing genes.** In most other organisms, such as *E. coli*, rmlABCD are on a single operon, often in the order B, D, A, C (24, 40, 43, 47). However, from the results above and the genome sequence (8), it is clear that in *M. tuberculosis* the four genes responsible for dTDP-Rha formation are in three different loci. Thus, *rmlA* (Rv0334) is separate from all the other genes involved in rhamnosyl metabolism and appears to be the fourth gene in an operon where the functions of the proteins encoded by the other three genes are not known. The genes *rmlB* and *rmlC* (Rv3464 and Rv3465) are the second and third genes in a complex operon with perhaps five genes, where the last two genes are part of an insertion sequence (25) and the first gene encodes a protein with an unknown function. Finally, *rmlD* (Rv3266c) is the first gene of a three-gene operon. In this case the second gene is *wbbL*, encoding rhamnosyl transferase, and the third is *manB* (designated *rmlA*-2 in the genome sequence [8]), whose function is revealed in this report. There is some logic in the coordination of expression of *manB* with the rhamnose genes, as *manB* is needed for all mannosyl glycolipids (4) and polysaccharides (7), which, like rhamnosyl residues, are an important part of the mycobacterium envelope (30). The finding, however, that the *rml* genes are so scattered throughout the genome is surprising.

**Comparison of rhamnosyl formation enzyme genes in *M. tuberculosis* and *Mycobacterium leprae*.** It is of interest to compare the rhamnosyl-forming enzymes in *M. tuberculosis* and *M. leprae*. The sequencing of the *M. leprae* genome is just being completed. The genome of *M. leprae* is smaller than that of *M. tuberculosis*, many potential ORFs are degraded, and half of the DNA is noncoding (20). Since the cell wall AGs of *M. leprae* and *M. tuberculosis* are nearly identical (10), it stands to reason that the genes involved in rhamnosyl formation enzymes should be amongst the intact genes found in the *M. leprae* genome. BLAST searches of the nearly completed *M. leprae* genome confirm that this is indeed true; *rmlABCD* and *wbbL* are all five intact in *M. leprae*, and, interestingly, the grouping of the genes in the various operons is the same in both mycobacteria, although the arrangement and orientation of the operons themselves along the genome are different. This result is consistent with the essential function of rhamnosyl residues in mycobacteria.

**Targeting dTDP-Rha formation in *M. tuberculosis*.** Targeting dTDP-Rha formation in *M. tuberculosis* has much to recommend it. Four enzymes are involved, and all of them catalyze reactions that are not found in humans (although...
the formation of UDP-glucose from α-D-glucose-1-phosphate and UTP is quite homologous to the formation of dTDP-Glc catalyzed by RmlA. The enzymes are soluble and can be readily prepared in large amounts. X-ray structural analysis is becoming available for the Salmonella homologs of these enzymes (1, 15–17), with efforts proceeding on the M. tuberculosis versions. Mechanisms of action and kinetic parameters are becoming known (18, 19, 34, 42, 45). The rhamnosyl transferase, WbbL, which uses dTDP-Rha as its substrate to insert the rhamnosyl residue in the cell wall, is essential for growth, as shown by the fact that an M. smegmatis TS mutant of WbbL differing in a single amino acid (GenBank accession numbers AAF04375 and AAF04376) do not grow at the nonpermissive temperature (Mills et al., unpublished). There is no known way in nature to form dTDP-Rha other than by these enzymes. Finally, three of the enzymes (RmlB, -C, and -D) are readily assayed together by monitoring the oxidation of NADPH, and this assay is readily adaptable to microtiter plates (Fig. 3) and can be used to identify inhibitors (Table 1 and Fig. 3 and 4).

Use of the microtiter plate assay. The microtiter plate assay for RmlB, RmlC, and RmlD described above has the advantage of screening for inhibitors of three key enzymes at the same time. In practice we have found that preparing the three enzymes from E. coli in sufficient purity for the assay is straightforward but does require some care in adequately washing nickel columns when they are used in the purification. Although the experiments reported herein were done using a non-His-tagged version of RmlB, for convenience His-tagged RmlB from M. tuberculosis can readily be cloned (see Materials and Methods) and purified by standard methods after expression in E. coli and used successfully in the assay (data not shown). In identifying active inhibitors we selected compounds that inhibited the formation of dTDP-Rha more than 60%. Compounds showing activity were then retested, and generally the percent inhibition was reproducible to roughly ±20%, allowing a clear separation of active and inactive inhibitors under the conditions of the assay.

The most important finding of the present investigation is the fact that the Rml enzyme assay is sufficiently robust to uncover potential enzyme inhibitors as a starting place for continued analysis. Most compounds selected by the screen were active against more than a single enzyme. Given the structural similarities of the substrates for all three enzymes, this is perhaps not surprising. It was interesting that three of the eleven compounds (5372, 6429, and 6432) had a rhodamine core structure and a fourth (2943) had a core structure very similar to that of rhodamine. There were at least six other compounds in the Nanosyn library that had rhodamine rings but were not active in the enzyme assay, suggesting some very preliminary structure-activity relationships. It was also encouraging that four of the eleven active compounds identified in this preliminary study inhibited the growth of M. tuberculosis in culture (albeit usually at high concentrations [Table 1]). Clearly, more work must be done to show that such compounds actually affect growth of M. tuberculosis via inhibition of the rhamnosyl enzymes. Also, much work remains to identify further classes of active compounds and to determine which molecules have appropriate properties for further development. On a somewhat different track, compounds identified as enzyme inhibitors are candidates for cocrystallization with the enzymes they inhibit to reveal useful binding structures, regardless of their other properties.

The assay described here should be suitable for screening very large numbers of compounds. Although it was performed here in a 96-well format, there is no reason not to use 384-well plates or even plates containing higher number of wells, as only the OD at 340 nm needs to be monitored. The most expensive reagent is the dTDP-glucose, and the amounts of it can be decreased if a smaller overall change in OD can be tolerated (as reported here, the utilization of all the dTDP-glucose will result an OD drop of about 0.3 units). Small well sizes will also allow the use of less dTDP-glucose and will require less enzyme as well.

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