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# Azaaurones as potent antimycobacterial agents active against MDR- and XDR-TB

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**Abstract:** Herein we report the screening of a small library of aurones and their isosteric counterparts, azaaurones and *N*-acetylazaaurones, against *Mycobacterium tuberculosis*. Aurones were inactive at 20 μM, while azaurones and *N*-acetylazaaurones emerged as the most potent compounds, with nine derivatives displaying $MIC_{99}$ values ranging from 0.4 to 2.0 μM. In addition, several *N*-acetylazaaurones were found to be active against multidrug resistant (MDR) and extensively drug resistant (XDR) clinical *M. tuberculosis* isolates. The anti-mycobacterial mechanism of action of these compounds remains to be determined, however a preliminary mechanistic study confirmed that they do not inhibit the mycobacterial cytochrome *bc1* complex. Additionally, microsomal metabolic stability and metabolite identification studies revealed that *N*-acetylazaaurones are deacetylated to their azaaurone counterparts. Overall, these results demonstrate that azaaurones and their *N*-acetyl counterparts represent a new entry in the toolbox of chemotypes capable of inhibiting *M. tuberculosis* growth.

## Introduction

Tuberculosis (TB) stands as one of the most lethal infectious diseases worldwide, ranking above HIV and malaria. It is estimated that 10 million people developed TB in 2017, and that TB caused more than 1.5 million deaths in the same period.[1] The current complexity and duration of the treatment often leads to misuse, mismanagement and poor adherence. These factors collectively contribute to an increased disease burden and the emergence of drug resistance.[2] Multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) pose serious health threats, with strains resistant to first-line and multiple second-line antibiotics. [3] It is now estimated that MDR-TB and XDR-TB account for 21% and 2%, respectively, of all TB cases worldwide, [1] and these estimates are likely to increase as resistant strains spread and proliferate.[4] It is also noteworthy that reports suggest a new emerging threat, totally drug-resistant tuberculosis (TDR-TB), which is resistant to every known treatment.[5] In the past forty years, only two drugs from novel

chemical classes were approved: bedaquiline, **1** (FDA) and delamanid, **2** (EMA) (Fig. 1), both active against MDR-TB.[6] However, these drugs present several drawbacks, including common adverse effects such nausea, arthralgia and cardiac toxicity that limit their clinical usefulness. [7]

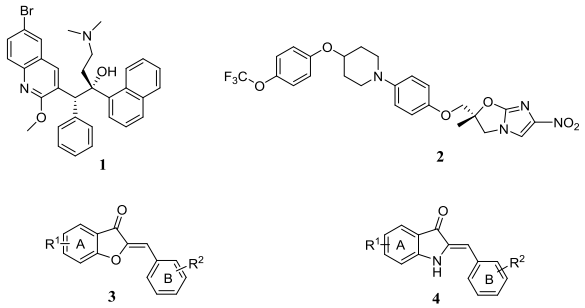


**Figure 1.** Recent FDA approved drugs bedaquiline, **1**, and delamanid, **2**, and chemical structures of aurones, **3**, and azaaurones, **4**.

Discovering new TB drugs remains a challenging task. Despite success in identifying active compounds through phenotypic screenings,[8] the conversion of hits into novel optimized leads suitable for selection as clinical candidates is hampered by the poor efficacy in eliminating *M. tuberculosis* within different host compartments, including macrophages, as well as a lack of knowledge about the specific target(s) inhibited and/or upregulated.[9] Furthermore, there is growing recognition that current TB screening programmes are characterized by significant redundancy in the nature of the compounds screened and, consequently, in the number (and variety) of mycobacterial targets identified.[10] Many high-throughput screening (HTS) campaigns performed so far have identified small, hydrophobic molecules affecting the function of essential membrane proteins such as DprE1 (subunit of the heteromeric decaprenylphosphoryl-beta-D-ribose-2'-epimerase), AtpE (membrane ATP synthase), MmpL3 (Trehalose monomycolate exporter) and QcrB (subunit of the cytochrome *c* oxireductase, *bc1* complex).[11] This highlights the prospect that highly lipophilic hit compounds may lead to toxicity that might be linked to inhibition of host membrane proteins or menaquinol membrane integrity. Interestingly, bedaquiline has been found to target the membrane protein AtpE, and its high lipophilicity (logP ca. 7) might be related to the liver and heart toxicity displayed by this compound.[12]

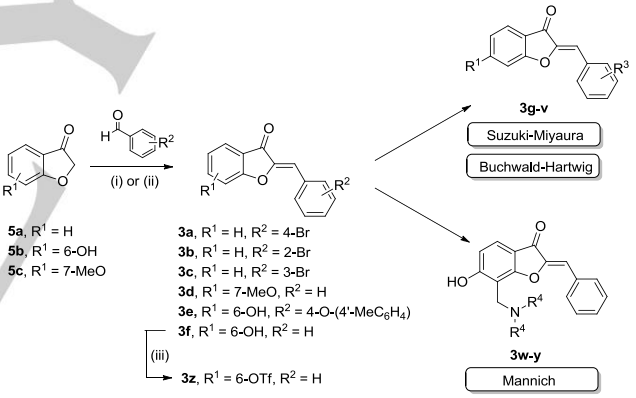
Aurones, **3** (Fig. 1), are heterocyclic compounds of the flavonoid family, with a benzofuran moiety linked to a benzylidene group at position C-2.[13] This scaffold has been studied for a variety of biological applications,[14] including as antimycobacterial agents.[15] However, the structure-activity relationship (SAR) studies of aurones are limited as a result of the narrow range of potencies displayed by these compounds. As a strategy to enrich the SAR, we hypothesised that the isosteric azaaurones, **4**, (Fig. 1), which present stereoelectronic and hydrogen-bonding properties significantly different from those of aurones, would broaden the range of potencies against *M. tuberculosis*. Although previously studied as antimalarial agents,[16] to the best of our knowledge azaaurones have not been screened for activity against *M. tuberculosis*. We now report the screening of our library comprising 70 aurones, azaaurones and their *N*-acetyl precursors against *M. tuberculosis*. Azaaurones and *N*-acetylazaaurones were found to be significantly more potent than their aurone counterparts. The most potent compounds were also tested against XDR- and MDR-TB strains. To better understand the pharmacokinetic behaviour of the azaaurone scaffold, preliminary in vitro ADME characterization was also performed.

## Results and Discussion

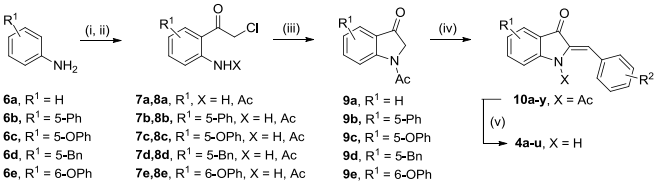
### Chemistry

Aurones, **3**, were previously developed by our group in order to probe their activities against *Plasmodium falciparum*. The synthesis of aurones involved the aldol condensation of benzofuranones **5** with the appropriately substituted benzaldehydes,[17] leading to the desired compounds with the more stable *Z* configuration (Scheme 1).[14a] Microwave assisted Suzuki-Miyaura and Buchwald-Hartwig cross-coupling reactions were also used to decorate the aurone scaffold.[18]

Azaaurone derivatives, **4**, were synthesized as reported previously and as depicted in Scheme 2.[16b] The *N*-acetyl precursors, **10**, were obtained as mixtures of *E* and *Z* isomers, while hydrolysis of **10** generated the thermodynamically more stable *Z* isomer of **4**.




**Scheme 1.** (i) $Al_2O_3$, MeOH, reflux under $N_2$, 48 hours; (ii) glacial AcOH, HCl, rt, 4 hours; (iii) $Tf_2O$, TEA, DCM.



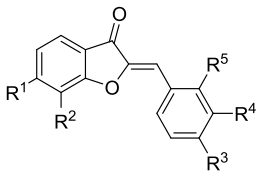

**Scheme 2.** (i) a) $BCl_3$ 1M in dichloromethane, chloroacetonitrile, $ZnCl_2$, dry 1,2-DCE, reflux; b) HCl 1M, reflux; (ii) AcOH, $Ac_2O$; (iii) NaH, dry DMF; (iv) ArCHO, piperidine, toluene, reflux; (v) KOH (50% in $H_2O$), MeOH, rt.

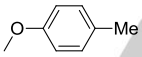
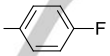
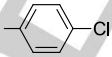
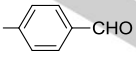
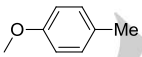
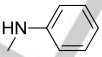
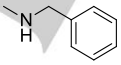
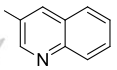
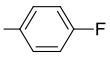
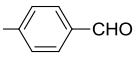
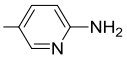
### Activity against *Mycobacterium tuberculosis* H37Rv

Aurones, **3**, azaaurones, **4**, and *N*-acetylazaaurones, **10**, were screened against the virulent *M. tuberculosis* H37Rv strain, and the corresponding $MIC_{90}$ and $MIC_{99}$ values are presented in

Tables 1-3. Cytotoxicity for selected compounds was determined against human embryonic kidney (HEK) 293T cells.

**Table 1.** *In vitro* antimycobacterial activities and cytotoxicity of aurone derivatives **3a-3y**.



| Cpd | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ | $MIC_{90}$ (μM) | $MIC_{99}$ (μM) |
|---|---|---|---|---|---|---|---|
| **3a** | H | H | 4-Br | H | H | >20 | >20 |
| **3b** | H | H | H | H | Br | >20 | >20 |
| **3c** | H | H | H | Br | H | >20 | >20 |
| **3d** | H | OMe | H | H | H | >20 | >20 |
| **3e** | OH | H | H | H | H | >20 | >20 |
| **3f** | OH | H |  | H | H | >20 | >20 |
| **3g** | H | H |  | H | H | >20 | >20 |
| **3h** | H | H |  | H | H | >20 | >20 |
| **3i** | H | H |  | H | H | >20 | >20 |
| **3j** | H | H |  | H | H | >20 | >20 |
| **3k** | H | H |  | H | H | >20 | >20 |
| **3l** | H | H |  | H | H | >20 | >20 |
| **3m** | H | H |  | H | H | >20 | >20 |
| **3n** | H | H |  | H | H | >20 | >20 |
| **3o** | H | H | H |  | H | >20 | >20 |
| **3p** | H | H | H |  | H | >20 | >20 |
| **3q** | H | H | H |  | H | >20 | >20 |
| **3r** | H | H | H |  | H | >20 | >20 |

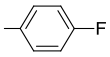
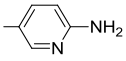
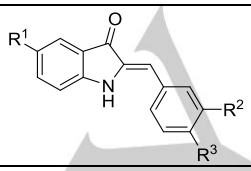
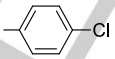
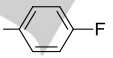
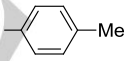
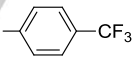
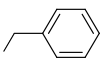
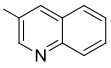
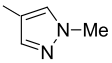
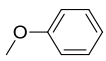
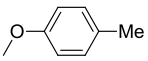
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **3s** |  | H | H | H | H | >20 | >20 |
| **3t** |  | H | H | H | H | >20 | >20 |
| **3u** |  | H | H | H | H | >20 | >20 |
| **3v** | OH |  | H | H | H | >20 | >20 |
| **3w** | OH |  | H | H | H | >20 | >20 |
| **3x** | OH |  | H | H | H | >20 | >20 |
| Rif | | - | - | | | 0.00291 | 0.00482 |

**Table 2.** *In vitro* antimycobacterial activities and cytotoxicity of azaaurones derivatives **4a-4u**.



| Compd | $R^1$ | $R^2$ | $R^3$ | $MIC_{90}$ H37Rv (µM) | $MIC_{99}$ H37Rv (µM) | HEK293T (µM) |
|---|---|---|---|---|---|---|
| **4a** | H | H | Br | 6.5 | 7.56 | >100 |
| **4b** | H | H |  | 1.78 | 2.4 | >100 |
| **4c** | H | H |  | 1.3 | 1.5 | >100 |
| **4d** | H | H |  | 2.47 | 2.74 | >100 |
| **4e** | H | H |  | 1.27 | 1.44 | >100 |
| **4f** | H | H |  | 1.28 | 1.53 | >100 |
| **4g** | H | H |  | >20 | >20 | >100 |
| **4h** | H | H | $N(Me)_2$ | >20 | >20 | >100 |
| **4i** | H | H |  | >20 | >20 | >100 |
| **4j** | H | H |  | 1.21 | 1.36 | >100 |
| **4k** | H | H |  | >20 | >20 | >100 |
| **4l** | H | H |  | >20 | >20 | >100 |
| **4m** | H | H |  | 3.07 | 4.09 | >100 |

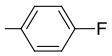
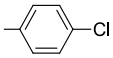
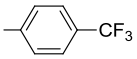
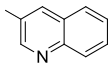
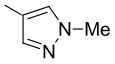
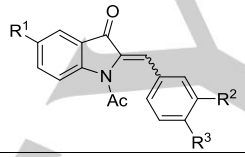
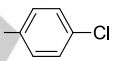
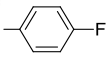
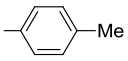
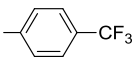
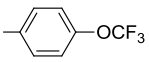
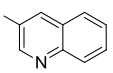
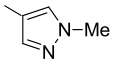
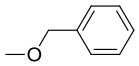
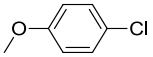
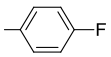
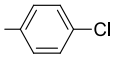
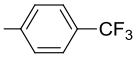
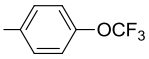
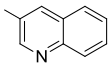
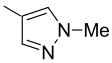
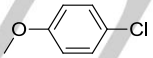
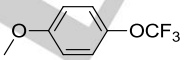
| | | | | | | |
|---|---|---|---|---|---|---|
| **4n** | H |  | H | >20 | >20 | >100 |
| **4o** | H |  | H | 15.9 | >20 | >100 |
| **4p** | H |  | H | 7.37 | 10.6 | 20 |
| **4q** | H |  | H | >20 | >20 | >100 |
| **4r** | H |  | H | >20 | >20 | >100 |
| **4s** | H |  | H | >20 | >20 | >100 |
| **4t** | H |  | H | >20 | >20 | >100 |
| **4u** |  | H | H | >20 | >20 | >100 |
| Rif | - | - | | 0.00291 | 0.00482 | - |

**Table 3**. In vitro antimycobacterial activities and cytotoxicity of *N*-acetylazaaurone derivatives **10a-y**.



| Cpd | $R^1$ | $R^2$ | $R^3$ | $MIC_{90}$ H37Rrv (µM) | $MIC_{99}$ H37Rv (µM) | HEK293T (µM) |
|---|---|---|---|---|---|---|
| **10a** | H | H | Br | 0.31 | 0.37 | ND |
| **10b** | H | H |  | 1.32 | 2.29 | ND |
| **10c** | H | H |  | 2.32 | 4.05 | ND |
| **10d** | H | H |  | 1.26 | 1.98 | 7 |
| **10e** | H | H |  | 2.71 | 4.37 | 9 |
| **10f** | H | H |  | 2.65 | 4.3 | ND |
| **10g** | H | H |  | 1.44 | 3.19 | ND |
| **10h** | H | H | $N(CH_3)_2$ | >20 | >20 | 8 |
| **10i** | H | H |  | 0.649 | 0.736 | 19 |
| **10j** | H | H |  | 0.336 | 0.6 | ND |

| | | | | | | |
|---|---|---|---|---|---|---|
| **10k** | H | H |  | 3.16 | 3.66 | ND |
| **10l** | H | H |  | 1.89 | 2.58 | ND |
| **10m** | H | H |  | 6.2 | 8.4 | ND |
| **10n** | H |  | H | 4.49 | 4.94 | ND |
| **10o** | H |  | H | 5.1 | 5.66 | ND |
| **10p** | H |  | H | 6.44 | 7.85 | 4 |
| **10q** | H |  | H | 11.6 | 17.5 | ND |
| **10r** | H |  | H | >20 | >20 | ND |
| **10s** | H |  | H | 2.65 | 3.13 | 14 |
| **10t** | H |  | H | 3.19 | 3.34 | ND |
| **10u** | H |  | H | 4.93 | 5.61 | ND |
| **10v** |  | H | H | 1.24 | 1.39 | 12 |
| **10w** | Cl | H |  | 6.02 | 6.87 | ND |
| **10x** | Cl | H |  | >20 | >20 | ND |
| **10y** | Me | H |  | >20 | >20 | ND |

Examination of the data in Table 1 reveals that all the aurone derivatives (**3a-y**) were found to be inactive against *M. tuberculosis* at the maximum concentration tested, 20 μM. The absence of active aurones in our library is consistent with other sreening campaigns. For example, the screening of a kinase-like inhibitor library, which contained ca. 60 aurones, identified only three hits based on this scaffold with reproducible anti-TB activity.[15a] In contrast, several azaaurones, **4** (Table 2), and N-acetylazaaurones, **10** (Table 3), emerged as active antimycobacterials, with $MIC_{90}$ and $MIC_{99}$ values under 10 μM, the cut-off value used in this study to select hits for further validation and optimization.[8c] Analysis of data presented in Tables 2 and 3 allows the following conclusions to be drawn:

First, *N*-acetylazaaurones generally exhibited higher potency than the corresponding azaaurones (e.g. **10a** vs **4a**, **10i** vs **4i**, **10k** vs **4k**, **10v** vs **4u**), a result suggesting that the more lipophilic *N*-acetylazaaurones **10** may cross the mycobacterial cell wall more efficiently than their deacetylated counterparts **4**. However, the different hydrogen-bonding properties of compounds **4** and **10** may also affect the antimycobacterial activity.

Second, substitution at C-4 of the B-ring is beneficial for the antimycobacterial activity when compared with substitution at C-3, both in *N*-acetylazaaurones (e.g. **10b** vs **10n**, **10c** vs **10p**, **10f** vs **10q**, **10g** vs **10r**, **10i** vs **10s**, **10j** vs **10t**) as well as in azaaurones (**4b** vs **4n**, **4f** vs **4q**, and **4j** vs **4s**). The most potent

compounds contained electron-withdrawing and/or lipophilic substituents at C-4 (e.g. **10a**, **10i** and **10j**).
Third, substitution at C-5 in ring A of *N*-acetylazaaurones was tolerated (e.g. **10m** vs **10w**).
Forth, azaaurones were shown to be non-cytotoxic against HEK293T cells, with $CC_{50}$ values >100 μM, while their *N*-acetyl counterparts displayed $CC_{50}$ values ranging from 4 to 20 μM, thus indicating selectivity against *M. tuberculosis*.

**Activity against *Mycobacterium tuberculosis cydKO* mutant**

Azaaurones were reported as potential inhibitors of *P. falciparum* cytochrome *bc1*, a key component of the mitochondrial electron-transport chain (mtETC).[16b] Cytochrome *bc1* complex is also an essential component of the respiratory electron transport chain required for ATP synthesis in *M. tuberculosis* and was identified as a high-confidence antimycobacterial drug target. [19] The cytochrome *bc1* transfers electrons from menaquinol to the cytochrome *c* oxidase, a process linked to proton translocation across the membrane and that ultimately is the most energetically favorable respiratory pathway in mycobacteria.[20] Hence, *N*-acetylazaaurones with $MIC_{99}$ < 20 μM were further assayed in order to ascertain whether the respiratory pathway of *M. tuberculosis* could be the potential target for these compounds. The assay used a *M. tuberculosis* cytochrome *bd* oxidase deletion mutant *cydKO*, which is hypersusceptible to compounds such as imidazopyridines that target the QcrB subunit of the qcrCAB-encoded cytochrome *bc1* complex.[20a,21] No MIC shift was observed when the *cydKO* mutant was tested against all selected *N*-acetylazaaurones (Table 4). The lack of hypersensitivity observed in the *cydKO* mutant strongly suggests that QcrB does not constitute the primary target for *N*-acetylazaaurones.[22]

**Table 4.** Antimycobacterial activity against the *M. tuberculosis cydKO* mutant lacking a cytochrome *bd* oxidase. Data determined at day 7 and day 14 in GAST/Fe media.

| Compd | $MIC_{99}$ H37Rrv (μM) | $MIC_{99}$ at day 7 (μM) *cydKO* | $MIC_{99}$ at day 14 (μM) *cydKO* |
|---|---|---|---|
| **10a** | 0.37 | 1.25 | 1.25 |
| **10b** | 2.29 | 1.25 | 1.25 |
| **10c** | 4.05 | 2.5 | 10 |
| **10d** | 1.98 | 1.25 | 1.25 |
| **10e** | 4.37 | 2.5 | 2.5 |
| **10f** | 4.3 | 2.5 | 2.5 |
| **10g** | 3.19 | 1.25 | 5 |
| **10i** | 0.736 | 1.25 | 1.25 |
| **10j** | 0.6 | 1.25 | 1.25 |
| **10k** | 3.66 | 2.5 | 5 |
| **10l** | 2.58 | 5 | 5 |
| **10m** | 8.4 | 5 | 5 |
| **10n** | 4.94 | 2.5 | 2.5 |
| **10o** | 5.66 | 5 | 10 |
| **10p** | 7.85 | 5 | 5 |
| **10q** | 17.5 | 5 | >10 |
| **10s** | 3.13 | 2.5 | 2.5 |
| **10t** | 3.34 | 5 | 5 |
| **10u** | 5.61 | 5 | 5 |
| **10v** | 1.39 | 1.25 | 2.5 |
| **10w** | 6.87 | 5 | 10 |

**Activity against drug-resistant clinical *M. tuberculosis* isolates**

A set of selected *N*-acetyl azaaurones was evaluated for the ability to suppress the growth of various patient-derived, genotyped drug-resistant (DR), multidrug resistant (MDR) and extensively drug resistant (XDR) *M. tuberculosis* clinical strains, which are part of the iMed.ULisboa's pathogenic mycobacterial strain collection (Table 5). Although there are differences in the assay methods to determine the inhibitory concentrations of the compounds against the bacterial pathogen in the primary screening and the poly-resistant strains, these methods have been extensively standardized. Pleasingly, we found that selected compounds were active against all resistant mutants, being equipotent against wild type, MDR and XDR isolates.

**Table 5.** Antimycobacterial activity against drug resistant (DR, resistant to isoniazid), multidrug resistant (MDR, resistant to isoniazid, rifampicin, ethambutol, pyrazinamide, capreomycin, and ethionamide), and extensively resistant (XDR, resistant to isoniazid, rifampicin, ethambutol, pyrazinamide, streptomycin, capreomycin, amikacin, kanamycin, ofloxacin, and ethionamide) *M. tuberculosis* clinical isolates.

| | MIC (μM) | | | |
|---|---|---|---|---|
| Compound | XDR-TB | MDR-TB | DR-TB | H37Rv |
| **10a** | 15.2 | 9.15 | 15.2 | 15.2 |
| **10b** | 12.3 | 12.3 | 12.3 | 12.3 |
| **10c** | 8.37 | 10.4 | 8.37 | 8.37 |
| **10e** | 17.7 | 17.7 | 17.7 | 14.8 |
| **10j** | 7.55 | 7.55 | 7.55 | 7.55 |
| Rifampicin | >12.2 | >12.2 | 0.377 | 0.377 |

***In vitro* metabolic studies.**

A microsomal metabolic stability study was performed on selected azaaurones and *N*-acetylazaaurones by incubating compounds in mouse liver microsomes activated in the presence of NADPH, at 37 ºC. Samples were analyzed by LC-MS/MS to determine the percentage of compounds remaining after 30 min of incubation.
Inspection of the results presented in Table 6 reveals that selected *N*-acetylazaaurones, **10**, underwent extensive NADPH-dependent degradation, with only 10 to 20% of the parent compounds remaining after 30 minutes of incubation. In contrast, selected compounds **4** were shown to be significantly more

stable in mouse liver microsomes. Interestingly, the most metabolically stable compound was the 3-thienyl azaaurone **4j**. Analysis of GlaxoSmithKline corporate database revealed that thiophene-containing compounds exhibited the highest CYP450 inhibition when compared to other 5-membered and 6-membered heterocycles,[23] and thus the predicted $E_H$ value of 0.37 for **4j** may reflect, at least partially, inhibition of this metabolizing enzyme system.

To better understand the metabolic pathway associated with azaaurones and *N*-acetylazaaurones, a metabolite identification study using LC-MS was carried out for compounds **4b**, **10j** and **10v**. Both compounds **10j** and **10v** underwent NADPH-independent deacetylation to form **4j** and **4u**, respectively (Fig. 2A), as revealed by the peaks at m/z M-43 in samples incubated in the presence or absence of NADPH. Various hydrolases are known to be present in microsomal preparations and may contribute to this metabolic activation. Importantly, deacetylation also occurred in pH 7.4 buffer, but at a lower extent than in microsomes.

For the azaaurone **4b**, the metabolites were tentatively identified by comparison of the fragmentation patterns of the parent compound with those from the incubation samples. The fragmentation pattern of **4b** showed two peaks, one at m/z 297 corresponding to the molecular ion and the other at m/z 165 corresponding to the biaryl side chain (Fig. 2B). After incubation with microsomes, the fragment at m/z 165 shifted to m/z 181, suggesting that hydroxylation had occurred on the side chain of the compound.

**Table 6.** *In vitro* metabolism of selected azaaurones and *N*-acetylazaaurones in mouse liver microsomes.

| Compd | % remaining (30 min) | $CL_{int}$ (µl/min/mg) | Predicted $E_H$ |
|---|---|---|---|
| **4b** | 49 | 101 | 0.73 |
| **4c** | 75 | 38 | 0.50 |
| **4e** | 78 | 33 | 0.46 |
| **4j** | 85 | 22 | 0.37 |
| **10a** | 20 | 220 | 0.85 |
| **10b** | 16 | 261 | 0.68 |
| **10c** | 9 | 335 | 0.90 |
| **10e** | 12 | 289 | 0.88 |
| **10j** | 11 | 293 | 0.89 |
| **10v** | 24 | 252 | 0.87 |
| Midazolam | <7 | 610 | 0.94 |

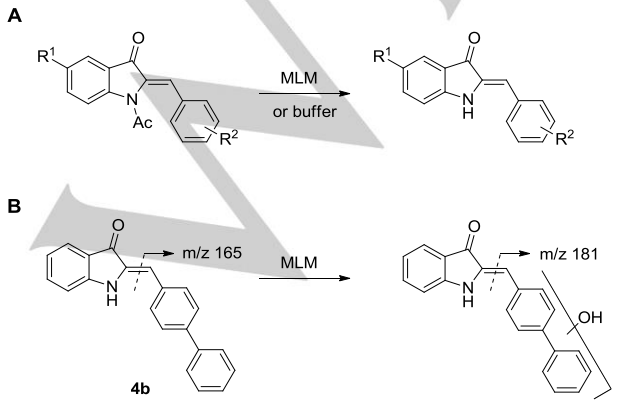



**Figure 2.** Metabolism of azaaurones and *N*-acetylazaaurones in mouse liver microsomes.

## Conclusions

There is an urgent need for new antimycobacterial agents preferably with novel mechanisms of action in order to tackle drug-resistant *M. tuberculosis* infection.[24] This work shows that azaaurones and their *N*-acetyl counterparts represent a new entry in the toolbox of chemotypes capable of inhibiting M. tuberculosis growth. In particular, *N*-acetylazaaurones **10a**, **10i** and **10j** were found to be active against *M. tuberculosis* with $MIC_{99}$ values in the submicromolar range, with **10i** showing excellent activity against MDR and XDR-TB from clinical isolates. The microsomal metabolic stability and metabolite identification studies showed that *N*-acetylazaaurones are rapidly and extensively deacetylated to their azaaurone counterparts. Moreover, a preliminary mechanistic study revealed that these compounds do not target the QcrB subunit of the mycobacterial cytochrome *bc1* complex, therefore eliminating one of several "promiscuous" targets increasingly identified in screening campaigns in vitro.[11h] Current work in our laboratory aims to elucidate the precise mechanism of action. Therefore, despite the fact that their molecular targets remain unknown, azaaurones may serve as leads for the generation of new anti-mycobacterial agents.

## Experimental Section

### Chemistry

General procedure for the synthesis of aurones **3a**, **3b**, **3c** and **3d**: To a solution of benzofuran-3(*2H*)-one (134 mg, 1 mmol) in dry methanol (20 mL) at room temperature was added the appropriate aldehyde (1.2 mmol) and $Al_2O_3$ (1 mmol). The mixture was refluxed, under $N_2$, for 48 hours. After, the solvent was removed and the solid residue was dissolved in $CH_2Cl_2$. The organic layer was washed with water, dried with anhydrous $Na_2SO_4$ and concentrated under reduced pressure to give the crude product.

General procedure for the synthesis of aurone **3e**: To a solution of 6-hydroxybenzofuran-3(*2H*)-one (0.57 mmol) in glacial acetic acid (5.7 mL) at room temperature was added the appropriate benzaldehyde (0.68 mmol) and HCl (3 drops). The reaction mixture was stirred for 4 hours at room temperature. After, the mixture was dropped in cold water and the precipitate formed was filtered and washed with water.

General procedure for the synthesis of ethers derivatives **3f**: To a solution of 6-hydroxybenzofuran-3(*2H*)-one (0.57 mmol) in glacial acetic acid (5.7 mL) at room temperature was added the appropriate aldehyde (0.68 mmol) and HCl (cat, 3 drops). The reaction mixture was stirred for 4 hours at room temperature. After, the mixture was dropped in cold water and the precipitate formed was filtered and washed with water.

General procedure for the synthesis of aurones derivatives **3g**, **3h**, **3i**, **3j**, **3n**, **3o**, **3p**, **3q**, **3r**, **3s**, **3t** and **3u** via Suzuki Coupling: To a solution of the appropriate aurone derivative (0.23 mmol) in

dioxane (2.3 mL) was added $Pd(PPh_3)_2Cl_2$ (0.023 mmol) and $Na_2CO_3$ 1M (690 µL) followed by the proper boronic acid (0.28 mmol). The resulting mixture was degassed and stirred at 100ºC for 3 hours under $N_2$. After cooling to room temperature, the reaction mixture was diluted with $CH_2Cl_2$, filtered under celite and concentrated under pressure to give the crude product.

General procedure for the synthesis of ethers derivatives **3k**: To a solution of benzofuran-3(*2H*)-one (0.57 mmol) in dry methanol (10 mL) at room temperature was added the appropriate aldehyde (0.68 mmol) and $Al_2O_3$ (0.57 mmol). The mixture was refluxed, under $N_2$, for 48 hours. After, the solvent was removed and the solid residue was dissolved in $CH_2Cl_2$. The organic layer was washed with water, dried with anhydrous $Na_2SO_4$ and concentrated under reduced pressure to give the crude product.

General procedure for the synthesis of aurones derivatives **3l** and **3m** via Buchwald Coupling: (*Z*)-2-(4-bromobenzylidene)benzofuran-3(*2H*)-one (5.27) (0.23 mmol), $Pd_2(dba)_3$ (0.0115 mmol), (*R*)-BINAP (0.075 mmol) and NaOtBu (0.322 mmol) were dissolved in dry toluene (2.3 mL). The resulting mixture was degassed and the appropriate amine (0.276 mmol) was added. The mixture was stirred at 100ºC for 15 minutes under MW conditions. After cooling to room temperature, the reaction mixture was diluted with $Et_2O$, filtered under celite and concentrated under pressure to give the crude product.

General procedure for the synthesis of Mannich Bases derivatives **3v**, **3w** and **3x**: To a solution of (*Z*)-2-benzylidene-6-hydroxybenzofuran-3(*2H*)-one (0.29 mmol) in absolute ethanol (1 mL) was added the appropriate amine (0.32 mmol) followed by formaldehyde solution (0.32 mmol). The mixture was refluxed for 3h30. After, the solvent was removed and the solid residue was dissolved in $CH_2Cl_2$ and extracted with HCl 1M. The aqueous layer was neutralized with $NaHCO_3$ saturated solution and extracted with $CH_2Cl_2$. The organic layer was dried with anhydrous $Na_2SO_4$ and concentrated under reduced pressure to give the crude product.

General procedure for the synthesis of azaaurone derivatives **10a to 10y**: To a solution of the appropriate 1-acetylindolin-3-one derivative (0.5 mmol) in toluene (5 mL) at room temperature was added the proper aldehyde (1.2 mmol) and piperidine (1 drop). The mixture was refluxed for 4 hours. After reaction completion, the solvent was removed to provide the crude product.

General procedure for the synthesis of azaaurone derivatives **4a to 4t**: To a solution of the proper acetylated azaaurone derivative (0.25 mmol) in MeOH (2.5 mL) at room temperature was added a solution of KOH 50% in water (375 µL). The mixture was stirred for 45 minutes. After reaction completion, the reaction mixture was neutralized with extracted with EtOAc. The organic layer was dried with anhydrous $Na_2SO_4$ and concentrated under reduced pressure to give the crude product.

**Antimycobacterial screening against Mycobacterium tuberculosis H37Rv**

The minimum inhibitory concentration (MIC) was determined using the standard broth micro dilution method, where a 10 mL culture of *M. tuberculosis* pMSp12::GFP,[25] was grown to an optical density ($OD_{600}$) of 0.6 – 0.7 in GAST-Fe (glycerol–alanine–salts) medium pH 6.6, supplemented with 0.05% Tween-80.[26] The culture was then diluted 1:100 in GAST-Fe. The compounds to be tested were reconstituted to a concentration of 10 mM in DMSO. Two-fold serial dilutions of the test compound were prepared in GAST-Fe, across a 96-well micro titre plate, after which, 50 µL of the 1:100 diluted *M. tuberculosis* culture was added to each well in the serial dilution. The concentration range assayed was 20 – 0.039 µM. The plate layout was a modification of the method previously described.[27] Controls used were a minimum growth control (Rifampicin at 2xMIC), and a maximum growth control (5% DMSO in GAST-Fe). The micro titre plate was sealed in a secondary container and incubated at 37 °C with 5% $CO_2$ and humidification. Relative fluorescence (excitation 485 nM; emission 520 nM) was measured using a plate reader (FLUOstar OPTIMA, BMG LABTECH), at day 7 and day 14. In the absence of a MIC shift between day 7 and day 14, day 14 data were analysed and reported. The raw fluorescence data were archived and analysed using the CDD Vault from Collaborative Drug Discovery, in which, data were normalised to the minimum and maximum inhibition controls to generate a dose response curve (% inhibition), using the Levenberg-Marquardt damped least-squares method, from which the $MIC_{90}$ was calculated.[28] The lowest concentration of drug that inhibits growth of more than 90 %, and 99%, of the bacterial population was considered to be the $MIC_{90}$ and $MIC_{99}$ respectively.

**Antimycobacterial activity against M. tuberculosis clinical strains**

Ten selected compounds were evaluated against three clinical *M. tuberculosis* isolates displaying different drug resistance spectra. All three isolates are part of the iMed.ULisboa *M. tuberculosis* strain collection, were previously characterized by classical genotyping methods (24-loci MIRU-VNTR and Spoligotyping) as previously described and, have further been subjected to whole genome sequencing with the raw sequence data made publicly available at the European Nucleotide Archive (Supplementary Table S1).[4a, 29] Phenotypic drug susceptibility testing was also previously performed for these strains through the standardized BACTEC Mycobacterial Growth Indicator Tube (MGIT) 960 system (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) using the standardized procedure according to the manufacturer's instructions.[30] Herein, MIC determination was carried out using the resazurin microtitre assay (REMA), a non-commercial drug susceptibility testing endorsed by the WHO, coupled with microscopic examination for growth under an inverted microscope.[31] Briefly, all compounds were 2-fold serially diluted in a 96-well microtitre plate as to achieve a final concentration ranging between 100.00-0.10 µg/ml in Middlebrook 7H9-S (Middlebrook 7H9 broth supplemented with 0.1% casitone, 0.5% glycerol and 10% OADC supplement). Positive (no drug) and negative/sterile controls were included at each plate. The bacterial inoculum was prepared by adjusting a bacterial suspension to a McFarland turbidity standard 1.0 and diluted 1:20 before inoculating 100 µl to each well (final volume: 200 µl). Plates were sealed in plastic bags, incubated at 37ºC for 7 days and growth observed under a microscope before addition of 30 µl of 0.01% resazurin, followed by an additional incubation period of 24h. Blue to pink colour change was indicative of bacterial growth through the reduction of resazurin (blue) to resorufin (pink). Resazurin sodium salt (Sigma-Aldrich) solution was prepared at a concentration of 0.01%, sterilized by filtration

using a 0.2µm syringe filter and stored at 4ºC for a maximum period of one week as recommended.[32] MIC was recorded as the lowest concentration yielding bacterial growth. All assays were also performed in parallel for reference strain *M. tuberculosis* H37Rv (ATCC 2794) and the activity of rifampicin (Sigma-Aldrich) also assayed in parallel. All assays were performed in triplicate and final MIC values are herein expressed as the result of at least two concordant values.

**Metabolic stability**

The metabolic stability study was performed for selected azaaurones and *N*-acetylazaaurones in triplicate by incubating compounds in mouse liver microsomes activated in the presence of the co-factor, NADPH, using the cosolvent method.[33] Compounds were incubated for 30 min at 37 ºC and then the reactions were quenched by adding cold ACN with carbamazepine as internal standard. The samples were kept at -20 ºC for 15 min. and then centrifuged. The supernatants were analyzed by LC-MS/MS using Multiple Reaction Monitoring (MRM) methods. The % remaining was calculated using the analyte:internal standard ratios at 0 min and 30 min and converted to intrinsic clearance and $E_H$.[34]

**Metabolite identification**

The compounds (10 µM) were incubated with a solution of mouse liver microsomes and NADPH (1 mM) in phosphate saline buffer (100 mM, pH 7.4) and $MgCl_2$ (5 mM) for 1 h while shaking at 37 ºC. An equal volume of acetonitrile was added at the end of the incubation to quench the reaction. The mixture was centrifuged at 14000 rpm for 30 min and the supernatant was transferred to an HPLC vial. Control samples were prepared without NADPH or microsomes. The NADPH and the microsomes volumes were replaced by the same volume of PBS. T0 samples were quenched with acetonitrile after microsomes were added and placed at 8ºC for the duration of the incubation.

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**Keywords:** azaaurones • drug discovery • tuberculosis • multidrug-resistant tuberculosis • *Mycobacterium tuberculosis*

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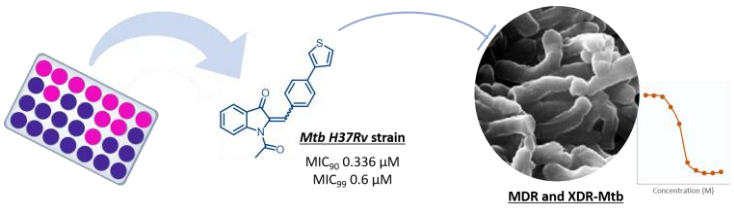


The screening of an in-house library against *M. tuberculosis* H37Rv identified azaaurones as novel antimycobacterial agents. In particular, several *N*-acetylazaaurones displayed $MIC_{99}$ values in the submicromolar range and showed to be active against MDR and XDR-TB clinical isolates. Although the primary mechanism of action remains undetermined, azaaurones stand as promising leads for further optimization.