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Usnic Acid Derivatives as Inhibitors of *Mycobacterium tuberculosis* Uracil–DNA Glycosylase

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Abstract

Tuberculosis (TB) remains a global health issue exacerbated by spreading drug resistance and lengthy treatment regimens. Targeting bacterial DNA-repair pathways, particularly those counteracting host-generated genotoxic stress, represents a promising strategy to sensitize *Mycobacterium tuberculosis* to existing antibiotics. Through structure-based virtual screening of a compound library, we identified novel small-molecule inhibitors of *M. tuberculosis* uracil–DNA glycosylase (MtbUng), an enzyme essential for the repair of DNA damage inflicted by macrophage-produced reactive nitrogen species. Experimental validation revealed that four derivatives of usnic acid, a lichen-derived metabolite, significantly inhibited MtbUng activity, with the most potent compound, OL10-88-1, exhibiting IC₅₀ 26 ± 7 µM. Molecular docking suggests that OL10-88-1 inhibits MtbUng by occupying both the active site and the DNA-binding groove, thereby disrupting multiple steps of uracil recognition. The compounds also showed variable inhibitory activity against uracil–DNA glycosylases from *Escherichia coli*, humans, and vaccinia virus. Our findings establish that the compound could potentially be used in combination therapies to enhance the efficacy of current anti-TB drugs by exploiting the vulnerability of DNA-repair-deficient mycobacteria.



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1. Introduction

Tuberculosis (TB), an infectious disease caused by the bacterium *Mycobacterium tuberculosis*, is one of the leading causes of death worldwide. According to the World Health Organization's *Global Tuberculosis Report 2025*, the number of cases increased from 10.1 million in 2020 to 10.7 million in 2024; the increase is mostly attributed to the COVID-19 pandemic's

burden on the public health system [1]. While the overall annual death toll has started to decline, the drug resistance of many *M. tuberculosis* strains, together with the length of the treatment regimens, greatly complicate TB management [1–4].

Since *M. tuberculosis* cells residing inside alveolar macrophages are constantly exposed to an aggressive host environment that, among other consequences, damages the pathogen's DNA, the integrity of the bacterial genome is protected by highly efficient DNA-repair systems. Mycobacteria possess DNA-repair pathways common to most prokaryotes, such as homologous recombination, non-homologous end-joining, nucleotide excision repair and base excision repair (BER) [5–7]. These mechanisms are of great interest as they may modulate the processes leading to drug resistance and thus new drug targets. The concept of synthetic lethality is successfully exploited in cancer therapy where interference with DNA repair and DNA damage response pathways sensitizes cancer cells to genotoxic agents: several inhibitors of poly(ADP-ribose) polymerase 1 are presently in clinical use [8], and many more protein targets are under assessment [9,10]. In the field of chemotherapy of bacterial infections, a new paradigm is gaining popularity, i.e., many antibiotics cause the exposed bacteria to spend energy in the attempt to cope, exhaust NAD(P)H supply and ultimately die from severe DNA and RNA damage due to oxidative stress [11–14]. Accordingly, disruption of DNA-repair pathways sensitizes bacterial pathogens, including *M. tuberculosis*, to the action of antibiotics [15–18].

Uracil–DNA glycosylase (Ung) is a DNA-repair enzyme that initiates BER by excising uracil (U), which arises by cytosine (C) deamination [19,20]. *M. tuberculosis* infects host macrophages that generate reactive nitrogen species (RNS), which are known to deaminate C in DNA [21]. Since the genome of *M. tuberculosis* is guanine (G):C-rich, it may be more susceptible to the deleterious consequences of U formation. Studies with mycobacteria have shown that Ung is a key enzyme for their survival in host cells [22,23]. Thus, inhibitors of *M. tuberculosis* Ung (MtbUng) could be used in the treatment of TB, either alone or in combination with other anti-TB drugs. At present, there are few works devoted to the synthesis and characterization of Ung inhibitors. Most of them are based on U or structurally similar moieties and show inhibition of Ung from various species in the range of micro- to millimolar concentrations [24]. *E. coli* Ung (EcoUng) is modestly inhibited by the aminoglycoside antibiotic gentamicin ($K_i = 0.4\text{--}1.5\text{ mM}$) [25,26]. A group of non-U inhibitors of MtbUng have been identified by in silico screening of several libraries of small-molecule compounds, the most potent one showing $IC_{50} \sim 0.14\text{ mM}$ [27]. Clearly, a wider range of pharmacophores capable of inhibiting Ung would be required to exploit the vulnerability of DNA-repair-suppressed bacteria to antibiotics and RNS-induced stress. Here, we report that derivatives of usnic acid, a secondary metabolite produced by the fungal symbionts of some lichens, can inhibit MtbUng, as well as uracil–DNA glycosylases of human, *E. coli* and viral origin.

2. Results

2.1. In Silico Screening for Potential MtbUng Inhibitors

With the availability of many MtbUng crystal structures [28–31], it is possible to make use of structural knowledge to identify novel inhibitors against MtbUng using in silico screening [32,33].

To investigate the reliability of the scoring functions and the reproducibility of the binding modes, we started from the crystal structure of MtbUng–uracil (PDB ID 4WPL [30]), removed the co-crystallized uracil, modeled and optimized it from scratch, and docked it back to the active site at 100% efficiency at 50 docking runs per molecule using the GOLD modeling software and at 50 runs per molecule and at SP and XP mode on Glide. The RMSD values of the heavy atoms of the best-ranking conformations were 0.41 Å, 0.31 Å

and 0.23 Å for the GS, CS and ChemPLP modes of GOLD; they were 0.23 and 0.16 Å for the SP and XP modes of Glide, respectively. The low RMSD values showed good overlays of the heavy atoms, thus validating the docking protocol. The co-crystallized uracil forms hydrogen bonds with Gln67, Phe81, Asn127 and His191, and π -stacking interactions with Tyr70 and Phe81 of MtbUng (Figure 1a).

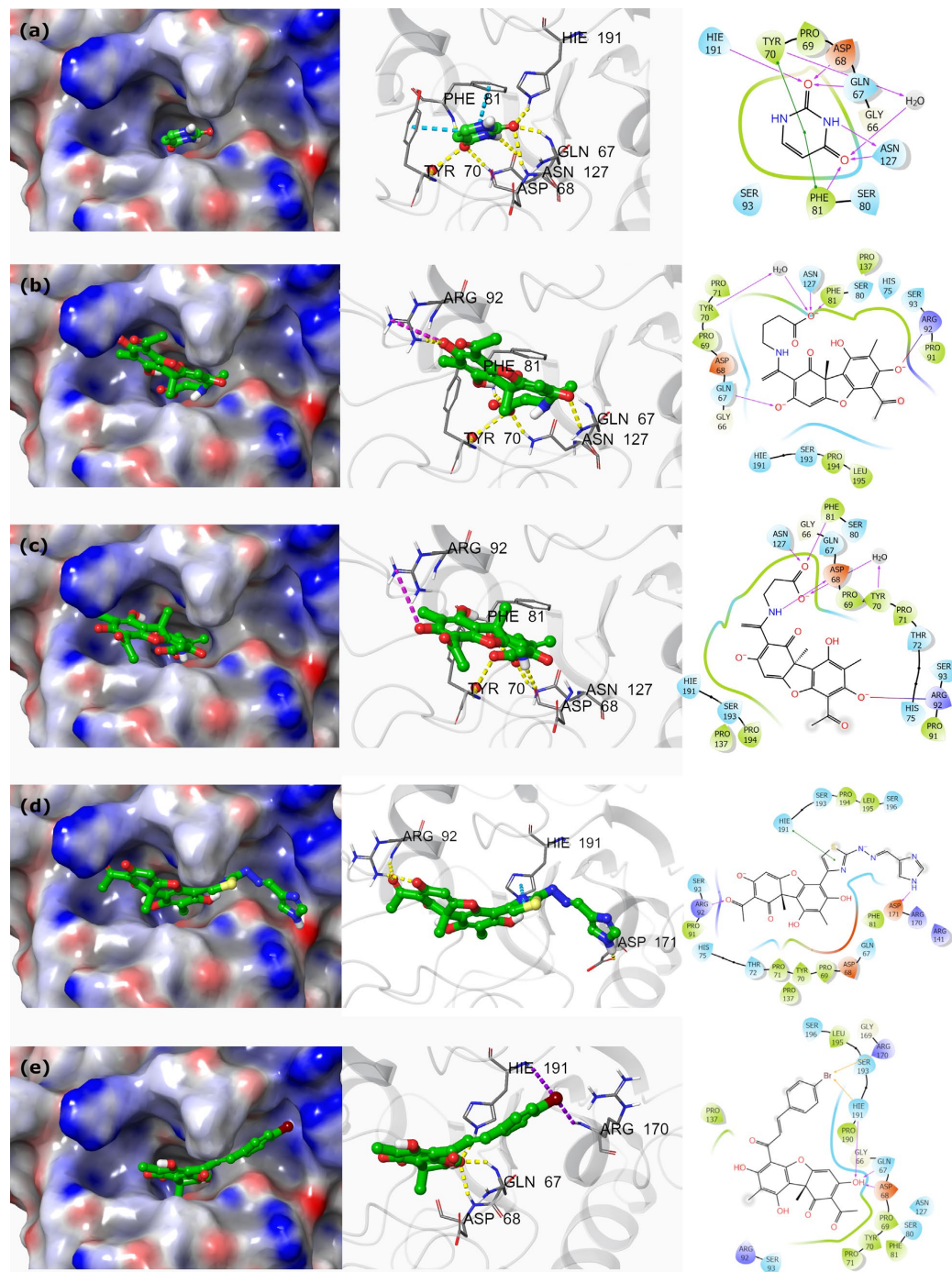


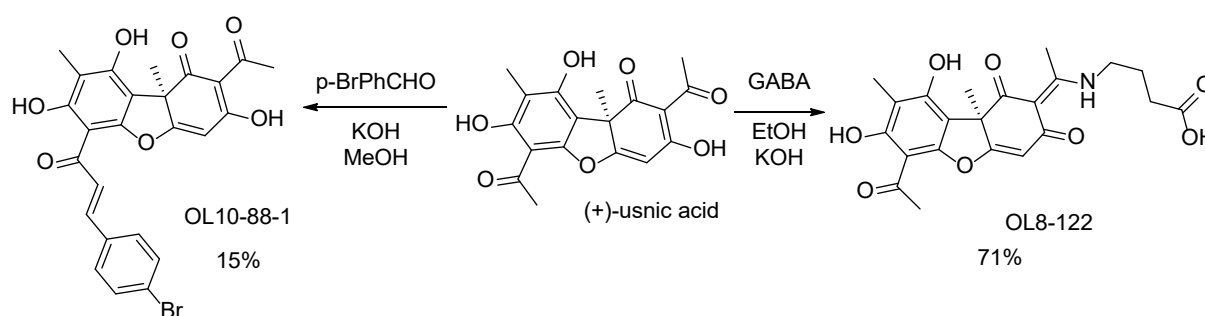
Figure 1. Binding interactions of the co-crystallized uracil (a) and predicted binding modes and interactions of OL8-122 (b), OL7-22 (c), AF-105 (d) and OL10-88-1 (e) with MtbUng. Ligands are shown in the ball-and-stick representation (carbon atoms, green); amino acid residues as sticks (carbon atoms, gray); nitrogen atoms are colored blue; oxygens, red; sulfur, yellow; and hydrogens, white. Water molecules are shown as red spheres, and electrostatic interactions as dashed lines. Protein surfaces are rendered according to the electrostatic potential.

Out of the entire 1447-compound library (in-house library of the Laboratory of Physiologically Active Compounds of the Novosibirsk Institute of Organic Chemistry), 297 molecules were selected from the first round of screening using the selection criteria $GS > 37$, $CS > 20$ and $ChemPLP > 44$, and hydrogen bonding scores of 1 or greater. The criteria were selected based on the scores of the co-crystallized uracil substrate equating to $GS = 37$, $CS = 20$, and $ChemPLP = 44$, with hydrogen bonding scores exceeding 1 across all three scoring functions, i.e., scores exceeding the criteria were predicted to have stronger binding. In the second round of screening, seven molecules were selected by visual inspection (Supplementary Table S1), i.e., a good fit to the binding site and hydrogen bond formations or stacking interactions with any of the amino acids important for the activity studied (Gln67, Asp68, Tyr70, Phe81, Asn127, and His191). In the case of Glide, 293 molecules were selected according to the criterion $Glide\ GScore \leq -5.5$, which were redocked and rescored in XP mode. Those with an $XP\ GScore \leq -6.0$ were selected and visually inspected; fifteen molecules with a good fit to the active site and that engaged in key interactions (hydrogen bonds, π - π , and π -cation, salt bridges, etc.) with the catalytic residues stated above were identified (Supplementary Table S2). Among the selected molecules, five were usnic acid derivatives, while the others were mostly derived from mono- or triterpenoids. Examples of the interactions of the high-scoring molecules with the MtbUng active sites are shown in Figure 1b–e.

2.2. Synthesis of the Inhibitors

Compounds for inhibitor screening were taken from the in-house library of the Laboratory of Physiologically Active Compounds of the Novosibirsk Institute of Organic Chemistry. Four active compounds, OL8-122, OL10-88-1, OL7-22, and AF-105 were then synthesized as described previously [34–37]. All these compounds are derivatives of usnic acid, the (+)- and (–)-enantiomers of which were used for the synthesis. The 1H -NMR spectra of OL8-122, OL10-88-1, OL7-22, and AF-105 are shown in Supplementary Figures S1–S4 and correspond to the literature data [34–37].

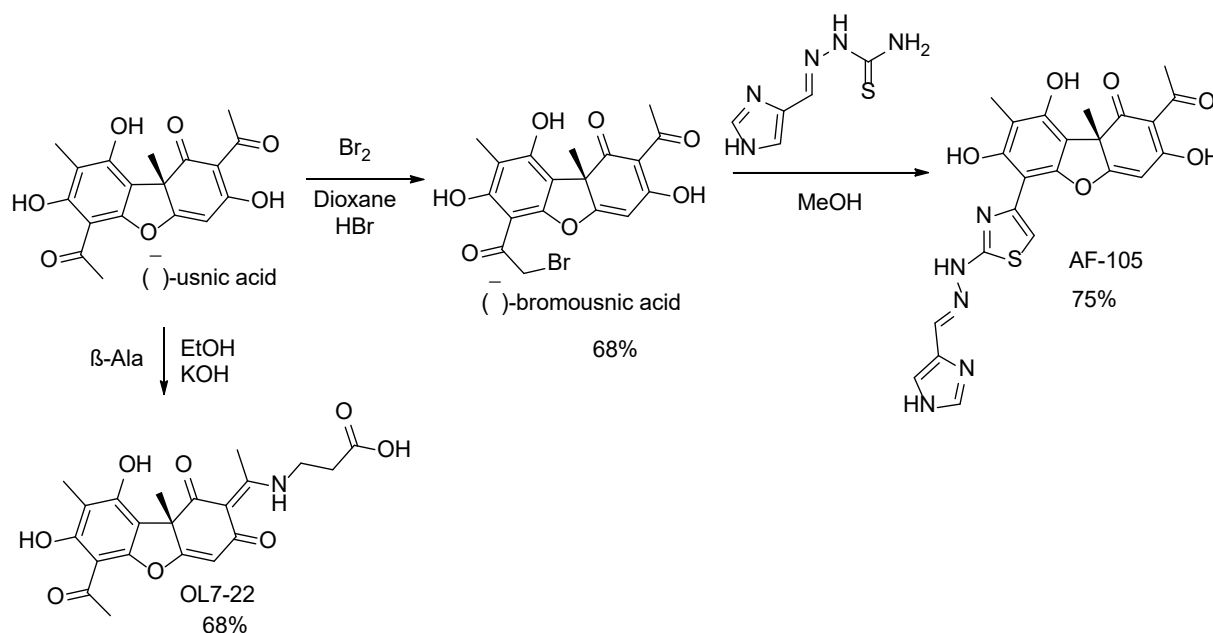
The synthesis of the enamino derivative OL8-122 was performed by boiling (+)-usnic acid with GABA (γ -aminobutyric acid) in ethanol in the presence of potassium hydroxide (Scheme 1) [34]. Compound OL8-122 was obtained with a yield of 71%.



Scheme 1. Synthesis of OL10-88-1 and OL8-122.

Derivative OL10-88-1 was synthesized by boiling (+)-usnic acid with *para*-bromobenzaldehyde in ethanol in the presence of 4 equivalent of KOH (Scheme 1), with a 15% yield [35]. The low yield was caused by losses during column chromatography purification.

Hydrazonothiazole AF-105 was synthesized by heating bromousnic acid (prepared from (–)-usnic acid) with thiosemicarbazone in methanol [36]. Compound AF-105 was obtained at a yield of 75% (Scheme 2). Compound OL7-22 was obtained by reacting (–)-usnic acid with β -alanine in ethanol in the presence of potassium hydroxide at 68% yield (Scheme 2) [37].



Scheme 2. Synthesis of OL7-22 and AF-105.

2.3. Inhibitor Screening

A custom panel of 22 low-molecular-weight compounds identified by virtual screening as potential inhibitors (Supplementary Table S1, Supplementary Table S2) was tested for MtbUng activity suppression. The enzyme was cloned and purified from overexpressing *E. coli* as a recombinant protein His₆-tagged at the C-terminus (Supplementary Figure S5). Since uracil–DNA glycosylases efficiently remove U from both single- and double-stranded DNA substrates, we used a single-stranded 23-mer oligodeoxyribonucleotide with a single U residue in position 11 as a substrate in all of the MtbUng activity assays. The enzyme showed excellent ability to process this substrate in a concentration-dependent manner, generating a 10-mer product after alkaline cleavage of the abasic site, which is the primary product formed by uracil–DNA glycosylases (Figure 2a). The activity of MtbUng was comparable with that of the catalytic fragment of human UNG (hUNG) (Figure 2b).

The full panel was first screened at the 1 mM concentration of the inhibitors using the same substrate (Figure 3). For comparison, bacteriophage PBS1 Ugi, a submicromolar-affinity protein inhibitor of all Ungs [38], was used as a positive control; Ugi fully suppressed the activity of MtbUng and hUNG. Of the 22 tested compounds, only four, OL8-122, OL7-22, AF-105 and OL10-88-1, decreased the enzyme activity more than twofold. The rest had either no effect or even apparently moderately (≤ 2 -fold) stimulated MtbUng. The nature of the stimulation was not further investigated at this time but it could have been due to the competition for lower-affinity unproductive binding to the excess normal DNA in the multiple-turnover reaction [39]. All four compounds that inhibited the reaction were usnic acid derivatives; the fifth molecule of this nature, DS-324, also suppressed enzyme activity but less efficiently (Figure 3) and was not included in the further analysis.

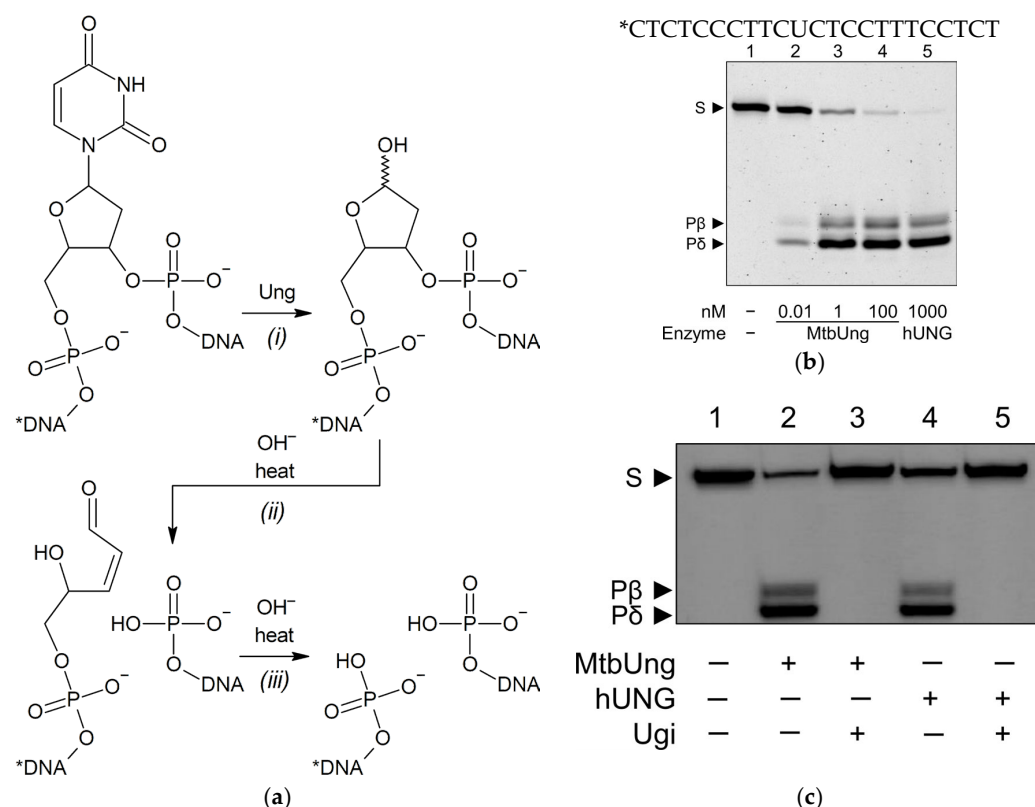


Figure 2. Cleavage of U-containing DNA by uracil–DNA glycosylases. **(a)** General reaction scheme. (i), U excision by the enzymatic activity of Ung produces an AP site; (ii), β -elimination at the AP site under alkaline conditions; (iii), δ -elimination at the AP site under alkaline conditions. Asterisk indicates the placement of a fluorescent label in the DNA substrate. **(b)** Substrate cleavage by MtbUng. Lane 1, no enzyme; lanes 2–4, 0.1–100 nM MtbUng; lane 5, 1000 nM hUNG. **(c)** Inhibition of MtbUng (0.5 nM) and hUNG (0.5 nM) by Ugi (0.02 U/ μ L). Arrowheads mark the oligonucleotide substrate (S) and the products of β -elimination (P β) and δ -elimination (P δ) at the AP site by hot alkali. The sequence of the substrate is shown above the gel image in panel (b).

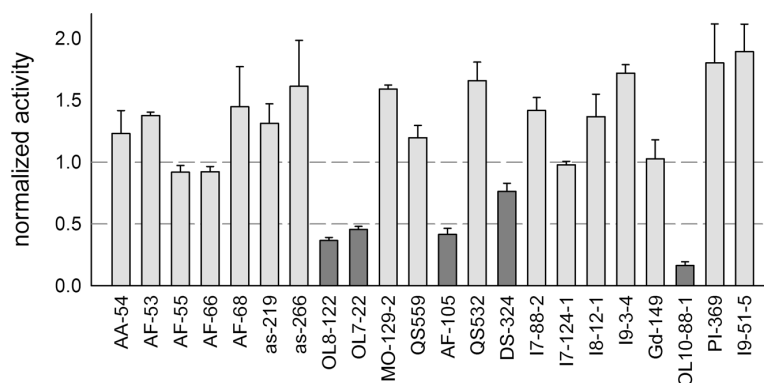


Figure 3. The effect of the compounds identified by molecular docking on the activity of MtbUng. The activity was normalized for the cleavage without the inhibitor (25–30% substrate cleaved). Usnic acid derivatives are shown as dark gray bars. Means and s.d. are shown ($n = 2$).

The compounds that showed >50% inhibition at 1 mM were investigated in more detail, using a concentration range of 10–1000 μ M (Figure 4). The best results were obtained with OL10-88-1, which demonstrated IC_{50} 26 ± 7 μ M and a clear sigmoidal dose–response curve extending into almost complete inhibition at ≥ 200 μ M (Figure 4a). This is at least 30-fold lower in comparison with free uracil, which has been reported to inhibit MtbUng with IC_{50} 0.8–2.0 mM [31]. AF-105 also showed an approximately sigmoidal dose–response

curve but never achieved full inhibition, even at 1000 μM (Figure 4b). Interestingly, OL8-122 and OL7-22 produced dose–response curves with a maximum between 10 and 1000 μM , although no bulk stimulation of MtbUng was observed, with the activity always remaining similar to or below that found in the absence of the inhibitors (Figure 4c,d). Notably, OL8-122 and OL7-22 are different in the chirality of the usnic acid moiety and the number of methylene groups in the substituting amino acid.

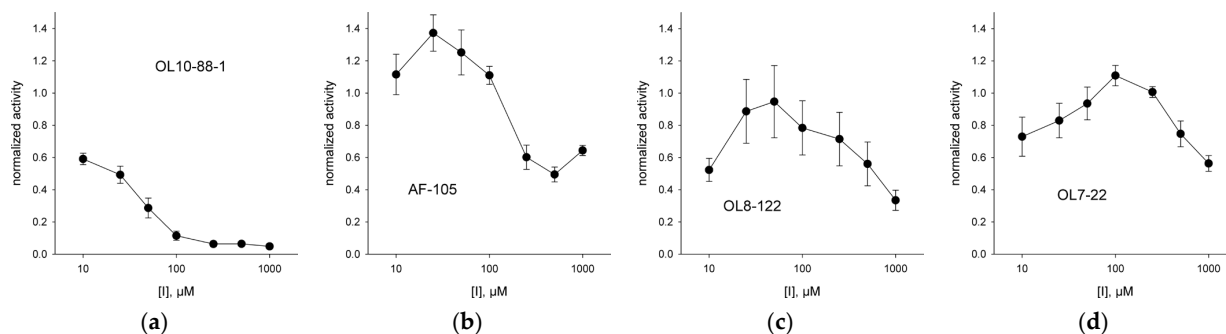


Figure 4. Dose–response relationship of the effect of compounds OL10-88-1 (a), AF-105 (b), OL8-122 (c) and OL7-22 (d) on the activity of MtbUng. The activity was normalized for the cleavage without the inhibitor. Means and s.d. are shown ($n = 3$).

2.4. Inhibition of Ungs from Other Species by Usnic Acid Derivatives

In order to assess the specificity of usnic acid derivatives as Ung inhibitors, they were tested using the enzymes from three other species, namely EcoUng, the catalytic fragment of hUNG, and UNG from the vaccinia virus (vvUNG) (Figure 5). All these enzymes share the same catalytic mechanism and substrate specificity [40,41]; however, they may respond to various inhibitors in a species-specific manner, likely due to the additional interactions of small molecules with non-conserved residues [42–44]. Of the four inhibitors studied with MtbUng, OL10-88-1 showed activity against all Ungs, albeit to a varying degree (Figure 5a–c): EcoUng was significantly affected only at 1 mM, whereas the human and viral enzymes were >50% inhibited at 100 μM already. vvUNG was the most sensitive of all of the tested enzymes, its activity being suppressed by all four usnic acid derivatives at 1 mM. hUNG showed some response to AF-105 but retained considerable activity even at 1 mM. OL7-22 and OL8-122 had no effect on hUNG and EcoUng at any concentration.

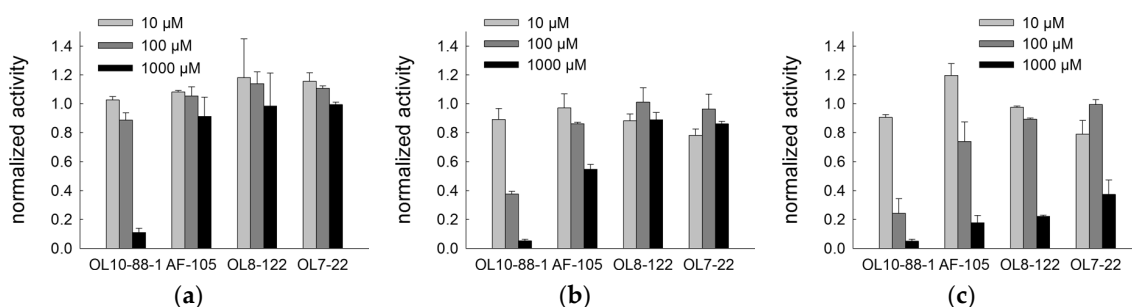


Figure 5. Inhibition of EcoUng (a), hUNG (b) and vvUNG (c) by OL10-88-1, AF-105, OL8-122 and OL7-22. The activity was normalized for the cleavage without the inhibitor. Means and s.d. are shown ($n = 3$). The concentrations of the inhibitors are indicated on the plots.

2.5. Structural Grounds for MtbUng Inhibition by Usnic Acid Derivatives

In an attempt to rationalize the variable inhibitory properties of usnic acid derivatives, we re-examined the structures of MtbUng with the docked OL8-122, OL7-22, AF-105 and OL10-88-1 in light of the structural mechanism of lesion recognition established for hUNG, assuming it is also conserved in MtbUng. In hUNG, the damaged base is captured in the

active site pocket when it spontaneously flips out of the DNA duplex, whereas normal pyrimidine bases that also occasionally flip out are bound in a so-called *exo*-site near the entry to the active site and do not proceed further [45–47]. OL8-122 was predicted to bind to the active site with the carboxylic acid moiety inserted deep into the pocket making hydrogen bond interactions with Ser80 and Asn127, and with Tyr70 through a water molecule, like U (Figure 1b). The tricyclic usnic acid moiety interacted with the *exo*-site and a hydrogen bond was formed with Gln67, like uracil, and with Arg92, which makes a hydrogen bond with uracil. A very similar binding mode was observed for OL7-22, with almost the same interactions as OL8-122 (Figure 1c). OL7-22 formed a hydrogen bond with Asp68, like uracil, instead of Asn67. Compound AF-105 was inserted along the active site crevice, with the usnic acid ring straddling the edge between the *exo*-site and the active site, attached to Arg92 via a double hydrogen bond, while the thiazole ring occupied the active site almost at the same position as U stabilized through π - π stacking, and the imidazole ring protruded even farther into the narrow gorge at the distant end of the pocket, forming a hydrogen bond with Asp171 (Figure 1d). OL10-88-1 placed the usnic acid moiety into the active site, while the *p*-bromocinnamoyl moiety protruded into the DNA-binding groove (Figure 1e). The hydroxyl at the far end of the usnic acid moiety made three strong hydrogen bonds with Gln67, Asp68, and His191 in a way similar to U. The bromine of the *p*-bromocinnamoyl moiety also made halogen contacts with the catalytic His191 residue and Arg170 in a “Gly-Ser” loop that pinches the DNA backbone to kink it and stabilize the flipped-out nucleotide conformation. Overall, OL10-88-1 binding seems to compete with almost all steps of uracil recognition by MtbUng.

3. Discussion

Despite significant progress in chemotherapy, TB remains a serious global health challenge due to the natural hardness of the infectious agent and the spread of a multidrug-resistant strain. The treatment schemes presently endorsed by the World Health Organization and many national regulatory bodies require several months of potent antibiotic cocktail administration; e.g., the 2HRZE/4HR regimen, the one most widely used for newly diagnosed drug-susceptible pulmonary TB, involves two months of isoniazid, rifampicin, pyrazinamide and ethambutol followed by four months of isoniazid and rifampicin, and the newer 2HPZM/2HPM regimen is two months of isoniazid, rifapentine, pyrazinamide, and moxifloxacin followed by isoniazid, rifapentine, and moxifloxacin for another two months [3,4]. For drug-resistant TB, the six-month bedaquiline, pretomanid, linezolid and moxifloxacin (BPaLM), or bedaquiline, delamanid, linezolid, levofloxacin and clofazimine (BDLLfxC) regimens may be used, or even longer treatment schedules depending on the particular strain [3,4]. Poor adherence due to pronounced side effects complicates treatment and promotes the evolution of drug resistance. Thus, the need is clear for drugs that potentiate the action of existing anti-TB antibiotics.

Since the seminal discovery of the role of DNA damage in antibiotic-triggered bacterial cell death (beyond DNA gyrase inhibitors that are long-known to generate DNA breaks) [15], many studies have attempted to clarify the mechanisms responsible and exploit them to sensitize bacteria to the action of antimicrobial drugs. This principle has been extensively validated in *M. tuberculosis* and related species, which face unique DNA damage stresses both from antibiotics and the hostile intracellular macrophage environment [48]. Within macrophages, *M. tuberculosis* encounters endogenous DNA damage from reactive oxygen species (ROS) and reactive nitrogen species (RNS). MtbUng, together with the BER AP endonucleases XthA and End and the recombination repair protein RecN, is essential for long-term survival of *M. tuberculosis* in infected mice [22]. In stationary phase *M. tuberculosis* cells, ROS-mediated oxidation of dCTP to 5-hydroxy-dCTP is lethal

because of massive incorporation into the chromosome saturating BER capacity, and the oxidized nucleotide has to be scavenged by the sanitizer protein MazG [18,49]. Resistance to rifampicin and streptomycin in the stationary phase also requires proteins of the nucleotide excision repair pathway (UvrA, UvrB), which are known to repair lesions inflicted by peroxydinitrite, a potent RNS [50,51]. Moreover, in *Mycobacterium smegmatis*, a model organism often used to study mycobacterial biology, accumulation of ROS-generated 8-oxoguanine in DNA and in the dNTP pool mediates cell death caused by rifampicin and isoniazid [7], and ambient O₂, mild chemical oxidizers or cellular antioxidant depletion efficiently eradicate antibiotic-tolerant persisters in both *M. tuberculosis* and *M. smegmatis* [52,53]. In vitro, the 2HRZE/4HR treatment strongly induces many BER and nucleotide pool sanitization genes in *M. smegmatis* in a pattern different from that caused by the gyrase inhibitor ciprofloxacin [54]. MtbUng was suggested as a possible target to compromise the fitness of the infectious agent, and attempts to develop small-molecule inhibitors based on virtual screening or fragment library crystallization have been reported, but the best compound, methyl 4-[(2,4,6-trioxotetrahydropyrimidin-5(2H)-ylidene)methyl]phenoxy}acetate (a barbituric acid derivative) still showed IC₅₀ ~ 300 µM [27,31]. On the other hand, BER suppression is a double-edged sword, since if DNA damage is insufficient to kill the cell, it may enhance mutagenesis and generate genetic diversity for selection of antibiotic-resistant alleles [55,56].

With this in mind, we conducted a virtual drug screening campaign to search for novel chemical scaffolds with therapeutic potential, aiming to impede U repair capabilities in *M. tuberculosis*. After evaluating the molecules from the in-house library by in silico docking and MtbUng inhibition at a single high concentration, four of the most active compounds were selected for follow up. Strikingly, all four were derivatives of usnic acid, a secondary metabolite found in lichens belonging to the genera *Usnea*, *Cladonia*, *Alectoria*, and many others. Usnic acid derivatives have a wide spectrum of biological activities, including antimicrobial, antitumor, anti-inflammatory and antiviral properties [57,58]. In particular, usnic acid derivatives have been developed into high-potency inhibitors of DNA-repair enzymes, including poly(ADP-ribose) polymerase 1 and tyrosyl-DNA phosphodiesterase [36,59–61], which show promising results combined with topoisomerase poisons in animal models [62–64]. Compounds with anti-TB activity have also been found among usnic acid derivatives [65,66]; however, their mechanism of action remains unknown. In this study, the best of the four compounds, OL10-88-1, showed IC₅₀ ~25 µM (an order of magnitude lower than the best MtbUng inhibitor reported thus far [31]) and an uncomplicated dose–response relationship. While this value is higher than the affinity of some of the above-listed anti-TB drugs (such as rifampicin, isoniazid or bedaquiline) for their targets, it is comparable, for example, with the IC₅₀ of moxifloxacin and levofloxacin against *M. tuberculosis* DNA gyrase (10–30 µM) [67] or of pretomanid against its molecular target, decaprenylphosphoribose-2′epimerase (~10 µM) [68], and is well below the IC₅₀ of linezolid for the translation initiation complex (~100–150 µM) [69]. Compared with other classes of uracil–DNA glycosylase inhibitors from different species (reviewed in [24]), OL10-88-1 is less active than the best bipartite or triskelion inhibitors (submicromolar to first micromolar) and is similar to the best inhibitors containing a single uracil moiety. While we did not specifically address the mechanism of inhibition, the advantage of OL10-88-1 over other usnic acid-based inhibitors seems to be due to its binding mode, in which the usnic acid moiety occupies the uracil-binding pocket while the rest of the molecule interacts with the *exo*-site and key DNA-binding residues, thus incapacitating MtbUng at several critical steps of its catalytic cycle. As the chemistry of usnic acid is quite well-advanced [57,58], there are plenty of opportunities to modify the scaffold to increase binding affinity and make the drug more selective for the bacterial enzyme in comparison with hUNG.

Could usnic acid derivatives be developed into more universal antimycobacterial agents? The importance of counteracting uracil appearance in DNA is underscored by the observation that the genomes of many mycobacterial species encode two or even three distinct uracil–DNA glycosylases. Besides regular Ung, mycobacteria harbor UdgB and UdgX, which are closer to the iron–sulfur-cluster-containing uracil–DNA glycosylases found in many thermophilic bacteria and some phages [70–72] (Figure 6a). However, not all species have the full complement, and, in particular, the two most important mycobacterial human pathogens, *M. tuberculosis* and *M. leprae*, lack UdgX (Figure 6a), leaving Ung and UdgB as their only ways to cope with cytosine deamination. Overlay of the predicted structures of Ung from *M. leprae* and two agents causing non-tuberculosis lung infections, *M. avium* and *Mycobacterioides abscessus*, show that their active sites are highly similar to that of MtbUng, so it is quite possible that OL10-88-1 and its analogs might inhibit them all (Figure 6b). UdgB is less similar to Ung in its 3D structure and differs in several key catalytic residues, and while OL10-88-1 can occupy approximately the same position in UdgB (Figure 6c), it remains to be seen whether the compound retains its inhibitory properties. However, UdgB could be less relevant as a drug target because phenotypes of the respective knockouts suggest that Ung is the main uracil repair enzyme, while UdgB has a back-up role [73–76].

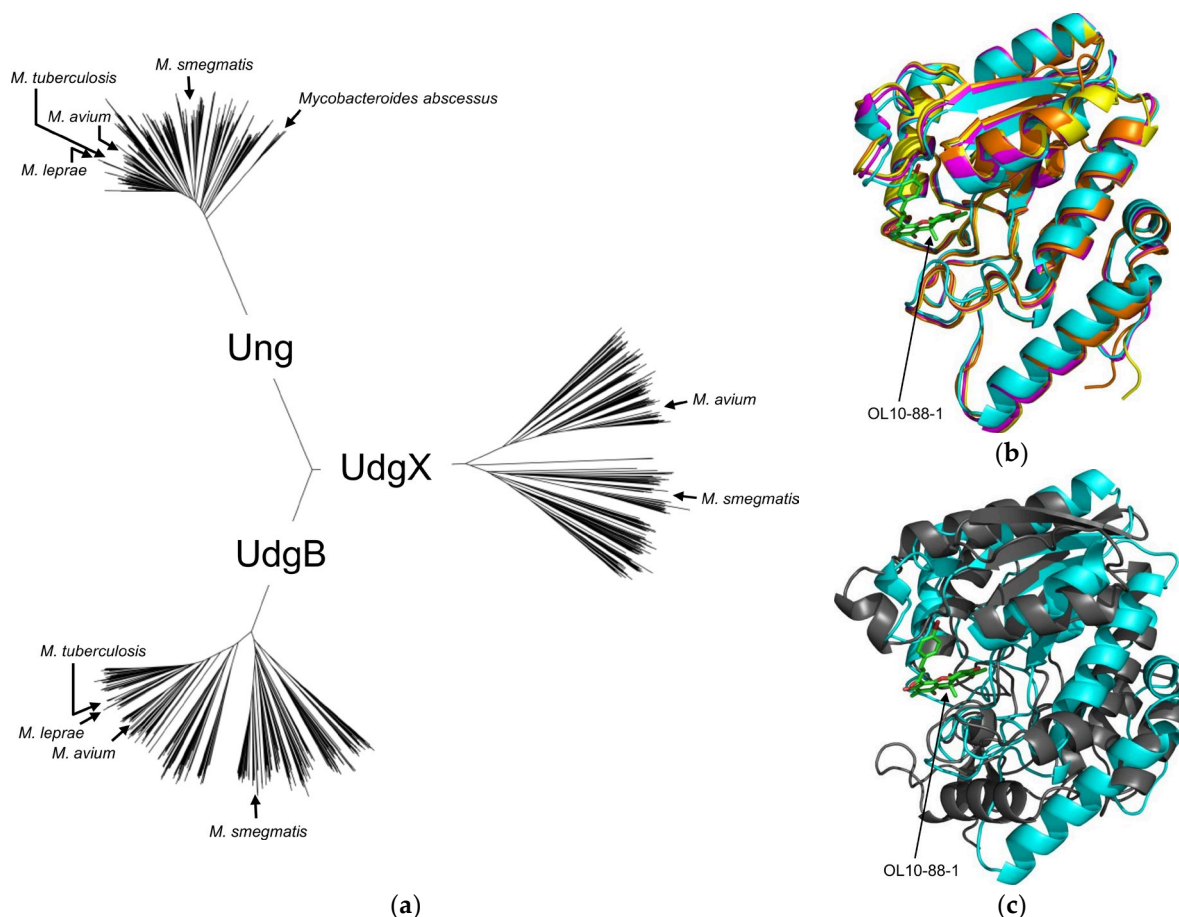


Figure 6. (a) Distribution of Ung, UdgB and UdgX proteins among Mycobacteriaceae. Homologs of MtbUng, MtbUngB and *Mycobacterium smegmatis* UdgX (2009 total) were retrieved from the US National Center for Biotechnology Information RefSeq database [77] (accessed on 8 January 2026), aligned with Clustal Omega v1.2.4 [78], and the tree was visualized in iTol v7.3 [79]. Several species of medical or laboratory importance are marked by arrows. (b) Overlay of the experimental structure of MtbUng (cyan; PDB ID 4WPL [30]) and AlphaFold models of Ung from *M. leprae* (magenta), *M. avium* (yellow) and *Mycobacterioides abscessus* (orange) OL10-88-1 (the stick representation, carbon atoms, green)

docked to MtbUng shown in a stick representation. (c) Overlay of the experimental structure of MtbUng (cyan) and the AlphaFold model of MtbUdgB (gray) with OL10-88-1 (the stick representation, carbon atoms, green) docked to MtbUng shown in a stick representation. The protein models used were taken from the AlphaFold Protein Structure Database v6 [80].

In conclusion, we have successfully established usnic acid derivatives as a novel class of inhibitors targeting MtbUng. This expands the limited repertoire of known Ung inhibitor chemotypes beyond uracil analogs and provides a new, synthetically tractable starting point for antibacterial drug discovery aimed at disabling bacterial DNA repair. While this study has limitations, most notably the moderate potency of the initial leads and the lack of in vivo validation in comparison with standard anti-TB antibiotics, such as isoniazid or rifampicin, it provides a clear proof-of-concept that MtbUng is pharmacologically targetable and that the usnic acid core can engage its active site. Rational structure-based optimization of this scaffold could be pursued to enhance binding affinity and selectivity for the bacterial enzyme over its human counterpart and to avoid human toxicity. Investigating the activity of these compounds against Ung orthologs in other clinically relevant mycobacterial species could reveal their potential as broader-spectrum therapeutic agents.

4. Materials and Methods

4.1. Molecular Docking

A total of 1670 chemical entities from the in-house synthetic library were optimized using the OPLS force field parameters [81] and conjugate gradients method with the MacroModel program (Schrödinger, New York, NY, USA). Counter ions and salts were removed as they were assumed to have minimal contribution to ligand–target interactions. Additionally, charged species were removed to be compatible with the QikProp computational tool (Schrödinger, New York, NY, USA) that was used to calculate pharmaceutically relevant molecular descriptors and properties for the molecules [82]. The library was filtered based on the Known Drug Space limits ($MW \leq 800 \text{ g}\cdot\text{mol}^{-1}$, $\log P \leq 6.5$, $HD \leq 7$, $HA \leq 15$, $PSA \leq 180 \text{ \AA}$ and $RB \leq 17$) [83], leaving 1447 ligands for docking.

Chemical entities were docked to the crystal structure of MtbUng (PDB ID 4WPL, resolution 1.15 Å) [30]. Hydrogen atoms were added and other ligands were removed; Lys and Arg residues were defined as protonated, while Asp and Glu were assumed to be deprotonated. The center of the binding pocket was defined as the centroid of the co-crystallized uracil with coordinates $x = 27.614$, $y = -9.729$, $z = 42.281$ with 10 Å radius. The molecules were docked at 30% efficiency in conjunction with 20 docking runs per molecule for the first round of the virtual screen, and 100% efficiency at 50 docking runs per molecule in the second round. The GoldScore (GS) [84], ChemScore (CS) [85] and ChemPLP [86] scoring functions were implemented to validate the predicted binding modes and relative energies of the ligands using the GOLD v5.7.1 software suite [85,87].

For the docking procedures in Glide [88–90], the receptor was prepared using the Protein Preparation module [91] of Maestro (Schrödinger, New York, NY, USA). In this process, the bond orders were set, hydrogens and partial charges were assigned, ionization and tautomeric states were generated, hydrogen bonds were assigned, and restrained minimization was performed. The Receptor Grid Generator procedure of Maestro was used to create a receptor grid for the binding pocket with the same coordinates and radius used for GOLD. Standard Precision (SP) and Extra Precision (XP) modes of Glide were used at 20 and 50 runs per molecule, respectively.

4.2. Chemistry

The ^1H NMR spectra for the solutions of the compounds in CDCl_3 or $\text{DMSO}-d_6$ were recorded on a Bruker AV-400 spectrometer (Bruker Corporation, Karlsruhe, Germany; oper-

ating frequency 400.13 MHz). The residual signals of the solvent were used as references (δ_{H} 7.27 for CDCl_3 and δ_{H} 2.50 for $\text{DMSO}-d_6$). Thin-layer chromatography was performed on TLC Silica gel 60F254 (Merck, Darmstadt, Germany).

The starting materials, reagents, and solvents used for synthesis (95–99% purity) were purchased from Sigma-Aldrich (Burlington, MA, USA), Acros Organics (Geel, Belgium), and AlfaAesar (Ward Hill, MA, USA). Reagent-grade solvents were redistilled prior to use. The *R*-(+)-usnic acid was obtained from Zhejiang Yixin Pharmaceutical (Lanxi, China). The (–)-Usnic acid was isolated from air-dried *Cladonia stellaris* thalli according to the procedure described in [92]. The (*E*)-2-((1*H*-Imidazol-4-yl)methylene)hydrazinecarbothioamide was synthesized according to the method in the literature [93]. The synthesis of the (–)-bromousnic acid was performed by reacting usnic acid with bromine in dioxane as described [94].

4.2.1. Synthesis of Enamine OL8-122

γ -Aminobutyric acid (309 mg, 3 mmol) was dissolved in aqueous ethanol (10 mL, 1:1 *v/v*). Potassium hydroxide was added to adjust the pH to ~9.5 and the mixture was refluxed for 10 min on a water bath. Then, a suspension of (+)-usnic acid (344 mg, 1 mmol) in ethanol (5 mL) was added in portions over a period of 30 min and the mixture was refluxed for 3 h on a water bath, with pH being maintained at ~9.5. The mixture was cooled and dilute HCl was added to pH ~5; this resulted in precipitation of a light-yellow solid. The reaction mixture containing a finely dispersed precipitate was extracted three times with ethyl acetate, the extract was dried with MgSO_4 , and the solvent was removed on a rotary evaporator. Compound OL8-122 was isolated by column chromatography on SiO_2 (gradient elution with CHCl_3 — EtOAc , 0 to 50%).

(*S*)-6-Acetyl-2-[1-(2-carboxyethylamino)ethylidene]-7,9-dihydroxy-8,9b-dimethyl-1,2,3,9b-tetrahydrodibenzo[*b,d*]furan-1,3-dione. Yellow amorphous powder. Yield 71%. Spectrum NMR ^1H ($\text{DMSO}-d_6$): 1.64 (3H, s), 1.85 (2H, tt, $J_1 = 7.3$ Hz, $J_2 = 7.3$ Hz), 1.96 (3H, s), 2.36 (2H, t, $J = 7.3$ Hz), 2.58 (3H, s), 2.63 (3H, s), 3.57 (2H, dt, $J_1 = 5.8$ Hz, $J_2 = 7.3$ Hz), 5.86 (1H, s), 12.26 (1H, bs), 12.31 (1H, s), 13.05 (1H, t, $J = 5.8$ Hz), 13.40 (1H, s). The spectrum of the substance corresponds to the literature data [34].

4.2.2. Synthesis of Compound OL10-88-1

A stirring suspension of (+)-usnic acid (100 mg, 0.29 mmol) in 10 mL of MeOH was treated with 650 μL of 10% KOH (1.16 mmol). The resulting solution was left to stir for 15 min at room temperature. *p*-Bromobenzaldehyde (0.29 mmol) was added to the mixture in one portion. The reaction mixture was left for 2 days at room temperature and periodically monitored by TLC. Solvents were evaporated under vacuum and the residue partitioned between 2% aqueous HCl and EtOAc (10 mL each). The organic layer was washed with brine and dried over anhydrous MgSO_4 . Compound OL8-122 was isolated by column chromatography on SiO_2 (elution with CHCl_3).

(*R,E*)-2-Acetyl-6-(3-(4-bromophenyl)acryloyl)-3,7,9-trihydroxy-8,9b-dimethyldibenzo[*b,d*]furan-1(9*bH*)-one. Orange amorphous powder. Yield 15%. Spectrum NMR ^1H (CDCl_3): 1.76 (3H, s), 2.10 (3H, s), 2.66 (3H, s), 6.01 (1H, s), 7.45 (2H, m), 7.55 (2H, m), 7.79 (2H, s), 11.10 (1H, s), 13.90 (1H, s), 18.84 (1H, s). The spectrum of the substance corresponds to the literature data [35].

4.2.3. Synthesis of Hydrazonothiazole AF-105

A mixture of (–)-bromousnic acid (423 mg, 1 mmol) and the corresponding thiosemicarbazone (169 mg, 1 mmol) were heated under reflux in 25 mL MeOH for 2 h. The reaction mixture was cooled and poured onto water (75 mL). After that the reaction mixture was cooled, the precipitate formed filtered off, and it was washed with MeOH and dried in air.

The precipitate was placed in a separatory funnel, 30 mL of methylene chloride was added, a suspension was formed, and 20 mL of a saturated solution of NaHCO₃ were added. The mixture was shaken vigorously several times until the suspension had completely turned into a dark red solution. The resulting solution was separated from the aqueous layer, washed with 20 mL water once, the extract was dried with MgSO₄, and the solvent was removed on a rotary evaporator.

5-[(1*E*)-(2-{4-[(1*S*)-12-Acetyl-3,5,11-trihydroxy-1,4-dimethyl-13-oxo-8-oxatricyclo[7.4.0.0^{2,7}]trideca-2(7),3,5,9,11-pentaen-6-yl]-1,3-thiazol-2-yl}hydrazin-1-ylidene)methyl]-1*H*-imidazole. Brown amorphous powder. Yield 75%. Spectrum NMR ¹H (DMSO-*d*₆): 1.72 (3H, s), 2.05 (3H, s), 2.60 (3H, s), 6.21 (1H, s), 7.37 (1H, s), 8.00 (1H, s), 8.09 (1H, s), 9.17 (1H, s), 10.31 (1H, s), 12.59 (1H, bs), 12.71 (1H, s). The spectrum of the substance corresponds to the literature data [36].

4.2.4. Synthesis of Enamine OL7-22

β-Alanine (267 mg, 3 mmol) was dissolved in aqueous ethanol (10 mL, 1:1, *v/v*). Potassium hydroxide was added (to adjust pH ~9.5) and the mixture was refluxed for 10 min on a water bath. Then a suspension of (–)-usnic acid (344 mg, 1 mmol) in ethanol (5 mL) was added in portions over a period of 30 min and the mixture was refluxed for 3 h on a water bath, pH being maintained at ~9.5. The mixture was cooled and dilute HCl was added to pH ~5; this resulted in precipitation of a light-yellow solid. The reaction mixture containing a finely dispersed precipitate was extracted three times with ethyl acetate, the extract was dried with MgSO₄, and the solvent was removed on a rotary evaporator. Compound OL7-22 was isolated by column chromatography on SiO₂ (gradient elution with CHCl₃–EtOAc, 0 to 50%).

(*S*)-6-Acetyl-2-[1-(2-carboxyethylamino)ethylidene]-7,9-dihydroxy-8,9b-dimethyl-1,2,3,9b-tetrahydrodibenzo[*b,d*]furan-1,3-dione. Yellow amorphous powder. Yield 68%. Spectrum NMR ¹H (CDCl₃): 1.68 (3H, s), 2.06 (3H, s), 2.64 (3H, s), 2.65 (3H, s), 2.80 (2H, t, *J* = 6.1 Hz), 3.80 (2H, dt, *J*₁ = 6.1 Hz, *J*₂ = 5.8 Hz), 5.82 (1H, s), 11.75 (1H, s), 13.32 (1H, s), 13.42 (1H, bs). The spectrum of the substance corresponds to the literature data [37].

4.3. Enzymes and Oligonucleotides

The fluorescently labeled substrate oligodeoxyribonucleotide 5'-Fluo-d(CTCTCCCTTC UCTCCTTTCCTCT)-3' (Fluo, 5(6)-carboxyfluorescein) was synthesized in-house from commercially available phosphoramidites (Glen Research, Sterling, VA, USA) and purified by denaturing polyacrylamide gel electrophoresis. EcoUng was purchased from Sibenzyme (Novosibirsk, Russia). The catalytic fragment of hUNG and full-length vvUNG were purified as described [41,44].

4.4. MtbUng Cloning and Purification

The coding sequence for uracil–DNA glycosylase from *M. tuberculosis* strain H37Rv (NCBI reference NP_217492.1) was optimized for expression in *E. coli* and synthesized by Gene Universal (Newark, DE, USA). The protein-coding sequence was flanked by NdeI and XhoI restriction sites. After verification of the insert by Sanger sequencing, the fragment was subcloned at NdeI–XhoI into the pET-24b expression vector. The resulting construct encodes full-length MtbUng with a C-terminal His₆ tag. The nucleotide and protein sequences and the expected protein parameters are given in the Supplementary Text. *E. coli* Arctic Express(DE3) cells (Agilent Technologies, Santa Clara, CA, USA) transformed with the expression plasmid were grown overnight at 30 °C in 25 mL of the LB medium supplemented with 100 µg/mL kanamycin and 20 µg/mL gentamicin with shaking at 250 rpm. The culture was transferred to 1 L of the 2 × YT medium, grown at 30 °C until A₅₉₅ = 0.6, shifted to 16 °C and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside

overnight. The cells were collected by centrifugation and frozen at -70°C until use. To purify MtbUng, the cells were thawed, resuspended in Buffer A (20 mM Na phosphate pH 7.6, 500 mM NaCl) supplemented with 1 mM phenylmethylsulfonyl fluoride, and disrupted by sonication. The debris was removed by centrifugation at $15,000\times g$, 4°C for 20 min; the supernatant was filtered and loaded onto a 10 mL Ni-NTA agarose column (Qiagen, Venlo, The Netherlands) equilibrated in Buffer A. The column was washed with Buffer A and then with Buffer A supplemented with 50 mM imidazole, and the proteins were eluted by a 50–500 mM imidazole gradient in Buffer A. The fractions were analyzed by electrophoresis in the Laemmli system with Coomassie staining, and those containing a protein band of the expected molecular mass were pooled, diluted 10-fold with Buffer B (20 mM Na phosphate pH 7.6, 1 mM EDTA, 1 mM dithiothreitol) and loaded onto a 1 mL HiTrap Heparin HP column (GE Healthcare, Chicago, IL, USA) equilibrated in the same buffer. The target protein was eluted by a 0–1000 mM NaCl gradient in Buffer B. The fractions containing the target protein at $>90\%$ purity (Supplementary Figure S5) were pooled and dialyzed against the storage buffer (20 mM Na phosphate pH 7.6, 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 50% glycerol) and kept at -20°C until use.

4.5. Ung Activity and Inhibition Assays

The reaction mixture (10 μL) contained 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 50 nM single-stranded fluorescently labeled substrate, and 0.01–10 nM MtbUng (0.01 nM in the inhibition assay). For the inhibition studies, stock solutions of all of the compounds (10 mM) were prepared in DMSO and kept frozen at -70°C . Immediately before the experiments, the inhibitors were diluted in DMSO (10% *v/v* in all final reaction mixtures) and added at 10–1000 μM as necessary. The reactions were allowed to proceed at 37°C for 10 min and terminated by adding 1 μL of 1 M NaOH followed by heating at 95°C for 2 min. After neutralization with equimolar HCl, the reactions were mixed with 6 μL of formamide and analyzed by electrophoresis in 20% polyacrylamide gel/7.2 M urea. The reaction products were visualized on a Typhoon FLA 9500 scanner (GE Healthcare, Chicago, IL, USA) and quantified using Quantity One v4.6.3 software (Bio-Rad Laboratories, Hercules, CA, USA). As a positive control, 1 μM hUNG was substituted for MtbUng in the otherwise-identical reaction mixture. Reactions with EcoUng, hUNG and vvUNG were performed under the same conditions. IC_{50} was calculated using SigmaPlot v11.0 (Grafiti, Palo Alto, CA, USA) for the one-site ligand binding model.

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