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Determination of the Bound Conformation of a Competitive Nanomolar Inhibitor of *Mycobacterium tuberculosis* Type II Dehydroquinase by NMR Spectroscopy

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In memory of Pierre Potier

The synergy between tuberculosis and the AIDS epidemic, along with the surge of multidrug-resistant isolates of M. tuberculosis*, has reaffirmed tuberculosis as a primary public health threat. It is therefore necessary to discover new, safe, and more efficient antibiotics against this disease. On the other hand, mapping the dynamic interactions of inhibitors of a target protein can provide information for the development of more potent inhibitors and consequently, more potent potential drugs. In this context, the conformational binding of our previously reported nanomolar inhibitor of* M. tuberculosis *type II dehydroquinase, the 3-nitrophenyl derivative* **1***, was studied using saturation transfer difference (STD) and transferred NOESY experiments. These studies have shown that in the bound state, one conformation of those present in solution of the competitive nanomolar inhibitor 3-nitrophenyl derivative* **1** *is selected. In the bound conformation, the aromatic ring is slightly shifted from coplanarity, with the double bond and the nitro group of* **1** *oriented towards the double bond side.*

Introduction

Since 1990 several highly virulent multidrug-resistant strains of *Mycobacterium tuberculosis,* the causative agent of tuberculosis (TB), have been identified among hospitalized patients with acquired immunodeficiency syndrome (AIDS).[1] These strains not only produced fulminant and fatal disease among patients infected with HIV, with a risk of death in 2–7 months (13% of AIDS deaths worldwide), but also proved highly infectious to exposed persons. This synergy between tuberculosis and the AIDS epidemic, along with the surge of multidrug-resistant isolates of *M. tuberculosis,* has reaffirmed tuberculosis as a primary public health threat. It has been predicted that by 2020 one billion people will be infected if new anti-TB treatments are not developed.[2] It is therefore necessary to discover new, safe, and more efficient antibiotics against this disease.

Recently, we reported a series of compounds that are active against *Mycobacterium tuberculosis* type II dehydroquinase (3-dehydroquinate dehydratase, EC 4.2.1.10).[3] This is the third enzyme in the shikimic acid pathway, which is the biosynthetic route to the aromatic amino acids L-phenylalanine, L-tryptophan, and L-tyrosine, as well as precursors of folate coenzymes, alkaloids, vitamins, and many other aromatic compounds.[4] This pathway is present in bacteria, fungi, plants, and has recently been discovered in the apicomplexan parasites *Plasmodium falciparum* (malaria), *Toxoplasma gondii*, and *Cryptosporidium parvum*.[5] The absence of the pathway in mammals combined with its essential nature in certain microorganisms makes the enzymes of the shikimic acid pathway attractive targets for the development of new antibiotics and herbicides. In fact, *N*-(phosphomethyl)glycine (glyphosphate), the active ingredient in the well-known herbicides RoundUp and Tumbleweed, is also a specific inhibitor of the sixth enzyme of the shikimate pathway (EPSP synthase),[6] and has proven to be active in vitro against malaria.[5a] In addition, (6*R*)- and (6*S*)-6-fluoroshikimic acids have shown antimicrobial activity against *Escherichia coli*.[7]

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Mapping the dynamic interactions of inhibitors of a target protein can provide information for the development of more potent inhibitors and, as a consequence, more potent potential drugs. For this reason, we attempted to study the conformational binding of our previously reported potent competitive inhibitor of *M. tuberculosis* type II dehydroquinase, nitro derivative **1** with a K_i value of 54 nM—considerably below the K_M value of the substrate (40 μM) (Figure 1).[8] The specific characterization of which parts of a ligand are in direct contact to a protein is usually left to X-ray crystallographic analyses of ligand–enzyme complexes. However, during the last few years, NMR-based methods to characterize binding processes have been used. For instance, transferred nuclear Overhauser enhancement (TR-NOE) may be employed to obtain conformational details on the three-dimensional structure of a ligand bound to the active site of a protein, and TR-NOESY experiments provide information about internuclear distances of a ligand bound to the receptor.[9]

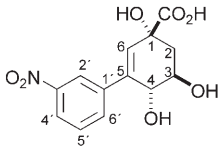

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Figure 1. Potent competitive inhibitor of *M. tuberculosis* type II dehydroquinase.

In addition, the saturation transfer difference (STD) NMR technique has become a very powerful tool in the characterization of ligand binding and, more importantly, of the binding epitope of the ligand. Examples of this type of study with a variety of ligands and receptors have been reported, such as carbohydrates to proteins,[10] inhibitors to enzymes,[11] cofactors to proteins,[12] peptides and proteins to proteins.[13] The STD NMR technique relies on the transfer of saturation from the protein to the ligand. When the protein is selectively irradiated, the ligand, in exchange between the bound and free forms, also becomes saturated when bound to the protein. Subtraction of the spectra acquired with off-resonance irradiation readily reveals the binding epitopes. The group epitope mapping analysis is carried out by integrating the signals of the ligand in the difference spectra and referencing these to the corresponding signals in the off-resonance spectrum. The differential STD effects within one ligand provide information on the proximity of the individual protons to the protein surface. This technique is rapid and does not require isotope labelling of the protein or excessively large quantities of protein.

For both the TR-NOE and STD techniques to be successful, there are certain requirements for the free–bound exchange process. In fact, the off-rate of the ligand should be faster than the relaxation rates for the free state.[9,14] Clearly, this condition excludes very tight binders from direct observation, and assuming that the on-rate is controlled by diffusion (10^7–$10^8\ M^{-1}s^{-1}$), the corresponding off-rate for micromolar affinities would be around 10–100 s^{-1}, which could lead to significant TR-NOE cross-peaks. Indeed, applications of TR-NOE experiments for describing the bound conformation of ligands with affinities (expressed as dissociation constants) ranging from 1 mM to 200 nM,[15] or even stronger (33 nM)[16] have been described. In these last cases, the on-rate is probably not diffusion-controlled, but faster than $10^8\ M^{-1}s^{-1}$.

We report herein the use of selective inversions, STD, and TR-NOESY experiments to determine the bound conformation of nitrophenyl derivative **1**, a nanomolar inhibitor of *M. tuberculosis* type II dehydroquinase.

Results

NMR studies

To avoid the extra proton signals due to buffer salts, all the NMR studies where performed in deuterated phosphate buffer at pD 7.2.[17] The inhibition behaviour under these new experimental conditions was tested for compound **1**. In fact, some decrease in the affinity was observed, since the K_i increased from 54 nM[3] to 400 nM. Nevertheless, this decrease in inhibition potency facilitated the NMR studies, since the K_i value was now within the range where fast exchange may occur.[18]

Selective inversion NOE experiments with nitrophenyl derivative **1** were performed to elucidate the possible conformations of the free ligand in solution. Positive cross-peaks (with different sign to the inverted signal) were observed, as expected for a small molecule tumbling rapidly in solution. It was found that inversion of H6 enhanced H2′ (8%) and H6′ (7%), and inversion of H4 enhanced H2′ (8%) and H6′ (6%) (Figure 1S, see Supporting Information). These results clearly show that fast rotation and thus conformational averaging around the C5–C1′ bond is taking place in solution.

To elucidate if rotation around the C5–C1′ bond could also exist in the bound state, 1D transient NOE experiments with nitrophenyl derivative **1** in the presence of *M. tuberculosis* type II dehydroquinase (20:1 molar ratio) were performed. Interestingly, negative NOE peaks (with the same sign as the inverted signals) were observed (Figure 2c). This change in the sign of the NOE peak corresponds to a slow tumbling molecule, and is expected for derivative **1** when bound to the dehydroquinase enzyme. As mentioned above, signals of inverted sign were observed for derivative **1** in the free state (Figure 2b), as expected for a low-molecular-weight molecule. It was found that inversion of proton H6 of the cyclohexene ring in the presence of the protein exclusively enhanced proton H2′ of the aromatic ring (14%) (Figure 2c), whereas, in the absence of protein, both aromatic protons, H2′ and H6′, that flank the bond between the nitrophenyl and cyclohexene rings were affected.

TR-NOESY experiments were also performed, and the resulting spectrum is shown in Figure 3. As in the 1D case, cross-peaks with the same sign as the diagonal were observed, again indicating that ligand **1** is indeed bound to the active site of the *M. tuberculosis* enzyme. The observed NOE peaks between protons H6 and H2′ and between H4 and H6′ (Figure 3) indicate that these protons are close in space, and

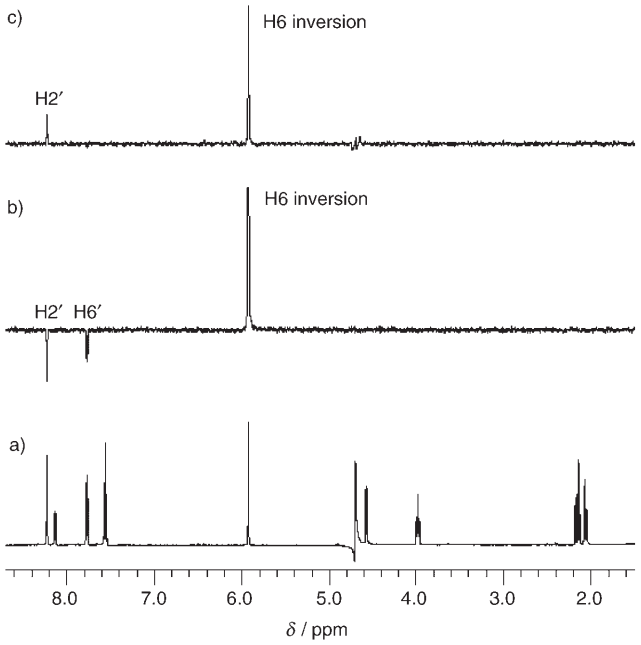

Figure 2. a) ^{1}H NMR spectrum of the free ligand **1**; b) 1D transient NOE spectrum of free ligand **1** obtained after selective irradiation of H6 (mixing time 1 s); c) 1D transient NOE spectrum of ligand **1** bound to *M. tuberculosis* type II dehydroquinase (20:1 molar ratio) obtained after selective irradiation of H6 (mixing time 300 ms). All experiments were recorded at 298 K in deuterated potassium phosphate buffer (50 mM, pD 7.2).

consequently the nitro group at the aromatic ring must be oriented to the same side of the double bond in the cyclohexene ring. NOE cross-peaks of alternative conformations were not detected.

On the other hand, NOE is proportional to the inverse of the sixth power of the interprotonic distance between the two nuclei. Assuming similar effective correlation times for both proton pairs and similar spin diffusion pathways, the ratio of the cross-relaxation rates was used to estimate distance limits (at least semiquantitatively) using the integrals of the 1D and 2D-NOESY cross-peaks for proton pairs H6–H2′/H4–H6′. The TR-NOESY data indicated that the interprotonic distance H4–H6′ is approximately 1.2–1.3 times longer than the H6–H2′ distance.

STD NMR experiments were also performed. The NMR spectrum of ligand **1** with the *M. tuberculosis* enzyme in a 20:1 molar ratio is shown in Figure 4a, and the corresponding STD NMR spectrum is shown in Figure 4b. The relative degree of saturation for the individual protons compared with the off-resonance spectra (see Experimental Section) is shown in

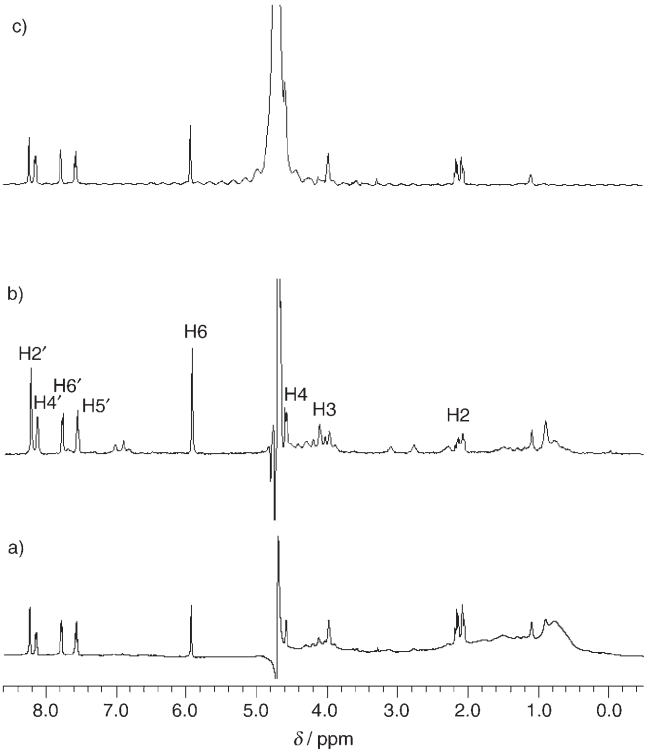

Figure 4. a) ^{1}H NMR spectrum of ligand **1** bound to *M. tuberculosis* type II dehydroquinase (20:1 molar ratio); b) ^{1}H NMR STD spectrum of ligand **1** bound to *M. tuberculosis* type II dehydroquinase (20:1 molar ratio, 3 s irradiation at 0.5 ppm); c) ^{1}H NMR STD off-resonance spectrum.

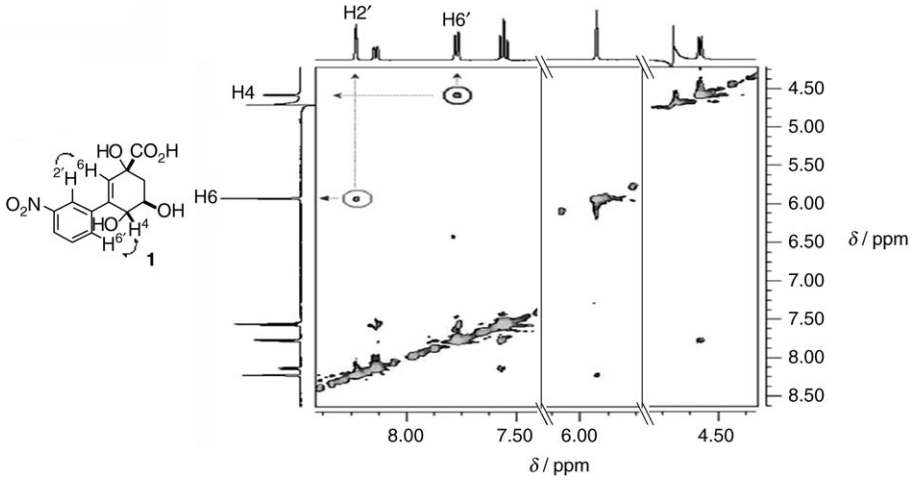

Figure 3. Section of the TR-NOESY spectrum of ligand **1** bound to *M. tuberculosis* type II dehydroquinase (20:1 molar ratio, mixing time 200 ms, 298 K). Relevant cross-peaks are indicated.

Figure 5. It was found that protons H2′ of the aromatic ring and H6 of the cyclohexene ring gave the highest STD effects. Lower STD effects were obtained for the other aromatic protons (H4′, H5′, and H6′), and progressively decreasing intensities were found for protons H4 to H2. These results indicate that the aromatic ring interacts with the protein and especially the region constituted by H2′ of the nitrophenyl group and H6 of the cyclohexene ring provide the best contact interactions with the receptor.

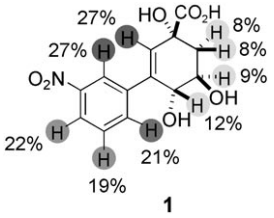

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Figure 5. Percentages of saturation transfer relative to the off-resonance spectra shown for ligand **1** in the 1D STD spectrum after irradiation at the region of the protein aliphatic protons (see Figure 4b).

Computational studies

To elucidate the conformational preference of the nitrophenyl group in inhibitor **1**, geometry optimizations and energy calculations were performed using the Gaussian 98W[19] program with the semiempirical AM1 method. The C4–C5–C1′–C2′ dihedral angle of derivative **1** was progressively incremented from 0° to 360°, and the resultant geometry was minimized (keeping the dihedral angle constant). As is shown in Figure 6a, these calculations predicted that the most stable conformations of ligand **1** should have the aromatic ring slightly shifted from coplanarity with the double bond of the cyclohexene moiety. Furthermore, the less-favoured conformations should have the aromatic ring coplanar with the double bond. Analysis of the interprotonic distance variation between protons H6–H2′ and between H4–H6′ in each resulting conformer (Figure 6b) indicates that only conformers with dihedral angles ranging between 170–240° could have both pairs of protons closer than 3.2 Å. This distance was estimated, in a very conservative manner, as the upper limit distance compatible with the observed H6–H2′ and H4–H6′ NOE effects in the bound state. Out of this range, either these effects should not be observed or NOE effects between protons H6–H6′ and between H4–H2′ should be obtained instead (Figure 6S, see Supporting Information).

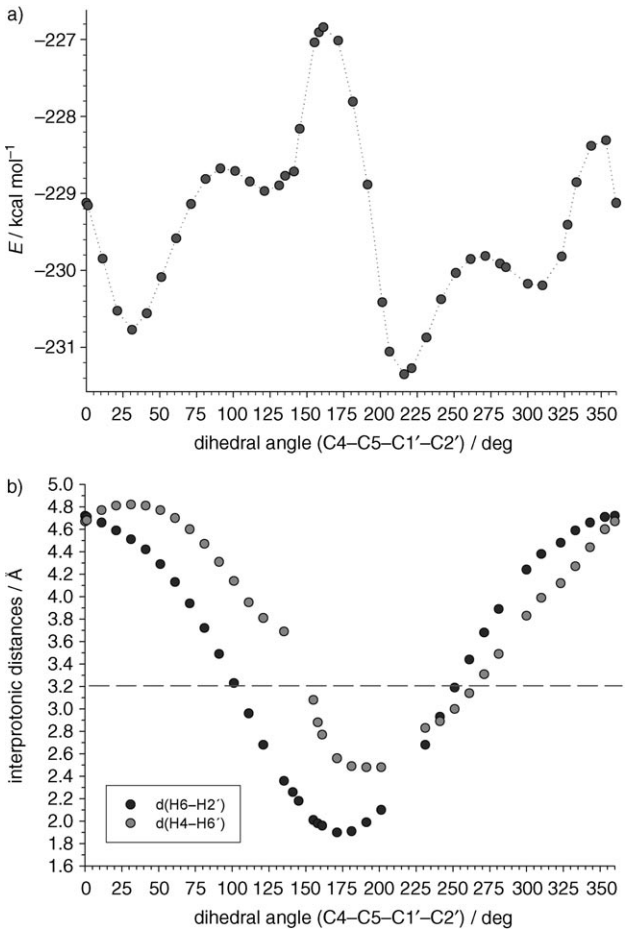

Figure 6. Rotation effect around the C5–C1′ bond of nitrophenyl derivative **1** in a) energy and b) interprotonic distances between protons H6–H2′ and H4–H6′.

Discussion and Conclusions

The reaction mechanism of the type II enzyme involves an elimination reaction by a stepwise E_1CB mechanism via an enol intermediate.[20] Two residues, Arg 19 and Tyr 24 in *M. tuberculosis*, have been identified by chemical modification and site-directed mutagenesis studies as being essential for enzyme activity.[21,22] Both residues are on the flexible loop that closes over the active site upon substrate binding. In the previously reported apo-enzyme structure, these important residues were not visible. However, in the recently solved crystal structures of the enzyme in complex with sulfate[17b] and with 2,3-dehydroquinic acid,[23] one of these residues, Tyr 24, is visible and is positioned in the active site of the enzyme. However, the crystal structure of the complex between 3-hydroxyiminoquinic acid and enzyme shows Arg 19 in the active site of the enzyme, but Tyr 24 is not visible.[24]

Docking studies carried out using GOLD 3.0.1[25] and the crystal structures of the 3-hydroxyiminoquinic acid–enzyme complex (PDB code: 1H0S) and the 2,3-dehydroquinic acid–enzyme complex (PDB code: 1H0R) suggest that the cyclohexene ring of inhibitor **1** occupies approximately the same site as known inhibitors in the crystal structures of the enzyme–inhibitor complexes. Furthermore, the aromatic ring of ligand **1** is predicted to be located within the pocket formed between the three Arg units of the active site: Arg 19, Arg 108, and Arg 112 (Figure 7). However, differences in the position of the aromatic ring were obtained depending on the enzyme–inhibitor crystal structure used for docking. These differences are probably a consequence of the absence of some important residues of the catalytic domain in both crystal structures (Tyr 24 in 1H0S and Arg 19 in 1H0R). In fact, the predicted orientation of the nitro group seems to be correlated with the differences in the observed arginine residues in the X-ray structures available. In the case of the structure 1H0S, the nitro group points to Arg 19 (Figure 7a) whereas in structure 1H0R, Arg 19 is absent and the nitro group is now rotated towards Arg 112 (Figure 7b). However, as TR-NOESY experiments clearly show the proximity of protons H2′ and H6 and also protons H6′ and H4 in the enzyme-bound state, the aromatic ring and thus the nitro group of **1** should be with the aromatic ring rotated towards the H6 proton. Figure 8 shows a proposed binding mode of ligand **1** in the active site of the enzyme, in a confor-

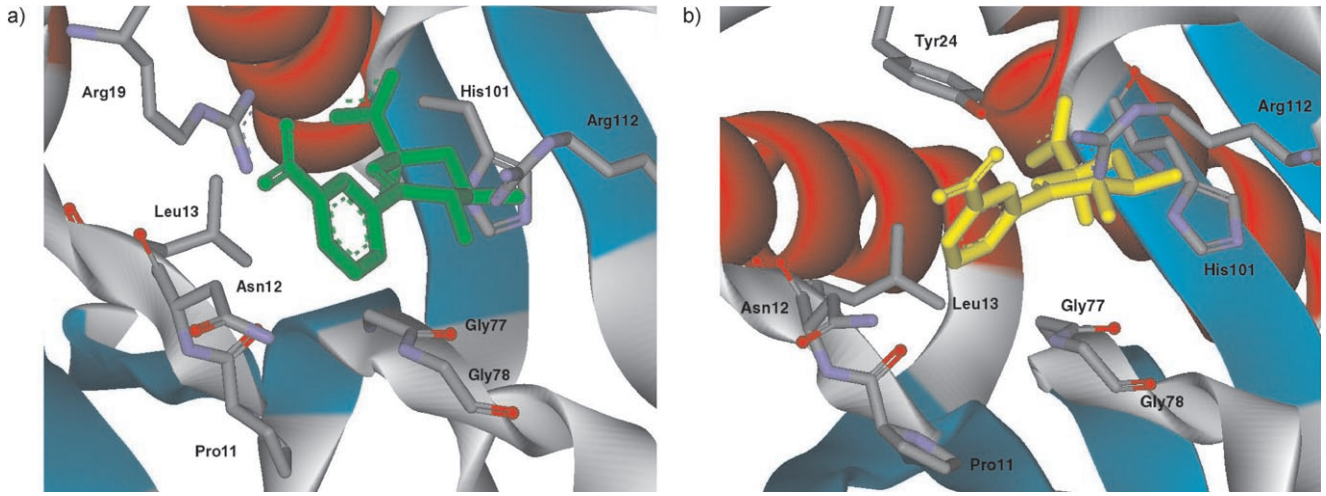

Figure 7. GOLD-predicted binding mode of ligand **1** to the active site of *M. tuberculosis* type II dehydroquinase using a) 1H0S (ligand **1**; green) and b) 1H0R (ligand **1**; yellow).

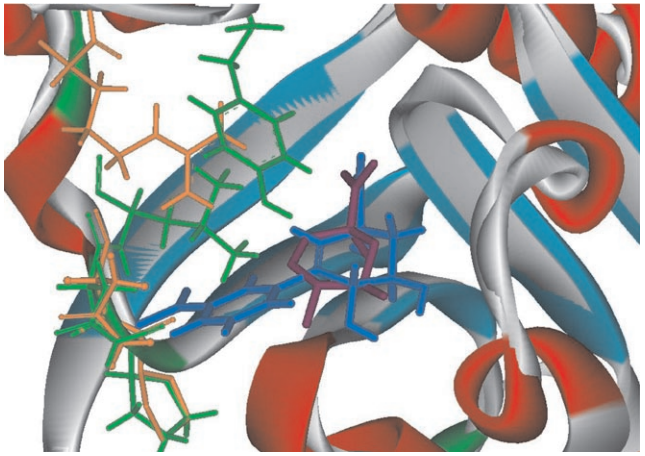

Figure 8. Proposed bound conformation of inhibitor **1** (blue) to the active site of *M. tuberculosis* type II dehydroquinase. Relevant catalytically important domains of crystal structures 1H0S (orange) and 1H0R (green) are indicated. The ligand 2,3-dehydroquinic acid (purple) co-crystallized with 1H0R is indicated.

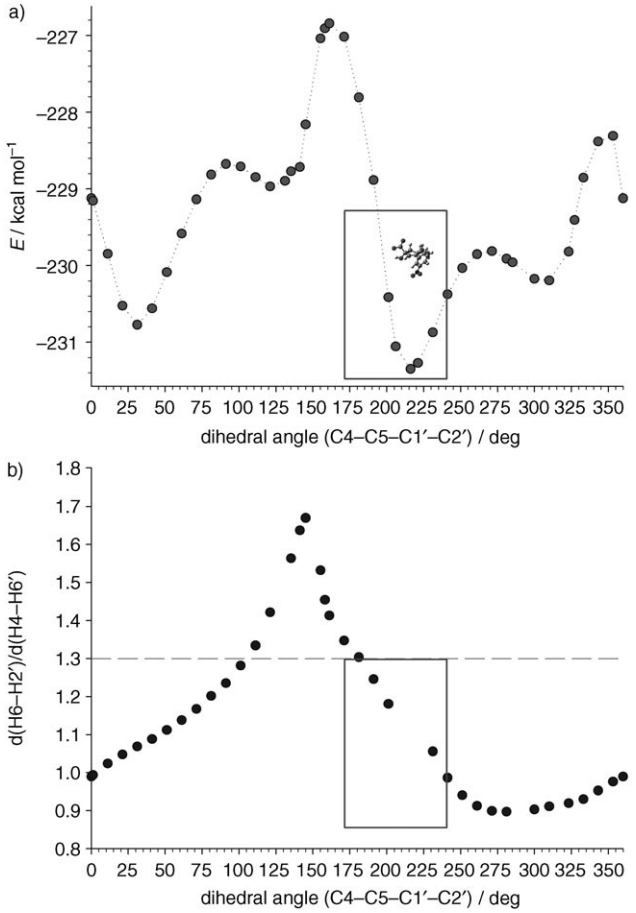

Figure 9. Comparison of the ratio between H6–H2′ and H4–H6′ interprotonic distances on the calculated energy curve versus dihedral angle.

mation close to the calculated energy minimum. In this geometry, the aromatic ring is slightly shifted from coplanarity with the double bond, with H2′ under the plane of the cyclohexene ring and therefore H6′ above this plane, leading to close proximity between protons H6′ and H4 of the ligand. The above geometry should be compatible with the observation of H6–H2′ and H4–H6′ NOE cross-peaks, which indicates that both pairs of protons are closer than 3.2 Å (corresponding to a dihedral angle between 170 and 240°). Moreover, if the ratio between both interprotonic distances is superimposed on the calculated energy curve (versus the corresponding dihedral angle), the resulting selected conformation resembles that of the calculated global minimum with the nitro group below the plane of the double bond, and a torsion angle slightly larger than 200° (Figure 9).

The proposed bound conformer is also supported by the STD experiments that show strong saturation transfer from the protein to proton H6 of the cyclohexene ring and to the aromatic proton H2′ of ligand **1**. The equivalent H6 proton in the

crystal structure of 2,3-dehydroquinic acid–enzyme (PDB code: 1H0R) is close to the aromatic protons of Tyr 24 and to a lesser extent to methyl groups of Leu 13. In the proposed binding mode of ligand **1**, similar contacts are found for H6, while H2′ is close to the Leu 13 side chain. Furthermore, H4′ is located in the vicinity of the methylene group of Asn 12.

In conclusion, a combined NMR and modelling approach has been used to study the conformational details of *M. tuberculosis* type II dehydroquinase inhibition. NMR data obtained from STD and 1D- and 2D-TR-NOESY experiments have shown that only one conformation of those present in solution for the competitive nanomolar inhibitor 3-nitrophenyl derivative **1** is selected when bound to the active site of the enzyme. In the bound state, the 3-nitrophenyl unit of the potent inhibitor **1** is frozen in one orientation relative to the cyclohexene ring, with the nitro group oriented towards the double-bond side. Despite the fact that the available structural data of the protein active site is incomplete and precludes a quantitative analysis of the experimental data with the CORCEMA suite of programmes,[26] the proposed bound conformation adequately explains the experimental data.

Experimental Section

General remarks: Nitro derivative **1** was prepared according to previously published procedures.[3] *M. tuberculosis* type II dehydroquinase, a 15.6 kDa dodecamer, was purified as described previously.[27] A concentrated stock solution of the protein (0.7 mg mL^{-1}, 44.75 μM stored in potassium phosphate buffer (50 mM, pH 7.2), DTT (1 mM), and NaCl (150 mM)), was exchanged to deuterated buffer by successive concentration and dilution using deuterated potassium phosphate buffer (50 mM, pD 7.2) at 4 °C in an Amicon Centricon-10 microconcentrator prior to NMR experiments.

Sample preparation for NMR analysis: NMR samples were prepared in a 1:20 molecular excess by adding 5 μL of a concentrated solution of inhibitor **1** (2 mM in an NMR tube) to 500 μL of *M. tuberculosis* type II dehydroquinase (100 μM in an NMR tube) both in deuterated potassium phosphate buffer (50 mM, pD 7.2).

NMR experiments: All NMR experiments were performed on a Bruker AVANCE 500 MHz spectrometer at 298 K. For regular 1D experiments, solvent suppression of the residual HDO peak was achieved by continuous low-power presaturation pulse during the relaxation delay. First, line broadening of the ligand protons was monitored in the presence and absence of protein.

1D transient NOE experiments were measured by using the double pulsed field gradient spin echo sequence proposed by Shaka and co-workers.[28] Selective pulses (Gaussian types) were 40 ms long. No water suppression scheme was applied, as the buffer was prepared in D_2O. 1D transient NOEs were performed with mixing times of 200, 400, 600, 800, and 1000 ms, for a ligand/protein molar ratio of 20:1. For STD experiments, two FIDs, one for off-resonance irradiation (at 50 ppm) and a second for on-resonance saturation (at 0.5 ppm) were obtained, with scans acquired in interleaved fashion. A purging spin lock period of 15 ms to remove the protein background signals was employed. A train of 20, 40, or 60 Gaussian-shaped pulses of 50 ms each was used to run different STD experiments with a total saturation time of the protein envelope of 1, 2, or 3 s, respectively. Solvent suppression was achieved using a Watergate module just before acquisition.

TR-NOESY experiments were performed with mixing times of 50, 100, 200, and 250 ms for a 20:1 molar ratio of ligand/protein. No purging spin lock period to remove the NMR signals of the macromolecule background was employed, since they were basically unobservable because of the large size of the receptor. First, line broadening of the ligand protons was monitored after the addition of the ligand. Strong negative NOE cross-peaks were observed, in contrast to the free state, indicating binding. TR-NOESY experiments were also carried out to exclude spin-diffusion effects. A continuous wave spin lock pulse was used during the mixing time of 250 ms. Key NOEs were shown to be direct cross-peaks, since they showed different signs to diagonal peaks. Two identical samples were used for the STD and TR-NOESY experiments with a 20:1 ligand:protein molar ratio and were repeated twice, each time giving the same results.

Docking: The ligand **1** was docked in the active site of *M. tuberculosis* type II dehydroquinase using the program GOLD (version 3.0.1).[25] Receptor and ligand were used as MOL2 files. The ligand structure was prepared using Gaussian 98W,[19] and the energy was minimized using AM1. No energy minimization was performed on the enzyme. The crystal structure of the complexes 3-hydroxyiminoquinic acid–enzyme (PDB code: 1H0S) and 2,3-dehydroquinic acid–enzyme (PDB code: 1H0R) were used. The ligand was docked using GOLD 3.0.1 in 25 independent genetic algorithm (GA) runs, and for each of these a maximum number of 100 000 GA operations were performed on a single population of 50 individuals. Operator weights for crossover, mutation, and migration in the entry box were used as default parameters (95, 95, and 10, respectively), as well as the hydrogen bonding (4.0 Å) and van der Waals (2.5 Å) parameters. The position of the active site was introduced, and the radius was set to 15 Å, with the automatic active-site detection on. The "flip ring corners" flag was switched on, while all the other flags were off. The GoldScore scoring function was used.

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