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# Discovery of an acyclic nucleoside phosphonate that inhibits M. Tuberculosis ThyX based on the binding mode of a 5-alkynyl substrate analogue.

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*((Dedication, optional))*

*The urgent need for new antibiotics poses a challenge to target un(der)exploited vital cellular processes. Thymidylate biosynthesis is one of these processes due to its crucial role in DNA replication and repair. Thymidylate synthases (TS) catalyze a crucial step in the biosynthesis of TTP, an elementary building block required for DNA synthesis and repair. To date, TS inhibitors are only successfully applied in anticancer therapy due to their lack of specificity for antimicrobial versus human enzymes. However the discovery of a new family of TS enzymes (ThyX) in a range of pathogenic bacteria that is structurally and biochemically different from the 'classic' TS (ThyA) opened possibilities to develop selective ThyX inhibitors as potent antimicrobial drugs. In this work we explored the interaction of the known inhibitor (compound 1) with M. tuberculosis ThyX enzyme using molecular modeling and confirmed our findings with NMR experiments. While the dUMP moiety of compound 1 occupies the cavity of the natural substrate in ThyX, the rest of the ligand (the '5-alkynyl tail') extends to the outside of the enzyme between two of its four subunits. The hydrophobic pocket that accommodates the alkyl part of the 'tail' is formed by displacement of Tyr44.C, Tyr108.A and Lys165.A. Changes of Lys165-$NH_3$ upon ligand binding were monitored in a titration experiment by 2D NMR HISQC. Inspired by the success of acyclic antiviral nucleosides, we have synthesized compounds where 5-alkynyl uracyl was coupled to acyclic nucleoside phosphonates (ANPs). One of these compounds showed 43% of inhibitory effect on ThyX at 50$\mu$M.*

## Introduction

The first antibiotic (penicillin) in 1928 was followed by fast discovery of other nowadays known antibiotics. Unfortunately, rapid emergence and spread of drug-resistant bacteria started a battle that is going on for more than 50 years. To overcome the resistance problem, there is an urgent need for new classes of antibiotics that target preferentially the most vital and vulnerable cell processes that are un(der)exploited so far.[1, 2] Hitting new bacterial targets slows down and reduces the probability of development cross-resistance with known drugs that are targeting other cellular processes.[3, 4] Despite many efforts in the last 50 years, only one new broad-spectrum class of antibiotics (fluoroquinolones) reached the market, targeting DNA (un)winding catalyzed by topoisomerase II. Usually, chemical modifications are applied to existing drugs to avoid known resistance mechanisms. Recently diarylquinolones targeting bacterial ATP synthase came into the picture. So far, this new class of antibiotics has a narrow spectrum, focusing on key gram-positive bacteria such as *M. Tuberculosis*.[5]

One of the cell processes that is currently underexploited by antibacterial drugs is DNA replication and repair. By blocking the synthesis of one of the DNA building blocks (dATP, dGTP, dCTP and TTP), a direct impact on cell survival is inevitable. Inhibition of TTP biosynthesis is a well-established therapeutic strategy: dihydrofolate reductase (DHFR) inhibitors[6, 7] are routinely applied in antibacterial (e.g. trimethoprim), antimalarial (e.g. proguanyl) and antitumoral therapy (e.g. methotrexate) while thymidylate synthase inhibitors are used for decades as a cytostatic agent.

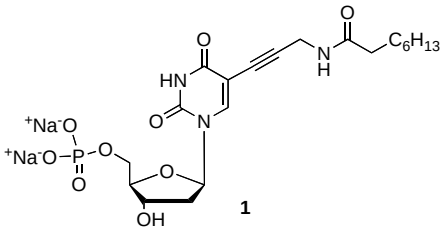
The lack of specificity for bacterial over human thymidylate synthase hampered application of the latter in the antibacterial field. The possibility for specific inhibition of bacterial thymidylate synthase activity was opened at the start of this century by the discovery of a flavin dependent thymidylate synthase (FDTS or ThyX) in a range of bacteria and mobile genetic elements as an alternative pathway for biosynthesis of the TMP precursor of TTP.[3] The classical thymidylate synthase, ThyA, is a well-studied and characterized enzyme that catalyzes the reductive methylation of dUMP to TMP using R-$N^5$-$N^{10}$-methylene-5,6,7,8-tetrahydrofolate ($CH_2$THF) as a source for methylene and hydride.[8] The newly discovered ThyX requires also $CH_2$THF as a methylene donor producing THF as by-product but a reduced flavin adenosine dinucleotide ($FADH_2$) serves as a hydride donor, making ThyX independent of DHFR activity in the recycling of its $CH_2$THF cofactor.[9, 10] ThyX not only uses a unique catalysis mechanism, it also lacks any structure or sequence homology with classical thymidylate synthase ThyA. Therefore it is an excellent target for developing selective antibacterial drugs which will have little or no effect on human ThyA-based thymidylate synthase activity. To date, there are only few compounds that

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influence ThyX activity, for example: 5-FdUMP and 5-BrdUMP, but these are non-selective since they inhibit also ThyA. Therefore they cannot be used in antibacterial therapy.[11-13]

It was shown that 30% of microorganisms depend on ThyX for their TMP production and many of them are severe human, animal and plant pathogens. In most organisms ThyX and ThyA are mutually exclusive, only few are known that carry the genetic code for both enzymes.[3, 14] *Mycobacterium tuberculosis*, the main cause of tuberculosis (TB), is one of the rare organisms that encode the genes for both TS in its genome.[3] Despite the presence of *thyA*, it is proven that the *thyX* gene is essential for *M. tuberculosis*.[15, 16] In this work it was chosen as a model organism since there is an urgent need for new anti-TB drugs due to the emergence of multi-drug and extreme drug-resistant strains (MDR and XDR resp.). Nowadays TB remains a global health priority worldwide with estimated nine million new cases and two million deaths each year.[17-19]

Several attempts have been made to synthesize ThyX inhibitors,[20-22] also some of recently synthesized anti-TB inhibitors may act by inhibiting ThyX enzyme.[23] In our strategy substrate analogues are prepared to obtain ThyX inhibitors. In a first stage we modified the nucleobase of dUMP and obtained a promising 5-alkynyl uridine analogue **1** (figure 1).[21] In this article we used computer modeling to dock our most promising inhibitor of *M. tuberculosis* ThyX (**1**) in the binding pocket of ThyX to determine the crucial parts of this compound with the target enzyme. NMR experiments were performed to probe structural changes upon binding expected from molecular modeling. To obtain more stable analogues of compound **1**, several compounds were synthesized with the nucleobase of compound **1** linked to a phosphonate group by an acyclic linker, replacing the labile glycosidic and phosphate ester bonds in nucleotides that can be enzymatically hydrolyzed to yield inactive compounds. Comparable acyclic nucleoside phosphonates (ANPs) are successful reverse transcriptase inhibitors: several ANP-based drugs are currently in clinical use for antiviral treatments.[24] A $^{3}H$ release assay was used to test the inhibitory effect of prepared compounds on ThyX activity in vitro.




**1**

Figure 1. Compound **1**

## Results and Discussion

### Modelling

Starting from an available crystal structure of *M. tuberculosis* ThyX (pdb: 2AF6)[25] a model was generated to explore the binding mode of our most promising inhibitor. While ThyX contains 4 active sites, the one at the interface of subunits A, C and D was selected to accommodate compound **1**. If the dUMP core of our compound is posed in the position of the natural substrate, its 5-alkynyl 'tail' extends between the subunits A and C to the outside of the enzyme. The wall of its binding pocket is formed by residues Tyr108.A, Val109.A, Lys165.A Ser105.A and Tyr44.C. Some 'induced fit' mechanisms at the binding site are required to accommodate this 'tail': displacement of the side chain in residues Tyr44.C, Tyr108.A and Lys165.A is needed to avoid steric clashes. In our model, the aliphatic parts of the Tyr108.A, Val109.A and Tyr44.C side chains form the wall of a hydrophobic pocket that accommodates the alkyl chain in compound **1**. The position of the inhibitor 'tail' is maintained by hydrogen bonding interactions with amino acid side chains that line the binding pocket (Figure 2). In the starting crystal structure, the amino group in the Lys165.A side chain is hydrogen bonded to a water molecule that resides at the interface between subunits A and C. After its displacement to accommodate compound **1**, this charged amino group is within hydrogen bonding distance to the oxygen of the amide linkage in the inhibitor 'tail' (2.45Å). The nitrogen of this amide is close enough to the terminal hydroxyl in the Ser105.A side chain to allow hydrogen bonding (2.59Å). This highly conserved amino acid was originally attributed a role in the mechanism of catalysis, highlighting the importance of this interaction.[10] The hydrogen bond that existed in the starting structure between the terminal hydroxyl of Ser105.A and Tyr108.A is lost due to 'induced fit' at the binding site. Structural changes observed in the crystal structure *T. Maritima* ThyX bound to a folate analogue (pdb code: 4GTB) also involve repositioning of corresponding Ser88 and Tyr108 amino acids.[26] The binding pocket of the 5-alkynyl 'tail' in the proposed model is different from that observed for the methoxybenzyl group of 2-hydroxy-3-(4-methoxybenzyl)-1,4-naphthoquinone bound to PBCV-1 ThyX (pdb: 4FZB).[27] In this crystal structure the aliphatic parts of the Gln75, Glu152 and Arg90 side chains form the wall of a hydrophobic pocket that accommodates the 4-methoxybenzyl group of the ligand while the rest of this compound occupies the cavity of uracyl (corresponding residues in our model: Glu92.D, Gln169.A and Arg107.A -> based on structure based sequence alignment in [28]).

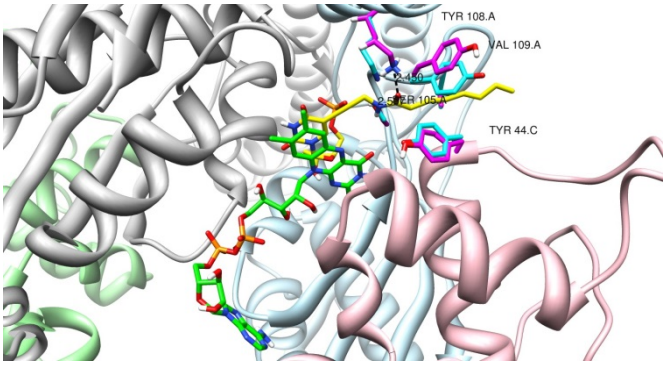



**Figure 2.** Compound **1** in the ThyX active site. Ribbon colors: chain A in blue, chain B in green, chain C in pink, chain D in grey. The labeled residues move a slightly to accommodate the inhibitor tail (induced fit). Their position in the is shown in cyan and magenta sticks while magenta sticks in structures before and after docking compound 1 respectively. The sugar and base part are on the same position as the original substrate analogue, maintaining the stacking with the FAD cofactor.

### NMR experiments

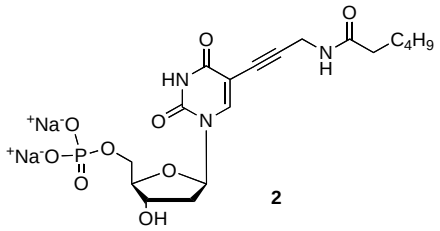
Since we were not able to obtain co-crystals of compound **1** in complex with *M. tuberculosis* ThyX, we used NMR to probe structural changes expected from molecular modelling. We focused on the proposed interaction of the Lys165-$NH_3$ group and CO of the amide in the tail of the inhibitor using a $^{1}H$-$^{15}N$ 2D

NMR experiment that enables to monitor the Lys165-$NH_3$ cross-peak behavior upon addition of the inhibitor. Signals of lysine-$NH_3$ groups in proteins are barely detected by NMR due to high water exchange rates[29] and also the fact that common $^1H$-$^{15}N$ 2D NMR experiments (HSQC, HMQC) are designed and optimized for the detection of backbone NH cross-peaks at high magnetic field. Recently Clore et al[30] proposed a new type of $^1H$-$^{15}N$ 2D experiment (HISQC), that is especially designed for the lysine-$NH_3$ groups. The main advantage of the HISQC experiment is that it is not affected by scalar relaxation in the $^{15}N$ dimension resulting in better resolution in this dimension and higher signal to noise ratio for detection of $NH_3$ cross-peaks. It means that $^{15}N$ transverse relaxation during $t_1$-evolution period is independent of water exchange rates, however rapid water exchange still causes significant line broadening in the $^1H$ dimension. Signals sharpen upon lowering temperature and pH. Unfortunately we couldn't go below pH 5 since protein precipitation occurred in more acidic conditions.

*M. tuberculosis* ThyX is a symmetric homotertamer (total weight: 110 kDa) and has 6 lysine residues in each subunit. According to the X-ray structure (pdb 2AF6)[25] side-chains of 5 lysine residues are directed towards outside of the protein. Hence the amino protons of their side chains have very rapid water exchange rates and therefore those amino cross-peaks are difficult to detect by NMR. It was found that lysine-$NH_3$ groups that are involved in hydrogen bonding or salt bridge interactions are much easier to observe because they are protected from water exchange.[30] The Lys165 is located in the binding pocket of the enzyme hydrogen bonded to a water molecule resulting in a slower water exchange rate and a better intensity of a NMR signal. According to the proposed model Lys165 has to change its side chain conformation upon binding of the inhibitor and form a hydrogen bond to the CO in the 'tail' of compound **1**. Such a change is expected to result in a different chemical shift of the $NH_3$ cross-peak, what allows for the visualization of the binding by NMR.

To experimentally confirm a change of Lys165 upon binding the compound **1** we performed the HISQC experiment as described by Clore et al[30] without using a coaxial NMR tube. $^{15}N$ labeled *M. tuberculosis* ThyX was prepared and purified as previously described.[25] The spectra were recorded at 5°C, pH 5. The NMR sample contained 0.47 mM ThyX, 50 mM TRIS, 10% $D_2O$ and increasing amount of inhibitor 0 μg, 30 μg, 50 μg, 80 μg (Figure 3). Compound **2** (Figure 3) was used instead of compound **1** as an inhibitor in these NMR studies due to insufficient available amount of the latter. Note that 'tail' at C5 position of the used inhibitor **2** is two carbon atoms shorter than that of compound **1**. In the absence of the inhibitor only one cross-peak with $^{15}N$ chemical shift 32.2 ppm is visible in the HISQC. After the addition of 30 μg of the inhibitor an extra peak with lower intensity appears slightly downfield from the original signal and increases if more inhibitor is added, while intensity of the first peak decreases (Figure 4). The resolved signals that are observed for ligand free and bound states of ThyX, indicate that the ligand is binding with high affinity and low dissociation constant ($K_D$ = μM or lower).[31]

In order to prove that this peak originates from the Lys165-$NH_3$ group, a $^{15}N$ labeled K165A mutant of *M. tuberculosis* ThyX was prepared and purified using affinity chromatography. The used cloning vector contained a C-terminal KGHHHHHH purification tag what resulted in one extra lysine residue per monomer. The HISQC experiments were performed using the same conditions as described above. One cross-peak appeared in the HISQC spectrum of our his-tagged mutant (Figure 5). Since the position of this signal is slightly different from that observed in the wild type protein and the fact that this signal did not change upon adding the inhibitor **2** (50 μg), we assigned it to the extra lysine preceding the his-tag. From the 2AF6 X-ray structure we can see that the last amino acids at the C-terminus of ThyX subunits are directed inwards the protein, and this could explain the visibility of the cross-peak of lysine $NH_3$ from purification tag.




**2**

**Figure 3.** Compound **2**

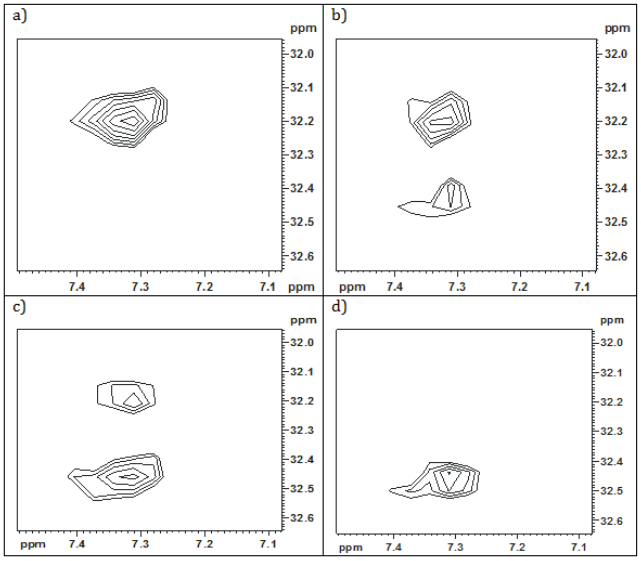



**Figure 4.** Results of $^1H$-$^{15}N$ HISQC NMR experiments of ThyX: a) cross-peak of Lys165 $NH_3$ b) cross-peaks after addition of 30 μg of the inhibitor, c) cross-peaks after addition of 50 μg of the inhibitor, d) cross-peaks after addition of 80 μg of the inhibitor

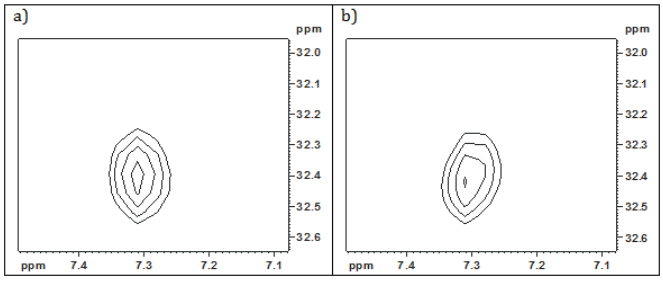
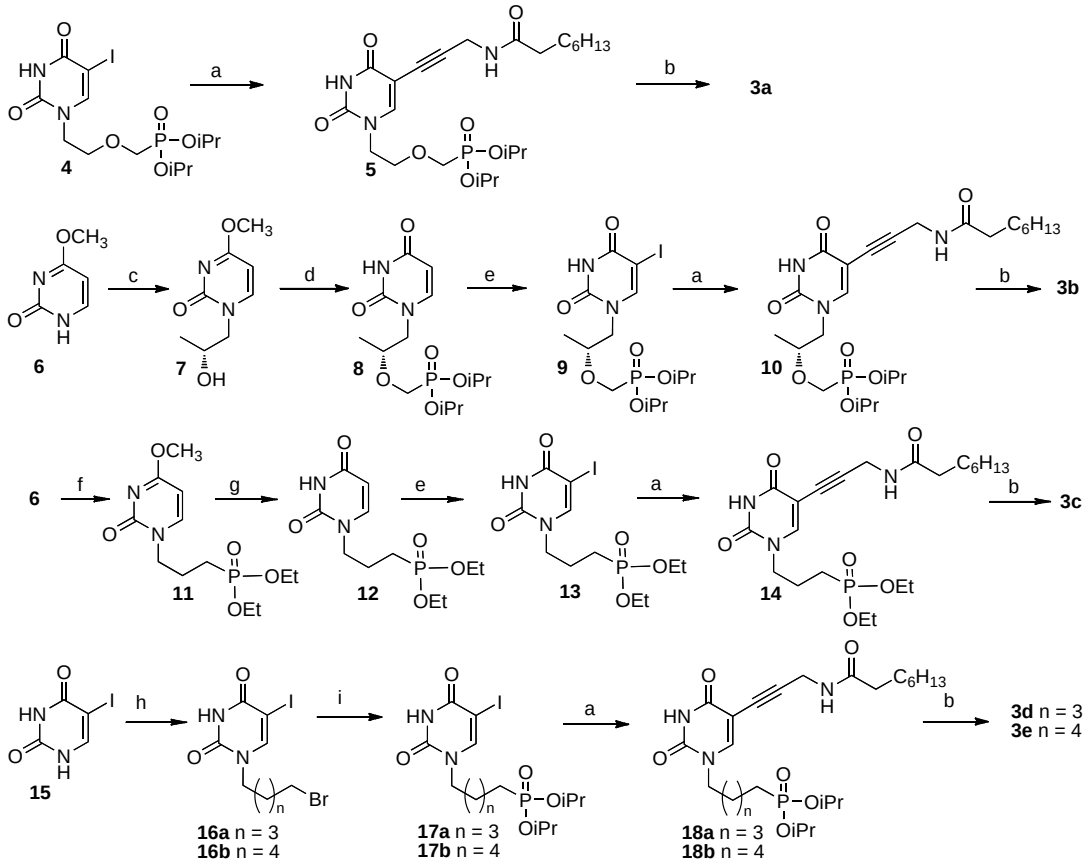



**Figure 5.** Results of 1H-15N HISQC NMR experiments of K165A ThyX: a) cross-peak of lysine NH3 from the purification tag b) cross-peak of lysine NH3 from the purification tag after addition of 50 μg of the inhibitor

## Chemistry

Based on molecular modelling and NMR results we concluded that the 'alkynyl tail' in compound **1** is important for its inhibitory activity. The 5' phosphate in this compound is required



**Scheme 1.** Reagents and conditions: a) alkyne, $Pd(PPh_3)_4$, CuI, DMF/$Et_3N$ 10:1, 2h 50°C; b) $BrSiMe_3$, $CH_3CN$, $Et_3N$, rt overnight; c) $Cs_2CO_3$, DMF, 20 min 120°C; R-propylene carbonate, 5h 120°C; d) $CF_3SO_3CH_2PO(OiPr)_2$, NaH, $CH_3CN$ 10 min 0°C, 30 min rt; e) $I_2$, CAN, $CH_3CN$, 80°C; f) NaH, DMF, 1h rt; $BrCH_2CH_2CH_2PO(OEt)_2$, 3h 100°C; g) Dowex50(H+), 90% aq MeOH, 3h reflux; h) *N,O*-Bis(trimethylsilyl)acetamide, DMF, 2h rt; 1,5-dibromopentane or 1,6-dibromohexane, TBAI, 130°C overnight; i) $P(OiPr)_3$, 4h 140°C

for its activity but prone to be removed by esterases. In analogy with successful development of antiviral nucleoside analogues[24, 32], we synthesized a series of acyclic nucleoside phosphonates (ANPs) that contain 5-alkynyl uracyl (Figure 6). Important advantages of ANPs are their catabolic stability and isopolarity with phosphate. The flexibility of the acyclic chain could improve target binding since its ability to adopt different conformations helps to find a suitable one for binding the active site of the enzyme.

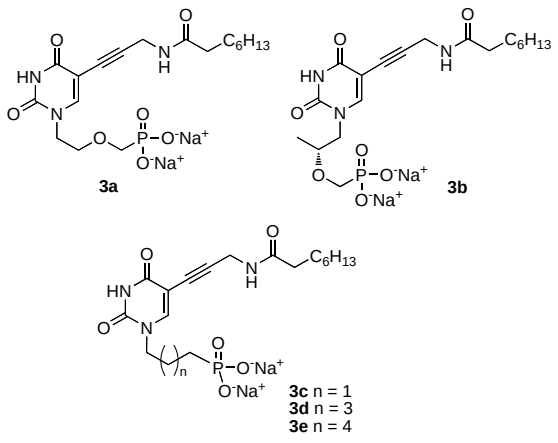


**Figure 6.** Structures of synthesized compounds

Compound **3a** was synthesized starting from compound **4** that was obtained as previously described (Scheme 1).[33-36] Sonogashira coupling of compound **4** with N-(Prop-2-ynyl)octanamide[21] was used for the introduction of the alkyne tail.[36] The sodium salt of phosphonic acid **3a** was prepared by treatment of the isopropyl diester with bromo(trimethyl)silane in acetonitrile.[37] For the synthesis of compound **3b** we used a commercially available 2,4-dimethoxypyrimidine that was transformed into 4-methoxypyrimidin-2(1*H*)-one **6** like previously described.[33] Then we carried out N-1 alkylation of this protected uracyl with R-propylene carbonate in DMF using $Cs_2CO_3$ as a base giving compound **7**.[37] The phosphonate moiety was introduced using a $C_1$-synthon ($CF_3SO_3CH_2PO(OiPr)_2$) with NaH as a base in acetonitrile resulting in compound **8**.[37] The latter one was iodinated at 5-position with $I_2$ and cerium(IV) ammonium nitrate (CAN) in acetonitrile giving compound **9**.[36] Following steps, Sonogashira coupling and deprotection of phosphodiesters, were done as described above.

For the synthesis of **3c**, 4-methoxypyrimidin-2(1*H*)-one **6** was alkylated with $BrCH_2CH_2CH_2PO(OEt)_2$ in DMF using NaH as a base, followed by deprotection of uracyl in 90% aqueous methanol using Dowex 50 ($H^+$ form),[35] resulting in compound **12**. The further steps were the same as for compound **3b**. 5-iodouracyl **15** was used as a starting product for the synthesis of **3d** and **3e**. Selective alkylation at N-1 position with 1,5-dibromopentane (**3d**) or 1,6-dibromohexane (**3e**) was performed

after in-situ silylation with *N*,*O*-Bis(trimethylsilyl)acetamide using TBAI as a phase-transfer catalyst.[38-41] The resulting compounds **16a** and **16b** were transformed into corresponding phosphonates by the Arbuzov reaction using $P(OiPr)_3$,[42] followed by Sonogashira coupling and deprotection with bromo(trimethyl)silane.

**Biological evaluation**

The synthesized compounds were tested for their activity against ThyX using a $^3H$ release assay that was optimized based on data found in the literature.[11, 21, 27, 43, 44] The total reaction mixture contained 20 μM mTHF, 1 μM ThyX, 41 μM FAD, 200 μM NADPH, 1% glycerol, 1 mM $MgCl_2$, 50 mM HEPES (pH = 7.5), 50 μM of tested inhibitor and 0.8 μM of [5-$^3$H] dUMP (25.5 Ci/mmol). 5F-dUMP was used as a reference compound. All of the tested compounds exhibit $IC_{50}$ value of more than 50 μM (0-3% inhibition at 50 μM), however compound **3e** showed 43% of inhibitory effect on ThyX at 50μM. A linker of 6 $CH_2$ groups connects nucleobase and phosphonate in **3e**, while other analogues have a shorter linker (3 atoms in **3c**, 4 atoms in **3a** and **3b**; and 5 atoms in **3d**). In a next stage the linker of **3e** can be optimized by a nitrogen or oxygen replacing one or more $CH_2$ and/or adding 'side chains' or functional groups to the linker.

## Conclusion

A molecular model was generated to explore the binding of compound **1** with ThyX. It was shown that the 5-alkynyl 'tail' of this compound is accommodated in a binding pocket that is formed by an induced fit mechanism. Interactions of the ligand occur with highly conserved amino acids of ThyX. Especially hydrogen bonding of the amide N in the inhibitor 'tail' with Ser105 is interesting since this is a highly conserved amino acid that was originally thought to initiate the reaction catalyzed by ThyX.[10] In our model, the carbonyl oxygen of this amide is within hydrogen bonding distance to amino group of Lys165.A. A $^1H$-$^{15}N$ 2D HISQC experiment was performed to monitor Lys side chain amino signals upon addition of the inhibitor. NMR results show changes in Lys165-$NH_3$ that are in agreement with predicted binding of the ligand in a 'slow exchange regime' on the NMR time scale, indicative for binding with high affinity and low dissociation constant.

Based on our findings, four new compounds were synthesized and tested for their activity against *M. tuberculosis* ThyX. Synthesized compounds are 5-alkynyl uridine analogues where the sugar moiety was replaced by acyclic phosphonates. One of the tested compounds (**3e**) exhibits 43% of inhibitory effect on ThyX at 50μM. The obtained results demonstrate that ANP can inhibit ThyX, though it is clear that further development is needed to improve the biological activity. We aim to determine the structure of ThyX in complex with **3e** to guide further modification of the latter that could generate more potent compounds.

## Experimental Section

**Modeling.** A static model was created using the pdb structure 2AF6 of *M. tuberculosis* ThyX.[25] The through base atom C5 connected tail of structure of compound **1** was sketched and created using chemdraw/chem3D. Using the quatfit program (available in the CCL archives, http://www.ccl.net/) this tail was then connected to atom C5 of the substrate analogue 5-bromo-2'-4 deoxyuridine-5'-monophosphate, present in the X-ray structure (the Br atom was removed). The ThyX enzyme has 4 subunits A, B, C and D. There are 4 active sites in ThyX and each site is located at the interface of 3 subunits.[3, 44] The inhibitor was then entered in one of those 4 active site pockets, the one at the interface of unit A, C and D (putting the sugar, base at the same position as the original inhibitor). The tail conformation had to be adjusted to reduce steric clashes using Chimera.[45] The structure was then energy minimized using the AMBER (version 10.0) program.[46] During minimization, some extra valence angle restraints at the neighbor atoms of the triple bond had to be activated to help the inhibitor keep the correct conformation. The interactions of the inhibitor with the surrounding protein matrix were then calculated by Ligplot[47] and highlighted using Chimera.[45]

**NMR experiments.** 2D $^1H$-$^{15}N$ HISQC spectra were recorded with a Bruker Advance 600 ($^1H$ NMR, 600 MHz; $^{13}C$ NMR, 150 MHz; $^{15}N$ NMR 60.8 MHz). The spectra were recorded at 5°C, pH 5. The NMR sample contained 0.47 mM ThyX, 50 mM TRIS, 10% D2O and increasing amount of compound **2** 0 μg, 30 μg, 50 μg, 80 μg.

**Making of the plasmid containing mutant K165A ThyX insert.** ThyX gene with a mutation in 165 position (AAG codon replaced by GCG codon) was synthesized by commercial supplier Integrated DNA Technologies. After the PCR amplification of the gene, the dA overhangs were created using Dream Taq Polymerase. The gene fragment was then cloned in a pEXP5-CT/TOPO® vector following the manufacturer's instructions and sequence was verified by standard sanger sequencing. The pEXP5-CT/TOPO® vector encodes a 6His-tag, connected to the C-terminus of the protein with two extra codons (for lysine and glycine) separating gene and the His tag (KGHHHHHH). *BL21* (*DE3*) *pLysS E. coli* RbCl chemical competent cells were transformed with the pEXP5-CT/TOPO® vector containing the ThyX K165A insert, grown during 1h at 37°C and 250 rpm in 900 μl LB-medium, then plated on the LB-agar plates and grown overnight at 37°C. Afterwards a colony was picked up into an overnight culture in LB-medium. A 25% glycerol stock was made from the overnight culture and stored at -80°C.

**Expression and purification of $^{15}N$ labeled ThyX and $^{15}N$ mutant K165A ThyX.** An overnight culture (50 μl glycerol stock in 20 ml $^{15}N$ enriched minimal medium) was prepared and the next day added into 1L $^{15}N$ enriched minimal medium. The bacteria were grown at 37°C and 250 rpm till $OD_{600}$= 0.6-0.8 then induced with 1 ml 1M IPTG solution. Afterwards the culture was let to grow overnight at 22°C and 250 rpm. In the morning bacteria were spun down in a precooled centrifuge (4 °C) for 10 minutes at 10000 rpm. The bacterial pellet was resuspended in 10 ml lysis buffer containing 5 mg lysozyme, 1mM DTT, one protease inhibitor cocktail tablet and incubated on ice for 30 minutes. After 5 times French Press, lysate was spun down in a precooled centrifuge (4°C) for 20 minutes at 10000 rpm. ThyX (or K165A ThyX) protein was purified from supernatants using His6-based affinity chromatography (NTA magnetic beads, Qiagen) according to the manufacturer's instructions.

**Chemistry.** Analytical grade solvents were used for the reactions. Dry acetonitrile was obtained by distillation over $CaH_2$. Dry DMF was purchased from commercial suppliers. NMR spectra ($^1H$, $^{13}C$, $^{31}P$) were recorded with a Bruker Advance 300 ($^1H$ NMR, 300mHz; $^{13}C$ NMR, 75 MHz, $^{31}P$ NMR 121.5 MHz), Bruker Advance 500 ($^1H$ NMR, 500mHz; $^{13}C$ NMR, 125 MHz), Bruker Advance 600 ($^1H$ NMR, 600mHz; $^{13}C$ NMR, 150 MHz). Tetramethylsilane was used as internal standard for $^1H$ NMR spectra while DMSO-d6 (39.52 ppm) and $CDCl_3$ (77.16 ppm) were used for $^{13}C$ NMR spectra. Following abbreviations were used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br s = broad signal. Chemical shifts are expressed in parts per million (ppm). Mass spectra were acquired on a quadrupole orthogonal acceleration time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, Milford, MA). Samples were infused at 3uL/min and spectra were obtained in positive (or: negative) ionization mode with a resolution of 15000 (FWHM) using leucine enkephalin as lock mass.

Fluka Silica gel/ TLC plates were used for TLC. ICN silica gel 63-200, 60Å was used for column chromatography.

**(*R*)-1-(2-hydroxypropyl)-4-methoxypyrimidin-2(1*H*)-one (7).** 4-methoxypyrimidin-2(1*H*)-one **6** (883 mg, 7 mmol), $Cs_2CO_3$ (137 mg, 0.42 mmol), dry DMF (30ml) were stirred under an argon atmosphere for 20 minutes at 120°C. After the addition of R-propylene carbonate (726.7 μl, 8.40 mmol), the reaction mixture was stirred for 5 h at 120°C. The solvent was evaporated in vacuo and two times coevaporated with toluene. The residue was extracted two times with boiling methanol and the filtrate was filtered through a celite path and evaporated in vacuo. The residue was purified using silica gel column chromatography ($CH_2Cl_2$/Acetone/MeOH 84:15:1) giving the compound **6** in 45%yield (590 mg). $^1$H NMR (300 MHz, $CDCl_3$) δ: 7.49 (d, 1 H, J = 7.1 Hz, H-6), 5.86 (d, 1H, J = 7.1 Hz, H-5), 4.19 (m, 1H, CH'), 3.93 (s, 3H, $OCH_3$), 3.45 (m, 2H, $CH_2$'), 3.0 (br s, 1H, OH), 1.25 (d, 3H, J = 6.3 Hz, $CH_3$'). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 172.1 (C-4), 157.5 (C-2), 148.4 (C-6), 95.4 (C-5), 66.3 (CH'), 57.6 ($CH_2$'), 54.5 ($OCH_3$), 21.2 ($CH_3$'). HRMS (ESI) for $C_8H_{12}N_2O_3$: calcd 185.0921 $[M + H]^+$, found 185.0926.

**(*R*)-diisopropyl(((1-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)propan-2-yl)oxy)methyl)phosphonate (8).** Compound **7** (287 mg, 1.56 mmol), $CF_3SO_3CH_2PO(OiPr)_2$ (511 mg, 1.56 mmol), 60% NaH (38 mg, 1.56 mmol), dry acetonitrile (6 ml) were stirred under an argon atmosphere for 10 min at 0°C, and 30 minutes at room temperature. Completion of the reaction was controlled by TLC analysis. After the addition of 1ml acetic acid, reaction mixture was evaporated in vacuo. The residue was dissolved in ethyl acetate and the organic layer was extracted with 1M HCl, saturated $NaHCO_3$, washed with brine, dried over $Na_2SO_4$, and evaporated in vacuo. The residue was purified using silica gel column chromatography (EtOAc/acetone 90:10) giving the compound **8** in 40% yield (219 mg). $^1$H NMR (300 MHz, DMSO) δ: 11.23 (s, 1H, NH), 7.49 (d, 1H, J = 7.8 Hz, H-6), 5.50 (d, 1H, J = 7.8, H-5), 4.55 (m, 2H, POCH), 3.7 (m, 5H, $CH_2$', CH', $CH_2$P), 1.22 (m, 12H, $CH_3$), 1.09 (d, 3H, J = 6.0 Hz, $CH_3$'). $^{13}$C NMR (75 MHz, DMSO) δ: 163.7 (C-4), 151.0 (C-2), 146.4 (C-6), 100.3 (C-5), 75.0 (d, 1C, $J_{C,P}$ = 11.9 Hz, CH'), 70.0 (d, 2C, $J_{C,P}$ = 6.1 Hz, POCH), 62.4 (d, 1C, $J_{C,P}$ = 165.4 Hz, $CH_2$P), 51.6 ($CH_2$'), 23.7 (m, 4C, $CH_3$), 16.3 ($CH_3$'). HRMS (ESI) for $C_{14}H_{25}N_2O_6P$: calcd 349.1523 $[M + H]^+$, found 349.1524.

**General procedure Iodination at C5 position.** Starting compound (1 mmol), CAN (0.5 mmol), $I_2$ (1.3 mmol) were dissolved in dry acetonitrile (10 ml) and refluxed for 3 hours under an argon atmosphere. Completion of the reaction was controlled by TLC analysis ($CH_2Cl_2$/MeOH 92.5:7.5). The reaction mixture was evaporated in vacuo and dissolved in EtOAc. The organic layer was washed with an ice-cold 5% $Na_2S_2O_3$ and afterwards with brine, dried over $Na_2SO_4$, and evaporated in vacuo. The residue was purified using silica gel column chromatography ($CH_2Cl_2$/MeOH 95:5).

**Diisopropyl ((2-(5-iodo-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)ethoxy)methyl)phosphonate (4).** This compound was obtained according to the general procedure described above in 95% yield (2.7 g). $^1$H NMR (300 MHz, DMSO) δ: 11.65 (s, 1H, NH), 8.06 (s, 1H, H-6), 4.57 (m, 2H, POCH), 3.87 (t, 2H, J = 4.7 Hz, $CH_2$-1'), 3.77 (d, 2H, J = 8.3 Hz, $CH_2$P), 3.69 (t, 2H, J = 4.7 Hz, $CH_2$-2'), 1.24 (s, 3H, $CH_3$), 1.22 (s, 6H, $CH_3$), 1.20 (s, 3H, $CH_3$). $^{13}$C NMR (75 MHz, DMSO) δ: 161.1 (C-4), 150.7 (C-2), 150.5 (C-6), 70.3 (d, 2C, $J_{C,P}$ = 6.3 Hz, POCH), 69.8 (d, 1C, $J_{C,P}$ = 11.4 Hz, $CH_2$-2'), 67.7 (C-5), 64.8 (d, 1C, $J_{C,P}$ = 163.1 Hz, $CH_2$P), 47.4 ($CH_2$-1'), 23.9 ($CH_3$), 23.85 ($CH_3$), 23.8 ($CH_3$), 23.75 ($CH_3$). HRMS (ESI) for $C_{13}H_{22}N_2O_6PI$: calcd 461.03347 $[M + H]^+$, found 461.0335.

**(*R*)-diisopropyl (((1-(5-iodo-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)propan-2-yl)oxy)methyl)phosphonate (9).** This compound was obtained according to the general procedure described above in 61% yield (176 mg). $^1$H NMR (300 MHz, $CDCl_3$) δ: 9.28 (br s, 1H, NH), 7.79 (s, 1H, H-6), 4.73 (m, 2H, POCH), 4.02 (d, 1H, J = 14.2 Hz, $CH_2$P), 3.9-3.45 (m, 5H), 1.31 (m, 12H, $CH_3$), 1.80 (d, 3H, J = 6.3 Hz, $CH_3$-3'). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 160.6 (C-4), 150.9 (C-2), 150.6 (C-6), 76.0 (d, 1C, $J_{C,P}$ = 11.5 Hz, CH-2'), 71.3 (t, 2C, $J_{C,P}$ = 5.5 Hz, POCH), 67.3 (C-5), 63.7 (d, 1C, $J_{C,P}$ = 169.7 Hz, $CH_2$P), 53.4 ($CH_2$-1'), 24.2 (4C, $CH_3$), 16.3 ($CH_3$-3'). HRMS (ESI) for $C_{14}H_{24}N_2O_6PI$: calcd 497.03109 $[M + Na]^+$, found 497.0311.

**Diethyl (3-(5-iodo-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)propyl)phosphonate (13).** This compound was obtained starting from compound **12** according to the general procedure described above in 38% yield (176 mg). $^1$H NMR (300 MHz, $CDCl_3$) δ: 10.22 (br s, 1H, NH-3), 7.78 (s, 1H, H-6), 4.1 (m, 4H, $POCH_2$), 3.84 (t, 2H, J = 7.1 Hz, $CH_2$-1'), 2.0 (m, 2H, $CH_2$), 1.75 (m, 2H, $CH_2$), 1.28 (m, 6H, $CH_3$). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 160.9 (C-4), 150.8 (C-2), 149.2 (C-6), 68.1 (C-5), 62.1 (d, 2C, $J_{C,P}$ = 6.7 Hz, $POCH_2$), 48.8 (d, 1C, $J_{C,P}$ = 14.9 Hz, $CH_2$-1'), 22.8 (d, 1C, $J_{C,P}$ = 71 Hz, $CH_2$-P), 21.8 (d, 1C, $J_{C,P}$ = 67.2 Hz, $CH_2$-2'), 16.6 ($CH_3$), 16.5 ($CH_3$). HRMS (ESI) for $C_{11}H_{18}N_2O_5PI$: calcd 438.98923 $[M + Na]^+$, found 438.9894.

**1-(5-bromopentyl)-5-iodopyrimidine-2,4(1*H*,3*H*)-dione (16a).** A mixture of 5-iodouracil (2.38 g, 10 mmol), BSA (5.4 ml, 22 mmol) in dry DMF (10 ml), was stirred under an argon atmosphere for 2 hours at room temperature. After the addition of 1,5-dibromopentane (6.90 g, 30 mmol) and TBAI (5 mg, 0.01 mmol), the reaction mixture was stirred overnight at 130°C. Completion of the reaction was controlled by TLC analysis ($CH_2Cl_2$/MeOH 95:5). The reaction was stopped by addition of 30 ml water and evaporated in vacuo. The residue was dissolved in $CH_2Cl_2$ and the organic layer was extracted with a saturated solution of $NaHCO_3$, washed with brine, dried over $Na_2SO_4$, and evaporated in vacuo. The residue was purified using silica gel column chromatography ($CH_2Cl_2$/MeOH 98:2) giving the desired compound in 60% yield (2.31 g). $^1$H NMR (300 MHz, $CDCl_3$) δ: 9.54 (br s, 1H, NH-3), 7.62 (s, 1H, H-6), 3.75 (t, 2H, J = 7.3 Hz, $CH_2$-1'), 3.40 (t, 2H, J = 6.6 Hz, $CH_2$-5'), 1.89 (m, 2H, $CH_2$), 1.71 (m, 2H, $CH_2$), 1.51 (m, 2H, $CH_2$). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 160.7 (C-4), 150.7 (C-2), 148.9 (C-6), 67.9 (C-5), 49.0 ($CH_2$-1'), 33.3 ($CH_2$), 32.0 ($CH_2$), 28.4 ($CH_2$), 24.9 ($CH_2$). HRMS (ESI) for $C_9H_{12}N_2O_2IBr$: calcd 384.90564 $[M - H]^-$, found 384.9053.

**1-(6-bromohexyl)-5-iodopyrimidine-2,4(1*H*,3*H*)-dione (16b).** A mixture of 5-iodouracil (2.38 g, 10 mmol), BSA (5.4 ml, 22 mmol) in dry DMF (10 ml), was stirred under an argon atmosphere for 2 hours at room temperature. After the addition of 1,6-dibromohexane (7.32 g, 30 mmol) and TBAI (5 mg, 0.01 mmol), the reaction mixture was stirred overnight at 130°C. Completion of the reaction was controlled by TLC analysis ($CH_2Cl_2$/MeOH 95:5). The reaction was stopped by addition of 30 ml water and evaporated in vacuo. The residue was dissolved in $CH_2Cl_2$ and the organic layer was extracted with a saturated solution of $NaHCO_3$, washed with brine, dried over $Na_2SO_4$, and evaporated in vacuo. The residue was purified using silica gel column chromatography ($CH_2Cl_2$/MeOH 98:2) giving the desired compound in 14% yield (552 mg). $^1$H NMR (300 MHz, $CDCl_3$) δ: 8.94 (br s, 1H, NH-3), 7.60 (s, 1H, H-6), 3.74 (t, 2H, J = 7.4 Hz, $CH_2$-1'), 3.41 (t, 2H, J = 6.7 Hz, $CH_2$-6'), 1.87 (m, 2H, $CH_2$-5'), 1.71 (m, 2H, $CH_2$-2'), 1.51 (m, 2H, $CH_2$-4'), 1.36 (m, 2H, $CH_2$-3'). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 159.5 (C-4), 149.6 (C-2), 147.9 (C-6), 66.8 (C-5), 48.2 ($CH_2$-1'), 32.7 ($CH_2$-6'), 31.5 ($CH_2$-5'), 28.2 ($CH_2$-2'), 26.7 ($CH_2$-4'), 24.7 ($CH_2$-3'). HRMS (ESI) for $C_{10}H_{14}N_2O_2IBr$: calcd 400.93584 $[M + H]^+$, found 400.9352.

**Diisopropyl (5-(5-iodo-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)pentyl)phosphonate (17a).** A mixture of **16a** (500 mg, 1.29 mmol) and triisopropyl phosphite (14 ml, 40 mmol) was stirred under an argon atmosphere for 4 hours at 140°C. Then it was evaporated in vacuo. The residue was purified using silica gel column chromatography ($CH_2Cl_2$/MeOH 98:2 -> 95:5) giving the desired compound in 50% yield (307 mg). $^1$H NMR (300 MHz, $CDCl_3$) δ: 9.33 (br s, 1H, NH-3), 7.61 (s, 1H, H-6), 4.69 (m, 2H, POCH), 3.72 (t, 2H, J = 7.3, $CH_2$-1'), 1.65 (m, 4H, 2x$CH_2$), 1.40 (m, 2H, $CH_2$), 1.29 (m, 14H, 1x$CH_2$, 4x$CH_3$). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 160.6 (C-4), 150.6 (C-2), 148.9 (C-6), 70.0 (d, 2C, $J_{C,P}$ = 6.7 Hz, POCH), 67.9 (C-5), 49.1 ($CH_2$-1'), 28.9 ($CH_2$), 27.3 (d, 1C, $J_{C,P}$ = 16.6 Hz, $CH_2$), 26.8 (d, 1C,

$J_{C,P}$ = 142.5 Hz, $CH_2$), 24.2 ($CH_3$), 24.1(2C, $CH_3$), 24.1 ($CH_3$), 22.3 (d, 1C, $J_{C,P}$ = 5.2 Hz, $CH_2$). HRMS (ESI) for $C_{15}H_{26}N_2O_5PI$: calcd 473.06985 $[M + H]^+$, found 473.0696.

**Diisopropyl (6-(5-iodo-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)hexyl)phosphonate (17b).** A mixture of **16b** (401 mg, 1 mmol) and triisopropyl phosphite (10 ml, 40 mmol) was stirred under an argon atmosphere for 4 hours at 140°C. Then it was evaporated in vacuo. The residue was purified using silica gel column chromatography ($CH_2Cl_2$/MeOH 98:2 -> 95:5) giving the desired compound in 90% yield (437 mg). $^1$H NMR (300 MHz, $CDCl_3$) δ: 9.88 (br s, 1H, NH-3), 7.61 (s, 1H, H-6), 4.7 (m, 2H, POCH), 3.69 (t, 2H, J = 7.4, $CH_2$-1'), 1.65 (m, 6H, 3x$CH_2$), 1.25 (m, 16H, 2x$CH_2$, 4x$CH_3$). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 160.8 (C-4), 150.7 (C-2), 148.9 (C-6), 70.0 (d, 2C, $J_{C,P}$ = 6.7 Hz, POCH), 67.9 (C-5), 49.1 ($CH_2$-1'), 30.0 (d, 1C, $J_{C,P}$ = 16.7 Hz, $CH_2$), 29.0 ($CH_2$), 27.8 ($CH_2$), 26.0 ($CH_2$), 24.2 ($CH_3$), 24.1(2C, $CH_3$), 24.1 ($CH_3$), 22.5 (d, 1C, $J_{C,P}$ = 5.2 Hz, $CH_2$). HRMS (ESI) for $C_{16}H_{28}N_2O_5PI$: calcd 487.08550 $[M + H]^+$, found 487.0855.

**General procedure Sonogashira.** A mixture of a 5-iodouracil derivative (1 mmol), $CHCCH_2NH$-CO-$C_7H_{15}$ (1.3 mmol), $Pd(PPh_3)_4$ (0.05 mmol), CuI (0.3 mmol) in 5 ml DMF/$Et_3N$ (10/1) was stirred under an argon atmosphere for 2 hours at 50°C. Completion of the reaction was controlled by TLC analysis ($CH_2Cl_2$/MeOH 95:5). The solvent was evaporated in vacuo and two times coevaporated with toluene. The residue was extracted two times with boiling methanol and the filtrate was filtered through a celite path and evaporated in vacuo. The residue was purified using silica gel column chromatography ($CH_2Cl_2$/MeOH 97:3 ->95:5).

**Diisopropyl ((2-(5-(3-octanamidoprop-1-yn-1-yl)-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)ethoxy)methyl)phosphonate (5).** This compound was obtained according to the general procedure described above in 71% yield (74 mg). $^1$H NMR (300 MHz, $CDCl_3$) δ: 10.47 (s, 1H, NH-3), 7.58 (s, 1H, H-6), 6.81 (br s, 1H, NH), 4.69 (m, 2H, POCH), 4.15 (m, 2H, $CH_2$-NH), 3.93 (m, 2H, $CH_2$-1'), 3.76 (m, 4H, $CH_2O$, $CH_2P$), 2.19 (t, 2H, J = 7.2 Hz, CO-$CH_2$), 1.59 (m, 2H, CO-$CH_2$-**$CH_2$**), 1.27 (m, 20H, 4x$CH_3$, 4x$CH_2$), 0.83 (br s, 3H, $CH_3$). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 173.2 (NH-CO), 162.9 (C-4), 150.1 (C-2), 149.3 (C-6), 98.5 (C-5), 89.7, 74.3, 71.4 (d, 2C, $J_{C,P}$ = 6.7 Hz, POCH), 70.8 (d, 1C, $J_{C,P}$ = 11.6 Hz, $CH_2$-2'), 66.1 (d, 1C, $J_{C,P}$ = 167.6 Hz, $CH_2P$), 48.6 ($CH_2$-1'), 36.4, 31.7, 30.1, 29.4, 29.1, 25.7, 24.1 (4C, $CH_3$), 22.6, 14.1 ($CH_3$). HRMS (ESI) for $C_{24}H_{40}N_3O_7P$: calcd 536.24962 $[M + Na]^+$, found 536.2491.

**(R)-diisopropyl (((1-(5-(3-octanamidoprop-1-yn-1-yl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)propan-2-yl)oxy)methyl) phosphonate (10).** This compound was obtained according to the general procedure described above in 75% yield (148 mg). $^1$H NMR (300 MHz, $CDCl_3$) δ: 10.28 (br s, 1H, NH-3), 7.61 (s, 1H, H-6), 6.79 (t, 1H, J = 4.6 Hz, NH), 4.68 (sep, 2H, J = 6.6 Hz, POCH), 4.13 (s, 2H, $CH_2$NH), 4.04 (d, 1H, J = 14.1 Hz, $CH_2P$), 3.85-3.45 (m, 5H), 2.18 (t, 2H, J = 7.4 Hz, CO-$CH_2$), 1.59 (m, 2H, CO-$CH_2$-**$CH_2$**), 1.35-1.2 (m, 20H, 4x$CH_3$, 4x$CH_2$), 1.17 (d, 3H, J = 6.2 Hz, $CH_3$-3'), 0.82 (t, 3H, J = 6.5 Hz, $CH_3$). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 173.2 (NH-CO), 162.8 (C-4), 150.3 (C-2), 149.6 (C-6), 98.4 (C-5), 89.6, 76.1 (d, 1C, $J_{C,P}$ = 12.5 Hz, CH-2'), 74.6, 71.4 (d, 1C, $J_{C,P}$ = 6.7 Hz, POCH), 71.2 (d, 1C, $J_{C,P}$ = 6.7 Hz, POCH), 63.5 (d, 1C, $J_{C,P}$ = 170.1 Hz, $CH_2P$), 53.0 ($CH_2$-1'), 36.3, 31.7, 30.2, 29.4, 29.1, 25.7, 24.1 ($CH_3$), 24.0 (2C, 2x$CH_3$), 23.9 ($CH_3$), 22.6, 16.3 ($CH_3$-3'), 14.1 ($CH_3$). HRMS (ESI) for $C_{25}H_{42}N_3O_7P$: calcd 528.28329 $[M + H]^+$, found 528.2833.

**Diethyl (3-(5-(3-octanamidoprop-1-yn-1-yl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)propyl)phosphonate (14).** This compound was obtained according to the general procedure described above in 81% yield (240 mg). $^1$H NMR (300 MHz, $CDCl_3$) δ: 10.29 (br s, 1H, NH-3), 7.59 (s, 1H, H-6), 6.98 (m, 1H, NH), 4.15 (d, 2H, J = 5.1 Hz, $CH_2$NH), 4.5 (m, 4H, $POCH_2$), 3.81 (t, 2H, J = 6.8 Hz, $CH_2$-1'), 2.18 (t, 2H, J = 7.4 Hz, CO-$CH_2$), 1.96 (m, 2H, $CH_2P$), 1.78 (m, 2H, $CH_2$-2'), 1.57 (m, 2H, CO-$CH_2$-**$CH_2$**), 1.3-1.18 (m, 14H, 2x$POCH_2$-**$CH_3$**, 4x$CH_2$), 0.80 (t, 3H, J = 6.5 Hz, $CH_3$). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 173.4 (NH-CO), 162.7 (C-4), 150.0 (C-2), 148.2 (C-6), 99.1 (C-5), 90.1, 73.8, 62.0 (d, 2C, $J_{C,P}$ = 6.6 Hz, $POCH_2$), 49.1 (d, 1C, $J_{C,P}$ = 16.2 Hz, $CH_2$-1'), 36.3 (CO-$CH_2$), 31.7 ($CH_2$), 29.8 ($CH_2$), 29.3 ($CH_2$), 29.0 ($CH_2$), 25.6 ($CH_2$), 22.7 (d, 1C, $J_{C,P}$ = 73.8 Hz, $CH_2P$), 22.6 ($CH_2$), 21.7 (d, 1C, $J_{C,P}$ = 63.9 Hz, $CH_2$-2'), 16.4 (d, 2C, $J_{C,P}$ = 6.0 Hz, $CH_3$), 14.0 ($CH_3$). HRMS (ESI) for $C_{22}H_{36}N_3O_6P$: calcd 470.24143 $[M + H]^+$, found 470.2417.

**Diisopropyl (5-(5-(3-octanamidoprop-1-yn-1-yl)-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)pentyl)phosphonate (18a).** This compound was obtained according to the general procedure described above in 79% yield (110 mg). $^1$H NMR (300 MHz, $CDCl_3$) δ: 9.67 (br s, 1H, NH-3), 7.47 (s, 1H, H-6), 6.36 (t, 1H, J = 5 Hz, NH), 4.67 (m, 2H, POCH), 4.20 (d, 2H, J = 5.2 Hz $CH_2$-NH), 3.71 (t, 2H, J = 7.2, $CH_2$-1'), 2.19 (t, 2H, J = 7.4 Hz, CO-$CH_2$), 1.65 (m, 8H, 4x$CH_2$), 1.42 (m, 4H, 2x$CH_2$), 1.25 (m, 18H, 3x$CH_2$, 4x$CH_3$), 0.84 (t, 3H, J = 6.6 Hz, $CH_3$). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 173.2 (NH-CO), 162.4 (C-4), 149.9 (C-2), 147.7 (C-6), 99.2 (C-5), 90.2, 73.9, 70.0 (d, 2C, $J_{C,P}$ = 6.7 Hz, POCH), 49.2 ($CH_2$-1'), 36.5 (CO-**$CH_2$**), 31.8 ($CH_2$), 30.0 ($CH_2$), 29.3 ($CH_2$), 29.1 ($CH_2$), 28.7 ($CH_2$), 27.30 (d, 1C, $J_{C,P}$ = 16.5 Hz, $CH_2$), 26.79 (d, 1C, $J_{C,P}$ = 142.5 Hz, $CH_2$), 25.7 ($CH_2$), 24.2 ($CH_3$), 24.2 (2C, $CH_3$), 24.1 ($CH_3$), 22.7 ($CH_2$), 22.3 (d, 1C, $J_{C,P}$ = 5.1 Hz, $CH_2$), 14.1 ($CH_3$). HRMS (ESI) for $C_{26}H_{44}N_3O_6P$: calcd 526.30402 $[M + H]^+$, found 526.3038.

**Diisopropyl (6-(5-(3-octanamidoprop-1-yn-1-yl)-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)hexyl)phosphonate (18b).** This compound was obtained according to the general procedure described above in 36% yield (39 mg). $^1$H NMR (300 MHz, $CDCl_3$) δ: 9.27 (br s, 1H, NH-3), 7.45 (s, 1H, H-6), 6.21 (br s, 1H, NH), 4.68 (m, 2H, POCH), 4.23 (d, 2H, J = 5.1 Hz $CH_2$-NH), 3.72 (t, 2H, J = 7.2, $CH_2$-1'), 2.19 (t, 2H, J = 7.4 Hz, CO-$CH_2$), 1.65 (m, 8H, 4x$CH_2$), 1.4 (m, 4H, 2x$CH_2$), 1.26 (m, 20H, 4x$CH_2$, 4x$CH_3$), 0.85 (t, 3H, J = 6.5 Hz, $CH_3$). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 173.1 (NH-CO), 162.3 (C-4), 149.8 (C-2), 147.6 (C-6), 99.3 (C-5), 90.3, 73.9, 70.0 (d, 2C, $J_{C,P}$ = 6.7 Hz, POCH), 49.3 ($CH_2$-1'), 36.6 (CO-**$CH_2$**), 31.8 ($CH_2$), 30.1 (d, 1C, $J_{C,P}$ = 16.6 Hz, $CH_2$), 30.0 ($CH_2$), 29.9 ($CH_2$), 29.4 ($CH_2$), 29.1 ($CH_2$), 28.9 ($CH_2$), 27.9 ($CH_2$), 26.0 ($CH_2$), 25.7 ($CH_2$), 24.3 ($CH_3$), 24.2 (2C, $CH_3$), 24.1 ($CH_3$), 22.7 ($CH_2$), 22.5 (d, 1C, $J_{C,P}$ = 5.2 Hz, $CH_2$), 14.2 ($CH_3$). HRMS (ESI) for $C_{27}H_{46}N_3O_6P$: calcd 538.30512 $[M - H]^-$, found 538.3051.

**General procedure. Deprotection.** A mixture of a protected phosphonate (1 mmol), $BrSiMe_3$ (4 mmol), $Et_3N$ (10 mmol) in 3 ml anhydrous acetonitrile was stirred overnight at room temperature under an argon atmosphere. Completion of the reaction was controlled by TLC analysis ($CH_2Cl_2$/MeOH 95:5). The solvent was evaporated in vacuo and two times coevaporated with acetonitrile. The residue was purified using silica gel column chromatography ($CH_2Cl_2$/MeOH/1M TEAB 50:25:3). The resulting compound was dissolved in water and applied to an ion-exchange column packed with Dowex $Na^+$. The compound was eluted with water and lyophilized.

**Sodium ((2-(5-(3-octanamidoprop-1-yn-1-yl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)ethoxy)methyl)phosphonate (3a).** This compound was obtained according to the general procedure described above in 90% yield (63 mg). $^1$H NMR (300 MHz, $D_2O$) δ: 7.91 (s, 1H, H-6), 4.13 (s, 2H, $CH_2$NH), 3.96 (t, 2H, J = 5.4 Hz, $CH_2$-1'), 3.78 (t, 2H, J = 5.2 Hz, $CH_2O$), 3.48 (d, 2H, J = 8.3 Hz, $CH_2P$), 2.25 (t, 2H, J = 7.3 Hz, CO-$CH_2$), 1.58 (t, 2H, J = 7.0 Hz, CO-$CH_2$-**$CH_2$**), 1.23 (m, 8H, 4x$CH_2$), 0.79 (t, 3H, J = 6.6 Hz, $CH_3$). $^{13}$C NMR (75 MHz, $D_2O$) δ: 176.9 (NH-CO), 160.7 (C-4), 155.7 (C-2), 149.7 (C-6), 97.8 (C-5), 88.6, 74.9, 69.6 (d, 1C, $J_{C,P}$ = 10.9 Hz, $CH_2$-2'), 68.6 (d, 1C, $J_{C,P}$ = 129.8 Hz, $CH_2P$), 48.5 ($CH_2$-1'), 35.3, 30.7, 29.3, 27.8, 27.7, 25.0, 21.6, 13.1 ($CH_3$). $^{31}$P NMR (121.5 MHz, $D_2O$) δ: 13.32. HRMS (ESI) for $C_{18}H_{28}N_3O_7P$: calcd 428.15919 $[M - H]^-$, found 428.1589.

**Sodium (R)-(((1-(5-(3-octanamidoprop-1-yn-1-yl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)propan-2-yl)oxy)methyl)phosphonate (3b).** This compound was obtained according to the general

procedure described above in 45% yield (60 mg). $^1$H NMR (300 MHz, $D_2O$) δ: 7.99 (s, 1H, H-6), 4.14 (s, 2H, $CH_2NH$), 3.95-3.76 (m, 3H, $CH_2P$, CH-2'), 3.71-3.46 (m, 2H, $CH_2$-1'), 2.26 (t, 2H, J = 7.2 Hz, CO-$CH_2$), 1.59 (m, 2H, CO-$CH_2$-**$CH_2$**), 1.32-1.2 (m, 8H, 4x$CH_2$), 1.18 (d, 3H, J = 5.9 Hz, $CH_3$-3'), 0.81 (t, 3H, J = 6.7, $CH_3$). $^{13}$C NMR (75 MHz, $D_2O$) δ: 177.3 (NH-CO), 165.2 (C-4), 151.4 (C-2), 150.9 (C-6), 98.1 (C-5), 89.9, 75.6 (d, 1C, $J_{C,P}$ = 11.4 Hz, CH-2'), 73.3, 65.2 (d, 1C, $J_{C,P}$ = 156.64 Hz, $CH_2P$), 52.8 ($CH_2$-1'), 35.6, 31.1, 29.5, 28.1, 25.3, 21.9, 16.0 ($CH_3$-3'), 13.4 ($CH_3$). $^{31}$P NMR (121.5 MHz, $D_2O$) δ: 15.58. HRMS (ESI) for $C_{19}H_{30}N_3O_7P$: calcd 442.17484 [M - H]$^-$, found 442.1748.

**Sodium (3-(5-(3-octanamidoprop-1-yn-1-yl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)propyl)phosphonate (3c).** This compound was obtained according to the general procedure described above in 58% yield (136 mg). $^1$H NMR (300 MHz, $D_2O$) δ: 7.94 (s, 1H, H-6), 4.06 (s, 2H, $CH_2NH$), 3.75 (t, 2H, J = 7.2 Hz, $CH_2$-1'), 2.19 (t, 2H, J = 7.2 Hz, CO-$CH_2$), 1.82 (m, 2H, $CH_2P$), 1.6-1.35 (m, 4H, $CH_2$-2', CO-$CH_2$-**$CH_2$**), 1.25-1.1 (m, 8H, 4x$CH_2$), 0.74 (t, 3H, J = 6.6 Hz, $CH_3$). $^{13}$C NMR (75 MHz, $D_2O$) δ: 177.0 (NH-CO), 165.0 (C-4), 151.3 (C-2), 150.0 (C-6), 97.9 (C-5), 89.5, 73.0, 50.1 (d, 1C, $J_{C,P}$ = 19.4 Hz, $CH_2$-1'), 35.3 (CO-$CH_2$), 30.7 ($CH_2$), 29.1 ($CH_2$), 27.7 ($CH_2$), 27.6 ($CH_2$), 25.4 (d, 1C, $J_{C,P}$ = 71.2 Hz, $CH_2P$), 24.6 (d, 1C, $J_{C,P}$ = 60.9 Hz, $CH_2$-2'), 23.1 ($CH_2$), 21.6 ($CH_2$), 13.1 ($CH_3$). $^{31}$P NMR (121.5 MHz, $D_2O$) δ: 22.06. HRMS (ESI) for $C_{18}H_{28}N_3O_6P$: calcd 412.16428 [M - H]$^-$, found 412.1643.

**Sodium (5-(5-(3-octanamidoprop-1-yn-1-yl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)pentyl)phosphonate (3d).** This compound was obtained according to the general procedure described above in 62% yield (63 mg). $^1$H NMR (300 MHz, $D_2O$) δ: 8.01 (s, 1H, H-6), 4.18 (s, 2H, $CH_2$-NH), 3.83 (t, 2H, J = 7.2, $CH_2$-1'), 2.30 (t, 2H, J = 7.1 Hz, CO-$CH_2$), 1.75 (m, 2H, $CH_2$), 1.60 (m, 6H, 3x$CH_2$), 1.45 (m, 2H, $CH_2$), 1.30 (m, 8H, 4x$CH_2$), 0.85 (t, 3H, J = 6.6 Hz, $CH_3$). $^{13}$C NMR (75 MHz, $D_2O$) δ: 177.0 (NH-CO), 165.0 (C-4), 151.1 (C-2), 149.9 (C-6), 97.9 (C-5), 89.7, 72.9, 49.0 ($CH_2$-1'), 35.2 ($CH_2$), 30.8 ($CH_2$), 29.1 ($CH_2$), 28.5 ($CH_2$), 27.8 ($CH_2$), 27.6 ($CH_2$), 27.1 (d, 1C, $J_{C,P}$ = 64.4 Hz, $CH_2$), 26.8 (d, 1C, $J_{C,P}$ = 7.1 Hz, $CH_2$), 25.0 ($CH_2$), 22.6 (d, 1C, $J_{C,P}$ = 7.1 Hz, $CH_2$), 21.6 ($CH_2$), 13.1 ($CH_3$). $^{31}$P NMR (121.5 MHz, $D_2O$) δ: 25.54. HRMS (ESI) for $C_{20}H_{32}N_3O_6P$: calcd 440.19558 [M - H]$^-$, found 440.1957.

**Sodium (6-(5-(3-octanamidoprop-1-yn-1-yl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)hexyl)phosphonate (3e).** This compound was obtained according to the general procedure described above in 43% yield (15 mg). $^1$H NMR (300 MHz, $D_2O$) δ: 7.97 (s, 1H, H-6), 4.14 (s, 2H, $CH_2$-NH), 3.80 (t, 2H, J = 7.1, $CH_2$-1'), 2.27 (t, 2H, J = 7.0 Hz, CO-$CH_2$), 1.69 (m, 2H, $CH_2$), 1.61 (m, 2H, $CH_2$), 1.54 (m, 2H, $CH_2$), 1.4 (m, 2H, $CH_2$), 1.34 (m, 2H, $CH_2$), 1.28 (m, 6H, 3x$CH_2$), 1.21 (m, 4H, 2x$CH_2$), 0.81 (t, 3H, J = 7.0 Hz, $CH_3$). $^{13}$C NMR (75 MHz, $D_2O$) δ: 177.1 (NH-CO), 166.6 (C-4), 152.7 (C-2), 151.7 (C-6), 99.5 (C-5), 91.4, 74.5, 50.8 ($CH_2$-1'), 36.9 (CO-**$CH_2$**), 32.5 ($CH_2$), 31.1 (d, 1C, $J_{C,P}$ = 16.9 Hz, $CH_2$), 30.8 ($CH_2$), 29.6 ($CH_2$), 29.5 ($CH_2$), 29.3 ($CH_2$), 29.2 ($CH_2$), 28.7 ($CH_2$), 26.8 ($CH_2$), 26.5 ($CH_2$), 23.8 (d, 1C, $J_{C,P}$ = 4.1 Hz, $CH_2$), 14.8 ($CH_3$). $^{31}$P NMR (121.5 MHz, $D_2O$) δ: 25.45. HRMS (ESI) for $C_{21}H_{34}N_3O_6P$: calcd 454.21122 [M - H]$^-$, found 454.2114.

**Tritium release assay.** The assay was performed in a 96-well flat bottom plate and in final reaction volume of 25 μl. The reaction mixture consisted of 20 μM mTHF, 1 μM ThyX, 41 μM FAD, 200 μM NADPH, 1% glycerol, 1 mM $MgCl_2$, 50 mM HEPES (pH = 7.5), 50 μM of tested inhibitor and 0.8 μM of [5-$^3$H] dUMP (25.5 Ci/mmol). 5-FdUMP was used as a positive control. The reaction was initiated by addition of [5-$^3$H] dUMP followed by 10 minutes incubation at room temperature. Hereafter 20 μl of stop solution (3:1 2M TCA and 4.3 mM dUMP) and 200 μl of 10% (w/v) activated charcoal were added to the reaction mixture. The plate was incubated on ice for 15 minutes and spun down at 4500 rpm for 10 minutes in a precooled centrifuge. From each well a 100 μl aliquot was transferred to a white OptiPlate (Perkin Elmer) together with 150 μl of Microscint 40. The amount of tritium-containing water produced during the reaction was determined using liquid scintillation counting. Each reaction was performed 5 times and the mean value of inhibition percentages of three independent experiments was calculated.

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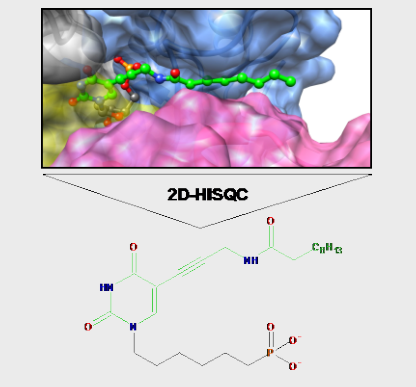
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**Entry for the Table of Contents**

Layout 1:

# FULL PAPERS

**Rational design:** The selective ThyX inhibitor 5-alkynyl dUMP was modeled into its target active site and NMR was used to monitor this binding mode. To increase stability of our lead compound some acyclic nucleoside phosphonate were synthesized and tested for ThyX inhibition in vitro. The active compound that was discovered will be used for further optimization that should lead to antibacterial thymidylate synthase inhibitors.




*Anastasia Parchina, Matheus Froeyen, Lia Margamuljana, Jef Rozenski, Steven De Jonghe, Yves Briers, Rob Lavigne, Piet Herdewijn, Eveline Lescrinier**

***Page No. – Page No.***

**Discovery of an acyclic nucleoside phosphonate that inhibits M. Tuberculosis ThyX based on the binding mode of a 5-alkynyl substrate analogue**