Journal of Medicinal Chemistry

ARTICLE

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Synthesis and Evaluation of 5-Substituted 2′-deoxyuridine Monophosphate Analogues As Inhibitors of Flavin-Dependent Thymidylate Synthase in *Mycobacterium tuberculosis*

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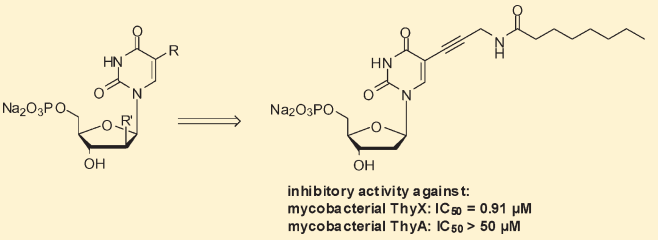
Supporting Information

ABSTRACT: A series of 5-substituted 2′-deoxyuridine monophosphate analogues has been synthesized and evaluated as potential inhibitors of mycobacterial ThyX, a novel flavin-dependent thymidylate synthase in *Mycobacterium tuberculosis*. A systematic SAR study led to the identification of compound **5a**, displaying an IC_{50} value against mycobacterial ThyX of 0.91 μM. This derivative lacks activity against the classical mycobacterial thymidylate synthase ThyA (IC_{50} > 50 μM) and represents the first example of a selective mycobacterial FDTS inhibitor.



INTRODUCTION

According to the WHO,[1] 1.7 million people died from tuberculosis (TB) in 2009 and more than 2 billion people (constituting one-third of the world's population) are infected with TB. Among the 9.4 million new TB cases reported in 2009, an estimated 1.1−1.2 million (11−13%) suffer from HIV coinfection. Hence, TB, a contagious infectious disease, is regarded as the leading cause of death due to an infectious agent among adults worldwide and a major threat to global health.[2] The disease establishes mainly in the pulmonary system and is caused by some mycobacteria of the *Mycobacterium tuberculosis* complex, predominantly *Mycobacterium tuberculosis*.[3]

Active TB is treated by chemotherapy with so-called first-line drugs.[4−6] From the many trials conducted in the past 50−60 years, two standard regimens emerged which are recommended by the WHO and consist of a two-month daily treatment of either isoniazid, rifampin, pyrazinamide, and streptomycin or isoniazid, rifampin, pyrazinamide, and ethambutol, followed by further four months daily or intermittent doses of isoniazid and rifampin. Unfortunately, the long treatment times and the side effects[7] of the drugs (especially hepatotoxicity) lead to a high proportion of noncompliance patients. This and the rise of the HIV pandemic[8] in the early 1980s led to the emergence of multidrug resistant[9] (MDR) and, more recently, even extensively drug resistant[10] (XDR) strains. MDR-TB is defined as resistance to the action of at least isoniazid and rifampin, and its treatment requires the inclusion of so-called second-line drugs such as aminoglycosides, fluoroquinolones, and second-line bacteriostatics. XDR bacteria are MDR and also resistant to fluoroquinolones and at least one injectable antibiotic. The XDR-tuberculosis is virtually untreatable. The increasing incidences of MDR- and XDR-tuberculosis urgently demand novel drugs for therapy of tuberculosis. The side-effects of current drugs, the high costs of second-line drugs, and the lack of well-established guidelines for the treatment of MDR-TB and XDR-TB require alternative approaches to target tuberculosis.[11,12]

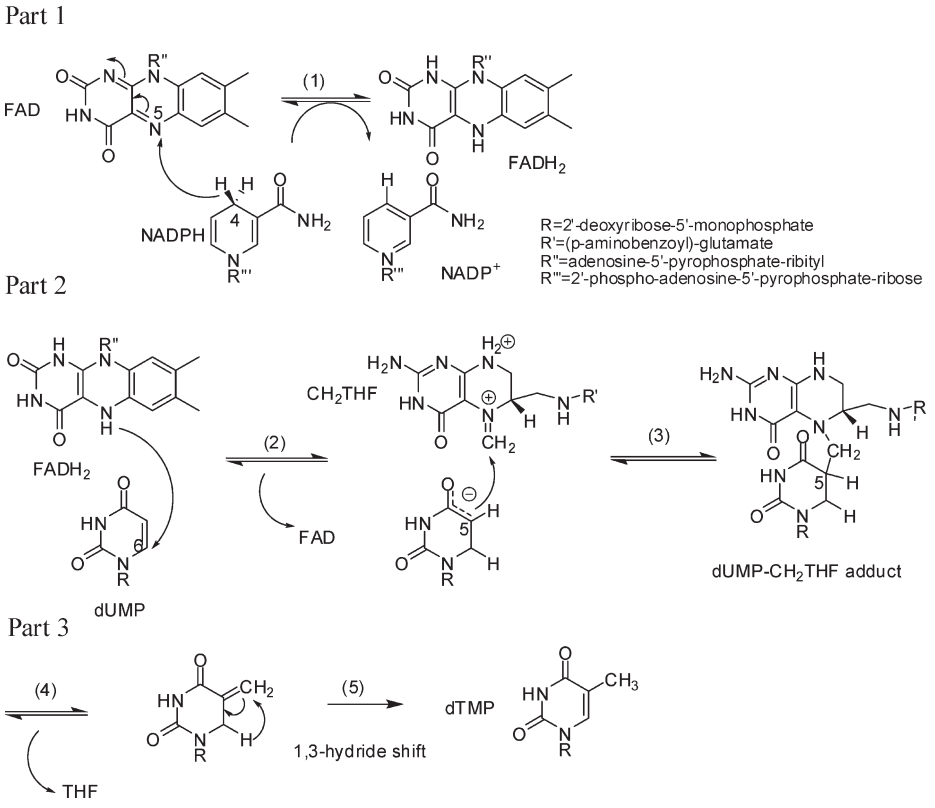
Traditionally, the only known pathway for de novo synthesis of thymidylate which is essential for most eubacteria, plants, and eukaryotic cells was by thymidylate synthase (TS or ThyA).[13] This enzyme catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to 2′-deoxythymidine-5′-monophosphate using *R*-N^5-N^{10}-methylene-5,6,7,8-tetrahydrofolate (CH_2THF) as a methylene and hydride donor, resulting in the formation of 7,8-dihydrofolate (DHF). In the classical ThyA cycle, formation of thymidylate must be coupled with reduction of DHF to THF catalyzed by dihydrofolate reductase (DHFR) and remethylenation by serinehydroxymethyl transferase (SHMT).

However, it has been shown that several microorganisms lack the genes encoding for ThyA and DHFR but are still fully viable in thymidine-deficient media. This led to the discovery of the ThyX gene, which encodes for the ThyX protein, a flavin-dependent

Received: April 19, 2011
Published: June 09, 2011

dx.doi.org/10.1021/jm2004688 | *J. Med. Chem.* 2011, 54, 4847–4862

Scheme 1. Current Biochemical Reaction Mechanism Involving Direct Hydride Transfer and 1,3-Hydride Shift



thymidylate synthase (FDTS) which is rare in eukaryotes and absent in humans.[14] ThyX uses CH_2THF only as one-carbon donor, whereas reduced flavin adenine dinucleotide ($FADH_2$) serves as a hydride donor. Members of the ThyX family are NAD(P)H-oxidases because catalysis requires the presence of NAD(P)H to furnish sufficient amounts of $FADH_2$ via reduction of FAD. Furthermore, ThyX shows no structural similarity with ThyA[15] and there is no sequence similarity between ThyA and ThyX proteins, allowing the design of selective ThyX-inhibitors as a novel approach to target tuberculosis. While some pathogens solely rely on ThyX, genetic analysis revealed that *M. tuberculosis* carries genes encoding for ThyA, ThyX, and DHFR.[14] Transposon site hybridization studies have shown that the ThyX gene is required for mycobacterial growth and survival within macrophages.[16,17] Nonetheless, it remains unclear under which conditions either gene is activated. Steady-state kinetic analyses of *M. tuberculosis* revealed that dTMP-synthesis proceeds with very low catalytic rates in both enzymes, thereby suggesting that the true biological substrates still remain to be found.[18] These findings may be in accordance with the extremely low growth rates of *M. tuberculosis* and could be an indicator for down-regulation of dTMP-production.

In the past few years, several studies to reveal the biochemical reaction mechanism were documented in literature.[19,20] Initially, an active site catalytic serine was assumed to nucleophilically attack C-6 of dUMP in a Michael-type reaction to give rise to an enolate at C-4 and C-5, which in turn attacks the iminium ion of CH_2THF to form the new C−C bond in dTMP. This dUMP−CH_2THF adduct subsequently undergoes β-elimination to install an *exo*-methylene bond between C-5 and C-7, which is reduced by $FADH_2$ in the final step to yield dTMP.

However, the following facts led to the supersession of this mechanism: (i) The active site serine is too far away from the C-6 of dUMP, as shown by Sampathkumar et al., who provided a crystal structure[21] of Mtb-ThyX in complex with the substrate analogue 5-Br dUMP and FAD, and only conformational changes in the enzyme would be able to lead to nucleophilic catalysis. (ii) No general base is located near this putative serine which would be required to enhance its otherwise too weak nucleophilicity. (iii) Point mutation studies in *Thermotoga maritima* have shown that the enzyme was still active after mutation of this serine to either an alanine or cysteine. Recently, Koehn et al. performed the reaction in 99% D_2O which resulted in incorporation of deuterium in the 6-position. These new intriguing findings led to the suggestion of an alternative reaction mechanism[22] (Scheme 1) which contains the following two distinctive key steps: (i) a hydride equivalent is transferred directly from $FADH_2$ to the C-6 of dUMP (part 2, step 2) and (ii) an unusual 1,3-hydride shift from C-6 to the *exo*-methylene C-7 in the last step furnishes dTMP (part 3, step 5). Steps 3 and 4 correspond to the previously proposed mechanism and are considered as logical on the mechanistic pathway toward dTMP. An alternative addition−elimination mechanism involved in the last step could be ruled out when the reaction was performed using 6D-dUMP in H_2O because the formation of nondeuterated dTMP could not be observed.

Although ThyX is an attractive target for antimycobacterial drug design, the only known inhibitors are 5-F and 5-BrdUMP.[23] However, both are known to act as human ThyA inhibitors and are therefore of no interest for antibacterial therapy.[24−28] Up to now, no selective inhibitors of mycobacterial FDTS are known in literature. In this paper, we describe the synthesis

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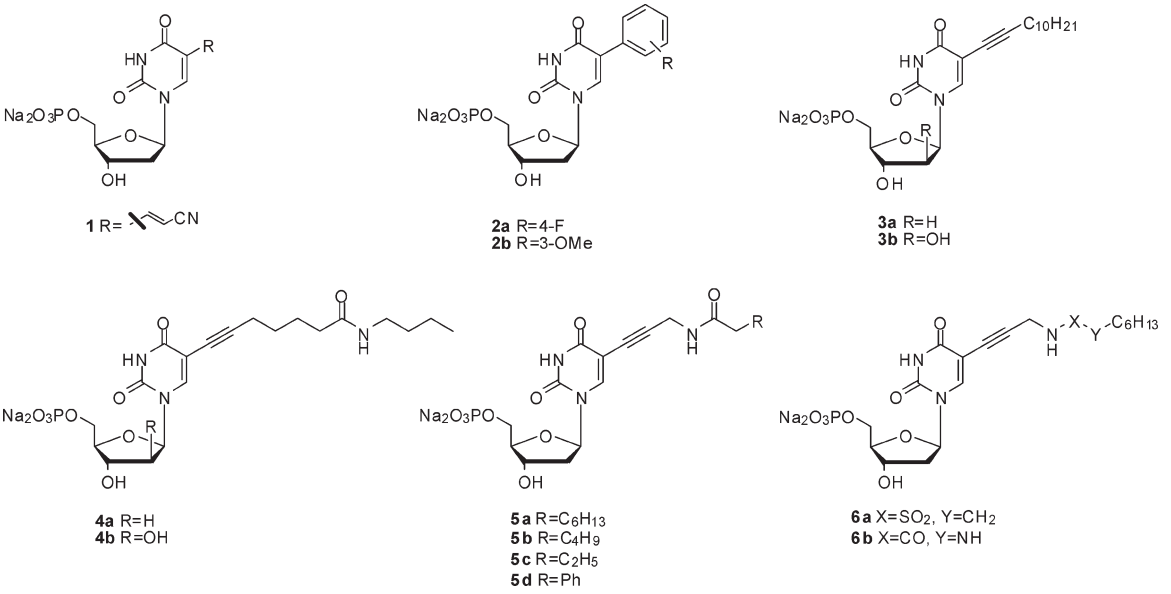
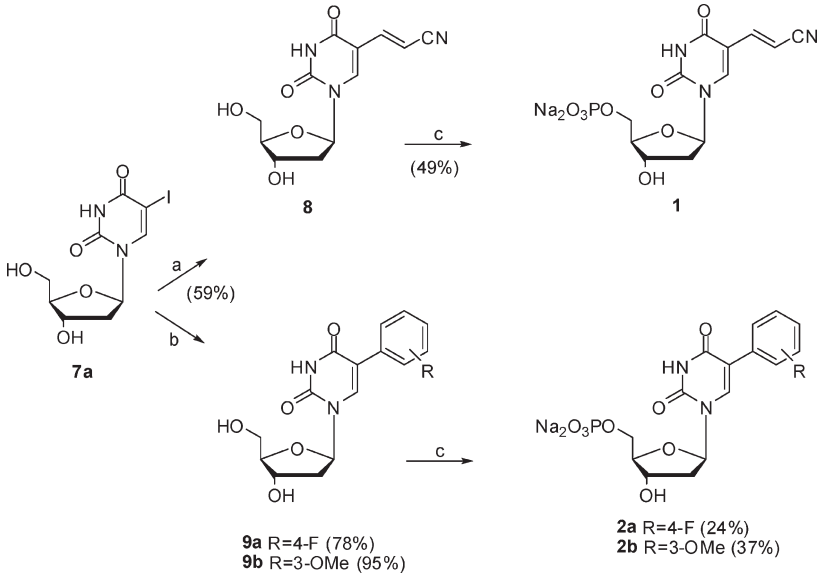
Scheme 2. Structures of Synthesized ThyX-Inhibitors



Scheme 3[a]



[a] Reagents and conditions: (a) CH_2CHCN, $Pd(PPh_3)_4$, $(n\text{-}Bu)_3N$, DMF, 80 °C; (b) boronic acid, $Pd(PPh_3)_4$, CsF, DMF/H_2O, 60 °C; (c) $POCl_3$, pyr, H_2O, $CH_3CN/PO(OMe)_3$, 0 °C.

of a novel series of C-5 substituted 2′-dUMP analogues and their evaluation as inhibitors of mycobacterial ThyX and ThyA (Scheme 2).

RESULTS AND DISCUSSION

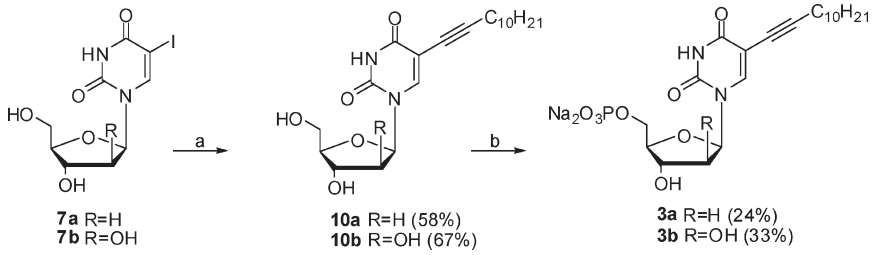
Chemistry. To have easy access to C-5 variation of the dUMP scaffold, 5-iodo-2′-deoxyuridine **7a** was considered as ideal starting material. It is commercially available or, alternatively, it can be prepared from 2′-deoxyuridine according to a protocol described in literature.[29]

5-(2-cyanovinyl)-deoxyuridine **8** was synthesized via a Heck reaction of **7a** with acrylonitrile in the presence of a Pd(0) catalyst and a tertiary amine in DMF at elevated temperature.[30−32] Phosphorylation of the primary hydroxyl group of compound **8** according to the procedure by Sowa and Ouchi[33] yielded the desired monophosphate derivative **1** (Scheme 3).

To introduce a phenyl group at position 5 of the uracil moiety, Pd-catalyzed Suzuki cross-coupling reaction[34] of **7a** has been performed with suitable boronic acids. Attempts using various bases (K_2CO_3, Na_2CO_3, NaOH, KF) in an organic solvent/H_2O mixture predominantly led to dehalogenation. After carefully

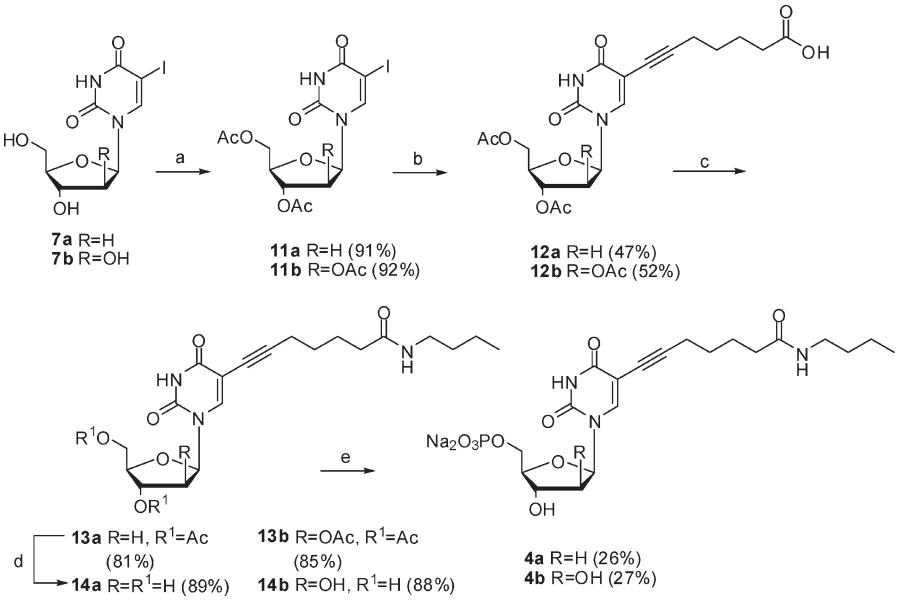
dx.doi.org/10.1021/jm2004688 |J. Med. Chem. 2011, 54, 4847–4862

Scheme 4[a]



[a] Reagents and conditions: (a) 1-dodecyne, $Pd(PPh_3)_4$, CuI, NEt_3, DMF, rt; (b) $POCl_3$, proton sponge, $PO(OMe)_3$, 0 °C.

Scheme 5[a]



[a] Reagents and conditions: (a) Ac_2O, DMAP, NEt_3, CH_3CN, rt; (b) 6-heptynoic acid, $Pd(PPh_3)_4$, CuI, DIPEA, DMF, rt; (c) *n*-butylamine, DIC, HOBt, DCM, 0 °C to rt; (d) NaOMe, MeOH, rt; (e) $POCl_3$, proton sponge, $PO(OMe)_3$, 0 °C.

tuning reaction conditions, it was found that successful cross coupling could be achieved with CsF as base in a mixture of DMF and water as solvent (Scheme 3). Under these reaction circumstances, only small amounts of 2′-deoxyuridine were detected. Phosphorylation of compounds **9a**−**b** afforded the 5-aryl-2′-dUMP analogues **2a**−**b**.

Long-chain acetylenic compounds **3a**−**b** have been synthesized via palladium-catalyzed Sonogashira cross-coupling of suitable acetylenes with unprotected 5-iodo nucleosides as the key step (Scheme 4).[35,36] Compounds **10a** and **10b** were accessible from unprotected 5-iodo-2′-deoxyuridine **7a** and 1-(β-D-arabinofuranosyl)-5-iodoracil **7b**, respectively, by Sonogashira cross-coupling with 1-dodecyne with a slightly modified workup than the one described in the literature. Yoshikawa phosphorylation[37,38] ($POCl_3$/proton sponge/trimethyl phosphate) finally furnished compounds **3a** and **3b** (Scheme 4). The arabino-based compounds were synthesized from 1-(β-D-arabinofuranosyl)-5-iodoracil,[39] **7b**, which was prepared by reaction of 1-(β-D-arabinofuranosyl)uracil[39,40] with I_2 in $HNO_3/CHCl_3$.

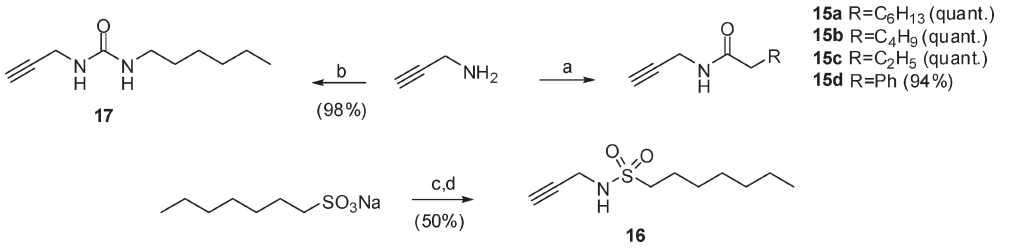
For the introduction of an amide functionality into this long chain, acetylated 5-iodo nucleosides **11a**[29] and **11b**[41,42] were used as starting material (Scheme 5). For the synthesis of derivatives **4a** and **4b**, bearing an *N*-butyl-6-heptynamide chain, compounds **11a** and **11b** were subjected to Pd-catalyzed cross-coupling with 6-heptynoic acid,[43] followed by DIC/HOBt mediated coupling with *n*-butylamine.[44] Deprotection of the acetyl groups under alkaline conditions,[29] followed by phosphorylation, yielded the desired final compounds **4a** and **4b**. On the other hand, the synthesis of 5-propargylamide derivatives **5a**−**d** and **6a**−**b** was performed in a divergent manner. The acetylene moieties were synthesized through condensation reaction of propargylamine with suitable acyl chlorides, yielding **15a**−**d** as shown in Scheme 6.[45]

The introduction of the amide-containing terminal acetylenes **15a**−**d** at position 5 of the uracil ring was achieved via a Sonogashira cross-coupling, followed by deacetylation affording nucleoside analogues **18a**−**d** (Scheme 7).

Phosphorylation of derivative **18a** was problematic. The standard method previously used ($POCl_3$/proton sponge/trimethyl phosphate) only gave unsatisfactory results with low yields and difficult purification due to the presence of side products. However, repeated purification (flash chromatography and HPLC) led to the isolation of the pure nucleoside

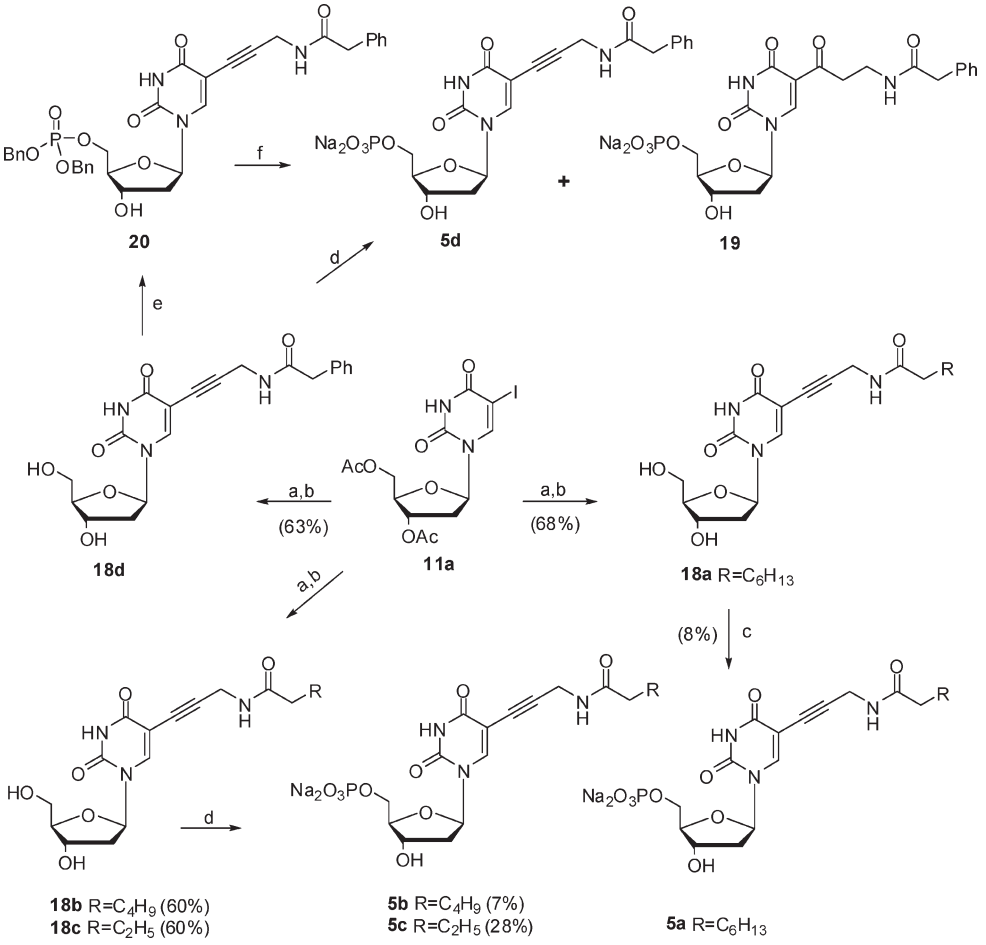
dx.doi.org/10.1021/jm2004688 |*J. Med. Chem.* 2011, 54, 4847–4862

Scheme 6[a]



[a] Reagents and conditions: (a) acyl chloride, DIPEA, DCM, 0 °C to rt; (b) hexyl isocyanate, DCM, 0 °C to rt; (c) thionyl chloride, reflux; (d) propargylamine, NEt_3, DCM, 0 °C to rt.

Scheme 7[a]



[a] Reagents and conditions: (a) acetylene **15a**−**d**, $Pd(PPh_3)_4$, CuI, DIPEA, DMF, rt; (b) NaOMe, MeOH, rt; (c) $POCl_3$, proton sponge, $PO(OMe)_3$, 0 °C; (d) $POCl_3$, H_2O, pyr, $CH_3CN/PO(OMe)_3$, 0 °C; (e) dibenzyl phosphate, PPh_3, DIAD, THF, rt; (f) TMSI, CH_3CN, −10 °C.

monophosphate derivative **5a**. For the phosphorylation of derivatives **18b**−**d**, the protocol employed by Sowa and Ouchi[33,46] was evaluated in which the addition of H_2O to the reaction mixture was reported to enhance the reaction rate. Because of the poor solubility of derivatives **18b**−**d** in CH_3CN, a mixture of acetonitrile and trimethyl phosphate was used as solvent. Unfortunately, these reaction conditions ($POCl_3$, pyridine, H_2O in $CH_3CN/PO(OMe)_3$) were not compatible with the acetylenic group. However, the desired analogues **5b** and **5c** could be obtained in 96% and 92% purity, respectively, albeit only after a second HPLC purification step. In the case of the desired phenacylamide **5d**, considerable amounts of the corresponding keto compound **19**, due to hydration of the triple bond, were identified by NMR spectroscopy (Scheme 7). The ^1H NMR spectrum shows two distinct singlets at δ = 7.98 and 8.36 ppm, which were assigned to H-6 in derivatives **5d** and **19** in a ratio of 3.26:1, respectively. Furthermore, as expected, the ^{13}C NMR spectrum shows a clear peak at δ = 199.2 ppm for the ketocarbonyl group, whereas both amide carbonyl peaks of **5d** and **19** overlap at δ = 174.2. These findings were confirmed by mass

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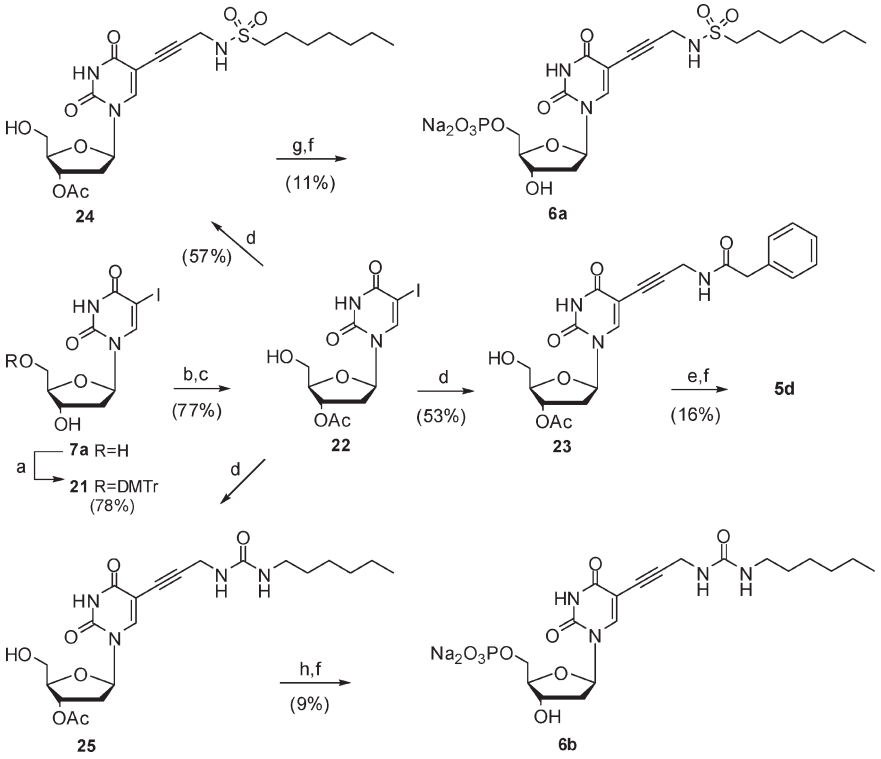
Scheme 8[a]



[a] Reagents and conditions: (a) DMTrCl, DMAP, NEt_3, pyr, rt; (b) Ac_2O, DMAP, NEt_3, CH_3CN, rt; (c) TCA, DCM, rt; (d) acetylene **15d**, **16**, or **17**, $Pd(PPh_3)_4$, CuI, DIPEA, DMF, rt; (e) 2-cyanoethyl phosphate, DCC, pyr, rt; (f) 1 M NaOH, 100 °C; (g) 2-cyanoethyl phosphate, DCC, HOBt, pyr, rt; (h) 2-cyanoethyl phosphate, DCC, HOBt, pyr, 40 °C.

spectrometry. The (−)-ESI mass spectrum of this mixture shows two peaks at $m/z = 478$ and 496, corresponding to the $[M - H^+]$ peak of **5d** and **19**, respectively.

Attempts to separate the desired acetylenic compound from its hydrated form by RP-HPLC were unsuccessful, and therefore alternatives were sought. Compound **18d** was coupled with dibenzyl phosphate under Mitsunobu conditions[47] (PPh_3, DIAD, THF), furnishing the corresponding nucleoside dibenzyl phosphate triester **20**. To our disappointment, TMSI-mediated debenzylation[48] equally resulted in an inseparable mixture of **5d** and **19** in a ratio of 2.14:1 according to ^{1}H NMR spectroscopy.

Finally the problem has been solved by using the method of Tener.[49] Thus, 5-iodo-3′-*O*-acetyl-2′-deoxyuridine **22**,[50−52] prepared in three steps from 5-iodo-2′-deoxyuridine **7a** (tritylation, acetylation, and detritylation), was coupled with acetylene **15d**, yielding 3′-*O*-acetylated derivative **23**. DCC-coupling of **23** with 2-cyanoethyl phosphate (pyridinium salt) in anhydrous pyridine, followed by deprotection with 1 M NaOH at 100 °C for a few minutes, finally gave pure **5d** after purification, however in only 16% yield (Scheme 8). The same strategy was used for the synthesis of dUMP derivatives with sulfonamide and urea-containing acetylene moieties. Reaction of sodium 1-heptanesulfonate in neat thionyl chloride,[53] followed by condensation of the resulting sulfonyl chloride with propargylamine,[54] furnished acetylene **16** (Scheme 6). Similarly, condensation of propargylamine with hexyl isocyanate yielded acetylenic urea **17**.[55] Cross-coupling reaction of **22** with **16** proceeded smoothly, affording compound **24**, whereas the corresponding urea derivative **25** could not be obtained in pure form but was used in the subsequent phosphorylation step without any further attempt of purification. Addition of HOBt (1 equiv) did not lead to any significant improvement; sulfonamide monophosphate **6a** was obtained in 11% yield. For the synthesis of urea monophosphate **6b**, the reaction was carried out at 40 °C together with the addition of HOBt (1 equiv). Again, no substantial increase in yield of the desired product (9%) could be achieved (Scheme 8).

Biological Evaluation and Structure−Activity Relationship Study. Compounds were evaluated for their inhibitory activity against mycobacterial ThyX and ThyA. Cloning of the ThyX gene, protein expression, and purification was done as published previously.[21] The inhibition of ThyX-catalyzed dTMP synthesis was measured in a standard radioactive assay[56,57] using purified ThyX-protein and [5-^{3}H]-dUMP as a radiolabeled substrate. The amount of tritiated water released during the reaction was measured by liquid scintillation counting. The *thyA* gene was expressed in *E. coli* and the expressed His(6)-tagged enzymes were purified by nickel-chelate chromatography. The inhibition of dTMP-synthesis by ThyA was measured in a standard photometric assay[58] on a purified ThyA-protein. The extent of inhibition found is based on a decrease of absorption of DHF (which is formed during the biochemical transformation) at 340 nm in the presence of an inhibitor (see Experimental Section).

The crystal structure[21] of the ThyX-FAD-5-BrdUMP complex shows that the substrate analogue is buried completely in a deep pocket. The oxygen atoms of the phosphate moiety as well as the nitrogen and oxygen atoms of the pyrimidine ring have crucial interactions with numerous highly conserved active site residues,

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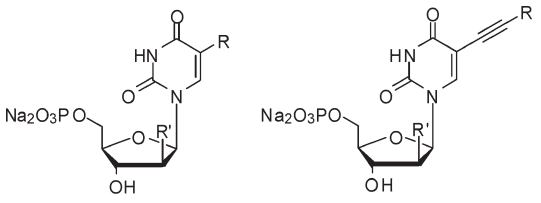
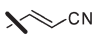
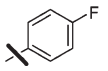
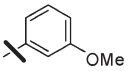
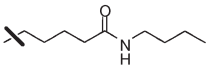
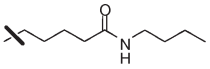
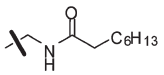
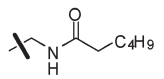
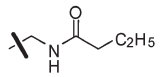
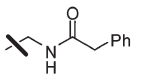
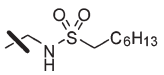
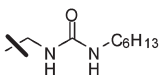
Table 1. Inhibition of Mtb-ThyX and Mtb-ThyA by Compounds 1−7



5-F-dUMP 1-2 | 3-6

			ThyX-inhibition[a]		ThyA-inhibition[a]	
cmpd	R	R'	% inhibition (50 μM)	IC_{50} (μM)	% inhibition (50 μM)	IC_{50} (μM)
5-F-dUMP[b]	F	H	95.1	0.29	100	0.57
1		H	81.3	7.4	8.8	>50
2a[c]		H	74	10	1.1	>50
2b		H	16	>50	0	>50
3a	$C_{10}H_{21}$	H	82.6	27.8	32.5	>50
3b	$C_{10}H_{21}$	OH	19	>50	26.2	>50
4a		H	74.6	8.03	6.4	>50
4b		OH	21	>50	5.5	>50
5a		H	92.8	0.91	15.6	>50
5b		H	89.9	8.61	14	>50
5c[d]		H	72	26.2	14.8	>50
5d		H	86.9	7.3	32.7	>50
6a		H	84.7	6.93	7.2	>50
6b		H	90.9	4.84	17.9	>50

[a] Values are means of three independent experiments. [b] 5-F dUMP used as positive control. [c] 93% purity. [d] 92% purity.

dx.doi.org/10.1021/jm2004688 |J. Med. Chem. 2011, 54, 4847–4862

notably arginine, glutamic acid, and aspartic acid. As this 5′-*O*-monophosphate group is critical for binding of 5-Br-dUMP, all synthesized nucleosides within this study have been phosphorylated, affording the corresponding nucleotides that have been evaluated as potential mycobacterial ThyX and ThyA inhibitors. Furthermore, the pyrimidine ring of 5-BrdUMP and the isoalloxazine ring of FAD are close enough to interact via hydrogen bonds and π-stacking. Therefore, the pyrimide ring system of dUMP has been kept intact in the search for novel mycobacterial ThyX inhibitors. The C-5 position of dUMP has been subjected to extensive structural modifications using human ThyA as drug target. In this paper, a similar strategy was followed in order to discover novel mycobacterial ThyX inhibitors based on a dUMP scaffold. Compounds were initially evaluated at a concentration of 50 μM in the ThyX as well as in the ThyA assay. Compounds displaying more than 50% inhibition were subjected to dose−response curves in order to determine IC_{50} values. In both assays, 5-F dUMP was included as reference compound and positive control.

The SAR study started with the exploration of relatively small modifications at the C-5-position of the uracil base. Introduction of a cyanovinyl moiety in compound **1** confers good inhibitory activity of mycobacterial ThyX (Table 1, IC_{50} = 7.4 μM), although no inhibitory effect on mycobacterial ThyA could be observed.

To probe if ThyX can accommodate sterically more demanding substituents at position 5, aryl groups were introduced via Suzuki cross-coupling reactions. The 5-(4-fluorophenyl) analogue **2a** exhibits good activity against ThyX (IC_{50} = 10 μM), whereas no activity could be determined against ThyA. On the other hand, the 5-(3-methoxyphenyl) analogue **2b** shows no activity against both enzymes. It indicates that the nature of the substitution pattern on the phenyl ring has a large influence on the biological activity.

Recently, Johar et al. disclosed arabinonucleoside analogues with a long alkynyl side chain at the 5-position of the uracil moiety that exhibited high levels of antimycobacterial activity in vitro (MIC_{90} = 1−5 μg/mL).[59] This observation prompted us to study the effect of a long-chain alkynyl group at C-5 of uridylate on mycobacterial ThyX and ThyA activity. Indeed, 5-dodecynyl-dUMP **3a** exhibits moderate ThyX inhibition (IC_{50} = 28 μM) but has no effect on ThyA. The corresponding arabino analogue **3b** completely lacks activity against both enzymes.

To further explore the SAR, functional groups were introduced into the alkyl chain of derivatives **3a** and **3b**. The insertion of an amide group in the center of the long alkyl chain afforded compound **4a**, which was 3-fold more potent in the ThyX assay when compared with its long-chain analogue **3a**. To our disappointment, again, the corresponding arabino congener **4b** was completely devoid of thymidylate synthase inhibitory activity (IC_{50} > 50 μM). Consequently, any further effort on the synthesis of corresponding arabino derivatives was discontinued and, in subsequent studies, we solely focused on the design and synthesis of dUMP analogues. Shifting the amide functional group closer toward the nucleobase furnished propargylamide derivative **5a**, which is 1 order of magnitude more potent (IC_{50} = 0.9 μM) than its congener **4a**.

To probe the influence of the arm length, the total number of carbon atoms of the alkynyl chain of **5a** (12 carbon atom) was decreased to a total number of 10 (compound **5b**) and 8 atoms (compound **5c**), resulting in a 10- to 30-fold decrease of ThyX inhibition, respectively. Similarly, the presence of a phenyl ring in *N*-propargyl phenacylamide derivative **5d** led to a decreased ThyX inhibitory activity, displaying IC_{50} values of 7.3 μM. None of these derivatives shows any appreciable ThyA inhibition.

A long chain consisting of 12 carbon atoms turned out to be optimal for ThyX inhibition. Therefore, the total lengths of 12 atoms, as well as the propargylamine moiety, were preserved and the functional group was modified in a further round of optimization. The amide group of compound **5a** was replaced by a sulfonamide moiety and a urea functionality, affording compounds **6a** and **6b**, respectively. Both analogues show a 5-fold drop in ThyX inhibition when compared to the amide congener **5a** and completely lack activity against ThyA.

CONCLUSION

The increasing incidences of resistant *M. tuberculosis* bacteria urgently demand novel drugs for therapy of tuberculosis. However, no new anti-TBC drugs have been brought to the market in the last 30 years. The last drug with a new mechanism of action approved for TB was rifampicin (which was already discovered in 1963). Therefore, there is an urgent need for new antimycobacterial drugs with new modes of action to potentially avoid cross-resistance. In this paper, mycobacterial ThyX has been selected as a promising target for the development of novel antibacterial drugs. Starting from the natural substrate, dUMP, a SAR study was carried out in which structural variation at C-5 of the uracil moiety was introduced. This work led to the discovery of a novel series of 5-alkynyl dUMP analogues from which the most potent congener (compound **5a**) displays an IC_{50} value of 0.91 μM against ThyX. The high polarity of this compound makes it very unlikely that it will be able to cross the cell membrane of *M. tuberculosis* and therefore it has not been tested for its antimicrobial activity. Compound **5a** completely lacks activity against mycobacterial ThyA. To our knowledge, this is the first time a selective mycobacterial FDTS inhibitor is reported. Up to now, we have no rationale to explain the observed selectivity. Co-crystallization of compound **5a** with the mycobacterial ThyX enzyme should reveal the binding mode of compound **5a** and might explain the selective mycobacterial ThyX inhibition. Although compound **5a** shows promising activity against the ThyX enzyme, the presence of the phosphate moiety hampers its further development. Further medicinal chemistry efforts will be necessary to improve its drug-like characteristics in order to have access to compounds which are useful for testing in a bacterial assay.

EXPERIMENTAL SECTION

Chemistry. For all reactions, analytical grade solvents were used. Dry acetonitrile, pyridine, and MeOH were obtained by distillation over CaH_2. Dry DCM, DMF, and THF were purchased from commercial suppliers. All moisture-sensitive reactions were carried out in oven-dried glassware (120 °C). ^{1}H and ^{13}C NMR spectra were recorded with a Bruker Advance 300 (^{1}H NMR, 300 MHz; ^{13}C NMR, 75 MHz; ^{31}P NMR, 121 MHz) or 500 MHz (^{1}H NMR, 500 MHz; ^{13}C NMR, 125 MHz) spectrometer using tetramethylsilane as internal standard for ^{1}H NMR spectra and DMSO-d_6 (39.52 ppm) or $CDCl_3$ (77.16 ppm) for ^{13}C NMR spectra. Abbreviations used are: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br s = broad signal. Chemical shifts are expressed in parts per million (ppm). Coupling constants are expressed in hertz (Hz). Mass spectra are obtained with a Finnigan LCQ advantage Max (ion trap) mass spectrophotometer from Thermo Finnigan, San Jose, CA, USA. Exact mass measurements are performed on a

quadrupole time-of-flight mass spectrometer (Q-tof-2, Micromass, Manchester, UK) equipped with a standard electrospray-ionization (ESI) interface. Samples were infused in *i*-PrOH/H_2O (1:1) at 3 μL/min. Melting points are determined on a Barnstead IA 9200 and are uncorrected. Precoated aluminum sheets (Fluka Silica gel/TLC-cards, 254 nm) were used for TLC. Column chromatography was performed on ICN silica gel 63−200, 60 Å. All final compounds possess a purity of at least 95% (see Supporting Information) except derivatives **2a** and **5c** (93 and 92% purity, respectively), as determined by analytical RP-HPLC analysis on a XBridge column (C-18, 5 μm, 4.6 mm × 150 mm) in combination with a Waters 600 HPLC system, a Waters 717 plus autosampler and a Waters 2996 photodiode array detector from Waters, Milford, Massachusetts, USA. Preparative HPLC purification was carried out on the same instrument using a preparative XBridge column (C-18, 5 μm, 19 mm × 150 mm) from Waters, Milford, Massachusetts, USA.

5-(2-Cyanovinyl)-2′-deoxyuridine (8)[30−32]**.** 5-Iodo-2′-deoxyuridine[29] **7a** (250 mg, 0.71 mmol) was dissolved in anhydrous DMF (10 mL), and the solution was flushed with nitrogen gas. To the solution were added acrylonitrile (468 μL, 7.1 mmol), tributylamine (185 μL, 0.78 mmol), and tetrakis(triphenylphosphine)palladium(0) (81 mg, 0.07 mmol), and the reaction mixture was heated at 80 °C for 9 h. The solution was evaporated in vacuo and purified using silica gel column chromatography (DCM/MeOH = 97:3→95:5) yielding compound **9** (116 mg, 59%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.74 (s, 1H, NH), 8.35 (s, 1H, H-6), 7.23 (d, 1H, J = 16.3 Hz, alkene H), 6.52 (d, 1H, J = 16.3 Hz, alkene H), 6.09 (t, 1H, J = 6.2 Hz, H-1′), 5.27 (s, 1H, OH), 5.13 (s, 1H, OH), 4.25 (m, 1H, H-3′), 3.82 (m, 1H, H-4′), 3.62 (ddd, 2H, J = 23.3, 12.0, 3.4 Hz, H-5′), 2.16 (m, 2H, H-2′). ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.65, 149.01, 144.50, 144.35, 119.25, 107.73, 94.27, 87.74, 85.17, 69.68, 60.80, 40.15. HRMS (ESI) calcd for $C_{12}H_{13}N_3O_5$ 278.0782 (M − H^+); found 278.0773.

General Procedure for the Suzuki Reaction of 5-Iodonucleosides 7a−b with Various Boronic Acids. 5-Iodo-2′-deoxyuridine[29] **7a** (250 mg, 0.71 mmol) (or 5-iodoracil 1-β-D-arabinofuranoside **7b**, 250 mg, 0.67 mmol) was dissolved in DMF (7.5 mL) and H_2O (3.75 mL). To the solution were added cesium fluoride (268 mg, 1.76 mmol) and a boronic acid (0.85 mmol), whereupon nitrogen gas was passed through the solution for 5 min. Tetrakis(triphenylphosphine)palladium(0) (81 mg, 0.07 mmol) was added, and the reaction mixture was heated at 60 °C until the reaction was complete (determined by TLC). The crude mixture was evaporated in vacuo and purified using silica gel column chromatography (DCM/MeOH = 98:2→90:10), yielding title compounds **9a−b**.

5-(4-Fluorophenyl)-2′-deoxyuridine (9a). This compound was prepared according to the general procedure described above starting from **7a** using 4-fluorophenylboronic acid (119 mg), yielding compound **9a** (178 mg, 78%). ^{1}H NMR (300 MHz, CD_3OD) δ 8.31 (s, 1H, H-6), 7.59 (dd, 2H, J = 8.9, 5.4 Hz, ArH), 7.11 (t, 2H, J = 8.9 Hz, ArH), 6.38 (t, 1H, J = 6.5 Hz, H-1′), 4.46 (dd, 1H, J = 8.6, 4.9 Hz, H-3′), 3.97 (q, 1H, J = 3.1 Hz, H-4′), 3.80 (ddd, 2H, J = 25.7, 11.9, 2.9 Hz, H-5′), 2.34 (m, 2H, H-2′). ^{13}C NMR (75 MHz, CD_3OD) δ 163.95, 161.93 (d, 1C, J_{CF} = 185.4 Hz), 150.47, 138.35, 129.87 (d, 2C, J_{CF} = 8.0 Hz), 129.14 (d, 1C, J_{CF} = 3.3 Hz), 114.59 (d, 2C, J_{CF} = 21.5 Hz), 113.67, 87.63, 85.40, 70.60, 61.08, 40.31. HRMS (ESI) calcd for $C_{15}H_{15}FN_2O_5$ 321.0892 (M − H^+); found 321.0894.

5-(3-Methoxyphenyl)-2′-deoxyuridine (9b). This compound was prepared according to the general procedure described above starting from **7a** using 3-methoxyphenylboronic acid (129 mg), yielding compound **9b** (223 mg, 95%). ^{1}H NMR (300 MHz, CD_3OD) δ 8.31 (s, 1H, H-6), 7.28 (t, 1H, J = 7.9 Hz, ArH), 7.17 (s, 1H, ArH), 7.13 (d, 1H, J = 7.9 Hz, ArH), 6.89 (dd, 1H, J = 6.9, 2.2 Hz, ArH), 6.37 (t, 1H, J = 6.5 Hz, H-1′), 4.46 (m, 1H, H-3′), 3.97 (m, 1H, H-4′), 3.82 (s, 3H, OCH_3), 3.79 (ddd, 2H, J = 24.3, 12.0, 3.1 Hz, H-5′), 2.35 (m, 2H, H-2′). ^{13}C NMR (75 MHz, DMSO-d_6) δ 162.47, 159.54, 150.36, 138.53, 135.00, 129.51, 120.66, 114.00, 113.72, 113.27, 88.09, 85.11, 70.77, 61.55, 55.51, 40.61. HRMS (ESI) calcd for $C_{16}H_{18}N_2O_6$ 333.1092 (M − H^+); found 333.1107.

3′,5′-di-*O*-Acetyl-2′-deoxyuridine-5-heptynoic Acid (12a). Compound **11a** (1.68 g, 3.83 mmol), $Pd(PPh_3)_4$ (433 mg, 0.382 mmol), and CuI (147 mg, 0.764 mmol) were dissolved in anhydrous DMF (20 mL) under an Ar atmosphere, and DIPEA (1.32 mL, 7.67 mmol) and heptynoic acid (1.45 mL, 11.50 mmol) were then added. After being stirred at rt for 22 h, the solvent was evaporated and coevaporated with xylene. The residue was taken up in DCM/MeOH = 1:1 (40 mL), BioRad XG-X8 (HCO_3^--form) resin was added, and the mixture was stirred at rt for 30 min. After filtration of the resin and evaporation of the solvents, the crude residue was taken up in EtOAc (70 mL), and the organic layer was washed with brine (3 × 70 mL), dried over $MgSO_4$, and evaporated. The residue was purified by silica gel column chromatography (heptane/EtOAc = 2:3 containing 1% formic acid), and early and late fractions were subjected to a second column using the same eluent to yield compound **12a** (783 mg, 47%) as an off-white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.05 (br s, 1H, COOH), 11.65 (br s, 1H, NH), 7.87 (s, 1H, H-6), 6.14 (t, 1H, J = 7.2 Hz, H-1′), 5.18 (m, 1H, H-3′), 4.26 (d, 2H, J = 4.3 Hz, H-5′), 4.19 (m, 1H, H-4′), 2.54−2.28 (m, 2H, H-2′), 2.39 (t, 2H, J = 6.9 Hz, α-CH_2), 2.24 (t, 2H, J = 7.2 Hz, CH_2COOH), 2.08 (s, 3H, CH_3), 2.06 (s, 3H, CH_3), 1.65−1.45 (m, 4H, 2 × CH_2). ^{13}C NMR (75 MHz, DMSO-d_6) δ 174.32, 170.05, 161.59, 149.42, 142.36, 99.54, 93.30, 81.46, 73.81, 72.69, 63.56, 36.09, 33.15, 27.63, 23.83, 20.76, 20.55, 18.54. MS (ESI) calcd for $C_{20}H_{23}N_2O_9$ 435.14 (M − H^+), found 435.16.

2′,3′,5′-tri-*O*-Acetyl-1-β-D-arabinofuranosyluracil-5-heptynoic Acid (12b). Compound **11b** (2 g, 4.03 mmol), $Pd(PPh_3)_4$ (455 mg, 0.403 mmol), and CuI (155 mg, 0.8 mmol) were dissolved in anhydrous DMF (20 mL) under an Ar atmosphere, and DIPEA (1.39 mL, 8.08 mmol) and heptynoic acid (1.52 mL, 12.09 mmol) were then added. After being stirred at rt overnight, the solvent was evaporated and coevaporated with xylene. The residue was taken up in DCM/MeOH = 1:1 (40 mL), BioRad XG-X8 (HCO_3^--form) resin was added, and the mixture was stirred at rt for 30 min. After filtration of the resin and evaporation of the solvents the crude residue was taken up in EtOAc (80 mL), and the organic layer was washed with brine (3 × 80 mL), dried over $MgSO_4$, and evaporated. The residue was purified by silica gel column chromatography (heptane/EtOAc = 2:3 containing 1% formic acid), and early and late fractions were subjected to a second column using the same eluent to yield compound **12b** (1.04 g, 52%) as an off-white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.02 (br s, 1H, COOH), 11.72 (br s, 1H, NH), 7.78 (s, 1H, H-6), 6.23 (d, 1H, J = 5.3 Hz, H-1′), 5.41 (m, 1H, H-2′), 5.25 (m, 1H, H-3′), 4.35 (d, 2H, J = 4.3 Hz, H-5′), 4.26 (m, 1H, H-4′), 2.38 (t, 2H, J = 6.8 Hz, α-CH_2), 2.24 (t, 2H, J = 7.2 Hz, CH_2COOH), 2.09 (s, 6H, 2 × CH_3), 1.96 (s, 3H, CH_3), 1.63−1.47 (m, 4H, 2 × CH_2). ^{13}C NMR (75 MHz, DMSO-d_6) δ 174.25, 169.99, 169.69, 168.79, 161.34, 148.95, 142.83, 98. 81, 93.28, 82.97, 78.08, 74.90, 74. 36, 72.38, 62.54, 33.13, 27.57, 23.80, 20.56, 20.54, 20.13, 18.51. MS (ESI) calcd (M − H^+) $C_{22}H_{25}N_2O_{11}$, 493.15; found, 493.14.

3′,5′-di-*O*-Acetyl-2′-deoxyuridine-5-(*N*-butyl)heptynamide (13a). To a solution of compound **12a** (350 mg, 0.802 mmol) and HOBt (124 mg, 0.924 mmol) in anhydrous DCM (13 mL) under an Ar atmosphere was added *n*-butylamine (92 μL, 0.924 mmol), and the mixture was cooled to 0 °C. DIC (125 μL, 0.807 mmol) was added, and the reaction mixture was stirred at 0 °C for 1 h and at rt for 5 h. The solvent was evaporated and the residue taken up in EtOAc (25 mL). The organic layer was washed with satd $NaHCO_3$ (3 × 20 mL), dried over $MgSO_4$, and evaporated. The residue was purified by silica gel column chromatography (heptane/EtOAc = 1:3 containing 1% formic acid), which gave compound **13a** (321 mg, 81%) as an off-white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.54 (br s, 1H, NH), 7.84 (s, 1H, H-6),

7.61 (t, 1H, J = 5.3 Hz, amide NH), 6.14 (t, 1H, J = 7 Hz, H-1′), 5.19 (m, 1H, H-3′), 4.26 (d, 2H, J = 4.3 Hz, H-5′), 4.19 (m, 1H, H-4′), 3.03 (m, 2H, CH_2NH), 2.53−2.28 (m, 2H, H-2′), 2.37 (t, 2H, J = 6.9 Hz, α-CH_2), 2.11−2.03 (m, 8H, 2 × CH_3 and CH_2CO), 1.65−1.21 (m, 8H, 4 × CH_2), 0.87 (t, 3H, J = 7.2 Hz). ^{13}C NMR (75 MHz, DMSO-d_6) δ 171.59, 170.02 (2C), 161.60, 149.40, 142.34, 99.55, 93.40, 84.70, 81.43, 73.78, 72.63, 63.55, 38.04, 36.08, 34.82, 31.28, 27.73, 24.64, 20.75, 20.54, 19.56, 18.55, 13.65. MS (ESI) calcd for $C_{24}H_{33}N_3O_8$ 492.23 (M + H^+), 514.22 (M + Na^+), 1005.44 (2M + Na^+); found 492.11, 514.10, 1004.86.

2′,3′,5′-tri-*O*-Acetyl-1-β-D-arabinofuranosyluracil-5-(*N*-butyl)-heptynamide (13b). To a solution of compound **12b** (350 mg, 0.712 mmol) and HOBt (110 mg, 0.82 mmol) in anhydrous DCM (12 mL) under an Ar atmosphere was added *n*-butylamine (82 μL, 0.82 mmol), and the mixture was cooled to 0 °C. DIC (117 μL, 0.758 mmol) was added, and the reaction mixture was stirred at 0 °C for 1 h and at rt for 4 h. The solvent was evaporated and the residue taken up in EtOAc (25 mL). The organic layer was washed with satd $NaHCO_3$ (3 × 20 mL), dried over $MgSO_4$, and evaporated. The residue was purified by silica gel column chromatography (heptane/EtOAc = 1:3 containing 1% formic acid), which gave compound **13b** (333 mg, 85%) as an off-white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.71 (br s, 1H, NH), 7.77 (s, 1H, H-6), 7.72 (t, 1H, J = 6.0 Hz, amide NH), 6.23 (d, 1H, J = 5.3 Hz, H-1′), 5.41 (t, 1H, J = 4.5 Hz, H-2′), 5.26 (t, 1H, J = 4.6 Hz, H-3′), 4.35 (m, 2H, H-5′), 4.25 (m, 1H, H-4′), 3.02 (m, 2H, CH_2NH), 2.37 (t, 2H, J = 6.9 Hz, α-CH_2), 2.11−2.03 (m, 8H, 2 × CH_3 and CH_2CO), 1.96 (s, 3H, CH_3), 1.63−1.20 (m, 8H, 4 × CH_2), 0.86 (t, 3H, J = 7.1 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 171.57, 169.97, 169.69, 161.35, 148.94, 142.80, 98.83, 93.40, 82.96, 78.03, 74.86, 74.36, 72.31, 62.53, 38.02, 34.80, 31.27, 27.69, 24.62, 20.53, 20.11, 19.54, 18.52, 13.64. MS (ESI) calcd for $C_{26}H_{35}N_3O_{10}$ 550.24 (M + H^+), 572.22 (M + Na^+), 1121.45 (2M + Na^+); found 550.10, 572.19, 1120.88.

2′-Deoxyuridine-5-(*N*-butyl)heptynamide (14a). Compound **13a** (276 mg, 0.562 mmol) was dissolved in dry MeOH (8 mL), NaOMe (30%-solution in MeOH, 152 μL, 0.8 mmol) was added, and the reaction mixture was stirred for 1 h at rt under an Ar atmosphere. The solution was neutralized with Dowex 50WX-8 resin (H^+-form), and the resin was filtered off and washed with MeOH. After evaporation of the filtrate the residue was purified by silica gel column chromatography (EtOAc/MeOH = 98:2), which yielded compound **14a** (204 mg, 89%) as an off-white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.55 (br s, 1H, NH), 8.15 (s, 1H, H-6), 7.76 (t, 1H, J = 5.4 Hz, amide NH), 6.11 (t, 1H, J = 6.6 Hz, H-1′), 5.24 (d, 1H, J = 4.2 Hz, OH), 5.14 (t, 1H, J = 4.8 Hz, OH), 4.23 (m, 1H, H-3′), 3.79 (m, 1H, H-4′), 3.59 (m, 2H, H-5′), 3.02 (m, 2H, CH_2NH), 2.36 (t, 2H, J = 6.9 Hz, α-CH_2), 2.13−2.05 (m, 4H, H-2′ and CH_2CO), 1.64−1.19 (m, 8H, 4 × CH_2), 0.86 (t, 3H, J = 7.2 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 171.81, 161.79, 149.48, 142.81, 99.00, 92.99, 87.53, 84.59, 73.00, 70.13, 60.97, 38.08, 34.86, 31.27, 27.74, 24.62, 19.57, 18.59, 13.67. MS (ESI) calcd for $C_{20}H_{29}N_3O_6$ 408.21 (M + H^+), 430.20 (M + Na^+), 837.40 (2M + Na^+); found 408.12, 430.12, 837.04. HRMS (ESI) calcd for $C_{20}H_{29}N_3O_6$ 446.1693 (M + K^+); found 446.1674.

1-β-D-Arabinofuranosyluracil-5-(*N*-butyl)heptynamide (14b). Compound **13b** (303 mg, 0.551 mmol) was dissolved in dry MeOH (8 mL), NaOMe (30%-solution in MeOH, 152 μL, 0.8 mmol) was added, and the reaction mixture was stirred for 1 h at rt under an Ar atmosphere. The solution was neutralized with Dowex 50WX-8 resin (H^+-form), and the resin was filtered off and washed with MeOH. After evaporation of the filtrate, the residue was purified by silica gel column chromatography (EtOAc/MeOH = 95:5), which yielded compound **14b** (206 mg, 88%) as an off-white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.55 (br s, 1H, NH), 7.84 (s, 1H, H-6), 7.75 (t, 1H, J = 5.4 Hz, amide NH), 5.96 (d, 1H, J = 4.4 Hz, H-1′), 5.60 (d, 1H, J = 5.1 Hz, OH), 5.47 (br s, 1H, OH), 5.15 (br s, 1H, OH), 4.00 (m, 1H, H-2′), 3.90 (m, 1H, H-3′), 3.74 (m, 1H, H-4′), 3.61 (m, 2H, H-5′), 3.02 (m, 2H, CH_2NH), 2.36 (t, 2H, J = 6.9 Hz, α-CH_2), 2.07 (t, 2H, J = 7.3 Hz, CH_2CO), 1.64−1.20 (m, 8H, 4 × CH_2), 0.86 (t, 3H, J = 7.2 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 171.77, 161.94, 149.44, 144.26, 97.45, 92.67, 85.29, 84.72, 75.21, 75.15, 73.06, 60.42, 38.07, 34.86, 31.27, 27.77, 24.65, 19.57, 18.57, 13.67. MS (ESI) calcd for $C_{20}H_{29}N_3O_7$ 424.21 (M + H^+), 446.19 (M + Na^+), 869.39 (2M + Na^+); found 424.06, 446.09, 868.96. HRMS (ESI) calcd for $C_{20}H_{29}N_3O_7$ 424.2078 (M + H^+), 446.1898 (M + Na^+), 462.1642 (M + K^+); found 424.2080, 446.1880, 462.1578.

***N*-(Prop-2-ynyl)heptane-1-sulfonamide (16).** Sodium 1-heptanesulfonate (1 g, 4.95 mmol) was refluxed in neat thionyl chloride overnight under an Ar atmosphere. The mixture was allowed to cool to rt, and then the volatiles were removed in vacuo and the residue was dissolved in dry DCM (15 mL) under an Ar atmosphere. A mixture of NEt_3 (705 μL, 5.01 mmol) and propargylamine (315 μL, 4.91 mmol) were subsequently added dropwise at 0 °C. The ice bath was removed, and the reaction mixture was stirred at rt overnight, diluted with DCM (25 mL), washed with H_2O, satd $NaHCO_3$, and again H_2O (40 mL each), dried over $MgSO_4$, and evaporated to yield sulfonamide **16** (535 mg, 50%) as a red oil. This product was used in the Sonogashira coupling step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.53 (m, 1H, NH), 3.95 (m, 2H, α-CH_2), 3.14 (m, 2H, CH_2SO_2), 2.35 (t, 1H, J = 2.5 Hz, acetylenic H), 1.85 (m, 2H, C$H_2CH_2SO_2$), 1.48−1.19 (m, 8H, 4 × CH_2), 0.89 (t, 3H, J = 6.9 Hz, CH_3) ^{13}C NMR (75 MHz, $CDCl_3$) δ 79.19, 73.09, 53.73, 32.76, 31.61, 28.87, 28.34, 23.74, 22.66, 14.15. MS (ESI) calcd for $C_{10}H_{19}NO_2S$ 216.11 (M − H^+); found 215.70.

1-Hexyl-3-(prop-2-ynyl)urea (17). Propargylamine (500 μL, 7.80 mmol) was dissolved in anhydrous DCM (20 mL) under an Ar atmosphere, and the resulting yellow solution was cooled to 0 °C. Hexyl isocyanate (995 mg, 7.82 mmol) dissolved in 10 mL of anhydrous DCM was added drop- to portionwise, the ice bath was removed, and the reaction mixture was stirred at rt for 45 min. The solvent was evaporated to yield compound **17** (1.39 g, 98%) as an off-white solid, which was used in the following step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.51 (br s, 1H, NH), 5.32 (br s, 1H, NH), 3.97 (m, 2H, α-CH_2), 3.16 (m, 2H, CH_2NH), 2.20 (t, 1H, J = 2.5 Hz, acetylenic H), 1.48 (m, 2H, CH_2CH_2NH), 1.29 (m, 6H, 3 × CH_2), 0.88 (t, 3H, J = 6.7 Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.43, 81.15, 70.91, 40.65, 31.69, 30.33, 30.12, 26.73, 22.71, 14.14. MS (ESI) calcd for $C_{10}H_{18}N_2O$ 183.15 (M + H^+); found 183.09.

***N*-(3-(5-(2′-Deoxyuridine))prop-2-ynyl)octanamide (18a).** Compound **11a** (400 mg, 0.913 mmol), $Pd(PPh_3)_4$ (103 mg, 0.091 mmol), and CuI (35 mg, 0.182 mmol) were dissolved in anhydrous DMF (5 mL) under an Ar atmosphere, and DIPEA (313 μL, 1.82 mmol) and acetylene **15a** (496 mg, 2.74 mmol) were then added. After being stirred at rt for 12 h, the solvent was evaporated and coevaporated with xylene. The residue was taken up in DCM/MeOH = 1:1 (20 mL), BioRad XG-X8 (HCO_3^--form) resin was added, and the mixture was stirred at rt for 30 min. After filtration of the resin and evaporation of the solvents, the crude residue was taken up in EtOAc (30 mL) and the organic layer was washed with brine (3 × 30 mL), dried over $MgSO_4$, and evaporated. The residue was purified by silica gel column chromatography (cyclohexane/EtOAc = 1:1 containing 1% formic acid), and the obtained intermediate was dissolved in dry MeOH (14 mL). NaOMe (30%-solution in MeOH, 265 μL, 1.4 mmol) was added, and the reaction mixture was stirred for 1 h at rt under an Ar atmosphere. The solution was neutralized with Dowex 50WX-8 resin (H^+-form), and the resin was filtered off and washed with MeOH. After evaporation of the filtrate, the residue was purified by silica gel column chromatography (EtOAc/MeOH = 98:2 containing 0.5% formic acid), which yielded compound **18a** (251 mg, 68% over 2 steps) as an off-white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.61 (br s, 1H, NH), 8.29 (t, 1H, J = 5.4 Hz, amide NH), 8.16 (s, 1H, H-6),

6.11 (t, 1H, J = 6.7 Hz, H-1′), 5.25 (d, 1H, J = 3.9 Hz, OH), 5.10 (t, 1H, J = 4.7 Hz, OH), 4.22 (m, 1H, H-3′), 4.06 (d, 2H, J = 5.4 Hz, α-CH_2), 3.79 (m, 1H, H-4′), 3.63−3.53 (m, 2H, H-5′), 2.10 (m, 4H, H-2′ and CH_2CO), 1.48 (m, 2H, CH_2CH_2CO), 1.24 (m, 8H, 4 × CH_2), 0.85 (t, 3H, J = 7.0 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 171.83, 161.59, 149.41, 143.60, 98.15, 89.78, 87.62, 84.70, 74.19, 70.24, 61.03, 35.07, 31.13, 28.60, 28.47, 28.41, 25.11, 22.03, 13.92. MS (ESI) calcd for $C_{20}H_{29}N_3O_6$ 408.21 (M + H^+), 430.20 (M + Na^+), 837.40 (2M + Na^+); found 408.09, 430.05, 836.95. HRMS (ESI) calcd for $C_{20}H_{29}N_3O_6$ 446.1693 (M + K^+); found 446.1670.

***N*-(3-(5-(2′-Deoxyuridine))prop-2-ynyl)hexanamide (18b).** Compound **11a** (400 mg, 0.913 mmol), $Pd(PPh_3)_4$ (103 mg, 0.091 mmol), and CuI (35 mg, 0.182 mmol) were dissolved in anhydrous DMF (5 mL) under an Ar atmosphere, and DIPEA (313 μL, 1.82 mmol) and acetylene **15b** (420 mg, 2.74 mmol) were then added. After being stirred at rt for 14 h, the solvent was evaporated and coevaporated with xylene. The residue was taken up in DCM/MeOH = 1:1 (10 mL), BioRad XG-X8 (HCO_3^--form) resin was added, and the mixture was stirred at rt for 30 min. After filtration of the resin and evaporation of the solvents, the crude residue was taken up in EtOAc (30 mL), and the organic layer was washed with brine (3 × 30 mL), dried over $MgSO_4$, and evaporated. The residue was purified by silica gel column chromatography (heptane/EtOAc = 2:3 containing 1% formic acid), and the obtained intermediate was dissolved in dry MeOH (14 mL). NaOMe (30% solution in MeOH, 265 μL, 1.4 mmol) was added, and the reaction mixture was stirred for 1 h at rt under an Ar atmosphere. The solution was neutralized with Dowex 50WX-8 resin (H^+-form), and the resin was filtered off and washed with MeOH. After evaporation of the filtrate, the residue was purified by silica gel column chromatography (EtOAc/MeOH = 98:2 containing 1% formic acid), which yielded compound **18b** (209 mg, 60% over 2 steps) as an off-white solid. ^{1}H NMR (300 MHz, $CDCl_3$:MeOD = 5:2) δ 8.33 (s, 1H, H-6), 6.25 (t, 1H, J = 6.5 Hz, H-1′), 4.43 (m, 1H, H-3′), 4.13 (br s, 2H, α-CH_2), 3.98 (m, 1H, H-4′), 3.87 (dd, 1H, J = 12, 2.9 Hz, H-5′), 3.77 (dd, 1H, J = 12, 2.9 Hz, H-5′), 2.40−2.34 (m, 1H, H-2′), 2.23−2.14 (m, 3H, H-2′ and CH_2CO), 1.63 (m, 2H, CH_2CH_2CO), 1.33 (m, 4H, 2 × CH_2), 0.90 (t, 3H, J = 7 Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$:MeOD = 5:2) δ 174.31, 163.12, 149.54, 143.91, 98.83, 88.98, 87.52, 85.71, 73.85, 70.36, 61.19, 40.79, 35.93, 31.17, 29.53, 25.10, 22.09, 13.48. MS (ESI) calcd for $C_{18}H_{25}N_3O_6$ 380.18 (M + H^+), 402.16 (M + Na^+), 781.33 (2M + Na^+); found 379.72, 401.67, 780.43. HRMS (ESI) calcd for $C_{18}H_{25}N_3O_6$ 402.1636 (M + Na^+), 418.1380 (M + K^+); found 402.1656, 418.1379.

***N*-(3-(5-(2′-Deoxyuridine))prop-2-ynyl)butanamide (18c).** Compound **11a** (400 mg, 0.913 mmol), $Pd(PPh_3)_4$ (103 mg, 0.091 mmol), and CuI (35 mg, 0.182 mmol) were dissolved in anhydrous DMF (4 mL) under an Ar atmosphere, and DIPEA (313 μL, 1.82 mmol) and then acetylene **15c** (343 mg, 2.74 mmol, dissolved in 1 mL anhydrous DMF) were then added. After being stirred at rt for 18 h, the solvent was evaporated and coevaporated with xylene. The residue was taken up in DCM/MeOH = 1:1 (10 mL), BioRad XG-X8 (HCO_3^--form) resin was added, and the mixture was stirred at rt for 30 min. After filtration of the resin and evaporation of the solvents, the crude residue was taken up in EtOAc (30 mL), and the organic layer was washed with brine (3 × 30 mL), dried over $MgSO_4$, and evaporated. The residue was purified by silica gel column chromatography (heptane/EtOAc = 1:2→1:3 each containing 1% formic acid), and the obtained intermediate was dissolved in dry MeOH (14 mL). NaOMe (30% solution in MeOH, 265 μL, 1.4 mmol) was added, and the reaction mixture was stirred for 1 h at rt under an Ar atmosphere. The solution was neutralized with Dowex 50WX-8 resin (H^+-form), and the resin was filtered off and washed with MeOH. After evaporation of the filtrate, the residue was purified by silica gel column chromatography (EtOAc/MeOH = 98:2 containing 1% formic acid), which yielded compound **18c** (193 mg, 60% over 2 steps) as an off-white solid. ^{1}H NMR (300 MHz, $CDCl_3$:MeOD = 5:2) δ 8.37 (s, 1H, H-6), 6.26 (t, 1H, J = 6.4 Hz, H-1′), 4.44 (m, 1H, H-3′), 4.14 (s, 2H, α-CH_2), 3.99 (m, 1H, H-4′), 3.89 (dd, 1H, J = 12, 2.6 Hz, H-5′), 3.78 (dd, 1H, J = 12, 2.6 Hz, H-5′), 2.41−2.34 (m, 1H, H-2′), 2.23−2.16 (m, 3H, H-2′ and CH_2CO), 1.66 (m, 2H, CH_2CH_2CO), 0.96 (t, 3H, J = 7.4 Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$:MeOD = 5:2) δ 174.14, 163.09, 149.53, 143.90, 98.82, 88.97, 87.51, 85.70, 73.82, 70.35, 61.19, 40.77, 37.79, 29.50, 18.81, 13.23. MS (ESI) calcd for $C_{16}H_{21}N_3O_6$ 352.15 (M + H^+), 374.13 (M + Na^+), 725.28 (2M + Na^+); found 351.74, 373.68, 724.37. HRMS (ESI) calcd for $C_{16}H_{21}N_3O_6$ 390.1067 (M + K^+); found 390.1058.

2-Phenyl-*N*-(3-(5-(2′-deoxyuridine))prop-2-ynyl)acetamide (18d). Compound **11a** (400 mg, 0.913 mmol), $Pd(PPh_3)_4$ (103 mg, 0.091 mmol), and CuI (35 mg, 0.182 mmol) were dissolved in anhydrous DMF (5 mL) under an Ar atmosphere, and DIPEA (313 μL, 1.82 mmol) and then acetylene **15d** (474 mg, 2.74 mmol) were then added. After being stirred at rt for 14 h, the solvent was evaporated and coevaporated with xylene. The residue was taken up in DCM:MeOH = 1:1 (10 mL), BioRad XG-X8 (HCO_3^--form) resin was added, and the mixture was stirred at rt for 30 min. After filtration of the resin and evaporation of the solvents, the crude residue was taken up in EtOAc (30 mL), and the organic layer was washed with brine (3 × 30 mL), dried over $MgSO_4$, and evaporated. The residue was purified by silica gel column chromatography (heptane:EtOAc = 1:2→1:3 each containing 1% formic acid) and the obtained intermediate was dissolved in dry MeOH (14 mL). NaOMe (30% solution in MeOH, 265 μL, 1.4 mmol) was added, and the reaction mixture was stirred for 1 h at rt under an Ar atmosphere. The solution was neutralized with Dowex 50WX-8 resin (H^+-form), the resin was filtered off and washed with MeOH. After evaporation of the filtrate, the residue was purified by silica gel column chromatography (EtOAc:MeOH = 98:2 containing 1% formic acid), which yielded compound **18d** (231 mg, 63% over 2 steps) as an off-white solid. ^{1}H NMR (300 MHz, MeOD) δ 8.29 (s, 1H, H-6), 7.35−7.19 (m, 5H, ArH), 6.23 (t, 1H, J = 6.6 Hz, H-1′), 4.38 (m, 1H, H-3′), 4.14 (s, 2H, α-CH_2), 3.94 (m, 1H, H-4′), 3.82−3.68 (ddd, 2H, J = 24.5, 12, 3.4 Hz, H-5′), 3.52 (s, 2H, CH_2Ph), 2.34−2.15 (m, 2H, H-2′). ^{13}C NMR (75 MHz, MeOD) δ 173.63, 164.53, 151.14, 145.44, 136.64, 130.13 (2C), 129.58 (2C), 127.93, 99.91, 89.92, 89.17, 87.06, 75.25, 72.05, 62.62, 43.63, 41.70, 30.63. MS (ESI) calcd for $C_{20}H_{21}N_3O_6$ 400.15 (M + H^+), 422.13 (M + Na^+), 821.28 (2M + Na^+); found 399.77, 421.63, 821.55. HRMS (ESI) calcd for $C_{20}H_{21}N_3O_6$ 438.1067 (M + K^+); found 438.1040.

2-Phenyl-*N*-(3-(5-(5′-*O*-acetyl-2′-deoxyuridine))prop-2-ynyl)-acetamide (23). Compound **22** (200 mg, 0.505 mmol), $Pd(PPh_3)_4$ (58 mg, 0.051 mmol), and CuI (20 mg, 0.104 mmol) were dissolved in anhydrous DMF (3 mL) under an Ar atmosphere, and DIPEA (174 μL, 1.01 mmol) and then acetylene **15d** (262 mg, 1.51 mmol) were then added. After being stirred at rt for 16 h, the solvent was evaporated and coevaporated with xylene. The residue was taken up in DCM/MeOH = 1:1 (10 mL), BioRad XG-X8 (HCO_3^--form) resin was added, and the mixture was stirred at rt for 30 min. After filtration of the resin and evaporation of the solvents, the crude residue was purified by a first column of silica gel (DCM/MeOH = 98:2→97:3 each containing 1% formic acid), and the obtained product was subjected to a second short column of silica gel (DCM/MeOH = 95:5 containing 1% formic acid), which yielded compound **23** (118 mg, 53%) as an off-white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.97 (br s, 1H, NH), 8.23 (s, 1H, H-6), 7.27 (m, 5H, ArH), 6.88 (t, 1H, J = 5 Hz, amide NH), 6.24 (t, 1H, J = 6.9 Hz, H-1′), 5.32 (m, 1H, H-3′), 4.12 (m, 3H, α-CH_2 and H-4′), 3.86 (m, 2H, H-5′), 3.57 (s, 2H, CH_2CO), 2.47 (m, 2H, H-2′), 2.08 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.14, 170.82, 162.69, 149.58, 144.58, 134.61, 129.54 (2C), 128.95 (2C), 127.40, 99.45, 89.65, 86.08, 85.82, 75.05, 74.26, 62.20, 43.20, 38.32, 30.44, 21.11. MS (ESI) calcd for $C_{22}H_{23}N_3O_7$ 442.16 (M + H^+), 464.14 (M + Na^+), 905.30 (2M + Na^+); found 441.68, 463.65, 904.37.

***N*-(3-(5-(3′-*O*-Acetyl-2′-deoxyuridine))prop-2-ynyl)heptane-1-sulfonamide (24).** Compound **22** (325 mg, 0.82 mmol), Pd-$(PPh_3)_4$ (91 mg, 0.082 mmol), and CuI (32 mg, 0.164 mmol) were dissolved in anhydrous DMF (4 mL) under an Ar atmosphere, and DIPEA (283 μL, 1.64 mmol) and then acetylene **16** (535 mg, 2.46 mmol) dissolved in anhydrous DMF (1 mL) were added. After being stirred at rt overnight, the solvent was evaporated and coevaporated with xylene. The residue was taken up in DCM/MeOH = 1:1 (10 mL), BioRad XG-X8 (HCO_3^--form) resin was added, and the mixture was stirred at rt for 30 min. After filtration of the resin and evaporation of the solvents, the crude residue was purified by a first column of silica gel (heptane/EtOAc = 1:1 containing 1% formic acid) and the obtained product was subjected to a second short column of silica gel (DCM/acetone =85:15 containing 1% formic acid), which yielded compound **24** (225 mg, 57%) as an off-white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.70 (br s, 1H, NH), 8.24 (s, 1H, H-6), 7.64 (br s, 1H, amide NH), 6.14 (t, 1H, J = 7.1 Hz, H-1′), 5.29 (br s, 1H, OH), 5.22 (m, 1H, H-3′), 4.04 (br m, 1H, H-4′), 3.98 (d, 2H, J = 5.2 Hz, α-CH_2), 3.64 (d, 2H, J = 2.5 Hz, H-5′), 3.13 (m, 2H, CH_2SO_2), 2.29 (m, 2H, H-2′), 2.06 (s, 3H, CH_3), 1.65 (m, 2H, $CH_2CH_2SO_2$), 1.49−1.12 (m, 8H, 4 × CH_2), 0.82 (t, 3H, J = 7.0 Hz, CH_3) ^{13}C NMR (75 MHz, DMSO-d_6) δ 169.98, 161.44, 149.38, 143.49, 98.21, 88.97, 85.07, 84.57, 75.80, 74.62, 61.16, 52.15, 37.30, 32.38, 30.97, 28.26, 27.47, 23.20, 21.92, 20.80, 13.85 MS (ESI) calcd for $C_{21}H_{31}N_3O_8S$ 486.19 (M + H^+), 508.17 (M + Na^+); found 486.3, 508.2.

1-Hexyl-(3-(5-(3′-*O*-acetyl-2′-deoxyuridine))prop-2-ynyl)-urea (25). Compound **22** (300 mg, 0.756 mmol), $Pd(PPh_3)_4$ (84 mg, 0.075 mmol), and CuI (29 mg, 0.151 mmol) were dissolved in anhydrous DMF (5 mL) under an Ar atmosphere, and DIPEA (261 μL, 1.52 mmol) and then acetylene **17** (415 mg, 2.27 mmol) were added. After being stirred at rt overnight, the solvent was evaporated and coevaporated with xylene. The residue was taken up in DCM/MeOH = 1:1 (10 mL), BioRad XG-X8 (HCO_3^--form) resin was added, and the mixture was stirred at rt for 30 min. After filtration of the resin and evaporation of the solvents, the crude residue was purified by a first column of silica gel (heptane/EtOAc = 1:2→1:3→1:4 each containing 1% formic acid), and the obtained product was subjected to a second short column of silica gel (DCM/acetone = 80:20→75:25 each containing 1% formic acid), which yielded compound **25** (259 mg) as an oil containing substantial amounts of impurities and was therefore used in the phosphorylation step without any further purification.

General Procedure A[33,46] for the Phosphorylation of 5-Substituted Derivatives 8, 9a−b, 18b−c. An unprotected 5-substituted derivative (1 equiv) was dissolved in $PO(OMe)_3/CH_3CN$ = 1:1, and dry pyridine (4.8 equiv) and H_2O (2.8 equiv) were added and the resulting solution was cooled to 0 °C. $POCl_3$ (4.4 equiv) was added in one portion, and the reaction mixture was stirred at 0 °C for 3 h. The reaction mixture was poured into ice/H_2O and brought to pH 8−9 with NH_4OH, and then the volatiles were removed in vacuo and the residue was purified by silica gel column chromatography using a gradient of 2-propanol/water/NH_4OH. The phosphates isolated after lyophilization were further purified by preparative RP-HPLC (C18, 5 μM, 19 mm × 150 mm) using a gradient of acetonitrile and TEAB (4−10 mM), yielding analytically pure compounds. Next, the phosphates were subjected to ion exchange (Dowex 50 WX-8, Na^+) and lyophilized, yielding the corresponding disodium salts.

General Procedure B[37,38] for the Phosphorylation of 5-Substituted Derivatives 10a−b, 14a−b, 18a. To a solution of an unprotected 5-substituted derivative (1 equiv) and proton sponge (1.5−3 equiv) in trimethyl phosphate was added $POCl_3$ (1.5−3 equiv) in one portion at 0 °C, and the reaction mixture was stirred at 0 °C for 2−3 h. The reaction mixture was poured into ice/H_2O and brought to pH 8−9 with NH_4OH or 1 M NaOH, and then the volatiles were removed in vacuo and the residue was purified by silica gel column chromatography using a gradient of 2-propanol/water/NH_4OH. The phosphates isolated after lyophilization were further purified by preparative RP-HPLC (C18, 5 μM, 19 mm × 150 mm) using a gradient of acetonitrile and TEAB (4−30 mM), yielding analytically pure compounds. Next, the phosphates were subjected to ion exchange (Dowex 50 WX-8, Na^+) and lyophilized, yielding the corresponding disodium salts.

5-(2-Cyanovinyl)-2′deoxyuridine-5′-monophosphate (1). This compound was prepared according to the general procedure A described above. The reaction mixture was worked up as described above including silica gel column chromatography (2-propanol/NH_4OH/H_2O = 90:5:5→75:15:15) and RP-HPLC (10 mM aq TEAB/CH_3CN = 95:5→80:20, 16 mL/min), yielding title compound **1** (49 mg, 49%) as a white solid. ^{1}H NMR (500 MHz, D_2O) δ 8.15 (s, 1H, H-6), 7.35 (d, 1H, J = 16.4 Hz, vinylic H), 6.52 (d, 1H, J = 16.4 Hz, vinylic H), 6.30 (t, 1H, J = 6.8 Hz, H-1′), 4.53 (m, 1H, H-3′), 4.12 (q, 1H, J = 3.6 Hz, H-4′), 3.92 (d, 2H, J = 4.4 Hz, H-5′), 2.35 (m, 2H, H-2′). ^{13}C NMR (125 MHz, D_2O) δ 161.82, 156.10, 146.12, 143.99, 120.61, 109.91, 94.10, 86.08 (d, 1C, J_{CP} = 8.3 Hz), 85.71, 71.03, 63.49, 39.17. ^{31}P NMR (121 MHz, D_2O) δ 3.97. MS (ESI) calcd for $C_{12}H_{14}N_3O_8P$ 358.0446 (M − H^+); found 358.0431.

5-(4-Fluorophenyl)-2′-deoxyuridine-5′-monophosphate (2a). This compound was prepared according to the general procedure A described above. The reaction mixture was worked up as described above, including silica gel column chromatography (2-propanol/NH_4OH/H_2O = 90:5:5→75:15:15) and RP-HPLC (10 mM aq TEAB:CH_3CN = 95:5→80:20, 16 mL/min), yielding title compound **2a** (53 mg, 24%) as a white solid. ^{1}H NMR (500 MHz, D_2O) δ 7.84 (s, 1H, H-6), 7.45 (dd, 2H, J = 8.6, 5.5 Hz, ArH), 7.14 (t, 2H, J = 8.9 Hz, ArH), 6.33 (t, 1H, J = 7.0 Hz, H-1′), 4.52 (m, 1H, H-3′), 4.16 (m, 1H, H-4′), 4.04 (m, 2H, H-5′), 2.39 (m, 2H, H-2′). ^{13}C NMR (125 MHz, D_2O) δ 164.74, 162.33 (d, 1C, J_{CF} = 244.5 Hz), 151.19, 138.48, 130.60 (d, 2C, J_{CF} = 8.4 Hz), 127.76 (d, 1C, J_{CF} = 2.2 Hz), 115.31 (d, 2C, J_{CF} = 21.8 Hz), 115.11, 85.55 (d, 1C, J_{CP} = 12.0 Hz), 85.45, 70.95, 64.83 (d, 1C, J_{CP} = 4.5 Hz), 38.76. ^{31}P NMR (121 MHz, D_2O) δ 0.015. MS (ESI) calcd for $C_{15}H_{16}FN_2O_8P$ 401.0555 (M − H^+); found 401.0569.

5-(3-Methoxyphenyl)-2′-deoxyuridine-5′-monophosphate (2b). This compound was prepared according to the general procedure A described above. The reaction mixture was worked up as described above, including silica gel column chromatography (2-propanol/NH_4OH/H_2O = 90:5:5→75:15:15) and RP-HPLC (10 mM aq TEAB/CH_3CN = 95:5→ 80:20, 16 mL/min), yielding title compound **2b** (102 mg, 37%) as a white solid. ^{1}H NMR (300 MHz, D_2O) δ 7.65 (s, 1H, H-6), 7.26 (t, 1H, J = 8.2 Hz, ArH), 6.94 (m, 2H, ArH), 6.87 (dd, 1H, J = 8.2, 1.6 Hz, ArH), 6.16 (t, 1H, J = 6.8 Hz, H-1′), 4.43 (m, 1H, H-3′), 4.09 (m, 1H, H-4′), 4.03 (m, 2H, H-5′), 3.73 (s, 3H, OCH_3), 2.29 (m, 2H, H-2′). ^{13}C NMR (75 MHz, D_2O) δ 163.93, 158.35, 150.65, 138.23, 132.91, 129.59, 120.90, 114.86, 113.78, 113.46, 85.60, 85.06 (d, 1C, J_{CP} = 8.2 Hz), 70.63, 65.00 (d, 1C, J_{CP} = 4.9 Hz), 55.02, 38.55. ^{31}P NMR (121 MHz, D_2O) δ 0.014. MS (ESI) calcd for $C_{16}H_{19}N_2O_9P$ 413.0755 (M − H^+); found 413.0731.

5-Dodecynyl-2′-deoxyuridine-5′-monophosphate (3a). This compound was prepared according to the general procedure B described above. The following amounts of starting material and reagents were employed: compound **10a** (500 mg, 1.29 mmol), $POCl_3$ (181 μL, 1.94 mmol), and proton sponge (416 mg, 1.94 mmol) in 5 mL of $PO(OMe)_3$. The reaction mixture was stirred at 0 °C for 2 h and worked up as described above, including silica gel column chromatography (2-propanol/NH_4OH/H_2O = 77.5:15:2.5→70:20:5) and RP-HPLC (4 mM aq TEAB/CH_3CN = 73:27→60:40, 16 mL/min), yielding title compound **3a** (159 mg, 24%) as a white solid; mp >151 °C (dec). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.77 (s, 1H, H-6), 6.08 (t, 1H, J = 6.9 Hz, H-1′), 4.27 (m, 1H, H-3′), 3.88 (m, 1H, H-4′), 3.79 (m, 2H, H-5′), 2.35 (t, 2H, J = 7.1 Hz, α-CH_2), 2.21−1.99 (m, 2H, H-2′), 1.48 (m, 2H, β-CH_2), 1.24 (m, 14H, 7 × CH_2), 0.85

(t, 3H, J = 6.7 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.72, 149.47, 142.25, 99.40, 93.68, 86.10 (d, 1C, J_{CP} = 6.3 Hz), 84.43, 72.54, 70.74, 64.11 (d, 1C, J_{CP} = 4.8 Hz), 39.40, 31.30, 28.98, 28.71, 28.56, 28.38, 28.19, 22.09, 18.88, 13.95. ^{31}P NMR (121 MHz, DMSO-d_6) δ 0.40. HRMS (ESI) calcd for $C_{21}H_{32}N_2O_8P$ 471.1902 ($M - H^+$); found 471.1884.

1-β-D-Arabinofuranosyl-5-dodecynyluracil-5′-monophosphate (3b). This compound was prepared according to the general procedure B described above. The following amounts of starting material and reagents were employed: compound **10b** (500 mg, 1.22 mmol), $POCl_3$ (171 μL, 1.83 mmol), and proton sponge (392 mg, 1.83 mmol) in 5 mL of $PO(OMe)_3$. The reaction mixture was stirred at 0 °C for 2 h and worked up as described above, including silica gel column chromatography (2-propanol/NH_4OH/H_2O = 74:17.5:3.5→70:20:5, 16 mL/min) and RP-HPLC (4 mM aq TEAB/CH_3CN = 75:25→60:40), yielding title compound **3b** (214 mg, 33%) as a white solid; mp >159 °C (dec). ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.59 (br s, 1H, NH), 7.57 (s, 1H, H-6), 5.94 (d, 1H, J = 3.6 Hz, H-1′), 4.71 (br s, 2H, 2′−OH and 3′−OH), 4.04 (m, 1H, H-2′), 3.92 (br m, 3H, H-3′ and H-5′), 3.80 (m, 1H, H-4′), 2.35 (t, 2H, J = 7.1 Hz, α-CH_2), 1.48 (m, 2H, β-CH_2), 1.24 (m, 14H, 7 × CH_2), 0.85 (t, 3H, J = 6.7 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.90, 149.40, 144.05, 97.47, 93.20, 85.73, 84.07, 76.34, 74.87, 72.72, 63.91, 31.32, 28.99, 28.72, 28.57, 28.42, 28.25, 22.11, 18.83, 13.97. ^{31}P NMR (121 MHz, DMSO-d_6) δ 1.70. HRMS (ESI) calcd for $C_{21}H_{32}N_2O_9P$ 487.1851 ($M - H^+$); found 487.1847.

2′-Deoxyuridine-5-(*N*-butyl)heptynamide-5′-monophosphate (4a). This compound was prepared according to the general procedure B described above. The following amounts of starting material and reagents were employed: compound **14a** (154 mg, 0.378 mmol), $POCl_3$ (53 μL, 0.567 mmol), and proton sponge (121 mg, 0.567 mmol) in 1.5 mL of $PO(OMe)_3$. The reaction mixture was stirred at 0 °C for 3 h and worked up as described above, including silica gel column chromatography (2-propanol/NH_4OH/H_2O = 77.5:15:2.5→74:17:3.5) and RP-HPLC (4 mM aq TEAB/CH_3CN = 85:15, isocratic, 16 mL/min), yielding title compound **4a** (52 mg, 26%) as a white solid; mp >192 °C (dec). ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.58 (br s, 1H, NH), 8.20 (br s, 1H, amide NH), 7.85 (s, 1H, H-6), 6.10 (t, 1H, J = 6.9 Hz, H-1′), 4.29 (s, 1H, H-3′), 3.88 (m, 1H, H-4′), 3.80 (m, 2H, H-5′), 3.01 (m, 2H, CH_2N), 2.36 (t, 2H, J = 6.9 Hz, α-CH_2), 2.19−2.03 (m, 2H, H-2′), 2.09 (t, 2H, J = 7.4 Hz, CH_2CO), 1.61 (m, 2H, CH_2), 1.47 (m, 2H, CH_2), 1.36 (m, 2H, CH_2), 1.24 (m, 2H, CH_2), 0.84 (t, 3H, J = 7.3 Hz, CH_3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.02, 161.84, 149.58, 142.57, 99.49, 93.52, 86.35 (d, 1C, J_{CP} = 6.7 Hz), 84.52, 72.81, 70.85, 64.15, 40.51, 38.16, 35.08, 31.38, 27.69, 24.93, 19.70, 18.68, 13.80. ^{31}P NMR (121 MHz, DMSO-d_6) δ 1.41. HRMS (ESI) calcd for $C_{20}H_{29}N_3O_9P$ 486.1647 ($M - H^+$); found 486.1630.

1-β-D-Arabinofuranosyluracil-5-(*N*-butyl)heptynamide-5′-monophosphate (4b). This compound was prepared according to the general procedure B described above. The following amounts of starting material and reagents were employed: compound **14b** (160 mg, 0.378 mmol), $POCl_3$ (53 μL, 0.567 mmol), and proton sponge (121 mg, 0.567 mmol) in 1.5 mL of $PO(OMe)_3$. The reaction mixture was stirred at 0 °C for 3 h and worked up as described above, including silica gel column chromatography (2-propanol/NH_4OH/H_2O = 74:17.5:3.5→70:20:5) and RP-HPLC (4 mM aq TEAB/CH_3CN = 90:10, isocratic, 16 mL/min), yielding title compound **4b** (56 mg, 27%) as a white solid; mp >186 °C (dec). ^{1}H NMR (500 MHz, D_2O) δ 7.81 (s, 1H, H-6), 6.13 (d, 1H, J = 4.7 Hz, H-1′), 4.30 (dd, 1H, J = 4.6, 3.6 Hz, H-2′), 4.17 (dd, 1H, J = 4.7, 3.6 Hz, H-3′), 4.06−3.95 (m, 3H, H-4′ and H-5′), 3.14 (t, 2H, J = 6.9 Hz, CH_2N), 2.40 (t, 2H, J = 7.1 Hz, α-CH_2), 2.25 (t, 2H, J = 7.3 Hz, CH_2CO), 1.73−1.66 (m, 2H, CH_2), 1.58−1.51 (m, 2H, CH_2), 1.46−1.39 (m, 2H, CH_2), 1.31−1.23 (m, 2H, CH_2), 0.85 (t, 3H, J = 7.3 Hz, CH_3). ^{13}C NMR (125 MHz, D_2O) δ 176.29, 174.03, 157.28, 143.57, 98.72, 93.66, 85.35, 81.81 (d, 1C, J_{CP} = 7.8 Hz), 75.79, 74.86, 73.46, 63.12 (d, 1C, J_{CP} = 4.4 Hz), 38.64, 34.90, 30.03, 26.79, 24.53, 18.97, 18.06, 12.55. ^{31}P NMR (121 MHz, DMSO-d_6) δ 1.61. HRMS (ESI) calcd for $C_{20}H_{30}N_3O_{10}P$ 502.1596 ($M - H^+$); found 502.1570.

***N*-(3-(5-(2′-Deoxyuridine-5′-monophosphate))prop-2-ynyl)-octanamide (5a).** This compound was prepared according to the general procedure B described above. The following amounts of starting material and reagents were employed: compound **18a** (147.2 mg, 0.361 mmol), $POCl_3$ (101 μL, 1.08 mmol), and proton sponge (235 mg, 1.09 mmol) in 2.5 mL of $PO(OMe)_3$. The reaction mixture was stirred at 0 °C for 3 h and worked up as described above, including silica gel column chromatography (2-propanol/NH_4OH/H_2O = 85:10:5→80:10:10→75:15:10) and RP-HPLC (30 mM aq TEAB/CH_3CN = 85:15→80:20→75:25, 16 mL/min), yielding title compound **5a** (16 mg, 8%) as a white solid; mp >171 °C (dec). ^{1}H NMR (500 MHz, D_2O) δ 8.12 (s, 1H, H-6), 6.23 (t, 1H, J = 6.7 Hz, H-1′), 4.51 (m, 1H, H-3′), 4.13 (br m, 3H, α-CH_2 and H-4′), 3.94 (t, 2H, J = 5 Hz, H-5′), 2.40−2.28 (br m, 2H, H-2′), 2.24 (t, 2H, J = 7.3 Hz, CH_2CO), 1.57 (m, 2H, CH_2CH_2CO), 1.29−1.11 (br m, 8H, 4 × CH_2), 0.78 (t, 3H, J = 7.0 Hz, CH_3). ^{13}C NMR (125 MHz, D_2O) δ 176.97, 164.33, 150.20, 144.51, 98.73, 89.65, 85.61 (d, 1C, J_{CP} = 8.5 Hz), 85.34, 72.99, 70.49, 63.52 (d, 1C, J_{CP} = 4.4 Hz), 38.27, 35.18, 30.64, 29.09, 27.68, 27.58, 24.89, 21.49, 12.97. ^{31}P NMR (121 MHz, D_2O) δ 2.68. HRMS (ESI) calcd for $C_{20}H_{30}N_3O_9P$ 486.1647 ($M - H^+$); found 486.1655.

***N*-(3-(5-(2′-Deoxyuridine-5′-monophosphate))prop-2-ynyl)-hexanamide (5b).** This compound was prepared according to the general procedure A described above. The reaction mixture was worked up as described above, including silica gel column chromatography (2-propanol/NH_4OH/H_2O = 70:20:5) and RP-HPLC (4 mM aq TEAB/CH_3CN = 88:12→75:25, 16 mL/min), followed by a second run (30 mM aq TEAB/CH_3CN = 87:13, isocratic, 7 mL/min), yielding title compound **5b** (15.7 mg, 7%) as a white solid; mp >161 °C (dec). ^{1}H NMR (500 MHz, D_2O) δ 8.17 (s, 1H, H-6), 6.28 (t, 1H, J = 6.7 Hz, H-1′), 4.56 (m, 1H, H-3′), 4.18 (br m, 1H, H-4′), 4.17 (s, 2H, α-CH2), 4.05 (m, 2H, H-5′), 2.47−2.34 (br m, 2H, H-2′), 2.29 (t, 2H, J = 7.4 Hz, CH_2CO), 1.61 (m, 2H, CH_2CH_2CO), 1.35−1.20 (br m, 4H, 2 × CH_2), 0.86 (t, 3H, J = 7.0 Hz, CH_3). ^{13}C NMR (125 MHz, D_2O) δ 177.29, 164.62, 150.52, 144.86, 99.07, 89.89, 85.86 (d, 1C, J_{CP} =8.4 Hz), 73.47, 70.79, 64.29 (d, 1C, J_{CP} = 4.3 Hz), 38.93, 35.59, 30.42, 29.55, 24.93, 21.66, 13.20. ^{31}P NMR (121 MHz, D_2O) δ 1.23. HRMS (ESI) calcd for $C_{18}H_{26}N_3O_9P$ 458.1334 ($M - H^+$); found 458.1338.

***N*-(3-(5-(2′-Deoxyuridine-5′-monophosphate))prop-2-ynyl)-butanamide (5c).** This compound was prepared according to the general procedure A described above. The reaction mixture was worked up as described above, including silica gel column chromatography (2-propanol/NH_4OH/H_2O = 70:20:5) and RP-HPLC (10 mM aq TEAB/CH_3CN = 94:6→80:20, 16 mL/min), followed by a second run (30 mM aq TEAB/CH_3CN = 92:8, isocratic, 7 mL/min), yielding title compound **5c** (53 mg, 28%) as a white solid; mp >168 °C (dec). ^{1}H NMR (500 MHz, D_2O) δ 8.12 (s, 1H, H-6), 6.27 (t, 1H, J = 6.8 Hz, H-1′), 4.55 (m, 1H, H-3′), 4.19 (s, 1H, α-CH2), 4.16 (m, 1H, H-4′), 4.01−3.90 (m, 2H, H-5′), 2.45−2.34 (br m, 2H, H-2′), 2.27 (t, 2H, J = 7.4 Hz, CH_2CO), 1.62 (m, 2H, CH_2CH_2CO), 0.92 (t, 3H, J = 7.4 Hz, CH_3). ^{13}C NMR (125 MHz, D_2O) δ 177.04, 160.24, 151.57, 144.77, 99.08, 89.73, 86.04 (d, 1C, J_{CP} = 8.2 Hz), 85.65, 73.84, 70.97, 63.70 (d, 1C, J_{CP} = 4.5 Hz), 38.50, 37.50, 29.55, 18.84, 12.70. ^{31}P NMR (121 MHz, D_2O) δ 4.01. HRMS (ESI) calcd for $C_{16}H_{22}N_3O_9P$ 430.1021 ($M - H^+$); found 430.1017.

2-Phenyl-*N*-(3-(5-(2′-deoxyuridine-5′-monophosphate))-prop-2-ynyl)acetamide (5d). Compound **23** (110 mg, 0.25 mmol) and 2-cyanoethyl phosphate (pyridinium salt, 0.1 M stock solution in H_2O/pyridine; 5 mL, 0.5 mmol) were evaporated and coevaporated with dry pyridine (2 × 2 mL) and then dissolved in dry pyridine (3 mL) under an Ar atmosphere. DCC (309 mg, 1.5 mmol) was then added, and the reaction mixture was stirred at rt for 2 1/2 days, after which time the

solvent was evaporated. The residue was taken up 1 M NaOH (2 mL) and stirred at 100 °C for 5 min. The mixture was allowed to cool down to rt, and the pH was adjusted to ≈8−9 (dropwise addition of AcOH) and the volatiles were removed in vacuo. The residue was subjected to a column of silica gel (2-PrOH/NH_4OH/H_2O = 90:5:5→80:10:10) and to RP-HPLC (C-18, 10 mM aq TEAB/CH_3CN = 90:10, isocratic, 16 mL/min). Appropriate fractions were pooled and lyophilized, and the obtained product was converted into its disodium salt by passage through a column of Dowex 50-WX8 (Na^+-form) and elution with H_2O. After evaporation of the solvent and lyophilization, compound **5d** (21 mg, 16%) was obtained as a white solid; mp >174 °C (dec). ^{1}H NMR (500 MHz, D_2O) δ 7.98 (s, 1H, H-6), 7.36 (m, 5H, ArH), 6.28 (t, 1H, J = 6.9 Hz, H-1′), 4.51 (m, 1H, H-3′), 4.16 (s, 2H, α-CH_2), 4.11 (m, 1H, H-4′), 3.92 (m, 2H, H-5′), 3.64 (s, 2H, CH_2Ph), 2.34 (m, 2H, H-2′). ^{13}C NMR (125 MHz, D_2O) δ 174.21, 172.60, 156.60, 143.64, 134.47, 128.92 (2C), 128.61 (2C), 126.99, 98.74, 88.01, 85.33 (d, 1C, J_{CP} = 8.2 Hz), 85.13, 75.78, 70.77, 63.44 (d, 1C, J_{CP} = 4.5 Hz), 41.91, 38.25, 29.63. ^{31}P NMR (121 MHz, D_2O) δ 3.99. HRMS (ESI) calcd for $C_{20}H_{22}N_3O_9P$ 478.1021 ($M - H^+$); found 478.1020.

***N*-(3-(5-(2′-Deoxyuridine-5′-monophosphate))prop-2-ynyl)-heptane-1-sulfonamide (6a).** Compound **24** (121 mg, 0.25 mmol), HOBt (38 mg, 0.25 mmol), and 2-cyanoethyl phosphate (pyridinium salt, 0.1 M stock solution in H_2O/pyridine; 6.25 mL, 0.625 mmol) were evaporated and coevaporated with dry pyridine (3 × 2 mL), then dissolved in dry pyridine (3 mL) under an Ar-atmosphere. DCC (309 mg, 1.5 mmol) was then added, and the reaction mixture was stirred at rt for 2 days, after which time the solvent was evaporated. The residue was taken up 1 M NaOH (2 mL) and stirred at 100 °C for 5 min. The mixture was allowed to cool down to rt and the pH was adjusted to ≈8−9 (dropwise addition of AcOH) and the volatiles were removed in vacuo. The residue was subjected to a column of silica gel (2-PrOH/NH_4OH/H_2O = 90:5:5→75:15:10) and to RP-HPLC (C-18, 10 mM aq TEAB/CH_3CN = 80:20, isocratic, 16 mL/min). Appropriate fractions were pooled and lyophilized, and the obtained product was converted into its disodium salt by passage through a column of Dowex 50-WX8 (Na^+-form) and elution with H_2O. After evaporation of the solvent and lyophilization, compound **6a** (15 mg, 11%) was obtained as a white solid; mp >175 °C (dec). ^{1}H NMR (500 MHz, D_2O) δ 8.19 (s, 1H, H-6), 6.21 (t, 1H, J = 6.8 Hz, H-1′), 4.50 (m, 1H, H-3′), 4.11 (m, 1H, H-4′), 4.08 (s, 2H, α-CH_2), 3.93 (m, 2H, H-5′), 3.28 (m, 2H, CH_2SO_2), 2.39−2.27 (m, 2H, H-2′), 1.72 (m, 2H, CH_2), 1.28 (m, 2H, CH_2), 1.19−1.09 (m, 6H, 3 × CH_2), 0.74 (t, 3H, J = 6.9 Hz, CH_3). ^{13}C NMR (125 MHz, D_2O) δ 164.14, 150.14, 144.77, 98.51, 89.36, 85.70 (d, 1C, J_{CP} = 7.5 Hz), 85.36, 74.63, 70.41, 63.41, 51.66, 38.43, 32.16, 30.56, 27.62, 27.06, 22.52, 21.52, 12.86. ^{31}P NMR (121 MHz, D_2O) δ 2.72. HRMS (ESI) calcd for $C_{19}H_{30}N_3O_{10}PS$ 522.1317 ($M - H^+$); found 522.1310.

1-Hexyl-(3-(5-(2′-deoxyuridine-5′-monophosphate))prop-2-ynyl)urea (6b). Crude **25** (228 mg, 0.51 mmol), HOBt (77 mg, 0.51 mmol), and 2-cyanoethyl phosphate (pyridinium salt, 0.1 M stock solution in H_2O/pyridine; 15 mL, 1.5 mmol) were evaporated and coevaporated with dry pyridine (3 × 5 mL) and then dissolved in dry pyridine (6 mL) under an Ar atmosphere. DCC (625 mg, 3.04 mmol) was then added, and the reaction mixture was stirred at 40−45 °C for 2 days, after which time the solvent was evaporated. The residue was taken up 1 M NaOH (7 mL) and stirred at 100 °C for 5 min. The mixture was allowed to cool down to rt and the pH was adjusted to ≈8−9 (dropwise addition of AcOH) and the volatiles were removed in vacuo. The residue was subjected to a column of silica gel (2-PrOH/NH_4OH/H_2O = 90:5:5→75:15:10) and to RP-HPLC (C-18, 10 mM aq TEAB/CH_3CN = 85:15, isocratic, 16 mL/min). Appropriate fractions were pooled and lyophilized, and the obtained product was converted into its disodium salt by passage through a column of Dowex 50-WX8 (Na^+-form) and elution with H_2O. After evaporation of the solvent and lyophilization, compound **6b** (26 mg, 9%) was obtained as a white solid; mp >169 °C (dec). ^{1}H NMR (500 MHz, D_2O) δ 8.19 (s, 1H, H-6), 6.29 (t, 1H, J = 6.8 Hz, H-1′), 4.57 (m, 1H, H-3′), 4.17 (m, 1H, H-4′), 4.09 (d, 2H, J = 1.8 Hz, α-CH_2), 3.97 (m, 2H, H-5′), 3.13 (td, 2H, J = 6.8, 1.3 Hz, CH_2NH), 2.45−2.36 (m, 2H, H-2′), 1.49 (m, 2H, CH_2), 1.34−1.24 (m, 6H, 3 × CH_2), 0.85 (t, 3H, J = 7.1 Hz, CH_3). ^{13}C NMR (125 MHz, D_2O) δ 164.60, 160.07, 150.41, 144.52, 98.90, 91.11, 85.76 (d, 1C, J_{CP} = 8.8 Hz), 85.28, 72.98, 70.50, 63.33 (d, 1C, J_{CP} = 3.8 Hz), 39.56, 38.28, 30.45, 30.01, 28.75, 25.26, 21.60, 12.92. ^{31}P NMR (121 MHz, D_2O) δ 3.43. HRMS (ESI) calcd for $C_{19}H_{29}N_4O_9P$ 487.1599 ($M - H^+$); found 487.1585.

Cloning, Expression, and Purification of Mycobacterial ThyX. This was done according to protocols described.[21]

ThyX-Inhibition Assay. The tritium-release assay was adapted from Hunter et al.[56] Briefly, the reaction was performed in a final volume of 25 μL, in a 96-well flat bottom plate, including 1.5 μg of M. tuberculosis ThyX, 20 μM homemade mTHF, 10 μM FAD and 500 μM NADPH, and compounds if required. Reaction was initiated by addition of 10 μL of 2 μM [5-^{3}H] dUMP (13 Ci/mmol). After a 10 min reaction period, at room temperature, the reaction was terminated by the addition of 20 μL of a stop solution (a 3:1 ratio of 2 N TCA:4.3 mM dUMP). To remove unreacted substrates, 150 μL of 10% (w/v) activated charcoal in water was added to the reaction mixture. The plate was incubated on ice for 15 min and then centrifuged at 3800*g* at 4 °C for 10 min. A 100 μL aliquot of the supernatant was assayed by liquid scintillation counting to determine the amount of tritium-containing water produced by the reaction. Percentages of activity compared to control points were calculated and IC_{50} values determined.

Cloning, Expression, and Purification of Mycobacterial ThyA. *Cloning.* PCR reactions were performed using a BioRad MJ Mini Personal Thermal Cycler. Primers were purchased from Eurogentech: 5′-CTTGACCATATGGGCCATCATCATCATCATCACG-GCACGCC ATACGAGGACCTGCTG, 5′-CTAGTCGGATCCT-CATACCGCGACTGGAGC cDNA containing ThyA gene was used as a template. PCR reactions were carried out using Ex Taq polymerase and standard reaction conditions (Ta 55 °C). Reactions were purified using QIAquick PCR purification kit (Qiagen). NdeI and *Bam*HI restrictions enzymes (BioLabs) were used to create sticky ends on the ThyA PCR-fragment, and manufacturer's standard reaction conditions were used. Pet3a vector was also cut with NdeI and *Bam*HI restriction enzymes and treated with alkaline phosphatase. Afterward, pet3a vector was purified from 0.8% agarose gel using QIAEXII Gel extraction kit (Qiagen). Ligation reaction (T4 DNA ligase, BioLabs) was performed according to manufacturer's instructions. TG1 *E. coli* RbCl chemical competent cells were transformed with the ligation mixture, grown during 1 h at 37 °C and 250 rpm in 900 μL LB-medium and then plated on the LB agar plates and grown overnight at 37 °C. Several colonies were picked up for colony PCR and also overnight cultures in LB medium were set up. Pet3a plasmid of a positive colony was purified from an overnight culture using QIAprep Spin Miniprep kit (Qiagen), and the concentration was measured using NANODROP 1000.

Expression. BL21 (DE3) pLysS *E. coli* RbCl chemical competent cells were transformed with pet3a vector containing the ThyA insert, grown during 1 h at 37 °C and 250 rpm in 900 μL LB-medium, then plated on the LB-agar plates and grown overnight at 37 °C. Afterward, a colony was picked up into an overnight culture. Bacteria from the overnight culture were grown in 1 L LB medium at 37 °C and 250 rpm until OD_{600} = 0.6−0.8 and then induced with 200 μL 1 M IPTG solution and grown overnight at 22 °C and 250 rpm. Bacteria were spun down for 10 min at 10000 rpm and 4 °C. The obtained bacterial pellet was stored at −20 °C.

Purification. The bacterial pellet was resuspended in 10 mL of lysis buffer containing 5 mg of lysozyme, 1 mM DTT, and protease inhibitor cocktail. After five times, French Press lysate was spun down for 20 min. ThyA protein was purified from supernatants using His6-based affinity

chromatography (NTA magnetic beads, Qiagen) according to the manufacturer's instructions.

ThyA-Inhibition Assay. ThyA activity was adapted from Kan et al.[58] In brief, the reaction mixture (100 μL) contained 1 μg of ThyA enzyme, 50 mM Tris-HCl (pH 7.5), 500 μM homemade mTHF, 10 mM $MgCl_2$, and compound if required. Reactions, performed at room temperature in a 96-well flat bottom plate, were initiated by addition of 20 μL of 2 mM dUMP. The increase in absorbance at 340 nm was measured directly after dUMP addition and after 30 min, using a spectrophotometer (envision, Perkin elmer). Percentages of activity compared to control points (no enzyme, no inhibitor) were calculated and IC_{50} values determined.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures for the synthesis of compounds **10a**−**b**, **11a**−**b**, **15a**−**d**, **21**−**22**, and purity of synthesized derivatives **1**−**2**, **3a**−**b**, **4a**−**b**, **5a**−**d**, and **6a**−**b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

We are grateful to Prof. Roger Busson for his kind assistance and his expertise regarding the interpretation of complex product mixtures by NMR spectroscopy and Luc Baudemprez for running 500 and 600 MHz NMR spectra. This work was partly funded by a collaborative project grant (NATT-project) from the European Commission. Martin Kögler is deeply indebted to the IWT (Agentschap voor Innovatie door Wetenschap en Technologie) for providing a Ph.D. scholarship.

■ ABBREVIATIONS USED

AcOH, acetic acid; Ac_2O, acetic anhydride; CAN, cerium(IV)-ammonium nitrate; CH_2THF, methylenetetrahydrofolic acid; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DHF, dihydrofolate; DHFR, dhydrofolate reductase; DIAD, diisopropyl azodicarboxylate; DIC, diisopropycarbodiimide; DIPEA, diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DMTrCl, 4,4′-dimethoxytrityl chloride; dTMP, 2′-deoxythymidine-5′-monophosphate; dUMP, 2′-deoxyuridine-5′-monophosphate; EDTA, ethylenediamine tetraacetic acid; EtOAc, ethyl acetate; EtOH, ethanol; FAD, flavin adenine dinucleotide; FDTS, flavin-dependent thymidylate synthase; HIV, human immunodeficiency virus; HOBt, hydroxybenzotriazole; IC_{50}, half maximal (50%) inhibitory concentration; MDR-TB, multi drug-resistant TB; MeOH, methanol; MIC_{90}, minimum inhibitory concentration 90%; NADPH, nicotinamide adenine nucleotide phosphate hydride; NEt_3, triethylamine; RP-HPLC, reversed-phase high performance liquid chromatography; rt, room temperature; SAR, structure−activity relationship; SHMT, serine hydroxymethyl transferase; TB, tuberculosis; TCA, trichloroacetic acid; TEAB, triethylammonium bicarbonate; THF, tetrahydrofuran; ThyA, thymidylate synthase; ThyX, flavin-dependent thymidylate synthase; TMSI, trimethylsilyl iodide; TS, thymidylate synthase; WHO, World Health Organization; XDR, extensively drug resistant

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