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Synthesis of fluorescent nucleoside analogs as probes for 2'-deoxyribonucleoside kinases

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Abstract

We are reporting on the synthesis of fluorescent nucleoside analogs with modified sugar moieties (*e.g.*, sugars other than ribose and 2'-deoxyribose). Four novel derivatives of the fluorescent thymidine analog 6-methyl-3-(β-D-2'-deoxyribofuranosyl) furano-[2,3-*d*]pyrimidin-2-one were synthesized via Sonogashira reaction and subsequent copper-catalyzed cycloaddition. These compounds represent promising tools for studying nucleoside metabolism inside living cells, as well as for screening directed evolution libraries of 2'-deoxyribonucleoside kinases with new and improved activity for the corresponding nucleoside analogs.

The ability to detect small-molecule metabolites with high sensitivity in complex mixtures such as a cell's cytoplasm greatly benefits studies of their cellular uptake and metabolism.[1,2] The application of fluorophors as reporters has proven a particularly versatile strategy and hence was recently adapted by our group to study the phosphorylation of nucleosides and nucleoside analogs by nucleoside kinases (dNKs).[3] The dNKs are part of the nucleoside salvage pathway and also play a critical role in activating nucleoside analog prodrugs used for antiviral and cancer therapy.

Although nucleosides possess intrinsic fluorescence properties through their pyrimidine and purine moieties, low quantum yields and overlapping absorption maxima with aromatic amino acids in proteins and small-molecule metabolites such as flavines and NADH make their selective detection *in vivo* impractical. To overcome cellular autofluorescence and improve signal-to-noise ratios, fluorescent substrates with absorption maxima of >300 nm are necessary.[4–6]

Fortunately, relatively small synthetic modifications of the nucleobase moieties can augment the spectral properties of nucleosides, red-shifting their absorption spectra and increasing quantum yields. In recent work, Berry and coworkers reported the decoration of pyrimidines with furano and pyrrolo moieties, creating fluorescent analogs of thymidine (**1**) and 2'-deoxycytidine (**2**) to study DNA structure.[7] The modification of the nucleobase shifted the excitation wavelength to 331 – 335 nm while the emission maximum reached 413 – 415 nm.

Our kinetic experiments with **1** showed that the expanded heterocycle does not have a major effect on the performance of dNK from *Drosophila melanogaster* (*Dm*dNK), a reference enzyme for the phosphorylation of 2'-deoxynucleosides and nucleoside analogs.[3] More

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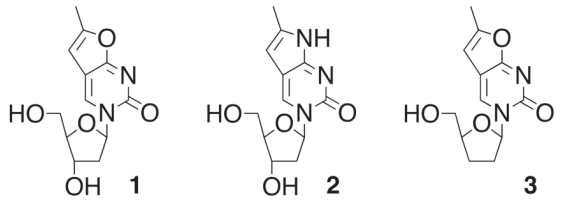
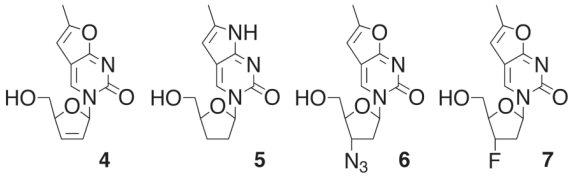
qualitatively, activation of the fluorescent nucleoside by the fruitfly dNK resulted in accumulation of the phosphorylated analog which could be monitored via fluorescence microscopy (Fig. 1). In the same study, we also prepared the fluorescent version of the nucleoside analog prodrug 3'-deoxy-thymidine (fddT, **3**). Fluorescent ddT was successfully used to screen directed evolution libraries of *Dm*dNK for variants with changed substrate specificity and enhanced activity for the prodrug. Host cells that express kinase variants with the highest activity for **3** were identified and sorted by flow cytometry and yielded enzymes with up to 10,000-fold change in substrate specificity. [3]



To explore the use of fluorescent nucleoside analogs more broadly, we herein report the successful preparation of four additional fluorescent nucleoside analogs, namely 6-methyl-3-(β-D-2',3'-dideoxy-2',3'-didehydro-ribofuranosyl)furano-[2,3-*d*]pyrimidin-2-one (**4** ; fd4T) , 6 -methyl-3-(β-D-2',3'-dideoxy-ribofuranosyl) pyrrolo-[2,3-*d*]pyrimidin-2-one (**5**; fddC), 6 -methyl-3-(β-D-3' azido - 2 ' , 3 ' - dideoxy-ribofuranosyl)furano-[2,3-*d*]pyrimidin-2-one (**6**; fAZT), and 6-methyl-3-(β-D-3'-fluoro-2',3'-dideoxy-ribofuranosyl) furano-[2,3-*d*] pyrimidin-2-one (**7**; fFT).



The synthesis of **4** and **5** is relatively straight forward and follows the preparation previously described for **3**.[3] For **4**, we started from the known 5-iodo intermediate **8**, assembling the furano pyrimidine via the established Sonogashira route using propyne in the presence of Pd/Cu[8,9] and subsequent Cu-catalyzed cycloaddition to afford the fluorescent moiety (Scheme 1).[10] The pyrrolo analog **5** can be prepared directly from **3** by treatment of the furano pyrimidine with ammonic methanol to yield the cytidine variant. [11,12]

A similar strategy to prepare **6** was complicated by the azido group's interference with the Pd chemistry, forcing a change in the synthetic approach as outlined in Scheme 2. Following selective protection of the 5'-OH group in **9** with trityl chloride and treatment of the intermediate with mesyl chloride, the configuration of the 3'-group was readily inverted upon refluxing in ethanolic sodium hydroxide to give the xylo compound **10**.[13] Successive treatment of **10** with propyne in the presence of Pd/Cu and Cu-catalyzed cyclization produced **11**.[8,9] Next, reaction with MsCl yielded the mesylate **12** and subsequent S_N2 substitution with LiN_3 and deprotection with acetic acid afforded the azido substituted fluorescent nucleoside analog **6**.[14, 15]

Alternatively, intermediate **10** could readily be converted to the 3'-fluoro analog **13** with DAST (Scheme 3). Subsequent substitution of the 5-iodo group with propyne, followed by cyclization again yielded the corresponding furano-pyrimidine **7**.[16]

The fluorescence properties of all four compounds were determined and found to be very similar to the previously reported **3**, having excitation wavelengths of 333 ± 2 nm with a molar absorptivity of 3460 M^{-1} cm^{-1} and emission maxima ranging from 413 – 415 nm. The quantum yield is approximately 0.3. Interestingly, the fluorescence intensity of **4** is approximately 2-fold higher than the other nucleosides and nucleoside analogs, presumably a reflection of the heterocycle's electronic interactions with the alkene bond in the sugar moiety.

Separately, we also tested the compounds' substrate properties with wild type *Dm*dNK (Table 1). Experiments were limited to specific activity measurements at 100 μM substrate concentration as the determination of accurate Michaelis-Menten parameters was complicated by limited solubility of fluorescent analogs above 1 mM and K_M values of >250 μM for some substrates.

Our data indicate a relatively minor impact on the substrate properties of 2'-deoxyribonucleosides **1** and **2** upon introduction of the furano and pyrrolo moieties. Beyond the overall lower activities for nucleoside analogs, the same trend is observed for ddT and its corresponding fluorescent analog **3**. Furano-pyrimidines with the 3'-azido (**6**) and 3'-fluoro (**7**) groups decline about 10-fold, possibly reflecting changes in substrate binding due to steric constraints in the enzyme active site and conformational changes in the substrate. Similar binding effects, albeit this time in favor of the substrate, could explain the 3-fold gain in activity for **4**, the fluorescent version of d4T. Unexpectedly, pyrrolo analog **5** shows a 40-fold drop in activity relative to the non-fluorescent ddC, a trend opposite to the slight gain observed with its 2'-deoxyribosoyl analog **2**. Conformational differences in the sugar moiety are unlikely to account for the differences as **3** possesses the same 2',3'-dideoxyribosyl portion, yet shows 10-fold higher activity. Future studies exploring the conformational preferences of furano and pyrrolopyrimidines in regard to their syn-anti orientation might assist in rationalizing their difference in performance.

In summary, the application of fluorescent nucleoside analogs as molecular probes provides a new tool for studying the uptake and metabolism of antiviral prodrugs as demonstrated in our laboratory and by other research groups.[3,17,18] Herein, we have shown that nucleoside analogs with furano and pyrrolo modification are synthetically readily accessible and can serve as substrates for the type-I 2'-deoxyribo-nucleoside kinase from *Drosophila melanogaster*. In bacterial and mammalian cell cultures, these fluorescent analogs have shown no unusual cytotoxicity, hence making them suitable reporters to study cellular uptake and phosphorylation, as well as to evaluate large combinatorial libraries of kinases for variants with substrate specificity for the corresponding prodrugs by fluorescence activated cell sorting (FACS).

Acknowledgments

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10. Compound **4**: UV (H_2O) λ_{max} 331 nm; IR (cm^{-1}) 3440 (br), 3195, 1670, 1642; ^1H NMR (400 MHz, DMSO-d_6) δ 2.29 (s, 3H), 3.61 (m, 2H), 4.88 (s, 1H), 5.05 (t, 1H, J = 5.6 Hz), 6.01 (d, 1H, J = 6.0 Hz), 6.38 (m, 2H), 6.95 (s, 1H), 8.53 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d^6) δ 14.27, 62.78, 88.88, 92.61, 100.95, 107.50, 127.22, 135.24, 138.19, 154.98, 155.60, 172.18; HRMS (FAB) *m/z* 271.0684, calcd for $C_{12}H_{12}O_4N_2Na$ 271.0689 (M^+Na).
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12. Compound **5**: UV (H_2O) λ_{max} 335 nm; IR (cm^{-1}) 3350 (br), 1670, 1560, 1090; ^1H NMR (400 MHz, $CDCl_3$/CD_3OD) δ 1.91–1.93 (m, 2H), 2.18 (m, 1H), 2.41(s, 3H), 2.54 (m, 1H) 3.82 (dd, 1H, J = 4.0, 12 Hz), 4.08 (dd, 1H, J = 2.8, 12.4 Hz), 4.26 (m, 1H), 4.80 (d, 1H, J = 1.6 Hz), 6.22 (dd, 1H, J = 2.4, 6.8 Hz), 8.44 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.5, 24.2, 33.9, 62.5, 83.2, 88.8, 97.9, 110.6, 134.9, 138.6, 155.5, 158.6; HRMS (FAB) m/z 250.1183, calcd for $C_{12}H_{16}O_3N_3$ 250.1186 (M+H).
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15. Compound **6** was crystallized from ethyl acetate. UV (H_2O) λ_{max} 331 nm; IR (cm^{-1}) 3469 (br), 2839, 1667, 1638, 1573, 1482; ^1H NMR (400 MHz, $CDCl_3$) δ 2.27 (d, 3H, J = 7.2 Hz), 2.30–2.36 (m, 1H), 2.57–2.64 (m, 1H), 3.29 (br, 1H), 3.71–3.75 (m, 1H), 3.90–3.95 (m, 2H), 4.18 (dd, 1H, J = 6.8, 13.2 Hz), 6.10–6.14 (m, 1H), 8.65 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 39.2 58.6, 60.5, 85.7, 88.0, 100.1, 108.4, 136.2, 155.2, 156.2, 171.9; HRMS (FAB) *m/z* 292.1036, calcd for $C_{12}H_{14}O_4N_5$ 292.1039 (M^+H).
16. Compound **7**: UV (H_2O) λ_{max} 335 nm; IR (cm^{-1}) 3350 (br), 1670, 1560, 1090; ^1H NMR (600 MHz, DMSO-d_6) δ 2.20 (m, 1H), 2.33 (s, 3H), 2.73 (m, 1H), 3.66 (m, 2H), 4.33 (dt, 1H, J = 25.8 Hz), 5.32 (dd, 1H, J = 56.4 Hz), 6.22 (q, 1H), 6.44 (s, 1H), 8.60 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 13.6, 60.6, 86.2, 87.6, 95.4, 100.4, 106.8,136.5, 154.3, 171.56. ^{19}F NMR (376 MHz, DMSO-d_6) δ-174.9; HRMS (FAB) *m/z* 269.0932, calcd for $C_{12}H_{14}O_4N_2F_1$ 269.0932 (M^+H).
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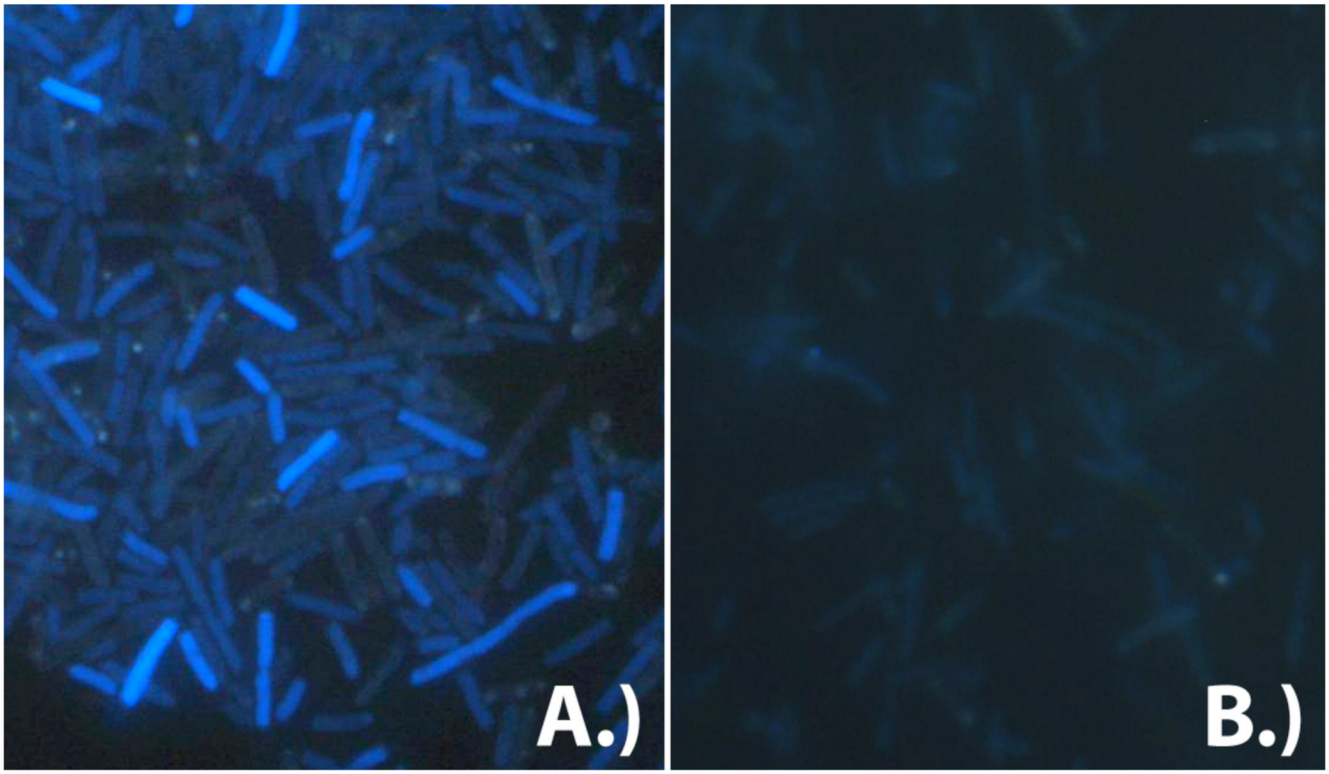
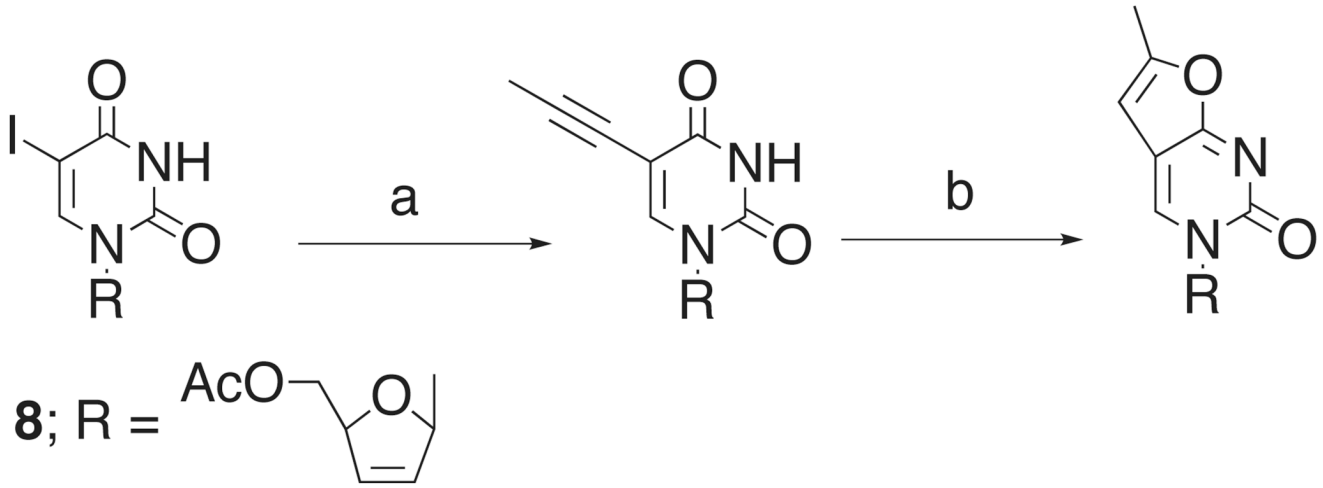
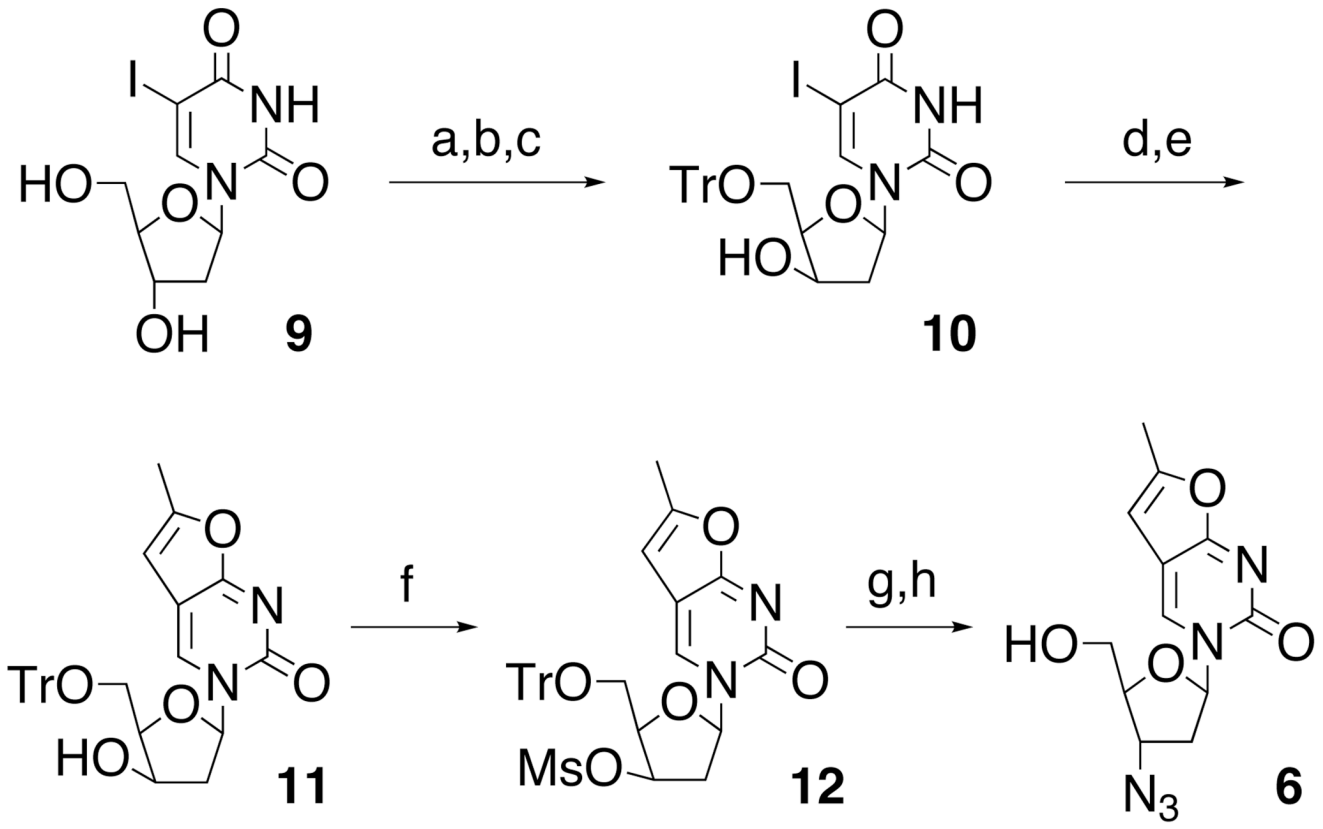


Figure 1.
Fluorescence microscopy images of *E. coli* KY895 expressing A.) an exogenous 2'-deoxyribonucleoside kinase from *Drosophila melanogaster* or B.) glycinamide ribonucleotide formyltransferase from *E. coli* (negative control) upon incubation with compound **1** (λ_{ex} = 331 nm, λ_{em} = 413 nm). In the presence of a kinase, intracellular accumulation of **1** is observed as reflected in higher fluorescence intensity.

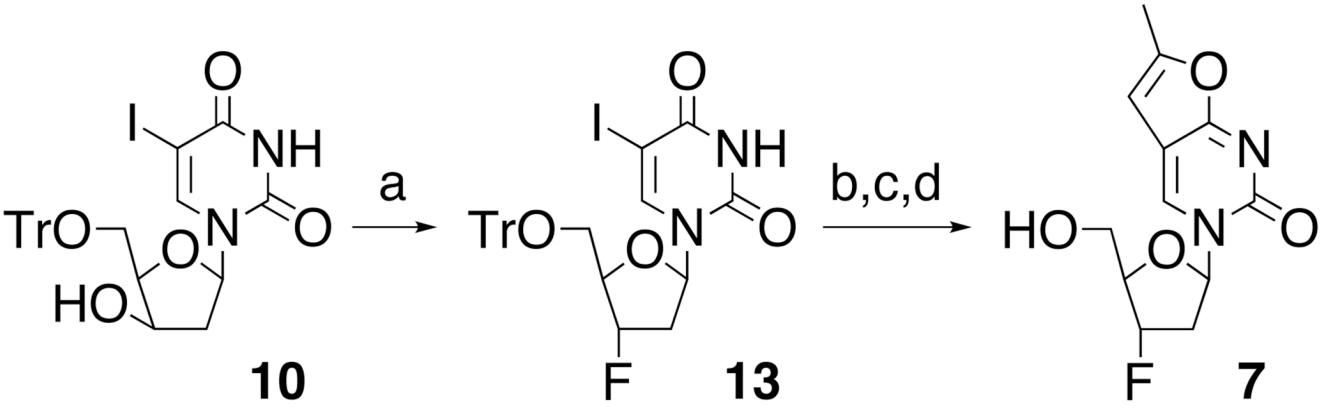


Scheme 1.
Introduction of furano moiety. (a) propyne, $Pd(PPh_3)_4$, CuI, NEt_3, DMF, rt, 22 h, 60%; (b) CuI, NEt_3, MeOH, reflux, 28–45%.



Scheme 2.

Preparation of fAZT. (a) TrCl, Py, reflux; (b) MsCl, CH_2Cl_2, 67%; (c) NaOH, EtOH, reflux, 65%; (d) propyne, $Pd(PPh_3)_4$, CuI, NEt_3, DMF, rt, 22 h, 62%; (e) CuI, NEt_3, MeOH, reflux, 70%;(f) MsCl, Et_3N, CH_2Cl_2, rt, 1h, 87%; (g) LiN_3, DMF, 110°C, 2.5h, 80%; (h) AcOH, 90° C, 15 min, 60%.



Scheme 3.

Fluorination of xylo-intermediate **10**. (a) DAST, CH_2Cl_2, rt, 1h, 52%; (b) propyne, Pd $(PPh_3)_4$, CuI, NEt_3, DMF, rt, 22 h, 65%; (c) CuI, NEt_3, MeOH, reflux, 38%; (d) AcOH, 90° C, 15 min, 60%.

Table 1

Substrate specificities of wild type DmdNK

substrate (100 μM)	specific activity (μM min^{-1} mg^{-1})
THY *	60,000 ± 1010
fTHY (**1**) *	25,511 ± 455 (43%)
dC *	43,734 ± 660
fdC (**2**)	56,034 ± 1050 (128%)
ddT	1310 ± 87
fddT (**3**)	397 ± 36 (30%)
ddC	2005 ± 148
fddC (**5**)	52 ± 9 (2.6%)
FT	1189 ± 17
fFT (**7**)	144 ± 27 (12%)
AZT	1054 ± 14
fAZT (**6**)	128 ± 7 (12%)
d4T	70 ± 15
fd4T (**4**)	232 ± 20 (330%)

Specific activities were measured by spectrophotometric coupled-enzyme assay with 100 μM substrate and 1 mM ATP except for substrates marked with an asterisk (measured at 10 μM substrate concentration under v_{max} conditions). Experiments were performed in triplicates. Percentage in parentheses is relative activity of fluorescent analog over non-fluorescent analog.