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Synthesis, anti-HIV and cytostatic evaluation of 3'-deoxy-3'-fluorothymidine (FLT) pro-nucleotides

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Abstract— A series of pro-nucleotide phosphoramidates and phosphorodiamidates of the antiviral lead compound 3'-deoxy-3'-fluorothymidine (FLT) have been designed and synthesized. *In vitro* antiretroviral and cytostatic studies revealed potent (sub-micromolar) inhibition of HIV-1 and HIV-2 replication, with retention of activity in thymidine kinase-negative cell models, as predicted by the ProTide concept.

Keywords: nucleosides; antiviral; anticancer; phosphoramidates; pro-nucleotides; pro-drugs

Despite their crucial and widespread role as approved therapeutics for antiviral¹ and anticancer² therapy, the activity of nucleoside analogues is compromised by their requirement for active transporter-mediated cell uptake and bioactivation, usually via three successive phosphorylation steps. Since initial nucleoside kinase-catalysed phosphorylation is frequently the rate-limiting step in nucleoside drug activation, a number of pro-drug (pro-nucleotide) monophosphate strategies have been developed to circumvent delivery, uptake and metabolic activation issues.

One of the most widely used pro-nucleotide strategies is the approach pioneered by McGuigan,^{3,4} in which the monophosphate is masked as a phosphoramidate,⁵ or in more recent modalities as a phosphorodiamidate.⁶ Following passive diffusion through the cell membrane, the pro-nucleotide (ProTide) breaks down within the cell via a well-studied mechanism to release the nucleoside monophosphate, trapped intracellularly and primed for further phosphorylation. The ProTide approach has been applied to improve efficacy for a wide range of antiviral and anticancer nucleosides. Recent highlights include the clinical trial candidates INX-189 (hepatitis C virus, to Phase 2)⁷ and a pro-

nucleotide of the cancer chemotherapeutic gemcitabine (NUC-1031, Phase 1, ongoing),⁸ shown in Figure 1.

Figure 1. Chemical structures of ProTides INX-189 and NUC-1031.

Fluorine is well known to impart rather special physicochemical and pharmacological properties on drug candidates, and is present in 15-20% of currently approved drugs overall.⁹ Several fluorinated nucleosides have been applied as both therapeutic agents and in diagnostic imaging. Examples of fluorinated nucleoside antivirals include emtricitabine (HIV),¹⁰ clevudine (HBV)¹¹ and trifluridine (HSV; cancer).^{12,13} Besides trifuridine, fluorinated nucleoside-based anticancer drugs include also the 5-fluorouracil pro-drug such as capecitabine¹⁴ and gemcitabine;¹⁵ and the related purine nucleosides fludarabine and clofarabine.¹⁶

3'-Deoxy-3'-fluorothymidine (FLT) is a particularly interesting fluorinated nucleoside derivative possessing a variety of biological properties. The major clinical application of FLT is in the non-invasive diagnostic imaging of tumour proliferation using Positron Emission Tomography (PET), where the ¹⁸F radiolabelled analogue (¹⁸FLT, $t_{1/2} = 110$ min.) is

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routinely employed as a highly sensitive imaging probe.¹⁷ In terms of therapeutic application, FLT (also known as alvodine) has been shown to be a more potent inhibitor of HIV replication than the well-known anti-retroviral agent AZT. *In vitro* studies have shown that FLT inhibits replication of nucleoside analogue reverse transcriptase inhibitor (NRTI)-resistant HIV strains.¹⁸ Further clinical development of FLT was halted, however, due to toxicological safety concerns.¹⁹ Previous preliminary work on FLT pro-nucleotides as HIV inhibitors suggested the possibility to retain the potent activity of the parent compound using a ProTide-based strategy.²⁰ In this paper, we describe the synthesis and *in vitro* evaluation of both phosphoramidate (**3a-m**) and phosphorodiamidate (**5a-b**) pro-nucleotide derivatives of FLT as antiretroviral and anticancer agents. These studies test the hypothesis that application of the ProTide delivery concept might generate highly potent new nucleotide analogues that are able to by-pass the requirement for a functional thymidine kinase (TK).

Synthesis of the FLT phosphoramidates was accomplished through application of known pro-nucleotide chemistry.⁵ Reaction of the commercially available FLT (**1**, Carbosynth U.K.) with a range of previously reported aryloxyphosphorochloridates (**2a-m**), promoted by *tert*-butylmagnesium chloride as a hindered base in THF, provided the target phosphoramidate products in low to moderate isolated yield (high yield based on recovered starting material, BORMS) following column chromatography (Scheme 1).^{21,22}

We then tried to synthesize diamidate prodrugs of FLT according to the trimethyl phosphate method used previously in our laboratory.²³ Unfortunately, after 5h of reaction at -78 °C in the presence of TMP and POCl₃, the signal of the desired intermediate **4** on ³¹P NMR was barely visible. After a further 15h in the presence of L-Ala neopentyl ester and DIPEA, no signal of the desired diamidate prodrug was observed on ³¹P NMR. We can explain this by a lack of solubility of FLT in the organic solvents used. Eventually symmetrical FLT phosphorodiamidates (**5a-b**) were synthesized according to literature methods^{6,24} by treating FLT with phosphoryl chloride under basic conditions to generate an intermediate dichloridate (**4**) which was not isolated. Reaction of FLT dichloridate with further triethylamine, followed by excess α -amino acid ester, gave the required FLT phosphorodiamidates after purification by column chromatography and preparative TLC (Scheme 1).^{25,26}

Scheme 1. Synthesis of FLT pro-nucleotides^{a,b}

The resulting FLT Pro-Tides were evaluated for their *in vitro* inhibitory effect on the replication of HIV-1 and HIV-2 in human T-lymphocyte (CEM) cell cultures, according to previously described methods.^{24,27} Importantly, TK⁻ mutant CEM cells were included to examine the hypothesis that the ProTide derivatives, unlike the parent compound FLT, would be able to by-pass the requirement for TK-directed phosphorylation and would be active in this test. The results of the HIV assays are shown in Table 1. FLT (**1**) was used as a positive control, confirming potent anti-HIV-1 and -HIV-2 activity in the TK-expressing CEM cells (EC₅₀ = 6.2 and 18 nM, respectively), but not in the TK knockout CEM mutant cells (EC₅₀ >50 μ M). The >1000 fold loss of activity of (**1**) in the TK⁻ assay is notable.

Cpd.	EC ₅₀ / μ M (CEM/0)		EC ₅₀ / μ M (CEM/TK ⁻)
	HIV-1	HIV-2	HIV-2
3a	0.55 \pm 0.21	1.3 \pm 0.14	4.7 \pm 4.8
3b	0.10 \pm 0.068	0.24 \pm 0.23	1.3 \pm 0.71
3c	0.75 \pm 0.0	1.1 \pm 0.23	28 \pm 2.1
3d	0.29 \pm 0.16	0.90 \pm 0.71	>50
3e	0.13 \pm 0.11	0.18 \pm 0.049	2.0 \pm 0.0
3f	0.43 \pm 0.35	0.65 \pm 0.35	3.0 \pm 1.3
3g	0.16 \pm 0.028	0.18 \pm 0.071	5.2 \pm 0.92
3h	0.56 \pm 0.23	1.0 \pm 0.27	>50
3i	2.3 \pm 1.4	4.7 \pm 2.4	>50
3j	0.048 \pm 0.078	0.14 \pm 0.0071	2.0 \pm 1.1
3k	0.032 \pm 0.0057	0.080 \pm 0.0	0.87 \pm 0.33
3l	0.12 \pm 0.014	0.18 \pm 0.078	2.1 \pm 1.2
3m	0.65 \pm 0.21	1.6 \pm 0.38	89 \pm 8.5
5a	0.036 \pm 0.0042	0.13 \pm 0.0071	1.0 \pm 0.87
5b	0.017 \pm 0.0092	0.045 \pm 0.012	20 \pm 0.71
1	0.0062 \pm 0.0021	0.018 \pm 0.014	>50

Table 1. Anti-HIV-1 and -HIV-2 activity (EC₅₀, μ M) values in human T-lymphocyte (CEM) cell cultures. Results are expressed as mean values of at least 2 to 3 independent experiments.

Examination of the *in vitro* antiretroviral activity indicates some interesting and novel findings. Although the new pro-nucleotides were not generally found to be as active as FLT in TK-positive cells (CEM/0), some phosphoramidates (e.g. **3j-k**) and phosphorodiamidates (e.g. **5a-b**) gave EC₅₀ values in the middle (\leq 100 nM) nanomolar concentration range for HIV-1 and/or HIV-2. Phosphorodiamidate (**5b**) (R = neopentyl) was found to be the most potent compound in the assays. Importantly, the test compounds were able to retain moderate activity in the CEM/TK⁻ (TK mutant) cell cultures in the lower micromolar range, unlike FLT (with the exception of ProTides **3d**, **3h-i**, **3m** and **5b**). Based on the overall profile across these three test assays, FLT phosphoramidate (**3k**, R = naphthyl; R' = neopentyl) and FLT phosphorodiamidate (**5a**, R = Bn) are the most potent and promising compounds for further study.

Importantly, compound (**3k**) shows only a 10-fold loss of activity in the **TK knockout CEM mutant cell** assay, versus >1000-fold for compound (**1**).

In vitro examination of the cytostatic potential of the FLT ProTides was studied in the human T-lymphocyte CEM cells (both wild-type TK and TK-deficient (TK⁻)), and the murine leukaemia cell line L1210, according to previously reported protocols.^{24,28} The results of these studies are shown in Table 2.

Cpd.	IC ₅₀ / μM		
	CEM/0	CEM/TK ⁻	L1210
3a	>250	>250	8.2 ± 3.7
3b	>250	>250	4.4 ± 1.4
3c	145 ± 38	>250	23 ± 1
3d	124 ± 58	>250	6.3 ± 0.6
3e	117 ± 88	172 ± 59	5.8 ± 0.6
3f	66 ± 10	112 ± 52	3.0 ± 1.2
3g	81 ± 1	138 ± 5	6.0 ± 0.9
3h	206 ± 62	>250	24 ± 2
3i	148 ± 17	>250	36 ± 3
3j	13 ± 2	20 ± 3	2.9 ± 1.2
3k	14 ± 1	20 ± 3	1.9 ± 1.1
3l	54 ± 5	57 ± 8	5.2 ± 3.3
3m	100 ± 12	>250	43 ± 13
5a	17 ± 6	22 ± 5	1.2 ± 1.1
5b	66 ± 11	43 ± 34	3.8 ± 2.7
1	39 ± 26	>250	0.081 ± 0.037

Table 2. Cytostatic activity (IC₅₀, μM) values in human T-lymphocyte (CEM) and murine leukaemia (L1210) cells. Results are expressed as mean values of at least 2 to 3 independent experiments.

Examination of Table 2 reveals few compounds with significantly potent cytostatic activity within these cell line models. The most potent activity was observed in the L1210/0 leukaemia cell line, where low micromolar IC₅₀ values was found for a variety of compounds, but where cytostatic activity was lower than for the parental FLT (**1**). Gratifyingly, compounds with moderate activity in the CEM/0 cell line (such as **3j**, **3k** and **5a**) were largely able to retain activity within the CEM/TK⁻ tumour cell model (unlike the parent FLT) and were non-toxic at antiviral concentrations.

In conclusion, the preparation of a series of phosphoramidate and phosphorodiamidate Pro-Tides of the antiviral lead compound FLT have been prepared. *In vitro* screening with anti-HIV and anticancer model systems revealed compounds with generally lower activity than the parent lead, but with the ability to retain activity within TK⁻ models. This retention of activity across the test panels provides further evidence for the potential of ProTides to, at least in part, by-pass the need of thymidine kinase-catalysed phosphorylation

and deliver the intact nucleoside monophosphate ready for further metabolic conversion to the biologically active form.

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Supplementary Data available: ¹H, ¹³C, ¹⁹F and ³¹P NMR, mass spectrometry and HPLC data for FLT pro-nucleotide analogues.

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21. *General method for synthesis of FLT phosphoramidates (3a-m)*. A solution of *tert*-butylmagnesium chloride (1M in THF, 1.3 eq.) was added dropwise to a stirring solution of 3'-deoxy-3'-fluorothymidine (FLT, **1**, 0.1 M) in anhydrous THF at room temperature, followed by stirring for 30 min. A solution of phosphochloridate (**2a-m**, 2.05 eq.) in anhydrous THF (1 mL) was then added dropwise to the reaction mixture followed by stirring for 14-22h. THF was then removed *in vacuo* and the residue purified by column chromatography (CHCl₃/MeOH 100/0 to 95/5) followed by preparative TLC (CHCl₃/MeOH 95/5) to give the required FLT phosphoramidates (**3a-m**) as colorless oils.
22. Representative characterisation data for FLT phosphoramidates: 3'-Deoxy-3'-fluorothymidine 5'-O-1-naphthyl-(neopentoxo-alaninyl)-phosphate (**3k**). This compound was synthesized according to the procedure above in 57% yield. The ratio of the diastereoisomers (d/s) at the phosphorous atom was 1/0.7 (¹H NMR analysis). ¹H-NMR (500 MHz, CDCl₃): δ 8.62 (br s, 1H, NH of one d/s), 8.58 (brs, 1H NH of one d/s), 8.05 (m, 1.7H, ArH), 7.86 (m, 1.7H, ArH), 7.68 (m, 1.7H, ArH), 7.56-7.49 (m, 5.1H, ArH), 7.42-7.35 (m, 2.7H, ArH + H5 of both d/s), 7.29 (dd, *J* = 6.0, 0.9 Hz, 0.7H, ArH), 6.29 (dd, *J* = 9.5, 5.2 Hz, 0.7H, H1' of one d/s), 6.23 (dd, *J* = 9.4, 5.2 Hz, 1H, H1' of one d/s), 5.17 (m, dd, *J* = 5.0, 3.5 Hz, 0.7H, H3' of one d/s), 5.11 (m, dd, *J* = 5.0, 3.5 Hz, 1H, H3' of one d/s), 4.50-4.45 (m, 0.7H, H4' of one d/s), 4.43-4.30 (m, 4.4H, H4' of one d/s, H5' of both d/s), 4.19-4.10 (m, 1.7H, CH Ala of both d/s), 4.00-3.96 (dd, *J* = 9.0, 11.2 Hz, 1H, NH of one d/s), 3.88, 3.76 (AB system, *J* = 10.5 Hz, 2H, CH₂ neopentyl of one d/s), 3.84-3.80 (m, 0.7H, NH of one d/s), 3.86, 3.69 (AB system, *J* = 10.5 Hz, 1.4H, CH₂ neopentyl of one d/s), 2.46-2.35 (m, 2H, H2'a of both d/s), 1.82 (d, *J* = 0.9 Hz, 2.1H, CH₃ base of one d/s), 1.80 (d, *J* = 1.0 Hz, 3H, CH₃ base of one d/s), 1.70-1.62 (m, 1.7H, H2'b of both d/s), 1.42 (d, *J* = 7.0 Hz, 3H, CH₃ Ala of one d/s), 1.37 (d, *J* = 7.1 Hz, 2.1H, CH₃ Ala of one d/s), 0.93 (s, 9H, CH₃ neopentyl of one d/s), 0.90 (s, 6.3H, CH₃ neopentyl of one d/s). ¹³C-NMR (126 MHz, CDCl₃): δ 173.5 (d, *J* = 7.3 Hz, C=O of one d/s), 173.3 (d, *J* = 7.3 Hz, C=O of one d/s), 163.39 (Cq), 163.32 (Cq), 150.07 (Cq), 150.05 (Cq), 146.3 (d, *J* = 6.9 Hz, Cq), 146.1 (d, *J* = 6.8 Hz, Cq), 135.0 (C6 of one d/s), 134.8 (C6 of one d/s), 134.7 (Cq), 128.06, 128.04, 126.8, 126.56, 126.55 (CAr), 126.28, 126.23, 126.21, 126.16 (Cq), 125.46, 125.42, 125.40, 125.3, 125.2, 121.0, 120.9, 115.40, 115.3, 115.1, 115.08 (CAr), 111.44 (C5 of one d/s), 111.39 (C5 of one d/s), 93.4 (d, *J* = 179.3 Hz, C3' of one d/s), 93.2 (d, *J* = 179.7 Hz, C3' of one d/s), 85.0 (C1' of one d/s), 84.8 (C1' of one d/s), 82.9 (dd, *J* = 7.7, 27.0 Hz, C4' of one d/s), 82.8 (dd, *J* = 7.7, 26.8 Hz, C4' of one d/s), 74.98 (CH₂ neopentyl), 74.95 (CH₂ neopentyl), 66.1 (dd, *J* = 5.1, 11.3Hz, C5' of one d/s), 65.9 (dd, *J* = 5.2, 10.9 Hz, C5' of one d/s), 50.58 (d, *J* = 1.5Hz, CH Ala of one d/s), 50.50 (CH Ala of one d/s), 37.9 (d, *J* = 20.9 Hz, C2' of one d/s), 37.8 (d, *J* = 20.9 Hz, C2' of one d/s), 31.3 (Cq of one d/s), 31.4 (Cq of one d/s), 26.27 (CH₃ neopentyl of one d/s), 26.26 (CH₃ neopentyl of one d/s), 21.2 (d, *J* = 4.6 Hz, CH₃ Ala of one d/s), 21.1 (d, *J* = 5.2 Hz, CH₃ Ala of one d/s), 12.42 (CH₃ base of one d/s), 12.24 (CH₃ base of one d/s). ¹⁹F-NMR (471 MHz, CDCl₃): δ -174.92, -175.21. ³¹P-NMR (202 MHz, CDCl₃): δ 3.18, 3.03. MS (ES+) *m/z*: 614.20 (M + Na⁺). Reverse HPLC eluting with H₂O/MeOH from 90/10 to 0/100 over 25 min: *t_R* = 39.91 and 58.53 min (98%).
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25. *General method for synthesis of FLT phosphorodiamidates (5a-b)*. Et₃N (1.05 eq) was added dropwise to a solution of FLT (**1**) in anhydrous THF (0.2 M). The reaction mixture was stirred at rt for 30 min and was then cooled down to -78°C before addition of POCl₃ (1.05 eq) dropwise. The reaction mixture was stirred at -78°C for 1h before addition of the appropriate amino acid (5 eq), DCM (4-5 mL) and Et₃N (10 eq). The reaction mixture was stirred at rt for 16-27 h before evaporation of solvents to dryness. Purification of the crude was done by column chromatography (CHCl₃/MeOH 100/0 to 95/5) followed by a preparative TLC (CHCl₃/MeOH 95/5) to give the desired diamidate prodrug as a colorless oil.
26. Representative characterisation data for FLT phosphorodiamidates: FLT-5'-O-bis((benzoxy-L-alaninyl)] phosphate (**5a**). This compound was synthesized according to the procedure above in 10% yield. ¹H-NMR (500 MHz, MeOD): δ 7.54 (d, *J* = 1.2 Hz, 1H, H6), 7.37-7.30 (m, 10H, ArH), 6.26 (dd, *J* = 9.3, 5.5 Hz, 1H, H1'), 5.24 (dd, *J* = 5.0, 3.5 Hz, 1H, H3'), 5.14-5.11 (m, 4H, 2xCH₂Ph), 4.33-4.26 (m, 1H, H4'), 4.14-4.06 (m, 2H, H5'), 3.98-3.92 (m, 2H, 2xCH Ala), 2.50-2.41 (m, 1H, H2'-a), 2.30-2.17 (m, 1H, H2'-b), 1.87 (d, *J* = 1.2 Hz, 3H, CH₃ base), 1.38 (dd, *J* = 7.2, 0.6 Hz, 3H, CH₃ Ala), 1.34 (dd, *J* = 7.2, 0.8 Hz, 3H, CH₃ Ala). ¹³C-NMR (126 MHz, MeOD): δ 175.3 (d, *J* = 4.7 Hz, C=O), 175.2 (d, *J* = 6.0 Hz, C=O), 166.2 (C=O), 152.2 (C=O), 137.3 (C6), 137.2 (Cq), 129.61, 129.59, 129.38, 129.34 (CAr), 112.15 (C5), 95.1 (d, *J* = 176.6 Hz, C3'), 86.3 (C1'), 84.6 (dd, *J* = 8.3, 26.3Hz, C4'), 67.98 (CH₂Ph), 67.96 (CH₂Ph), 66.0 (dd, *J* = 11.0, 5.2 Hz, C5'), 51.1 (CH Ala), 38.5 (d, *J* = 20.9 Hz, C2'), 20.7 (d, *J* = 6.2 Hz, CH₃ Ala), 20.6 (d, *J* = 6.5 Hz, CH₃ Ala), 12.6 (CH₃ base). ¹⁹F-NMR (471 MHz, CDCl₃): δ -176.58. ³¹P-NMR (202 MHz, CDCl₃): δ 13.69. MS (ES+) *m/z*: 669.23 (M + Na⁺). Reverse HPLC eluting with H₂O/MeOH from 90/10 to 0/100 in 25 min: *t_R* 20.97 min (95%).
27. *Anti-HIV Activity Assays*: Inhibition of HIV-1(III_B)- and HIV-2(ROD)-induced cytopathicity in CEM cell cultures was measured in microtiter 96-well plates containing ~ 3 x 10⁵ CEM cells/mL infected with 100 CCID₅₀ of HIV per milliliter and containing appropriate dilutions of the test compounds. After 4-5 days of incubation at 37 °C in a CO₂-controlled humidified atmosphere, CEM giant (syncytium) cell formation was examined microscopically. The EC₅₀ (50% effective concentration) was defined as the compound concentration required to inhibit HIV-induced giant cell formation by 50%.

28. *Cytostatic Activity Assays*: All assays were performed in 96-well microtiter plates. To each well were added $(5-7.5) \times 10^4$ tumour cells and a given amount of the test compound. The cells were allowed to proliferate for 48 h (murine lymphocytic CEM and human leukaemia L1210 cells) at 37 °C in a humidified CO₂-controlled atmosphere. At the end of the incubation period, the cells were counted in a Coulter counter. The IC₅₀ (50% inhibitory concentration) was defined as the concentration of the compound that inhibited cell proliferation by 50%.

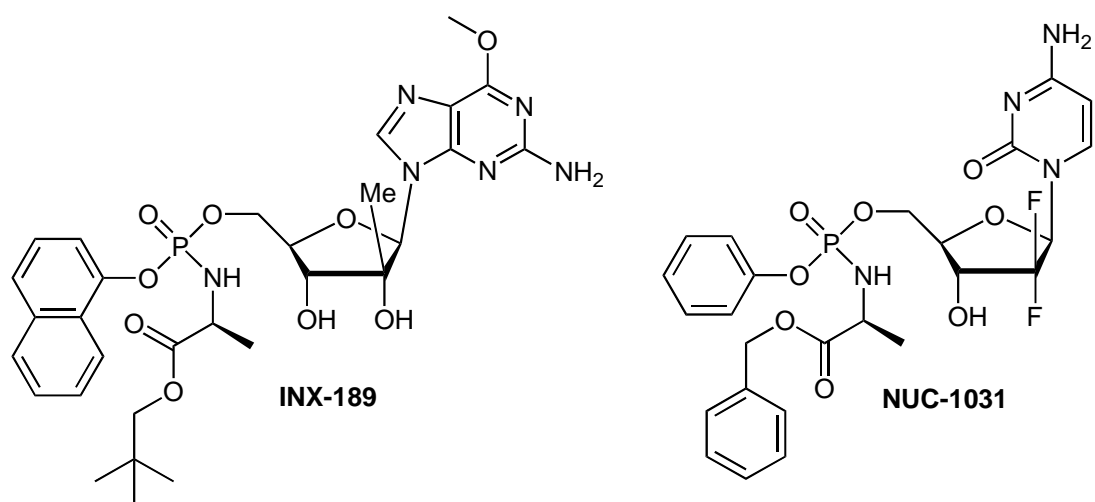
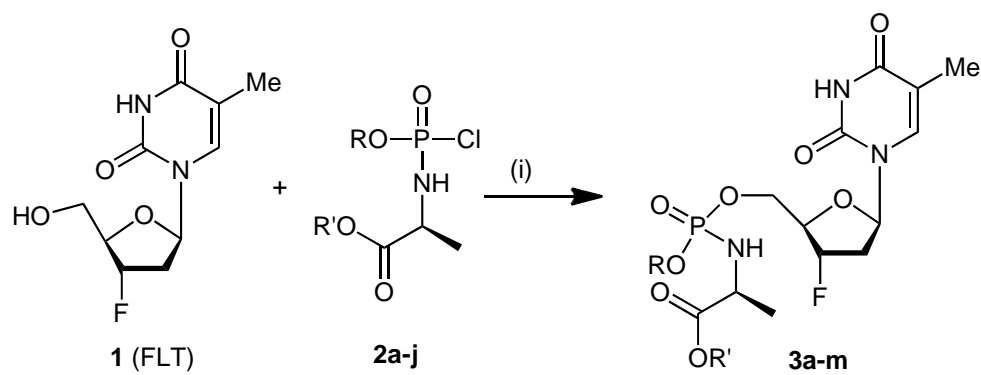
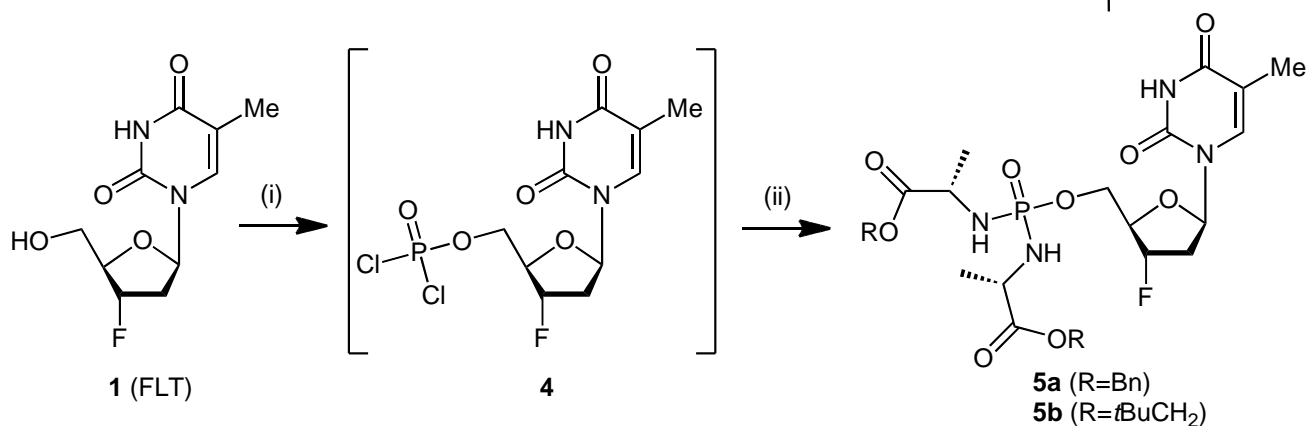


Figure 1. Chemical structures of ProTides INX-189 and NUC-1031.



^aReagents and conditions: (i) *t*-BuMgCl, anhydrous THF, 16h

	R	R'
a	Ph	Me
b	Ph	Et
c	Ph	<i>i</i> -Pr
d	Ph	<i>t</i> -Bu
e	Ph	Bn
f	1-Naph	Me
g	1-Naph	Et
h	1-Naph	<i>i</i> -Pr
i	1-Naph	<i>t</i> -Bu
j	1-Naph	<i>c</i> -Hex
k	1-Naph	<i>t</i> -BuCH ₂
l	1-Naph	Bn
m	Me	Bn



^bReagents and conditions: (i) POCl₃, Et₃N, anhydrous THF; (ii) amino ester (5 eq), Et₃N (10 eq), anhydrous CH₂Cl₂

Scheme 1. Synthesis of FLT pro-nucleotides.