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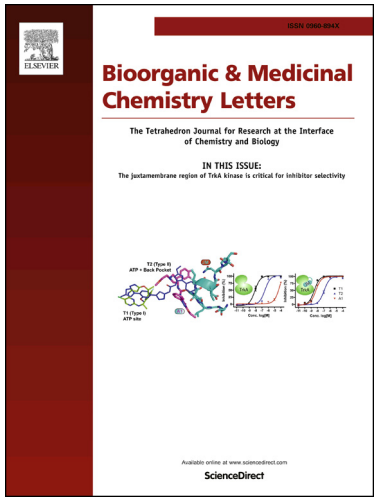

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Synthesis and anti-HIV activities of unsymmetrical long chain dicarboxylate esters of dinucleosides reverse transcriptase inhibitors

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Abstract— A series of 11 unsymmetrical dinucleoside dicarboxylate conjugates of nucleoside reverse transcriptase inhibitors were synthesized. Three dicarboxylic acids, succinic acid, suberic acid and 1,14-tetradecandioc acid, were diesterified with either 3′-azido-2′,3′-dideoxythymidine (AZT), 3′-fluoro-2′,3′-dideoxythymidine (FLT), 2',3'-dideoxy-3'-thiacytidine (3TC) or 5-fluoro-2',3'-dideoxy-3'-thiacytidine (FTC). The anti-HIV activity of synthesized compounds was evaluated against HIV-1 X4 (IIIB) and R5 (BaL) viral strains in single-round infection assays. Results indicated that the tetradecandioate esters of nucleosides were more active against HIV than the corresponding parent nucleosides and nucleoside conjugates. The tetradecandioate conjugate of FLT and FTC (**5**) was found to be the most potent compound with EC_{50} values of 47 and 75 nM against X4 and R5 HIV-1 strains, respectively, while the EC_{50} values for the parent analogs, FLT and FTC, ranged from 700 to 3,300 nM.

Acquired immunodeficiency syndrome (AIDS) is an immune disorder caused by the attack of human immunodeficiency virus (HIV) on immune cells, such as T-lymphocytes dendritic cells, and macrophages.[1,2] HIV uses its two envelope proteins gp120 and gp41 to bind to these immune cells via the CD4 receptor and the chemokine coreceptors CCR5 and CXCR4.[1-4] Depending on the type of coreceptors used for viral binding to the cell membrane, HIV can be classified into two categories: R5 and X4 strains, which bind to CCR5 and CXCR4 coreceptors, respectively.[4] R5 strains predominate among those establishing primary infection at cervicovaginal and colorectal mucosa.[5] After entry into the cell, the virus uses a reverse transcriptase (RT) to copy its RNA and produce viral DNA copies which integrate in the cellular genome, transcribe and produce new virions.[1,2]

Even though the efforts to find proper treatment against HIV started more than three decades ago, the search for effective drugs still continues.[2] Since the beginning, the RT enzyme has been a target for development of effective therapy and several antiretroviral drugs have been introduced and approved as inhibitors of RT Nucleoside reverse transcriptase inhibitors (NRTIs) such as zidovudine (3'-azido-3'-deoxythymidine, AZT), lamivudine (2',3'-dideoxy-3'-thiacytidine, 3TC) and stavudine (2',3'-didehydro-2',3'-dideoxythymidine, d4T) were the first and the only category of antiretroviral drugs (ARVs) approved by FDA until 1995 for HIV treatment (Figure 1).[6] In following years, several other categories of drugs such as non-nucleoside reverse transcriptase inhibitors, integrase strand transfer inhibitors, and protease inhibitors were introduced in the market.[2,6] Currently, NRTIs such as emtricitabine (2',3'-dideoxy-5-fluoro-3'-thiacytidine, FTC) and tenofovir are used in combination with these other categories of ARVs as part of highly active antiretrovrial therapy (HAART) to improve synergy and reduce resistance.. FTC (Figure 1) is a highly potent NRTI and the last approved by FDA in this category. Alovudine (3'-azido-3' deoxythymidine, FLT, Figure 1) is also another highly potent NRT inhibitor. Even though FLT has never been approved for its clinical use against HIV, it remains a drug of interest due to its potency and resistance profile.[7-9]

Lipophilic derivatives of nucleoside analogues have been used to modulate the pharmacological behavior of NRTIs for better biodistribution and improved accumulation at target sites.[10,11] Prodrug modifications of NRTI drugs could be used to improve pharmacokinetics and to enhance their delivery to difficult-to-reach HIV reservoirs.[12,13] In our earlier studies, we have reported the synthesis of long-chain acyl esters of 3TC,[14] d4T,[10] and FTC.[15] These studies indicated a significant increase in the antiviral activities of fatty acyl esters against their parent nucleosides. In

[1]*Both authors contributed equally.*

later studies, we incorporated 2-3 nucleosides in one molecule using various spacers such as amino acids and peptides.[16] Synthesis of lipophilic derivatives containing two different anti-HIV nucleosides would allow for the generation of asymmetrical conjugates that can provide simultaneous delivery of two nucleoside analogs, improve their resistance profile, and generate synergistic activity. The selection of the nucleosides was based on their potency and resistance profile.

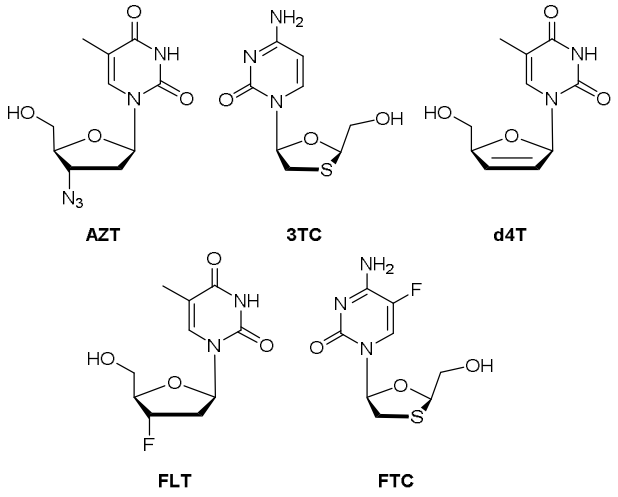
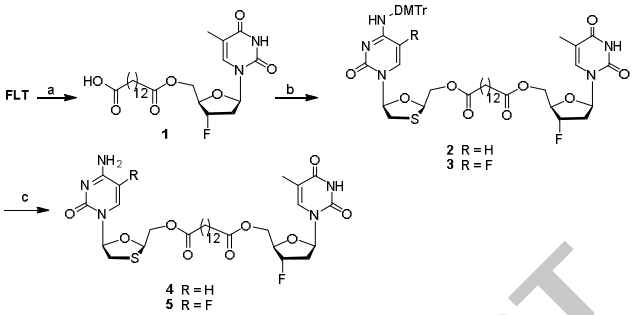


Figure 1. Chemical structures of zidovudine (AZT), lamivudine (3TC), stavudine (d4T), alovudine (FLT), and emtricitabine (FTC).

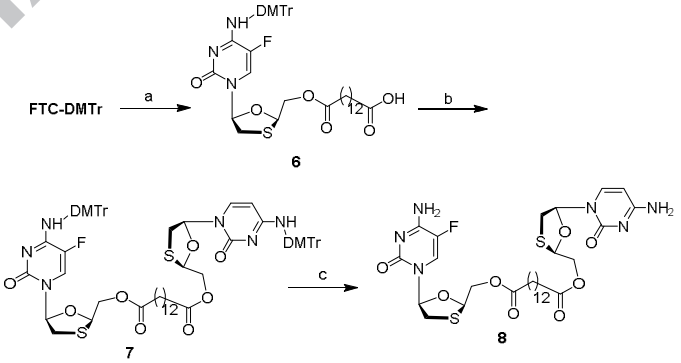
In a recent study, we screened symmetrical dinucleoside dicarboxylate conjugates against HIV.[17] We found that in most cases, the presence of two nucleosides on a fatty acyl chain did not affect the overall anti-HIV activity. However, the diesters of tetradecanoic acid were more active than the parent nucleoside and the corresponding shorter chain nucleoside conjugates. Based on these results, in the current study, we have synthesized unsymmetrical dinucleoside conjugates of 1,14-tetradecandioic acid (C_{14}) with three highly active antiviral agents FLT, FTC, and 3TC. We also synthesized similar symmetrical and unsymmetrical short (C_4) and intermediate chain (C_8) conjugates for comparative studies against X4 and R5 HIV-1 strains. Lipophilic conjugates were expected to display better membrane interaction, show improved cellular uptake, release two different nucleosides intracellularly, and provide an overall synergistic effect.

Schemes 1-5 depict the synthesis of dinucleoside-fatty acid conjugates. Unsymmetrical dinucleoside 1,14-tetradecandioate derivatives of FLT, 3TC and FTC were synthesized by reaction of nucleosides with 1,14-tetradecandioic acid in the presence of HBTU as a coupling agent (Schemes 1 and 2). Both 3TC-DMTr and FTC-DMTr were synthesized using the previously published procedure.[14,15]



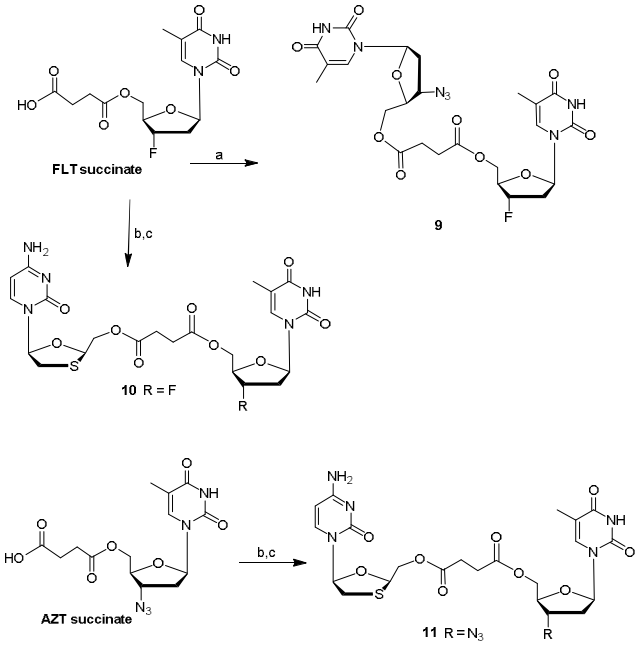
Scheme 1. Synthesis of unsymmetrical 5•,5•-dinucleoside 1,14-tetradecanoate esters of FLT with FTC (**4**) and 3TC (**5**): (a) 1,14-tetradecandioic acid, HBTU, DIPEA, DMF, rt, 4 h; (b) 3TC-DMTr/FTC-DMTr, HBTU, DIPEA, DMF, rt, 6 h; (c) TFA in acetic acid (2%, v/v), rt, 1 h.

FLT and N4-protected FTC (FTC-DMTr) were first coupled with an excess of 1,14-tetradecandioic acid to obtain the mono-substituted product (**1** and **6**, respectively). FLT-tetradecanoate (**1**) was further coupled with either FTC-DMTr or 3TC-DMTr followed by removal of protecting group to produce conjugates FLT-C_{14}-FTC (**4**) and FLT-C_{14}-3TC (**5**; Scheme 1). DMTr-FTC-tetradecanoate (**6**) was coupled with 3TC-DMTr, and the protective group was removed to produce FTC-C_{14}-3TC (**8**, Scheme 2).



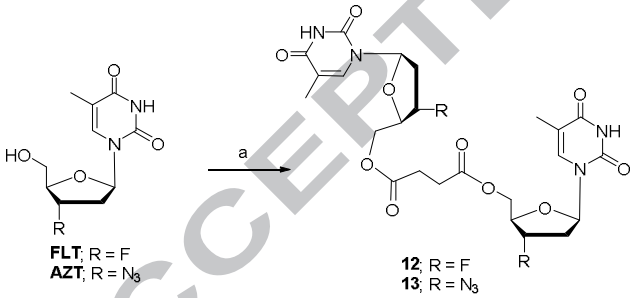
Scheme 2. Synthesis of unsymmetrical 5•,5•-dinucleoside 1,14-tetradecandioate esters of FTC with 3TC (**8**): (a) 1,14-tetradecandioic acid, HBTU, DIPEA, DMF, rt, 4 h; (b) 3TC-DMTr, HBTU, DIPEA, DMF, rt, 6 h; (c) TFA in acetic acid (2%, v/v), rt, 1 h.

Similarly, succinate esters of AZT, FLT and 3TC were synthesized by coupling FLT-succinate with AZT (FLT-C_4-AZT, **9**) and 3TC-DMTr and by coupling AZT-succinate with 3TC-DMTr. Protective group (DMTr) was removed in situ to produce FLT-C_4-3TC (**10**) and AZT-C_4-3TC (**11**) (Scheme 3).

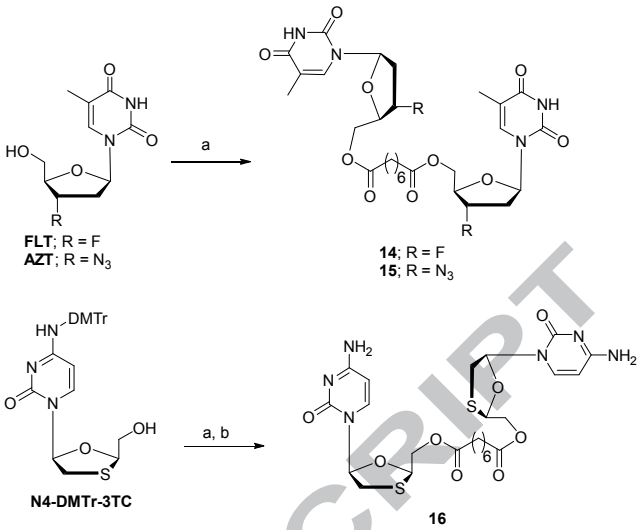


Scheme 3. Synthesis of unsymmetrical 5•,5•-dinucleoside 1,4-succinate derivatives of FLT, AZT, and 3TC. (a) AZT, HBTU, DIPEA, DMF, rt, overnight; (b) 3TC-DMTr, HBTU, DIPEA, DMF, rt, overnight; (c) acetic acid (80% in water, v/v), 80 °C, 1 h.

Corresponding symmetrical dinucleoside 1,4-succinate derivatives of FLT (FLT-C_4-FLT, **12**) and AZT (AZT-C_4-AZT, **13**) were synthesized by reaction of nucleosides with succinyl chloride (Scheme 4). Symmetrical dinucleoside 1,8-suberates (**14**-**16**, Scheme 5) were synthesized by reacting two equivalents of nucleosides, FLT, AZT, or DMTr-3TC, with one equivalent of suberic acid and removing protecting group in 3TC conjugate (**16**).



Scheme 4. Synthesis of symmetrical 5•,5•-dinucleoside 1,4-succinate derivatives of FLT (**12**) and AZT (**13**). (a) FLT/AZT, succinyl chloride, DMAP, benzene, rt, overnight.



Scheme 5. Synthesis of symmetrical 5•,5•-dinucleoside 1,8-suberate derivatives of FLT (**14**), AZT (**15**), and 3TC (**16**). (a) HBTU, suberic acid, DIPEA, DMF, rt, overnight; (b) acetic acid (80% in water, v/v), 80 °C, 1 h.

All the intermediates and final conjugates were purified by silica gel normal phase chromatography using DCM/methanol eluent. Final conjugates were further purified for higher purity by reverse phase high performance liquid chromatography using water/methanol as gradient solvent eluents. The purity of the final products was confirmed by analytical HPLC (95-99% pure) with detection at 265 nm wavelength.

Targeted synthesized compounds were tested for their cytotoxicity and anti-HIV activity against cell-free virus (X4 and R5 strains) (Table 1). In general, the synthesized compounds showed no significant cytotoxic effects against target cells as shown in MTS cytotoxic assays.

As shown in Table 1, the unsymmetrical conjugates of the FLT, 3TC and FTC with tetradecandioic acid (**4**, **5** and **8**) showed high antiviral activity (0.047-0.18 μM), being significantly more potent than their parent nucleoside analogues (0.7-32.7 μM). Although all three conjugates were highly active against R5 strains of HIV (EC_{50} ~ 0.07 μM), only conjugate **5** was very active against X4 strains with an EC_{50} value of 0.047 μM. FLT-C_{14}-FTC (**5**) was the most potent molecule among the studied conjugates with an EC_{50} of 47 nM and 75 nM against IIIB and BaL strains of HIV, respectively. It was clear that the conjugates of nucleosides with higher activity against HIV such as FLT and FTC resulted in high antiviral activity. Also, simply introducing a fatty acid group on a nucleoside drug did not improve its activity. Conjugate **5** was 38 and 7 times more potent when compared to mono FLT-tetradecanoate derivative (**1**) with EC_{50} values of 1.8 μM against HIV IIIB and 0.55 μM against HIV BaL, respectively.

Table 1. Anti-HIV Activity of dicarboxylic acid nucleoside conjugates.

Compd.	Description	Cytotoxicity[a] CC_{50} (μM)	HIV-1 IIIb[b] EC_{50} (μM)	HIV-1 BaL[c] EC_{50} (μM)
AZT	3'-azido-2',3'-dideoxythymidine	>374	34.4	129
FLT	3'-fluoro-2',3'-deoxythymidine	>410	0.8	3.3
3TC	2',3'-dideoxy-3'-thiacytidine	80.3	32.7	11.3
FTC	5-fluoro-2',3'-dideoxy-3'-thiacytidine	>200	1.9	0.7
1	FLT-C_{13}-COOH	>61	1.8	0.55
4	FLT-C_{14}-3TC	>43	0.1	0.070
5	FLT-C_{14}-FTC	>42	0.047	0.075
8	3TC-C_{14}-FTC	>43	0.18	0.073
9	AZT-C_4-FLT	>168	14.7	17.5
10	3TC-C_4-FLT	>180	44.5	42.2
11	3TC-C_4-AZT	>173	16.1	19.2
12	FLT-C_4-FLT	>175	3.7	3.0
13	AZT-C_4-AZT	>162	14.0	68.1
14	FLT-C_8-FLT	>160	0.4	0.5
15	AZT-C_8-AZT	>148	0.2	<0.15
16	3TC-C_8-3TC	>168	26.6	4.7

[a]Cytotoxicity assay (MTT), CC_{50} (50% toxic concentration); [b]Single-round infection assay (lymphocytotropic strain; X4-strain), EC_{50} (50% effective concentration; [c]Single-round infection assay (monocytotropic strain; R5-strain). In the single-round infection assay, compounds, virus and cells were incubated for 2 hours. Cells were then washed and cultured for additional 48 h. Infection was measured by HIV-LTR driven galactosidase expression. DMSO and dextran sulfate were used as negative and positive controls, respectively. All the assays were carried out in triplicate (n = 3).

Anti-HIV activities of tetradecandioic acid esters were compared with the short chain (C_4), symmetrical and unsymmetrical succinate esters of FLT, AZT and 3TC. The unsymmetrical dioate nucleoside conjugates containing the succinate linker (**9**-**13**) (EC_{50} = 3-68 μM) were not as potent as those with the longer spacers. These results indicate that the prodrug formation through succinate linker approach is not efficient enough to improve the antiviral activity of nucleosides. Results were in agreement with our previous studies where long chain fatty acid esters showed higher activity than those with short chain esters. Conjugates **4**, **5** and **8** were also compared with moderate chain length (C_8) conjugates of dinucleosides FLT, AZT and 3TC (**14**-**16**). Even though suberate esters of dinucleoside (**14-16**, EC_{50} <0.15-26 μM) showed higher activity against HIV than the parent nucleosides (EC_{50} = 0.8-129 μM) and the corresponding succinate derivatives (**9-13**, EC_{50} = 3-68 μM), their activity was still weaker than that of tetradecanoate diesters (**4**, **5**, **8**; EC_{50} = 0.047-0.18 μM). Suberate derivatives of AZT (**15**, EC_{50} <0.15-0.2 μM) showed a significant improvement in antiviral activity in comparison with both AZT (EC_{50} = 34-129 μM) and the corresponding succinate derivative (**13**, EC_{50} = 14-68 μM).

In conclusion, conjugation of two different NRTIs through 1,14-tetradecandioic acid spacer resulted in conjugates with antiviral activities in the 0.047-0.18 μM range. Conjugates with C_{14} chain showed significantly improved antiviral activity in comparison to their parent nucleosides and suberic acid (C_8 chain) and succinic acid (C_4 chain). Conjugate FLT-C_{14}-FTC (**5**, EC_{50} = 0.047-0.075 μM) was the most active conjugate in this series. In our earlier studies on FLT, d4T, FTC, and 3TC esters of long chain fatty acids, we explained the effect of the lipophilic nature of the synthesized esters in improving cellular uptake and antiviral activity. In this study, tetradecandioic acid esters have longer carbon chain length spacer and are more lipophilic than suberic acid and succinate esters. Based on these findings, within the boundaries of the analogs tested, it can be concluded that longer chain length spacers between nucleosides analogues improved antiviral activity. Tetradecandioic acid was found to be an optimal linker for the improved potency of synthesized dinucleoside prodrugs, possibly due to the improved cellular uptake.

Acknowledgements

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Supplementary data

Supplementary data (experimental synthetic procedures and characterization of compounds using ^{1}H NMR, ^{13}C NMR, and HR–MS (ESI–TOF), and anti-HIV assays) can be found in the online version of this article.

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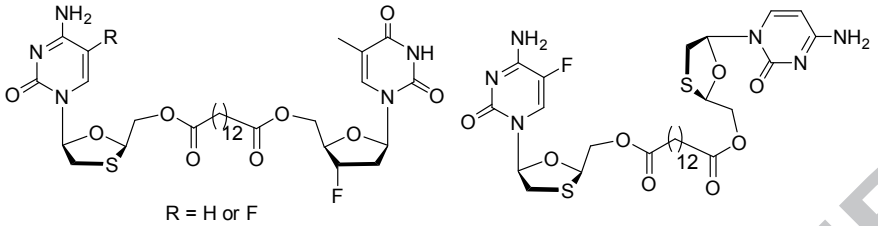
Graphical Abstract

The synthesis of unsymmetrical long chain dicarboxylate esters of dinucleoside reverse transcriptase inhibitors and their anti-HIV activities are reported.



[1]*Both authors contributed equally.*