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Anti-retroviral and cytostatic activity of 2′,3′-dideoxyribonucleoside 3′-disulfides

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ABSTRACT

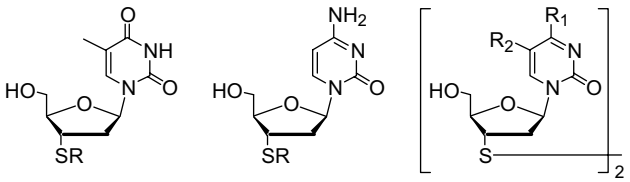
Herein, we report the synthesis, antiviral and cytostatic effects of nucleosides bearing a 3′-disulfide function as prodrugs of potentially active 3′-mercaptonucleotides. The lack of the anti-HIV effects in mutant CEM/TK-cells for most of the thymidine disulfides suggests that a phosphorylation step involving thymidine kinase is necessary for the eventual antiviral activity of the thymidine nucleosides. The comparable anti-HIV activities of most of the disulfides and their rapid reduction in CEM cell extracts imply an inhibitory effect of the 2′,3′-dideoxy-3′-mercaptothymidine 5′-triphosphate metabolite. The cytostatic effects of the disulfides in CEM/0 and Molt4/C8 cells appeared to be strongly dependent on the nature of the non-nucleosidic disulfide moiety and were decreased in preserving the anti-retroviral activity.

1. Introduction

In the field of nucleosides, nucleotides, and oligonucleotides, sulfur chemistry appears particularly fruitful in the development of therapeutic agents and tools for biology.[1–4] The explosion of interest in thionucleosides began by the discovery in the early 1990's of the L-isomer of 2′-deoxy-3′-thiacytidine [(−)-β-L-3TC, Lamivudine)] that is among the most potent inhibitors of HIV (Human Immunodeficiency Virus) and HBV (Hepatitis B Virus).[5–7]

To exhibit therapeutic effects through disruption in the biosynthesis of the viral nucleic acids, the modified nucleosides have to be phosphorylated (activated) by cellular kinases. For example, the antiviral drug 3′-azido-2′,3′-dideoxythymidine (3′-azidodT, AZT) inhibits HIV reverse transcription after phosphorylation to lead to the corresponding 5′-triphosphate.[8]

In the search for antiviral agents acting as inhibitors of the viral DNA chain elongation, 2′,3′-dideoxynucleosides bearing at the 3′-position a sulfur atom such as 3′-thiocyanato- or 3′-methylthio-2′,3′-dideoxythymidine (3′-methylthiodT) appeared inactive against HIV.[9] However, 3′-mercapto 2′,3′-dideoxynucleoside 5′-triphosphates (T, C, A, G) were reported to irreversibly inhibit DNA chain elongation by AMV (Avian Myeloblastosis Virus) and HIV reverse transcriptases.[10–12] More recently, these 5′-triphosphates were found able to function as substrates for the Y410F mutant of Deep Vent (exo⁻) polymerase.[13] In the evaluation of the antiviral properties of the corresponding nucleosides, 3′-mercaptodT **1** (Fig. 1) appeared inactive in CEM cells infected with HIV;[14] however, 3′-mercapto-2′,3′-dideoxycytidine (3′-mercaptodC) **3** has shown activity against HIV-1 in MT4 cell cultures (EC_{50} = 20 μM).[15] The easy oxidation of the thiol function in such nucleosides leading rapidly to the corresponding symmetrical disulfides is a key problem in their evaluation and use as antiviral agents. The symmetrical 3′-disulfides dT **5** and dC **6**, respectively, were found inactive in HIV-infected MT4 cells.[15] However, an anti-HIV effect of **5** has been observed in infected MT4 cells (EC_{50} = 16 μM), albeit at a concentration that is close to its 50%-cytostatic concentration (CC_{50} = 34 μM).[9]



1: R = H
2: R = SCH_3
3: R = H
4: R = SCH_3
5: R_1= OH, R_2 = CH_3
6: R_1= NH_2, R_2 = H
7: R_1= OH, R_2 = H

Figure 1. Structures of the 3′-mercaptonucleosides and the corresponding methyl and symmetrical disulfides.

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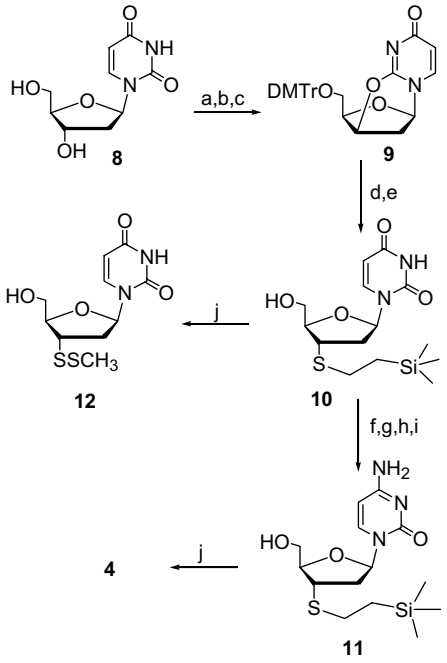
In order to evaluate the antiviral potential of a stable precursor of 3′-mercaptodT **1**, we have synthesized its methyl disulfide prodrug **2**[16] (Fig. 1) using the 2-(trimethylsilyl)ethanethiol chemistry.[17] Compound **2** has been found able to reduce HIV-1 replication by 50% at 5–10 μM concentrations in CEM cell cultures with a CC_{50} near 150 μM.[14] It showed anti-HIV effects much higher than the corresponding symmetrical disulfide **5** and thiol **1**, the latter being ineffective.

Therefore, the methyl disulfide **2** appeared to be an interesting prodrug which should be reduced and phosphorylated in vivo to generate the active 3′-mercaptodT 5′-triphosphate probably acting at the HIV reverse transcription level. The nature of the side methyl group in the disulfide function should be involved in the efficiency of the cellular uptake and in the toxicity of the prodrug and limit the formation of the active nucleotide. We report here the synthesis and the antiviral activities of nucleosides mixed disulfides as prodrugs of 3′-mercapto-dT **1** and -dC **3** and their 5′-phosphates.



Scheme 1. Preparation of the 2′,3′-dideoxyuridine and cytidine 3′-methyl disulfides. Reagents and conditions: (a) DMTrCl, Py; (b) MsCl, Py; (c) K_2CO_3, CH_3CN, reflux (78% for the three steps a,b,c); (d) 2-trimethylsilylethanethiol, DMF, NaH; (e) dichloroacetic acid, CH_2Cl_2 (56% for the steps d,e); (f) Ac_2O, Py; (g) 1,2,4-triazole, $POCl_3$, Et_3N, CH_3CN; (h) NH_3, dioxane; (i) NH_3, EtOH, H_2O (80% for the four steps f,g,h,i); (j) dimethyl(methylthio)sulfonium tetrafluoroborate, dimethyl disulfide, THF (**4**: 75%, **12**: 74%).

2. Results and discussion

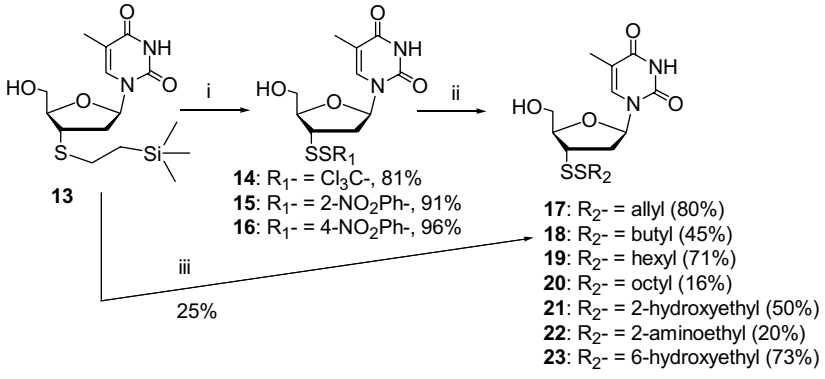
2.1. Synthesis

The dT methyl 3′-disulfide **2** (Fig. 1) was prepared from the corresponding 3′-(2-(trimethylsilyl)ethyl) (TMSE) sulfide **13** through reaction with dimethyl(methylthio)sulfonium tetrafluoroborate in the presence of a large excess of dimethyl disulfide.[16] The same methodology was used for preparing the dC methyl 3′-disulfide **4** (Scheme 1) through the synthesis of 3′-TMSE sulfide **10** in the 2′,3′-dideoxyuridine (dU) series and conversion of the uracil base to cytosine. Reaction of the anhydro dU derivative **9**[18,19] at 90 °C for 1 h with sodium 2-trimethylsilylethanethiolate and detritylation led to the 2′-TMSE sulfide **10** as the major product (56%, two steps). This sulfide was converted to the corresponding cytosine sulfide **11** in four steps using a classical procedure (Scheme 1, 80% yield).[16] The 3′-TMSE sulfides dU **10** and dC **11** led to the corresponding methyl disulfides **12** and **4** in 74% and 75% yields, respectively (Scheme 1).

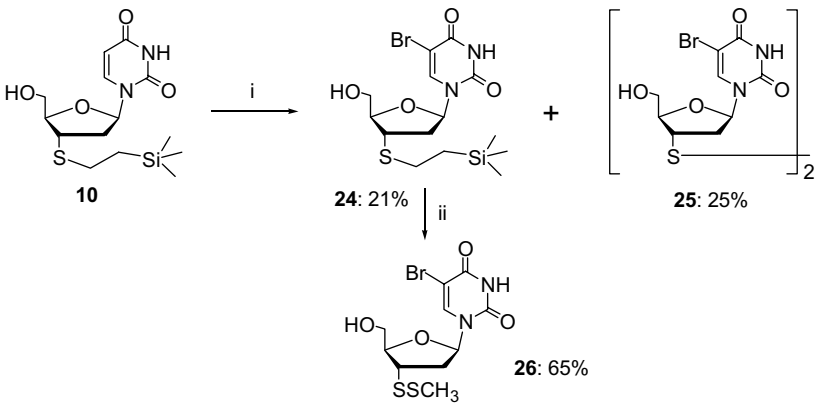
According to the reaction previously developed for preparing nucleoside vinyl disulfides,[20,21] the dT 3′-disulfides **14**, **15**, and **16** were prepared by reaction of the sulfide **13** with trichloromethylsulfenyl, 2- and 4-nitrobenzenesulfenyl chlorides in 81%, 91%, and 96% yields, respectively (Scheme 2). This reaction should be interesting in the preparation of oligonucleotides incorporating 2′,3′-dideoxy-3′-mercaptonucleotides[22–24] and in the synthesis of 2′,3′-dideoxy-3′-mercaptonucleoside triphosphates.[13]

The 4-nitrophenyl disulfide **16** was used for preparing various mixed alkyl disulfides through reaction with aliphatic thiols in aqueous solution at pH 9.5 (phosphate buffer). Disulfides carrying hydrophobic allyl (**17**), butyl (**18**), hexyl (**19**), octyl (**20**) groups, hydrophilic 2-hydroxyethyl (**21**) and 2-aminoethyl (**22**), and miscellaneous 6-hydroxyhexyl (**23**) side chains were prepared in low to good yields (16–80%). The formation of the dT 3′-symmetrical disulfide **5** as a side product was observed in most of the experiments.

We had previously observed that the reaction of 2′-deoxy-2′-(2-(trimethylsilyl)ethylthio)uridine with cyanogen bromide in dichloromethane leads to the corresponding uridine symmetrical disulfide.[25] Such a reaction should proceed through formation of the intermediate 2′-deoxyuridine 2′-sulfenyl bromide which then reacts with the starting silyl sulfide. Alkylsulfenyl halides are unstable and are not commercially available. They should be generated in situ by treatment of aliphatic symmetrical disulfides with bromine or cyanogen bromide.



Scheme 2. Preparation of the 3′-deoxythymidine 3′-mixed disulfides. Reagents and conditions: (i) R_1SCl (Cl_3CSCl, 4-NO_2PhSCl or 2-NO_2PhSCl), CH_2Cl_2, rt, 4 h; (ii) R_2SH, aqueous phosphate buffer, pH 9.5, THF, rt; (iii) dibutyl disulfide, Br_2, CH_2Cl_2, rt.



Scheme 3. Preparation of the 5-bromouracil nucleosides. Reagents and conditions: (i) BrCN, CH_2Cl_2, reflux, 48 h; (ii) dimethyl(methylthio)sulfonium tetrafluoroborate, dimethyl disulfide, THF.

These assumptions were confirmed through the preparation of the dT butyl 3′-disulfide **18** which was obtained in 25% yield after reaction of the TMSE sulfide **13** at room temperature in dichloromethane with dibutyl disulfide in excess and bromine.

This reaction and this procedure represent a new route for preparing rapidly mixed disulfides from TMSE sulfides.[21] The dT symmetrical disulfide **5** also was obtained in 43% yield using this approach after reaction of the corresponding TMSE sulfide **13** with cyanogen bromide in dichloromethane (reflux). In order to prepare the corresponding dU symmetrical disulfide **7**, the dU 3′-TMSE sulfide **10** was treated with cyanogen bromide under the same conditions (Scheme 3). The concomitant bromination of the uracil base occurred to lead to the 5-bromo symmetrical disulfide **25** (25%) and 5-bromodU TMSE sulfide **24** (21%). This latter led to the 5-bromodU 3′-methyl disulfide **26** in 65% yield (Scheme 3).

The symmetrical disulfides dU **7** and dC **6** were finally prepared from the corresponding methyl disulfides **4** and **12**, respectively, through their reduction in methanol with dithiothreitol (DTT) and in situ oxidation by aeration.

2.2. Reduction of the disulfides

In order to study the reduction of the prepared disulfides **2**, **4**, **14**–**23**, DTT in excess was used as a reducing agent. Complete and clean reductions were observed in methanol or DMSO leading to the corresponding thiol **1** or **3** (TLC, in situ ^{1}H NMR spectrometry in CD_3OD, mass spectrometry, characteristic yellow coloration in the presence of the Ellman's reagent). In the presence of an excess of DTT in methanol (5 equiv, 10 mM disulfide concentration, 20 °C), the reduction of the aromatic disulfides **15** and **16** was rapid and complete ($t_{1/2}$ < 10 min). The reaction was slower for the non-aromatic disulfides ($t_{1/2}$ (**14**) < 90 min, $t_{1/2}$ (**2**, **17**–**23**) < 8 h). The velocity of reduction of the dT disulfides **2**, **14**–**23** and the dC methyl disulfide **4** increased strongly at pH 7.2 in a methanol–water mixture 50:50 ($t_{1/2}$ < 5 min). Under the same conditions using cysteine or glutathione as reducing agents, the reaction appeared slower than in the presence of DTT.

At 20 °C, in the presence of a concentrated CEM cell extract in PBS (Phosphate Buffered Saline) and methanol, the reduction of the aromatic dT disulfide **15** or **16** led to the immediate appearance of a yellow coloration corresponding to the formation of the nitrothiophenolate also detected by TLC on silica gel. The presence of the dT symmetrical disulfide **5** was observed after centrifugation and concentration of the supernatant. These experiments showed that the aromatic disulfides are reduced rapidly in cell extracts to generate the thiol **1** which is rapidly oxidized in the presence of air. The reduction was not complete but quantitative experiments cannot be performed in regard to the excess of disulfide (1 mg for 100 μL of cell extract supernatant) and to the cell treatment modifying probably the concentration of the reducing agents in the cell extract (reaction with dioxygen).

The formation of the dT symmetrical disulfide **5** and traces of thiol **1** also were detected by TLC after reaction for 10 min of the dT disulfides **14**, **17**–**23** and CEM cell extract in a methanol–water mixture. A rapid reduction of the dC methyl disulfide **4** also was observed under the same conditions.

2.3. Antiviral properties

The mixed disulfides were evaluated for their anti-HIV-1 and -HIV-2 activities in CEM cell cultures. In a first set of experiments (Table 1), we could show that the methyl disulfides dT **2** and dC **4** induce similar anti-HIV-1 and -2 effects (EC_{50} HIV-1: 12 and 10 μM) whereas the dU methyl disulfide **12** and the corresponding 5-bromo derivative **26** are inactive. The symmetrical disulfides dC **6** and 5-bromodU **25** showed much lower antiviral activities than the methyl disulfides dT **2** and dC **4**. The symmetrical disulfide dU **7** appeared inactive as reported in the literature.[15]

In a second set of experiments, the anti-HIV activity of a first series of dT disulfides (**2**, **14**–**18**, **21**, and **22**) were compared (Table 2). The antiviral effects appeared close for most of these disulfides except for the trichloromethyl (**14**) and 2-aminomethyl (**22**) disulfides which showed weaker effects.

The third set of experiments (Table 3) revealed that, in comparison to the methyl disulfides dT **2** and dC **4**, the antiviral activity was increased for the hydrophobic hexyl (**19**) and octyl (**20**) disulfides. However, these disulfides appeared, as most of the disulfides,

Table 1
Anti-HIV-1 and -HIV-2 activities and cytostatic properties of the mixed disulfides and symmetrical disulfides in human T-lymphocyte CEM cells

Disulfide (side chain)	Base	EC_{50} HIV-1[a] (μM)	EC_{50} HIV-2[a] (μM)	CC_{50}[b] (μM)
2 (methyl)	T	12 ± 8.5	12 ± 6.5	102 ± 16
4 (methyl)	C	10 ± 9.9	14 ± 9.1	111 ± 11
6 (symmetrical)	C	73 ± 25	65 ± 21	216 ± 49
12 (methyl)	U	>50	⩾50	170 ± 57
7 (symmetrical)	U	>250	>250	⩾250
26 (methyl)	5-BrU	⩾50	⩾50	103 ± 7
25 (symmetrical)	5-BrU	127 ± 25	⩾250	>250
Tenofovir	—	5.5 ± 2.1	2.6 ± 2.0	103 ± 7

[a] 50% Effective concentration or compound concentration required to protect cells against the cytopathicity of HIV by 50%.
[b] 50% Inhibitory concentration or compound concentration required to inhibit cell proliferation by 50%.

Table 2
Anti-HIV-1 and anti-HIV-2 activities and cytostatic properties of the mixed disulfides in human T-lymphocyte CEM cells

Disulfide dT (side chain)	EC_{50} HIV-1[a] (μM)	EC_{50} HIV-2[a] (μM)	CC_{50}[b] (μM)
2 (methyl)	12 ± 8.5	12 ± 6.5	102 ± 16
14 (trichloromethyl)	25 ± 0	17.5 ± 3.5	103 ± 4
15 (2-nitrophenyl)	10.0 ± 0.0	>10	18.5 ± 0.2
16 (4-nitrophenyl)	⩾10	7.3 ± 2.3	23 ± 2
17 (allyl)	12.5 ± 3.5	15 ± 7	53 ± 7
18 (butyl)	8 ± 3	>10	23.3 ± 0.6
21 (2-hydroxyethyl)	13.5 ± 3.0	14 ± 7	>250
22 (2-aminoethyl)	27.5 ± 3.5	27.5 ± 3.5	>250

[a] 50% Effective concentration or compound concentration required to protect cells against the cytopathicity of HIV by 50%.
[b] 50% Inhibitory concentration or compound concentration required to inhibit cell proliferation by 50%.

more antiproliferative than the disulfides **2** and **4** in CEM/0 and Molt4/C8 cells (Tables 1–3). Interestingly, the 2-hydroxyethyl (**21**) and 2-aminoethyl (**22**) disulfides appeared devoid of measurable cytostatic activity in CEM cell cultures. The disulfide **21** also showed interesting antiviral effects similar to those of the methyl disulfides **2** and **4** that are more antiproliferative than **21** (Tables 2 and 3).

The antiviral effects of the disulfides also were evaluated in wild-type CEM/0 and mutant CEM/TK-cells that are deficient for cellular cytosolic thymidine kinase (TK, Table 3). The antiviral (HIV-2) activities observed appeared markedly lower than the values previously obtained in wild-type CEM cells except for the trichloromethyl (**14**) and 4-nitrophenyl (**16**) derivatives for which the effects were quite similar in both cell lines.

Decreased activities of the compounds against HIV-2 in the CEM/TK-cell line point to dT kinase dependence of the compounds to exert their eventual antiviral activity. It is therefore somewhat puzzling that the compounds **14** and **16** are endowed with similar antiviral potencies in CEM/0 and CEM/TK-cells, pointing to a potential different mechanism of antiviral action.

The inhibitory activity of the dT disulfides **2** (methyl), **14** (trichloromethyl), **21** (2-hydroxyethyl), and **22** (2-aminoethyl) against recombinant purified TK-1-catalyzed phosphorylation of dT was studied under reducing (50 mM DTT) and non-reducing conditions (Table 4). The IC_{50} values obtained revealed a higher inhibition of TK-1-catalyzed dT phosphorylation in the absence of DTT for the disulfides than under reducing conditions leading to the thiol **1** (similar values for the four disulfides in the presence of DTT). These results as well as the rapid reduction of the disulfides to generate the thiol **1** in CEM cell extracts, and the strong decreasing of the anti-HIV activity in CEM/TK-cells for most of the disulfides, suggest that the anti-HIV effects most likely result from the phosphorylation of the thiol derivatives. It would be interesting to investigate the inhibitory activity of the 5′-triphosphate metabolites of the 3′-disulfides and the 3′-thiol derivatives against HIV-1 reverse transcriptase.

Table 4
Inhibitory activity of thymidine disulfides against TK-1 catalyzed phosphorylation of dT under reducing and non-reducing conditions

	IC_{50}[a] (μM)	
	Without DTT	In the presence of DTT (50 mM)
2 (methyl)	73 ± 22	333 ± 37
14 (trichloromethyl)	13 ± 9	365 ± 110
21 (2-hydroxyethyl)	18 ± 2	405 ± 85
22 (2-aminoethyl)	10 ± 8	418 ± 65

[a] 50% Inhibitory concentration or compound concentration required to inhibit 1 μM [CH_3-^{3}H]dT phosphorylation by recombinant purified TK-1 by 50%.

Table 5
Inhibitory effect of the disulfides against MSV-induced transformation of C3H/3T3 embryo murine fibroblasts in vitro

Compound	EC_{50}[a] (μM)	MIC[b] (μg/μM)
2 (methyl)	8.3 ± 2.3	>100
4 (methyl)[c]	48 ± 2	>100
14 (trichloromethyl)	>20	100 (>20)[d]
15 (2-nitrophenyl)	1.1 ± 0.8	20 (>4)
16 (4-nitrophenyl)	0.89 ± 0.36	20 (>4)
17 (allyl)	2.2 ± 0.3	100 (>20)
18 (butyl)	1.0 ± 0.5	100 (>20)
21 (2-hydroxyethyl)	5.9 ± 0.5	>100
22 (2-aminoethyl)	63 ± 4	>100
(R)-PMPA	0.23 ± 0.03	>10
PMEA	0.53 ± 0.13	> 20

[a] EC_{50}: 50% effective dose.
[b] MIC: Minimal Inhibitory Concentration.
[c] dC, dT for the other disulfides.
[d] Values between brackets are the compound concentrations at which no alteration of cell morphology was observed.

When evaluated for their inhibitory effect against MSV-induced transformation of C3H/3T3 embryo murine fibroblasts in vitro (Table 5), the nitrophenyl (**15** and **16**), allyl (**17**) and butyl (**18**) derivatives showed the most pronounced antiviral efficiencies (EC_{50} values between 1 and 2 μM) which appeared close to the activities found for PMEA (adefovir, EC_{50}: 0.53 μM) and (R)-PMPA (tenofovir, EC_{50}: 0.23 μM). In this assay, the dC methyl disulfide **4** was found

Table 3
Anti-HIV-1 and anti-HIV-2 activities and cytostatic properties of the mixed disulfides in human T-lymphocyte CEM/0, CEM/TK$^-$, and Molt4/C8 cells

Disulfide (side chain)	EC_{50} HIV-1[a] (μM)	EC50 HIV-2[a] (μM)	EC50 HIV-2 CEM/TK$^-$[a] (μM)	CEM/0[b]	Molt4/C8[b]
2 (methyl)	12 ± 8.5	12 ± 6.5	>50	102 ± 16	66 ± 9
4 (methyl)[c]	10 ± 9.9	14 ± 9.1	45 ± 21	111 ± 11	149 ± 21
14 (trichloromethyl)	20 ± 0	27.5 ± 3.5	22.5 ± 3.5	121 ± 18	55 ± 19
15 (2-nitrophenyl)	>10	>10	>10	32 ± 1	31 ± 5
16 (4-nitrophenyl)	8.0 ± 2.8	8.5 ± 2.1	⩾10	34 ± 0	29 ± 2
17 (allyl)	17.5 ± 3.5	20 ± 7	>50	128 ± 18	34 ± 6
18 (butyl)	>10	>10	>10	44 ± 1	28 ± 7
19 (hexyl)	⩾10	6.5 ± 0.7	>10	39 ± 1	27 ± 4
20 (octyl)	3.0 ± 0.0	4.5 ± 2.1	>10	21 ± 12	26 ± 4
21 (2-hydroxyethyl)	17.5 ± 3.5	15 ± 7	>50	347 ± 71	128 ± 33
22 (2-aminoethyl)	20 ± 0	27.5 ± 3.5	150 ± 0	437 ± 71	163 ± 41
23 (6-hydroxyhexyl)	15.0 ± 0.0	15.0 ± 0.0	>50	52 ± 5	42 ± 6

Also mentioned, cytostatic properties in human T-lymphocyte cells Molt4/C8.
[a] 50% Effective concentration or compound concentration required to protect cells against the cytopathicity of HIV by 50%.
[b] 50% Inhibitory concentration or compound concentration required to inhibit cell proliferation by 50%.
[c] dC, dT for the other disulfides.

much less efficient than the methyl (**2**) and hydroxyethyl (**21**) dT disulfides (EC_{50} = 48, 8, and 6 μM, respectively).

3. Conclusion

The drug delivery approach employing conjugation through a disulfide linkage has been reported fruitfully in the literature.[26] The first linkage-employing drug conjugate that exploits the reversible nature of this unique covalent linkage, the anti-CD33 antibody-*S*-*S*-calicheamicin, Mylotarg, developed for the treatment of acute myeloid leukemia, was approved for human use by the FDA in March 2000.[27]

In order to generate in cells, the easily oxidizable 3′-mercaptodT **1** and -dC **3** and their triphosphates, the corresponding mixed disulfides **2**, **14**–**23**, and **4** were prepared. Most of the disulfides showed interesting anti-HIV EC_{50} equivalent to twice the EC_{50} of Tenofovir (Tables 1–3). The anti-MSV activities of the most active derivatives were found not far from the activity of PMEA (Table 5).

In comparison to the dT methyl disulfide **2**, it was not possible to strongly increase the anti-HIV effect through modification of the side chain of the disulfide function without increasing the cytostatic effect in CEM and Molt4/C8 cells. However, the cytostatic effect was decreased with the 2-hydroxyethyl disulfide **21** in preserving interesting anti-HIV-1, anti-HIV-2, and anti-MSV activities. This nucleoside appears interesting for a further evaluation in vivo.

The lack of the anti-HIV effects in mutant CEM/TK- cells for most of the thymidine disulfides suggests that a phosphorylation step involving thymidine kinase is necessary for the eventual antiviral activity of the thymidine nucleosides. It was indeed ascertained that cytosolic TK showed affinity for the disulfide and the thiol derivatives. We showed that the reduction of the disulfides occurs rapidly in CEM cell extracts to generate the corresponding thiols **1** and **3**. Therefore, the similar antiviral effects of most of the disulfides result probably from the rapid reduction of the disulfide function in cells and a likely comparable inhibitory effect of the 2′,3′-dideoxy-3′-mercaptothymidine 5′-triphosphate metabolite.

4. Experimental

4.1. Synthesis

All starting materials were commercially available research-grade chemicals and used without further purification. Reactions were monitored by analytical TLC on silica gel with fluorescent indicator UV_{254} from Machery-Nagel. Melting points were determined in open glass capillaries using a Büchi 510 apparatus and are reported uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Bruker avance 400 (400 and 100 MHz). Chemical shifts are reported in ppm (parts per million) relative to the residual signal of the solvent, and the signals are described as singlet (s), doublet (d), triplet (t), doublet of doublet (dd), quartet (q), and multiplet (m); coupling constants are reported in Hertz (Hz).

4.1.1. 2′,3′-Dideoxy-3′-(methyldithio)cytidine (4)

To a solution of **11** (65 mg, 0.180 mmol) and methyl disulfide (650 μL, 7.23 mmol) in anhydrous THF (4 mL) was added dimethyl(thiomethyl)sulfonium tetrafluoroborate (57 mg, 0.360 mmol). The mixture was stirred for 48 h under argon, then sodium bicarbonate (10%, 100 μL) was added. After evaporation, the residue was dissolved in water and chromatographed on C18 reversed phase silica gel (1 g) in water/methanol (9:1). After concentration to dryness, the residue was chromatographed on silica gel in dichloromethane/methanol (90:10) to give **4** (39 mg, 0.135 mmol, 75%) as a yellow oil; ^1H NMR (100 MHz, CD_3OD) δ 8.17 (1H, d, J = 7.6 Hz, 6-H), 6.15 (1H, dd, J = 6.8 Hz, J = 4 Hz, 1′-H), 5.92 (1H, d, J = 7.6 Hz, 5-H), 4.03 (1H, m, 4′-H), 3.95–3.82 (2H, m, 5′-H × 2), 3.53 (1H, m, 3′-H), 2.67 (1H, m, 2′-H), 2.42 (1H, m, 2′-H), 2.48 (3H, s, CH_3–S); ^{13}C NMR (100 MHz, CD_3OD) δ 163.4 (C4), 150.2 (C2), 143.7 (C6), 96.4 (C5), 88.0 (C1′), 87.9 (C4′), 62.5 (5′-CH_2), 46.8 (C3′), 40.9 (2′-CH_2), 25.3 (S–CH_3); MS (FAB$^+$, glycerol) m/z 290 $[M+H]^+$; HRMS for $C_{10}H_{15}N_3O_3NaS_2$, $[M+Na]^+$, calculated 312.0453, found 312.0437.

4.1.2. 2′,3′-Dideoxy-3′-(2-(trimethylsilyl)ethyl)thiouridine (10)

To a suspension of sodium hydride (60%, 234 mg, 7.02 mmol) in dry DMF (8 mL) was added a solution of 2-(trimethylsilyl)ethanethiol (980 μL, 7.02 mmol) in dry DMF (8 mL). This mixture was stirred for 15 min under argon and then compound **9** (3.00 g, 5.85 mmol) was added. After 24 h at 90 °C, methanol (3 mL) was added and the mixture was concentrated to dryness. The residue was taken up in dichloromethane (100 mL), washed with an aqueous solution of NaH_2PO_4 (10%, 10 mL), then with water (100 mL), and the organic phase was dried over sodium sulfate, and concentrated. The residue was chromatographed on silica gel in dichloromethane/ethyl acetate (80:20 with 1% triethylamine) to give the protected sulfide as a yellow foam. This foam was dissolved in a solution of dichloroacetic acid in dichloromethane (2%, 80 mL) and stirred for 4 h at rt. The solution was neutralized by an aqueous sodium bicarbonate solution (5%, 30 mL). The water phase was extracted with dichloromethane (50 mL) and the combined organic phases were dried over sodium sulfate, then evaporated. The residue was chromatographed on silica gel in dichloromethane/ethyl acetate (60:40) to give **10** (1.06 g, 3.08 mmol, 56% (two steps)) as a white solid; mp 148 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 9.47 (1H, s, NH), 7.82 (1H, d, J = 8.4 Hz, 6-H), 6.12 (1H, dd, J = 7.0 Hz, J = 3.6 Hz, 1′-H), 5.73 (1H, d, J = 8.4 Hz, 5-H), 4.05 (1H, m, 3′-H), 3.85 (2H, m, 5′-H × 2), 3.47 (1H, m, 4′-H), 2.64–2.50 (4H, m, 2′-H × 2, S–CH_2), 0.84 (2H, m, CH_2–Si), −0.02 (9H, s, Si(CH_3)$_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.9 (CO), 150.4 (CO), 140.9, 101.9, 86.2, 85.7, 61.0 (5′-CH_2), 40.7, 40.1 (2′-CH_2), 27.5 (S–CH_2), 17.4 (CH_2–Si), −1.8 (Si(CH_3)$_3$); MS (FAB$^+$, glycerol) m/z = 345 $[M+H]^+$; elemental analysis for $C_{14}H_{24}N_2O_4SSi$, 1/3 H_2O, calculated C 47.97, H 7.09, N 7.99, S 9.15, found C 47.82, H 7.13, N 7.79, S 9.66.

4.1.3. 2′,3′-Dideoxy-3′-(2-(trimethylsilyl)ethyl)thiocytidine (11)

To a solution of **10** (300 mg, 0.87 mmol) in dry pyridine (3 mL) at 0 °C was added dropwise acetic anhydride (1.23 mL). The resulting solution was stirred at rt for 24 h and then ethanol (1 mL) was added. After 30 min, the mixture was evaporated and the residue was taken up in dichloromethane (10 mL). The solution was washed with water (10 mL), the organic phase was dried over sodium sulfate (15 mL), evaporated and coevaporated with toluene (10 mL) to give the acetylated nucleoside as a yellow solid.

1,2,4-Triazole (902 mg, 13.1 mmol) was added to a solution of $POCl_3$ (292 μL, 2.87 mmol) in anhydrous acetonitrile (5 mL). The solution was stirred for 15 min at 0 °C and then triethylamine (5 mL, 35.8 mmol) was added dropwise. The obtained mixture was allowed to warm up at rt overnight, and then a solution of the previously obtained acetylated nucleoside in acetonitrile (5 mL) was added. The mixture was stirred at rt for 48 h, then water (1 mL) was added before concentration. The residue was dissolved in dichloromethane (10 mL), neutralized with an aqueous solution of sodium bicarbonate (10%, 2 mL), and washed with water (20 mL). The organic phase was dried over sodium sulfate, evaporated, and coevaporated with toluene (10 mL).

The residue was dissolved in a solution of ammonia in dioxane (50 mL, 0,5 M) and stirred under argon for 48 h before concentration to dryness.

The yellow solid obtained was dissolved in ethanol (10 mL), and a solution of concentrated ammonia (30%, 10 mL) was added. After 12 h the solvents were evaporated, coevaporated with ethanol, and the obtained residue was chromatographed on silica gel in dichloromethane/methanol (90:10) to give **11** (250 mg, 0.695 mmol, 80%) as a pale yellow oil; ^{1}H NMR (400 MHz, CD_3OD) δ 7.45 (1H, d, J = 7.5 Hz, 6-H), 6.10 (1H, dd, J = 10.0 Hz, J = 4.8 Hz, 1′-H), 5.99 (1H, d, J = 7.5 Hz, 5-H), 3.95 (1H, m, 5′-H), 3.81 (1H, m, 4′-H), 3.80 (1H, m, 5′-H), 3.38 (1H, m, 3′-H), 2.70–2.50 (2H, m, 2′-H × 2), 2.71–2.42 (2H, m, S–CH_2), 0.89 (2H, m, CH_2–Si), 0.05 (9 H, s, Si(CH_3)$_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 166.2 (C4), 156.7 (C2), 141.3 (C6), 94.0 (C5), 86.5 (C1′), 85.8 (C4′), 60.3 (5′-CH_2), 40.7 (2′-CH_2), 39.9 (C4′), 20.6 (S–CH_2), 16.9 (CH_2–Si), −3.2 (Si(CH_3)$_3$); MS FAB$^+$, glycerol) m/z = 291 [M+H]$^+$; elemental analysis for $C_{14}H_{25}N_2O_3S_2$, 2/3 H_2O, calculated C 47.46, H 7.45, N 11.86, S 9.05, found C 47.65, H 7.45, N 11.64, S 9.28.

4.1.4. 2′,3′-Dideoxy-3′-(methyldithio)uridine (12)

To a solution of **10** (200 mg, 0.58 mmol) and methyl disulfide (2.1 mL, 23.2 mmol) in anhydrous THF (5 mL) was added dimethyl(thiomethyl)sulfonium tetrafluoroborate (183 mg, 1.16 mmol). The mixture was stirred for 48 h under argon, then sodium bicarbonate (10%, 100 μL) was added. After evaporation, the residue was dissolved in water and chromatographed on C18 reversed phase silica gel (1 g) in water/methanol (9:1). After concentration to dryness, the residue was chromatographed on silica gel in dichloromethane/methanol (80:20) to give **12** (125 mg, 0.43 mmol, 74%) as a white foam; mp 74–76 °C; ^{1}H NMR (100 MHz, $CDCl_3$) δ 7.51 (1H, d, J = 8.0 Hz, 6-H), 6.09 (1H, dd, J = 7.0 Hz, J = 3.0 Hz, 1′-H), 5.74 (1H, d, J = 8.1 Hz, 5-H), 4.07–4.03 (2H, m, 5′-H × 2), 3.91 (1H, m, 4′-H), 3.61 (1H, m, 3′-H), 2.70–2.50 (2H, m, 2′-H × 2), 2.50 (3H, s, CH_3–S); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.4 (C2), 150.2 (C4), 140.7 (C6), 102.1 (C5), 85.7 (C1′), 85.5 (C4′), 60.9 (5′-CH_2), 44.5 (C3′), 38.5 (2′-CH_2), 24.4 (S–CH_3); MS (FAB$^+$, glycerol) m/z = 291 [M+H]$^+$; elemental analysis for $C_{10}H_{14}N_2O_4S_2$, 1/3H_2O, calculated C 41.80, H 5.24, N 8.68, found C 41.88, H 5.17, N 8.62.

4.1.5. 2′,3′-Dideoxy-3′-(trichloromethyldithio)thymidine (14)

To a solution of **13** (40 mg, 0.11 mmol) in anhydrous dichloromethane (1.5 mL), under argon, was added trichloromethanesulfenyl chloride (60 μL, 0.56 mmol), and the mixture was stirred at rt for 4 h. The solvent was then evaporated by using a nitrogen stream, and the obtained residue was chromatographed on silica gel in dichloromethane/methanol (95:5) to give **14** (37 mg, 0.09 mmol, 81%) as a white powder; mp 68–70 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.36 (1H, s, NH), 7.48 (1H, s, 6-H), 6.13 (1H, dd, J = 6.4 Hz, J = 12.7 Hz, 1′-H), 4.26 (1 H, m, 3′-H), 4.18 (1H, m, 4′-H), 4.09–3.88 (2H, m, 5′-H × 2), 2.77–2.56 (2H, m, 2′-H × 2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.9 (C2), 150.4 (C4), 136.7 (C6), 115.2 (C5), 100.4 (C–Cl_3), 86.2 (C1′), 85.4 (C4′), 61.3 (5′-CH_2), 47.9 (C3′), 38.4 (2′-CH_2), 12.5 (5-CH_3); MS (DCI, NH_3-isobutane) m/z 409 [M+H]$^+$, 429 [M+NH_3]$^+$; elemental analysis for $C_{11}H_{13}N_2O_4S_2$, 0.5 MeOH, calculated C 32.60, H 3.57, N 6.61, S 15.13, found C 32.40, H 3.21, N 6.87, S 14.97.

4.1.6. 2′,3′-Dideoxy-3′-(2-nitrophenyldithio)thymidine (15)

To a solution of **13** (300 mg, 0.84 mmol) in anhydrous dichloromethane (1.5 mL), under argon, was added 2-nitrobenzenesulfenyl chloride (476 mg, 2.50 mmol), and the mixture was stirred at rt for 4 h. The solvent was evaporated and the obtained residue was chromatographed on silica gel in dichloromethane/methanol (98:2 then 95:5) to give **15** (314 mg, 0.76 mmol, 91%) as a yellow powder; mp 65–67 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.26 (1H, s, NH), 8.29 (1H, m, Ar), 8.25 (1H, m, Ar), 7.74 (1H, m, Ar), 7.52 (1H, s, 6-H), 7.42 (1H, m, Ar), 6.04 (1H, dd, J = 4.8 Hz, J = 11.6 Hz, 1′-H), 4.08–4.05 (2H, m, 4′-H, 5′-H), 3.85 (1H, m, 5′-H), 3.50 (1H, m, 3′-H), 2.56–2.44 (2H, m, 2′-H), 1.89 (3H, s, 5-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.9 (C2), 150.3 (C4), 145.6 (C–NO_2), 136.8 (C6), 136.6 (C–SS), 134.3, 127.1, 126.8, 126.3 (4 C Ar), 110.8 (C5), 85.9 (C1′), 85.0 (C4′), 61.1 (5′-CH_2), 45.3 (C3′), 37.8 (2′-CH_2), 12.4 (5-CH_3). MS (DCI, NH_3-isobutane) m/z 412 [M+H]$^+$, 429 [M+H+NH_3]$^+$, elemental analysis for $C_{16}H_{17}N_3O_6S_2$, 0.5 H_2O, calculated C 45.70, H 4.31, N 9.99, found C 45.70, H 4.36, N 9.75; HRMS for $C_{16}H_{17}N_3O_6NaS_2$ [M+Na]$^+$, calculated 434.0456, found 434.0450.

4.1.7. 2′,3′-Dideoxy-3′-(4-nitrophenyldithio)thymidine (16)

To a solution of **13** (300 mg, 0.84 mmol) in anhydrous dichloromethane, under argon, was added 4-nitrobenzenesulfenyl chloride (476 mg, 2.50 mmol). The mixture was stirred at rt for 4 h, then concentrated before purification by chromatography on silica gel in dichloromethane/methanol (98:2 then 95:5) to give **16** (331 mg, 0.80 mmol, 96%) as a pale yellow powder; mp 64–66 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.48 (1H, s, NH), 8.20 (2H, d, Ar), 7.70 (2H, d, Ar), 7.46 (1H, s, 6-H), 6.07 (1H, dd, J = 5.2 Hz, J = 11.6 Hz, 1′-H), 4.05 (1H, m, 5′-H), 4.02 (1H, m, 4′-H), 3.88 (1H, m, 5′-H), 3.50 (1H, m, 3′-H), 2.60–2.51 (2H, m, 2′-H × 2), 1.89 (3H, s, 5-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.4 (C2), 150.1 (C4), 145.6 (C–NO_2), 136.8 (C6), 136.6 (C–SS), 126.1 (2 C Ar), 124.3 (2 C Ar), 111.1 (C5), 85.9 (C1′), 84.7 (C4′), 61.2 (5′-CH_2), 45.8 (C3′), 37.6 (2′-CH_2), 12.5 (5-CH_3); MS (DCI, NH_3-isobutane) m/z 412 [M+H]$^+$, 429 [M+H+NH_3]$^+$; elemental analysis for $C_{16}H_{17}N_3O_6S_2$, calculated C 46.71, H 4.16, N 10.21, found C 46.69, H 4.20, N 9.88; HRMS for $C_{16}H_{17}N_3O_6NaS$ [M+Na]$^+$ calculated 434.0456, found 434.0457.

4.1.8. General procedure for the preparation of 3′-mixed disulfides 17–23 from 16

Argon was bubbled into a solution of thiol in the appropriated buffer (phosphate buffer: 5%, pH 9.5; or imidazole buffer: 10^{-3} M, pH 6.0). Argon was also bubbled into a solution of **16** in a mixture of THF and appropriated buffer, then this solution was added to the first solution of thiol. The mixture was then stirred at rt under argon, concentrated, and chromatographed on silica gel in dichloromethane/methanol (98:2 to 90:10) to give the expected disulfide.

4.1.9. 2′,3′-Dideoxy-3′-(allyldithio)thymidine (17)

Compound **17** (45 mg, 0.14 mmol, 80%) was obtained as a yellow oil from **16** (70 mg, 0.17 mmol), allylthiol (476 μL, 2.50 mmol), THF (10 mL), and phosphate buffer (425 μL), 2 h; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.56 (1H, s, NH), 7.53 (1H, s, 6-H), 6.10 (1H, dd, J = 4.8 Hz, J = 6.8 Hz, 1′-H), 5.90–5.81 (1H, m, H allyl), 5.26–5.20 (2H, m, 2H allyl), 4.09–4.01 (2H, m, 4′-H, 5′-H), 3.90 (1H, m, 5′-H), 3.62 (1H, m, 3′-H), 3.90 (2H, m, CH_2–S allyl), 2.55 (2H, m, 2′-H × 2), 1.93 (3H, s, 5-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.3 (C2), 150.0 (C4), 136.5 (C6), 134.1 (CH-allyl), 117.2 (CH_2-allyl), 110.8 (C5), 85.8 (C1′), 85.4 (C4′), 45.2 (C3′), 61.5 (5′-CH_2), 38.2 (2′-CH_2), 34.7 (CH_2–S), 12.5 (5-CH_3); MS (DCI, NH_3-isobutane) m/z 331 [M+H]$^+$; HRMS for $C_{13}H_{18}N_2O_4NaS_2$ [M+Na]$^+$, calculated 353.0605, found 353.0615.

4.1.10. 2′,3′-Dideoxy-3′-(butyldithio)thymidine (18)

Compound **18** (15 mg, 0.043 mmol, 45%) was obtained as a white powder from **16** (40 mg, 0.097 mmol), butanethiol 107 μL, 0.97 mmol), THF (6 mL) and phosphate buffer (243 μL), 2 h; mp 71 °C (decomposition); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.56 (1H, s, NH), 7.54 (1H, s, 6-H), 6.12 (1H, dd, J = 4.4 Hz, J = 6.8 Hz, 1′-H), 4.10–4.02 (2H, m, 4′-H, 5′-H), 3.90 (1H, m, 5′-H), 3.60 (1H, m, 3′-H), 2.75 (2H, m, CH_2–S), 2.64–2.51 (2H, m, 2′-H × 2), 1.93 (3H, s, 5-CH_3), 1.68 (2H, m, S–CH_2–CH_2), 1.43 (2H, m, CH_2–CH_3), 0.95

(3H, m, CH_2–CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.3 (C2), 150.0 (C4), 136.5 (C6), 110.8 (C5), 85.8 (C1′), 85.4 (C4′), 61.5 (5′-CH_2), 45.2 (C3′), 39.8 (CH_2–S), 38.2 (2′-CH_2), 31.2 (S–CH_2–CH_2), 21.5 (CH_2–CH_3), 13.6 (CH_2–CH_3), 12.5 (5-CH_3); MS (FAB$^+$, NBA) m/z 347 $[M+H]^+$, 369 $[M+Na]^+$; HRMS for $C_{14}H_{22}N_2O_4NaS_2$ $[M+Na]^+$, calculated 369.0919, found 369.0918. The nucleoside symmetrical disulfide **5** formed under these conditions was isolated (23%).

Compound **18** was also obtained directly from TMSE sulfide **13**. To a stirred solution of **13** (40 mg, 0.112 mmol) in dichloromethane were added successively dibutyl disulfide (106 μL, 0.56 mmol) and a solution of Br_2 in dichloromethane (1 M, 1.12 mmol). The resulting solution was stirred under argon at rt overnight, then an aqueous solution of sodium thiosulfate (5%, 2 mL) was added. The organic phase was washed with water (10 mL), dried over $MgSO_4$, and evaporated. Methanol was added to precipitate **18** (10 mg, 0.029 mmol, 26%). The non-precipitated fraction was evaporated to give the symmetrical disulfide **5** (10 mg, 0.019 mmol, 33%).

4.1.11. 2′,3′-Dideoxy-3′-(hexyldithio)thymidine (19)

Compound **19** (22 mg, 0.059 mmol, 71%) was obtained as a white powder from **16** (34 mg, 0.083 mmol), hexanethiol (123 μL, 0.83 mmol), THF (6 mL) and phosphate buffer (243 μL), 2 h; mp 64–66 °C (decomposition); ^1H NMR (400 MHz, $CDCl_3$) δ 8.66 (1H, s, NH), 7.56 (1H, s, 6-H), 6.12 (1H, dd, J = 4.7 Hz, J = 7.2 Hz, 1′-H), 4.10–4.01 (2H, m, 4′-H, 5′-H), 3.90 (1H, m, 5′-H), 3.60 (1H, m, 3′-H), 2.70 (2H, m, CH_2–S), 2.64–2.48 (2H, m, 2′-H × 2), 1.92 (3H, s, 5-CH_3), 1.68 (2H, m, CH_2–CH_2–S), 1.44–1.25 (6H, m, CH_2–CH_2–CH_2), 0.91 (3H, m, CH_2–CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.4 (C2); 150.1 (C4), 133.4 (C6), 110.8 (C5), 85.8 (C1′), 85.5 (C4′), 61.5 (5′-CH_2), 45.3 (C3′), 40.2 (CH_2–S), 38.2 (2′-CH_2), 31.3 (CH_2), 29.1 (CH_2–CH_2–S), 28.0, 22.4 (2 CH_2), 13.9 (CH_2–CH_3), 12.4 (5-CH_3); MS (DCI, NH_3-isobutane) m/z 375 $[M+H]^+$; elemental analysis for $C_{16}H_{26}N_2O_4S_2$, calculated C 51.31, H 7.00, N 7.48, found C 51.26, H 6.70, N 7.57; HRMS for $C_{16}H_{26}N_2O_4NaS_2$ $[M+Na]^+$, calculated 397.1232, found 397.1230.

4.1.12. 2′,3′-Dideoxy-3′-(octyldithio)thymidine (20)

Compound **20** (12 mg, 0.030 mmol, 16%) was obtained as a white powder from **16** (75 mg, 0.18 mmol), octanethiol (316 μL, 1.82 mmol), a mixture of THF/imidazole buffer (2.5/2.5 mL) and imidazole buffer (10^{-3} M, pH 6.0, 5 mL), 15 h; ^1H NMR (400 MHz, $CDCl_3$) δ 8.66 (1H, s, NH), 7.68 (1H, s, 6-H), 6.13 (1H, dd, J = 4.4 Hz, J = 6.8 Hz, 1′-H), 4.09–3.88 (3H, m, 4′-H, 5′-H × 2), 3.60 (1H, m, 3′-H), 2.73 (2H, m, CH_2–S), 2.74–2.51 (2H, m, 2′-H × 2), 1.93 (3H, s, 5-CH_3), 1.68 (2H, m, CH_2–CH_2–S), 1.39–1.20 (10H, m, CH_2–CH_2–CH_2–CH_2–CH_2), 0.90 (3H, m, CH_2–CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.5 (C2), 150.1 (C4), 136.5 (C6), 110.8 (C5), 85.8 (C1′), 85.4 (C4′), 61.4 (5′-CH_2), 45.2 (C3′), 40.1 (CH_2–S), 38.1 (2′-CH_2), 31.7 (CH_2), 29.2 (CH_2–CH_2–S), 29.1, 28.4, 22.6 (4 CH_2), 14.0 (CH_2–CH_3), 12.5 (5-CH_3); MS (DCI, NH_3-isobutane) m/z 403 $[M+H]^+$; HRMS for $C_{18}H_{30}N_2O_4NaS_2$ $[M+Na]^+$, calculated 425.1545, found 425.1548. The nucleoside symmetrical disulfide **5** formed under these conditions was isolated (65%).

4.1.13. 2′,3′-Dideoxy-3′-(2-hydroxyethyldithio)thymidine (21)

Compound **21** (41 mg, 0.12 mmol, 50%) was obtained as a white powder from **16** (100 mg, 0.24 mmol), mercaptoethanol (170 μL, 2.43 mmol), imidazole buffer (10^{-3} M, pH 6.0, 5 mL), and a mixture of THF/imidazole buffer (2.5/2.5 mL), 15 h; mp 58–61 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 8.25 (1H, s, NH), 7.59 (1H, s, 6-H), 6.08 (1H, dd, J = 5.2 Hz, J = 6.4 Hz, 1′-H), 4.12–4.00 (3H, m, 4′-H, 5′-H × 2), 3.94 (2H, m, CH_2–OH), 3.73 (1H, m, 3′-H), 3.01–2.93 (2H, m, CH_2–S), 2.54 (2H, m, 2′-H × 2), 1.94 (3H, s, 5-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.0 (C2), 150.8 (C4), 136.8 (C6), 109.7 (C5), 85.6 (C4′), 84.5 (C1′), 60.6 (5′-CH_2), 59.8 (CH_2–OH), 45.4 (C3′), 41.8 (CH_2–S), 37.9 (2′-CH_2), 11.0 (5-CH_3); MS (DCI, NH_3-isobutane) m/z 335 $[M+H]^+$; HRMS for $C_{12}H_{18}N_2O_5NaS_2$, $[M+Na]^+$, calculated 357.0555, found 357.0549. The nucleoside symmetrical disulfide **5** formed under these conditions was isolated (24%).

4.1.14. 2′,3′-Dideoxy-3′-(2-aminoethyldithio)thymidine (22)

Compound **22** (9 mg, 0.024 mmol, 20%) was obtained as a white powder from **16** (50 mg, 0.12 mmol), 2-aminoethanethiol hydrochloride (28 mg, 0.24 mmol), imidazole buffer (1 M, pH 6.0, 400 μL), and a mixture of THF/imidazole buffer (2.0/2.0 mL), 15 h; ^1H NMR (400 MHz, D_2O) δ 7.54 (1H, s, 6-H), 6.08 (1H, dd, J = 4.4 Hz, J = 7.2 Hz, 1′-H), 4.02 (1H, m, 4′-H), 3.87 (1H, dd, J = 2.8 Hz, J = 12.8 Hz, 5′-H), 3.76 (1H, dd, J = 2.8 Hz, J = 12.8 Hz, 5′-H), 3.52 (1H, m, 3′-H), 3.30 (2H, m, CH_2–NH_2), 2.93 (2H, m, CH_2–S), 2.60–2.43 (2H, m, 2′-H × 2), 1.80 (3H, s, 5-CH_3); ^{13}C NMR (100 MHz, D_2O) δ 166.4 (C2), 151.5 (C4), 137.5 (C6), 111.1 (C5), 85.1 (C4′), 84.8 (C1′), 60.5 (5′-CH_2), 45.1 (C3′), 37.8 (CH_2–NH_2), 36.9 (2′-CH_2), 35.0 (CH_2–S), 11.5 (5-CH_3); MS (DCI, NH_3-isobutane) m/z 334 $[M+H]^+$; HRMS for $C_{12}H_{20}N_3O_4NaS_2$, $[M+Na]^+$, calculated 356.0715, found 356.0721. The nucleoside symmetrical disulfide **5** formed under these conditions was isolated (48%).

4.1.15. 2′,3′-Dideoxy-3′-(6-hydroxyhexyldithio)thymidine (23)

Compound **23** (37 mg, 0.094 mmol, 73%) was obtained as a white powder from **16** (55 mg, 0.13 mmol), 6-mercaptohexanol (183 μL, 1.34 mmol), phosphate buffer (5%, pH 9.5, 334 μL), and THF (6 mL), 2 h; mp 106–108 °C; ^1H NMR (400 MHz, CD_3OD) δ 7.94 (1H, s, 6-H), 6.16 (1H, dd, J = 4.8 Hz, J = 6.8 Hz, 1′-H), 4.02–3.92 (2H, m, 4′-H, 5′-H), 3.81 (1H, m, 5′-H), 3.58 (3H, m, 3′-H + CH_2-OH), 2.79 (2H, m, CH_2–S), 2.62–2.42 (2H, m, 2′H × 2), 1.90 (3H, s, 5-CH_3), 1.74 (2H, m, CH_2–CH_2–S), 1.56 (2H, m, CH_2–CH_2-OH), 1.44 (4H, m, CH_2–CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ 162.2 (C2), 150.8 (C4), 136.8 (C6), 109.8 (C5), 85.6 (C4′), 84.5 (C1′), 61.4 (CH_2–OH), 60.6 (5′-CH_2), 45.4 (C3′), 39.2 (CH_2–S), 38.0 (2′-CH_2), 32.0 (CH_2–CH_2–OH), 28.7 (CH_2–CH_2–S), 27.8, 25.0 (2 CH_2), 11.0 (5-CH_3); MS (DCI, NH_3-isobutane) m/z 391 $[M+H]^+$; elemental analysis for $C_{16}H_{26}N_2O_5S_2$, calculated C 49.21, H 6.71, N 7.17, S 16.42, found C 49.29, H 6.99, N 7.06, S 16.69; HRMS for $C_{16}H_{26}N_2O_5NaS_2$ $[M+Na]^+$, calculated 413.1181, found 413.1193.

4.1.16. 5-Bromo-2′,3′-dideoxy-3′-(2-(trimethylsilyl)ethyl) thiouridine (24) and bis-(2′,3′-dideoxy-5-bromouridin-3′-yl) disulfide (25)

To a solution of **10** (150 mg, 0.44 mmol) in dry dichloromethane (4 mL) was added cyanogen bromide (230 mg, 2.17 mmol). The solution was stirred under argon at 40 °C for 96 h, then hydrolyzed with a phosphate buffer solution (0.5 M, pH 7, 2 mL) for 30 min, and concentrated to dryness. The residue was chromatographed on silica gel in dichloromethane/methanol (98:2 then 95:5) to give the symmetrical disulfide **25** (27 mg, 0.04 mmol, 25%) and compound **24** (42 mg, 0.10 mmol, 21%) as white powders. Compound **24**: mp 81–83 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 8.41 (1H, s, 6-H), 6.09 (1H, dd, J = 6.7 Hz, J = 3.2 Hz, 1′-H), 4.14 (1H, s, 5′-H), 4.93 (2H, m, 4′-H, 5′-H), 3.95 (1H, m, 3′-H), 2.66 (2H, m, S–CH_2), 2.60–2.44 (2H, m, 2′-H × 2), 1.28 (1H, t, 5′-OH), 0.88 (2H, m, CH_2–Si), −0.02 (9H, s, Si(CH_3)$_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.8 (C2), 149.3 (C4), 140.4 (C6), 96.1 (C5), 86.3 (C1′), 86.2 (C4′), 60.8 (5′-CH_2), 41.2 (2′-CH_2), 39.6 (C3′), 27.6 (S–CH_2), 17.5 (CH_2–Si), −1.5 (Si(CH_3)$_3$); MS (FAB$^+$, NBA) m/z 423 $[M+H]^+$; HRMS for $C_{14}H_{22}N_2O_4^{79}BrNa_2SiS$, $[M+2Na]^+$, calculated 467.0048, found 467.0049. **25**: ^1H NMR (400 MHz, $CDCl_3$) δ 8.63 (1H, s, 6-H), 6.12 (1H, dd, J = 6.5 Hz, J = 4.0 Hz, 1′-H), 4.05 (1H, s, 4′-H), 3.97 (1H, dd, J = 2.4 Hz, J = 12.3 Hz, 5′-H), 3.86 (1H, dd, J = 2.7 Hz, J = 12.3 Hz, 5′-H), 3.66 (1H, m, 3′-H), 2.61 (2H, m, 2′-H × 2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.3 (C2), 150.1 (C4), 140.7 (C6), 96.3 (C5), 86.2 (C1′), 85.3 (C4′), 60.0 (5′-CH_2), 45.3 (2′-CH_2), 38.3 (C3′);

MS (FAB$^+$, NBA) m/z 645 [M+H]$^+$; HRMS for $C_{18}H_{20}N_4O_8^{79}Br_2NaS_2$, [M +Na]$^+$, calculated 664.8987, found 664.8987.

4.1.17. 5-Bromo-2′,3′-dideoxy-3′-(methyldithio)uridine (26)

To a solution of **24** (20 mg, 0.005 mmol) and methyl disulfide (170 μL, 1.90 mmol) in anhydrous THF (500 μL) was added dimethyl(thiomethyl)sulfonium tetrafluoroborate (22 mg, 0.014 mmol). The mixture was stirred for 24 h under argon, then sodium bicarbonate (10%, 100 μL) was added. After evaporation, the residue was dissolved in water and chromatographed on C18 reversed phase silica gel (1 g) in water/methanol (9:1). After concentration to dryness, the residue was chromatographed on silica gel in dichloromethane/methanol (95:5) to give **26** (11 mg, 0.003 mmol, 65%) as a white foam; mp 74–77 °C; ^{1}H NMR (400 MHz, CD_3OD) δ 8.67 (1H, s, 6-H), 6.11 (1H, dd, J = 6.8 Hz, J = 3.5 Hz, 1′-H), 4.01 (2H, m, 4′-H, 5′-H), 3.84 (1H, m, 5′-H), 3.62 (1H, m, 3′-H), 2.64–2.52 (2H, m, 2′-H × 2), 2.48 (3H, s, S–CH_3); ^{13}C NMR (100 MHz, CD_3OD) δ 160.3 (C2), 150.1 (C4), 140.7 (C6), 95.2 (C5), 86.0 (C4′), 85.3 (C1′), 59.7 (5′-CH_2), 44.1 (C3′), 39.5 (2′-CH_2), 23.2 (S–CH_3); MS (DCI, NH_3-isobutane) m/z 369 [M+H]$^+$; HRMS for $C_{10}H_{13}N_2O_4{}^{79}BrNaS_2$, [M+Na]$^+$, calculated 390.9398, found 390.9404.

4.1.18. UV absorption characteristics (MeOH)

Compound **4**: λ_{max} 272 nm (ε 12,800); **12**: λ_{max} 261 nm (ε 8800); **14**: λ_{max} 266 nm (ε 26,250); **15**–**16**: λ_{max} 266 nm (ε 17,800); **17**–**23**: λ_{max} 266 nm (ε 8200 ± 700); **25**–**26**: λ_{max} 278 nm (ε 12,350 ± 300).

4.2. Biological assays

4.2.1. Preparation of the cell extract

To prepare a crude CEM cell extract, cell cultures were grown in 75 cm^2-culture bottles until cell density reached ~1–1.5 × 10^6 cells/mL. Then, the cells were carefully washed twice with PBS and suspended in PBS at a cell density of 10^8 cells/mL. The suspension was sonicated 3× for 10 s to lyse >99% of the cells and stored in aliquots at −80 °C before use.

4.2.2. Anti-HIV activity assays[28]

Inhibition of HIV-1(III_B) and HIV-2(ROD)-induced cytopathicity in CEM or CEM/TK$^-$ cell cultures was measured in microtiter 96-well plates containing ~3 × 10^5 CEM cells/mL, infected with 100 $CCID_{50}$ of HIV per mL and containing appropriate dilutions of the test compounds. After 4–5 days of incubation at 37 °C in a CO_2-controlled humidified atmosphere, CEM giant (syncytium) cell formation was examined microscopically. The EC_{50} (50% effective concentration) was defined as the compound concentration required to inhibit HIV-induced giant cell formation by 50%.

4.2.3. Affinity of test compounds for TK-1

The 50% inhibitory concentration (IC_{50}) of the test compounds against phosphorylation of [CH_3-^{3}H]dT as the natural substrate for recombinant purified cytosolic TK-1 was determined as described by Balzarini et al.[28] Briefly, the activity of the nucleoside kinase was assayed in a 50 μL reaction mixture containing 50 mM Tris–HCl, pH 8.0, 2.5 mM $MgCl_2$, 10 mM dithiothreitol (DTT) (or no DTT), 2.5 mM ATP, 1.0 mg/mL bovine serum albumin, 10 mM NaF. [CH_3-^{3}H]dThd (0.1 μCi in 5 μL; 1 μM final concentration), and 5 μL of recombinant enzyme (containing 455 ng of TK-1 protein). The samples were incubated at 37 °C for 30 min in the presence or absence of different concentrations of the test compounds. During this time period, the enzyme reaction proceeded linearly. Aliquots of 45 μL of the reaction mixtures were spotted on Whatman DE-81 filter paper disks (Whatman, Maidstone, UK). The filters were washed three times for 5 min in 1 mM ammonium formate and once for 5 min in ethanol. The radioactivity on the filters was determined by scintillation counting.

4.2.4. Cytostatic activity assays[28]

The assays were performed in 96-well microtiter plates. To each well were added 5–7.5 × 10^4 CEM cells and a given amount of the test compound. The cells were allowed to proliferate for 72 h at 37 °C in a humidified CO_2-controlled atmosphere. At the end of the incubation period, the cells were counted in a Coulter counter. The CC_{50} was defined as the concentration of the compound that inhibited cell proliferation by 50%.

4.2.5. Anti-Moloney murine sarcoma virus (MSV) assays

The inhibitory effect of a variety of test compound concentrations on MSV-induced transformation of murine embryo fibroblast C3H/3T3 cell cultures was examined microscopically at day 6 post-infection. MSV was added at 75 focus-forming units to monolayer cell cultures in 48-well microtiter plates.

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