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Original article

Synthesis and evaluation of fatty acyl ester derivatives of cytarabine as anti-leukemia agents

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ABSTRACT

Cytarabine is a chemotherapeutic agent predominately used for the treatment of acute myeloid leukemia and lymphoblastic leukemia. Cytarabine is a polar nucleoside, has a short plasma half-life, and its use is associated with severe side effects. Fatty acyl derivatives of cytarabine were synthesized with the expectation to improve cellular uptake and generate derivatives with a longer duration of action. Multi-step protection and deprotection reactions of hydroxyl and amino groups and conjugation with a fatty acid (i.e., myristic acid and 12-thioethyldodecanoic acid) afforded 5′-*O*-substituted, 2′-*O*-substituted, and 2′,5′-disubstituted fatty acyl derivatives of cytarabine. 2′,5′-Dimyristoyl derivative of cytarabine was found to inhibit the growth of CCRF-CEM cells by approximately 76% at concentration of 1 μM after 96 h incubation.

1. Introduction

Cytarabine (cytosine arabinoside, 1-β-D-arabinofuranosylcytosine, ara-C), a pyrimidine nucleoside analog, is predominantly used against acute myelogenous leukemia and non-Hodgkin's lymphoma [1,2]. It is also used in combination with other anticancer drugs for the treatment of leukemia and solid tumors [3,4]. Cytarabine is a polar nucleoside and has a short plasma half-life. The low bioavailability of cytarabine is created by its low permeability across membrane and rapid conversion into inactive 1-β-D-arabinofuranosyluracil. Thus, continuous intravenous infusion of higher doses is required to maintain constant plasma level of the drug in 8–24 h. The higher doses of cytarabine lead to toxicity to normal organs and side effects [5].

Recently there has been emphasis on the development of cytarabine derivatives to obtain compounds with a higher therapeutic index for the treatment of leukemia and lymphoma. Among the explored alternatives, the prodrug strategy by introducing modifications on the parent drug to enhance plasma half-life or delivery to cancer cells is a subject of major interest. These design approaches included designing amino acid–cytarabine [6,7], amino acid–fatty acid–cytarabine [8], cytarabine phosphate derivatives [9], and 5′-*O*-unsaturated fatty acid derivatives [10].

ValCytarabine [7,11] and CP-4055 as amino acid and fatty acid prodrugs of cytarabine, respectively, have been evaluated for the treatment of leukemia and solid tumors. CP-4055, an elaidic acid derivative of cytarabine (ara-C-5′-elaidic acid ester), enhanced the cellular uptake of ara-C in tumor cells possibly via passive diffusion through the cellular membrane [12]. Repeated treatment with CP-4055 in human leukemia and in solid tumor models *in vivo* enhanced the antitumor effect [13]. CP-4055 [14] is currently undergoing phase 2 studies in patients with solid tumors under the brand name Elacyst™. Among other lipophilic derivatives, a simple stearyl phosphate diester prodrug, cytarabine ocfosfate, was approved in 1992 for leukemia treatment [15]. Phosphoramidate derivative of cytarabine metabolite 2-β-D-arabinouridine (AraU) has also been evaluated [16].

The biological activities of synthesized conjugates depended upon the rate of intracellular release of cytarabine. Once inside the cells, cytarabine is converted into cytarabine triphosphate derivative to show its cytotoxic effect. Cytarabine triphosphate is incorporated into the growing DNA chain by DNA polymerases and leads to the termination of chain elongation process of DNA synthesis [17]. The phosphorylation process occurs on the 5′-hydroxyl group of the arabinose sugar. Prodrug strategies used either the substitution at amine group of cytosine base or the protected 5′-monophosphate

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derivatives have resulted in compromised activities against various cancers and generally leading to reduced anticancer activity [15].

Alternatively, cytarabine encapsulation in delivery systems [18] like nanoparticles [19], chitosan [20], hydrogels [21], niosome vesicles of tween 20 [22,23], and liposomes [24] has been explored. Liposomal cytarabine was approved by the FDA for the treatment of lymphomatous meningitis [25]. Lipophilic liposomal encapsulated formulation of cytarabine passes across the blood brain barrier, enhances delivery of the drug, and thus therapeutic effect. Our earlier efforts of designing lipophilic antifungal and antiviral agents indicated that designed lipophilic prodrugs, by conjugating of parent analogs with fatty acid derivatives, led to enhanced biological activities of the respective parent drugs [26–30]. Herein, we report the synthesis of lipophilic fatty acyl derivatives of cytarabine with the expectation to improve the cellular uptake. Furthermore, intracellular hydrolysis of the conjugate may result in the sustained release of cytarabine and increasing the duration of biological activity. Three classes of 5′-*O*-substituted, 2′-*O*-substituted, and 2′,5′-disubstituted fatty acyl derivatives of cytarabine were synthesized. The anti-leukemia activity of compounds were evaluated and compared in CCRF-CEM cells.

2. Results and discussion

2.1. Chemistry

Three classes of 5′-*O*-substituted, 2′-*O*-substituted, and 2′,5′-disubstituted fatty acyl derivatives of cytarabine were synthesized from cytarabine (**1**) using multi-step protection and deprotection reactions. Two fatty acids, myristic acid and 12-thioethyldodecanoic acid, were used for the conjugation with cytarabine.

2.1.1. Synthesis of 5′-*O*-fatty acyl derivatives of cytarabine

Scheme 1 depicts the synthesis of 5′-*O*-fatty acyl ester derivatives of cytarabine. Subsequent protection of 5′- and 3′-hydroxyl groups with *tert*-butyldimethylsilyl chloride (TBDMS-Cl, 2.5 equiv) in the presence of imidazole as a base afforded 5′,3′-TBDMS-protected cytarabine (**2**) in a controlled reaction. The 2′-hydroxyl group of cytarabine was unaffected, but using a larger amount of TBDMS-Cl led to protection of all three hydroxyl groups. 4-Amino group of **2** was protected with the 4,4′-dimethoxytrityl (DMTr) protecting group in the presence of DMTr-Cl and pyridine to afford **3**. Selective deprotection of 5′-*O*-TBDMS group in **3** in the presence of 0.1 Methanolic potassium hydroxide solution [31] generated **4**. The potassium salt was neutralized with dilute acetic acid. The neutralization was carried out carefully in a controlled fashion and addition of excess acetic acid was avoided, thus highly acid labile DMTr group remained intact under these conditions. Conjugation reaction of **4** with the fatty acid (i.e., myristic acid and 12-thioethyldodecanoic acid) in the presence of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), and *N*,*N*-diisopropylethylamine (DIPEA) afforded protected fatty acyl derivatives of cytarabine, **5** and **6**. Deprotection of 4-amino and 3′-hydroxyl groups in the presence of acetic acid and tetrabutylammonium fluoride (TBAF), respectively, generated 5′-*O*-(fatty acyl) ester derivatives of cytarabine **9** and **10**.

2.1.2. Synthesis of 2′,5′-dimyristoyl derivative of cytarabine

The synthesis of 2′,5′-dimyristoyl derivative of cytarabine was initiated with the reaction of **4** with an excess amount of myristoyl chloride in the presence of 4-dimethylaminopyridine (DMAP) to generate **11**. When the coupling reaction of **4** with myristic acid was carried out using HBTU and HOBt, only the 5′-monosubstituted analog was obtained possibly because the sterical hindrance at 2′-hydroxyl group prevents the proximity of bulky HBTU-activated fatty acid. On the other hand, smaller size acyl chloride was able to form a conjugate ester with 2′ hydroxyl group to yield **11**. Dimyristoyl substituted cytarabine **13** was obtained from **11** by deprotection of N_4-DMTr and 3′-*O*-TBDMS groups with acetic acid/TFA and TBAF, respectively (Scheme 2).

2.1.3. Synthesis of 2′-*O*-fatty acyl derivatives of cytarabine

Similar to the 2′,5′-disubstitution, the fatty acyl chloride was chosen to achieve the esterification at 2′-hydroxyl group. Reaction of **3** with the fatty acyl chloride in the presence of DMAP afforded **14** and **15**. Excess of DMAP base was maintained during the coupling (esterification) reaction with **3** to avoid cleavage of DMTr because of the formation of HCl during the reaction. The DMTr group was removed by using acetic acid and 1–2% trifluoroacetic acid. While removing the DMTr group from product **14**, the 5′-TBDMS group was also cleaved as indicated by the observed MS peak at 568 $[M + 1]^+$ to generate **16**. Similar results were observed during deprotection of **15** [32]. Compounds **16** and **17** were treated with TBAF in THF to afford 2′-*O*-(fatty acyl) ester derivatives of cytarabine (**18** and **19**) (Scheme 3). Myristoyl chloride is commercially available. 12-Thioethyldodecanoic acid was synthesized from 12-bromododecanoic acid and thioethanol as described previously [33]. 12-Thioethyldodecanoic acid was treated with oxalyl chloride under nitrogen atmosphere to afford crude 12-thioethyldodecanoyl chloride in situ after removal of excess oxalyl chloride and was immediately used in the esterification reaction with **3**.

2.1.4. Purification and characterization

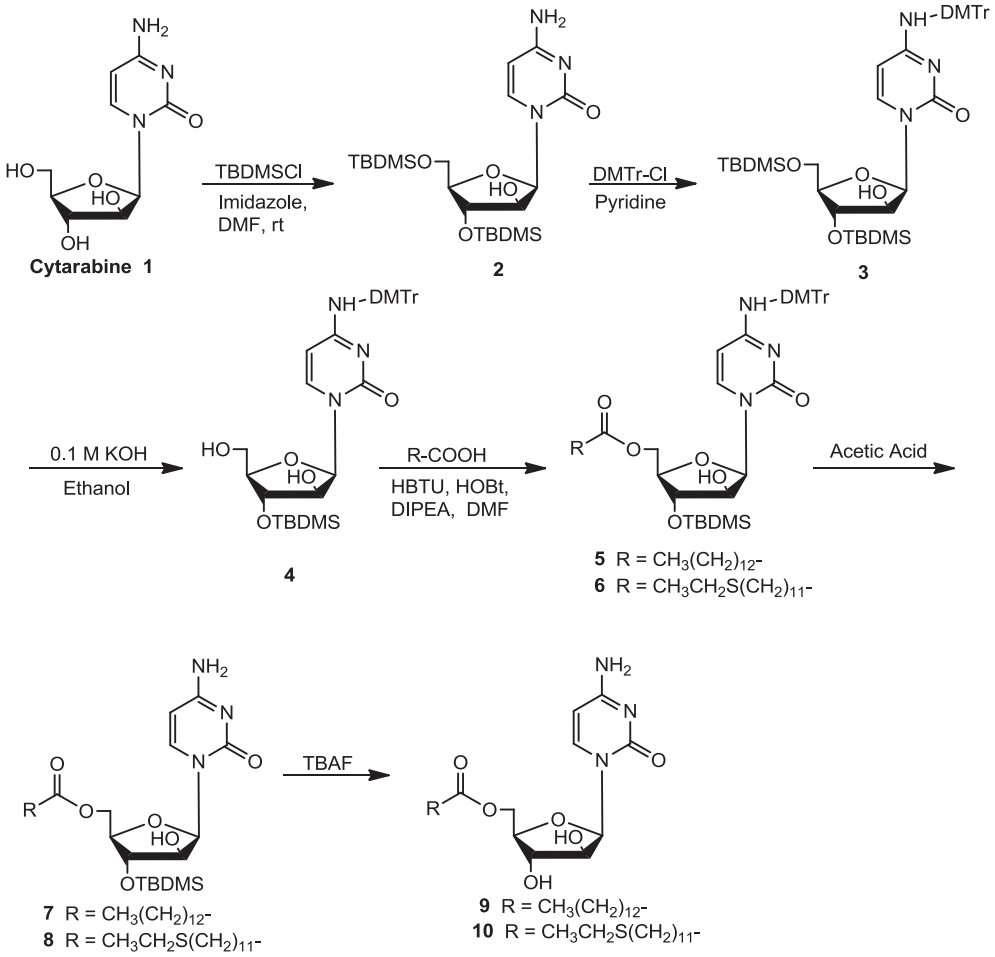
The final products were purified on a reverse phase high performance liquid chromatography (HPLC) using a C18 column. The chemical structures of the synthesized fatty acyl derivatives of cytarabine and intermediate products were confirmed with mass-spectrometry (MS) and/or nuclear magnetic resonance (NMR). The purity of the final products (**9**, **10**, **13**, **18**, **19**) was in the range of 95.0–99.1% as determined by the analytical HPLC using water/acetonitrile as eluents with a flow rate of 1 mL/min.

2.2. Biological activity

The effect of the compounds (1 μM) on the cell proliferation of leukemia cells was evaluated in CCRF-CEM cell line after 24 h and 96 h (Fig. 1). Cytarabine (1 μM) and DMSO were used as the positive and negative controls, respectively [34,35].

The cytotoxic mechanism of cytarabine involves phosphorylation of 5′-hydroxyl into triphosphate, which on incorporation in nucleotide chain synthesis results into termination of chain elongation and thus inhibition of ribonucleotide synthesis and ultimately cell division inhibition [36]. The effectiveness of the cytarabine and cytarabine derivatives depends on the concentration of drug delivered intracellularly and availability of 5′-hydroxyl group. 5′-Monosubstituted fatty acyl derivatives of cytarabine (**9** and **10**) were not able to inhibit significantly the cell proliferation even after 96 h. The cell proliferation inhibitory activity at 24 h and after 96 h was not significantly different for both derivatives, suggesting that the compounds were probably stable intracellularly and did not release cytarabine effectively. Because of high lipophilicity, these compounds were expected to have enhanced cellular uptake. Thus, the lack of inhibitory activity is postulated to be because of limited intracellular hydrolysis of 5′-ester.

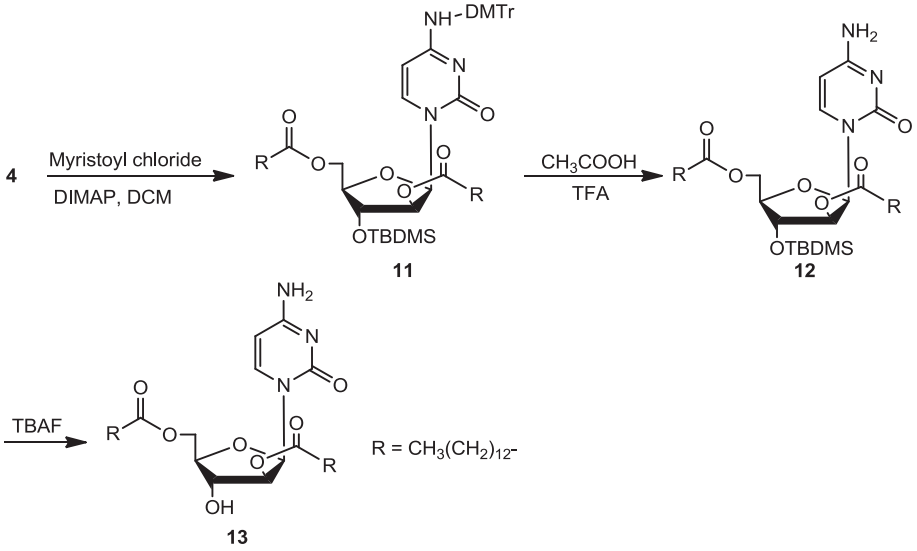
On the other hand, 2′,5′-dimyristoyl derivative of cytarabine (**13**) and 2′-fatty acyl derivatives of cytarabine (**18** and **19**) were able to inhibit the growth of cancer cells by approximately 36–76% at a concentration of 1 μM after 96 h incubation. The 2′-fatty acyl



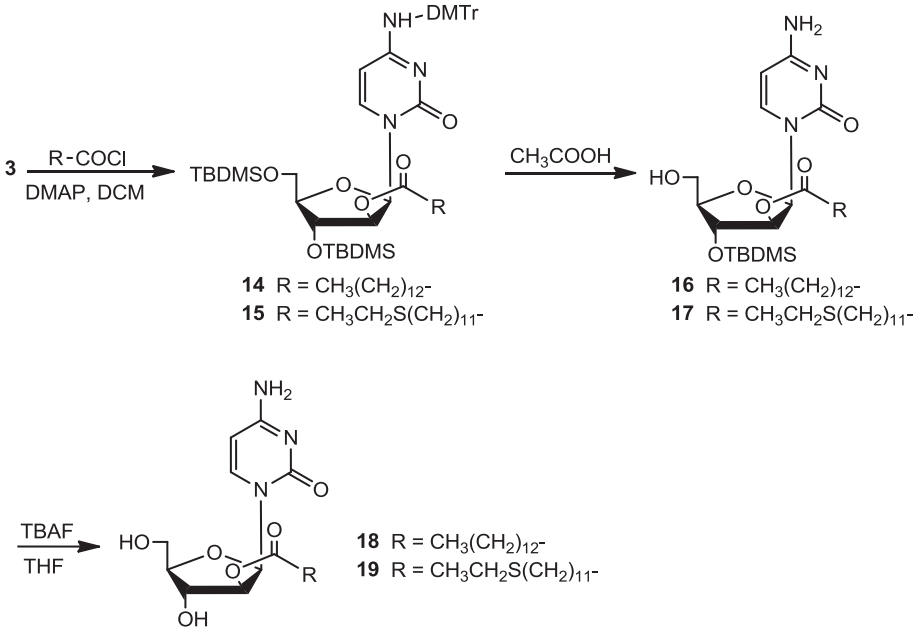
Scheme 1. Synthesis of 5′-*O*-fatty acyl derivatives of cytarabine.

derivatives (**18** and **19**) showed better inhibitory activity when compared to 5′-fatty acyl derivatives (**9** and **10**) probably because of readily available 5′-hydroxyl for phosphorylation and eventual cytotoxic activity.

2′,5′-Disubstituted derivative **13** showed comparable activity to that of parent drug **1** and physical mixtures after 96 h and significantly higher activity when compared with less lipophilic monosubstituted analogs, **9**, **10**, **18**, and **19**. The inhibitory activity for **13** at



Scheme 2. Synthesis of 2′,5′-dimyristoyl derivative of cytarabine.



Scheme 3. Synthesis of 2′-*O*-fatty acyl derivatives of cytarabine.

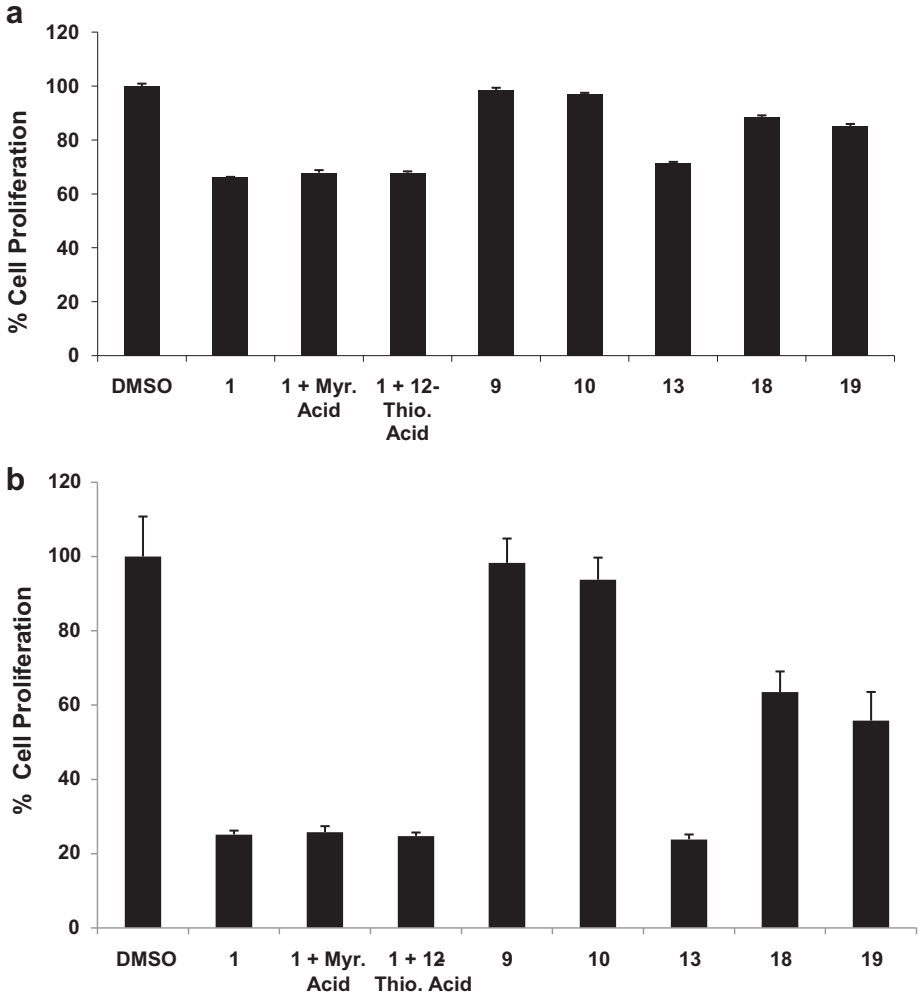


Fig. 1. Inhibition of CCRF-CEM cell proliferation by the compounds (1 μM) and cytarabine (1 μM) after (a) 24 h and (b) 96 h. The results are shown as the percentage of the control that has no compound (set at 100%). All the experiments were performed in triplicate.

24 h and 96 h suggests that the intracellular hydrolysis of the prodrug to cytarabine leads to improved antiproliferative activity after longer incubation period. Enhanced activities of **13**, **18**, and **19** after 96 h compared to 24 h indicate that the conjugates release cytarabine slowly and may behave as a prodrug for sustained delivery of the parent nucleoside. Although the anticancer activities of these compounds were only comparable to that of cytarabine after 96 h, their sustained release effect may be beneficial similar to liposomal cytarabine. Furthermore, these prodrugs are expected to have higher cellular uptake when compared to cytarabine, because of the presence of lipophilic fatty acyl chains.

3. Conclusions

Three classes of fatty acyl derivatives of cytarabine were synthesized as fatty acyl prodrugs and evaluated for the inhibition of CCRF-CEM cell proliferation. The data indicate that designing molecules with comparable anti-leukemia activity to cytarabine with sustained release effect is possible by structure modification. A number of compounds may have potential application for slow delivery of cytarabine. Further optimization is required to generate lead compounds with improved cell proliferation inhibition versus cytarabine.

4. Experimental protocols

4.1. Materials and methods

Cytarabine, HBTU, HOBt, anhydrous dichloromethane, anhydrous pyridine, *N*,*N*-dimethylformamide (DMF) and other chemicals and reagents were purchased from Sigma–Aldrich Chemical Co. (Milwaukee, WI). 12-Thioethyldodecanoic acid was synthesized from 12-bromododecanoic acid and thioethanol as described previously [33]. HBTU and HOBt in *N*,*N*-dimethylformamide (DMF) were used as coupling and activating reagents, respectively. The chemical structures of final products were characterized by nuclear magnetic resonance spectra (^{1}H NMR, ^{13}C NMR) determined on a Bruker NMR spectrometer (400 MHz) or a Varian NMR spectrometer (500 MHz). ^{13}C NMR spectra are fully decoupled. Chemical shifts were reported in parts per million (ppm) using deuterated solvent peak as the standard. The chemical structures of final products were confirmed by a high-resolution Biosystems QStar Elite time-of-flight electrospray mass spectrometer. Details of procedures and spectroscopic data of the respective compounds are presented below. Final compounds were purified on a Phenomenex Prodigy 10 μm ODS reversed-phase column (2.1 cm × 25 cm) with a Hitachi HPLC system using a gradient system of acetonitrile or methanol and water ($CH_3CN/CH_3OH/H_2O$, 0–100%, pH 7.0, 60 min). The purity of final products (>99%) was confirmed by analytical HPLC. The analytical HPLC was performed on the Hitachi analytical HPLC system using a C18 Shimadzu Premier 3 μm column (150 cm × 4.6 mm) using two different isocratic systems, and a flow rate of 1 mL/min with a UV detection at 265 nm.

4.2. Chemistry

4.2.1. 1-[3′,5′-Di(O-tert-butyldimethylsilyl)-β-D-arabinofuranosyl] cytosine (**2**)

tert-Butyldimethylsilyl chloride (TBDMS-Cl) (247 mg, 1.64 mmol) and imidazole (112 mg, 1.64 mmol) were added to a solution of cytarabine (100 mg, 0.41 mmol) in dry DMF (20.0 mL). The reaction mixture was stirred at room temperature for 14 h. After the completion of the reaction, the solvent was removed under reduced pressure, and the crude compound was purified by column chromatography over silica gel using dichloromethane/methanol (0–10%) as the eluents to afford product **2** (178 mg, 92%). ^{1}H NMR (CD_3OD, 400 MHz, δ ppm): 7.71 (d, J = 10 Hz, 1H, H-6), 6.20 (d, J = 5 Hz, 1H, H-1′), 5.86 (d, J = 10 Hz, 1H, H-5), 4.31 (dd, J = 5 Hz, J = 5 Hz, 1H, H-2′), 4.09 (t, J = 5 Hz, 1H, H-3′), 3.93–3.97 (m, 1H, H-4′), 3.85–3.92 (m, 2H, H-5′), 0.96 (s, 9H, SiC(CH_3)$_3$), 0.83 (s, 9H, SiC(CH_3)$_3$), 0.15 (s, 3H, SiCH_3), 0.13 (s, 3H, SiCH_3), 0.11 (s, 3H, SiCH_3), −0.05 (s, 3H, SiCH_3); ^{13}C NMR (CD_3OD, 100 MHz, δ ppm): 161.93 (C-4), 149.38 (C-2), 147.18 (C-6), 93.84 (C-5), 88.17 (C-1′), 86.71 (C-4′), 77.42 (C-3′), 77.06 (C-2′), 63.01 (C-5′), 26.65, 26.38 (2 × SiC(CH_3)$_3$), 19.16, 18.97 (2 × SiC(CH_3)$_3$), −4.07, −4.41, −5.08, −5.13 (2 × Si(CH_3)$_2$); HR-MS (ESI-TOF) (m/z): calcd for $C_{21}H_{41}N_3O_5Si_2$: 471.2585; found, 472.2620 $[M + 1]^+$, 943.5228 $[2M + 1]^+$.

4.2.2. 1-[3′,5′-Di(O-tert-butyldimethylsilyl)-N-(4,4′-dimethoxytrityl)-β-D-arabinofuranosyl]cytosine (**3**)

Compound **2** (170 mg, 0.36 mmol) was dissolved in dry pyridine (5 mL). To the solution was added 4,4′-dimethoxytrityl chloride (DMTr-Cl, 166 mg, 0.49 mmol) dissolved in dry pyridine (10 mL) dropwise at 0 °C. The mixture was stirred for 4 h at 0 °C and then for an additional 14 h at room temperature. The solution was then neutralized with aqueous sodium bicarbonate (5.0%) and extracted with dichloromethane (3 × 50 mL). The organic layer was dried with anhydrous sodium sulfate, and the solvent was removed under reduced pressure. The crude compound was purified by column chromatography over silica gel using hexane/dichloromethane containing 1.0% triethylamine as the eluents to yield **3** (240 mg, 86%). ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm): 7.82 (d, J = 7.7 Hz, 1H, H-6), 7.40–7.53 (m, 5H, DMTr-Ar-H), 7.32 (br s, 2H, DMTr-Ar′-C2-H), 7.31 (br s, 2H, DMTr-Ar′-C6-H), 7.01 (br s, 2H, DMTr Ar'-C5-H), 7.00 (s, 2H, DMTr Ar'-C3-H), 6.28 (d, J = 4.7 Hz, 1H, H-1′), 5.21 (d, J = 7.7 Hz, 1H, H-5), 4.46–4.53 (m, 1H, H-2′), 4.27–4.32 (m, 1H, H-3′), 4.04–4.08 (m, 2H, H-5′), 3.90 (s, 6H, 2 × OCH_3), 3.86–3.92 (m, 1H, H-4′), 1.07 (s, 9H, SiC(CH_3)$_3$), 0.97 (s, 9H, SiC(CH_3)$_3$), 0.31 (s, 3H, SiCH_3), 0.27 (s, 3H, SiCH_3), 0.20 (s, 3H, SiCH_3), 0.17 (s, 3H, SiCH_3); ^{13}C NMR ($CDCl_3$, 125 MHz, δ ppm): 165.63 (C-4), 158.80 (DMTr Ar-C4-O), 156.22 (C-2), 144.73 (DMTr-C1-Ar), 142.04 (C-6), 136.44 (DMTr-C1-Ar′-OCH_3), 130.24, 130.13, 128.75, 128.41, 127.90, 127.52, 113.68 (DMTr-Ar carbons), 94.24 (C-5), 87.29 (C-1′), 84.64 (C-4′), 78.33 (C-3′), 75.86 (C-2′), 70.43 (DMTr C-Ar$_3$), 62.07 (C-5′), 55.38 (DMTr Ar-O-CH_3), 26.03, 25.92 (TBDMS 2 × SiC(CH_3)$_3$), 18.37, 18.14 (TBDMS 2 × SiC(CH_3)$_3$), −4.16, −4.92, −5.47, −5.48 (TBDMS 4 × SiCH_3); HR-MS (ESI-TOF) (m/z): calcd for $C_{42}H_{59}N_3O_7Si_2$: 773.3892; found, 774.0265 $[M + 1]^+$.

4.2.3. 1-[3′-O-(tert-Butyldimethylsilyl)-N-(4,4′-dimethoxytrityl)-β-D-arabinofuranosyl]cytosine (**4**)

Compound **3** (200 mg, 0.26 mmol) was treated with 0.1 M solution of potassium hydroxide in ethanol (20 mL). The mixture was stirred for 1 h at room temperature. After completion of reaction, the solution was neutralized with 0.01% acetic acid in ethanol to pH 7 at 0 °C. The solvent was evaporated under reduced pressure and the crude sticky product was purified on column chromatography over silica gel using dichloromethane/methanol containing 1.0% triethylamine as the eluents to yield **4** (152 mg, 89%). ^{1}H NMR (CD_3OD, 400 MHz, δ ppm): 7.58 (d, J = 8.0 Hz, 1H, H-6), 7.25–7.40 (m, 5H, DMTr-Ar-H), 7.17 (br s, 4H, DMTr-Ar′-C2-H, DMTr-Ar′-C6-H), 6.88 (br s, 4H, DMTr Ar'-C3-H, DMTr Ar'-C5-H), 6.05–6.20 (m, 1H, H-1′), 5.22 (d, J = 8.0 Hz, 1H, H-5), 4.08–4.13 (m, 2H, H-2′, H-3′), 3.81–3.83 (m, 1H, H-4′), 3.79 (s, 6H, 2 × OCH_3), 3.69–3.75 (m, 2H, H-5′), 0.93 (s, 9H, SiC(CH_3)$_3$), 0.13 (s, 6H, Si(CH_3)$_2$); ^{13}C NMR (CD_3OD, 100 MHz, δ, ppm): 165.9 (C-4), 158.96, 158.68 (DMTr Ar-C4-O), 156.21 (C-2), 144.57 (DMTr-C1-Ar), 142.92 (C-6), 136.16, 136.07 (DMTr-C1-Ar-OCH_3), 130.13, 129.88, 129.79, 128.41, 127.93, 127.11, 113.19, 112.61, 112.37 (DMTr-Ar carbons), 94.42 (C-5), 87.23 (C-1′), 86.21

(C-4′), 78.12 (C-3′), 75.41 (C-2′), 70.13 (DMTr *C*-Ar$_3$), 61.10 (C-5′), 54.38 (DMTr Ar-O-*C*H$_3$), 54.31 (DMTr Ar′-O-*C*H$_3$), 24.96 (TBDMS SiC(*C*H$_3$)$_3$), 17.14 (TBDMS SiC(CH$_3$)$_3$), −5.92, −6.18 (TBDMS 2 × SiCH$_3$); HR-MS (ESI-TOF) (*m*/*z*): calcd for $C_{36}H_{45}N_3O_7Si$: 659.3027; found, 660.0500 $[M + 1]^+$, 697.9557 $[M + K]^+$, 1319.0863 $[2M + 1]^+$.

4.2.4. 1-[3′-O-(tert-Butyldimethylsilyl)-5′-O-tetradecanoyl-N-(4,4′-dimethoxytrityl)-β-D-arabinofuranosyl]cytosine (**5**)

Compound **4** (100 mg, 0.15 mmol) was dissolved in dry DMF (20 mL). Myristic acid (34.8 mg, 0.15 mmol), HBTU (115 mg, 0.30 mmol), and HOBt (40.9 mg, 0.30 mmol) were added to the reaction mixture. DIPEA (100 mg, 0.77 mmol) was added to the reaction mixture at room temperature. The mixture was stirred for 10 h under nitrogen atmosphere. After completion of the reaction, the solvent was removed under vacuum. Water (50 mL) was added to the mixture and the crude product was extracted with dichloromethane (3 × 40 mL). After removal of dichloromethane, the crude product was directly carried forward to the next deprotection steps. HR-MS (ESI-TOF) (*m*/*z*): calcd for $C_{50}H_{71}N_3O_8Si$: 869.5010; found, 870.2031 $[M + 1]^+$.

4.2.5. 1-[3′-O-(tert-Butyldimethylsilyl)-5′-O-tetradecanoyl-β-D-arabinofuranosyl]cytosine (**7**)

Compound **5** was treated with acetic acid (30 mL) and was stirred for 3 h under nitrogen atmosphere. After the completion of the reaction, acetic acid was removed under vacuum and concentrated crude product was directly carried forward to the next reaction. HR-MS (ESI-TOF) (*m*/*z*): calcd for $C_{29}H_{53}N_3O_6Si$: 567.3704; found, 568.0838 $[M + 1]^+$.

4.2.6. 1-[5′-O-Tetradecanoyl-β-D-arabinofuranosyl]cytosine (**9**)

Compound **7** was treated with tetrabutylammonium fluoride (TBAF, 1 M) solution in tetrahydrofuran (20 mL) and stirred for 6 h at room temperature under nitrogen atmosphere. After completion of the reaction, the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography over silica gel using hexane/dichloromethane and then dichloromethane/methanol (0–10%) as the eluents to afford product **9** (21 mg, 27% yield from **4**). The product was further purified on a reverse phase HPLC using C18 column and gradient methanol/water as mobile phase as described above. ^{1}H NMR (CD$_3$OD, 500 MHz, δ, ppm): 7.84 (d, *J* = 7.5 Hz, 1H, H-6), 6.18 (d, *J* = 3.9 Hz, 1H, H-1′), 5.84 (d, *J* = 7.5 Hz, 1H, H-5), 4.18 (dd, *J* = 3.9 Hz, *J* = 2.4 Hz, 1H, H-2′), 4.08 (t, *J* = 2.8 Hz, 1H, H-3′), 3.94–3.98 (m, 1H, H-4′), 3.76–3.86 (m, 2H, H-5′), 2.28 (t, *J* = 7.5 Hz, 2H, C*H*$_2$CO), 1.56–1.67 (m, 2H, C*H*$_2$CH$_2$CO), 1.28 (br m, 20H, methylene protons), 0.89 (t, *J* = 6.9 Hz, 3H, C*H*$_3$); ^{13}C NMR (CD$_3$OD, 100 MHz, δ, ppm): 173.81 (*C*OO), 166.22 (C-4), 156.76 (C-2), 143.12 (C-6), 93.21 (C-5), 87.36 (C-1′), 82.83 (C-4′), 77.15 (C-2′), 74.91 (C-3′), 64.30 (C-5′), 34.01 (*C*H$_2$COO), 31.65, 29.36, 29.33, 29.27, 29.17, 29.05, 29.97, 29.89, 28.71, 24.87, 22.31 (methylene −CH$_2$− carbons), 13.00 (*C*H$_3$); HR-MS (ESI-TOF) (*m*/*z*): calcd for $C_{23}H_{39}N_3O_6$: 453.2839; found, 454.3192 $[M + 1]^+$.

4.2.7. 1-[3′-O-(tert-Butyldimethylsilyl)-5′-O-(12-thioethyldodecanoyl)-N-(4,4′-dimethoxytrityl)-β-D-arabinofuranosyl]cytosine (**6**)

Compound **4** (100 mg, 0.15 mmol), 12-thioethyldodecanoic acid (39.0 mg, 0.15 mmol), HBTU (115 mg, 0.30 mmol), HOBt (40.9 mg, 0.30 mmol) were dissolved in dry DMF (20 mL). DIPEA (100 mg, 0.77 mmol) was added to the reaction mixture at room temperature. The mixture was stirred for 12 h under nitrogen atmosphere. After the completion of reaction, the solvent was removed under vacuum. Water (50 mL) was added to the mixture. The crude product was extracted with dichloromethane (3 × 40 mL). After removal of dichloromethane, the crude product was directly carried forward to the next deprotection reaction. HR-MS (ESI-TOF) (*m*/*z*): calcd for $C_{50}H_{71}N_3O_8SSi$: 901.4731; found, 902.1630 $[M + 1]^+$.

4.2.8. 1-[3′-O-(tert-Butyldimethylsilyl)-5′-O-(12-thioethyldodecanoyl)-β-D-arabinofuranosyl]cytosine (**8**)

Synthesis of **8** followed the same procedure as that of **7** as described above. HR-MS (ESI-TOF) (*m*/*z*): calcd for $C_{29}H_{53}N_3O_6SSi$: 599.3424; found, 600.0838 $[M + 1]^+$.

4.2.9. 1-[5′-O-(12-Thioethyldodecanoyl)-β-D-arabinofuranosyl]cytosine (**10**)

A similar procedure to that of **9** was used for the synthesis and purification of **10** using **8** as the starting material instead of **7** as described above. ^{1}H NMR (CD$_3$OD, 500 MHz, δ, ppm): 7.57 (d, *J* = 7.5 Hz, 1H, H-6), 5.97 (d, *J* = 4.2 Hz, 1H, H-1′), 5.70 (d, *J* = 7.5 Hz, 1H, H-5), 3.92–3.96 (m, 1H, H-2′), 3.81–3.86 (m, 1H, H-3′), 3.69–3.75 (m 1H, H-4′), 3.53–3.58 (m, 2H, H-5′), 2.35–2.40 (m, 4H, C*H*$_2$SC*H*$_2$), 2.11 (t, *J* = 7.4 Hz, 2H, C*H*$_2$CO), 1.40–1.48 (m, 4H, SCH$_2$C*H*$_2$, C*H*$_2$CH$_2$CO), 1.15–1.30 (br m, 14H, methylene protons), 1.09 (t, *J* = 7.4 Hz, 3H, C*H*$_3$); ^{13}C NMR (CD$_3$OD, 125 MHz, δ, ppm): 173.50 (*C*OO), 166.21 (C-4), 155.91 (C-2), 143.37 (C-6), 93.94 (C-5), 86.27 (C-1′), 84.91 (C-4′), 76.31 (C-2′), 74.99 (C-3′), 64.17 (C-5′), 31.03 (*C*H$_2$COO), 29.18, 29.13, 28.98, 28.85, 28.80, 28.44, 28.31, 25.36, 24.85 (methylene −CH$_2$− carbons), 15.06 (*C*H$_3$); HR-MS (ESI-TOF) (*m*/*z*): calcd for $C_{23}H_{39}N_3O_6S$: 485.2560; found, 485.4508 $[M]^+$.

4.2.10. 1-[3′-O-(tert-Butyldimethylsilyl)-2′,5′-di(O-tetradecanoyl)-N-(4,4′-dimethoxytrityl)-β-D-arabinofuranosyl]cytosine (**11**)

Compound **4** (65 mg, 0.1 mmol) and 4-dimethylaminopyridine (DMAP, 50 mg, 0.41 mmol) were dissolved in dry dichloromethane (5 mL) under nitrogen atmosphere. Myristoyl chloride (60 mg, 0.24 mmol) diluted with dry DCM (5 mL) was added dropwise to the reaction mixture at room temperature. The reaction mixture was stirred for 16 h under nitrogen atmosphere. After completion of the reaction, additional DCM (30 mL) was added to the reaction mixture, which was washed with 0.1% HCl (20 mL × 2) and then with water (20 mL × 1). The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. After removal of dichloromethane, the crude product was directly carried forward to the next deprotection reaction. HR-MS (ESI-TOF) (*m*/*z*): calcd for $C_{64}H_{97}N_3O_9Si$: 1079.6994; found, 1080.7453 $[M + 1]^+$.

4.2.11. 1-[3′-O-(tert-Butyldimethylsilyl)-2′,5′-di(O-tetradecanoyl)-β-D-arabinofuranosyl]cytosine (**12**)

Compound **11** was treated with acetic acid (30 mL) and trifluoroacetic acid (1 mL). The reaction mixture was stirred for 4 h under nitrogen atmosphere. After the completion of the reaction, acetic acid/TFA was removed under vacuum and concentrated product was directly carried to next reaction. HR-MS (ESI-TOF) (*m*/*z*): calcd for $C_{43}H_{79}N_3O_7Si$: 777.5687; found, 778.6055 $[M + 1]^+$.

4.2.12. 1-[2′,5′-di(O-Tetradecanoyl)-β-D-arabinofuranosyl]cytosine (**13**)

Compound **12** was treated with TBAF (1 M solution in THF, 20 mL). The mixture was stirred for 12 h at room temperature under nitrogen atmosphere. After completion of the reaction, the solvent was evaporated under reduced pressure. The liquid crude product compound was purified by column chromatography over silica gel using hexane/dichloromethane and then dichloromethane/methanol (0–10%) as the eluents to afford product **13** (18 mg, 27.6% yield from **4**). The product was further purified on a reverse phase HPLC using C18 column and gradient water/acetonitrile as mobile phase as described above. ^{1}H NMR (DMSO, 500 MHz, δ, ppm): 7.69 (d, *J* = 7.5 Hz, 1H, H-6), 6.09–6.12 (br s, 1H, H-1′), 5.81 (d, *J* = 7.5 Hz, 1H, H-5), 4.10–4.23 (m,

1H, H-2′), 3.97–4.05 (m, 1H, H-3′), 3.96–3.97 (m, 1H, H-4′), 3.66–3.70 (m, 2H, H-5′), 2.22–2.28 (m, 4H, 2 × CH_2CO), 1.50–1.57 (m, 4H, 2 × CH_2CH_2CO), 1.10–1.35 (br m, 40H, methylene protons), 0.92 (dt, J = 6.7 Hz, 6H, 2 × CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz, δ, ppm): 173.86 (COO), 166.23 (C-4), 156.76 (C-2), 143.13 (C-6), 93.21 (C-5), 87.35 (C-1′), 82.83 (C-4′), 77.15 (C-2′), 74.93 (C-3′), 62.09 (C-5′), 31.65 (CH_2COO), 29.19, 29.33, 29.27, 29.17, 29.05, 29.97, 29.89, 28.83, 24.81, 22.45 (methylene $-CH_2-$ carbons), 14.33 (CH_3); HR-MS (ESI-TOF) (m/z): calcd for $C_{37}H_{65}N_3O_7$: 663.4823; found, 664.5155 $[M + 1]^+$.

4.2.13. 1-[3′,5′-di-(O-tert-Butyldimethylsilyl)-2′-O-myristoyl-N-(4,4′-dimethoxytrityl)-β-D-arabinofuranosyl]cytosine (**14**)

Compound **3** (120 mg, 0.16 mmol) and DMAP (45 mg, 0.37 mmol) were dissolved in dry dichloromethane (15 mL) under nitrogen atmosphere. Myristoyl Chloride (60 mg, 0.24 mmol) diluted with dry DCM (5 mL) was added dropwise to the reaction mixture at room temperature. The mixture was stirred for 18 h under nitrogen atmosphere. After the completion of the reaction, additional DCM (30 mL) was added to the reaction mixture, which was washed with 0.1% HCl (20 mL × 2) to remove DMAP and then with water (20 mL × 1). The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. After removal of dichloromethane, product was directly carried forward to the next deprotection reaction. HR-MS (ESI-TOF) (m/z): calcd for $C_{56}H_{85}N_3O_8Si_2$: 983.5875; found, 984.6040 $[M + 1]^+$.

4.2.14. 1-[3′-O-(tert-Butyldimethylsilyl)-2′-O-tetradecanoyl-β-D-arabinofuranosyl]cytosine (**16**)

DMTr group was removed from compound **14** by adding acetic acid (20 mL) and stirring at room temperature for 3 h. Evaporation of acetic acid under reduced pressure gave crude product **16**, which was directly carried further to the next reaction without further purification. HR-MS (ESI-TOF) (m/z): calcd for $C_{29}H_{53}N_3O_6Si$: 567.3704; found, 568.3944 $[M + 1]^+$.

4.2.15. 1-[2′-O-Tetradecanoyl-β-D-arabinofuranosyl]cytosine (**18**)

Crude compound **16** was treated with TBAF (1 M solution in THF, 20 mL). The reaction mixture was stirred for 8 h at room temperature under nitrogen atmosphere. After completion of the reaction, THF was removed under reduced pressure and the liquid crude product was purified by column chromatography over silica gel using hexane/dichloromethane and then dichloromethane/methanol (0–20%) as eluents to afford white solid product **18**. The product was further purified on reverse phase HPLC using C18 column and gradient water/methanol as mobile phase as described above. Overall yield obtained from **3** (22 mg, 31.4%). ^{1}H NMR (CD_3OD, 500 MHz, δ, ppm): 7.68 (d, J = 10 Hz, 1H, H-6), 6.16 (d, J = 5 Hz, 1H, H-1′), 5.88 (d, J = 10 Hz, 1H, H-5), 4.22–4.25 (m, 1H, H-2′), 4.11–4.17 (m, 1H, H-3′), 4.01–4.07 (m, 1H, H-4′), 3.95–4.00 (m, 1H, H-5″), 3.75–3.80 (m, 1H, H-5′), 2.21 (t, J = 7.5 Hz, 2H, CH_2CO), 1.50–1.62 (m, 2H, CH_2CH_2CO), 1.24 (br m, 20H, methylene protons), 0.85 (t, J = 6 Hz, 3H, CH_3); ^{13}C NMR (CD_3OD, 125 MHz, δ, ppm): 173.81 (COO), 166.22 (C-4), 156.76 (C-2), 143.12 (C-6), 93.21 (C-5), 87.36 (C-1′), 82.83 (C-4′), 77.15 (C-2′), 74.91 (C-3′), 64.30 (C-5′), 34.01 (CH_2COO), 31.65, 29.36, 29.33, 29.27, 29.17, 29.05, 29.97, 29.89, 28.71, 24.87, 22.31 (methylene $-CH_2-$ carbons), 13.00 (CH_3); HR-MS (ESI-TOF) (m/z): calcd for $C_{23}H_{39}N_3O_6$: 453.2839; found, 454.2857 $[M + 1]^+$.

4.2.16. 1-[3′,5′-Di(O-tert-butyldimethylsilyl)-2′-O-(12-thioethyldodecanoyl)-N-(4,4′-dimethoxytrityl)-β-D-arabinofuranosyl]cytosine (**15**)

12-Thioethyldodecanoic acid (70 mg, 0.24 mmol) was added to oxalyl chloride (20 mL, 210.4 mmol). The reaction mixture was stirred for 2 h at room temperature and then at 60 °C for 4 h. Oxalyl chloride was removed under vacuum. Dry DCM (20 mL) was added and evaporated under vacuum again to remove traces of oxalyl chloride. 12-Thioethyldodecanoyl chloride obtained was diluted with dry DCM (10 mL) and was added dropwise at room temperature to the mixture of compound **3** (120 mg, 0.16 mmol) and DMAP (50 mg, 0.41 mmol) dissolved in dry DCM. The reaction mixture was stirred for 7 h at room temperature. After the completion of the reaction, the crude product was washed with 0.1% HCl (2 × 20 mL) and then with water (2 × 20 mL). The organic layer was dried over sodium sulpfate and evaporated under reduced pressure. The crude product **15** thus was carried to the next deprotection reaction without further purification. HR-MS (ESI-TOF) (m/z): calcd for $C_{56}H_{85}N_3O_8SSi$: 1015.5596; found, 1016.6177 $[M + 1]^+$.

4.2.17. 1-[3′-O-(tert-Butyldimethylsilyl)-2′-O-(12-thioethyldodecanoyl)-β-D-arabinofuranosyl]cytosine (**17**)

Crude compound **17** was synthesized by a similar procedure described above for that of **16**. HR-MS (ESI-TOF) (m/z): calcd for $C_{29}H_{53}N_3O_6SSi$: 599.3424; found, 600.3065 $[M + 1]^+$.

4.2.18. 1-[2′-O-(12-Thioethyldodecanoyl)-β-D-arabinofuranosyl]cytosine (**19**)

Compound **19** was synthesized and purified by a similar procedure described above for that of **18**. ^{1}H NMR (CD_3OD, 500 MHz, δ, ppm): 7.82 (d, J = 7.6 Hz, 1H, H-6), 6.16 (d, J = 4.2 Hz, 1H, H-1′), 5.93 (d, J = 7.6 Hz, 1H, H-5), 4.22–4.27 (m, 1H, H-2′), 4.05–4.10 (m, J = 3.6 Hz, 1H, H-3′), 3.92–3.96 (m, 1H, H-4′), 3.75–3.87 (m, 2H, H-5′), 2.47–2.56 (m, 4H, CH_2SCH_2), 2.28 (t, J = 7.4 Hz, 2H, CH_2CO), 1.50–1.63 (m, 2H, m, 4H, SCH_2CH_2, CH_2CH_2CO), 1.36–1.41 (m, 2H, $CH_2CH_2CH_2$CO), 1.25–1.35 (br m, 12H, methylene protons), 1.23 (t, J = 7.4 Hz, 3H, CH_3); ^{13}C NMR (CD_3OD, 125 MHz, δ, ppm): 173.81 (COO), 166.21 (C-4), 156.77 (C-2), 143.14 (C-6), 93.90 (C-5), 86.66 (C-1′), 84.44 (C-4′), 76.31 (C-2′), 75.31 (C-3′), 61.10 (C-5′), 30.94 (CH_2COO), 29.11, 29.03, 29.00, 28.84, 28.73, 28.70, 28.31, 25.24, 24.75 (methylene $-CH_2-$ carbons), 13.86 (CH_3); HR-MS (ESI-TOF) (m/z): calcd for $C_{23}H_{39}N_3O_6S$: 485.2560; found, 486.2244 $[M + 1]^+$.

4.3. Cell culture

Human leukemia cell line CCRF-CEM was obtained from American Type Culture Collection (ATCC no. CCL-119). Cells were grown on 75 cm^2 cell culture flasks with RPMI-16 medium, supplemented with 10% Fetal bovine serum (FBS), and 1% penicillin–streptomycin solution (10,000 units of penicillin and 10 mg of streptomycin in 0.9% NaCl) in a humidified atmosphere of 5% CO_2, 95% air at 37 °C.

4.4. Cell proliferation assay

Cell proliferation assay of cytarabine derivatives synthesized was evaluated in CCRF-CEM cells, and was compared with that of cytarabine. Cell proliferation assay was carried out using CellTiter 96 aqueous one solution cell proliferation assay kit (Promega, USA). Briefly, CCRF-CEM cells were suspended at 5 × 10^5/mL and 100 μL of the cell suspension were placed in each well of the 96 well culture plate. Cells were incubated with cytarabine and fatty acyl derivatives (1 μM) in 4% DMSO and tests were in triplicates. Incubation was carried out at 37 °C in an incubator supplied with 5% CO_2 for 24–96 h. At the end of the sample exposure period (24–96 h), 20 μL CellTiter 96 aqueous solution was added. The plate was returned to the incubator for 1 h in a humidified atmosphere at 37 °C. The absorbance of the formazan product was measured at 490 nm using microplate reader. The percentage of cell survival was calculated as OD value of cells treated with test

compound – OD value of culture medium/(OD value of control cells – OD value of culture medium) × 100%.

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