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Research paper

Disulfide-incorporated lipid prodrugs of cidofovir: Synthesis, antiviral activity, and release mechanism

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ABSTRACT

The double-stranded DNA (dsDNA) viruses represented by adenovirus and monkeypox virus, have attracted widespread attention due to their high infectivity. In 2022, the global outbreak of mpox (or monkeypox) has led to the declaration of a Public Health Emergency of International Concern. However, to date therapeutics approved for dsDNA virus infections remain limited and there are still no available treatments for some of these diseases. The development of new therapies for treating dsDNA infection is in urgent need. In this study, we designed and synthesized a series of novel disulfide-incorporated lipid conjugates of cidofovir (**CDV**) as potential candidates against dsDNA viruses including vaccinia virus (VACV) and adenovirus (AdV) 5. The structure-activity relationship analyses revealed that the optimum linker moiety was C_2H_4 and the optimum aliphatic chain length was 18 or 20 atoms. Among the synthesized conjugates, **1c** exhibited more potency against VACV (IC_{50} = 0.0960 μM in Vero cells; IC_{50} = 0.0790 μM in A549 cells) and AdV5 (IC_{50} = 0.1572 μM in A549 cells) than brincidofovir (**BCV**). The transmission electron microscopy (TEM) images revealed that the conjugates could form micelles in phosphate buffer. The stability studies in the GSH environment demonstrated that the formation of micelles in phosphate buffer might protect the disulfide bond from glutathione (GSH) reduction. The dominant means of the synthetic conjugates to liberate the parent drug **CDV** was by enzymatic hydrolysis. Furthermore, the synthetic conjugates remained sufficiently stable in simulated gastric fluid (SGF), simulated intestinal fluid (SIF), and pooled human plasma, which indicated the possibility for oral administration. These results indicated **1c** may be a broad-spectrum antiviral candidate against dsDNA viruses with potential oral administration. Moreover, modification of the aliphatic chain attached to the nucleoside phosphonate group was involved as an efficient prodrug strategy for the development of potent antiviral candidates.

1. Introduction

Since May 2022, the outbreak of monkeypox (recently renamed mpox), a zoonotic viral disease, has been ongoing worldwide. Mpox has been declared a Public Health Emergency of International Concern (PHEIC) by the World Health Organization (WHO) on July 23, 2022 [1]. As of January 09, 2023, 84,415 laboratory confirmed cases in 110 countries and 76 deaths have been reported to WHO since January 01, 2022 [2]. Mpox is caused by the monkeypox virus (MPXV) which belongs to the orthopoxvirus genus of the family Poxviridae [3]. Variola virus (VARV), another member of the orthopoxvirus genus, is the causative agent of smallpox which is one of the most devastating human diseases resulting in 300 million to 500 million deaths in the 20th century alone [4]. While officially declared to be eliminated in 1980 by the WHO, smallpox remains to be a significant threat, especially under the circumstance of the loss of herd immunity in public [5]. VARV is considered a potential biological weapon candidate and is defined as a category A bioterrorism agent by US Centers for Disease Control and Prevention (CDC) [6]. MPVX, VARV, and other members of the orthopoxvirus genus are all double-stranded DNA (dsDNA) viruses. Some other dsDNA viruses, including adenovirus (AdV), cytomegalovirus (CMV), human herpesvirus (HHV), Epstein-Barr virus (EBV), and BK virus (BKV), are associated with severe and life-threatening viral infectious diseases in immunosuppressed patients who received allogeneic

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hematopoietic cell transplant (allo-HCT) or solid organ transplantation [7]. Multiple dsDNA viruses can be detected frequently in allo-HCT recipients resulting in a dose-response relationship with increased mortality [8]. For example, the mortality rate is as high as 80% for patients with disseminated disease caused by AdV [9]. The infectious diseases caused by dsDNA viruses have greatly threatened public health and attracted global attention. However, approved therapeutics for the treatment of the infectious diseases caused by dsDNA viruses are largely limited and there are even no treatment options available for some of these diseases. The development of novel broad-spectrum antiviral agents for treating dsDNA virus infections is urgently needed and significant to combat viral threats.

Cidofovir (**HPMPC**, **CDV**, Fig. 1), an acyclic nucleoside phosphonate (ANP), was originally described by De Clercq et al., in 1987 [10] and officially approved for the treatment of cytomegalovirus (CMV) retinitis in AIDS patients in 1996 [11]. CDV demonstrates broad-spectrum antiviral activity against the dsDNA viruses, including CMV, AdV, HHV, EBV, BKV, VARV, MPVX, and others [11,12]. Owing to the intrinsic phosphonate of **CDV**, the rate-limiting first phosphorylation step is bypassed and the unstable nature of the P–O bonds of phosphate groups is eliminated [11,13]. **CDV** enters the cell through a fluid endocytosis process and is converted to the active antiviral metabolite, cidofovir diphosphate (**CDV-PP**, Fig. 1) by intracellular kinases. By acting as chain terminators of the viral DNA synthesis catalyzed by viral DNA polymerase, **CDV-PPs** exert antiviral effects [11]. However, the negatively charged character of the phosphonate group under physiological pH results in poor cell membrane permeability and inadequate oral bioavailability, leading to the need for intravenous administration of **CDV** [11]. Moreover, as a substrate for the human organic anion transporter 1 (hOAT1), **CDV** demonstrates substantial nephrotoxicity, limiting the dosage and demanding co-administration with probenecid [14]. Thus, the broad clinical utility of **CDV** is limited. The prodrug strategy can be one of the robust alternatives to eliminate these drawbacks. Hostetler et al. designed and synthesized a series of alkoxyalkyl esters of **CDV** by disguising the phosphonate group as lysophosphatidylcholine (LPC) analogs [15–17]. The alkoxyalkyl ester modification results in enhanced cellular uptake and more efficient conversion to the active **CDV-PP** form by intracellular enzymes [18]. The ether-lipid-**CDV** conjugates display remarkable increases in antiviral activities, reductions in toxicity, and promises in oral bioavailability [18,19]. Among these conjugates, the hexadecyloxypropyl-cidofovir (**HDP-CDV**), also named brincidofovir (**BCV**) or **CMX001** (Fig. 1), has shown satisfying antiviral potency against dsDNA viruses and favorable pharmacokinetic and safety profile [20,21]. **BCV** has been evaluated in clinical trials for the treatment of AdV and BKV [22,23] and approved by the U.S. Food and Drug Administration (FDA) for the treatment of smallpox disease in all age groups in 2021 [24]. Thus, the alkoxyalkyl ester modification approach is demonstrated as a reliable prodrug strategy.

The disulfide bond (-S-S-) has been applied to various biological applications due to its attractive redox-responsive character [25,26]. Disulfide bonds are vulnerable to reductive agents, including glutathione (GSH), the most abundant antioxidant in cells and tissues. The GSH concentration varied drastically between intracellular (1–10 mM) and extracellular fluids (20–40 μM) [25]. Therefore, the disulfide bond can be employed as a brilliant tool to design prodrugs. By taking advantage of the disulfide bond characters and the alkoxyalkyl ester prodrug strategy, Liotta and collaborators designed and synthesized a series of disulfide-linked lipid tenofovir analogies that can permeate the biological membrane efficiently and release the nucleoside trigged by intracellular GSH rather than enzymes [27]. The compound **12c** (Fig. 1) exhibits a sub-nanomolar EC_{50} value of 0.5 nM to inhibit HIV-1 with a therapeutic index (TI) of 28,000 and is not susceptible to human plasma. In continuation of this work, they developed the next-generation lipid conjugates of tenofovir, in which the disulfide moiety varies in length and conformational flexibility of the spacer [28]. Compound **2b** (Fig. 1), the distance of the disulfide moiety is 4 carbon atoms from the phosphonic acid group, demonstrating potent antiviral activity against HIV-1 and HBV and with reduced cytotoxicity (TI > 100,000). Distinguished from the disulfide reduction induced release of **12c**, the enzymatic hydrolysis (phospholipase C and/or sphingomyelinase) was predominantly responsible for the activity and cleavage of **2b**. In a disclosed patent, Liotta and collaborators also claimed that the lipid disulfide prodrug strategy could be applied to design other nucleoside or nucleobase prodrugs [29]. However, the preparations, characterizations, and biological activities of the disclosed compounds other than tenofovir derivatives are not described in this patent. The disulfide lipid strategy shows great promise to develop novel phosphonate nucleoside antiviral prodrugs.

With the aim to obtain more efficient and safer oral bioavailable antiviral agents against dsDNA viruses, we herein applied the disulfide lipid prodrug strategy to design and synthesize CDV-conjugated derivates (Fig. 2). The antiviral activities of all the title compounds against dsDNA viruses including vaccinia virus (VACV) and AdV5 are evaluated. Several of the conjugates demonstrate more potent antiviral activities

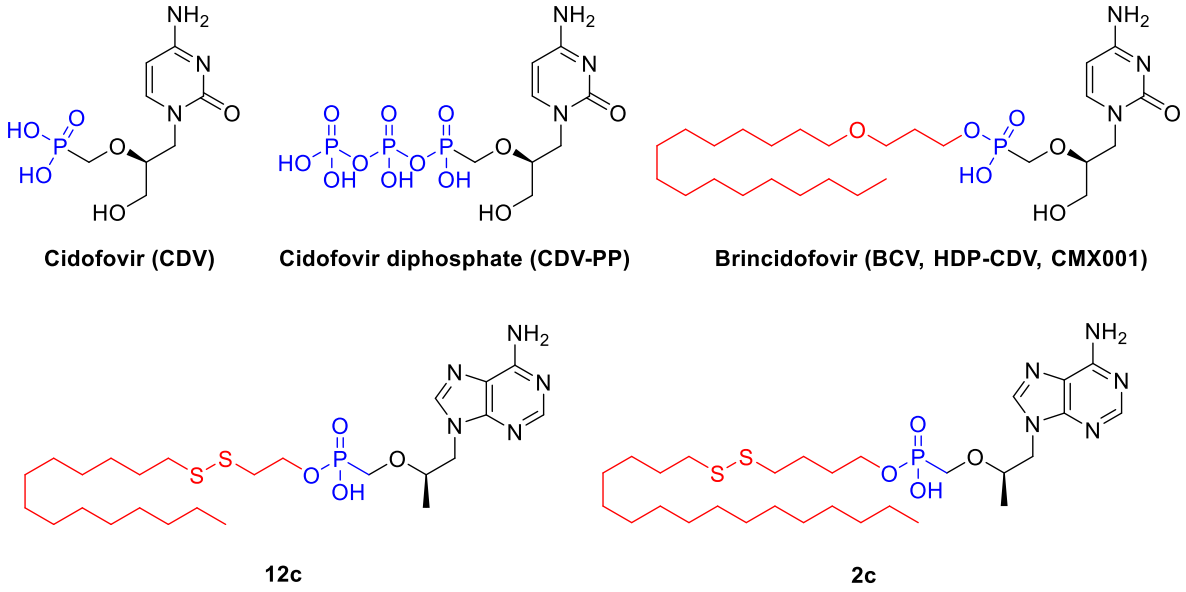


Fig. 1. Chemical structures of cidofovir, cidofovir diphosphate, brincidofovir, and the reported tenofovir prodrug **12c** [27] and **2c** [28].

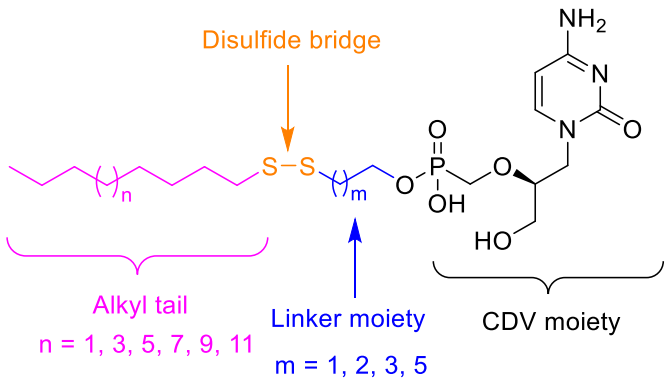


Fig. 2. Design of disulfide-incorporated lipid prodrugs of **CDV**.

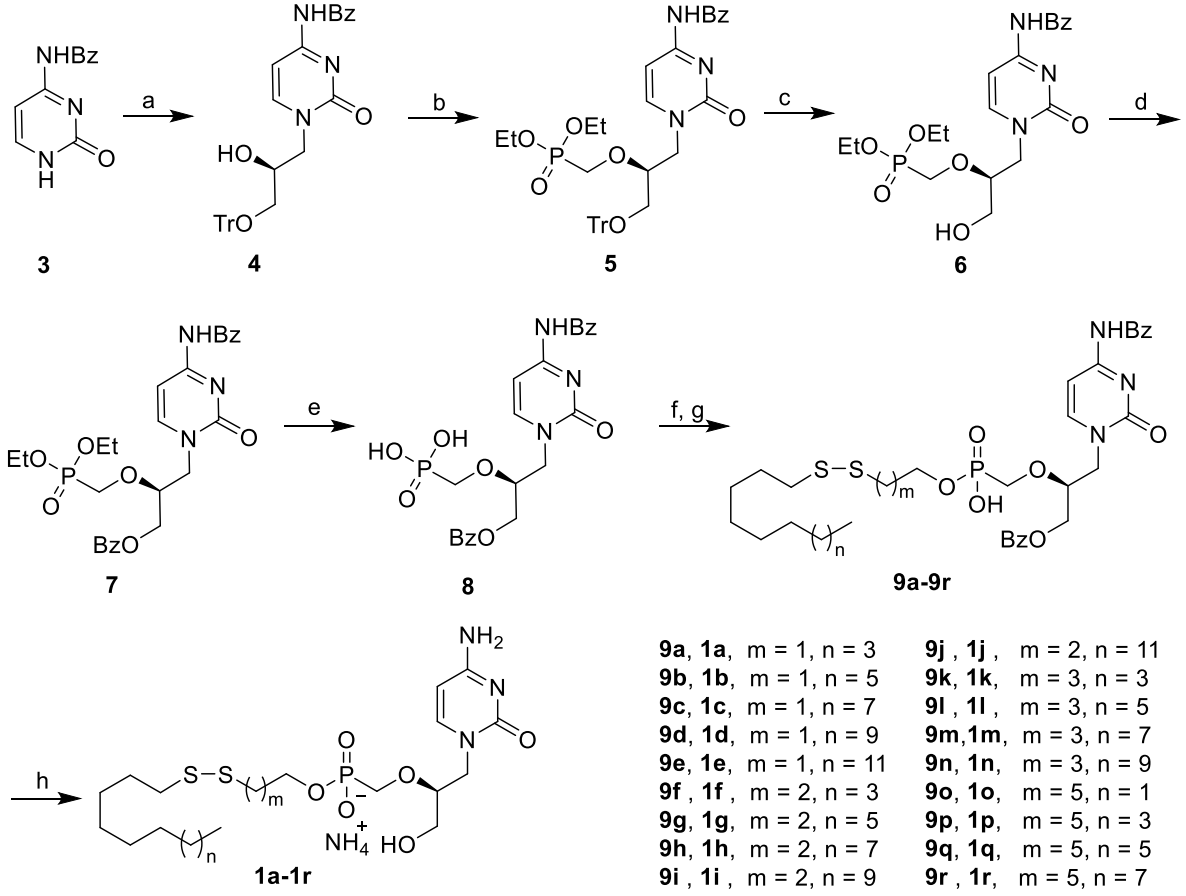
and higher TI values. Additionally, the mechanism of prodrug release is studied by treating the conjugates with GSH. Furthermore, the stability study in biorelevant media suggests that the disulfide-incorporated lipid prodrugs of CDV could be potential oral bioavailable antiviral candidates.

2. Results and discussion

2.1. Design and synthesis

As illustrated by Liotta and co-workers, the linker length between the disulfide moiety and the phosphonate moiety is not only associated with antiviral activity and cytotoxicity but also decisive for the mechanism of drug release [27,28]. Therefore, in this study, varying linkers with 2, 3, 4, or 6 carbon atoms along with a disulfide bridge were employed to conjugate the **CDV** with different alkyl tails. To identify the optimal chain length, the disulfide-incorporated aliphatic chains were designed to range from 14 to 23 atoms when all parts are taken into account. **BCV** and other alkyloxyalkyl ester ANPs are modified by conjugating a single lipid tail to the phosphonate group by mimicking lysophospholipids which are inverted cone-shaped [18]. This structural feature enables the analogies to disturb lipid bilayers, facilitating more efficient diffusion. By contrast, if the phosphonate group is masked by attaching two lipid tails that resemble cylinder-shaped diacyl phospholipids, the translocation tendency from the outer to the inner leaflet of the plasma membranes will be limited to a large extent [18]. These regularities were further demonstrated in Liotta's work [27]. In light of these findings, only a single aliphatic tail was attached to the phosphonate group resulting in an anionic phosphonic acid moiety exposed in this study (Fig. 2).

Compounds **1a-r** were synthesized as outlined in Scheme 1. Firstly, the disulfides **2a-r** were prepared according to a previously reported procedure in similar yields (Scheme S1 in the Supporting Information). [27,28]. Briefly, selected alkyl thiols (C_8–C_{18}) were oxidized with 2-mercapto-1-ethanol, 3-mercapto-1-propanol, 4-mercapto-1-butanol, or 6-mercapto-1-hexanol respectively in the presence of iodine to afford the disulfides **2a-r** in 30–48% yields. All the disulfide analogies were purified by silica gel column chromatography except for **2e** which was purified by crystallization. Then, intermediate **6** was obtained referring to the procedure to synthesize **CDV** [30]. Briefly, the commercially available materials *N*4-benzoylcytosine (**3**) and (*S*)-2-((trityloxy) methyl)oxirane were condensed, followed by coupling with



Scheme 1. Reagents and conditions: (a) (*S*)-(−)-trityl glycidyl ether, NaH, DMF, r. t. to 110 °C; (b) diethyl (tosyloxy) methylphosphonate, NaH, DMF, 0 °C; (c) 5% hydrogen chloride–methanol, CH_2Cl_2, 0 °C to r. t., 58% in three steps; (d) benzoyl cyanide, Et_3N, CH_3CN, r. t., 78%; (e) Me_3SiBr, CH_2Cl_2, r. t., 95%; (f) $(COCl)_2$, DMF, CH_2Cl_2, r. t.; (g) **2a-r**, pyridine, CH_2Cl_2, N_2, 0 °C, 46–59% in two steps; (h) NH_3 (7 M in MeOH), r. t., 40–60%.

(diethoxyphosphoryl)methyl 4-methylbenzenesulfonate to afford **5**. Acid-mediated deprotection of triphenylmethyl group afforded **6** in 58% yield in 3 steps, which was corresponding to the reported yield (56%) [30]. All the intermediates were used without further chromatography purification for the following reaction. The primary hydroxyl of **6** was further protected with acid-insensitive benzoyl moiety by condensation with benzoyl cyanide to produce **7** in 78% yield. Deprotection of the phosphonate esters of **7** with bromotrimethylsilane (TMSBr) gave the key intermediate **8**, which could crystalize in dichloromethane and be separated by filtration in a yield of 95%. The key intermediate **8** was chloride acetylated with excess oxalyl chloride to convert to the bis-chloridate followed by coupling with equivalent **2a-r** subsequently to afford monoesters **9a**−**r** in moderate yields (46–59%). **9a**−**r** were treated with a solution of ammonia in methanol (7.0 M) and purified on silica gel using DCM: MeOH: NH_4OH as eluent to afford the title compounds as their ammonium salts in 40–60% yields. All intermediates and title compounds were fully characterized by HRMS, ^{1}H NMR, and ^{13}C NMR. The compounds constituted with phosphorus atoms were further characterized by ^{31}P NMR. The analytical high-performance liquid chromatography (HPLC) results indicated the >95% purity of all the title conjugates.

2.2. In vitro antiviral activity and cytotoxicity evaluation

The orthopoxviruses share the conserved core viral function. VACV, the prototypical orthopoxvirus, which can be handled in Biosafety Level 2 facilities, has been widely used to screen novel antiviral compounds against orthopoxviruses including VARV and MAPV [31–34]. In this study, the VACV Tiantan strain (TT) was employed as the representative to evaluate the antiviral activity of the synthetic conjugates (**1a-r**) against orthopoxvirus. The antiviral activity and cytotoxicity assays for all title compounds were conducted both in Vero cells and A549 cells. **BCV** was used as a reference in both antiviral activity and cytotoxicity assays. The results are detailed in Table 1 and Fig. 3. Generally, the novel conjugates displayed comparable antiviral activity in both cell lines and moderately reduced cytotoxicity in A549 cells compared to Vero cells. To determine the correlation of the aliphatic chain length with the antiviral activity and cytotoxicity, we compared the cytotoxicity and antiviral activity of compounds **1a-e** featured with the same C_2H_4 linker. Both the cytotoxicity and antiviral activity of the compounds **1a-d** increased corresponding to the extended alkyl chain length from decyl to hexadecyl, while compound **1e**, which was installed with octadecyl, was almost equipotent to compound **1d**. This cytotoxicity tendency was corresponding to the phenomenon that increasing chain length was associated with a concomitant increase in cytotoxicity for a variety of surfactants [35]. Among **1a-e**, the most potent compounds were **1c** (IC_{50} = 0.0960 μM in Vero cells, Fig. 3A, and IC_{50} = 0.0790 μM in A549 cells, Figs. 3C) and **1d** (IC_{50} = 0.0684 μM in Vero cells, Fig. 3A, and IC_{50} = 0.0904 μM in A549 cells, Fig. 3C), of which the total chain lengths were 18 and 20 atoms respectively. Compounds **1f-j**, **1k-n**, or **1o-r** with the C_3H_6, C_4H_8, and C_6H_{12} linker respectively demonstrated comparable tendencies. Compounds **1a**, **1f**, **1k**, and **1o**, in which the total chain length is 14∼16 atoms, showed 10- to 250-fold less potent than **BCV**. We also noted that if the conjugates were featured with C_4H_6 or C_6H_{12} linkers, the conjugates with 20 atoms chain length (**1m**, **1q**) exhibited more potent antiviral activity than the conjugates with 18 atoms chain length (**1l**, **1p**). The observed dependence of antiviral activity on chain length was previously detailed by Kern's [36] and Hostetler's [37] work which illustrated the optimum chain length of the alkoxyalkyl modified **CDV** prodrugs was 20 atoms, and shorter or longer chain length resulted in markedly reduced antiviral activity. Thus, we concluded that the chain length played a vital role in the antiviral activities of the disulfide-incorporated lipid prodrugs of **CDV**, of which the ideal chain length was 18 or 20 atoms for potent antiviral activities. With respect to selectivity, however, compound **1c** (CC_{50} = 6.530 μM in Vero cells, Fig. 3B, and CC_{50} = 14.73 μM in A549 cells, Fig. 3D) with 18 atoms chain length was less toxic relative to compound **1d** (CC_{50} = 2.578 μM in Vero cells, Fig. 3B, and CC_{50} = 8.689 μM in A549 cells, Fig. 3D) with 20 atoms chain length, which contributed that the TI of compound **1c** was 2.8-fold less toxic in A549 cells (TI = 186.5 vs 66.99) and comparable in Vero cells (TI = 68.02 vs 70.66) relative to **BCV**.

We also noted that the linker moiety was an important determinant of antiviral activity. The compounds **1a-e** with C_2H_4 linker moiety between the phosphonate moiety and disulfide bridge displayed enhanced antiviral activity relative to the compounds with C_3H_6, C_4H_8, and C_6H_{12} linkers. For example, compounds **1c** demonstrated a 2.8- to 7.6-fold boost in potency relative to **1h** (IC_{50} = 0.2826 μM in Vero cells, and IC_{50} = 0.2233 μM in A549 cells), **1m** (IC_{50} = 0.5165 μM in Vero cells,

Table 1
In vitro activity against VACV and cytotoxicity of conjugates **1a-r**.

Compd.	M	n	chain length[a]	Vero cells			A549 cells		
				IC_{50}[b] (μM)	CC_{50}[c] (μM)	TI[d]	IC_{50}[b] (μM)	CC_{50}[c] (μM)	TI[d]
BCV	–	–	20	0.1963	13.87	70.66	0.2996	20.07	66.99
1a	1	3	14	2.490	81.33	32.66	6.272	65.16	10.39
1b	1	5	16	0.3654	14.31	39.16	0.2174	46.98	216.1
1c	1	7	18	0.0960	6.530	68.02	0.0790	14.73	186.5
1d	1	9	20	0.0684	2.578	37.69	0.0904	8.689	96.12
1e	1	11	22	0.1410	4.903	34.77	0.1035	14.11	136.3
1f	2	3	15	31.49	181.7	5.77	21.96	129.8	5.91
1g	2	5	17	6.535	33.29	5.09	2.020	47.55	23.54
1h	2	7	19	0.2826	13.94	49.33	0.2233	50.74	227.2
1i	2	9	21	0.2737	12.67	46.29	0.4007	46.69	116.5
1j	2	11	23	0.4356	9.870	22.66	0.7243	32.31	44.61
1k	3	3	16	40.64	95.83	2.36	45.66	161.9	3.546
1l	3	5	18	0.8611	34.96	40.60	1.050	80.62	76.78
1m	3	7	20	0.5165	18.10	35.04	0.5991	44.34	74.01
1n	3	9	22	0.3042	12.48	41.03	1.648	31.04	18.83
1o	5	1	16	49.34	> 200	4.054	>50	> 200	>4
1p	5	3	18	6.274	115.4	18.39	4.286	193.8	45.22
1q	5	5	20	0.8539	15.21	17.81	0.8375	134.3	160.4
1r	5	7	22	0.5492	8.561	15.59	0.5927	80.80	136.3

[a] The number of atoms (H is not counted) of the aliphatic chain attached to the phosphonate group of **CDV**.
[b] IC_{50}: half-maximal inhibitory concentration. The samples were examined in triplicate. Vero or A549 cells were incubated with test compounds and VACV.
[c] CC_{50}: half-maximal cytotoxic concentration. The samples were examined in triplicate. Values indicated as > 200 μM mean that 50% cytotoxicity was not reached at the highest concentration tested.
[d] TI: therapeutic index (TI = CC_{50}/IC_{50}).

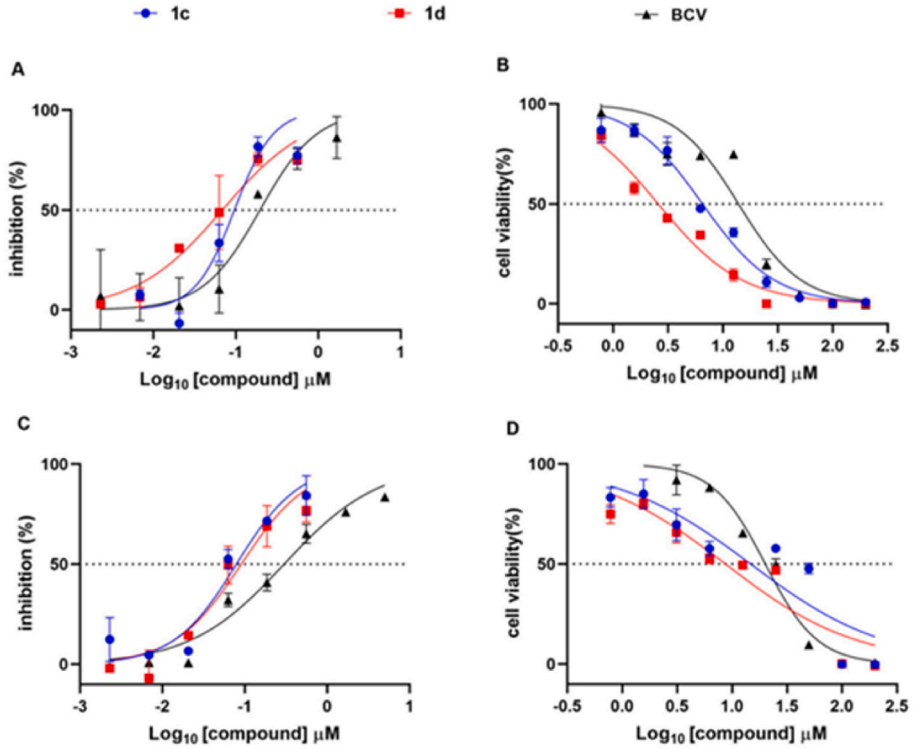


Fig. 3. The in vitro anti-VACV activity and cytotoxicity of **1c**, **1d**, and **BCV**. (A) Antiviral activity in Vero cells. (B) Cytotoxicity in Vero cells. (C) Antiviral activity in A549 cells. (D) Cytotoxicity in A549 cells. Both antiviral and cytotoxicity assays were determined by the neutral red uptake and each experiment was performed in triplicated.

and IC_{50} = 0.5991 μM in A549 cells), and **1r** (IC_{50} = 0.5492 μM in Vero cells, and IC_{50} = 0.5927 μM in A549 cells) featuring the same alkyl tail (tetradecyl). Additionally, compounds **1c** demonstrated a 9.0- to 65.4-fold increase in activity compared to its isomers **1l** (IC_{50} = 0.8611 μM in Vero cells, and IC_{50} = 1.050 μM in A549 cells) and **1p** (IC_{50} = 6.274 μM in Vero cells, and IC_{50} = 4.286 μM in A549 cells) which featured the same full chain length (18 atoms). These data suggest that C_2H_4 tended to be an optimum linker moiety of the disulfide-incorporated lipid prodrugs of **CDV** for activity against VACV.

As is **BCV** a broad-spectrum antiviral agent against pathogenic human dsDNA viruses, we hypothesize the designed disulfide-incorporated lipid prodrugs of **CDV** should have broad-spectrum antiviral activity as well. To verify the broad-spectrum antiviral potential, the synthetic **CDV** prodrugs were evaluated in vitro for anti-AdV5 activity in A549 cells. The results are outlined in Table 2. In general, the synthetic **CDV** prodrugs displayed similar regularities in activity against AdV5 relative to against VACV. As anticipated, conjugates **1c** and **1d** exhibited relatively potent anti-AdV5 activity while conjugate **1c** showed a broader TI relative to **1d**. To our surprise, conjugate **1n** (IC_{50} = 0.1337 μM) demonstrated 2-fold more potent than **BCV** (IC_{50} = 0.2453 μM) with reduced cytotoxicity and a broader TI value of 232.2. And an 8.9-fold increase in TI value was observed of compound **1q** (TI = 724.8) compared to **BCV** (TI = 81.82), in which two compounds showed comparable potent activity. Noting the linker of **1n** and **1q** were C_4H_8 and C_6H_{12} respectively, we inferred that the linker moiety of the synthetic conjugates also plays an important role in the antiviral activity against AdV5. These results suggested that the linker and the lipid chain length should be carefully studied in the development of lipid prodrug of the nucleoside phosphonates.

Table 2
In vitro activity against AdV5 and cytotoxicity of conjugates **1a-r**.

Compd.	m	N	chain length[a]	IC_{50}[b] (μM)	CC_{50}[c] (μM)	TI[d]
BCV	–	–	20	0.2453	20.07	81.82
1a	1	3	14	12.52	65.16	5.204
1b	1	5	16	0.7951	46.98	59.09
1c	1	7	18	0.1572	14.73	93.70
1d	1	9	20	0.1573	8.689	55.24
1e	1	11	22	0.1931	14.11	73.07
1f	2	3	15	–	129.8	–
1g	2	5	17	1.261	47.55	37.71
1h	2	7	19	0.5479	50.74	92.61
1i	2	9	21	0.2997	46.69	155.8
1j	2	11	23	1.865	32.31	17.32
1k	3	3	16	–	161.9	–
1l	3	5	18	0.2545	80.62	316.8
1m	3	7	20	0.3844	44.34	115.3
1n	3	9	22	0.1337	31.04	232.2
1o	5	1	16	–	> 200	–
1p	5	3	18	2.309	193.8	83.93
1q	5	5	20	0.1853	134.3	724.8
1r	5	7	22	0.2736	80.80	295.3

[a] The number of atoms (H is not counted) of the aliphatic chain attached to the phosphonate group of **CDV**.
[b] IC_{50}: half-maximal inhibitory concentration. The samples were examined in triplicate. A549 cells were incubated with test compounds and AdV5.
[c] CC_{50}: half-maximal cytotoxic concentration. The samples were examined in triplicate. Values indicated as > 200 μM mean that 50% cytotoxicity was not reached at the highest concentration tested.
[d] TI: therapeutic index (TI = CC_{50}/IC_{50}).

2.3. The drug release mechanism study

As is known that the disulfide bond is biodegradable in the presence of reductive agents. On the other hand, the lipid prodrug of **CDV** could form micelles in solution [38]. The micelles could hinder the disulfide bond from approaching GSH and other reductive agents [28,39]. Thus, we wondered whether it was the reduction of the disulfide bond or enzymatic hydrolysis of the phosphonate group of the synthetic conjugates that governed the liberation of the parent drug **CDV**. Liotta and

co-workers investigated the mechanism of the disulfide-incorporated lipid conjugates of tenofovir **(TFV)** and concluded that enzymatic hydrolysis played a vital role in liberating **TFV**, and the disulfide reduction mechanism worded only when 3-*exo-tet* intramolecular cyclization could be produced [28]. However, due to the poor aqueous solubility, the **TFV** conjugates failed to be evaluated directly and the corresponding thiol conjugates of **TFV** were employed. Furthermore, considering the identity of the parent nucleoside plays a vital role in lipid prodrug cleavage, the release mechanism of the synthetic lipid CDV conjugates in this work was studied. To verify the formation of micelles of the synthetic conjugates, transmission electron microscopy (TEM) experiments were conducted. As shown in Fig. 4, at a concentration of 50.0 μM, the conjugates with varied alkyl tails (**1c**, **1d**, **1e**) and varied linkers (**1e**, **1n**, and **1r**) could form micelles in pH 7.4 phosphate buffer. The TEM images also showed that the micelles were nanospherical in morphology with good dispersion. Then, the stability of the synthetic lipid **CDV** conjugates in the GSH environment was studied to probe the release mechanism in our study. Thanks to the satisfying solubility in phosphate buffer [40], the synthetic lipid **CDV** conjugates were enabled to be treated with GSH and monitor the stability by the HPLC method to probe the release mechanism. As shown in Fig. 5A, when incubated with 20 mM GSH in pH 7.4 phosphate buffer at 37 °C, conjugate **1c** with a C_2H_4 linker moiety, which could perform the 3-*exo-tet* cyclization post reduction, was approximately 13% remaining after 360 min. In contrast, conjugates **1h**, **1m**, and **1r** featured the same lipid tail ($C_{14}H_{29}$) exhibited significant enhancement in stability with the expansion of the linker length (C_3H_6, C_4H_8, C_6H_{12} respectively). Additionally, conjugates **1d** and **1e**, which featured the same linker (C_2H_4) with **1c**, exhibited significant enhancement in stability. **1e** (93% remaining, Fig. 5A) with a longer lipid tail was more stable than **1d** (81% remaining, Fig. 5A). And the TEM image showed that the micelles of **1e** could keep stable after incubated for 4 h in GSH environment (Fig. 4F). Furthermore, The CMC of conjugates **1c**, **1d**, **1e**, and **1m** were measured by colorimetric method [38] (Fig. S1 in the Supporting Information). As shown in Fig. 5B, **1c**, **1d**, and **1e**, the CMC values decreased as the expansion of the alkyl tail length. Conjugates **1d** and **1m**, which are isomers with the same chain length, had similar CMC values. They demonstrated similar stability in GSH environments as well (Fig. 5A). As demonstrated before, in a homologous series of n-alkyl surfactants, the increase in the length of the alkyl chain resulted in the increase of the log *P* and the decrease of the critical micellization concentration (CMC) [41–43]. The driving force of micellization is governed by hydrophobic effects partly [43,44]. Taken together, these results illustrated that the tendency to form micelles was predominantly responsible for the stability of the synthetic **CDV** conjugates. The longer hydrophobic chain resulted in a lower CMC value and could keep the disulfide bond away from GSH more efficiently.

As mentioned above, the conjugates featuring the C_2H_4 linker, such as **1c**, could perform the 3-*exo-tet* cyclization to liberate the parent drug **CDV** after disulfide reduction. Although the TEM image verified that **1c** could form micelles in buffer, **1c** showed poor stability in the GSH environment. Accordingly, we wondered whether these conjugates liberate **CDV** by enzymatic hydrolysis or by reductive cleavage. According to the reported literature [45], the level of GSH in cancer cells is several times higher than in normal cells. However, the observed anti-VACV activities of **1c** and **1d** in A549 cells were comparable to those in Vero cells. The distinction of GSH concentration appears not to be relevant to the antiviral activity, which exemplifies that the synthetic conjugates with C_2H_4 linker moiety are inclined to release the parent drug CDV by enzymatic hydrolysis in cells as well. Thus, we tentatively conclude that, like **BCV**, the predominating means to liberate **CDV** of the synthetic conjugates is enzymatic hydrolysis. We also declared that the detailed parent drug release mechanism of these synthetic conjugates should be elucidated by more experiments.

2.4. Chemical stability studies in biorelevant media

For oral administration, the synthetic conjugates must be stable in the gastrointestinal tract and plasma. Integrating the results of antiviral

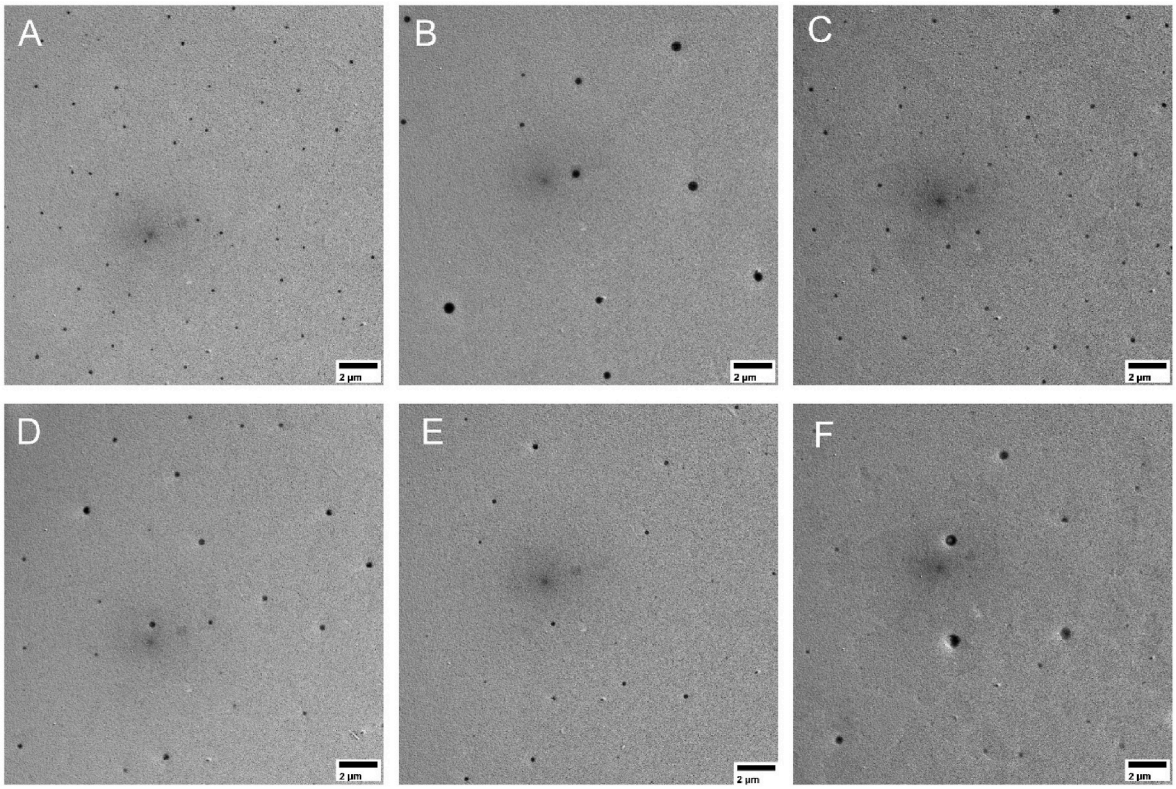
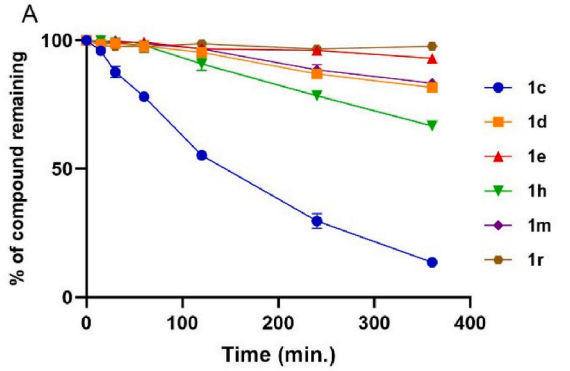


Fig. 4. The TME images of the synthetic conjugates. (A) **1c**. (B) **1d**. (C) **1e**. (D) **1n**. (E) **1r**. (F) **1e** incubated in GSH environment for 4 h.



B

Compd.	linker moiety	alkyl tail	chain length	CMC (μM)
1c	C_2H_4	$C_{14}H_{28}$	18	5.28
1d	C_2H_4	$C_{16}H_{32}$	20	3.93
1e	C_2H_4	$C_{18}H_{36}$	22	3.48
1m	C_4H_8	$C_{14}H_{28}$	20	3.87

Fig. 5. (A) Stability of compounds **1c**, **1d**, **1e**, **1h**, **1m**, and **1r** in GSH environment. (B). The CMC values of compounds **1c**, **1d**, **1e**, and **1m**.

activities, conjugates **1c**, **1d**, **1n**, and **1q** were selected as model compounds for chemical stability studies in biorelevant media. To identify whether these conjugates display pre-absorption degradation in the gastrointestinal tract, simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) containing the most relevant enzymes were used to evaluate the stability under conditions of gastric and intestinal digestion. As shown in Fig. 6A, conjugates **1n**, and **1q** were markedly stable in SGF which were not decomposed within 120 min at 37 °C. Conjugates **1c** and **1d** exhibited good stability in SGF with approximately 77% and 68% remaining respectively after 120 min. Conjugates **1c**, **1d**, **1n**, and **1q** were more stable than **BCV** (43% remaining). Upon incubation in SIF at 37 °C, all the tested compounds were less affected, with > 90% remaining after 360 min (Fig. 6B). Overall, the satisfied chemical stability indicated that the conjugates were relatively stable in the gastrointestinal tract and should be absorbed mainly in the non-degradable form by the small intestine.

The in vitro metabolic stability of conjugates is the foundation for exerting and maintaining their effectiveness. Therefore, the stabilities of the conjugates **1c**, **1d**, **1n**, and **1q** in pooled human plasma at 37 °C were determined. As indicated in Table 3, although the test conjugates were less stable than **BCV**, they exhibited favorable plasma stability, with a half-life value greater than 2 h, which was stable enough for oral dosing. We also noted that the chemical stability of conjugates in biorelevant media is in correlation with the length of the hydrophobic chain, which was corresponding to the stability of the conjugates in the GSH environment. Taken together, these stability properties in biorelevant media promise the potential of the synthetic conjugates to be developed as oral-dosing candidates.

3. Conclusion

In this study, a series of novel disulfide-incorporated lipid prodrugs of **CDV** with a varied length of linker and alkyl tail was rationally designed and synthesized. Several synthetic conjugates showed potent in vitro anti-VACV and anti-AdV5 activities. By structure-activity relationship analysis, we concluded that both the length of the aliphatic chain and the linker moiety played a vital role in the antiviral activities. The C_2H_4 linker and a chain length of 18–20 atoms (**1c** and **1d**) were demonstrated as ideal options for achieving potent antiviral activity. Especially, **1c** exhibited more potent antiviral activity against both VACV and AdV5 and a higher TI value than **BCV**. As a prodrug of **CDV**, we rationally inferred that **1c** promises broad-spectrum antiviral activities against orthopoxviruses including VARV and MPXV and other dsDNA viruses as well. Additionally, the conjugates demonstrate satisfied stability in SGF, SIF, and pooled human plasma, indicating the potential to be developed as candidates for oral administration. Furthermore, by systematic stability study of the parent drug release mechanism of the conjugates, we concluded that although the disulfide bond was susceptible to reductive agents, the disulfide-incorporated lipid prodrugs of **CDV** tend to form micelles in solution that can prevent the disulfide bond from reduction by GSH. Enzymatic hydrolysis was the predominant way for the disulfide-incorporated lipid conjugates to release the parent drug **CDV**, just like **BCV**. Considering the improved antiviral activity and TI value, optimization of the lipid chain attached to phosphonate or phosphate nucleoside analogue promises an efficient prodrug strategy to develop potent antiviral candidates. Further

Table 3
Stability of model compounds **1c**, **1d**, **1n**, and **1q** in pooled human plasma.

Compd.	m	n	chain length	$t_{1/2}$ (h)
BCV	–	–	20	> 24
1c	1	7	18	2.144
1d	1	9	20	3.237
1n	3	9	22	4.375
1q	5	5	20	4.450

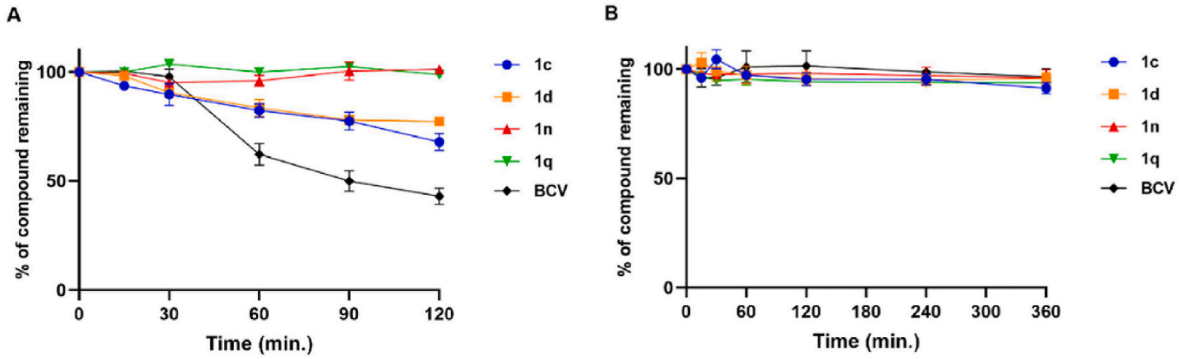


Fig. 6. Stability of model compounds **1c**, **1d**, **1n**, **1q**, and reference drug **BCV** in (A) SGF and (B) SIF.

development of novel series of **CDV** prodrugs by optimizing the lipid chain is in progress.

4. Experimental section

4.1. Chemistry

All reagents and solvents were purchased from commercial suppliers (Sigma-Aldrich, Adamas, Sinoreagent, Energy Chemical, 9dingchem) and used without further purification. Evaporation of the solvents was performed with a rotary evaporator (IKA, RV-8) under reduced pressure. All reactions were monitored by thin layer chromatography (TLC) with Huanghai GF254 glass silica gel plates Flash column chromatography was carried out using Biotage Selekt automated flash chromatography system with Biotage Rening columns or Biotage Sfär silica gel columns. The ^{1}H, ^{13}C, and ^{31}P NMR spectra of the synthetic compounds were recorded on a Bruker AVANCE 600 MHz NMR spectrometer in CD_3OD, $CDCl_3$, DMSO-d_6, or $CD_3OD/CDCl_3$ with tetramethylsilane (TMS) as the internal standard. Chemical shifts (δ) and coupling constant (J) values are expressed in parts per million (ppm) and hertz (Hz), with the following spectral pattern designations: s, singlet; d, doublet; t, triplet, q, quartet; m, multiplet; dd, doublet of doublets. High-resolution mass spectra (HRMS) were acquired on a Thermo Scientific Q Exactive instrument by electron spray ionization (ESI) ionization sources. Analysis of the sample purity was performed on an Agilent 1100 HPLC system with a photometric diode array (PDA) detector and a SunFire C18 column (4.6 mm × 250 mm × 5 μm, Waters). Detailed HPLC conditions and experiment methods are provided in Table S1. The purity of all target conjugates was > 95% (Table S1, Supporting Information).

4.1.1. Diethyl (S)-(((1-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-3-(trityloxy)propan-2-yl)oxy)methyl)phosphonate (5)

To a stirred solution of N^4-benzoylcytosine (25.80 g, 120.0 mmol) in anhydrous DMF (200 mL) was slowly added NaH (60% in mineral oil, 1.000 g, 25.00 mmol) at r. t. After the reaction mixture was heated to 110 °C, (*S*)-(−)-trityl glycidyl ether (31.70 g, 100.0 mmol) was added. The reaction mixture was stirred for an additional 5 h at this temperature to afford the crude intermediate **4** and was used directly for the next step.

After chilling to 0 °C, to the resulting reaction mixture was gradually added NaH (60% in mineral oil, 12.00 g, 300.0 mmol), followed by the addition of diethyl (tosyloxy) methylphosphonate (48.50 g, 150.0 mmol). After stirring vigorously at 0 °C for 3 h, the reaction mixture was diluted with EtOAc (500 mL) and quenched with ice water (500 mL). The organic layer was collected, washed with saturated aqueous $NaHCO_3$ and brine, and dried with anhydrous Na_2SO_4. The mixture was filtered and evaporated to afford the crude intermediate **5** as light-yellow oil. A small portion of crude intermediate **5** was purified on a silica column using a DCM/DCM: MeOH (80: 20) gradient (0−40%) for structure identification. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.76 (s, 1H), 7.90–7.89 (m, 2H), 7.74 (d, J = 7.2 Hz, 1H), 7.62–7.59 (m, 1H), 7.52–7.50 (m, 2H), 7.45–7.44 (m, 7H), 7.33–7.30 (m, 6H), 7.26–7.23 (m, 3H), 4.37 (dd, J = 3.6, 13.8 Hz, 1H), 4.12–4.06 (m, 4H), 3.96–3.93 (m, 2H), 3.81 (dd, J = 5.6, 13.8 Hz, 1H), 3.65 (dd, J = 9.6, 13.8 Hz, 1H), 3.42 (dd, J = 3.0, 10.2 Hz, 1H), 3.12 (dd, J = 4.2, 10.8 Hz, 1H), 1.31–1.28 (m, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 162.35, 150.80, 143.40, 133.20, 129.07, 128.59, 128.02, 127.55, 127.30, 96.15, 86.94, 78.62 (d, J = 11.1 Hz), 63.99 (d, J = 166.2 Hz), 62.45 (d, J = 6.5 Hz), 62.33 (d, J = 6.5 Hz), 62.20, 52.07, 16.54 (d, J = 2.3 Hz), 16.50 (d, J = 2.3 Hz); ^{31}P NMR (240 MHz, $CDCl_3$) δ 20.99. HRMS (ESI) *m/z*: calculated for $C_{38}H_{40}N_3O_7PNa$ $[M + Na]^+$ 704.2496, found 704.2477.

4.1.2. Diethyl (S)-(((1-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (6)

Crude **5** was dissolved in anhydrous DCM (200 mL) and MeOH (100 mL), followed by the addition of HCl (4.0 M in MeOH, 100 mL). After stirred at r. t. for 7 h, the reaction mixture was extracted with water (200 mL, 1 ×) and hydrochloric acid (2.5 M, 100 mL, 2 ×). The combined water phase was added $NaHCO_3$ to adjust the pH to 8 in an ice bath. After being transferred to a separatory funnel, the water phase was extracted with DCM (150 mL, 3 ×). The organic phase was combined, washed with brine (500 mL, 2 ×), dried over anhydrous Na_2SO_4, filtered, and evaporated to afford the crude **6** as pale yellow oil (25.30 g, 58% yield over 3 steps). A small portion of crude intermediate **5** was purified on a silica column using a DCM/DCM: MeOH (80: 20) gradient (0−50%) for structure identification. ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.20 (s, 1H), 7.94–7.93 (m, 2H), 7.84 (d, J = 7.2 Hz, 1H), 7.62–7.59 (m, 1H), 7.54–7.50 (m, 3H), 4.38 (s, 1H), 4.23 (dd, J = 3.6, 13.8 Hz, 1H), 4.18–4.10 (m, 4H), 4.05 (dd, J = 6.6, 13.8 Hz, 1H), 3.95–3.86 (m, 3H), 3.74–3.71 (m, 1H), 3.64–3.61 (m, 1H), 1.36–1.31 (m, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.96, 162.79, 156.55, 150.82, 133.16, 133.01, 128.96, 127.72, 96.63, 80.26 (d, J = 8.85 Hz), 64.03 (d, J = 166.9 Hz), 62.81 (d, J = 6.3 Hz), 62.62 (d, J = 6.6 Hz), 60.11, 51.04, 16.48 (t, J = 5.6 Hz); ^{31}P NMR (240 MHz, $CDCl_3$) δ 21.59. HRMS (ESI) *m/z*: calculated for $C_{19}H_{27}N_3O_7P$ $[M + H]^+$ 440.1581, found 440.1567, $C_{19}H_{26}N_3o_7PNa$ $[M + Na]^+$ 462.1401, found 462.1389.

4.1.3. (S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-((diethoxyphosphoryl)methoxy)propyl benzoate (7)

Crude **6** (25.30 g, 55.00 mmol) was dissolved in acetonitrile (500 mL), and to the mixture was added triethylamine (16.70 g, 165.0 mmol). After stirring for 30 min, benzoyl cyanide (10.82 g, 82.50 mmol) was added, and the mixture was stirred at r. t. for 4 h. After evaporated under reduced pressure to remove the volatile components, the resulting residue was purified on a silica column using a DCM/DCM: MeOH (80: 20) gradient (0−50%) to afford the intermediate **7** as pale yellow oil (23.40 g, 78% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.96 (s, 1H), 8.06–8.04 (m, 2H), 7.93–7.92 (m, 2H), 7.81 (d, J = 7.2 Hz, 1H), 7.63–7.45 (m, 7H), 4.70 (dd, J = 3.6, 12.0 Hz, 1H), 4.50 (dd, J = 3.0, 13.8 Hz, 1H), 4.38 (dd, J = 4.2, 12.0 Hz, 1H), 4.25–4.22 (m, 1H), 4.11–4.05 (m, 5H), 3.84 (dd, J = 9.6, 13.8 Hz,1H), 3.79 (dd, J = 8.4, 13.8 Hz, 1H), 1.29–1.26 (m, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.03, 162.66, 155.75, 150.55, 133.39, 133.18, 133.04, 129.70, 129.46, 129.00, 128.54, 127.65, 96.36, 77.52 (d, J = 10.8 Hz), 64.14 (d, J = 166.1 Hz), 62.95, 62.50 (d, J = 6.5 Hz), 62.38 (d, J = 6.2 Hz), 52.12, 16.44 (t, J = 5.6 Hz); ^{31}P NMR (240 MHz, $CDCl_3$) δ 20.49. HRMS (ESI) *m/z*: calculated for $C_{26}H_{31}N_3O_8P$ $[M + H]^+$ 544.1843, found 544.1814, $C_{26}H_{30}N_3o_8PNa$ $[M + Na]^+$ 566.1663, found 566.1639.

4.1.4. (S)-(((1-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-3-(benzoyloxy)propan-2-yl)oxy)methyl)-phosphonic acid (8)

Intermediate **7** (6.160 g, 11.00 mmol) was dissolved in anhydrous DCM (70 mL), and to this solution was added bromotrimethylsilane (6.740 g, 44.00 mmol). The mixture was stirred at r. t. for 5 h, and then the solvent and excess bromotrimethylsilane were evaporated under reduced pressure. The resulting residue was redissolved in DCM (100 mL) and washed with 5% aqueous sodium dihydrogen phosphate. The organic layer was collected and kept at r. t. The solid was crystallized from DCM and collected by filtration. The filter cake was washed with DCM (20 mL, 2 ×) and dried in vacuo to give the key intermediate **8** as a white solid (6.482 g, 95% yield). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.08 (d, J = 7.2 Hz, 1H), 7.95–7.93 (m, 4H), 7.61–7.55 (m, 2H), 7.48–7.45 (m, 4H), 7.23 (d, J = 7.2 Hz, 1H), 4.43 (dd, J = 4.2, 12.0 Hz, 1H), 4.24 (dd, J = 4.8, 12.0 Hz, 1H), 4.17 (dd, J = 4.2, 13.2 Hz, 1H), 4.08–4.98 (m, 2H), 3.71 (dd, J = 9.0, 13.2 Hz, 1H), 3.61 (dd, J = 9.6, 13.2 Hz, 1H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 167.96, 165.98, 163.54, 155.74, 151.75, 133.91, 133.71, 133.17, 129.88, 129.80, 129.17, 128.93, 128.87, 96.20, 77.22 (d, J = 11.3 Hz), 66.36 (d, J = 159.9 Hz), 64.38, 50.65; ^{31}P NMR (240 MHz, DMSO-d_6) δ 16.06. HRMS (ESI^+) *m/z*: calculated for $C_{22}H_{23}N_3O_8P$ $[M + H]^+$ 488.1217 found 488.1227, $C_{22}H_{22}N_3o_8PNa$ $[M + Na]^+$ 510.1037, found 510.1043.

4.1.5. General procedure to afford the intermediates 9a-r

To a stirring solution of key intermediate **8** (0.585 g, 1.20 mmol) in anhydrous DCM (8 mL) was gradually added excess oxalyl chloride (2.0 M in DCM, 2.5 mL, 4.800 mmol) followed by the addition of DMF (0.020 mL, 0.25 mmol). After the mixture was stirred at r. t. for an additional 2 h, the solvent and excess oxalyl chloride were removed under reduced pressure. The resulting residue was suspended in DCM (8 mL) under a nitrogen atmosphere and stirred at 0 °C. A mixture of intermediate **2a-r** (1.00 mmol) and pyridine (0.316 mg, 4.00 mmol) in anhydrous DCM was slowly added dropwise to the reaction mixture. The solution was stirred at 0 °C for 1 h and then naturally warmed to r. t. After stirring at r. t. for an additional 3 h, the reaction mixture was quenched with water and washed with aqueous HCl (0.2 M, 20 mL,2 ×) and brine (20 mL, 2 ×). The organic layer was collected, dried with anhydrous Na_2SO_4, filtered, concentrated, and purified on a silica column using a DCM/DCM: MeOH (80: 20) gradient (0−60%) to afford the intermediates **9a-r** in 46–59% yields.

4.1.6. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-(((2-(decyldisulfanyl)ethoxy)(hydroxy)phosphoryl)methoxy)propyl benzoate (9a)

9a was obtained as a white solid (0.384 g, 53% yield). ^{1}H NMR (600 MHz, $CD_3OD/CDCl_3$) δ 8.20 (d, J = 13.8 Hz, 1H), 8.09–7.99 (m, 4H), 7.65–7.48 (m, 6H), 4.62 (dd, J = 9.6, 45.6 Hz, 1H), 4.44–4.29 (m, 3H), 4.15–3.98 (m, 4H), 3.84–3.72 (m, 1H), 2.88–2.80 (m, 2H), 2.65–2.58 (m, 2H), 1.62–1.56 (m, 2H), 1.38–1.27 (m, 14H), 0.90 (t, J = 15.6 Hz, 3H); ^{13}C NMR (150 MHz, $CD_3OD/CDCl_3$) δ 134.07, 130.34, 129.41, 129.22, 128.90, 128.83, 39.33, 39.31, 32.60, 30.20 (d, J = 3.6 Hz), 29.99, 29.87, 29.69, 29.04, 23.28, 14.01; ^{31}P NMR (240 MHz, $CD_3OD/CDCl_3$) δ 19.71, 19.40. HRMS (ESI^+) m/z: calculated for $C_{34}H_{47}N_3O_8PS_2$ $[M + H]^+$ 720.2537, found 720.2529, $C_{34}H_{46}N_3O_8PS_2Na$ $[M + Na]^+$ 742.2356, found 742.2302.

4.1.7. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-(((2-(dodecyldisulfanyl)ethoxy)(hydroxy)phosphoryl)methoxy)propyl benzoate (9b)

9b was obtained as a white solid (0.363 g, 49% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.00–7.97 (m, 4H), 7.56–7.36 (m, 6H), 4.62–3.85 (m, 9H), 2.82–2.72 (m, 2H), 2.54–2.51 (m, 2H), 1.53–1.51 (m, 2H), 1.33–1.13 (m, 18H), 0.87 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.11, 133.26, 129.72, 129.57, 128.71, 128.51, 38.83, 31.93, 29.69, 29.66, 29.65, 29.58, 29.37, 29.28, 29.10, 28.55, 22.70, 14.14; ^{31}P NMR (240 MHz, $CDCl_3$) δ 15.62. HRMS (ESI^+) m/z: calculated for $C_{36}H_{51}N_3O_8PS_2$ $[M + H]^+$ 748.2850, found 748.2834, $C_{36}H_{50}N_3O_8PS_2Na$ $[M + Na]^+$ 770.2669, found 770.2652.

4.1.8. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-((hydroxy(2(tetradecyldisulfanyl)ethoxy)phosphoryl)methoxy)propyl benzoate (9c)

9c was obtained as a white solid (0.440 g, 57% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.06–7.94 (m, 4H), 7.55–7.36 (m, 6H), 4.59–3.86 (m, 9H), 2.83–2.71 (m, 2H), 2.54–2.52 (m, 2H), 1.53–1.51 (m, 2H), 1.41–1.21 (m, 22H), 0.88 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.11, 133.25, 129.72, 129.57, 128.72, 128.51, 63.41, 63.08, 38.84, 38.74, 31.94, 29.72, 29.70, 29.68, 29.66, 29.59, 29.40, 29.38, 29.29, 29.10, 28.56, 22.70, 14.14; ^{31}P NMR (240 MHz, $CDCl_3$) δ 15.73. HRMS (ESI^+) m/z: calculated for $C_{38}H_{55}N_3O_8PS_2$ $[M + H]^+$ 776.3163, found 776.3146, $C_{38}H_{54}N_3O_8PS_2Na$ $[M + Na]^+$ 798.2982, found 798.2965.

4.1.9. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-(((2(hexadecyldisulfanyl)ethoxy)(hydroxy)phosphoryl)methoxy)propyl benzoate (9d)

9d was obtained as a white solid (0.413 g, 51% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.94–7.91 (m, 4H), 7.47–7.33 (m, 6H), 4.52–3.78 (m, 9H), 2.75–2.71 (m, 2H), 2.50–2.47 (m, 2H), 1.50–1.45 (m, 2H), 1.26–1.14 (m, 26H), 0.81 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.09, 133.29, 129.73, 129.55, 128.74, 128.61, 128.53, 128.46, 127.44, 63.46, 63.06, 38.87, 31.94, 30.95, 29.73, 29.71, 29.68, 29.66, 29.59, 29.38, 29.30, 29.11, 28.56, 22.70, 14.14; ^{31}P NMR (240 MHz, $CDCl_3$) δ 16.17. HRMS (ESI^+) m/z: calculated for $C_{40}H_{59}N_3O_8PS_2$ $[M + H]^+$ 804.3476, found 804.3470, $C_{40}H_{58}N_3O_8PS_2Na$ $[M + Na]^+$ 826.3295, found 826.3289.

4.1.10. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-((hydroxy(2-(octadecyldisulfanyl)ethoxy)phosphoryl)methoxy)propyl benzoate (9e)

9e was obtained as a white solid (0.458 g, 55% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.00–7.97 (m, 4H), 7.55–7.39 (m, 6H), 4.59–3.85 (m, 9H), 2.80–2.73 (m, 2H), 2.54–2.51 (m, 2H), 1.55–1.50 (m, 2H), 1.31–1.20 (m, 30H), 0.88 (t, J = 7.2 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.11, 133.25, 129.72, 129.58, 128.70, 128.51, 63.41, 63.03, 38.82, 31.94, 29.74, 29.73, 29.68, 29.60, 29.38, 29.31, 29.10, 28.57, 22.70, 14.14; ^{31}P NMR (240 MHz, $CDCl_3$) δ 15.58. HRMS (ESI^+) m/z: calculated for $C_{42}H_{63}N_3O_8PS_2$ $[M + H]^+$ 832.3789, found 832.3787, $C_{42}H_{62}N_3O_8PS_2Na$ $[M + Na]^+$ 854.3608, found 854.3613.

4.1.11. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-(((2-(decyldisulfanyl)ethoxy)(hydroxy)phosphoryl)methoxy)propyl benzoate (9f)

9f was obtained as a white solid (0.368 g, 50% yield). ^{1}H NMR (600 MHz, $CD_3OD/CDCl_3$) δ 8.18 (d, J = 10.8 Hz, 1H), 8.04–7.87 (m, 4H), 7.64–7.47 (m, 6H), 4.62 (dd, J = 9.6, 45.6 Hz, 1H), 4.43–4.09 (m, 4H), 4.02–3.94 (m, 3H), 3.78–3.64 (m, 1H), 2.71–2.56 (m, 4H), 1.94–1.92 (m, 2H), 1.66–1.58 (m, 2H), 1.38–1.27 (m, 14H), 0.89 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, $CD_3OD/CDCl_3$) δ 167.10, 134.04, 133.78, 130.55, 129.42, 129.25, 129.21, 129.12, 98.00, 64.06, 64.00, 39.17, 39.15, 35.02, 32.62, 30.93, 30.22 (d, J = 2.9 Hz), 30.01, 29.90, 29.88, 29.86, 29.77, 29.74, 29.07, 29.05, 23.29, 14.06; ^{31}P NMR (240 MHz, $CD_3OD/CDCl_3$) δ 20.69, 20.08. HRMS (ESI^+) m/z: calculated for $C_{35}H_{49}N_3O_8PS_2$ $[M + H]^+$ 734.2693, found 734.2697, $C_{35}H_{48}N_3O_8PS_2Na$ $[M + Na]^+$ 756.2513, found 756.2516.

4.1.12. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-(((3-(dodecyldisulfanyl)propoxy)(hydroxy)phosphoryl)methoxy)propyl benzoate (9g)

9g was obtained as a white solid (0.383 g, 50% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.99–7.87 (m, 4H), 7.54–7.41 (m, 6H), 4.60–3.86 (m, 9H), 2.60–2.53 (m, 4H), 1.90–1.82 (m, 2H), 1.56–1.53 (m, 2H), 1.34–1.23 (m, 18H), 0.87 (t, J = 7.2 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.11, 133.31, 129.72, 129.54, 128.74, 128.54, 38.81, 34.43, 31.93, 29.69, 29.66, 29.64, 29.58, 29.37, 29.29, 29.18, 28.57, 22.70, 14.14; ^{31}P NMR (240 MHz, $CDCl_3$) δ 15.97. HRMS (ESI^+) m/z: calculated for $C_{37}H_{53}N_3O_8PS_2$ $[M + H]^+$ 762.3006, found 762.3000, $C_{37}H_{52}N_3O_8PS_2Na$ $[M + Na]^+$ 784.2826, found 784.2820.

4.1.13. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-((hydroxy(3-(tetradecyldisulfanyl)propoxy)phosphoryl)methoxy)propyl benzoate (9h)

9h was obtained as a white solid (0.450 g, 57% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.26–7.96 (m, 4H), 7.59–7.39 (m, 6H), 4.65–3.69 (m, 9H), 2.69–2.48 (m, 4H), 2.05–1.20 (m, 26H), 0.88 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.01, 133.46, 129.74, 129.71, 128.62, 128.51, 38.89, 34.12, 31.94, 29.74, 29.72, 29.69, 29.68, 29.65, 29.62, 29.58, 29.38, 29.33, 29.30, 29.23, 29.17, 28.59, 22.71, 14.15; ^{31}P NMR (240 MHz, $CDCl_3$) δ 20.73. HRMS (ESI^+) m/z: calculated for $C_{39}H_{57}N_3O_8PS_2$ $[M + H]^+$ 790.3319, found 790.3305, $C_{39}H_{56}N_3O_8PS_2Na$ $[M + Na]^+$ 812.3139, found 812.3127.

4.1.14. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-(((3-(hexadecyldisulfanyl)propoxy)(hy-droxy)phosphoryl)methoxy)propyl benzoate (9i)

9i was obtained as a white solid (0.460 g, 56% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.00–7.86 (m, 4H), 7.55–7.38 (m, 6H), 4.83–3.85 (m, 9H), 2.64–2.51 (m, 4H), 1.85–1.22 (m, 30H), 0.87 (t, J = 7.2 Hz, 3H);

^{13}C NMR (150 MHz, $CDCl_3$) δ 166.12, 133.28, 129.71, 129.55, 128.72, 128.52, 63.48, 62.99, 38.78, 34.39, 31.94, 30.00, 29.73, 29.71, 29.68, 29.60, 29.38, 29.31, 29.18, 28.58, 22.71, 14.14; ^{31}P NMR (240 MHz, $CDCl_3$) δ 15.76. HRMS (ESI^+) *m/z*: calculated for $C_{41}H_{61}N_3O_8PS_2$ $[M + H]^+$ 818.3632, found 818.3605, $C_{41}H_{60}N_3O_8PS_2Na$ $[M + Na]^+$ 840.3452, found 840.3420.

4.1.15. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-((hydroxy(3-(octadecyldisulfanyl)pro-poxy)phosphoryl)methoxy)propyl benzoate (9j)

9j was obtained as a white solid (0.433 g, 51% yield) ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.99–7.87 (m, 4H), 7.56–7.35 (m, 6H), 4.60–3.72 (m, 9H), 2.61–2.53 (m, 4H), 1.90–1.83 (m, 2H), 1.55–1.53 (m, 2H), 1.41–1.23 (m, 30H), 0.88 (t, J = 7.2 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.11, 133.30, 129.72, 129.55, 128.71, 128.53, 38.80, 34.42, 31.94, 29.73, 29.68, 29.60, 29.38, 29.31, 29.19, 28.59, 22.70, 14.14; ^{31}P NMR (240 MHz, $CDCl_3$) δ 15.90. HRMS (ESI^+) *m/z*: calculated for $C_{43}H_{65}N_3O_8PS_2$ $[M + H]^+$ 846.3945, found 846.3935, $C_{43}H_{64}N_3O_8PS_2Na$ $[M + Na]^+$ 868.3765, found 868.3755.

4.1.16. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-(((4-(decyldisulfanyl)butoxy)(hydroxy)phosphoryl)methoxy)propyl benzoate (9k)

9k was obtained as a white solid (0.400 g, 53% yield). ^{1}H NMR (600 MHz, $CD_3OD/CDCl_3$) δ 8.17 (d, J = 7.2 Hz, 1H), 8.04–7.98 (m, 4H), 7.65–7.46 (m, 6H), 4.62 (dd, J = 12.0, 45.0 Hz, 1H), 4.39 (q, J = 15.6 Hz, 2H), 4.25 (dd, J = 12.6, 33.6 Hz, 1H), 4.14–3.96 (m, 2H), 3.91–3.89 (m, 2H), 3.80–3.69 (m, 1H), 2.65–2.59 (m, 4H), 1.72–1.57 (m, 6H), 1.38–1.27 (m, 14H), 0.89 (t, J = 7.2 Hz, 3H); ^{13}C NMR (150 MHz, $CD_3OD/CDCl_3$) δ 164.28, 134.13, 134.02, 133.78, 130.57, 130.37, 129.41, 129.26, 129.21, 129.12, 128.88, 64.05, 39.22, 39.20, 38.69, 38.65, 32.61, 30.21 (d, J = 3.8 Hz), 30.00, 29.89, 29.89, 29.76, 29.74, 29.03, 29.02, 26.07, 25.98, 23.29, 14.03; ^{31}P NMR (240 MHz, $CD_3OD/CDCl_3$) δ 20.89, 20.24. HRMS (ESI^+) *m/z*: calculated for $C_{36}H_{51}N_3O_8PS_2$ $[M + H]^+$ 748.2850, found 748.2835, $C_{36}H_{50}N_3O_8PS_2Na$ $[M + Na]^+$ 770.2669, found 770.2661.

4.1.17. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-(((4-(dodecyldisulfanyl)butoxy)(hydroxy)phosphoryl)methoxy)propyl benzoate (9l)

9l was obtained as a white solid (0.357 g, 46% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.99–7.90 (m, 4H), 7.53–7.40 (m, 6H), 4.61–4.13 (m, 5H), 3.91–3.83 (m, 4H), 2.61–2.54 (m, 4H), 1.60–1.57 (m, 4H), 1.43–1.24 (m, 20H), 0.87 (t, J = 7.2 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.10, 133.31, 129.71, 129.54, 128.75, 128.53, 64.81, 38.86, 38.20, 31.92, 29.68, 29.65, 29.63, 29.56, 29.36, 29.29, 29.20, 28.58, 25.25, 22.70, 14.13; ^{31}P NMR (240 MHz, $CDCl_3$) δ 16.16. HRMS (ESI^+) *m/z*: calculated for $C_{38}H_{55}N_3O_8PS_2$ $[M + H]^+$ 776.3163, found 776.3152, $C_{38}H_{54}N_3O_8PS_2Na$ $[M + Na]^+$ 798.2982, found 798.2978.

4.1.18. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-((hydroxy(4-(tetradecyldisulfanyl)buto-xy)phosphoryl)methoxy)propyl benzoat. (9 m)

9m was obtained as a white solid (0.388 g, 48% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.01–7.81 (m, 4H), 7.56–7.33 (m, 6H), 4.62–3.77 (m, 9H), 2.64–2.52 (m, 4H), 1.58–1.55 (m, 4H), 1.44–1.24 (m, 24H), 0.88 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.11, 133.28, 129.70, 129.57, 128.70, 128.52, 38.81, 38.20, 31.94, 31.83, 29.72, 29.70, 29.67, 29.65, 29.59, 29.38, 29.31, 29.20, 28.59, 25.26, 22.70, 14.14; ^{31}P NMR (240 MHz, $CDCl_3$) δ 15.61. HRMS (ESI^+) *m/z*: calculated for $C_{40}H_{59}N_3O_8PS_2$ $[M + H]^+$ 804.3476, found 804.3474, $C_{40}H_{58}N_3O_8PS_2Na$ $[M + Na]^+$ 826.3295, found 826.3291.

4.1.19. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-(((4-(hexadecyldisulfanyl)butoxy)(hy-droxy)phosphoryl)methoxy)propyl benzoate (9n)

9n was obtained as a white solid (0.492 g, 59% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.01–8.00 (m, 4H), 7.57–7.42 (m, 6H), 4.62–3.85 (m, 9H), 2.61–2.56 (m, 4H), 1.63–1.59 (m, 6H), 1.35–1.26 (m, 26H), 0.90 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.10, 133.33, 132.44, 129.71, 129.53, 128.77, 128.54, 128.47, 64.80, 63.00, 38.86, 38.18, 31.94, 29.72, 29.70, 29.67, 29.65, 29.58, 29.38, 29.31, 29.20, 28.59, 25.23, 22.70, 14.14; ^{31}P NMR (240 MHz, $CDCl_3$) δ 16.43. HRMS (ESI^+) *m/z*: calculated for $C_{42}H_{63}N_3O_8PS_2$ $[M + H]^+$ 832.3789, found 832.3761, $C_{42}H_{62}N_3O_8PS_2Na$ $[M + Na]^+$ 854.3608, found 854.3588.

4.1.20. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-((hydroxy((6-(octyldisulfanyl)hexyl)oxy)phosphoryl)methoxy)propyl benzoate (9o)

9o was obtained as a white solid (0.362 g, 48% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.01–7.87 (m, 4H), 7.60–7.33 (m, 6H), 4.62–3.65 (m, 9H), 2.67–2.52 (m, 4H), 1.68–1.19 (m, 20H), 0.87 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.13, 133.27, 133.12, 129.70, 129.60, 128.72, 128.61, 128.50, 128.36, 65.19, 63.11, 39.07, 39.04, 38.82, 31.81, 30.68, 30.20, 29.71, 29.22, 29.19, 29.06, 28.55, 28.20, 25.29, 22.65, 14.12; ^{31}P NMR (240 MHz, $CDCl_3$) δ 16.00. HRMS (ESI^-) *m/z*: calculated for $C_{36}H_{49}N_3O_8PS_2$ $[M - H]^-$ 746.2704, found 746.2729.

4.1.21. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-((((6-(decyldisulfanyl)hexyl)oxy)(hydro-xy)phosphoryl)methoxy)propyl benzoate (9p)

9p was obtained as a white solid (0.376 g, 48% yield). ^{1}H NMR (600 MHz, $CD_3OD/CDCl_3$) δ 8.19 (d, J = 7.8 Hz, 1H), 8.04–7.87 (m, 4H), 7.65–7.46 (m, 6H), 6.02 (d, J = 7.8 Hz, 1H), 4.65–4.56 (m, 1H), 4.42–4.35 (m, 2H), 4.29–4.08 (m, 2H), 3.99–3.93 (m, 1H), 4.65–4.56 (m, 1H), 3.86–3.83 (m, 2H), 3.72–3.68 (m, 1H), 2.67–2.56 (m, 4H), 1.67–1.27 (m, 24H), 0.89 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, $CD_3OD/CDCl_3$) δ 167.24, 134.21, 130.68, 130.47, 129.62, 129.37, 128.95, 64.13, 39.50, 39.32, 32.76, 30.36 (d, J = 4.5 Hz), 30.15, 30.04, 29.93, 29.82, 29.19, 28.83, 26.06, 23.45, 14.24; ^{31}P NMR (240 MHz, $CD_3OD/CDCl_3$) δ 16.67. HRMS (ESI^+) *m/z*: calculated for $C_{38}H_{55}N_3O_8PS_2$ $[M + H]^+$ 776.3163, found 776.3158, $C_{38}H_{54}N_3O_8PS_2Na$ $[M + Na]^+$ 798.2982, found 798.2979.

4.1.22. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-((((6(dodecyldisulfanyl)hexyl)oxy)(hy-droxy)phosphoryl)methoxy)propyl benzoate (9q)

9q was obtained as a white solid (0.378 g, 47% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.00–7.91 (m, 4H), 7.55–7.36 (m, 6H), 4.74–4.32 (m, 5H), 3.95–3.81 (m, 4H), 2.66–2.54 (m, 4H), 1.65–1.19 (m, 28H), 0.88 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.09, 133.31, 132.51, 129.70, 129.55, 128.76, 128.53, 65.29, 63.01, 39.03, 38.79, 31.92, 30.61 (d, J = 6.3 Hz), 29.67, 29.64, 29.62, 29.55, 29.36, 29.28, 29.24, 29.03, 28.57, 28.15, 25.25, 22.70, 14.13; ^{31}P NMR (240 MHz, $CDCl_3$) δ 16.46. HRMS (ESI^+) *m/z*: calculated for $C_{40}H_{59}N_3O_8PS_2$ $[M + H]^+$ 804.3476, found 804.3472, $C_{40}H_{58}N_3O_8PS_2Na$ $[M + Na]^+$ 826.3295, found 826.3302.

4.1.23. (2S)-3-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-2-((hydroxy((6-(tetradecyldisulfanyl)hexyl)oxy)phosphoryl)methoxy)propyl benzoate (9r)

9r was obtained as a white solid (0.437 g, 53% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.00–7.71 (m, 4H), 7.54–7.31 (m, 6H), 4.61–3.72 (m, 9H), 2.62–2.49 (m, 4H), 1.64–1.17 (m, 32H), 0.88 (t, J = 7.2 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.12, 133.25, 133.06, 129.69, 129.61, 128.69, 128.59, 128.51, 128.40, 38.99, 38.78, 31.94, 30.68, 30.20, 29.71, 29.69, 29.67, 29.64, 29.57, 29.37, 29.30, 29.24, 29.04, 28.59, 28.23, 25.30, 22.70, 14.14; ^{31}P NMR (240 MHz, $CDCl_3$) δ 15.10. HRMS (ESI^-) *m/z*: calculated for $C_{42}H_{61}N_3O_8PS_2$ $[M - H]^-$ 830.3643, found 830,3664.

4.2. General procedure for the preparation of the target compounds 1a-r

One of the intermediates **9a-r** (0.450 mmol) was treated with a solution of ammonia in methanol (7 M, 20 mL) and stirred at r. t. for 48 h. The solvents were evaporated under reduced pressure to afford a yellow

residue, which was purified on a silica column using a DCM/DCM: MeOH: NH_4OH (49: 49: 2) gradient (20−80%) to afford the corresponding target conjugates **1a-r** in 40–60% yields.

4.2.1. 2-(decyldisulfanyl)ethyl hydrogen((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxyxypropan -2-yl)oxy)methyl) phosphonate (1a)

1a was prepared from intermediate **9a** as a white solid (0.113 g, 48% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.81 (d, J = 7.8 Hz, 1H), 5.96 (d, J = 7.2 Hz, 1H), 4.16–4.10 (m, 3H), 3.84 (dd, J = 7.8, 14.4 Hz, 1H), 3.79–3.75 (m, 2H), 3.72–3.68 (m, 1H), 3.64 (dd, J = 9.0, 12.6 Hz, 1H), 3.56 (dd, J = 4.2, 12.6 Hz, 1H), 2.92 (t, J = 6.6 Hz, 2H),2.74 (t, J = 7.2 Hz, 2H), 1.72–1.67 (m, 2H), 1.42–1.39 (m, 2H), 1.36–1.29 (m, 12H), 0.92 (t, J = 7.2 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 164.02, 154.30, 149.09, 93.73, 80.08 (d, J = 11.6 Hz), 65.34 (d, J = 158.7 Hz), 63.00 (d, J = 5.6 Hz) 60.17, 50.03, 39.01 (d, J = 6.0 Hz), 38.55, 31.67, 29.30, 29.28, 29.06, 28.98, 28.79, 28.12, 22.34, 13.05; ^{31}P NMR (240 MHz, CD_3OD) δ 16.12. HRMS (ESI^+) m/z: calculated for $C_{20}H_{39}N_3O_6PS_2$ $[M + H]^+$ 512.2012, found 512.2009, $C_{20}H_{38}N_3O_6PS_2Na$ $[M + Na]^+$ 534.1832, found 534.1837.

4.2.2. 2-(dodecyldisulfanyl)ethyl hydrogen ((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (1b)

1b was prepared from intermediate **9b** as a white solid (0.132 g, 53% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.83 (d, J = 7.2 Hz, 1H), 5.96 (d, J = 7.2 Hz, 1H), 4.17–4.10 (m, 3H), 3.84 (dd, J = 7.2, 13.8 Hz, 1H), 3.79–3.75 (m, 2H), 3.72–3.69 (m, 1H), 3.64 (dd, J = 9.0, 12.6 Hz, 1H), 3.56 (dd, J = 4.2, 12.0 Hz, 1H), 2.92 (t, J = 7.2 Hz, 2H), 2.74 (t, J = 7.2 Hz, 2H), 1.72–1.67 (m, 2H), 1.43–1.29 (m, 18H) 0.92 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 163.75, 153.86, 149.26, 93.64, 80.04 (d, J = 11.7 Hz), 65.32 (d, J = 159.0 Hz), 62.99 (d, J = 5.7 Hz), 60.16, 50.04, 39.01 (d, J = 6.0 Hz), 38.55, 31.68, 29.39, 29.36, 29.33, 29.27, 29.08, 28.98, 28.79, 28.12, 22.34, 13.04; ^{31}P NMR (240 MHz, CD_3OD) δ 16.11. HRMS (ESI^+) m/z: calculated for $C_{22}H_{43}N_3O_6PS_2$ $[M + H]^+$ 540.2325, found 540.2323, $C_{22}H_{42}N_3O_6PS_2Na$ $[M + Na]^+$ 562.2145, found 562.2142.

4.2.3. 2-(tetradecyldisulfanyl)ethyl hydrogen ((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (1c)

1c was prepared from intermediate **9c** as a white solid (0.128 g, 49% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.74 (d, J = 7.2 Hz, 1H), 5.90 (d, J = 7.2 Hz, 1H), 4.14–4.10 (m, 3H), 3.83 (dd, J = 7.2, 13.8 Hz, 1H), 3.76–3.73 (m, 2H), 3.70–3.66 (m, 1H), 3.64 (dd, J = 9.0, 12.6 Hz, 1H), 3.54 (dd, J = 4.2, 12.0 Hz, 1H), 2.92 (t, J = 6.6 Hz, 2H),2.74 (t, J = 7.2 Hz, 2H), 1.72–1.67 (m, 2H), 1.43–1.40 (m, 2H), 1.36–1.29 (m, 20H), 0.92 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 165.56, 156.46, 148.26, 93.94, 80.39 (d, J = 11.7 Hz), 65.55 (d, J = 159.0 Hz), 63.04 (d, J = 5.4 Hz), 60.25, 50.00, 39.00 (d, J = 6.2 Hz), 38.55, 31.67, 29.39, 29.38, 29.36, 29.31, 29.26, 29.07, 28.97, 28.78, 28.11, 22.33, 13.03; ^{31}P NMR (240 MHz, CD_3OD) δ 16.10. HRMS (ESI^+) m/z: calculated for $C_{24}H_{47}N_3O_6PS_2$ $[M + H]^+$ 568.2638, found 568.2636, $C_{24}H_{46}N_3O_6PS_2Na$ $[M + Na]^+$ 590.2458, found 590.2456.

4.2.4. 2-(hexadecyldisulfanyl)ethyl hydrogen ((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxylpropan-2-yl)oxy)methyl)phosphonate (1d)

1d was prepared from intermediate **9d** as a white solid (0.110 g, 40% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.71 (d, J = 7.2 Hz, 1H), 5.88 (d, J = 7.2 Hz, 1H), 4.14–4.09 (m, 3H), 3.83 (dd, J = 7.2, 13.8 Hz, 1H), 3.76–3.72 (m, 2H), 3.69–3.66 (m, 1H), 3.64 (dd, J = 9.0, 12.6 Hz, 1H), 3.53 (dd, J = 4.2, 12.6 Hz, 1H), 2.92 (t, J = 7.2 Hz, 2H), 2.74 (t, J = 7.8 Hz, 2H), 1.72–1.67 (m, 2H), 1.43–1.40 (m, 2H), 1.36–1.29 (m, 24H), 0.92 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 166.08, 157.19, 147.98, 94.01, 80.49 (d, J = 11.9 Hz), 65.61 (d, J = 159.6 Hz), 63.05 (d, J = 5.5 Hz), 60.27, 50.00, 38.97 (d, J = 5.5 Hz), 38.55, 31.67, 29.37, 29.35, 29.31, 29.26, 29.07, 28.97, 28.78, 28.11, 22.33, 13.03; ^{31}P NMR (240 MHz, CD_3OD) δ 16.11. HRMS (ESI^+) m/z: calculated for $C_{26}H_{51}N_3O_6PS_2$ $[M + H]^+$ 596.2951, found 596.2946, $C_{26}H_{50}N_3O_6PS_2Na$ $[M + Na]^+$ 618.2771, found 618.2767.

4.2.5. 2-(octadecyldisulfanyl)ethyl hydrogen ((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (1e)

1e was prepared from intermediate **9e** as a white solid (0.135 g, 47% yield). ^{1}H NMR (600 MHz, $CD_3OD/CDCl_3$) δ 7.69 (d, J = 7.2 Hz, 1H), 5.87 (d, J = 7.2 Hz, 1H), 4.13–4.08 (m, 3H), 3.83 (dd, J = 7.2, 13.8 Hz, 1H), 3.76–3.71 (m, 2H), 3.68–3.61 (m, 2H), 3.52 (dd, J = 4.2, 12.6 Hz, 1H), 2.90 (t, J = 6.6 Hz, 2H),2.72 (t, J = 7.2 Hz, 2H), 1.71–1.66 (m, 2H), 1.42–1.38 (m,2H), 1.35–1.29 (m, 26H), 0.90 (t, J = 6.6 Hz, 3H); δ ^{13}C NMR (150 MHz, $CD_3OD/CDCl_3$) δ 166.27, 148.30, 95.05, 80.43 (d, J = 11.2 Hz), 60.20, 50.03, 38.66, 31.71, 29.42, 29.40, 29.37, 29.31, 29.24, 29.12, 29.04, 28.86, 28.21, 22.39, 13.26; ^{31}P NMR (240 MHz, CD_3OD/$CDCl_3$) δ 16.07. HRMS (ESI^+) m/z: calculated for $C_{28}H_{55}N_3O_6PS_2$ $[M + H]^+$ 624.3264, found 624.3262, $C_{28}H_{54}N_3O_6PS_2Na$ $[M + Na]^+$ 646.3084, found 646.3084.

4.2.6. 3-(decyldisulfanyl)propyl hydrogen ((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (1f)

1f was prepared from intermediate **9f** as a white solid (0.123 g, 50% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.77 (d, J = 7.2 Hz, 1H), 5.93 (d, J = 7.8 Hz, 1H), 4.12 (dd, J = 3.6, 13.8 Hz, 1H), 3.99–3.96 (m, 2H), 3.84 (dd, J = 7.2, 13.8 Hz, 1H), 3.77–3.73 (m, 2H), 3.71–3.67 (m, 1H), 3.63 (dd, J = 9.6, 13.2 Hz, 1H), 3.55 (dd, J = 3.6, 12.0 Hz, 1H), 2.80 (t, J = 7.2 Hz, 2H), 2.71 (t, J = 7.2 Hz, 2H), 2.01–1.97 (m, 2H), 1.71–1.66 (m,2H), 1.43–1.29 (m, 14H), 0.92 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 164.79, 155.41, 148.65, 93.82, 80.18 (d, J = 11.6 Hz), 65.30 (d, J = 158.6 Hz), 62.81 (d, J = 5.9 Hz), 60.18, 50.04, 38.22, 34.42, 31.66, 30.35 (d, J = 6.2 Hz), 29.28, 29.27, 29.05, 28.95, 28.83, 28.12, 22.33, 13.04; ^{31}P NMR (240 MHz, CD_3OD) δ 16.09. HRMS (ESI^+) m/z: calculated for $C_{21}H_{41}N_3O_6PS_2$ $[M + H]^+$ 526.2169, found 526.2165, $C_{21}H_{40}N_3O_6PS_2Na$ $[M + Na]^+$ 548.1988, found 548.1984.

4.2.7. 3-(dodecyldisulfanyl)propyl hydrogen ((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (1g)

1g was prepared from intermediate **9g** as a white solid (0.122 g, 48% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.80 (d, J = 7.2 Hz, 1H), 5.95 (d, J = 7.2 Hz, 1H), 4.14 (dd, J = 3.0, 13.8 Hz, 1H), 3.99–3.96 (m, 2H), 3.84 (dd, J = 7.8, 13.8 Hz, 1H), 3.78–3.74 (m, 2H), 3.71–3.68 (m, 1H), 3.62 (dd, J = 9.0, 12.6 Hz, 1H), 3.55 (dd, J = 4.2, 12.0 Hz, 1H), 2.80 (t, J = 7.2 Hz, 2H),2.71 (t, J = 7.2 Hz, 2H), 2.01–1.97 (m, 2H), 1.71–1.66 (m,2H), 1.42–1.29 (m, 18H), 0.92 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 164.11, 154.39, 149.05, 93.67, 80.06 (d, J = 11.7 Hz), 65.23 (d, J = 158.6 Hz), 62.80 (d, J = 5.6 Hz), 60.15, 50.05, 38.23, 34.42, 31.68, 30.34 (d, J = 6.2 Hz), 29.38, 29.36, 29.31, 29.26, 29.07, 28.96, 28.84, 28.12, 22.34, 13.04; ^{31}P NMR (240 MHz, CD_3OD) δ 16.11. HRMS (ESI^+) m/z: calculated for $C_{23}H_{45}N_3O_6PS_2$ $[M + H]^+$ 554.2482, found 554.2475, $C_{23}H_{44}N_3O_6PS_2Na$ $[M + Na]^+$ 576.2301 found 576.2296.

4.2.8. 3-(tetradecyldisulfanyl)propyl hydrogen ((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (1h)

1h was prepared from intermediate **9h** as a white solid (0.149 g, 55% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.80 (d, J = 7.2 Hz, 1H), 5.95 (d, J = 7.2 Hz, 1H), 4.14 (dd, J = 3.6, 14.4 Hz, 1H), 3.99–3.96 (m, 2H), 3.84 (dd, J = 7.8, 14.4 Hz, 1H), 3.78–3.74 (m, 2H), 3.71–3.68 (m, 1H), 3.62 (dd, J = 9.0, 12.6 Hz, 1H), 3.55 (dd, J = 4.2, 12.6 Hz, 1H), 2.80 (t, J = 7.2 Hz, 2H),2.71 (t, J = 7.2, 2H), 2.01–1.97 (m, 2H), 1.71–1.66 (m,2H),

1.43–1.39 (m, 2H), 1.36–1.29 (m, 20H), 0.92 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 164.15, 154.47, 149.02, 93.69, 80.06 (d, J = 11.7 Hz), 65.20 (d, J = 158.1 Hz), 62.80 (d, J = 5.6 Hz), 60.13, 50.05, 38.21, 34.40, 31.69, 30.33 (d, J = 6.2 Hz), 29.41, 29.40, 29.38, 29.33, 29.28, 29.09, 28.97, 28.84, 28.13, 22.35, 13.06; ^{31}P NMR (240 MHz, CD_3OD) δ 16.13. HRMS (ESI$^+$) *m/z*: calculated for $C_{25}H_{49}N_3O_6PS_2$ [M + H]$^+$ 582.2795, found 582.2792, $C_{25}H_{48}N_3O_6PS_2Na$ [M + Na]$^+$ 604.2614, found 604.2611.

4.2.9. 3-(hexadecyldisulfanyl)propyl hydrogen ((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (1i)

1i was prepared from intermediate **9i** as a white solid (0.145 g, 51% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.72 (d, J = 7.2 Hz, 1H), 5.89 (d, J = 7.2 Hz, 1H), 4.10 (dd, J = 3.6, 14.4 Hz, 1H), 3.99–3.94 (m, 2H), 3.83 (dd, J = 7.2, 13.8 Hz, 1H), 3.76–3.71 (m, 2H), 3.69–3.66 (m, 1H), 3.62 (dd, J = 9.0, 12.6 Hz, 1H), 3.53 (dd, J = 4.2, 12.6 Hz, 1H), 2.80 (t, J = 7.2 Hz, 2H),2.71 (t, J = 7.2, 2H), 2.01–1.97 (m, 2H), 1.72–1.67 (m,2H), 1.43–1.40 (m, 2H), 1.36–1.29 (m, 24H), 0.92 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 165.75, 156.72, 148.17, 93.91, 80.36 (d, J = 11.9 Hz), 65.39 (d, J = 158.7 Hz), 62.83 (d, J = 5.6 Hz), 60.21, 50.04, 38.20, 34.40, 31.68, 30.36 (d, J = 6.3 Hz), 29.39, 29.37, 29.36, 29.30, 29.26, 29.08, 28.95, 28.83, 28.12, 22.34, 13.04; ^{31}P NMR (240 MHz, CD_3OD) δ 16.12. HRMS (ESI$^+$) *m/z*: calculated for $C_{27}H_{53}N_3O_6PS_2$ [M + H]$^+$ 610.3108, found 610.3099, $C_{27}H_{52}N_3O_6PS_2Na$ [M + Na]$^+$ 632.2927, found 632.2921.

4.2.10. 3-(octadecyldisulfanyl)propyl hydrogen ((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (1j)

1j was prepared from intermediate **9j** as a white solid (0.142 g, 48% yield) .^{1}H NMR (600 MHz, $CD_3OD/CDCl_3$) δ 7.74 (d, J = 7.2 Hz, 1H), 5.91 (d, J = 7.2 Hz, 1H), 4.11 (dd, J = 3.6, 14.4 Hz, 1H), 3.99–3.95 (m, 2H), 3.83 (dd, J = 7.8, 13.8 Hz, 1H), 3.77–3.71 (m, 2H), 3.69–3.66 (m, 1H), 3.62 (dd, J = 9.6, 13.2 Hz, 1H), 3.53 (dd, J = 4.2, 12.6 Hz, 1H), 2.79 (t, J = 7.2 Hz, 2H),2.70 (t, J = 7.2, 2H), 2.01–1.97 (m, 2H), 1.71–1.66 (m,2H), 1.42–1.39 (m, 2H), 1.35–1.30 (m, 28H), 0.91 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, $CD_3OD/CDCl_3$) δ 164.90, 148.56, 93.91, 80.20 (d, J = 11.6 Hz), 65.33 (d, J = 158.3 Hz), 62.83 (d, J = 5.6 Hz), 60.14, 50.05, 38.32, 34.45, 31.71, 30.35 (d, J = 6.0 Hz), 29.43, 29.41, 29.40, 29.35, 29.31, 29.11, 29.01, 28.89, 28.20, 22.38, 13.20; ^{31}P NMR (240 MHz, $CD_3OD/CDCl_3$) δ 16.08. HRMS (ESI$^+$) *m/z*: calculated for $C_{29}H_{57}N_3O_6PS_2$ [M + H]$^+$ 638.3421, found 638.3419, $C_{29}H_{56}N_3O_6PS_2Na$ [M + Na]$^+$ 660.3240, found 660.3230.

4.2.11. 4-(decyldisulfanyl)butyl hydrogen ((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (1k)

1k was prepared from intermediate **9k** as a white solid (0.141 g, 56% yield).^{1}H NMR (600 MHz, CD_3OD) δ 7.82 (d, J = 7.8 Hz, 1H), 5.96 (d, J = 7.2 Hz, 1H), 4.15 (dd, J = 3.6, 14.4 Hz, 1H), 3.92–3.88 (m, 2H), 3.84 (dd, J = 7.8, 14.4 Hz, 1H), 3.78–3.75 (m, 2H), 3.71–3.68 (m, 1H), 3.62 (dd, J = 9.6, 13.2 Hz, 1H), 3.56 (dd, J = 4.2, 12.6 Hz, 1H), 2.74 (t, J = 6.6 Hz, 2H),2.70 (t, J = 7.2, 2H), 1.82–1.77 (m, 2H), 1.74–1.66 (m,4H), 1.44–1.39 (m, 2H), 1.36–1.29 (m, 12H), 0.92 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 163.71, 153.81, 149.31, 93.57, 79.97 (d, J = 11.6 Hz), 65.20 (d, J = 158.3 Hz), 63.96 (d, J = 5.7 Hz), 60.12, 50.05, 38.30, 37.92, 31.67, 29.50 (d, J = 6.2 Hz), 29.29, 29.27, 29.06, 28.97, 28.83, 28.10, 25.27, 22.34, 13.06; ^{31}P NMR (240 MHz, CD_3OD) δ 16.06. HRMS (ESI$^+$) *m/z*: calculated for $C_{22}H_{43}N_3O_6PS_2$ [M + H]$^+$ 540.2325, found 540.2323, $C_{22}H_{42}N_3O_6PS_2Na$ [M + Na]$^+$ 562.2145, found 562.2145.

4.2.12. 4-(dodecyldisulfanyl)butyl hydrogen ((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (1l)

1l was prepared from intermediate **9l** as a white solid (0.133 g, 51% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.82 (d, J = 7.8 Hz, 1H), 5.95 (d, J = 7.2 Hz, 1H), 4.15 (dd, J = 3.0, 13.8 Hz, 1H), 3.92–3.88 (m, 2H), 3.84 (dd, J = 7.8, 14.4 Hz, 1H), 3.78–3.74 (m, 2H), 3.71–3.68 (m, 1H), 3.62 (dd, J = 9.6, 13.2 Hz, 1H), 3.56 (dd, J = 4.2, 12.6 Hz, 1H), 2.74 (t, J = 7.2 Hz, 2H),2.70 (t, J = 7.2, 2H), 1.82–1.77 (m, 2H), 1.74–1.66 (m,4H), 1.42–1.39 (m, 2H), 1.36–1.29 (m, 16H), 0.92 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 163.78, 153.92, 149.26, 93.59, 79.98 (d, J = 11.4 Hz), 65.20 (d, J = 158.4 Hz), 63.95 (d, J = 5.6 Hz), 50.05, 38.30, 37.92, 31.68, 29.50 (d, J = 6.5 Hz), 29.39, 29.37, 29.32, 29.26, 29.08, 28.97, 28.83, 28.10, 25.27, 22.35, 13.06; ^{31}P NMR (240 MHz, CD_3OD) δ 16.06. HRMS (ESI$^+$) *m/z*: calculated for $C_{24}H_{47}N_3O_6PS_2$ [M + H]$^+$ 568.2638, found 568.2632, $C_{24}H_{46}N_3O_6PS_2Na$ [M + Na]$^+$ 590.2458, found 590.2454.

4.2.13. 4-(tetradecyldisulfanyl)butyl hydrogen ((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (1 m)

1m was prepared from intermediate **9m** as a white solid (0.131 g, 48% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.80 (d, J = 7.2 Hz, 1H), 5.94 (d, J = 7.2 Hz, 1H), 4.14 (dd, J = 3.6, 13.8 Hz, 1H), 3.92–3.87 (m, 2H), 3.84 (dd, J = 7.8, 13.8 Hz, 1H), 3.78–3.74 (m, 2H), 3.71–3.68 (m, 1H), 3.62 (dd, J = 9.6, 13.2 Hz, 1H), 3.55 (dd, J = 4.2, 12.6 Hz, 1H), 2.74 (t, J = 7.2 Hz, 2H),2.70 (t, J = 7.2, 2H), 1.82–1.78 (m, 2H), 1.74–1.66 (m,4H), 1.43–1.40 (m, 2H), 1.36–1.29 (m, 20H), 0.92 (t, J = 7.2 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 164.15, 154.48, 149.05, 93.67, 80.05 (d, J = 11.6 Hz), 65.24 (d, J = 158.6 Hz), 63.96 (d, J = 5.9 Hz), 60.13, 50.05, 38.30, 37.92, 31.69, 29.50 (d, J = 6.5 Hz), 29.41, 29.40, 29.38, 29.32, 29.27, 29.09, 28.97, 28.84, 28.11, 25.27, 22.35, 13.06; ^{31}P NMR (240 MHz, CD_3OD) δ 16.05. HRMS (ESI$^+$) *m/z*: calculated for $C_{26}H_{51}N_3O_6PS_2$ [M + H]$^+$ 596.2951, found 596.2947, $C_{26}H_{50}N_3O_6PS_2Na$ [M + Na]$^+$ 618.2771, found 618.2770.

4.2.14. 4-(hexadecyldisulfanyl)butyl hydrogen ((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (1n)

1n was prepared from intermediate **9n** as a white solid (0.172 g, 60% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.80 (d, J = 7.2 Hz, 1H), 5.94 (d, J = 7.2 Hz, 1H), 4.14 (dd, J = 3.6, 14.4 Hz, 1H), 3.92–3.87 (m, 2H), 3.84 (dd, J = 7.8, 14.4 Hz, 1H), 3.78–3.74 (m, 2H), 3.71–3.67 (m, 1H), 3.61 (dd, J = 9.6, 13.2 Hz, 1H), 3.55 (dd, J = 4.2, 12.6 Hz, 1H), 2.74 (t, J = 7.2 Hz, 2H),2.70 (t, J = 7.2, 2H), 1.82–1.78 (m, 2H), 1.74–1.66 (m,4H), 1.43–1.40 (m, 2H), 1.36–1.31 (m, 24H), 0.92 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 164.26, 154.63, 149.01, 93.68, 80.07 (d, J = 11.9 Hz), 65.24 (d, J = 157.8 Hz), 63.96 (d, J = 6.0 Hz), 60.13, 50.05, 38.29, 37.91, 31.69, 29.50 (d, J = 6.0 Hz), 29.42, 29.39, 29.38, 29.32, 29.27, 29.10, 28.98, 28.84, 28.11, 25.27, 22.36, 13.07; ^{31}P NMR (240 MHz, CD_3OD) δ 16.06. HRMS (ESI$^+$) *m/z*: calculated for $C_{28}H_{55}N_3O_6PS_2$ [M + H]$^+$ 624.3264, found 624.3257, $C_{28}H_{54}N_3O_6PS_2Na$ [M + Na]$^+$ 646.3084, found 646.3080.

4.2.15. 6-(octyldisulfanyl)hexyl hydrogen ((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (1o)

1o was prepared from intermediate **9o** as a white solid (0.113 g, 45% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.80 (d, J = 7.2 Hz, 1H), 5.94 (d, J = 7.2 Hz, 1H), 4.14 (dd, J = 3.6, 14.4 Hz, 1H), 3.90–3.82 (m, 3H), 3.78–3.74 (m, 2H), 3.71–3.67 (m, 1H), 3.61 (dd, J = 9.0, 12.6 Hz, 1H), 3.55 (dd, J = 4.2, 12.6 Hz, 1H), 2.71–2.68 (m, 4H), 1.73–1.66 (m, 4H), 1.65–1.60 (m,2H), 1.46–1.40 (m, 6H), 1.36–1.31 (m, 8H), 0.93 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 164.13, 154.44, 149.10, 93.63, 80.02 (d, J = 11.9 Hz), 65.22 (d, J = 158.4 Hz), 64.40 (d, J = 5.9 Hz), 60.12, 50.03, 38.34, 38.24, 31.59, 30.70 (d, J = 6.0 Hz), 28.94,

28.92, 28.83, 28.82, 28.09, 27.84, 25.16, 22.33, 13.05; ^{31}P NMR (240 MHz, CD_3OD) δ 16.03. HRMS (ESI^-) *m/z*: calculated for $C_{22}H_{41}N_3O_6PS_2$ $[M-H]^-$ 538.2180, found 538.2183.

4.2.16. 6-(decyldisulfanyl)hexyl hydrogen ((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (1p)

1p was prepared from intermediate **9p** as a white solid (0.121 g, 46% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.82 (d, J = 6.0 Hz, 1H), 5.94 (d, J = 7.8 Hz, 1H), 4.16–4.13 (m, 1H), 3.89–3.81 (m, 3H), 3.78–3.74 (m, 2H), 3.70–3.68 (m, 1H), 3.63–3.58 (m, 1H), 3.57–3.53 (m, 1H), 2.72–2.68 (m, 4H), 1.72–1.67 (m, 4H), 1.64–1.61 (m,2H), 1.45–1.39 (m, 6H), 1.36–1.28 (m, 12H), 0.92 (t, J = 5.4 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 163.86, 154.04, 149.24, 93.58, 79.97 (d, J = 11.9 Hz), 65.19 (d, J = 158.9 Hz), 64.40 (d, J = 6.2 Hz), 60.10, 50.03, 38.34, 38.24, 31.67, 30.70 (d, J = 6.2 Hz), 29.28, 29.26, 29.06, 28.94, 28.83, 28.07, 27.84, 25.16, 22.35, 13.06; ^{31}P NMR (240 MHz, CD_3OD) δ 15.97. HRMS (ESI^+) *m/z*: calculated for $C_{24}H_{47}N_3O_6PS_2$ $[M+H]^+$ 568.2638, found 568.2634, $C_{24}H_{46}N_3O_6PS_2Na$ $[M+Na]^+$ 590.2458, found 590.2459.

4.2.17. 6-(dodecyldisulfanyl)hexyl hydrogen ((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (1q)

1q was prepared from intermediate **9q** as a white solid (0.140 g, 51% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.82 (d, J = 7.2 Hz, 1H), 5.95 (d, J = 7.8 Hz, 1H), 4.15 (dd, J = 3.0, 13.8 Hz, 1H), 3.90–3.82 (m, 3H), 3.78–3.74 (m, 2H), 3.71–3.68 (m, 1H), 3.61 (dd, J = 9.0, 12.6 Hz, 1H), 3.55 (dd, J = 3.6, 12.0 Hz, 1H), 2.71–2.68 (m, 4H), 1.73–1.66 (m, 4H), 1.65–1.60 (m,2H), 1.46–1.39 (m, 6H), 1.36–1.29 (m, 16H), 0.92 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 163.85, 154.02, 149.26, 93.58, 79.97 (d, J = 11.7 Hz), 65.19 (d, J = 158.3 Hz), 64.40 (d, J = 5.6 Hz), 60.10, 50.04, 38.35, 38.24, 31.69, 30.71 (d, J = 6.0 Hz), 29.39, 29.37, 29.32, 29.25, 29.09, 28.94, 28.84, 28.82, 28.07, 27.84, 25.17, 22.36, 13.07; ^{31}P NMR (240 MHz, CD_3OD) δ 16.03. HRMS (ESI^+) *m/z*: calculated for $C_{26}H_{51}N_3O_6PS_2$ $[M+H]^+$ 596.2951, found 596.2949, $C_{26}H_{50}N_3O_6PS_2Na$ $[M+Na]^+$ 618.2771, found 618.2772.

4.2.18. 6-(tetradecyldisulfanyl)hexyl hydrogen ((((S)-1-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (1r)

1r was prepared from intermediate **9r** as a white solid (0.151 g, 52% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.81 (d, J = 7.8 Hz, 1H), 5.94 (d, J = 7.2 Hz, 1H), 4.15 (dd, J = 3.6, 14.4 Hz, 1H), 3.90–3.82 (m, 3H), 3.78–3.74 (m, 2H), 3.71–3.68 (m, 1H), 3.60 (dd, J = 9.6, 12.6 Hz, 1H), 3.55 (dd, J = 4.2, 12.6 Hz, 1H), 2.71–2.68 (m, 4H), 1.73–1.60 (m, 6H), 1.46–1.40 (m, 6H), 1.35–1.31 (m, 20H), 0.92 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 163.93, 154.16, 149.21, 93.62, 79.97 (d, J = 11.7 Hz), 65.18 (d, J = 158.7 Hz), 64.40 (d, J = 5.9 Hz), 60.10, 50.05, 38.34, 38.24, 31.70, 30.71 (d, J = 6.0 Hz), 29.42, 29.40, 29.39, 29.38, 29.31, 29.25, 29.10, 28.94, 28.84, 28.83, 28.08, 27.85, 25.17, 22.36, 13.08; ^{31}P NMR (240 MHz, CD_3OD) δ 16.04. HRMS (ESI^-) *m/z*: calculated for $C_{28}H_{53}N_3O_6PS_2$ $[M-H]^-$ 622.3119, found 622.3124.

4.3. Biology

4.3.1. Cells and viruses

A549 cells and Vero cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, ExCell Bio) and 1% penicillin/streptomycin (Hyclone) at 37 °C under 5% CO_2. The AdV5 and VACV-TT used in this study were amplified in A549 cells and Vero cells maintained in DMEM supplemented with 2% FBS and 1% penicillin/streptomycin, respectively. After three to five days, viral fluid was collected by lysing virus-infected cells at −80 °C and stored in a refrigerator at the same temperature. The virus titer was based on the Karber calculation method to determine the 50% infectious dose.

4.3.2. Cytotoxicity assay

The cytotoxicity of the synthetic conjugates was estimated by the neutral red uptake assay [46]. Briefly, A549 cells and Vero cells were seeded in 96 well plates at a density of 3×10^4 cells/well and cultured at 37 °C in a 5% CO_2 incubator for 24 h, followed by exposure to the two-fold dilution of tested compounds starting at 200.0 μM for 72 h. Afterward, the supernatant was removed, followed by incubation with neutral red medium (40 μg mL^{-1}, 100μL/well) at 5% CO_2 at 37 °C for 4 h. Then the supernatant was discarded, and the cells were washed with PBS. Finally, the cells were added 100 mL/well neutral red destain solution (50% ethyl alcohol, 49% deionized water, 1% glacial acetic acid) and the OD value of neutral red extract was measured at 540 nm in a microtiter plate reader spectrophotometer (SpectraMax i3x, Molecular Devices), using blanks which contain no cells as references. The data were analyzed through GraphPad Prism 8 software to calculate the half-maximal cytotoxic concentration (CC_{50}) of compounds. The cytotoxicity experiments for each tested compound were performed in triplicate.

4.3.3. Antiviral assay

The antiviral activity of the synthetic conjugates was evaluated against AdV 5 and VACV by neutral red uptake assay [46,47]. A549 (2×10^4 cells/well) and Vero cells (3×10^4 cells/well) in 96-well flat-bottom plates were infected with a viral inoculum (100 μL/well) ranging from 10 to 100 $TCID_{50}$. Meantime, DMEM (2% FBS) containing serial three-fold dilutions of the test compounds (100 μL/well, interest at concentrations 2-fold greater than those indicated) were added in triplicate. Each experiment also set up positive drug control (**BCV**), viral control, and normal cell control group. At 72 (anti-AdV5 assay) or 96 h (anti-VACV assay), the cytopathic effect was recorded via microscopy, and then the supernatant was displaced by neutral red medium (40 μg mL^{-1}, 100 μL/well). After incubating for 4 h at 5% CO_2 at 37 °C, the supernatant was removed, and the cells were washed with PBS. Ultimately, 100 mL/well neutral red destain solution (50% ethyl alcohol, 49% deionized water, 1% glacial acetic acid) was added, and neutral red ingestion was determined using a microtiter plate reader spectrophotometer (SpectraMax i3x, Molecular Devices) at 540 nm. Half-maximal inhibitory concentration (IC_{50}) values were calculated with GraphPad Prism 8 using nonlinear regression analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2023.115601.

Abbreviations

dsDNA	double-stranded DNA
CDV	Cidofovir
VACV	vaccinia virus
SAR	structure-activity relationship
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
PHEIC	Public Health Emergency of International Concern
WHO	World Health Organization
CDC	US Centers for Disease Control and Prevention

MPXV	monkeypox virus
VARV	variola virus
AdV	adenovirus
CMV	cytomegalovirus
HHV	human herpesvirus
EBV	Epstein-Barr virus
BKV	BK virus
allo-HCT	allogeneic hematopoietic cell transplantacyclic
ANP	acyclic nucleoside phosphonate
TFV	Tenofovir
AIDS	AcquiredImmune Deficiency Syndrome
CDV-PP	cidofovir diphosphate
hOAT1	human organic anion transporter 1
LPC	lysophosphatidylcholine
HDP-CDV	hexadecyloxypropyl-cidofovir
BCV	Brincidofovir
FDA	U.S. Food and Drug Administration
TT	Tiantan strain
GSH	glutathione
TI	therapeutic index
TMSBr	bromotrimethylsilane
Vero cell	African Green Monkey kidney epithelial cell
A549 cell	human lung carcinoma cell
TDP-CDV	tetradecyloxypropyl-CDV
TEM	transmission electron microscopy
CMC	critical micellization concentration
SDS	sodium dodecyl sulfate
TLC	layer chromatography
TMS	tetramethylsilane
δ	chemical shifts
J	coupling constant
ppm	parts per million
Hz	hertz
HRMS	high-resolution mass spectra
HPLC	high-performance liquid chromatography
ATCC	American Type Culture Collection
FBS	fetal bovine serum
DMEM	Dulbecco' s modified Eagle' s medium
CC_{50}	half-maximal cytotoxic concentration
IC_{50}	half-maximal inhibitory concentration

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