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# Study of Hydrolysis Kinetics and Synthesis of Single Isomer of Phosphoramidate ProTide-Acyclovir

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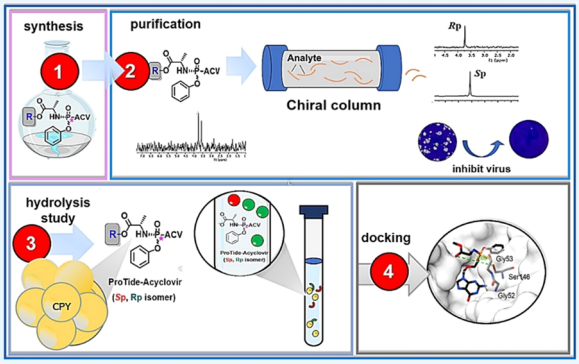
**ABSTRACT:** Acyclovir (ACV) is a vital treatment for herpes simplex (HSV) and varicella-zoster virus (VZV) infections that inhibit viral DNA polymerase. Phosphoramidate ProTides-ACV, a promising technology, circumvents the reliance on thymidine kinase (TK) for activation. Twelve novel single isomers of phosphoramidate ProTide-ACV were synthesized. Successful isomer separation was achieved, emphasizing the importance of single isomers in medical advancements. The enzymatic hydrolysis kinetics of the synthesized compounds were investigated by using carboxypeptidase Y (CPY). The results revealed a faster conversion for the isomer *Rp*- than for the *Sp*-diastereomer. Hydrolysis experiments confirmed steric hindrance effects, particularly with the *tert*-butyl and isopropyl groups. Molecular modeling elucidated the mechanisms of hydrolysis, supporting the results of the experiments. This research sheds light on the potential of phosphoramidate ProTides-ACV, bridging the gap in understanding their biological and metabolic properties, while supporting future investigations into anti-HSV activity. Preliminary screening revealed that three of the four single isomers demonstrated superior antiviral efficacy against wild-type HSV-1 compared to acyclovir, with isomer **24a** ultimately reducing the viral yield at 200 $\mu$M. These findings emphasize the importance of isolating racemic ACV-ProTides as pure single isomers for future drug development.




## 1. INTRODUCTION

Acyclovir (ACV, **1**) or acycloguanosine, an acyclic nucleoside analog of deoxyguanosine, is a pivotal medication employed in the treatment of herpes simplex (HSV) and varicella-zoster virus (VZV) infections.[1,2] The mechanism of action of ACV hinges on its unique ability to inhibit the activity of viral DNA polymerase, resulting in a marked reduction in HSV infections.[3−5] ACV becomes biologically active through a multistep process involving phosphorylation by thymidine kinase (TK) to produce 5′-monophosphate, a pivotal step in the activation process, with further conversion to 5′-di and finally to 5′-triphosphate-ACV by cellular enzymes. 5′-Triphosphate-ACV is the biologically active form that effectively inhibits the functioning of HSV viral DNA polymerase, resulting in chain termination.[6−8] Additionally, the emergence of HSV-1 mutations, often prevalent in individuals with weakened immune systems, along with the human hepatitis B virus (HBV), is a critical consideration. In previous research, there have been reports related to the mutation of HSV-1 involving two enzymes: the TK enzyme and DNA polymerase.[9−11] A significant proportion of these mutations was found to occur within the TK enzyme. As a result, these TK enzyme mutations render ACV incapable of undergoing conversion into 5′-triphosphate-ACV once it infiltrates a host cell.[12−14]

In this study, we described the ProTide approach for synthesizing a new ACV-phosphoramidate. The concept of ProTide was formally initiated by Professor Chris McGuigan's pioneering research team and has since shown great promise in enhancing its therapeutic effectiveness.[15] This technique presents an intriguing approach for generating nucleotide prodrugs with demonstrated antiviral and anticancer activities.[16,17] The structure comprises three essential components: (i) an aryl moiety, (ii) an amino acid, and (iii) esters (Figure 1). Therefore, using this method, we modified ACV analogs, to avoid the initial stage of bioactivation in cells that require the TK enzyme.

After the cellular uptake of phosphoramidates, a sequence of reactions involving esterase-catalyzed hydrolysis, ring closure, P−N bond cleavage, and phosphorylation leads to the formation of the crucial bioactive metabolite in the triphosphate form (Scheme 1).[18,19] The pathway is initiated by the production of

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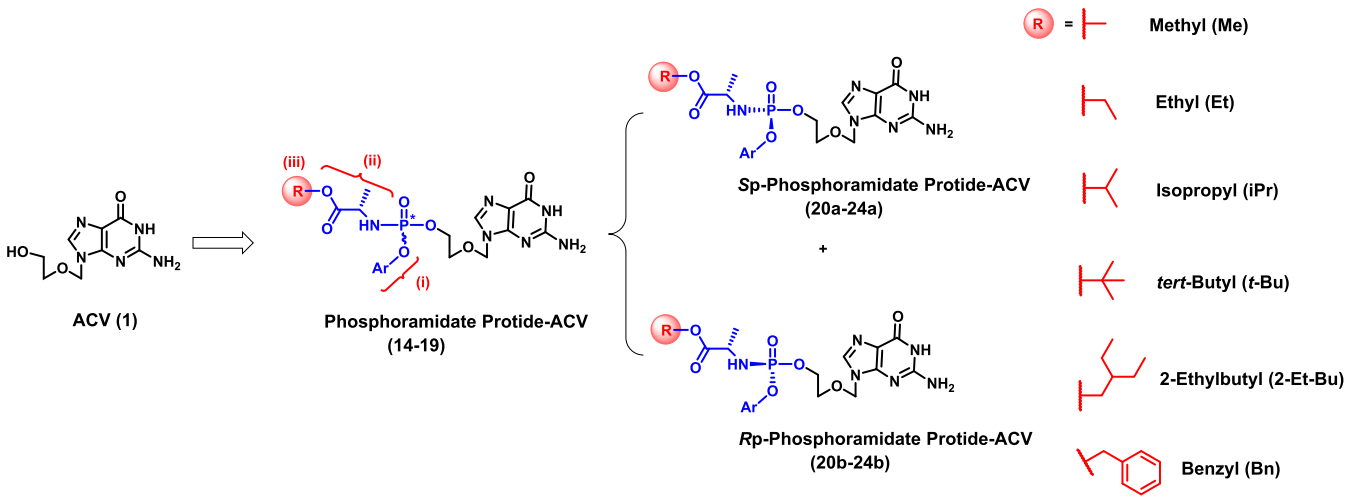
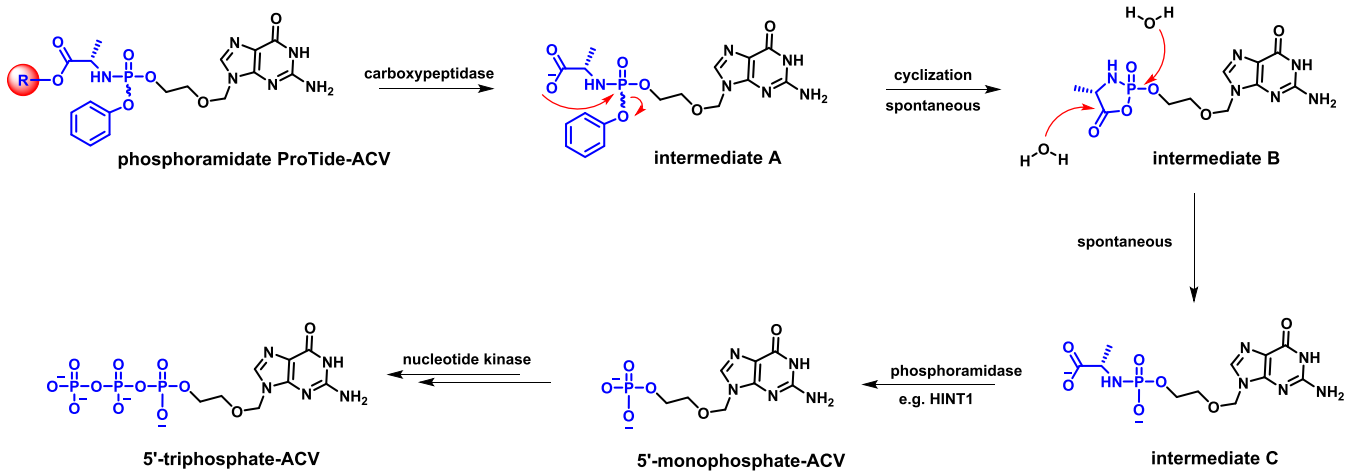



**Figure 1.** Overall structures of single *Sp*- and *Rp*-isomers of phosphoramidate ProTide-ACV.

**Scheme 1. Proposed Mechanism of Bioactivation of Phosphoramidate ProTide-ACV via Esterase and Phosphoramidase**




intermediate A, followed by the formation of an essential five-membered ring via the negative charge of oxygen to give intermediate B. Subsequently, a nucleophilic reaction yields intermediate C, a crucial precursor leading to the biologically active compound through the generation of monophosphates, diphosphates, and triphosphates. This process holds great significance, as it ensures the generation of active metabolites, highlighting the pivotal role of esterase and the hydrolysis step in the efficacy of phosphoramidate ProTide. The configurations at the phosphorus atom position, resulting in *Rp*- and *Sp*-isomers in phosphoramidate ProTides, are essential, as shown by significant variations in bioactivity, prompting in-depth research.[20] This has been successfully applied to FDA-approved drugs such as tenofovir alafenamide fumarate (TAF) and sofosbuvir for the treatment of human immunodeficiency virus (HIV) and hepatitis C virus (HCV), respectively. When evaluating potency against HIV and HCV, a comparison of the *Sp* and *Rp*-diastereomers exhibited that the *Sp*-TAF is more inhibit (10-fold) against HIV and the *Sp*-sofosbuvir is more potent (18-fold) against HCV, comparing to their corresponding *Rp*-isomer.[21−23] In addition, based on improvement of bioavailability and activity, *Sp*-remdesivir shows superior activity against the Ebola virus (EBOV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in comparison to the *Rp*-isomer.[24−27]

However, the biological activity of *Sp* is not always superior to that of the *Rp*-isomer, and there is relatively limited research explicitly studying the biological properties of *Sp* and *Rp*-phosphoramidates. Notably, reports on *Sp* or *Rp*-phosphoramidate ProTides-ACV demonstrating significant biological activity against HSV and the separation of single ProTides from a diastereomeric mixture are scarce. In general, it is extremely difficult to separate these compounds as single diastereomers using standard chromatography. Therefore, in this study, we aimed to isolate *Sp*- and *Rp*-phosphoramidate ProTides-ACV using chiral columns to investigate how the substituent groups in the ester groups of phosphoramidate affect the hydrolysis rate by using carboxypeptidase Y (CPY) as a model metabolic enzyme.

In this study, we attempted to synthesize phosphoramidate ProTide-ACVs from six different commercial L-alanine esters including methyl (Me), ethyl (Et), isopropyl (iPr), *tert*-butyl (*t*-Bu), 2-ethylbutyl (2-Et-Bu), and benzyl (Bn) ester groups. The derivatives were synthesized according to the procedure described by Siegel et al.[25] Most of the single diastereomers were separated by preparative chiral column chromatography. This study also investigated the effect of substituent groups in the ester moieties of phosphoramidate on the hydrolysis rate via carboxypeptidase Y (CPY), a metabolic enzyme, as well as some of the antiviral efficacy against wild-type HSV-1.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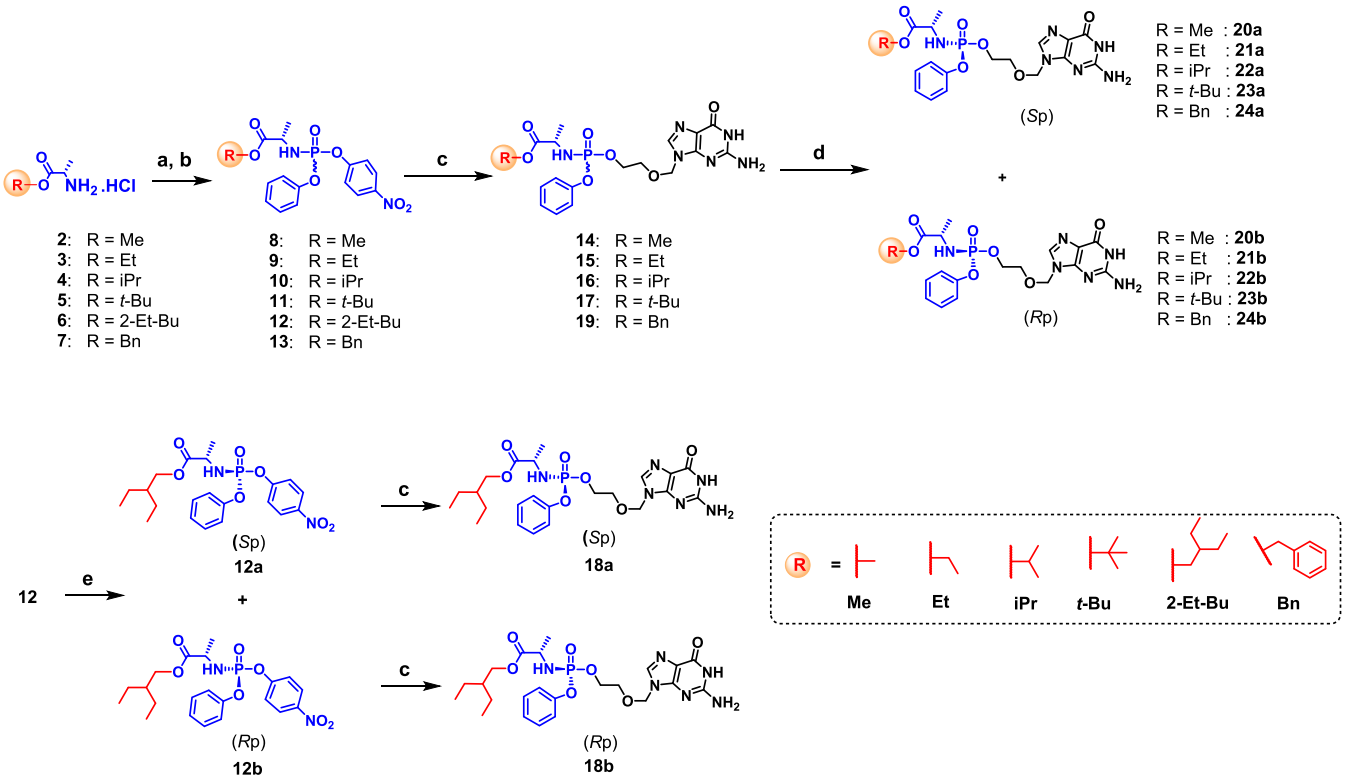
**Scheme 2. Synthesis of Phosphoramidate ProTide-ACV**[a]




[a]Reagents and conditions: (a) $PhOP(O)Cl_2$, $Et_3N$, $CH_2Cl_2$, −50 °C, 12 h; (b) 4-nitrophenol, $Et_3N$, $CH_2Cl_2$, 0 °C, 4 h, 37−48%; (c) ACV (**1**), $MgCl_2$, $iPrNEt_3$, DMF, 60 °C, 8−12 h, 18−49% for **14**−**17** and **19**; 54% for **18a**; 61% for **18b**; (d) preparative chiral chromatography; and (e) $i$-$Pr_2O$, 30% for **12a**, and preparative chiral chromatography, 60% for **12b**.

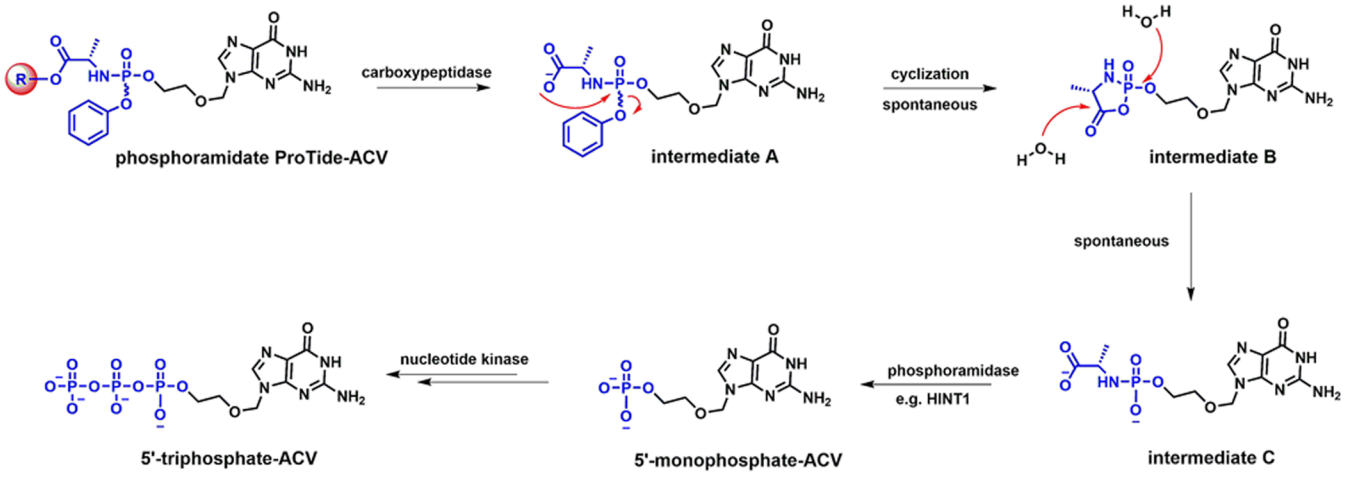



**Figure 2.** (A) Preparative chiral column chromatograms of **14**, **15**, **16**, and **19** (conditions: Lux 5 $\mu$m Cellulose-2 in 2%$H_2O$/$CH_3CN$). (B) Preparative chiral column chromatogram of **17** (conditions; Lux 5 $\mu$m i-Amylose-3 in 5%IPA/$CH_3OH$). (C) $^{31}P$ NMR ($CD_3OD$, 162 MHz) of **14** (*Rp*:*Sp* d.r. = 1.4 : 1.0) (d.r = diastereomer ratio). (D) $^{31}P$ NMR ($CD_3OD$, 162 MHz) spectra of **20b** and **20a**.

## 2. RESULTS AND DISCUSSION

**2.1. Synthesis.** The synthesis of a single *Sp* and *Rp* of phosphoramidate ProTide-ACV (**18a, 18b, 20a−24a**, and **20b−24b**) began with the synthesis of a mixture of isomers **8−13** through a two-step procedure (Scheme 2). In the first step, L-alanine ester (**2−7**) was reacted with phenyl phosphorodichloridate using triethylamine as the base at a low temperature, as monitored by $^{31}P$ NMR. The obtained crude products were subjected to the next step without purification. In the second step, 4-nitrophenol was added under the same conditions, resulting in a diastereomeric mixture at the phosphorus atom of **8−13** with yields ranging from 37 to 84%. Subsequently, racemic compounds **8−11** and **13** were synthesized through a reaction with ACV under Lewis acid conditions, using $MgCl_2$ as a catalyst, yielding compounds **14−17** and **19** with yields of 18−49% and a mixture of isomers in a 1:1 ratio of the diastereomeric mixture at the phosphorus atom.

In this study, the conformations of *Sp* and *Rp* were observed by using a resourceful strategy. We began with the purification of compounds **12a** (*Sp*-) and **12b** (*Rp*-) to afford **18a** (*Sp*-) and



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**18b** (*Rp*-), respectively. The step to obtain the desired single isomer **12** was a pivotal to assigning *Sp* and *Rp* arrangements because **12a** served as a precursor for the synthesizing remdesivir (GS-5734), a known conformation.[23] Racemic **12** was crystallized using diisopropyl ether to isolate the *Sp*-isomer following a previous procedure,[26] yielding the pure *Sp* isomer in 30% yield of **12a** ($^{31}$P NMR, downfield; see Supporting Information). However, *Rp*-isomer **12b** could not be separated by crystallization and was separated using a chiral column, Lux 5 $\mu$m i-Amylose-3 (yielding 60%, $^{31}$P NMR, upfield; see Supporting Information). These purified starting substances were then subjected to $S_N2$ substitution, resulting in pure isomers **18a** and **18b** with 54 and 61% yield, respectively (*Sp*-upfield and *Rp*-downfield in $^{31}$P NMR).

The confirmation of the chiral P atom in the phosphoramidate ProTide-ACV was conducted using $^{31}$P NMR. Previous research indicated that the chemical shift signals of the *Sp*-isomer were observed upfield, whereas those signals of the *Rp*-isomer were found downfield. This trend has been described in other ProTides analogs,[28,29] including remdesivir[24] and sofosbuvir.[21]

**2.2. Isolation of Single Isomer.** To obtain a single pure diastereomer, **14**−**17** and **19** were purified by using preparative chiral column chromatography and recrystallization. However, the purity of each compound was unsatisfactory. The successful separation of **14** was achieved by utilizing a cellulose-2 chiral column with 2% water in an acetonitrile eluent, yielding 22% **20a** and 83% **20b** with a resolution of 1.27 (Figure 2A). Likewise, the separation of **15** and **19** resulted in a high yield of the single isomer with a resolution greater than 1. Notably, **16** was effectively separated, even though the resolution was less than 1. In contrast, **17** was applied to the cellulose-2 chiral column; however, the separation was ineffective, leading to a transition to amylose-3 (Figure 2B). Separation was achieved with 5% isopropyl alcohol in methanol eluent, demonstrated by a resolution of 1 (Table 1). However, **18a** and **18b** were not obtained by chiral chromatography; these compounds were alternatively prepared starting from the single diastereomer **12a** and **12b** rather than the diastereomer mixture **12.** The pure isomers were examined by the $^{31}$P NMR spectrum (Figure 2C,2D).

**Table 1. Percentage of Yield of All Single Isomers from Chiral Chromatography and Selectivity Factor ($\alpha$), and Resolution ($R_s$) of Single Isomer Phosphoramidate ProTide-ACV**

| mixture isomer ProTide-ACVs | ester groups (*R*) | $\alpha$ | $R_s$ | single isomer ProTide-ACVs | % yield of single isomer[a] |
|---|---|---|---|---|---|
| **14** | methyl | 1.07 | 1.27[b] | **20a** | 22 |
| | | | | **20b** | 83 |
| **15** | ethyl | 1.13 | 1.64[b] | **21a** | 76 |
| | | | | **21b** | 88 |
| **16** | isopropyl | 1.07 | 0.78[b] | **22a** | 57 |
| | | | | **22b** | 47 |
| **17** | *tert*-butyl | 1.17 | 1.00[c] | **23a** | 73 |
| | | | | **23b** | 53 |
| **19** | benzyl | 1.14 | 1.25[b] | **24a** | 80 |
| | | | | **24b** | 60 |

[a]Yield from the isolated method. [b]Lux 5 $\mu$m Cellulose-2 in water:acetonitrile (2:98) as a mobile phase. [c]Lux 5 $\mu$m i-Amylose-3 in isopropyl alcohol:methanol (5:95) as a mobile phase.

From chiral chromatography part, the distinctive structural features of cellulose and amylose in the column were essential factors influenced the separation between *Rp*- and *Sp*-isomers.[30−32] Specifically, **14** and **15**, featuring methyl and ethyl groups, respectively, showed no structural hindrance, allowing effective separation on the cellulose column. However, **16**, with increased steric hindrance, exhibited a reduced separation efficiency and resolution on the cellulose column. Compound **17**, characterized by significant steric hindrance, could not be separated using cellulose, but demonstrated successful separation using amylose. The inability to separate **18a** and **18b** may be attributed to the excessive steric hindrance of the 2-ethylbutyl group, which prevented the successful separation in both column types.

**2.3. *In Vitro* Enzymatic Hydrolysis.** All single diastereomers were determined *in vitro* using the enzymatic hydrolysis of ester bonds by CPY. The hydrolysis pattern employed the $^{31}$P NMR technique to monitor the phosphoramidate ProTides-ACV, intermediate A, and intermediate C. In our experimental findings, most *Rp*-isomers were reported to be significantly more favorable to enzymes than *Sp*-isomers such as **20a, 20b** and **22a, 22b**, as shown in Figure 3. The isomers were then converted to intermediates A and C. Furthermore, the enzymatic mechanism demonstrated its ability to rapidly catalyze the hydrolysis reaction at the ester bond of both *Rp*- and *Sp*-isomers with an ethyl group (**21a** and **21b**), a 2-ethylbutyl group (**18a, 18b**), and a benzyl group (**24a** and **24b**). The appearance rates for intermediate C of these compounds were approximately greater than 1 Δ/min (Table 2). However, compounds with very small groups like methyl groups (**20a** and **20b**) displayed an appearance rate for intermediate C, which was less than that of the ethyl and benzyl groups. Notably, compounds with carbon extending from ethyl to 2-ethylbutyl were correlated with a reduction in the appearance rate of intermediate C, as outlined in Table 2. This decline was attributed to heightened steric hindrance, mainly observed with the *tert*-butyl groups (**23a** and **23b**) that did not undergo metabolism.

**2.4. Docking Studies.** To better understand the enzymatic results, molecular modeling studies were performed for these single isomers at the binding site of CPY (PDB entry 1YSC). According to Jung et al. and Dobó et al.,[33,34] ester hydrolysis requires the carbonyl moiety in the enzyme pocket to be suitably positioned for nucleophilic attack from the catalytic residue Ser146. The results showed that the carbonyl group of the *Rp*-isomers was closer to Ser146 than to that of the *Sp*-isomers, as observed for compounds **22a** and **22b** (Figure 4); more details are provided in the Supporting Data. Despite this, the binding affinities of all compounds were within the same range, approximately −7.3 to −8.6 kcal/mol. The oxyanion hole, including the backbone amides Gly53 and Tyr147,[35] was in the proper position in the *Rp*-isomers to stabilize the tetrahedral intermediate formed during the process, rather than in the *Sp*-isomers. Moreover, Tyr147 formed a $\pi-\pi$ stacking interaction with the phenyl group in the core structure of the *Sp*-isomers, leading to a loss of stabilization from the oxyanion hole during the process, while Ser146 exhibited an H-bond to the carbonyl group of the *Rp*-isomers. These findings support a faster ester-hydrolysis process of the *Rp*-isomers of phosphoramidate ProTide-ACVs against CPY because of the appropriate orientation of the carbonyl group and key residues in the binding pocket. Unexpectedly, the benzyl compounds showed a rapid hydrolysis profile for the *Rp*- and *Sp*-isomers. The reason might have resulted from a charge-relay system of geometrical positioning of Ser146, His397, and Asp338 which were close to the benzene ring and caused hydrolysis.[33,35]


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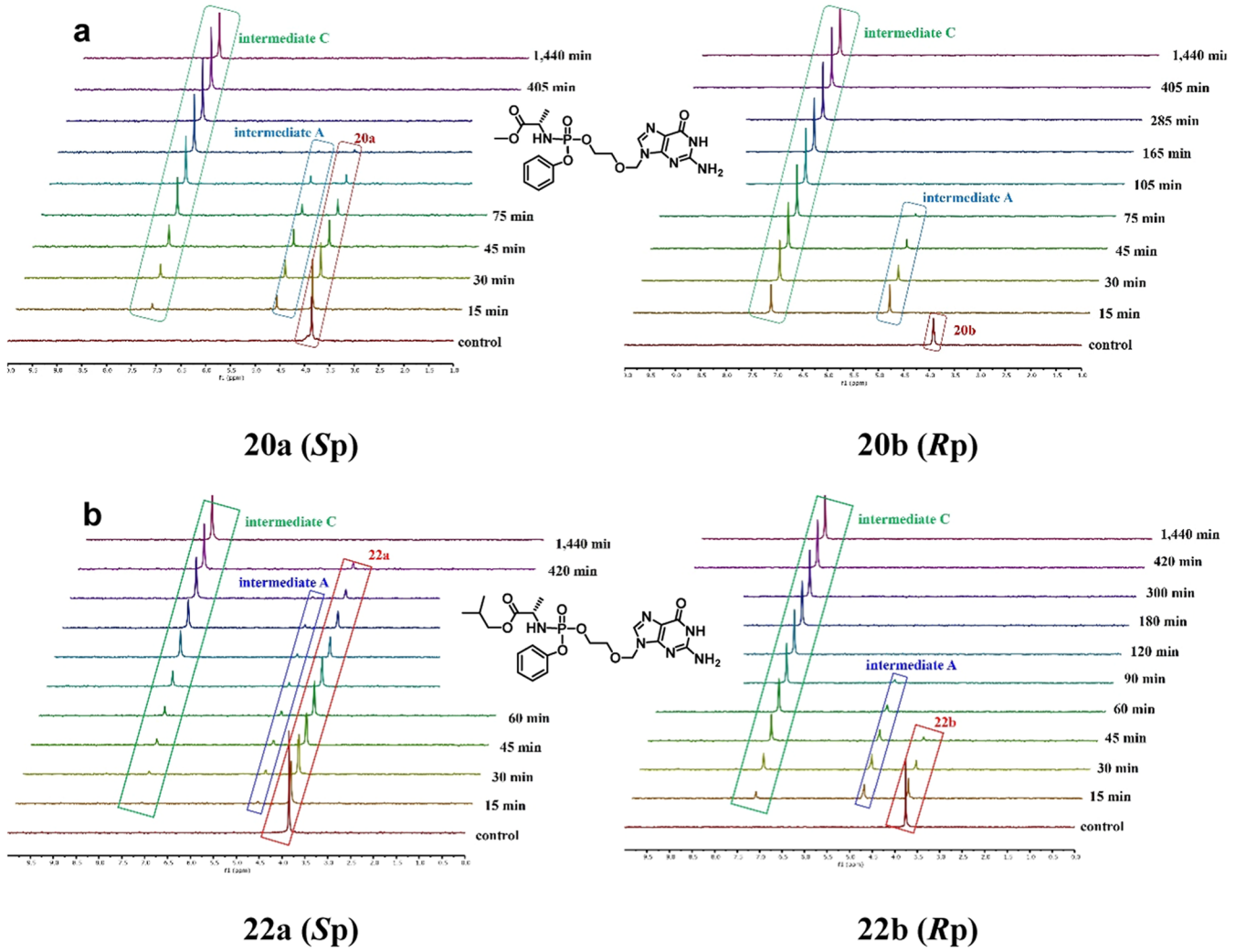


**Figure 3.** Enzymatic hydrolysis study of **20a**, **20b** (a) and **22a**, **22b** (b) in the presence of CPY determined by $^{31}$P NMR.

**Table 2. Summary of Enzymatic Hydrolysis Results of the Disappearance Rate of Single Isomers ProTide-ACVs and Formation Rate of Intermediate C, against CPY**

| single isomer ProTide-ACVs | *R* groups | disappearance rate of single isomers (Δ/min) | appearance rate of intermediate C (Δ/min) |
|---|---|---|---|
| **20a** | methyl | 0.60 | 0.35 |
| **20b** | | >6.67 | 0.95 |
| **21a** | ethyl | 6.67 | 1.67 |
| **21b** | | 6.67 | 1.11 |
| **22a** | isopropyl | 0.24 | 0.069−0.24 |
| **22b** | | 2.22 | 0.83 |
| **23a** | *tert*-butyl | <0.069 | <0.069 |
| **23b** | | 0.69−0.24 | 0.069−0.24 |
| **18a** | 2-ethylbutyl | >6.67 | 1.11 |
| **18b** | | 3.33−6.67 | 0.83 |
| **24a** | benzyl | >6.67 | 1.67 |
| **24b** | | >6.67 | 1.67 |

**2.5. Antiviral Activities.** Four single isomers, **20a**, **20b**, **24a**, and **24b**, were selected to assess their antiviral efficacy against wild-type HSV-1 (KOS strain, VR-1493) in the A549 lung cancer cell line, a permissive cell line for HSV infection and isolation.[36] Following a 2-h inoculation of cells with wild-type HSV1 and subsequent removal of the inoculum, the infected cells were treated with ACV or isomer compounds by substituting the culture media with or without the specified compounds at 100 and 200 $\mu$M concentrations. The antiviral activities were assessed by measuring the viral yield at 2 days postinfection (dpi) compared to the untreated infected cells. Figure 5 illustrates that ACV treatment at a dose of 200 $\mu$M significantly diminished viral production by nearly one log10 in comparison to the untreated cells. Among the four single isomers, three isomers—**20a**, **24a**, and **24b**—exhibited superior antiviral activities at a concentration of 200 $\mu$M compared to ACV treatment at the same concentration, as evidenced by a viral yield reduction of approximately two log10 for the 200 $\mu$M treatments of **20a** and **24b**, and a complete reduction of viral yield for the 200 $\mu$M treatment of **24a**. These results indicated that pure single diastereomers provide different distinct bioactivities. This preliminary biological function study facilitated our comprehension of the significance of isolating racemic ACV-ProTides to a pure single isomer, potentially rendering them a promising candidate for drug development.

## 3. CONCLUSIONS

In summary, we successfully separated all of the single diastereomers of ProTide-ACV, marking this the first successful attempt. The enzymatic hydrolysis kinetics study of phosphoramidate ProTides-ACV revealed that *R*p-isomer was more ester


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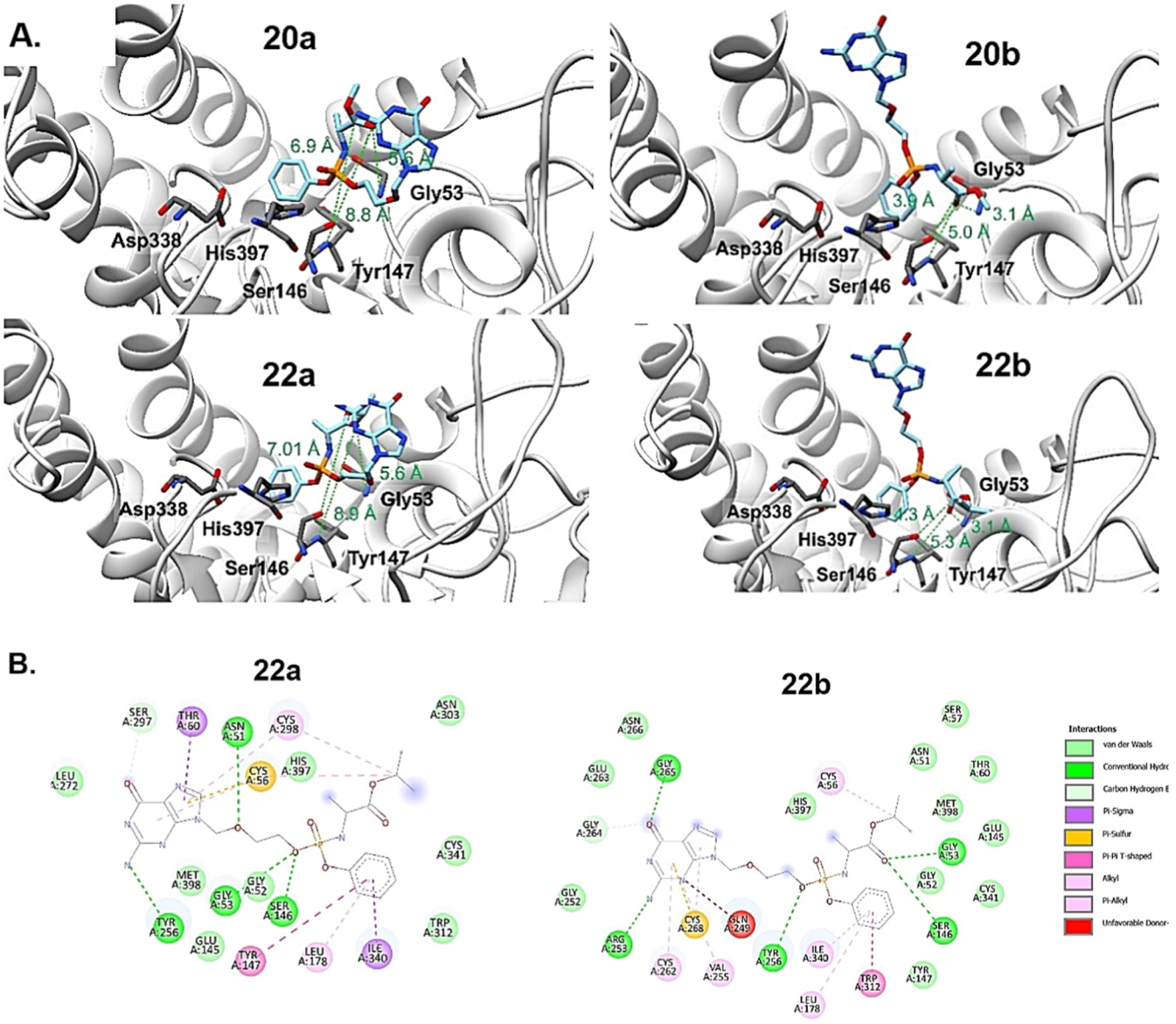


**Figure 4.** Representative poses from molecular docking of **20a**, **20b**, **22a**, and **22b** in the binding site of CPY, interacting with Ser146, Gly53, and Tyr147. The distances between the compound and the key residues are shaded in green. The backbone of CPY is shown as a gray ribbon representation (A). The two-dimensional (2D) interactions of **22a** and **22b** with amino acids of CPY (PDB ID: 1YSC) from molecular docking (B).

hydrolyzed than *Sp*-isomer. The structure with *tert*-butyl and isopropyl groups showed significant steric hindrance and resulted in slower hydrolysis compared to that with the ethyl, methyl, 2-ethylbutyl, and benzyl groups. Enzymatic docking studies of *Sp*- and *Rp*-isomers also showed conformation with most *Rp*-isomers consistently exhibiting favorable complexes with proteins than *Sp*-isomers. Taken together, the hydrolysis of *Rp*-isomers via CPY is preferred over that of the *Sp*-isomers. Recognizing the importance of single isomers, we successfully separated most of them using chiral column chromatography. Future research should explore the biological properties of the separated isomers, particularly their anti-HSV and other biological activities. The current study underscores the significance of successfully separating single isomers of phosphoramidate ProTides-ACV, as isomer **24a** demonstrated superior antiviral activity against wild-type HSV-1, ultimately reducing the viral yield at 200 $\mu$M and highlighting the potential of pure single isomers in future drug development.

## 4. EXPERIMENTAL SECTION

**4.1. Materials and Methods.** All chemicals were purchased from Sigma-Aldrich Fluka, Merck, and Acros Organic and used as received without further purification. $^{1}$H, $^{13}$C, and $^{31}$P NMR spectra were reported in ppm and recorded on a Bruker Mercury-400 plus or Bruker Avance 400 NMR spectrometer operating at 500 MHz $^{1}$H, 126 and 101 MHz $^{13}$C, and 162 and 202 MHz for $^{31}$P NMR. Ultra-performance flash purification chromatography (UPFP), Interchim Generation 5-PF-5.125 were separated by preparative chiral column chromatography using Lux 5 $\mu$m Cellulose-2, column size 250 mm × 21.2 mm, and Lux 5 $\mu$m i-Amylose-3, LC column 250 mm × 21.2 mm. Infrared (IR) spectra were recorded on an $\alpha$ FR-IR spectrometer. The ESI technique was used to record high-resolution mass spectrometry (HRMS) on a micrOTOF mass spectrometer.

*4.1.1. General Synthesis of Intermediates (**8**−**13**).* A suitable solution of L-alanine ester (**2**−**7**, 1.0 equiv) in dichloromethane


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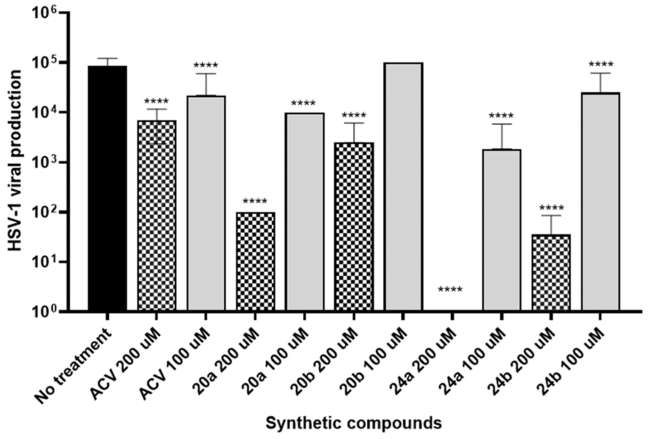


**Figure 5.** Level of viral production in wild-type HSV-1 infected cells, treated with or without synthetic compounds, is indicated. The viral yields were assessed after 2 dpi by determining the highest dilution of the collected media for each sample that exhibited cell lysis on Vero cells. Each bar indicates the mean viral production in each group, and the error bar represents the standard deviation. Antiviral efficacies were assessed by comparing the reduction of viral yields to those of untreated infected cells. Statistical analysis was performed using one-way ANOVA, followed by Tukey's multiple comparisons test in GraphPad Prism 10. **** symbolizes significant differences in comparison to the nontreatment group ($P < 0.0001$).

was added to a chilled solution of phenyl phosphorodichloridate (1.0 equiv) in dichloromethane and stirred under a nitrogen atmosphere. Triethylamine (2.0 equiv) was added dropwise at −50 °C. The mixture was stirred until −50 °C for 12 h or until the reaction was complete, as monitored by $^{31}$P NMR. Without purification, the resulting solution was further reacted with 4-nitrophenol (1.0 equiv). Triethylamine (1.0 equiv) was added dropwise under a nitrogen atmosphere and stirred at 0 °C until room temperature. The reaction was monitored by TLC with a suitable eluent (5−10% MeOH in $CH_2Cl_2$). After 12 h, the reaction was diluted with ethyl acetate (100 mL). The organic layer was washed with 5−10% bicarbonate (×5). The organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was purified by flash column chromatography using a suitable gradient (5−10% MeOH in $CH_2Cl_2$) for elution to afford intermediates **8**−**13**.

*4.1.2. Methyl [(4-Nitrophenoxy)(phenoxy)phosphoryl]-L-alaninate (**8**).* White solid (12.50 g, 65.6% yield, ∼1:1 diastereomeric mixture at phosphorus); $R_f$ = 0.85 (10% MeOH/$CH_2Cl_2$); $^1$H NMR (DMSO-$d_6$, 500 MHz): $\delta$ (ppm) 8.32 (d, $J$ = 1.7 Hz, 1H), 8.30 (d, $J$ = 1.7 Hz, 1H), 7.51 (d, $J$ = 9.0 Hz, 1H), 7.46 (d, $J$ = 9.0 Hz, 1H), 7.42 (dd, $J$ = 13.3, 7.4 Hz, 2H), 7.28 (d, $J$ = 8.6 Hz, 1H), 7.24 (d, $J$ = 7.9 Hz, 2H), 6.71 (dd, $J$ = 24.2, 10.8 Hz, 1H), 4.06−3.95 (m, 1H), 3.55 (d, $J$ = 6.3 Hz, 3H), 1.23 (dd, $J$ = 6.9, 3.8 Hz, 3H); $^{31}$P NMR (DMSO-$d_6$, 202 MHz): $\delta$ (ppm) −1.40 (*Sp*), −1.62 (*Rp*).

*4.1.3. Ethyl [(4-Nitrophenoxy)(phenoxy)phosphoryl]-L-alaninate (**9**).* White solid (2.38 g, 37.0% yield, ∼1:1 diastereomeric mixture at phosphorus); $R_f$ = 0.86 (10% MeOH/$CH_2Cl_2$); $^1$H NMR (DMSO-$d_6$, 500 MHz): $\delta$ (ppm) 8.30 (d, $J$ = 9.1 Hz, 2H), 7.50 (t, $J$ = 6.2 Hz, 1H), 7.46 (d, $J$ = 9.1 Hz, 1H), 7.41 (ddd, $J$ = 7.3, 4.8, 2.0 Hz, 2H), 7.28 (d, $J$ = 8.6 Hz, 1H), 7.23 (dd, $J$ = 9.3, 8.3 Hz, 2H), 6.70 (dt, $J$ = 13.6, 9.9 Hz, 1H), 4.05−3.94 (m, 3H), 1.23 (dd, J = 6.9, 3.2 Hz, 3H), 1.11 (td, $J$ = 7.1, 1.6 Hz, 3H); $^{31}$P NMR (DMSO-$d_6$, 202 MHz): $\delta$ (ppm) −1.41 (*Sp*), −1.60 (*Rp*).

*4.1.4. Isopropyl [(4-Nitrophenoxy)(phenoxy)phosphoryl]-L-alaninate (**10**).* Yellow oil (7.1 g, 58.0% yield, ∼1:1 diastereomeric mixture at phosphorus); $R_f$ = 0.88 (10% MeOH/$CH_2Cl_2$); $^1$H NMR (DMSO-$d_6$, 500 MHz): $\delta$ (ppm) 8.30 (d, $J$ = 9.1 Hz, 2H), 7.51 (d, $J$ = 9.1 Hz, 1H), 7.46 (d, $J$ = 9.0 Hz, 1H), 7.44−7.38 (m, 2H), 7.28 (d, $J$ = 8.3 Hz, 1H), 7.23 (d, $J$ = 8.2 Hz, 2H), 6.73−6.63 (m, 1H), 4.89−4.78 (m, 1H), 3.98−3.88 (m, 1H), 1.25−1.20 (m, 3H), 1.12 (dd, $J$ = 6.2, 2.5 Hz, 3H); $^{31}$P NMR (DMSO-$d_6$, 202 MHz): $\delta$ (ppm) −1.41 (*Sp*), −1.57 (*Rp*).

*4.1.5. tert-Butyl [(4-Nitrophenoxy)(phenoxy)phosphoryl]-L-alaninate (**11**).* Yellow oil (6.42 g, 70.6% yield, ∼1:1 diastereomeric mixture at phosphorus); $R_f$ = 0.80 (10% MeOH/$CH_2Cl_2$); $^1$H NMR (DMSO-$d_6$, 500 MHz): $\delta$ (ppm) 8.30 (d, $J$ = 8.9 Hz, 2H), 7.51 (d, $J$ = 9.0 Hz, 1H), 7.46 (d, $J$ = 9.1 Hz, 1H), 7.41 (td, $J$ = 8.3, 3.7 Hz, 2H), 7.27 (d, $J$ = 8.6 Hz, 1H), 7.24 (d, $J$ = 7.9 Hz, 2H), 6.60 (dt, $J$ = 13.6, 9.6 Hz, 1H), 3.83 (dt, $J$ = 14.3, 7.1 Hz, 1H), 1.34 (s, 9H), 1.19 (d, $J$ = 8.3 Hz, 3H); $^{31}$P NMR (DMSO-$d_6$, 202 MHz): $\delta$ (ppm) −1.34 (*Sp*), −1.51 (*Rp*).

*4.1.6. 2-Ethylbutyl [(4-Nitrophenoxy)(phenoxy)-phosphoryl]-L-alaninate (**12**).* Yellow oil (12.90 g, 70.6% yield, ∼1:1 diastereomeric mixture at phosphorus); $R_f$ = 0.85 (10% MeOH/$CH_2Cl_2$); $^1$H NMR (DMSO-$d_6$, 500 MHz): $\delta$ (ppm) 8.30 (d, $J$ = 9.1 Hz, 2H), 7.50 (d, $J$ = 9.1 Hz, 1H), 7.45 (d, $J$ = 9.1 Hz, 1H), 7.44−7.38 (m, 2H), 7.27 (d, $J$ = 7.9 Hz, 1H), 7.23 (d, $J$ = 7.6 Hz, 2H), 6.70 (dd, $J$ = 23.1, 12.3 Hz, 1H), 4.05−3.95 (m, 1H), 3.95−3.88 (m, 2H), 1.44−1.35 (m, 1H), 1.24 (dd, $J$ = 13.8, 8.0 Hz, 7H), 0.79 (t, $J$ = 7.4 Hz, 6H); $^{31}$P NMR (DMSO-$d_6$, 202 MHz): $\delta$ (ppm) −1.40 (*Sp*), −1.63 (*Rp*).

To provide the single *Sp* isomer **12a**, and the single *Rp*-isomer **12b**, the mixture of isomers **12** was separated by crystallization in *i*-$PrO_2$ and was then separated using preparative chiral column chromatography (chiral column, Lux 5 $\mu$m i-Amylose-3, LC Column 250 mm × 4.6 mm, mobile phase ratio 5−10% of methanol in acetonitrile).

*4.1.6.1. **12a**.* White solid (0.20 g, 30.0% yield, *Sp* isomer) $^1$H NMR (DMSO-$d_6$, 500 MHz): $\delta$ (ppm) 8.32−8.25 (m, 2H), 7.50 (d, $J$ = 8.8 Hz, 2H), 7.43−7.37 (m, 2H), 7.26−7.19 (m, 3H), 6.73−6.66 (m, 1H), 4.07−3.96 (m, 1H), 3.92 (d, $J$ = 5.6 Hz, 2H), 1.43−1.35 (m, 1H), 1.30−1.17 (m, 7H), 0.79 (t, $J$ = 7.4 Hz, 6H); $^{31}$P NMR (DMSO-$d_6$, 202 MHz): $\delta$ (ppm) −1.40 (*Sp*).

*4.1.6.2. **12b**.* Viscous liquid (0.30 g, 60.0% yield, *Rp*-isomer) $^1$H NMR (DMSO-$d_6$, 500 MHz): $\delta$ (ppm) $^1$H NMR (500 MHz, $CD_3OD$) $\delta$ (ppm) 8.29 (d, $J$ = 9.1 Hz, 2H), 7.44 (d, $J$ = 8.6 Hz, 2H), 7.40 (t, $J$ = 7.9 Hz, 2H), 7.29 (d, $J$ = 8.4 Hz, 2H), 7.24 (t, $J$ = 7.3 Hz, 2H), 4.06 (dt, $J$ = 14.4, 7.2 Hz, 1H), 4.01 (d, $J$ = 5.7 Hz, 2H), 1.46 (dt, $J$ = 12.4, 6.3 Hz, 1H), 1.33 (dq, $J$ = 17.4, 8.0 Hz, 7H), 0.87 (t, $J$ = 7.4 Hz, 6H); $^{31}$P NMR ($CD_3OD$, 202 MHz): $\delta$ (ppm) −1.74 (*Rp*).

*4.1.7. Benzyl [(4-Nitrophenoxy)(phenoxy)phosphoryl]-L-alaninate (**13**).* Yellow oil (8.96 g, 84.7% yield, ∼1:1 diastereomeric mixture at phosphorus); $R_f$ = 0.85 (10% MeOH/$CH_2Cl_2$); $^1$H NMR (DMSO-$d_6$, 500 MHz): $\delta$ (ppm) 8.25 (dd, $J$ = 9.3, 2.8 Hz, 2H), 7.47 (d, $J$ = 9.0 Hz, 1H), 7.43−7.36 (m, 3H), 7.36−7.28 (m, 5H), 7.28−7.18 (m, 3H), 6.78 (td, $J$ = 13.7, 10.2 Hz, 1H), 5.11−5.03 (m, 2H), 4.13−4.03 (m, 1H), 1.25 (t, $J$ = 6.3 Hz, 3H); $^{31}$P NMR (DMSO-$d_6$, 202 MHz): $\delta$ (ppm) −1.39 (*Sp*), −1.67 (*Rp*).

*4.1.8. General Synthesis of Phophoramidate ACV-ProTides (**14**−**17** and **19**).* Dimethylformamide was added to a mixture of **8**−**11** and **13** (1.5 equiv), ACV (**1**, 1.0 equiv), and $MgCl_2$ (1.5 equiv) at room temperature. The solution was heated for 10


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min, and *N,N*-diisopropyl ethylamine (2.5 equiv) was added and stirred at the same temperature. The reaction was monitored using TLC chromatography. After overnight, the reaction was allowed to cool at room temperature, and then diluted with ethyl acetate (100 mL). The organic layer was washed with 5% citric acid (40 mL), saturated $NH_4Cl$ (40 mL), 5% $K_2CO_3$ (2 mL× 40 mL), and brine (40 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was purified by flash column chromatography using suitable gradient elution of MeOH/$CH_2Cl_2$ to give racemic **14**−**17**, **19** and monitored by $^1$H NMR to examine the desired product. The ratio of *Rp*/*Sp* isomers was determined by $^{31}$P NMR.

*4.1.9. Acyclovir-[1-phenoxy(methoxy-L-alaninyl)]-phosphoramidate (**14**).* White solid (197.3 mg, 28.2% yield); $^1$H NMR (DMSO-$d_6$, 500 MHz): $\delta$ (ppm) 10.63 (s, 1H), 7.80 (d, $J$ = 13.7 Hz, 1H), 7.35 (t, $J$ = 7.2 Hz, 2H), 7.15 (dd, $J$ = 15.7, 8.2 Hz, 3H), 6.50 (s, 2H), 6.01−5.88 (m, 1H), 5.35 (d, $J$ = 10.0 Hz, 2H), 4.05−3.80 (dt, $J$ = 16.7, 6.1 Hz, 2H), 3.71−3.62 (m, 1H), 3.57 (d, $J$ = 5.9 Hz, 3H), 1.19 (dd, $J$ = 14.4, 7.1 Hz, 3H); $^{31}$P NMR (DMSO-$d_6$, 202 MHz): $\delta$ (ppm) 3.74 (*Rp*), 3.51 (*Sp*).

*4.1.10. Acyclovir-[1-phenoxy(ethoxy-L-alaninyl)]-phosphoramidate (**15**).* White solid (261.8 mg, 36.4% yield); $^1$H NMR (DMSO-$d_6$, 500 MHz): $\delta$ (ppm) 10.94 (s, 1H), 7.80 (d, $J$ = 7.4 Hz, 1H), 7.34 (t, $J$ = 7.6 Hz, 2H), 7.16−7.06 (m, 3H), 6.83 (s, 2H), 5.95 (dd, $J$ = 22.7, 11.7 Hz, 1H), 5.35 (d, $J$ = 10.4 Hz, 2H), 4.04 (dd, $J$ = 22.0, 14.7 Hz, 4H), 3.68 (ddd, $J$ = 45.6, 29.4, 7.5 Hz, 4H), 1.34−1.24 (m, 3H), 1.22−1.05 (m, 3H); $^{31}$P NMR (DMSO-$d_6$, 202 MHz): $\delta$ (ppm) 3.76 (*Rp*), 3.55 (*Sp*).

*4.1.11. Acyclovir-[1-phenoxy(isopropyloxy-L-alaninyl)]-phosphoramidate (**16**).* White solid (299.0 mg, 40.3% yield); $^1$H NMR (DMSO-$d_6$, 500 MHz): $\delta$ (ppm) 10.62 (s, 1H), 7.81 (d, $J$ = 7.4 Hz, 1H), 7.34 (t, $J$ = 7.7 Hz, 2H), 7.15 (dd, $J$ = 15.5, 8.0 Hz, 3H), 6.50 (s, 2H), 5.90 (dd, $J$ = 26.1, 12.1 Hz, 1H), 5.36 (d, $J$ = 9.9 Hz, 2H), 4.84 (dq, $J$ = 12.3, 6.2 Hz, 1H), 4.13−3.97 (m, 2H), 3.82−3.60 (m, 3H), 1.16 (dt, $J$ = 13.1, 6.9 Hz, 9H); $^{31}$P NMR (DMSO-$d_6$, 202 MHz): $\delta$ (ppm) 3.79 (*Rp*), 3.61 (*Sp*).

*4.1.12. Acyclovir-[1-phenoxy(tert-butyloxy-L-alaninyl)]-phosphoramidate (**17**).* White solid (494.1 mg, 48.6% yield); $^1$H NMR (DMSO-$d_6$, 500 MHz): $\delta$ (ppm) 10.65 (s, 1H), 7.80 (d, $J$ = 6.9 Hz, 1H), 7.34 (t, $J$ = 7.7 Hz, 2H), 7.27−7.09 (m, 3H), 6.52 (s, 2H), 5.96−5.72 (m, 1H), 5.36 (d, $J$ = 8.5 Hz, 2H), 4.18−3.95 (m, 2H), 3.76−3.52 (m, 2H), 1.36 (d, $J$ = 5.4 Hz, 9H), 1.28−1.11 (m, 3H); $^{31}$P NMR (DMSO-$d_6$, 202 MHz): $\delta$ (ppm) 3.82 (*Rp*), 3.69 (*Sp*).

*4.1.13. Acyclovir-[1-phenoxy(2-ethylbutyl-L-alaninyl)] (S)-Phosphoramidate (**18a**).* White solid (130.0 mg, 54.1% yield); $^1$H NMR (DMSO-$d_6$, 500 MHz): $\delta$ (ppm) 10.66 (s,1H), 7.80 (s, 1H), 7.35 (d, $J$ = 8.9 Hz, 2H), 7.16 (d, $J$ = 7.2 Hz, 3H), 6.52 (s, 2H), 5.96 (dd, $J$ = 13.0, 10.1 Hz, 2H), 5.34 (s, 2H), 4.10−4.00 (m, 2H), 3.96 (dd, $J$ = 10.9, 5.8 Hz, 1H), 3.90 (dd, $J$ = 10.9, 5.7 Hz, 1H), 3.87−3.77 (m, 1H), 3.65 (t, $J$ = 4.7 Hz, 2H), 1.44 (p, $J$ = 6.1 Hz, 1H), 1.31−1.20 (m, 7H), 0.81 (t, $J$ = 7.5 Hz, 6H). $^{31}$P NMR (202 MHz, DMSO-$d_6$): $\delta$ (ppm) 3.61. HRMS (AP-ESI) $m/z$ calcd for $C_{23}H_{33}N_6O_7P$ $[M + Na]^+$ 559.2046, found 559.2045.

*4.1.14. Acyclovir-[1-phenoxy(2-ethylbutyl-L-alaninyl)] (R)-Phosphoramidate (**18b**).* White solid (91.9 mg, 61.2%yield); $^1$H NMR (400 MHz, $CD_3OD$): $\delta$ 7.84 (s, 1H), 7.41−7.27 (m, 2H), 7.17 (ddd, $J$ = 8.8, 7.2, 1.1 Hz, 3H), 5.48 (s, 2H), 4.27−4.15 (m, 2H), 4.07−4.01 (m, 2H), 4.06−3.86 (m, 1H), 3.80 (td, $J$ = 4.6, 1.3 Hz, 2H), 1.49 (dt, $J$ = 12.3, 6.1 Hz, 1H), 1.40−1.26 (m, 8H), 0.88 (t, $J$ = 7.5 Hz, 6H). $^{31}$P NMR (162 MHz, $CD_3OD$): $\delta$ (ppm) 3.78. HRMS (AP-ESI) $m/z$ calcd for $C_{23}H_{33}N_6O_7P$ $[M + Na]^+$ 559.2046, found 559.2058.

*4.1.15. Acyclovir-[1-phenoxy(benzyloxy-L-alaninyl)]-phosphoramidate (**19**).* White solid (494.1 mg, 48.5%yield); $^1$H NMR (DMSO-$d_6$, 500 MHz): $\delta$ (ppm) 10.63 (s, 1H), 7.80 (d, $J$ = 8.3 Hz, 1H), 7.37−7.27 (m, 7H), 7.18−7.09 (m, 3H), 6.50 (s, 2H), 6.05−5.94 (m, 1H), 5.34 (d, $J$ = 10.4 Hz, 2H), 5.12−5.05 (m, 2H), 4.09−3.96 (m, 2H), 3.92−3.80 (m, 1H), 3.67−3.59 (m, 2H), 1.22 (dd, $J$ = 15.5, 7.1 Hz, 3H). $^{31}$P NMR (DMSO-$d_6$, 202 MHz): $\delta$ 3.80 (*Rp*), 3.54 (*Sp*).

*4.1.15.1. Protocol for Separation of Single Phosphoramidate ProTides-ACV from **14**−**17** and **19** Using Preparative Chiral Column Chromatography.* **14** (90.0 mg) was separated by preparative chiral column chromatography to obtain a single *Sp* isomer of **20a** (10.1 mg, 22.4%) and single *Rp*-isomer of **20b** (37.2 mg, 82.6%).

*4.1.16. Acyclovir-[1-phenoxy(methoxy-L-alaninyl)] (S)-Phosphoramidate (**20a**).* Amorphous white solid; $^1$H NMR ($CD_3OD$, 500 MHz): $\delta$ (ppm) 7.84 (s, 1H), 7.33 (dd, $J$ = 8.5, 7.3 Hz, 2H), 7.17 (d, $J$ = 7.4 Hz, 3H), 5.46 (s, 2H), 4.20−4.15 (m, 2H), 3.92 (dq, $J$ = 9.8, 7.1 Hz, 1H), 3.79−3.76 (m, 2H), 3.67 (s, 3H), 1.33 (dd, $J$ = 7.1, 0.8 Hz, 3H). $^{13}$C NMR ($CD_3OD$, 126 MHz): $\delta$ 175.46, 159.34, 155.67, 153.41, 152.23, 139.76, 130.71, 126.05, 121.42, 121.38, 117.50, 73.67, 69.38, 66.97, 52.76, 51.42, 20.45, 20.40. $^{31}$P NMR ($CD_3OD$, 202 MHz): $\delta$ (ppm) 3.56. IR 935, 1049, 1145, 1216, 1723, 3189. HRMS (ESI) $m/z$ calcd for $C_{18}H_{23}N_6O_7P$ $[M + Na]^+$ 489.1264, found 489.1255.

*4.1.17. Acyclovir-[1-phenoxy(methoxy-L-alaninyl)] (R)-Phosphoramidate (**20b**).* amorphous white solid; $^1$H NMR ($CD_3OD$, 500 MHz): $\delta$ (ppm) 7.84 (s, 1H), 7.33 (t, $J$ = 7.9 Hz, 2H), 7.19−7.13 (m, 3H), 5.48 (s, 2H), 4.58 (s, 2H), 4.27−4.17 (m, 2H), 3.91 (dq, $J$ = 14.4, 7.1 Hz, 1H), 3.81 (dd, $J$ = 6.5, 2.6 Hz, 2H), 3.66 (s, 3H), 1.30 (dd, $J$ = 7.2, 0.9 Hz, 3H). $^{13}$C NMR ($CD_3OD$, 101 MHz): $\delta$ (ppm) 174.14, 157.96, 154.26, 152.01, 150.81, 138.36, 129.30, 124.67, 120.05, 120.00, 116.10, 72.24, 68.08, 65.65, 51.30, 50.10, 18.97, 18.90. $^{31}$P NMR ($CD_3OD$, 202 MHz): $\delta$ (ppm) 3.73. IR 943, 1057, 1221, 1723, and 3177. HRMS (ESI) $m/z$ calcd for $C_{18}H_{23}N_6O_7P$ $[M + Na]^+$ 489.1264, found 489.1257.

**15** (85.0 mg) was separated by preparative chiral column chromatography to obtain **21a** (32.2 mg, 75.8%) and **21b** (37.2 mg, 87.5%).

*4.1.18. Acyclovir-[1-phenoxy(ethoxy-L-alaninyl)] (S)-Phosphoramidate (**21a**).* Amorphous white solid; $^1$H NMR ($CD_3OD$, 400 MHz): $\delta$ (ppm) 7.83 (s, 1H), 7.37−7.30 (m, 2H), 7.17 (t, $J$ = 7.1 Hz, 3H), 5.46 (s, 2H), 4.21−4.16 (m, 2H), 4.16−4.09 (m, 2H), 3.90 (dq, $J$ = 9.7, 7.2 Hz, 1H), 3.80−3.76 (m, 2H), 1.33 (d, $J$ = 7.1 Hz, 3H), 1.23 (t, $J$ = 7.1 Hz, 3H). $^{13}$C NMR ($CD_3OD$, 101 MHz): $\delta$ 173.61, 157.94, 154.26, 152.00, 150.77, 138.35, 129.31, 124.66, 124.64, 120.03, 119.98, 116.07, 72.25, 68.03, 65.61, 60.95, 50.12, 19.10, 13.06. $^{31}$P NMR ($CD_3OD$, 162 MHz): $\delta$ (ppm) 3.62. IR 935, 1050, 1150, 1214, 1725, 3190. HRMS (ESI) $m/z$ calcd for $C_{19}H_{25}N_6O_7P$ $[M + Na]^+$ 503.1420, found 503.1412.

*4.1.19. Acyclovir-[1-phenoxy(ethoxy-L-alaninyl)] (R)-Phosphoramidate (**21b**).* Amorphous white solid; $^1$H NMR ($CD_3OD$, 400 MHz): $\delta$ (ppm) 7.86 (s, 1H), 7.33 (t, $J$ = 7.9 Hz, 2H), 7.17 (t, $J$ = 7.3 Hz, 3H), 5.48 (s, 2H), 4.23 (td, $J$ = 7.4, 3.2 Hz, 2H), 4.11 (q, $J$ = 7.1 Hz, 2H), 3.89 (dq, $J$ = 14.4, 7.2 Hz, 1H), 3.81 (t, $J$ = 4.0 Hz, 2H), 1.30 (dd, $J$ = 7.1, 0.7 Hz, 3H), 1.22 (t, $J$ = 7.1 Hz, 3H). $^{13}$C NMR ($CD_3OD$, 101 MHz): $\delta$ (ppm) 173.70, 157.90, 154.28, 151.99, 150.82, 138.35, 129.30, 124.68,


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124.66, 120.06, 120.01, 115.98, 72.28, 68.09, 65.64, 60.92, 50.20, 18.93, 13.0. $^{31}$P NMR ($CD_3OD$, 162 MHz): $\delta$ (ppm) 3.79. IR 935, 1050, 1150, 1215, 1725, and 3190. HRMS (ESI) $m/z$ calcd for $C_{19}H_{25}N_6O_7P$ $[M + Na]^+$ 503.1420, found 503.1419.

**16** (100.0 mg) was separated by preparative chiral column chromatography to obtain **22a** (28.5 mg, 57.0%) and **22b** (23.6 mg, 47.2%).

*4.1.20. Acyclovir-[1-phenoxy(isopropyloxy-L-alaninyl)] (S)-Phosphoramidate (**22a**).* Amorphous white solid; $^1$H NMR ($CD_3OD$, 500 MHz): $\delta$ (ppm) 7.95 (s, 1H), 7.33 (t, $J$ = 7.7 Hz, 2H), 7.17 (t, $J$ = 8.0 Hz, 3H), 5.47 (s, 2H), 4.96 (dt, $J$ = 12.4, 6.3 Hz, 1H), 4.18 (s, 2H), 3.87 (dd, $J$ = 15.9, 7.5 Hz, 1H), 3.78 (s, 2H), 1.32 (d, $J$ = 7.1 Hz, 3H), 1.22 (d, $J$ = 6.1 Hz, 6H). $^{13}$C NMR ($CD_3OD$, 126 MHz): $\delta$ (ppm) 174.56, 174.52, 159.35, 155.78, 152.19, 130.71, 126.05, 121.42, 121.39, 73.83, 70.14, 69.45, 69.39, 67.00, 66.96, 51.65, 21.96, 21.88, 20.53, 20.48. $^{31}$P NMR ($CD_3OD$, 202 MHz): $\delta$ (ppm) 3.66. IR 937, 1050, 1151, 1212, 1724, and 3189. HRMS (ESI) $m/z$ calcd for $C_{20}H_{25}N_6O_7P$ $[M + Na]^+$ 517.1577, found 517.1579.

*4.1.21. Acyclovir-[1-phenoxy(isopropyloxy-L-alaninyl)] (R)-Phosphoramidate (**22b**).* Amorphous white solid; $^1$H NMR ($CD_3OD$, 500 MHz): $\delta$ (ppm) 7.84 (s, 1H), 7.33 (t, $J$ = 7.7 Hz, 2H), 7.17 (t, $J$ = 7.4 Hz, 3H), 5.48 (s, 2H), 4.95 (dt, $J$ = 12.5, 6.2 Hz, 1H), 4.28−4.17 (m, 2H), 3.87 (dd, $J$ = 15.4, 7.6 Hz, 1H), 3.83 (d, $J$ = 18.4 Hz, 2H), 1.29 (d, $J$ = 7.0 Hz, 3H), 1.24−1.18 (m, 6H). $^{13}$C NMR ($CD_3OD$, 126 MHz): $\delta$ (ppm) 174.66, 159.38, 155.68, 153.41, 139.74, 130.70, 126.07, 121.48, 121.44, 73.64, 70.10, 69.46, 69.40, 67.08, 67.03, 51.74, 21.94, 21.88, 20.40, 20.34. $^{31}$P NMR ($CD_3OD$, 202 MHz): $\delta$ (ppm) 3.81. IR 937, 1050, 1151, 1214, 1724, 3189. HRMS (ESI) $m/z$ calcd for $C_{20}H_{25}N_6O_7P$ $[M + Na]^+$ 517.1577, found 517.1573.

**17** (267.6 mg) was separated by preparative chiral column chromatography to obtain **23a** (97.6 mg, 72.9%) and **23b** (70.7 mg, 52.8%).

*4.1.22. Acyclovir-[1-phenoxy(tert-butyloxy-L-alaninyl)] (S)-Phosphoramidate (**23a**).* Amorphous white solid; $^1$H NMR ($CD_3OD$, 400 MHz): $\delta$ (ppm) 7.84 (s, 1H), 7.36−7.28 (m, 2H), 7.20−7.13 (m, 3H), 5.48 (s, 2H), 4.25−4.18 (m, 2H), 3.84−3.74 (m, 3H), 1.42 (s, 9H), 1.27 (dd, $J$ = 7.1, 1.0 Hz, 3H). $^{13}$C NMR (DMSO-$d_6$, 101 MHz): $\delta$ 152.01, 138.34, 129.32, 124.64, 120.04, 119.99, 81.24, 72.24, 67.94, 65.53, 50.70, 26.80, 19.26, 19.20. $^{31}$P NMR ($CD_3OD$, 162 MHz): $\delta$ (ppm) 3.77. IR 935, 1050, 1145, 1216, 1723, 3188. HRMS (ESI) $m/z$ calcd for $C_{21}H_{29}N_6O_7P$ $[M + Na]^+$ 531.1733, found 531.1722.

*4.1.23. Acyclovir-[1-phenoxy(tert-butyloxy-L-alaninyl)] (R)-Phosphoramidate (**23b**).* Amorphous white solid; $^1$H NMR ($CD_3OD$, 400 MHz): $\delta$ (ppm) 7.83 (s, 1H), 7.36−7.29 (m, 2H), 7.20−7.13 (m, 3H), 5.46 (d, $J$ = 0.4 Hz, 2H), 4.22−4.14 (m, 2H), 3.83−3.74 (m, 3H), 1.43 (s, 9H), 1.30 (dd, $J$ = 7.1, 0.8 Hz, 3H). $^{13}$C NMR (DMSO-$d_6$, 101 MHz): $\delta$ 151.21, 137.55, 128.50, 123.86, 119.28, 119.23, 80.37, 71.43, 67.26, 64.86, 50.00, 25.98, 18.32, 18.25. $^{31}$P NMR ($CD_3OD$, 162 MHz): $\delta$ (ppm) 3.88. IR 934, 1050, 1146, 1216, 1723, 3189. HRMS (ESI) $m/z$ calcd for $C_{21}H_{29}N_6O_7P$ $[M + Na]^+$ 531.1733, found 531.1717.

**19** (200.0 mg) was separated by preparative chiral column chromatography to obtain **24a** (80.0 mg, 80.0%) and **24b** (60.0 mg, 60.0%).

*4.1.24. Acyclovir-[1-phenoxy(benzyloxy-L-alaninyl)] (S)-Phosphoramidate (**24a**).* Amorphous white solid; $^1$H NMR ($CD_3OD$, 400 MHz): $\delta$ (ppm) 7.82 (s, 1H), 7.36−7.25 (m, 7H), 7.19−7.10 (m, 3H), 5.44 (s, 2H), 5.11 (s, 2H), 4.18−4.12 (m, 2H), 3.97 (dq, $J$ = 14.3, 7.2 Hz, 1H), 3.73 (t, $J$ = 4.0 Hz, 2H), 1.31 (dd, $J$ = 7.2, 0.9 Hz, 3H). $^{13}$C NMR ($CD_3OD$, 101 MHz): $\delta$ (ppm) 174.91, 174.86, 159.36, 155.65, 153.40, 139.76, 137.27, 130.70, 129.57, 129.35, 129.32, 126.09, 121.51, 121.46, 117.49, 73.60, 69.39, 69.32, 67.93, 67.07, 67.02, 51.69, 20.32, 20.25. $^{31}$P NMR ($CD_3OD$, 202 MHz): $\delta$ (ppm) 2.69. IR 935, 1050, 1147, 1213, 1725, 3189. HRMS (ESI) $m/z$ calcd for $C_{24}H_{27}N_6O_7P$ $[M + Na]^+$ 565.1577 found 565.1573.

*4.1.25. Acyclovir-[1-phenoxy(benzyloxy-L-alaninyl)] (R)-Phosphoramidate (**24b**).* Amorphous white solid; $^1$H NMR ($CD_3OD$, 400 MHz): $\delta$ (ppm) 7.78 (s, 1H), 7.38−7.27 (m, 7H), 7.15 (dd, $J$ = 7.5, 6.6 Hz, 3H), 5.40 (s, 2H), 5.13 (s, 2H), 4.15−4.07 (m, 2H), 3.95 (dq, $J$ = 9.8, 7.1 Hz, 1H), 3.73−3.68 (m, 2H), 1.34 (d, $J$ = 7.1 Hz, 3H). $^{13}$C NMR ($CD_3OD$, 101 MHz): $\delta$ (ppm) 174.76, 174.71, 159.36, 155.64, 153.38, 139.73, 137.29, 130.71, 129.59, 129.38, 129.35, 126.05, 121.42, 121.38, 117.48, 73.59, 69.36, 69.28, 67.94, 66.97, 66.92, 51.56, 20.41, 20.34. $^{31}$P NMR ($CD_3OD$, 202 MHz): $\delta$ (ppm) 3.79. IR 935, 1050, 1147, 1213, 1725, 3189. HRMS (ESI) $m/z$ calcd for $C_{24}H_{27}N_6O_7P$ $[M + Na]^+$ 565.1577, found 565.1575.

**4.2. *In Vitro* Enzymatic Hydrolysis.** Single isomer phosphoramidate ProTide-ACVs 5 mg were dissolved in $d_6$-acetone (150 $\mu$L) and Trizma buffer (300 $\mu$L, pH = 7.4) and a $^{31}$P NMR was recorded (starting material). Then a solution of carboxypeptidase Y (0.1 mg) in Trizma buffer (150 $\mu$L) was added, and a $^{31}$P NMR was performed recording the experiment every 15 min to 24 h.

**4.3. Docking Studies.** Molecular modeling studies were performed to assess the potential pose of these single isomers in the binding site of CPY (PDB entry 1YSC). Docking studies were carried out using AutoDock Vina (https://vina.scripps.edu/)[37,38] to visualize the protein−ligand interactions. Note that the ligands were performed geometry optimization using Gaussian 16 with HF/6-31G(d) level of theory before docking, following the previous procedure.[33] The grid box was set to 25 × 25 × 25 Å$^3$, covering the binding pocket and key residues, including Ser146, His397, and Asp338,[35,37] for each ligand. The binding affinity of the protein and ligand complexes was computed in three replicates with a maximum number of binding modes of 100 for each enantiomer. The best binding mode of each compound with the lowest binding affinity was selected, and the corresponding binding interactions were visualized by Discovery Studio Visualizer (BIOVIA, Dassault Systems, Discovery Studio Visualizer, v21.1.0.20298, San Diego: Dassault Systèmes, 2020) and compared.

**4.4. Antiviral Activities.** The antiviral efficacy of the synthesized compounds was assessed on the A549 cell line (ATCC CCL-185), originating from human lung cancer. A549 cells were cultivated in RPMI medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37 °C in a 5% $CO_2$ humidified incubator. Cells were preseeded on 48-well plates at a density of 4 × 10$^6$ cells per plate 1 day before the test. The cells were infected with wild-type HSV-1 at a multiplicity of infection (MOI) of 0.001 for 2 h at 37 °C with 5% $CO_2$. The inoculum was eliminated, and the infected cells were rinsed with 1× PBS three times. The cells were subsequently treated with the compounds by adding growth media containing the compounds as indicated at concentrations of 100 or 200 $\mu$M to the infected cells (500 $\mu$L per well). At 48 h postinfection (hpi), culture media containing released viral progeny were collected to assess the antiviral activities of the compounds by comparing viral production between the compounds treated and untreated infected cells, based on the extent of cell lysis in Vero


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cells. The antiviral assay was conducted in triplicate. Vero (ATCC CCL-81), an epithelial cell line derived from the African green monkey kidney, was maintained at 37 °C with 5% $CO_2$ in Minimum Essential Medium (MEM, Gibco) supplemented with 5% fetal bovine serum (FBS). For the cell lysis experiment, Vero cells were preseeded in 96-well plates at a density of $4 \times 10^6$ cells per plate (100 $\mu$L of cells per well) 1 day prior. The samples underwent a 10-fold serial dilution using serum-free medium, and 100 $\mu$L of each diluted sample was inoculated into a well. The cells infected with different dilutions of samples were conducted in duplicate and subsequently incubated at 37 °C with 5% $CO_2$. At 3 days postinfection (dpi), the cells were fixed using a 4% formaldehyde solution in PBS and subsequently stained with a 1% weight/volume (W/V) concentration of crystal violet. The intact cells appeared as blue-stained entities in the wells, whereas the lysed cells were represented as partially or entirely clear wells. The mean of the highest dilution with cell lysis capability for each sample was plotted with the standard deviation and assessed as the level of viral production. Statistical analysis was conducted utilizing one-way ANOVA, succeeded by Tukey's multiple comparisons test via GraphPad Prism 10. Differences were considered significant when $P < 0.0001$.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c06645.

Additional experimental details, materials, and methods; $^1H$ and $^{13}C$ NMR spectra, HPLC, and HR-MS spectra; and hydrolysis profile (PDF)

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### Author Contributions

#T.K. and P.M.-u.: contributed equally to this work. T.K.: Conception of the work, experiments, interpretation of data, draft manuscript. P.M.-u, M.U.: Computer modeling, N.S., S.T.: Supervision, conception of the work, revising manuscript, funding acquisition. C.T.: Bioactivity screening test.

### Notes

The authors declare no competing financial interest.


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