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Controllable synthesis of polymerizable ester and amide prodrugs of acyclovir by enzyme in organic solvent

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Abstract—A facile control of the acylation position at the primary hydroxyl and amino of acyclovir, respectively, was achieved and five polymerizable acyclovir prodrugs were synthesized. Various reaction conditions were studied in detail. Thus, lipase acrylic resin from *Candida antarctica* (CAL-B) in pyridine or acetone showed high chemo-selectivity toward the primary hydroxyl of acyclovir. However, lipase PS 'Amano' (PS) in DMSO selectively acylated the amino group. The selectivity of PS could be adjusted by changing reaction solvents. The acyclovir vinyl derivatives obtained would be important monomers used for the preparation of macromolecular nucleoside drugs.

1. Introduction

Acyclovir (9-(2-hydroxyethoxymethyl) guanine) is an acyclic nucleoside analogue that has shown a potent antiviral activity, and it is known to inhibit the replication of herpesviruses including herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), varicella-zoster virus (VZV), and Epstein-Barr virus (EBV) in cell cultures and in animals.[1,2] To retain the safety and efficacy profiles of acyclovir while greatly improving the bioavailability, some acyclovir prodrugs have been synthesized. Because the reactivity of the 4′-hydroxyl group is higher than that of the 2-amino group, most of these prodrugs were acylated at the primary hydroxyl by the chemical method.[2,3] Following the protection/deprotection steps of the 4′-hydroxyl group, *N*-acyl derivatives of acyclovir could be synthesized by a traditional chemical method.[4] However, this process was tedious and the operation was difficult to be controlled.

In selective structural modification of such polyfunctional substrates as nucleosides, enzymes, as environmentally friendly catalysts, play an important role due to broad substrate specificities, high selectivity, and mild reaction conditions.[5,6] Since Riva et al. reported the regioselective acylation of nucleosides at the primary hydroxyl groups using subtilisin in DMF, extensive modified nucleoside analogues have been prepared by enzymatic synthesis.[7,8] And, we have reported the control of regioselectivity in enzymatic modification of 2′-deoxyuridine at 3′- or 5′-hydroxy by the change of reaction conditions.[9] However, the control of enzymatic synthesis was almost focused on the regioselectivity of the same group such as hydroxyls in the sugar moiety of nucleosides. Few reports about the control of enzymatic chemo-selectivity between hydroxyl and amino groups of nucleosides were available.

In this study, we demonstrated a facile control of the acylation position at the primary hydroxyl and amino of acyclovir to obtain polymerizable 4′-*O*-acyl and 2-*N*-acyl acyclovir vinyl derivatives, respectively. And, by changing the reaction solvents, 4′-*O*-acyl and 2-*N*-acyl acyclovir vinyl derivatives can be obtained, respectively, using the same enzyme as catalyst. Moreover, the acyclovir prodrugs obtained would be useful as monomers for the preparation of macromolecular nucleoside drugs, which can improve drug delivery and solubility, prolong drug release, reduce doses and dosing intervals or achieve target ability by choosing appropriate copolymerizable monomers.[10]

Keywords: Chemo-selectivity; Enzymatic synthesis; Transesterification; Acyclovir.
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2. Results and discussion

2.1. Enzymatic synthesis of acyclovir vinyl derivatives

By choosing appropriate enzymes and organic solvents, controllable selective acylation at the primary hydroxyl or amino of acyclovir, respectively, was realized and five polymerizable acyclovir prodrugs, 2-*N*-acyl and 4′-*O*-acyl acyclovir vinyl derivatives, were synthesized by controllable selective enzymatic transesterification of acyclovir and divinyl dicarboxylates. The reaction route is shown in Figure 1. The acylation position of acyclovir was determined by ^{1}H NMR and ^{13}C NMR, according to the general strategy described by Yoshimoto et al., acylation of a hydroxyl group of substrate results in a downfield shift of the peak corresponding to the O-acylated carbon and an upfield shift of the peak corresponding to the neighboring carbon.[11] NMR characterization revealed that products **3a**–**3c** were substituted at the primary hydroxyl of acyclovir and the products **4b**–**4c** at amino position. The ^{13}C NMR (DMSO-d_6) data of the products **3a**–**3c**, **4b**, and **4c** are shown in Table 1.

2.2. Influence of enzyme source on the chemo-selectivity and yield

One of the most important parameters for enzyme-catalyzed reactions is the selection of enzyme sources. Enzymes derived from different sources always exhibit different properties, such as stability, activity, specificity, and so on. In order to choose the appropriate enzymes to selectively catalyze the acylation on the primary hydroxyl and amino groups of acyclovir, respectively, 10 commercially available enzymes were tested for the transesterification of acyclovir with divinyl adipate in two organic solvents, pyridine and DMSO, which can dissolve both acyclovir and acyl donor substrates well. The results are shown in Figure 2. The yields of acyclovir vinyl derivatives were determined by HPLC. In the absence of enzyme, no anticipant products were observed. From Figure 2a, it can be found that in pyridine, both CAL-B and Subtilisin showed a preference for the primary hydroxyl group of acyclovir. In DMSO, most enzymes, especially PS and MJL*, selectively catalyzed the acylation on the amino group of acyclovir (Fig. 2b). Among the enzymes studied CAL-B gave the best result. When CAL-B was screened as catalyst, 4′-*O*-vinyladipyl-acyclovir was obtained in pyridine with the best yield and selectivity. And, 2-*N*-vinyladipyl-acyclovir was synthesized using PS as catalyst in DMSO with better yield and selectivity. Interestingly, when PS was chosen as catalyst in different organic solvents, 4′-*O*-vinyladipyl-acyclovir and 2-*N*-vinyladipyl-acyclovir can be obtained, respectively.

2.3. Influence of organic solvent on the chemo-selectivity and yield

The choice of solvents is also significant for enzyme-catalyzed reactions, for solvent plays an important role in maintaining enzyme catalytic activity and stability. To increase the selectivity exhibited by CAL-B and PS, 10 organic solvents were tested for the transesterification of acyclovir with divinyl adipate. The results are shown in Figure 3. In all solvents chosen, only DMF, DMSO, and pyridine can dissolve acyclovir. From Figure 3a, it can be found that when CAL-B was chosen to catalyze the transesterification, 4′-*O*-vinyladipyl-acyclovir can be obtained with better yield and selectivity in a variety of organic solvents such as pyridine, acetone, THF, and so on. In general, enzymatic transesterification was not effective in DMSO that can dissolve enzyme and was regarded as enzyme denaturant. However, the activity

Table 1. Chemical shifts of ^{13}C NMR (DMSO-d_6) of acyclovir and acyclovir derivatives

Carbon	1	3a	3b	3c	4b	4c
2	153.42	153.91	154.99	153.93	147.88	148.61
4	150.99	151.42	152.06	151.46	149.21	149.35
5	116.01	116.52	116.91	116.51	121.12	120.62
6	156.40	156.76	157.89	156.87	155.12	155.39
8	137.36	137.61	138.17	137.69	139.89	140.57
1′	71.60	71.85	72.37	71.85	73.24	73.11
3′	69.94	66.45	67.14	66.60	70.89	71.01
4′	59.46	63.07	63.23	62.57	59.81	60.38
–CH_2–		28.31	33.49	33.31	33.51	36.39
		28.19	33.20	33.05	33.28	33.51
			24.21	28.48	24.54	28.99
			23.96	28.33	24.01	28.93
				28.23		28.87
				28.15		28.81
				24.35		24.84
				24.04		24.51
C=O		171.68	173.26	172.82	175.85	176.89
		169.50	170.91	170.44	170.11	170.91
–CH=CH_2–		141.17	141.78	141.24	141.21	141.72
		98.24	98.74	97.93	98.15	98.45

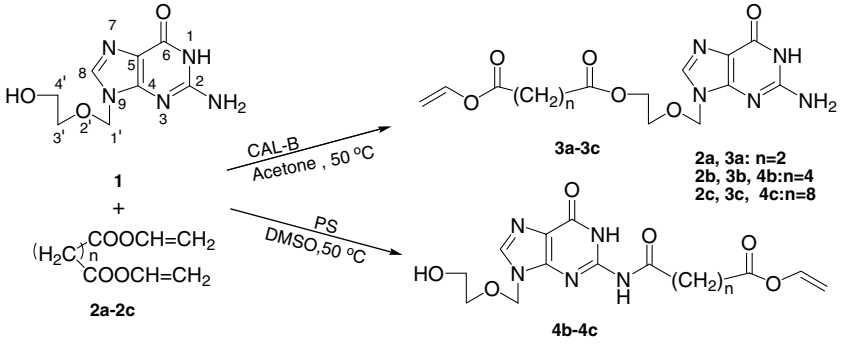

Figure 1. Synthesis of 2-*N*-acyl and 4′-*O*-acyl acyclovir vinyl derivatives.

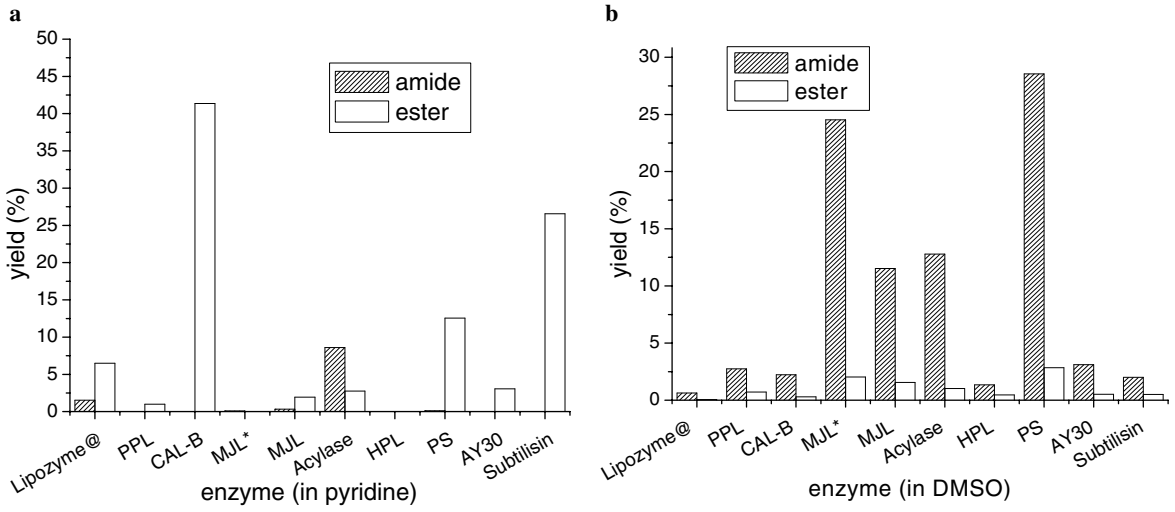

Figure 2. Influence of enzyme source on chemo-selectivity and yield of the transesterification in different organic solvents. Conditions: enzyme (15 mg mL^{-1}), acyclovir (1 mmol), divinyl adipate (6 mmol), pyridine (20 mL) (a) or DMSO (20 mL) (b), 50 °C, 250 rpm, 3 days.

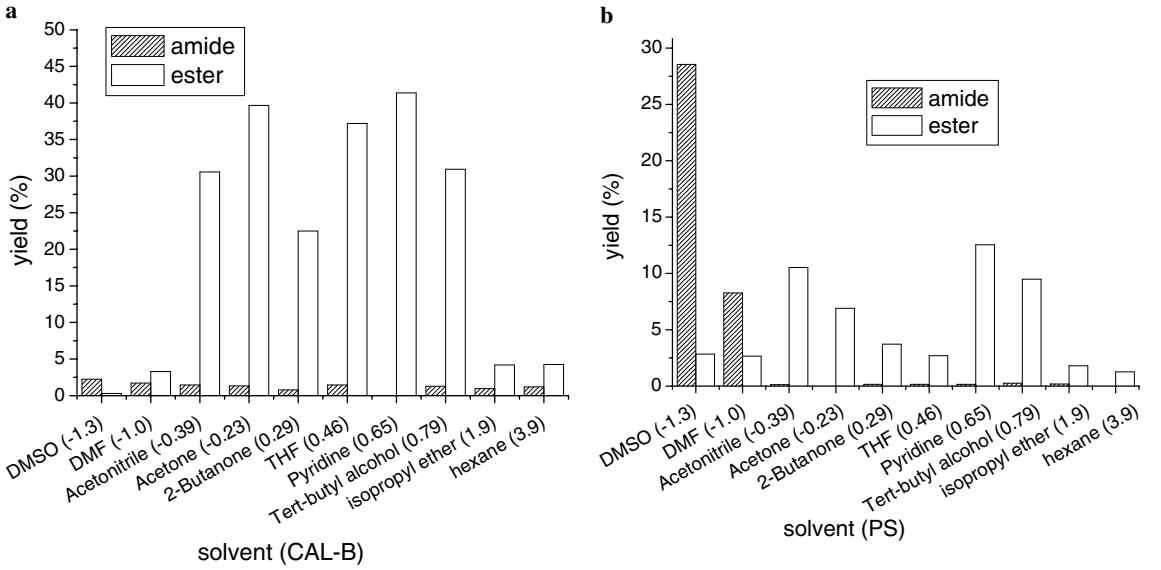

Figure 3. Influence of organic solvent on chemo-selectivity and yield of the transesterification in different enzymes. Conditions: CAL-B (15 mg mL^{-1}) (a) or PS (15 mg mL^{-1}) (b), acyclovir (1 mmol), divinyl adipate (6 mmol), and organic solvent (20 mL), 50 °C, 250 rpm, 3 days.

of the PS for the synthesis of 2-*N*-vinyladipyl-acyclovir was the highest in DMSO (Fig. 3b).

In Figure 3b, it was obviously observed that the chemo-selectivity of PS could be adjusted, even reversed, by changing solvents. In DMSO, PS selectively acylated the amino group of acyclovir, while it acylated the primary hydroxyl group in such solvents as pyridine, acetonitrile, and so on. The chemo-selectivity of CAL-B did not change with the variation of solvents (Fig. 3a).

2.4. Influence of water content in organic solvent on yield

It is known that all enzymes need essentially bound water, and enzymatic activity in the organic solvent depends on water content. The water content required to reach the maximal activity is different in various organic solvents.[12] Figure 4 shows the effect of water content in acetone and pyridine on transesterification of acyclovir and divinyl adipate catalyzed by CAL-B. In anhydrous acetone, CAL-B brought about the highest yield of acyclovir into 4′-*O*-vinyladipyl-acyclovir. Then, the yield decreased with increasing the content of water. However, in pyridine, the optimum water content was 0.1%. Optimum water content of the reaction in pyridine was higher than that in acetone. It accorded with the report that hydrophilic organic solvents were known to strip essential water bound to protein and inactivate enzymes.

2.5. Influence of reaction time on yield

In order to understand the mechanism of enzymatic transesterification, the time course of acylation of

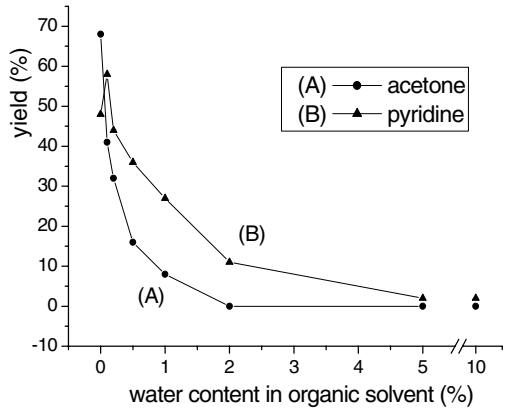

Figure 4. Influence of water content in organic solvent on the yield of the transesterification. Conditions: CAL-B (15 mg mL^{-1}), acyclovir (1 mmol), divinyl adipate (6 mmol), acetone (20 mL) (A) or pyridine (20 mL) (B), 50 °C, 250 rpm, 3 days.

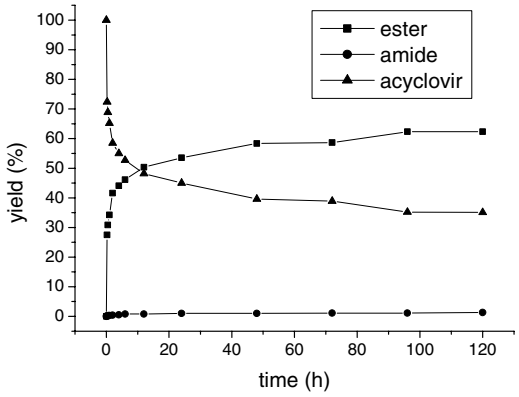

Figure 6. Time course of acyclovir with divinyl adipate catalyzed by CAL-B. Conditions: CAL-B (15 mg mL^{-1}), acyclovir (1 mmol), divinyl adipate (6 mmol), and pyridine (0.1% water, 20 mL), 50 °C, 250 rpm.

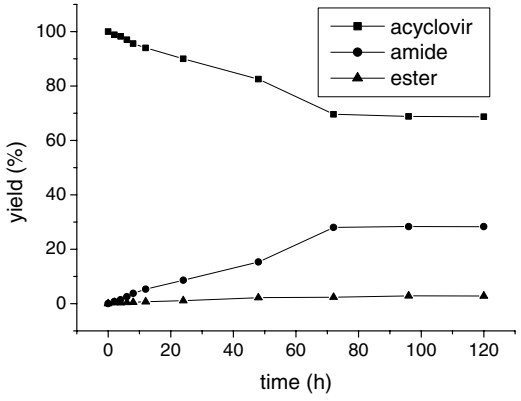

Figure 5. Time course of acyclovir with divinyl adipate catalyzed by PS. Conditions: PS (15 mg mL^{-1}), acyclovir (1 mmol), divinyl adipate (6 mmol), and DMSO (20 mL), 50 °C, 250 rpm.

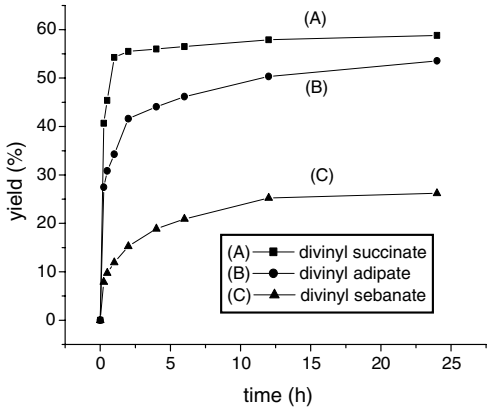

Figure 7. Influence of chain length of acylating agent on the initial reaction rate and yield of the transesterification. Conditions: CAL-B (15 mg mL^{-1}), acyclovir (1 mmol), divinyl dicarboxylates (6 mmol), and pyridine (0.1% water, 20 mL), 50 °C, 250 rpm.

acyclovir with divinyl adipate using PS as catalyst in DMSO was studied, which is shown in Figure 5. From the diagram, it can be found that PS showed a preference for the amino group of acyclovir and only trace of *O*-acyl acyclovir derivative was obtained during the reaction process. Furthermore, the reaction process of acyclovir and divinyl adipate catalyzed by CAL-B in pyridine was also studied and the time course is shown in Figure 6. Similarly, CAL-B in pyridine showed high chemo-selectivity toward the primary hydroxyl of acyclovir throughout the reaction. The optimum reaction time of the transesterification of acyclovir and divinyl adipate was 3 days.

2.6. Influence of chain length of acylating agent on initial reaction rate and yield

The chain length of acylating agents also affected the results of enzymatic transesterification. In this study, we selected divinyl succinate, divinyl adipate, and divinyl sebacate to investigate the influence. The result is shown in Figure 7 and Table 2. From Figure 7, it can be found that the chain length of acylating agents affected the yield and initial reaction rate of transesterification of acyclovir and divinyl dicarboxylates. When divinyl succinate was chosen as acylating agent, the initial reaction rate was the highest and the initial reaction rate is up to 115.59 mM h^{-1}. The reactivity of the transesterification using divinyl adipate as acylating agent was lower than that using divinyl succinate. And the reactivity of divinyl sebacate in the transesterification was the lowest.

Table 2. Influence of chain length of acylating agent on initial reaction rate and yield of the transesterification

Acylating agents	Time (h)	Yield[a] (%)	Initial rate (mM h^{-1})
2a	12	58.0	115.59
2b	12	50.4	39.66
2c	12	25.3	5.58

Conditions: CAL-B (15 mg mL^{-1}), acyclovir (1 mmol), divinyl dicarboxylates (6 mmol), and pyridine (0.1% water, 20 mL), 50 °C, 250 rpm.
[a] Determined by HPLC.

3. Conclusion

In this study, the selectivity of enzymatic synthesis of acyclovir with divinyl dicarboxylates was described. 4′-

O-acyl acyclovir vinyl derivatives could be prepared by CAL-B-catalyzed transesterification in anhydrous acetone or pyridine. And, PS and MJL* in DMSO selectively acylated the amino group of acyclovir. The influence of enzyme source, organic solvent, water content in organic solvent, reaction time, and chain length of acylating agent was systematically investigated. It was known that the most important parameters affecting selectivity of enzyme-catalyzed reactions were enzyme sources and solvents. And, water content in organic solvent and chain length of acylating agent showed important effect on the yield and initial reaction rate. The chemical polymerization of acyclovir vinyl derivatives obtained and further application to polymeric nucleoside controlled release systems are in progress.

4. Experiments

4.1. Materials

Lipozyme® immobilized lipase from *Mucor miehei* (Lipozyme®), lipase from *hog pancreas* (HPL), and lipase from *Mucor javanicus* (MJL) were purchased from Fluka. Lipase from *porcine pancreas* (PPL), Amano lipase M from *Mucor javanicus* (MJL*), and lipase acrylic resin from *Candida antarctica* (CAL-B) were purchased from Sigma. Lipase AY30 (AY30) was purchased from Acros. Lipase PS 'Amano' (PS) was purchased from Aldrich. Alkaline protease from *Bacillus subtilis* (Subtilisin) was purchased from Wuxi Enzyme Co. Ltd. (Wuxi, PR China). Immobilized penicillin G. Acylase from *Escherichia coli* (Acylase) was purchased from Hunan. Flag Biotech Co. (PR China). Divinyl succinate, divinyl adipate, and divinyl sebacate were produced and purified as described in the patent.[13] Acyclovir, vinyl acetate, and all other chemicals were of analytical grade.

4.2. Analytical methods

All reactions were monitored by TLC on silica gel plates eluted with ethyl acetate/methanol/ammonia (20/4/1, by vol). ^{1}H NMR and ^{13}C NMR spectra were recorded with TMS as internal standard using a Bruker AMX-500 MHz spectrometer. Chemical shifts were expressed in ppm and coupling constants (*J*) in Hz. The yields were determined by Shimadzu SPD-10Avp HPLC with a UV–vis detector ($\lambda = 254$ nm) and a reversed-phase Shim-Pack VP-ODS column (150 × 4.6 mm). Infrared spectra were measured with a Nicolet Nexus FTIR 670 spectrophotometer. Mass spectrometry data were obtained on Bruker Esquire-LC for electrospray (ESI-MS) measurements.

4.3. Synthesis of 4′-*O*-vinyl acyclovir derivatives (3a, 3b, and 3c)

The reaction was initiated by adding CAL-B (15 mg mL^{-1}) to anhydrous acetone (20 mL) containing acyclovir (0.225 g, 1 mmol) and divinyl dicarboxylates (6 mmol). The suspension was kept at 50 °C and stirred at 250 rpm. Formation of 4′-*O*-vinyl acyclovir derivatives was confirmed by TLC. The reaction was terminated by filtering the enzyme. Because the products were undissolvable in acetone, DMF was used to wash the filter paper to assure that products obtained were all dissolved in the filtrate. Then DMF and acetone were evaporated under reduced pressure. The product was separated by silica gel chromatography with an eluent consisting of ethyl acetate/methanol/ammonia (50/8/1, by vol).

4.4. 4′-*O*-Vinylsuccinyl-acyclovir (3a)

The reaction time was 12 h and the product (**3a**) was a light yellow solid (0.3 g, 95%); elemental analysis (Found: C, 47.8; H, 5.0; N, 19.9. $C_{14}H_{17}N_5O_6$ requires C, 47.9; H, 4.9; N, 19.9%); IR (KBr) ν_{max} 3436, 3320, 3121, 2925, 1736, 1691, 1647 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, Me_4Si) δ 10.63 (1H, s, NH), 7.82 (1H, s, NCH), 7.22 (1H, dd, $J = 6.25$ and 13.93, CH), 6.50 (2H, s, NH_2), 5.36 (2H, s, NCH_2O), 4.91 (1H, d, $J = 13.95$, CH_2), 4.67 (1H, d, $J = 6.20$, CH_2), 4.13 (2H, t, $J = 4.40$, OCH_2), 3.68 (2H, t, $J = 4.40$, CH_2O), 2.67 (2H, t, $J = 6.30$, CH_2CO), 2.27 (2H, t, $J = 6.30$, CH_2CO); ^{13}C NMR (125 MHz, DMSO-d_6) are shown in Table 1 (**3a**); ESI-MS (*m*/*z*): 373.9 [M+Na]$^+$.

4.5. 4′-*O*-Vinyladipyl-acyclovir (3b)

The reaction time was 3 days and the product (**3b**) was a light yellow solid (0.2 g, 53%); mp 156 °C; elemental analysis (Found: C, 50.7; H, 5.6; N, 18.5. $C_{16}H_{21}N_5O_6$ requires C, 50.7; H, 5.6; N, 18.5%); IR (KBr) ν_{max} 3432, 3319, 3125, 2935, 1738, 1689, 1647 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, Me_4Si) δ 10.82 (1H, s, NH), 7.81 (1H, s, NCH), 7.25 (1H, dd, $J = 5.90$ and 13.20, CH), 6.61 (2H, s, NH_2), 5.35 (2H, s, NCH_2O), 4.90 (1H, d, $J = 14.25$, CH_2), 4.65 (1H, d, $J = 5.40$, CH_2), 4.09 (2H, t, $J = 4.70$, OCH_2), 3.65 (2H, t, $J = 4.80$, CH_2O), 2.44 (2H, t, $J = 6.90$, CH_2CO), 2.27 (2H, t, $J = 6.80$, CH_2CO), 1.52–1.50 (4H, m, $2 \times CH_2$); ^{13}C NMR (125 MHz, DMSO-d_6) are shown in Table 1 (**3b**); ESI-MS (*m*/*z*): 401.9 [M+Na]$^+$.

4.6. 4′-*O*-Vinylsebacyl-acyclovir (3c)

The reaction time was 3 days and the product (**3c**) was a light yellow solid (0.1 g, 30%); mp 180 °C; elemental analysis (Found: C, 55.1; H, 6.8; N 16.1. $C_{20}H_{29}N_5O_6$ requires C, 55.2; H, 6.7; N, 16.1%); IR (KBr) ν_{max} 3437, 3327, 3118, 2932, 1738, 1689, 1647 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, Me_4Si) δ 10.75 (1H, s, NH), 7.84 (1H, s, NCH), 7.23 (1 H, dd, $J = 6.30$ and 13.99, CH), 6.55 (2H, s, NH_2), 5.38 (2H, s, NCH_2O), 4.89 (1H, d, $J = 13.99$, CH_2), 4.65 (1H, d, $J = 6.25$, CH_2), 4.10 (2H, t, $J = 4.48$, OCH_2), 3.68 (2H, t, $J = 4.54$, CH_2O), 2.42 (2H, t, $J = 7.36$, CH_2CO), 2.22 (2H, t, $J = 7.38$, CH_2CO), 1.48 (2H, t, $J = 7.04$, CH_2), 1.47 (2H, t, $J = 6.61$, CH_2), 1.23–1.24 (8 H, m, 4× CH_2); ^{13}C NMR (125 MHz, DMSO-d_6) was shown in Table 1 (**3c**); ESI-MS (*m*/*z*): 458.0 [M+Na]$^+$.

4.7. Synthesis of 2-*N*-vinyl acyclovir derivatives (4b, 4c)

Acyclovir (0.225 g, 1 mmol) and divinyl dicarboxylates (6 mmol) were added to DMSO (20 mL) containing PS

(15 mg mL^{-1}) as catalyst. The suspension was kept at 50 °C and stirred at 250 rpm. Formation of acyclovir vinyl amides was confirmed by TLC. The reaction was terminated by filtering the enzyme, and DMSO was evaporated under reduced pressure. The methods about monitoring and separation of products were mentioned above.

4.8. 2-*N*-Vinyladipyl-acyclovir (4b)

The reaction time was 3 days and the product (**4b**) was a light yellow solid (0.1 g, 28%); mp 135 °C; elemental analysis (Found: C, 50.7; H, 5.6; N, 18.5. $C_{16}H_{21}N_5O_6$ requires C, 50.7; H, 5.6; N, 18.5%); IR (KBr) ν_{max} 3161, 2934, 1750, 1647, 1556 cm^{-1}; ^1H NMR (500 MHz, CD_3OD, Me_4Si) δ 8.07 (1 H, s, NCH), 7.26 (1 H, dd, $J = 6.28$ and 13.99, CH), 5.57 (2 H, s, NCH_2O), 4.86 (1 H, d, $J = 13.76$, CH_2), 4.56 (1H, d, $J = 6.33$, CH_2), 3.62 (4H, t, 2× CH_2O), 2.52 (2H, t, CH_2CO), 2.47 (2H, t, CH_2CO), 1.73–1.74 (4 H, m, 2× CH_2); ^{13}C NMR (125 MHz, DMSO-d_6) are shown in Table 1 (**4b**); ESI-MS (*m*/*z*): 401.9 $[M+Na]^+$.

4.9. 2-*N*-Vinylsebacyl-acyclovir (4c)

The reaction time was 3 days and the product (**4c**) was a light yellow solid (0.08 g, 20%); mp 101 °C; elemental analysis (Found: C, 55.1; H, 6.8; N, 16.1. $C_{20}H_{29}N_5O_6$ requires C, 55.2; H, 6.7; N, 16.1%); IR (KBr) ν_{max} 3163, 2931, 1751, 1642, 1561 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6, Me_4Si) δ 12.09 (1 H, s, NH), 11.72 (1 H, s, NH), 8.12 (1H, s, NCH), 7.20 (1 H, dd, $J = 6.13$ and 13.83, CH), 5.46 (2H, s, NCH_2O), 4.87 (1H, d, $J = 13.95$, CH_2), 4.66 (1H, s, OH), 4.63 (1H, d, $J = 6.05$, CH_2), 3.47 (4H, t, 2× CH_2O), 2.39–2.46 (4H, m, 2× CH_2CO), 1.54–1.57 (4H, m, 2× CH_2), 1.23–1.26 (8H, m, 4× CH_2); ^{13}C NMR (125 MHz, DMSO-d_6) are shown in Table 1 (**4c**); ESI-MS (*m*/*z*): 458.0 $[M+Na]^+$.

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