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Promiscuous Zinc-Dependent Acylase-Mediated One-Pot Synthesis of Monosaccharide-Containing Pyrimidine Derivatives in Organic Medium

Qi Wu,[a] Jian-Ming Xu,[a] Li Xia,[a] Jun-Liang Wang,[a] and Xian-Fu Lin[a,*]

[a] Department of Chemistry, Zhejiang University, Hangzhou 310027, Peoples Republic of China
Fax: (+86)-571-8795-2618; e-mail: LLC123@zju.edu.cn

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Abstract: A facile one-pot synthesis route to monosaccharide-containing pyrimidine derivatives was developed by combining the two types of catalytic activities of one enzyme in an organic medium, i.e., the Michael addition/acylation activities of zinc-dependent D-aminoacylase (DA) from *Escherichia coli*. First, the stepwise approach was investigated. DA showed higher activity towards the Michael addition than acylation in this reaction system. The enzymatic Michael additions of pyrimidines to vinyl acrylate proceeded very rapidly and the initial reaction rates for the Michael addition of pyrimidines to vinyl acrylate were 7.2–16.5 mM min^{-1}. The catalytic specificity of aminoacylases toward Michael addition was demonstrated by the combination of different control experiments. Then, the two steps could be performed in one pot and a single aminoacylase catalyzed one-pot biotransformation was constructed. Using this strategy, a series of saccharide-pyrimidine complexes with potentially biological and pharmacological applications was prepared efficiently. This high Michael addition activity of zinc-dependent aminoacylases and the novel single aminoacylase-catalyzed one-pot synthesis combining two catalytic activities *in vitro* is of practical significance in expanding the application of enzymes and in the evolution of new biocatalysts.

Keywords: aminoacylase; catalytic promiscuity; enzyme catalysis; Michael addition; one-pot synthesis; pyrimidine derivatives

Introduction

The development of one-pot conversions is considered as one of the important future directions for carrying out sustainable organic synthesis. It will be more efficient than the usual stepwise approach, by saving the effort of isolating intermediates, reducing the production of waste, and avoiding the accumulation of reactive or unstable intermediates. A variety of elegant and promising one-pot conversions has been described in the literature, involving different combinations of enzymes, homogeneous and heterogeneous catalysts, and of uncatalyzed organic chemical conversions.[1] In particular, as efficient catalysts in synthetic chemistry due to their unique catalytic characteristics compared with chemical catalysts, exploitation of enzymatic reactions in one-pot synthesis as it is taking place in living cells, has attracted a great deal of attention of researchers. Recently, multienzyme cascade reactions have fully exhibited the great advantages and potentials in the synthesis of complex carbohydrate-related compounds,[2] amino acid derivatives,[3] β-lactam antibiotics,[4] vancomycin,[5] riboflavin,[6] statin intermediates,[7] as well as in the synthesis of sialosides.[8] Although multienzyme conversions can be accomplished successfully even using up to eight different enzymes in one pot for the synthesis of riboflavin,[6] and the record until now is the 12-step enzymatic conversion in one pot of the corrin moiety of vitamin B12,[9] the incompatibility of many enzymatic steps in terms of reagents, solvent, pH and/or temperature often hinder the combined use of these tools in one-pot conversions or in a cascade mode. It will be better to accomplish a specific complicated process with fewer enzymes. Specially, single-enzyme multistep conversions can fully overcome these obstacles. However, the natural enzyme that is capable of catalyzing multiple reactions is very scarce, and only few single-enzyme multistep conversions have been reported. For example, single lipase-mediated multistep techniques have been used in lipase LIP-catalyzed domino kinetic resolution/intramolecular Diels–Alder reactions.[10] Given the understanding of the state of the art and the need, it is desirable to devise new one-pot single-enzyme multistep transformations.

Recently a growing number of enzymes have been found to be capable of catalyzing secondary reactions at an active site that is specialized to catalyze a pri-

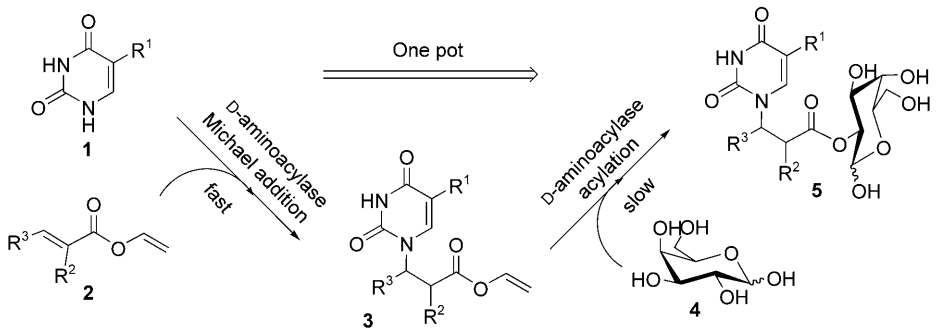
mary reaction. This catalytic promiscuity enriches largely the application of biocatalyst in organic synthesis.[11] More single enzyme multistep transformations will be expected. Several elegant works have been achieved successfully. Klaas et al. reported a combined multistep process of deprotection, acetylation and epoxidation catalyzed by Novozym 435.[12] Our group also reported the two-step enzymatic synthesis of imidazole derivatives containing glucose mediated by protease.[13] However, in many cases the promiscuous activity was much lower and the transformation generally requires several days. The stubborn low activity limits strongly the scope of application of biocatalysts. Thus exploration of biocatalysts with high promiscuous activity becomes particularly fascinating and remains a great challenge.

Recently, Berglund and co-workers reported that the promiscuous Ser105Ala mutant of *Candida antarctica* lipase B could catalyze the fast carbon-carbon bond formation reaction.[14] The Michael addition of acetylacetone to acrolein could be completed in several minutes by this promiscuous enzyme. Herein, we also found that two natural zinc binding metallo-acylases possess extremely high promiscuous Michael activity. Aminoacylase enzymes are zinc binding metallo-acylases and generally catalyze the hydrolysis of an acyl group from *N*-α-acylamino acids. With the help of site-directed mutagenesis and crystal structures of enzymes, the hydrolysis mechanism was postulated, in which the zinc ion of the active site stabilizes the negative charge of the transition states and Asp366, His67 and His69 serve as a proton shuttle.[15] Considering this mechanism and the special structure of aminoacylase, we envisioned that this enzyme could efficiently catalyze the Michael addition. We have found that D-aminoacylase (DA) from *Escherichia coli* displayed a promiscuous activity to catalyze the carbon-carbon bond formation reaction of 1,3-dicarbonyl compounds to methyl vinyl ketone in organic media.[16] Furthermore, in this paper, we surprisingly found that the two acylases DA and acylase "Amano" (AA) from *Aspergillus oryzae* exhibited extremely high activity for the Michael addition of pyrimidines to vinyl acrylate. The initial reaction rates for the Michael addition of pyrimidines to vinyl acrylate were 7.2-16.5 mM min^{-1} and the reaction can be finished in 5–10 minutes with quantitative yields. This high Michael addition activity of zinc-dependent acylases is of practical significance in expanding the application of enzymes and in the evolution of new biocatalysts.[17]

As part of our ongoing interest in a single enzyme-catalyzed combined conversions in one-pot for complicated organic synthesis, in this paper, we combined the high activity of aminoacylase enzymes for the Michael addition of pyrimidines to vinyl acrylate, with their native activity as an efficient catalyst for regioselective acylation of carbohydrates in organic media. The two steps could be performed in one pot and a single aminoacylase catalyzing the two-step one-pot biotransformation was constructed. A series of complicated pyrimidine-saccharide complexes with potentially biological and pharmacological application were prepared efficiently.

Results and Discussion

We firstly examined the Michael addition of fluorouracil (**1a**) to vinyl acrylate (**2a**). When the reaction was carried out with 2 equiv. of vinyl acrylate at 25 °C in the presence of 8 mg D-aminoacylase for 25 min, a single product was prepared in 96% isolated yield after flash chromatography. The structure of this compound was confirmed by IR, ^{1}H NMR, ^{13}C NMR and HR-MS. We then examined the enzymatic Michael addition of vinyl acrylate with other pyrimidine derivatives such as bromouracil (**1b**), uracil (**1c**) and thymine (**1d**). The enzymatic Michael additions of bromouracil, uracil and thymine also proceeded smoothly and could reach good yield in a short reaction time (Scheme 1). All the reactions in the presence of D-



1a R^1 = F; **1b** R^1 = Br; **1c** R^1 = H; **1d** R^1 = CH_3
2a R^2 = R^3 = H; **2b** R^2 = CH_3, R^3 = H; **2c** R^2 = H, R^3 = CH_3

Scheme 1.

aminoacylase led to the corresponding Michael adduct faster than that in the absence of the biocatalyst. We also detected the reaction process by HPLC and the time courses are shown in Figure 1. The reac-

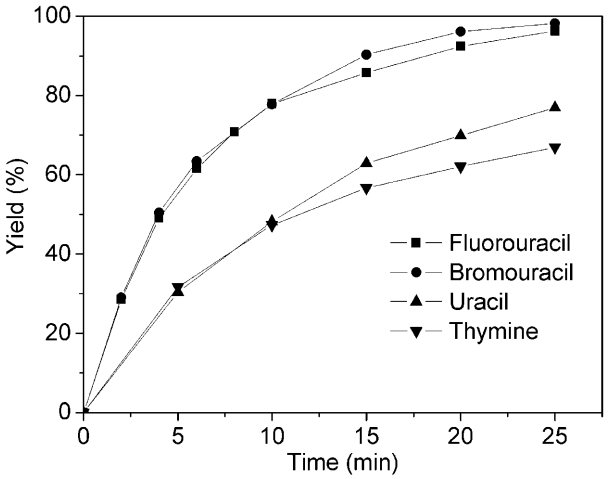


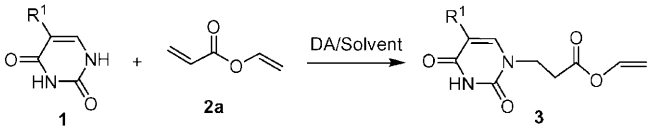
Figure 1. Progress curves of the reaction of different pyrimidines to vinyl acrylate by DA.

tions of vinyl acrylate with fluorouracil and bromouracil proceeded extremely fast with excellent yield (>96%) in 25 min. The initial reaction rates were up to 16.5 mM min^{-1}. The reactions of uracil and thymine could also achieve reaction rates of 7.2 mM min^{-1}. The difference in activities among the four uracil derivatives was partly caused by electron-withdrawing groups, which can reduce the electron cloud density of N-1 in uracil rings.

We have found that D-aminoacylase could also catalyze the Markovnikov addition of N-heterocycles and vinyl esters.[18] In the enzymatic Michael additions of fluorouracil, bromouracil, uracil and thymine with vinyl acrylate, it is worthwhile to note that no Markovnikov adduct was detected by TLC and HPLC under the current conditions. The Michael addition activity of vinyl acrylate was much more favored than the Markovnikov addition. Because vinyl acrylate was present in excess in the reaction, thus only the Michael adduct was obtained.

We then examined the influence of reaction temperature, and organic solvents on the enzymatic Michael addition reaction. The results are shown in Table 1. At room temperature (25 °C) fluorouracil and bromouracil could achieve quantitative yields in 25 min (entries 1 and 2, Table 1). However, the Michael additions of uracil and thymine to vinyl acrylate required 60 min for completion (entries 3 and 4, Table 1). When the reactions were carried out at 50 °C, the Michael addition could reach good yields in several minutes (entries 5–8, Table 1). We also observed that the Michael additions of pyrimidines to

Table 1. The enzymatic Michael addition in different conditions.[a]



Entry	R^1		Solvent	T [°C]	t [min]	Yield [%][b]
1	**1a**	F	**3a**	DMSO	25	25	96
2	**1b**	Br	**3b**	DMSO	25	25	95
3	**1c**	H	**3c**	DMSO	25	60	96
4	**1d**	CH_3	**3d**	DMSO	25	60	94
5	**1a**	F	**3a**	DMSO	50	5	96
6	**1b**	Br	**3b**	DMSO	50	5	95
7	**1c**	H	**3c**	DMSO	50	5	91
8	**1d**	CH_3	**3d**	DMSO	50	10	84
9	**1a**	F	**3a**	DMF	50	10	89

[a] *Reaction conditions:* pyrimidine (0.2 mmol), vinyl acrylate (0.4 mmol), solvent 2 mL, D-aminoacylase (8 mg).
[b] HPLC yields.

vinyl acrylate only could be performed in some highly polar solvents such as DMSO and DMF. The reaction rate in DMSO was faster than that in DMF. The reaction of fluorouracil and vinyl acrylate in DMF also could provide an 89% yield in 10 min at 50 °C (entry 9, Table 1).

Encouraged by these results, we designed some control experiments to demonstrate the catalytic specificity of acylases (Table 2). The reaction of fluorouracil (**1a**) with vinyl acrylate led to the Michael adduct in low yield in the absence of enzyme (entry 1, Table 2). In contrast, the reactions in the presence of DA and AA (acylase "Amano") were up to 40-fold and 70-fold, respectively (entries 2 and 6, Table 2). Besides, the initial reaction rate was practically proportional to the enzyme amount, also suggesting the catalytic effect of the enzyme (entries 2 and 3, Table 2). When DA was pre-treated with urea at 100 °C to completely denaturalize the enzyme, the rate was only 2.5-fold of the background reaction (entry 4, Table 2), suggesting that the tertiary structure of the biocatalyst is necessary to catalyze the reaction. When the reaction was incubated in bovine serum albumin (BSA), the rate was almost equal to the level of the denatured DA (entry 7, Table 2), also ruling out the possibility that the similar amino acid distribution on the protein surface has promoted the process. To further demonstrate that the active site of enzyme was responsible for the reaction, a control experiment was run with covalently inhibited DA by adding 1.5 mM of the non-competitive inhibitor $ZnCl_2$. The added zinc ion would anchor at the α_1 subsite and lower the pK_a values of its ligand residues, perturbing the proton shuttle and intermediate stabilization.[15a] The inhibited enzyme did not show any

Table 2. Michael addition between fluorouracil and vinyl acrylate in the presence of different catalysts.

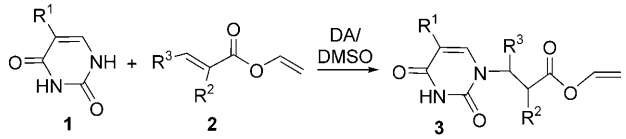
Entry	Catalyst	*t* [min]	Yield [%]	V_0 [mM min^{-1}]	Vr[a]
1	–	25	10	0.4	1
2	D-Aminoacylase	25	96	16.5	41.3
3	D-Aminoacylase[b]	25	89	8.5	21
4	Denatured[c]	25	25	1.0	2.5
5	Inhibited[d]	25	8	0.3	0.8
6	Acylase "Amano"	25	91	27.7	69.3
7	BSA	25	28	1.2	2.9
8	CAL B	25	11	0.5	1.1
9	CCL	25	16	0.6	1.5

[a] Relative initial reaction rate to the control reaction.
[b] 2 mg mL^{-1} DA were used.
[c] DA predenatured with urea at 100 °C for 6 h.
[d] DA inhibited by 1.5 mM $ZnCl_2$.

acylase activity and the specific activity for the Michael addition was the same as that of background reaction (entry 5, Table 2). All these facts suggest that the reaction must take place in a specific fashion at the catalytic site similar to other reported enzymes.[14,19]

In order to extend the scope of this methodology, α,β-substituted Michael acceptors like vinyl methacrylate and vinyl crotonate were tested in DMSO at 50 °C in the presence D-aminoacylase, and the results are shown in Table 3. Both of the acceptors showed moderate reactivity because the strong steric hindrance. Accordingly, higher temperature (50 °C) and longer reaction time were required. All the reactions could only obtain moderate yields even after 24 h. Comparing with vinyl crotonate, the reactions of pyrimidines with vinyl methacrylate proceeded more rapidly and could attain higher yields. To our surprise, none or little optical activity was observed for any tested Michael adducts. This result was similar with the Michael-type addition reaction by CAL-B.[14] The reasons for this will be investigated further and the mutation of some important amino acids around the active site and oxyanion hole will be done to improve the stereoselectivity of these enzymes in the future.

Table 3. Promiscuous acylase-catalyzed Michael addition of pyrimidines to vinyl esters.[a]



1 2 3

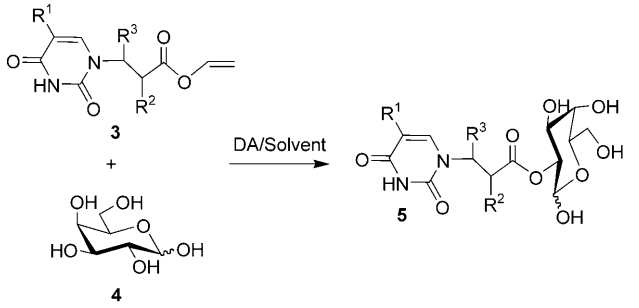
Entry		R^1	R^2	R^3	Product	Yield [%][b]
1	**1a**	F	CH_3	H	**3e**	70
2	**1b**	Br	CH_3	H	**3f**	63
3	**1c**	H	CH_3	H	**3g**	68
4	**1d**	CH_3	CH_3	H	**3h**	50
5	**1a**	F	H	CH_3	**3i**	44
6	**1b**	Br	H	CH_3	**3j**	35
7	**1c**	H	H	CH_3	**3k**	43
8	**1d**	CH_3	H	CH_3	**3l**	32

[a] *Reaction conditions:* pyrimidine (0.2 mmol), vinyl ester (0.4 mmol), DMSO 2 mL, D-aminoacylase (8 mg), 50 °C, 24 h.
[b] Isolated yield.

Hydrolases can carry out transesterification reactions toward a variety of substrates in non-aqueous media as is well known since the seminal work of Klibanov in the early 1980s.[20] Considering the enzymatic selectivity of carbohydrates as multifunctional substrates and the potential biomolecular activity of carbohydrate-pyrimidine complexes, we then investigated the acylation of carbohydrates catalyzed by the D-aminoacylase using the Michael adducts of the first step as the acyl donors. After a screening of DA-catalyzed acylations in different solvents, we found that DMSO was not suitable for the acylation of carbohydrates, although this is where the DA showed high activity of Michael addition. Fortunately, the acylation of carbohydrates could be performed efficiently in DMF, and we knew the DA also had high activity of Michael addition in DMF. The acylation results of D-galactose with different pyrimidine derivatives are shown in Table 4. From entries 1–3, it is seen that the substituent groups of the pyrimidine rings did not have a great influence on the reaction, and the yields were 36%, 40%, 42%, respectively. Moreover, the adducts of fluorouracil with α,β-substituted acrylates can also acylate D-galactose, but the yields are lower than those of other adducts without substitution on acrylate. The structures of these compounds were confirmed by IR, ^{1}H NMR, ^{13}C NMR and MS. These data were in agreement with the desired structure.

The acylation position of D-galactose was verified by ^{13}C NMR according to the general strategy described by Yoshimoto et al.[21] The ^{13}C NMR data are shown in Table 5. The chemical shifts for C-2α and C-2β of product **5a** were downfield shifted from 68.53 to

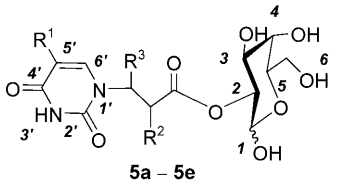
Table 4. The acylation reaction of D-galactose with Michael adducts catalyzed by DA.[a]



Entry		R^1	R^2	R^3	Product	Yield [%][b]
1	**3a**	F	H	H	**5a**	36
2	**3b**	Br	H	H	**5b**	40
3	**3d**	CH_3	H	H	**5c**	42
4	**3e**	F	CH_3	H	**5d**	30
5	**3i**	F	H	CH_3	**5e**	28

[a] *Reaction conditions:* pyrimidine adduct (0.5 mmol), D-galactose (0.25 mmol), DMF 2 mL, D-aminoacylase (10 mg), 50 °C, 48 h.
[b] Isolated yield.

Table 5. Chemical shifts of ^{13}C NMR of pyrimidine derivatives containing a galactose branch.

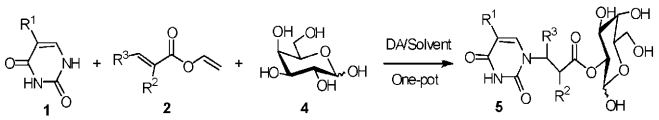


Carbon atom	D-Galactose	**5a**	**5b**	**5c**	**5d**	**5e**
C-6α	61.17	60.83	61.17	60.86	61.08	61.07
C-6β	61.37	60.93	61.30	61.05	61.27	61.13
C-4α	69.50	69.44	69.20	69.34	69.64	69.68
C-4β	68.93	68.83	68.61	68.74	69.02	69.05
C-2α	68.53	72.99	73.05	70.98	71.94	73.00
C-2β	72.07	75.06	75.11	73.98	74.14	75.04
C-3α	69.36	67.11	67.27	66.98	67.20	67.33
C-3β	72.99	71.69	71.81	71.67	71.60	71.95
C-5α	70.66	70.95	70.69	70.40	71.10	71.06
C-5β	75.33	75.69	75.43	75.34	75.50	75.70
C-1α	92.47	89.90	89.92	89.64	89.89	90.06
C-1β	96.44	95.02	95.57	94.53	94.70	95.41

72.99, and 72.07 to 75.06 ppm, and those for C-1α and C-1β were upfield shifted from 92.47 to 89.90 ppm and from 96.44 to 95.20 ppm, meanwhile that for C-3α and C-3β were upfield shifted from 69.36 to 67.11 ppm and from 72.99 to 71.69 ppm, respectively. Similar change was found in the ^{13}C NMR data of product **5b–5e**. These results revealed that esterification of D-galactose regioselectively occurred at the C-2 hydroxy position.

In order to avoid the intermediate purification, single DA-catalyzed one-pot synthetic methods were performed. Three components, **1a, 2a** and **4** were simultaneously added to the reaction system of DMF at 50 °C. After 48 h, the product was isolated and the total yield of **5a** was 40%. The results of the one-pot processes are shown in Table 6. The yields of products

Table 6. DA-catalyzed one pot synthesis of carbohydrate-pyrimidine complexes.[a]



Entry		R^1	R^2	R^3		Product	Yield [%][b]
1	**1a**	F	H	H	**2a**	**5a**	40
2	**1b**	Br	H	H	**2a**	**5b**	42
3	**1d**	CH_3	H	H	**2a**	**5c**	43
4	**1a**	F	CH_3	H	**2b**	**5d**	15
5	**1a**	F	H	CH_3	**2c**	**5e**	13

[a] *Reaction conditions:* pyrimidine (1 mmol), vinyl ester (1 mmol), D-galactose (0.5 mmol), DMF 2 mL, D-aminoacylase (10 mg), 50 °C, 48 h.
[b] Isolated yield.

5d and **5e** are lower than the others because the α,β-substitution of acrylates may cause some hindrance to the binding of substrates at the active site of the enzyme. The structures of the products from the one-pot reactions were confirmed to be the same as those from the separate steps. The process of the one-pot reaction catalyzed by the aminoacylase was monitored by HPLC (Figure 2). After 30 min, the Michael

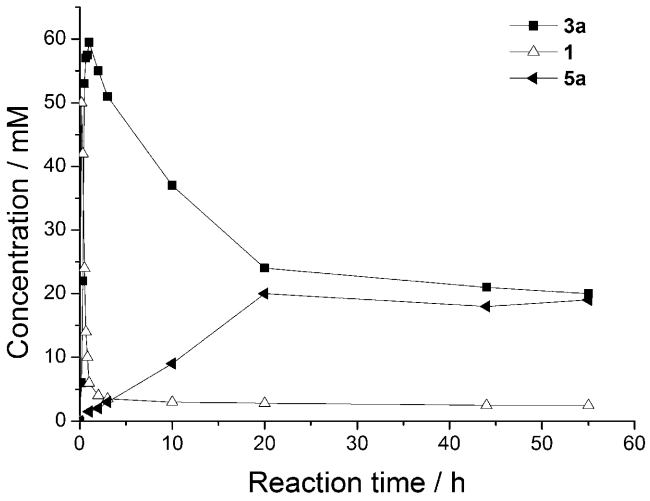


Figure 2. Concentration curve of **1a**, **3a** and **5a** in the process of D-aminoacylase-catalyzed, two-step, one-pot Michael addition/acylation reaction. Reaction was monitored by HPLC.

reaction was finished and most of **1a** was consumed. At this moment, the concentration of **3a** reached to the maximum, while the acylation product between **3a** and **4** appeared after 2 h. A quick increase of final product was observed during 5–20 h. After 20 h, the final product reached to the equilibrium and the concentration of adduct product **3a** did not continue to decrease. The results showed that the catalytic activity of the D-aminoacylase for Michael addition is higher than the activity for acylation. The phenomenon that a natural enzyme had the more active promiscuous activity than its native activity was interesting. It was also confirmed that the two-step one-pot synthesis of Michael addition/acylation could be efficiently performed by the D-aminoacylase. More efficient enzymes for promiscuous Michael addition are being rationally redesigned using directed evolution strategy.

Conclusions

In the present work we investigated the high Michael addition activity of aminoacylase enzymes for the addition of pyrimidines to vinyl acrylate, and the combination of this catalytic promiscuity with its native activity of regioselective acylation of carbohydrates in organic media. The combination provided a facile one-pot synthesis route to monosaccharide-containing pyrimidine derivatives. The initial reaction rates for the Michael addition were high and the reaction could be finished in several minutes under optimal conditions. After the optimization of the stepwise process, a series of saccharide-pyrimidine complexes with potentially biological and pharmacological applications was prepared efficiently using this single aminoacylase catalyzed one-pot biotransformation.

Experimental Section

Materials and General Methods

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE DMX-500 spectrometer at 500 MHz and 125 MHz in $CDCl_3$, respectively. Chemical shifts are reported in ppm (δ), relative to the internal standard of tetramethylsilane (TMS). IR spectra were measured with a Nicolet Nexus FTIR 670 spectrophotometer. D-Aminoacylase from *Escherichia coli* (DA) (EC 3.5.1.81, lyophilized powder, acylase activity 5MU/g) and acylase "Amano" from *Aspergillus* sp. (AA) (EC 3.5.1.14, lyophilized powder, acylase activity 30 KU/g) were purchased from Amano Enzyme Inc. (Japan). All chemicals were obtained from commercial suppliers. Solvents for column chromatography were distilled before use.

General Procedure for the Michael Addition of Pyrimidines and Vinyl Esters

A suspension of pyrimidine (**1**) (0.2 mmol) and 8 mg D-aminoacylase in 2 mL DMSO was incubated at 25 °C and 200 r.p.m. (orbitally shaken) for 5 min. Then, 2 equivalents of vinyl ester (**2**) were added in order to initiate the reaction. After a period of time, the enzyme was filtered off to terminate the reaction and washed with MeOH (3–5 mL). Solvent was evaporated under vacuum to dryness. The crude residue was purified by flash column chromatography on silica gel using petroleum/ethyl mixtures. Product-containing fractions were combined, concentrated, and dried to give **3**. All the compounds were spectroscopically characterized (IR, ^{1}H NMR, ^{13}C NMR and HR-MS). All the reactions were run at least twice.

3-(1′-Fluorouracil)-propionic acid vinyl ester (3a): White solid, mp 154–155 °C. IR: ν = 1728 (O-C=O), 1640 cm^{-1} (C=C); ^{1}H NMR (DMSO-d_6: δ = 11.7 (s, 1H, N3-H, fluorouracil), 8.05 (d, 1H, J = 6.86 Hz, C6-H, fluorouracil), 7.20 (m, 1H, O-CH=C), 4.91,4.68 (m, 2H, O-C=CH_2), 3.89 (t, 2H, J = 6.75 Hz, -CH_2-N), 2.84 (t, 2H, J = 6.74 Hz, -CH_2-C=O); ^{13}C NMR (DMSO-d_6):, δ = 168.80 (C=O), 158.03, 157.82 (C4, fluorouracil), 149.97 (C2, fluorouracil), 141.62 (-O-CH=), 140.69, 138.88 (C5, fluorouracil), 131.11, 130.84 (C6, fluorouracil), 98.98 (=CH_2), 44.21 (-CH_2-N), 32.58 (-CH_2-C=O); HR-MS (EI) m/z = 228.0550, calcd. for $C_9H_9FN_2O_4$: 228.0546.

3-(1′-Bromouracil)-propionic acid vinyl ester (3b): White solid, mp 178–179 °C. IR: ν = 1729 (O-C=O), 1643 cm^{-1} (C=C); ^{1}H NMR (DMSO-d_6): δ = 11.8 (s, 1H, N3-H, bromouracil), 8.19 (s, 1H, C6-H, bromouracil), 7.21 (m, 1H, O-CH=C), 4.91, 4.68 (m, 2H, O-C=CH_2), 3.95 (t, 2H, J = 6.65, -CH_2-N), 2.85 (t, 2H, J = 6.65, -CH_2-C=O); ^{13}C NMR ([DMSO-d_6): δ = 168.80 (C=O), 160.16 (C4, bromouracil), 150.72 (C2, bromouracil), 146.25 (-O-CH=), 141.60 (C5, bromouracil), 94.77 (=CH_2), 89.96 (C6, bromouracil), 44.38 (-CH_2-N), 32.61 (-CH_2-C=O); HR-MS (EI): m/z = 287.9745, 289.9727, calcd. for $C_9H_9BrN_2O_4$: 287.9746, 289.9725.

3-(1′-Uracil)-propionic acid vinyl ester (3c): White solid, mp 144–145 °C. IR: ν = 1732 (O-C=O), 1642 cm^{-1} (C=C); ^{1}H NMR ($CDCl_3$): δ = 9.17 (s, 1H, N3-H, uracil), 7.37 (d,1H, J = 7.90, C6-H, uracil), 7.24 (m, 1H, O-CH=C), 5.68 (d, 1H, J = 7.80, C5-H, uracil), 4.93, 4.64 (m, 2H, O-C=CH_2), 4.02 (t, 2H, J = 5.85, -CH_2-N), 2.89 (t, 2H, J = 5.90, -CH_2-C=O), ^{13}C NMR (DMSO-d_6): δ = 168.84 (C=O), 164.16 (C4, uracil), 151.31 (C2, uracil), 146.41 (-O-CH=), 141.60 (C5, uracil), 101.19 (=CH_2), 99.00 (C6, uracil), 44.12 (-CH_2-N), 32.81 (-CH_2-C=O); HR-MS (EI): m/z = 210.0643, calcd. for $C_9H_{10}N_2O_4$: 210.0641.

3-(1′-Thymine)-propionic acid vinyl ester (3d): White solid, mp 152–153 °C. IR: n = 1733 (O-C=O), 1641 cm^{-1} (C=C); ^{1}H NMR ($CDCl_3$): δ = 8.9 (m, 1H, N3-H, thymine), 7.24 (m, 1H, OCH=C), 7.19 (s, 1H, C6-H, thymine), 4.93, 4.64 (m, 2H, O-C=CH_2), 3.99 (t, 2H, J = 5.96, -CH_2-N), 2.89 (t, 2H, J = 5.97, -CH_2-C=O); ^{13}C NMR (DMSO-d_6): δ = 168.82 (C=O), 164.72 (C4, thymine), 151.26 (C2, thymine), 142.15 (-O-CH=), 141.60 (C5, thymine), 108.73 (=CH_2), 99.00 (C6, thymine), 43.86 (-CH_2-N), 32.90 (-CH_2-C=O), 12.38 (-CH_3); HR-MS (EI): m/z = 224.0795, calcd. for $C_{10}H_{12}N_2O_4$: 224.0797.

3-(1′-Fluorouracil)-2-methyl-propionic acid vinyl ester (3e): White solid, mp 108–109 °C. IR: ν = 1745 (-O-C=O), 1639 cm^{-1} (C=C); ^1H NMR ($CDCl_3$): δ = 7.43 (d, 1H, *J* = 5.60 Hz, C6-H, fluorouracil), 7.23 (m, 1H, -O-CH=C), 4.95, 4.67 (m, 2H, -O-CH=CH_2), 3.99, 3.71 (m, 2H, -CH_2-N), 3.10 (m, 1H, -CH-), 1.32 (d, 3H, *J* = 7.35 Hz, -CH_3); ^{13}C NMR ($CDCl_3$): δ = 171.37 (-OC=O), 157.98, 157.77 (C4, fluorouracil), 150.18 (C2, fluorouracil), 141.80 (-O-CH=), 140.79, 138.97 (C5, fluorouracil), 131.05, 130.79 (C6, fluorouracil), 99.10 (=CH_2), 50.31 (-CH_2-N), 38.15 (-CH-C=O); 14.23 (-CH_3); HR-MS (EI): *m/z* = 242.0706, calcd. for $C_{10}H_{11}FN_2O_4$: 242.0703.

3-(1′-Bromouracil)-2-methyl-propionic acid vinyl ester (3f): White solid, mp 130–131 °C. IR: ν = 1730 (-O-C=O), 1689, 1647 cm^{-1} (C=C); ^1H NMR ($CDCl_3$): δ = 9.08 (s, 1H, N-H of bromouracil), 7.68 (s, 1H, C6-H, bromouracil), 7.24 (m, 1H, -O-CH=C), 4.96, 4.67 (m, 2H, -OCH=CH_2), 3.98, 3.77 (m, 2H, -CH_2-N), 3.13 (m, 1H, -CH-), 1.32 (d, 3H, *J* = 7.24 Hz, -CH_3); ^{13}C NMR ($CDCl_3$): δ = 171.60 (-O-C=O), 159.41 (C4, bromouracil), 150.30 (C2, bromouracil), 145.04 (C6, bromouracil), 140.96 (-O-CH=), 99.14 (=CH_2), 96.33 (C5, bromouracil), 51.87 (-CH_2-N), 38.64 (-CH-C=O), 15.05 (-CH_3); HR-MS (EI): *m/z* = 301.9900, 303.9904, calcd. for $C_{10}H_{11}BrN_2O_4$: 301.9902, 303.9882.

3-(1′-Uracil)-2-methyl-propionic acid vinyl ester (3g): White solid, mp 70–71 °C. IR: ν = 1749 (-O-C=O), 1685, 1641 (C=C), 949 cm^{-1}; ^1H NMR ($CDCl_3$): δ = 9.65 (s, 1H, N-H of uracil), 7.28 (d, 1H, *J* = 7.93 Hz, C6-H, uracil), 7.22 (m, 1H, -O-CH=C), 5.68 (s, 1H, *J* = 7.93 Hz, C5-H, uracil), 4.93, 4.64 (m, 2H, -O-CH=CH^2), 3.98, 3.77 (qq, 2H, -CH_2-N), 3.15 (m, 1H, -CH-), 1.29 (d, 3H, *J* = 7.28 Hz, -CH_3); ^{13}C NMR ($CDCl_3$): δ = 171.88 (-O-C=O), 164.14 (C4, uracil), 151.25 (C2, uracil), 145.72 (C6, uracil), 141.06 (-O-CH=), 102.14 (C5, thymine), 99.06 (=CH_2), 51.68 (-CH_2-N), 38.72 (-CH-C=O), 15.11 (-CH_3); HR-MS (EI): *m/z* = 224.0795, calcd. for $C_{10}H_{12}N_2O_4$: 224.0797.

3-(1′-Thymine)-2-methyl-propionic acid vinyl ester (3h): White solid, mp 84–85 °C. IR: ν = 1744 (-O-C=O), 1683, 1642 cm^{-1} (C=C); ^1H NMR ($CDCl_3$): δ = 9.29 (s, 1H, N-H of bromouracil), 7.24 (m, 1H, -O-CH=C), 7.09 (s, 1H, C6-H, thymine), 4.93, 4.64 (m, 2H, -O-CH=CH_2), 3.96, 3.74 (m, 2H, -CH_2-N), 3.13 (m, 1H, -CH-), 1.90 (s, 3H, CH_3 of thymine), 1.29 (d, 3H, *J* = 7.20 Hz, -CH_3); ^{13}C NMR ($CDCl_3$): δ = 171.93 (-O-C=O), 164.53 (C4, thymine), 151.27 (C2, thymine), 141.55 (C6, thymine), 141.11 (-O-CH=), 110.67 (C5, thymine), 98.97 (=CH_2), 51.47 (-CH_2-N), 38.84 (-CH-C=O), 15.11 (-CH_3), 12.50 (CH_3 of thymine); HR-MS (EI): *m/z* = 238.0956, calcd. for $C_{11}H_{14}N_2O_4$: 238.0954.

3-(1′-Fluorouracil)-butyric acid vinyl ester (3i): White solid, mp 158–159 °C. IR: n = 1744 (O-C=O), 1647 cm^{-1} (C=C); ^1H NMR ($CDCl_3$): δ = 8.85 (s, 1H, N3-H, fluorouracil), 7.37(d, 1H, *J* = 5.96 Hz, C6-H, fluorouracil), 7.24 (m, 1H, -O-CH=C), 4.94, 4.65 (m, 2H, -O-CH=CH_2), 4.82 (m, 1H, -CH-N), 3.01, 2.81 (m, 2H, -CH_2-C=O), 1.53 (d, 3H, *J* = 7.03 Hz, -CH_3); ^{13}C NMR ($CDCl_3$): δ = 168.15 (-O-C=O), 157.55, 157.34 (C4, fluorouracil), 149.84 (C2, fluorouracil), 141.60 (-O-CH=), 141.31, 139.49 (C5, fluorouracil), 127.49, 127.23 (C6, fluorouracil), 99.09 (=CH_2), 48.95 (-CH-N), 38.33 (-CH_2-C=O), 19.07 (-CH_3); HR-MS (EI): *m/z* = 242.0700, calcd. for $C_{10}H_{11}FN_2O_4$: 242.0703.

3-(1′-Bromouracil)-butyric acid vinyl ester (3j): White solid, mp 168–169 °C. IR: ν = 1748 (O-C=O), 1697, 1648 cm^{-1} (C=C); ^1H NMR ($CDCl_3$): δ = 8.90 (s, 1H, N3-H, bromouracil), 7.60 (s, 1H, C6-H, bromouracil), 7.22 (m, 1H, -O-CH=C), 4.93, 4.64 (m, 2H, -O-CH=CH_2), 4.81 (m, 1H, -CH-N), 3.01, 2.81 (m, 2H, -CH_2-C=O), 1.54 (d, 3H, *J* = 7.03 Hz, -CH_3); ^{13}C NMR ($CDCl_3$): δ = 167.57 (-O-C=O), 159.02 (C4, bromouracil), 150.00 (C2, bromouracil), 142.19 (C5, bromouracil), 140.87 (-O-CH=), 96.76 (C6, bromouracil), 99.16 (=CH_2), 52.19 (-CH-N), 38.60 (-CH_2-C=O), 18.97 (-CH_3); HR-MS (EI): *m/z* = 301.9906, 303.9894, calcd. for $C_{10}H_{11}BrN_2O_4$: 301.9902, 303.9882.

3-(1′-Uracil)-butyric acid vinyl ester (3k): White solid, mp 133–134 °C. IR: ν = 1745 (OC=O), 1691, 1648 (C=C), 951 cm^{-1}; ^1H NMR ($CDCl_3$): δ = 9.51 (s, 1H, N3-H, uracil), 7.27 (d, 1H, *J* = 8.02 Hz, C6-H, uracil), 7.21 (m, 1H, -O-CH=C), 5.73 (d, 1H, *J* = 8.02 Hz, C5-H, uracil), 4.92, 4.62 (m, 2H, -O-CH=CH^2), 4.80 (m, 1H, -CH-N), 3.03, 2.80 (m, 2H, -CH^2-C=O), 1.52 (d, 3H, *J* = 7.05 Hz, -CH_3); ^{13}C NMR ($CDCl_3$): δ = 167.72 (-O-C=O), 163.59 (C4, uracil), 150.87 (C2, uracil), 142.51 (-O-CH=), 140.87 (C5, uracil), 102.47 (C6, uracil), 98.93 (=CH_2), 51.44 (-CH-N), 38.66 (-CH_2-C=O), 18.88 (-CH_3); HR-MS (EI): *m/z* = 224.0782, calcd. for $C_{10}H_{12}N_2O_4$: 224.0797.

3-(1′-Thymine)-butyric acid vinyl ester (3l): White solid, mp 137–138 °C. IR: ν = 1752 (OC=O), 1693, 1647 (C=C), 949 cm^{-1}; ^1H NMR ($CDCl_3$): δ, = 9.20 (s, 1H, N3-H, thymine), 7.27 (m, 1H, -O-CH=C), 7.07 (s, 1H, C6-H, thymine), 4.89, 4.63 (m, 2H, -O-CH=CH_2), 4.81 (m, 1H, -CHN), 3.05, 2.79 (m, 2H, -CH_2-C=O), 1.93 (s, 3H, C5-CH_3 of thymine), 1.51 (d, 3H, *J* = 6.73 Hz, -CH_3); ^{13}C NMR ($CDCl_3$): δ = 167.78 (-O-C=O), 164.07 (C4, thymine), 150.87 (C2, thymine), 140.92 (-O-CH=), 138.28 (C6, thymine), 110.97 (C5, thymine), 98.88 (=CH_2), 51.11 (-CH-N), 38.85 (-CH_2-C=O), 18.98 (-CH_3), 12.72 (CH_3 of thymine); HR-MS (EI): *m/z* = 238.0950, calcd. for $C_{11}H_{14}N_2O_4$: 238.0954.

General Procedure for the Transesterification of D-Galactose and Michael adducts

The reaction was initiated by adding 5 mg mL^{-1} D-aminoacylase to 2 mL anhydrous DMF containing 0.25 mmol D-galactose and 0.5 mmol Michael adducts of pyrimidines (**3a**, **3b**, **3d**, **3i**, **3e**), respectively. The suspension was kept at 50 °C and shaken at 200 r.p.m. The reaction was monitored by TLC (ethyl acetate/methanol/water, 17/4/1, v/v). The reactions were terminated by filtering off the enzyme and then the filtrate was evaporated under reduced pressure. The products were isolated by silica gel column chromatography.

2-*O*-[3′-(1″-Fluorouracil)-propionyl]-D-galactose (5a): Yellow oil. IR: n = 3380 (O-H), 1686 cm^{-1} (C=O); ^1H NMR (DMSO-d_6): δ = 11.8 (s, 1H, N3-H, fluorouracil), 8.04 (m, 1H, C6-H, fluorouracil), 6.77, 6.50, 5.06 (1H, m, 1H, H1 of D-galactose), 4.92–4.35, 3.90–3.50 (m, 12H, C1-OH, C2-H, C2-OH, C3-H, C3-OH, C4-H, C4-OH, C5-H, C6-H of D-galactose, -*N*-CH_2), 2.72 (m, 2H, -CH_2-C=O); ^{13}C NMR (DMSO-d_6): δ = 171.3, 170.5 (C=O), 158.2, 158.0 (C4, fluorouracil), 150.0 (C2, fluorouracil), 140.7, 138.9 (C5, fluorouracil), 131.4, 131.2 (C6, fluorouracil), 95.2 (C1 of β-D-galactose), 89.9 (C1 of α-D-galactose), 75.7 (C5 of β-D-galactose), 75.06 (C2 of β-D-galactose), 72.99 (C2 of α-D-galactose), 71.69 (C3 of β-D-galactose), 70.95 (C5 of α-D-galactose), 69.44 (C-4 of α-D-galactose), 68.83 (C-4 of β-D-galactose), 67.11 (C-3 of α-D-galactose), 60.93 (C-6 of β-D-galactose),

60.83 (C-6 of α-D-galactose), 44.79 (-CH_2-N), 33.12 (-CH_2-C=O); ESI-MS: $m/z = 387$ $[M+Na]^+$.

2-*O*-[3′-(1″-Bromouracil)-propionyl]-D-galactose (5b): Yellow oil. IR: n = 3382 (O-H), 1693 cm^{-1} (C=O); ^1H NMR (D_2O): $\delta = 8.05$ (s, 1H, C6-H, bromouracil), 5.24, 4.83, 4.50 (1H, H1 of D-galactose), 4.20–3.30 (8H, C2-H, C3-H, C4-H, C5-H, C6-H of D-galactose, -*N*-CH_2), 2.88–2.75 (m, 2H, -CH_2-C=O); ^{13}C NMR (D_2O): δ = 174.08 (C=O), 162.76 (C4, bromouracil), 151.82 (C2, bromouracil), 147.33 (C6, bromouracil), 96.72 (C5, bromouracil), 95.2 (C1 of β-D-galactose), 89.92 (C1 of α-D-galactose), 75.43 (C5 of β-D-galactose), 75.11 (C2 of β-D-galactose), 73.05 (C2 of α-D-galactose), 71.81 (C3 of β-D-galactose), 70.69 (C5 of α-D-galactose), 69.20 (C4 of α-D-galactose), 68.61 (C4 of β-D-galactose), 67.27 (C3 of α-D-galactose), 61.30 (C6 of β-D-galactose), 61.17 (C6 of α-D-galactose), 45.64 (-CH_2-N), 32.98 (-CH_2-C=O); ESI-MS: $m/z = 423$ $[M-1]^+$.

2-*O*-[3′-(1″-Uracil)-propionyl]-D-galactose (5c): Yellow oil. IR: ν = 3420 (O-H), 1685 cm^{-1} (C=O); ^1H NMR (D_2O): $\delta = 7.40$ (1H, C6-H, thymine), 5.24, 4.79, 4.75, 4.58 (1H, H1 of D-galactose), 4.15–3.30 (8H, C2-H, C3-H, C4-H, C5-H, C6-H of D-galactose, -*N*-CH_2), 2.80 (2H, -CH_2-C=O); ^{13}C NMR (D_2O): δ = 172.64, 172.48 (C=O), 167.0 (C4, thymine), 152.14 (C2, thymine), 143.56, 143.52, 142.44 (C6, thymine), 110.15, 110.38 (C5, thymine), 94.53 (C1 of β-D-galactose), 89.64 (C1 of α-D-galactose), 75.34 (C5 of β-D-galactose), 73.98 (C2 of β-D-galactose), 71.67 (C3 of β-D-galactose), 70.98 (C2 of α-D-galactose), 70.40 (C5 of α-D-galactose), 69.34 (C4 of α-D-galactose), 68.74 (C4 of β-D-galactose), 66.98 (C3 of α-D-galactose), 61.05 (C6 of β-D-galactose), 60.86 (C6 of α-D-galactose), 44.72 (-CH_2-N), 32.84 (-CH_2-C=O), 11.27 (-CH_3); ESI-MS: $m/z = 383$ $[M+Na]^+$.

2-*O*-[3′-(1″-Fluorouracil)-2-methyl propionyl]-D-galactose (5d): Yellow oil. IR: ν = 3391 (O-H), 1699 cm^{-1} (C=O); ^1H NMR (D_2O): $\delta = 7.78$ (1H, C6-H, fluorouracil), 5.31–5.23, 4.89–4.81 (m, 1H, H1 of D-galactose), 4.70–4.60, 4.20–3.30 (m, 8H, C2-H, C3-H, C4-H, C5-H, C6-H of D-galactose, -*N*-CH_2), 3.05 [m, 1H, -CH(CH_3)-C=O], 1.13 [s, 3H, -CH(C*H*$_3$)-C=O]; ^{13}C NMR (D_2O: δ = 175.5, 175.7, (C=O), 160.7, 160.5 (C4, fluorouracil), 151.49 (C2, fluorouracil), 141.40, 139.59 (C5, fluorouracil), 131.93, 131.83, 131.44 (C6, fluorouracil), 94.53 (C1 of β-D-galactose), 89.64 (C1 of α-D-galactose), 75.50 (C5 of β-D-galactose), 74.14 (C2 of β-D-galactose), 71.94 (C2 of α-D-galactose), 71.60 (C3 of β-D-galactose), 71.10 (C5 of α-D-galactose), 69.64 (C4 of α-D-galactose), 69.02 (C4 of β-D-galactose), 67.20 (C3 of α-D-galactose), 61.27 (C6 of β-D-galactose), 61.08 (C6 of a-D-galactose), 51.44 (-CH_2-N), 38.77 [-CH(CH_3)-C=O], 14.18 [-CH(CH_3)-C=O]; ESI-MS: $m/z = 401$ $[M+Na]^+$.

2-*O*-[3′-(1″-Fluorouracil)-3-methyl propionyl]-D-galactose (5e): Yellow oil. IR: ν = 3360 (O-H), 1695 cm^{-1} (C=O); ^1H NMR (DMSO-d_6): $\delta = 11.65$ (s, 1H, N3-H, fluorouracil), 8.15 (1H, C6-H, fluorouracil), 6.80, 6.50, 5.10 (m, 1H, H1 of D-galactose), 4.92–4.30, 3.80–3.50 [m, 11H, C1-OH, C2-H, C2-OH, C3-H, C3-OH, C4-H, C4-OH, C5-H, C6-H of D-galactose, -*N*-CH(CH_3)], 2.90 (d, 2H, -CH_2-C=O), 1.27 [s, 3H, -CH(C*H*$_3$)-N]; ^{13}C NMR (DMSO-d_6): $\delta = 170.97$, 170.08 (C=O), 157.99, 157.78 (C4, fluorouracil), 150.13 (C2, fluorouracil), 141.54, 139.72 (C5, fluorouracil), 127.86, 127.60 (C6, fluorouracil), 95.41 (C1 of β-D-galactose), 90.16 (C1 of α-D-galactose), 75.70 (C5 of β-D-galactose), 75.04 (C2 of β-D-galactose), 73.00 (C2 of α-D-galactose), 71.95 (C3 of β-D-galactose), 71.06 (C5 of α-D-galactose), 69.68 (C4 of α-D-galactose), 69.05 (C4 of β-D-galactose), 67.33 (C3 of α-D-galactose), 61.13 (C6 of β-D-galactose), 61.07 (C6 of α-D-galactose), 49.56 [-CH(CH_3)-N], 36.64 (-CH_2-C=O), 19.18 [-CH(CH_3)-*N*-]; ESI-MS: $m/z = 401$ $[M+Na]^+$.

General Procedure for One-Pot Synthesis

The reaction in a suspension of pyrimidine (1 mmol) and vinyl ester (1 mmol), D-galactose (0.5 mmol) in 2 mL DMF was initiated by the addition of 5 mg mL^{-1} D-aminoacylase. The system was incubated at 50 °C and 200 r.p.m. for 48 h. Then, the enzyme was filtered off to terminate the reaction. Solvent was evaporated under reduced pressure. The products were isolated by silica gel column chromatography. All the compounds were spectroscopically characterised.

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