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MANIPULATION OF ENZYME REGIOSELECTIVITY BY SOLVENT ENGINEERING: ENZYMATIC SYNTHESIS OF 5'-O-ACYLRIBONUCLEOSIDES[1]

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Abstract: Regioselectivity of enzyme can be manipulated by solvent engineering. By this technique, a simple and convenient method was developed for the synthesis of 5'-O-acylribonucleosides in high yields by the protease-catalyzed regioselective esterification of the primary hydroxyl groups of ribonucleosides in anhydrous pyridine.

Regioselective acylation of nucleosides is an interesting and challenging area of research. Successful developments in this area may lead to: (i) development of new methods for the synthesis of nucleoside analogues, (ii) introduction of base labile protecting group; useful factor in synthesis of oligonucleotides,[2] (iii) development of acylated derivatives (prodrugs) with enhanced therapeutic properties of the parent nucleoside drug.[3] Although selective chemical acylation of nucleosides have been reported in few cases, it is not a preferred method due to many associated problems, such as tedious separation process and low yields of mono-O-acyl derivatives following nonselective acylation, and time-consuming protection and deprotection steps.[4]

The use of enzymes in organic synthesis has become popular in recent years. Particularly, hydrolytic enzymes such as lipases and proteases have found interesting applications in regioselective acylation[5] of nucleosides and deacylation[6] of acylated nucleosides. There are many possible advantages of using enzymatic methods for example: (i) protection and deprotection steps are not required, (ii) regioselective acylation at desired position under mild conditions is possible, (iii) the extent of side reactions can be reduced, resulting in easy separation and high yield, (iv) regioselection can be controlled by solvent engineering, and/or by appropriate substrate selection, and (vi) enzyme can be recovered easily through filtration and reused.

Klibanov group[5b] made the first attempt to carry out the enzymatic regioselective acylation of ribonucleosides. They used the protease subtilisin in anhydrous dimethyl formamide (DMF) to acylate uridine and adenosine by the transesterification with activated ester such as trichloroethyl butyrate. This method, however, produced low yields (21-29%) of the desired 5'-O-acyl derivative with moderate to good (55-85%) regioselectivity. Wong *et al.*[5c] have used modified subtilisin (subtilisin 83500 derived from subtilisin BPN' via six site-specific mutations) with enol esters as acylating agents and obtained excellent regioselectivity with high yields (65-90%) for 5'-O-acyl derivatives of uridine, adenosine, and cytidine. This report describes a new and simple technique to achieve high regioselectivity in subtilisin catalyzed regioselective esterification of the primary hydroxyl group of ribonucleosides.[7]

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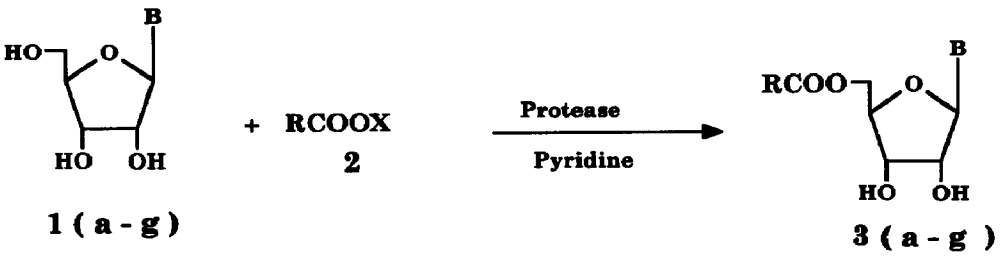
Over the years there has been much interest in manipulating the selectivity of enzymes, and until recently, the only approach has been to alter the protein itself either by chemical modification or through site-directed mutagenesis. Though protein engineering is a powerful tool, it requires that the protein of interest has been cloned and a detailed understanding of its native structure be known. Thus, in many cases, alteration of enzyme is not feasible, and alternate approaches must be found. Enzyme selectivity can be controlled by selection of solvent in non-aqueous media, a technique commonly referred as "Solvent Engineering".[8,9] While the use of solvent engineering to control enzyme enantioselectivity has been described in great detail,[8] there are only few studies on controlling regioselectivity.[9] We found that it is possible to alter and even reverse (from primary to secondary) the regioselectivity of enzyme by changing the solvent.[10] Using this technique, we were able to obtain high yields of 5'-O-acyl derivatives for most of pyrimidine and purine ribonucleosides in subtilisin catalyzed reactions described above by Klibanov[5b] and Wong.[5c] To our knowledge this report is the first to show that the regioselectivity can be optimized by solvent engineering in carbohydrate chemistry.

To study the effect of solvent on regioselectivity of enzyme, we examine the reaction described by Klibanov[5b] in various solvents.[10] From the results of these experiments, it appears that DMF was a poor choice as a solvent system as it provided low regioselectivity of subtilisin and therefore low yield of 5'-O-acyl derivatives of ribonucleoside. Further experiments revealed that pyridine was a better choice as an alternate solvent, selectively yielding 5'-O-acyl derivatives of ribonucleosides in high yields (Scheme 1). Keeping the cost effectiveness of this process in mind, it was also decided to investigate the catalytic properties of two crude (inexpensive) preparations of subtilisin[11] (Proleather and Protease N; Amano) which also showed better selectivity for 5'-position in pyridine under similar experimental conditions.

Table 1 shows that the acyl donor (2) trifluoroethyl butyrate selectively acylates the 5'-hydroxyl of ribonucleosides (1) giving excellent yield of 5'-O-acylribonucleosides (3) in reactions carried out in pyridine and catalyzed by subtilisin (entries 1,7,13,15,17). To overcome the insolubility of ribonucleosides such as adenosine, cytidine, and guanosine in pyridine, their corresponding amino groups were benzoylated. This protection of amino group greatly enhanced the solubility of parent nucleosides in pyridine, increasing reaction rates and yields of 5'-O-acylated products (entries 13,15,17). In case of protease Proleather, the yield of final products were almost the same as with subtilisin (entries 2,8), but with Protease N yields of 5'-O-acylated products were found to be lower under similar condition (entries 3,9). Proleather proved to be a better substitute for subtilisin than protease N in pyridine. In addition, acylation was also conducted using an enol ester such as vinyl butyrate as acylating agent and unmodified subtilisin under the similar reaction conditions which gave 5'-O-acyl derivatives in excellent yields (entries 4,9). In this case also, Proleather proved to be a better substitute for subtilisin (entries 5,10) than Protease N (entries 6,12). In these studies when vinyl acetate was used as acyl donor reactions were slower and gave slightly lower yields (data not shown). Synthesis of butyryl derivative was always a likely choice over the acetyl derivative due to better yield and simplicity of the procedure (data not shown).

Typically, experiments (Scheme 1) were performed by dissolving 0.245 g (1mmole) of uridine in 5mL of anhydrous pyridine to which either 1.70 g (10mmoles) of trifluoroethyl butyrate or 1.14 g (10mmoles) of vinyl butyrate was added. About 200mg of subtilisin[11] was added to this and the reaction mixture was incubated at 47°C with gentle shaking. The reaction was monitored by TLC and HPLC.[6] After 24 hours, the insoluble enzyme was removed by filtration and pyridine was removed by evaporation at reduced pressure. The resulting residue was subjected to flash column chromatography on silica gel to isolate 5'-O-butyryluridine (3a). Similarly (3a) was also prepared by using either 1g. of Proleather or Protease N replacing subtilisin (Table 1).[11] The same procedure was adopted for the synthesis of 5'-O-

Scheme 1



a B = Uracil-1-yl b B = Adenin-9-yl c B = N-6-Benzoyladenin-9-yl
d B = Cytosin-1-yl e B = N-4-Anisoylcytosin-1-yl f B = Hypoxanthin-9-yl
g B = N-2-Benzoylguanin-9-yl

R = $CH_3(CH_2)_2$, X = CH_2CF_3 or $CH{=}CH_2$, Protease = Subtilisin, Proleather, and Protease N

Table 1

Enzymatic Synthesis of 5'-O-Butyrylribonucleosides (3) in Various Reaction Conditions in Pyridine

Entry	Nucleoside (1)[l]	Acyl Donor (2)[m]	Wt. of Enzyme (g)	Vol. of Pyridine (mL)	Time (Days)	5'-O-Butyryl-Nucleoside (3)	Yield[n] (%)
1	1a	TFEB	Subtilisin (0.20)	5	1.83	3a	78
2	1a	TFEB	Proleather (1.00)	5	1.0	3a	79
3	1a	TFEB	Protease N (1.00)	5	3.75	3a	32
4	1a	VB	Subtilisin (0.20)	5	1.0	3a	83
5	1a	VB	Proleather (1.00)	5	1.0	3a	88
6	1a	VB	Protease N (1.00)	5	1.0	3a	29
7	1b	TFEB	Subtilisin (0.35)	25	6.75	3b	82
8	1b	TFEB	Proleather (4.50)	25	7.0	3b	71
9	1b	TFEB	Protease N (1.50)	25	5.0	3b	63
10	1b	VB	Subtilisin (0.35)	25	5.0	3b	68
11	1b	VB	Proleather (4.50)	25	5.0	3b	69
12	1b	VB	Protease N (4.50)	25	5.0	3b	27
13	1c	TFEB	Subtilisin (0.30)	5	3.0	3c	67
14	1d	TFEB	Proleather (4.00)	60	5.0	3d	44
15	1e	TFEB	Subtilisin (0.30)	5	3.0	3e	68
16	1f	TFEB	Proleather (6.00)	100	7.0	3f	63
17	1g	TFEB	Subtilisin (0.30)	5	3.0	3g	85

[l] All the reactions were started with 1 m mol of ribonucleosides except in entries 13, 15, and 17 where amounts of starting nucleosides (1c, 1e, and 1g) were only 0.5 m moles

[m] TFEB = trifluoroethyl butyrate, VB = vinyl butyrate; ratio of nucleoside (1) and acyl donor (2) was always 1:10.

[n] pure isolated product.

butyryladenosine (3b), 5'-O-butyryl-N^6-benzoyladenosine (3c), 5'-O-butyrylcytidine (3d), 5'-O-butyryl-N^4-anisoylcytidine (3e), 5'-O-butyrylinosine (3f), and 5'-O-butyryl-N^2-benzoylguanosine (3g) under the condition described in Table 1.[12]

In conclusion, 5'-O-acylribonucleosides can be synthesized in good yields by the reactions of ribonucleosides with activated esters and enol esters catalyzed by subtilisin in anhydrous pyridine, and to make this process cost-effective subtilisin can be replaced by crude protease Proleather. Proleather works particularly well for those nucleosides which are not very soluble in pyridine.

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11. The highly purified preparation of subtilisin obtained from Sigma costs $ 130/g. The crude enzymes (Proleather and Protease N) were purchased from Amano at $ 1/g. Prior to use, subtilisin and protease N were dissolved in phosphate buffer, the solution was adjusted to pH 7.8 and was freeze-dried. Proleather was also adjusted to pH 10 in borate buffer and was freeze-dried.
12. ^1H and ^{13}C NMR (300 MHz) data are provided for the following new compounds: (i) **3e**: ^1H NMR (DMSO-d_6/D_2O) δ 0.85 (3H, t, CH_3), 1.50 (2H, m, CH_2), 2.30 (2H, t, CH_2CO-), 4.10-4.40 (4H, m, H-3', H-4', H-5'), 4.75 (1H, t, H-2'), 6.05 (1H, d, J= 4.6 Hz, H-1'), 7.50-7.70 (3H, m, ArH), 8.05 (2H, d, ArH), 8.65 (1H, s, H-2) 8.75 (1H, s, H-8). ^{13}C NMR (DMSO-d_6) (partial) δ 13.33, 17.85, 35.16 (butyryl moiety), 63.59, 70.29, 72.93, 81.78, 87.91 (C-5', C-3', C-2', C-4', C-1'), 125.82, 128.23, 132.38, 133.34, 143.22, 150.42, 151.62, 152.10 (aromatic and adenine ring). (ii) **3d**: ^1H NMR (DMSO-d_6/D_2O) δ 0.90 (3H, t, CH_3), 1.55 (2H, m, CH_2), 2.35 (2H, t, CH_2CO-), 3.90-4.30 (5H, m, H-2', H-3', H-4', H-5'), 5.75 (2H, m, H-1', H-5), 7.60 (1H, d, J= 7.5 Hz, H-6). ^{13}C NMR (DMSO-d_6) (partial) δ 13.39, 17.88, 35.21 (butyryl moiety), 63.48, 69.63, 73.38, 80.38, 89.98 (C-5', C-3', C-2', C-4', C-1'), 94.07, 141.12, 155.07, 165.49 (cytosine ring). (iii) **3c**: ^1H NMR (DMSO-d_6/D_2O), δ 0.90 (3H, t, CH_3), 1.55 (2H, m, CH_2), 2.35 (2H, t, CH_2CO-), 3.85 (3H, s, OCH_3) 3.90-4.40 (5H, m, H-2', H-3', H-4', H-5'), 5.80 (1H, d, J= 2.8 Hz, H-1'), 7.05 (2H, d, J= 8.9 Hz, ArH), 7.35 (1H, d, J=7.6, H-5), 8.05 (1H, d, J= 8.9 Hz, ArH), 8.15 (1H, d, J= 7.6 Hz, H-6). ^{13}C NMR (DMSO-d6) (partial) δ 13.41, 17.89, 35.24 (butyryl moiety), 55.50 (MeO), 63.22, 69.30, 73.79, 80.80, 90.98 (C-5', C-3', C-2', C-4' C-1'), 96.37, 125.13, 130.69, 144.73, 154.38, 162.80, 163.11 (aromatic and cytosine ring). (iv) **3g**: ^1H NMR (DMSO-d_6/D_2O) δ 0.90 (3H, t, CH_3), 1.50 (2H, m, CH_2), 2.30 (2H, t, CH_2CO-), 4.05-4.40 (4H, m, H-3', H-4', H-5'), 4.55 (1H, m, H-2'), 5.90 (1H, d, J= 5.5 Hz, H-1'), 7.55-7.70 (3H, m, ArH), 8.05 (2H, d, J= 7.4 Hz, ArH), 8.20 (1H, s, H-8). ^{13}C NMR (DMSO-d_6) (partial) 13.34, 17.86, 35.16 (butyryl moiety), 63.71, 70.28, 73.11, 81.69, 86.88 (C-5', C-3', C-2', C-4', C-1'), 120.68, 128.45, 132.20, 133.11, 137.99, 148.12, 148.74, 154.97 (aromatic and guanine ring).

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