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Novel enzymatic synthesis of levulinyl protected nucleosides useful for solution phase synthesis of oligonucleotides

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Abstract—An efficient synthesis of 3′- and 5′-*O*-levulinyl-2′-deoxy- and 2′-*O*-alkylribonucleosides has been developed from appropriate nucleosides by enzyme-catalyzed regioselective acylation in organic solvents. Several lipases were screened in combination with acetonoxime levulinate as an acylating agent. Immobilized *Pseudomonas cepacia* lipase (PSL-C) was selected for acylation of the 3′-hydroxyl group in nucleosides, furnishing 3′-*O*-levulinylated products **1** and **3** in excellent yields. Similarly, *Candida antarctica* lipase B (CAL-B) provided 5′-*O*-levulinyl nucleosides **2** and **4** in high yields. Base-protected cytidine and adenosine analogs were found to be good substrates for lipase-mediated acylations. To demonstrate the industrial utility of this method, 3′-*O*-levulinyl thymidine and N^2-Ibu-5′-*O*-levulinyl-2′deoxyguanosine were synthesized on a 25 g scale. Additionally, PSL-C was reused to make the processes further economical.

1. Introduction

Chemical synthesis of therapeutic oligonucleotides[1] has undergone a renaissance during the last decade due to their potential use in the treatment of a wide range of human diseases.[2] Although classical solid-support methodology for the synthesis of oligonucleotides is well established, the solution-phase synthesis is perhaps the next best alternative should any of these drugs become commercially successful and are required in ton quantity. Therefore, establishment of the solution-phase synthesis of oligonucleotides is an important task.[3] In order to make large quantities of oligonucleotides via solution-phase synthesis, one would require appropriate nucleosidic building-blocks on an industrial scale.[4] The key building blocks for solution-phase oligonucleotide synthesis are appropriately protected nucleosidic monomers. Among the limited protecting groups available, the levulinyl group is frequently chosen to protect the 3′- and/or 5′-hydroxyl of the nucleosides. This group is stable during oligonucleotide coupling reactions and can be selectively cleaved without affecting other protecting groups in the same molecule.[5] Until recently, the preparation of these building blocks has been carried out through several tedious chemical protection/deprotection steps.[6]

The potential of enzymes in organic synthesis is well recognized, in particular when substrates possess several functional groups of similar reactivity.[7] The chiral nature of enzymes allows selective acylation and/or deacylation processes on a variety of molecules avoiding additional chemical protection/deprotection steps.

In our ongoing research related to the synthesis of selectively modified 3′-*O*-levulinyl and 5′-*O*-levulinyl nucleosides from 2′-deoxy and 2′-*O*-alkyl nucleosides, we have recently developed a general chemoenzymatic synthesis of monolevulinyl protected nucleosides using commercial lipases (Chart 1).[8] The original approach was a two-step method based on chemical acylation of both hydroxyl groups followed by the regioselective enzymatic hydrolysis of one of the levulinyl groups. *Candida antarctica* lipase B (CAL-B) was found to selectively hydrolyze the 5′-*O*-levulinate esters furnishing 3′-*O*-levulinyl-2′-deoxynucleosides **1** in >80% isolated yields. Immobilized *Pseudomonas cepacia* lipase (PSL-C) and *C. antarctica* lipase A (CAL-A) exhibited the opposite selectivity toward the hydrolysis at 3′-position affording 5′-*O*-levulinyl derivatives **2** in >70% isolated yields. A similar hydrolysis procedure was successfully extended to the synthesis of 3′- and 5′-*O*-

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levulinyl protected 2′-*O*-alkylribonucleosides **3** and **4**. Unfortunately, attempts to extend this methodology to nucleosides with *N*-benzoylated bases using CAL-B or unprotected bases using PSL-C were not successful.

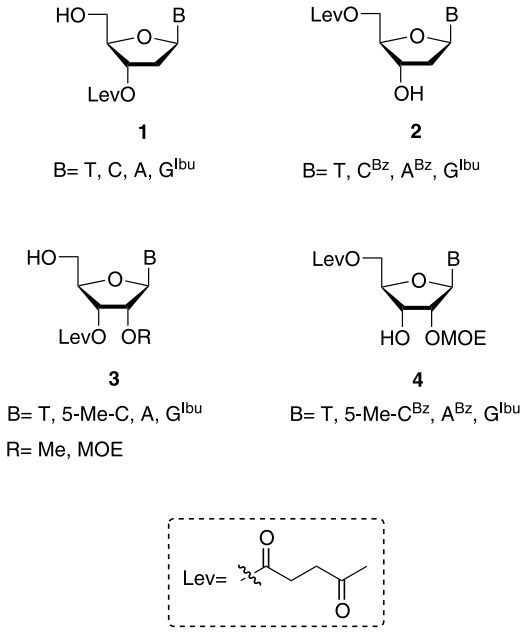
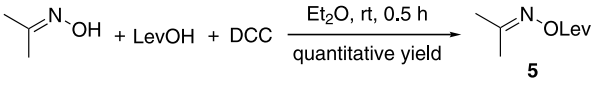


Chart 1.

Herein we describe a new and concise approach for the synthesis of levulinyl protected building blocks through regioselective enzymatic acylation of base-protected nucleosides overcoming the limitations of our original method. The improved method is a one step enzymatic protocol that utilizes the complementary behavior shown by enzymes in acylation and hydrolysis processes.

2. Results and discussion

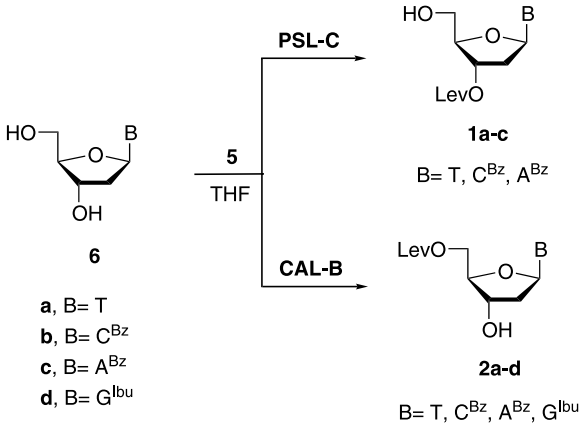
We had previously reported that oxime esters are excellent substrates towards regioselective enzymatic acylations of various natural products, including nucleosides.[9] Therefore, acetonoxime levulinate **5** was selected as an acylating agent for the present study. Synthesis of **5** was accomplished in quantitative yield in a single step starting from inexpensive industrial quality levulinic acid and acetonoxime (Scheme 1).[10]



Scheme 1.

2.1. Acylation studies of 2′-deoxynucleosides

The overall enzymatic approach for the protection of 2′-deoxynucleosides is shown in Scheme 2 and the experimental data is summarized in Table 1. Treatment of **5** with thymidine **6a** in the presence of PSL-C at 30°C in THF indicated (TLC) that after 7 h the starting material was completely consumed (entry 1, Table 1). After work-up, ^{1}H NMR data of the product confirmed the formation of 3′-*O*-levulinylthymidine **1a** as the sole product due to regioselective acylation of the 3′-hydroxyl group. Protected **1a** was isolated in 85% yield as a crystalline product without chromatography. Similar conditions were utilized for *N*-benzoylcytidine **6b** to obtain total regioselectivity at the 3′-position, however, the reaction was much slower (34 h, entry 2, Table 1) compared to thymidine. This could be attributed to the limited solubility of the starting material **6b** in THF.



Scheme 2.

Similarly, PSL-C regioselectively acylated the 3′-hydroxyl group of nucleoside **6c**, furnishing *N*-benzoyl-3′-*O*-levulinyl-2′-deoxyadenosine **1c** in 89% isolated yield (entry 3, Table 1). It is noteworthy that nucleosides **6b** and **6c** bearing *N*-benzoyl protected bases were completely and selectively acylated to form *N*-benzoyl-3′-*O*-levulinyl-2′-deoxycytidine **1b** and *N*-benzoyl-3′-*O*-levulinyl-2′-deoxyadenosine **1c**, respectively. The ease of preparation of **1b** and **1c** indicates the compatibility of the lipase toward acylation. This attribute overcomes the previous limitations of our method where we could not make **1b** and **1c** via enzymatic hydrolysis of 3′,5′-dilevulinyl nucleosides.[8] The acylation reaction catalyzed by PSL-C on N^2-isobutyryl-2′-deoxyguanosine **6d** afforded a mixture of 3′-*O*-levulinyl **1d**, 5′-*O*-levulinyl **2d**, and 3′,5′-di-*O*-levulinyl derivatives, in addition to unreacted nucleoside **6d** (entry 4, Table 1). Increasing the reaction temperature to 60°C did not improve the selectivity (entry 5, Table 1). Next, we evaluated the influence of co-solvents on the catalytic activity of the enzyme using different ratios of organic solvents. In this case, 10% of pyridine or acetonitrile were employed as co-solvents, 1,4-dioxane, acetonitrile and DMF were used as solvents without much success. Nevertheless, we are able to make **6d** via our original enzymatic hydrolysis protocol in good yields should there be a need for this nucleoside.[8]

Selective 5′-*O*-acylation of thymidine was accomplished with CAL-B at 30°C in a facile manner (entry 6, Table 1) with a trace amount of di-levulinyl product. In order to increase the selectivity, the reaction temperature was reduced to 10°C. The low temperature reaction did not

Table 1. Regioselective enzymatic acylation of 2′-deoxynucleosides **6**

Entry	Substrate	Enzyme	*T* (°C)	*t* (h)	**1** (%)[a,b]	**2** (%)[a,b]	**6** (%)[a]	diLev[c] (%)[a]
1	**6a**	PSL-C[d]	30	7	>97 (85)			
2	**6b**	PSL-C[d]	30	34	98 (77)		2	
3	**6c**	PSL-C[d]	30	11	96 (89)		4	
4	**6d**	PSL-C[d]	30	24	44	23	11	22
5	**6d**	PSL-C[d]	60	14	35	12	22	31
6	**6a**	CAL-B[e]	30	3	5	92 (74)		3
7	**6a**	CAL-B[e]	10	8	3	91		6
8	**6b**	CAL-B[e]	30	18	6	92 (76)		2
9	**6c**	CAL-B[e]	30	11	5	94 (71)		1
10	**6d**	CAL-B[e]	30	16		>97 (79)		
11	**6a**	PSL-C[f]	40	4.5	>97 (83)			Traces
12	**6a**	PSL-C[f,g]	40	8	>97 (80)			
13	**6b**	PSL-C[f]	40	5	>97 (86)			
14	**6c**	PSL-C[f]	40	10	94 (82)		6	
15	**6a**	PSL-C[f,h]	30	14	>97 (92)			
16	**6b**	PSL-C[f,h]	30	14	>97 (81)			
17	**6c**	PSL-C[f,h]	30	14	>97 (90)			

[a] Based on ^{1}H NMR signal integration.
[b] Percentages of isolated yields are given in parenthesis.
[c] diLev = 3′,5′-di-*O*-Levulinyl derivative.
[d] Ratio **6**:PSL-C is 1:3 (w/w); 0.1 M concentration.
[e] Ratio **6**:CAL-B is 1:1 (w/w); 0.1 M concentration.
[f] Ratio **6**:PSL-C is 1:2 (w/w); 0.2 M concentration.
[g] Recycled PSL-C from entry 11.
[h] Scale-up to 5 g of **6**.

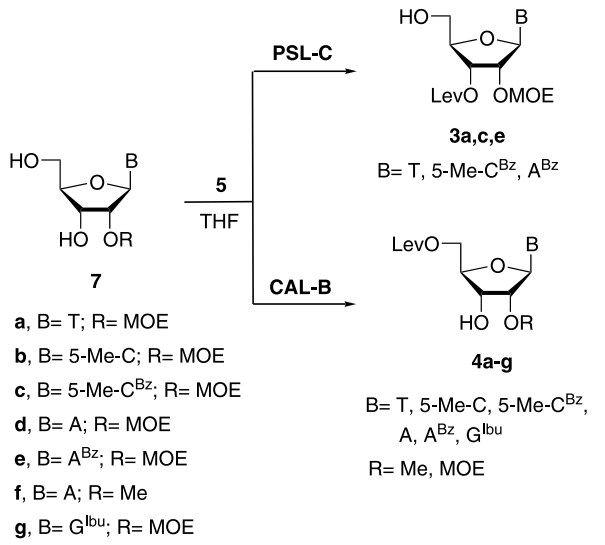
reduce the formation of the byproduct (entry 7, Table 1). Good selectivity towards the 5′-hydroxyl group was accomplished when nucleosides **6b–d** reacted with CAL-B at 30°C in THF, furnishing 5′-*O*-levulinyl derivatives **2b–d** in high yields (entries 8, 9, and 10, Table 1). Although there were minor byproducts formed during the acylation with CAL-B, we were able to isolate **6b–d** in high purity and acceptable yields (71–79%) after chromatography. In order to obtain total regioselectivity, Chirazyme L-2, c-f, C3, lyo (lipase comp. B from *C. antarctica* on a different carrier than Novozym SP435) were tried, without much avail.

Scale-up studies. All of the above PSL-C mediated acylation experiments were carried out with a high enzyme ratio (**6**:PSL-C, 1:3, w/w) to insure the completion of the reaction. For cost-efficient scale-up it was necessary to reduce the enzyme ratio (1:2) and increase the reaction concentration to 0.2 M, more appropriate for industrial processes. Additionally, the reaction temperature was increased to 40°C with the aim being ability to reduce the reaction times and plant occupancy. Treatment of **5** with **6a** (0.2 M) in the presence of PSL-C at 40°C provided 3′-*O*-levulinylnucleoside **1a** in 83% isolated yield with complete regioselectivity in 4.5 h (entry 11, Table 1). The industrial productivity of the biocatalytic reactions could be further increased when immobilized enzymes are utilized for chemical transformations. Since PSL-C is an immobilized lipase, we further explored the possibility of reusing the enzyme. In entry 12 (Table 1), the data confirmed that reusing PSL-C was possible and it worked as well as in our previous experiment (entry 11, Table 1) except an extended time was necessary to drive the reaction to completion. Similarly, nucleosides **6b** and **6c** were subjected to enzyme catalysis at 40°C in 0.2 M concentration, allowing isolated preparation of **1b** (86%) and **1c** (82%), respectively (entries 13 and 14, Table 1). Finally, the optimized processes with PSL-C at 30°C and 0.2 M concentration were scaled up to 5 g of nucleoside, furnishing **1a** (92%), **1b** (81%), and **1c** (90%), respectively, in 14 h (entries 15, 16, and 17, Table 1). It is noteworthy that 3′-*O*-Lev- and 5′-*O*-Lev-deoxynucleosides **1** and **2** were isolated by precipitation from the crude reaction mixture to avoid any expensive chromatographic steps. Only in those exceptional cases where the mixture of acylated byproducts were obtained and precipitation attempts failed, flash chromatography was employed to isolate pure products.

2.2. Acylation of 2′-*O*-alkyl nucleosides

We also became interested in the preparation of levulinyl protected 2′-*O*-alkyl nucleosides **3** and **4** (Scheme 3) as building blocks for solution-phase synthesis of second-generation oligonucleotides. The incorporation of 2′-*O*-alkyl residue in therapeutic oligonucleotides provided enhanced stability, higher binding affinity, and improved potency relative to their 2′-deoxy predecessors in vivo.[11]

The experimental data in Table 2 shows that PSL-C was selective towards the acylation of the 3′-position of 2′-*O*-MOE nucleosides which bears T, 5-Me-C^{Bz}, and A^{Bz} as bases (entries 1, 3, and 5, Table 2). However, no reaction occurred when both 5-Me-C and A unprotected bases were used (entries 2 and 4, Table 2). All the reactions took place at 30°C, except for 2′-*O*-MOE-A^{Bz}



Scheme 3.

(**7e**), where 40°C was needed to reach conversion close to 100%. Interestingly, when the reaction was carried out at 30°C it stalled at 50% conversion and did not proceed further even after longer reaction times. Similar stalling behavior was observed with 2′-*O*-Me-A **7f**. Although PSL-C catalyzed the acylation of **7f**, the selectivity was moderate due to the absence of the *N*-benzoyl group, a plausible enzyme recognition site for facile reactions (entries 6 and 7, Table 2). In the case of 2′-*O*-MOE-G^{Ibu} **7g**, PSL-C showed total selectivity toward the 3′-position, but low conversions were observed at 30 or 60°C even during extended period of reaction time (entries 8 and 9, Table 2).

Scale-up studies. The utility of PSL-C for large-scale reactions was demonstrated with nucleosides **7c** and **7e** as pyrimidine and purine examples, respectively. Thus, 3′-*O*-Lev-2′-*O*-MOE-C^{Bz} **3c** and 3′-*O*-Lev-2′-*O*-MOE-A^{Bz} **3e** were conveniently synthesized using similar enzymatic acylation method (1:2 substrate: enzyme ratio at 0.2 M) in 87 and 88% isolated yields, respectively (entries 10 and 11, Table 2).

As observed for 2′-deoxynucleosides, CAL-B exhibited opposite selectivity from PSL-C, catalyzing the acylation at the 5′-position of 2′-*O*-alkylribonucleosides **7** with absolute regioselectivity (entries 12–18, Table 2). The acylation process was complete at 40°C within 2.5–8 h to afford 5′-*O*-Lev-2′-*O*-alkylribonucleosides **4a**–**g**. The increased efficiency of 5′-*O*-acylations on 2′-*O*-alkylnucleosides compared to the 2′-deoxynucleoside with CAL-B may be attributed to the ability of the enzyme to recognize the sugar conformation (North versus South). From these results it appears that CAL-B prefers acylation of sugar with North conformation (e.g. 2′-*O*-alkylnucleosides) relative to sugar with South conformation (e.g. 2′-deoxynucleoside).[12]

Flash chromatography was used to isolate some of the levulinyl derivatives (Table 2) wherever precipitation of the crude was not practical due to solubility related issues. When enzymatic reactions gave mainly one acylated product, dry column vacuum chromatography described by Pedersen and Rosenbohm[13] was an excellent alternative for large-scale isolation. This technique

Table 2. Regioselective enzymatic acylation of 2′-*O*-alkylribonucleosides **7**

Entry	Substrate	Enzyme	*T* (°C)	*t* (h)	**3** (%)[a,b]	**4** (%)[a,b]	**7** (%)[a]	diLev[c] (%)[a]
1	**7a**	PSL-C[d]	30	23	80 (70)	16	4	
2	**7b**	PSL-C[d]	30	23			>99	
3	**7c**	PSL-C[d]	30	2.5	>97 (82)			
4	**7d**	PSL-C[d]	30	23			>99	
5	**7e**	PSL-C[d]	40	18	>95 (82)		4	
6	**7f**	PSL-C[d]	30	37	64	6	22	8
7	**7f**	PSL-C[d]	60	38	57	11	10	22
8	**7g**	PSL-C[d]	30	172	32		68	
9	**7g**	PSL-C[d]	60	172	36		64	
10	**7c**	PSL-C[e,f]	30	2.5	>97 (87)			
11	**7e**	PSL-C[d,g]	40	23	96 (88)		4	
12	**7a**	CAL-B[h]	40	7		>97 (70)		
13	**7b**	CAL-B[h]	40	2.5		>97 (61)		
14	**7c**	CAL-B[h]	40	5.5		>97 (86)		
15	**7d**	CAL-B[h]	40	8		>97 (86)		
16	**7e**	CAL-B[h]	40	2.5		>97 (84)		
17	**7f**	CAL-B[h]	40	2.5		>97 (82)		
18	**7g**	CAL-B[h]	40	3.5		>97 (88)		

[a] Based on ^{1}H NMR signal integration.
[b] Percentage of isolated yields are given in parenthesis.
[c] diLev = 3′,5′-di-*O*-levulinyl derivative.
[d] Ratio **7**:PSL-C is 1:3 (w/w).
[e] Scale-up to 4 g of **7**.
[f] Ratio **7**:PSL-C is 1:2 (w/w).
[g] Scale-up to 3 g of **7**.
[h] Ratio **7**:CAL-B is 1:1 (w/w).

improved the yield of 5′-*O*-Lev-2′-*O*-MOE ca. 20% and also reduced the amount of solvent and silica gel used, making it cost-efficient for scale-up.

2.3. Large-scale acylation studies

The industrial utility of these synthetic processes was proved via scale-up of the enzymatic acylation of thymidine. The reaction was carried out with 25 g (0.1 mol) of thymidine with 1:2 ratio of acylating agent (**6a**:**5**) and 1:2 (w/w) ratio of lipase (**6a**:PSL-C) (run 1, Table 3). The reaction was completed in 12.5 h and work-up of the reaction provided crude 3′-*O*-Lev-T **1a**, which was isolated in pure state (87%) via precipitation. An extra 9% product was recovered after flash chromatography of the mother liquors from the precipitation step. In the mother liquor, trace amounts of dilevulinate derivative were found. Four consecutive reactions were carried out with the recycled PSL-C making the acylation process more economical (runs 2–4, Table 3). Importantly, PSL-C maintained total selectivity toward acylation of the 3′-position with the exception being the reaction time, which got longer as the enzyme was recycled in subsequent runs.

Table 3. Large scale acylation of thymidine with PSL-C[a]

Run	*t* (h)	**1a** (%)[b]	**6a** (%)[b]	Yield (%)[c]
1	12.5	98.3	1.7	87 (9)
2[d]	15.5	98.2	1.8	90 (7)
3[d]	17	>99		88 (9)
4[d]	24.5	98.7	1.3	88 (9)

[a] The reaction was carried out at 30°C, 220 rpm, and under nitrogen in 0.2 M concentration with 25 g of T in a ratio 1:2 of acylating agent (T:oxime ester) and 1:2 (w/w) of lipase (T:PSL-C).
[b] Based on HPLC signal integration.
[c] Percentages of isolated yields by precipitation of 3′-*O*-Lev-T. Isolated yields by flash chromatography of the ether mother liquors are given in parenthesis.
[d] Recycled enzyme from the previous run.

Next, we focused our attention to the levulinate oxime ester **5** that was used in 1:2 excess with respect to the starting material. In order to recapture the excess of **5** after completion of the reaction, the desired product **1a** was collected via precipitation and the filtrate subjected to a dry column vacuum chromatography to furnish unreacted **5**.[13] The enzymatic acylation of thymidine with recaptured oxime ester **5** took place in a similar manner as if it was fresh acylation agent, with the exception of the extended reaction time (22 h versus 12.5 h). The fact that both the acylating agent and lipase can be recycled after each acylation reaction makes the new process atom efficient[14] and very attractive for industrial production.

To further demonstrate the scalability of enzymatic acylation with CAL-B, *N*-isobutyryl-2′-deoxyguanosine **6d** was chosen. The initial experiment was carried out with 5 g of **6d** at 0.2 M concentration instead of 0.1 M (entry 10 of Table 1), in a ratio of 1:2 acylating agent (**6d**:**5**) and 1:1 (w/w) of lipase (**6d**:CAL-B). Under these conditions, HPLC analysis showed that the reaction did not proceed after 30 h (65% conversion) probably due to the poor solubility of the starting material. In order to increase the conversion rate and to try and maintain the high concentration useful for the industrial scale a higher temperature (40°C) was selected to increase the solubility of the starting material. Increased temperature gave a small improvement in conversion rate (67%) in 40 h. The best results were obtained (100% conversion at 40°C) when a large excess of acylating agent **5** (1:3) was utilized. Next, the reaction was carried out with 25 g of **6d**. As indicated (entry 1, Table 4), CAL-B catalyzed the acylation process with total selectivity furnishing **2d** in 82% isolated yield. Again with the exception of longer reaction time, similar behavior was observed in a successive run with recycled enzyme from the previous reaction. Since direct precipitation was not satisfactory, isolation of 5′-*O*-Lev-dG^{Ibu} **2d** was accomplished by dry column vacuum chromatography from the crude reaction mixture.

Table 4. Large scale acylation of N^2-isobutyryl-2′-deoxyguanosine with CAL-B[a]

Run	*t* (h)	**2d** (%)[b]	**6d** (%)[b,c]	Yield (%)[d]
1	24	87.5	12.5	82
2[e]	216	77	23	86

[a] The reaction was carried out at 40°C, 220 rpm, and under nitrogen in 0.2 M concentration with 25 g of dG^{Ibu} in a ratio 1:3 of acylating agent (dG^{Ibu}:oxime ester) and 1:1 (w/w) of lipase (dG^{Ibu}:CAL-B).
[b] Based on HPLC signal integration.
[c] **6d** is ≅91% pure by HPLC (impurity: *N*-Ibu-guanine). In the HPLC the impurity and **6d** have very close retention times and when the ratio between them are similar it is not possible to integrate both signals separately, therefore the value is the sum of both. In addition, the recycled enzyme contains the impurity from previous run, consequently, processes were allowed to react until no evolution was observed.
[d] Percentages of isolated yields.
[e] Recycled enzyme from the previous run.

Next, large-scale acylation of N^6-benzoyl-2′-deoxyadenosine **6c** was studied with recycled enzyme. Treatment of **6c** (5 g) with 3 equivalents of oxime ester and CAL-B (entry 1, Table 5) furnished >90% (HPLC) of 5′-*O*-Lev nucleoside **2c** after 24 h, with a trace of starting material and minor amounts of other acyl derivatives. Standard work-up provided pure **2c** in 87% yield. Crystallization of **2c** from MeOH provided analytically pure sample. Two consecutive runs were carried out with the recycled enzyme from run 1 (entries 2 and 3, Table 5). A conversion of ∼80% was reached after 112 h during the third experiment, where *N*-benzoyl-5′-*O*-levulinyl-2′-deoxyadenosine was isolated in 61% yield after work-up.

3. Summary

3′- and 5′-*O*-Levulinyl protected derivatives of 2′-deoxy and 2′-*O*-alkyl nucleosides have been prepared by regioselective enzymatic acylation using a variety of

Table 5. Large scale acylation of 2′-deoxyadenosine with CAL-B[a]

Run	t (h)	**1c** (%)[b]	**2c** (%)[b]	**6c** (%)[b]	DiLev[c] (%)[b]	Yield (%)[d]
1	24	Traces	>90	7	Traces	87 (78)
2[e]	64	Traces	>89	7	Traces	85 (79)
3[e]	112	Traces	>75	19	Traces	61 (53)

[a] The reaction was carried out at 30°C, 250 rpm, and under nitrogen in 0.2 M concentration with 5 g of dA^{Bz} in a ratio 1:3 of acylating agent (dA^{Bz}:oxime ester) and 1:1 (w/w) of lipase (dA^{Bz}:CAL-B).
[b] Based on HPLC and ^{1}H NMR signal integration.
[c] diLev = 3′,5′-di-*O*-Lev-dA^{Bz}.
[d] Percentages of isolated yields by precipitation of 5′-*O*-Lev-dA^{Bz}. Isolated yields by recrystallization of the products are given in parenthesis.
[e] Recycled enzyme from the previous run.

lipases and acetonoxime levulinate as acylating agent. *N*-Benzoyl-3′-*O*-levulinyl cytidine and adenosine derivatives were obtained in good yield overcoming the limitations of our original hydrolysis protocol. The new and improved method is shorter than the one described before, allowing greater atom efficiency[14] and lowering the cost of enzyme and reagents via recycling. To the best of our knowledge this is the first example of enzyme directed regioselective levulinylation reaction of base protected nucleosides. The industrial suitability of this process was proven via large-scale acylation of several natural and unnatural nucleosides. Additionally, we have demonstrated that the biosynthesis of protected nucleosides has the potential to allow synthesis under environmentally friendly conditions while achieving high specificity, yield and generated significantly less waste products than alternate chemical processes.[15]

4. Experimental[16]

Candida antarctica lipase B (CAL-B, Novozym 435, 10000 PLU/g), Chirazyme L-2, c-f, C3, lyo (lipase from *C. antarctica* B, 400 U/g), and immobilized *Pseudomonas cepacia* lipase (PSL-C, 904 U/g) were purchased from Novo Nordisk Co., Roche Diagnostics, and Amano Pharmaceuticals, respectively. Solvents were distilled over an adequate desiccant under nitrogen. High performance liquid chromatography analyses (HPLC) were carried out at 254 nm using a Kromasil 100, C18, 5μ column (150×46 mm) and two mobile phase (A: MeCN; B: 1.54 g ammonium acetate in 1-L water containing 1% MeCN). Gradient condition:

t (min)	A	B
0.0	1	99
10.0	4	96
15.0	4	96
20.0	10	90
25.0	20	80
30.0	50	50
40.0	70	30

Compounds **1a**, **2a**–**d**, **3a**, **4a**, **4c**, **4e**, and **4g** have been previously obtained by enzymatic hydrolysis.[8]

4.1. Procedure for *O*-levulinyl acetoxime from levulinic acid and acetonoxime

To a solution of acetonoxime (4.60 g, 63 mmol) in Et_2O (70 mL) under nitrogen was added levulinic acid (6.44 mL, 63 mmol) and dicyclohexylcarbodiimide (12.98 g, 63 mmol). The suspension was stirred at rt during 0.5 h. After separation of the solid by filtration, the filtrate was evaporated under vacuum to afford 10.77 g (quantitative yield) of acetonoxime levulinate[10] as pale yellow viscous oil, which solidified on cooling. R_f (EtOAc): 0.37; IR (NaCl): ν 1756 and 1719 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.99 (s, 3H, Me), 2.01 (s, 3H, Me), 2.19 (s, 3H, *Me*CO), 2.66 (t, 2H, CH_2-Lev, $^3J_{HH}$ 6.6 Hz), and 2.82 (t, 2H, CH_2-Lev, $^3J_{HH}$ 6.8 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 16.9 (Me), 21.8 (Me), 26.5 (*C*H_2-Lev), 29.8 (Me), 37.7 (*CH*$_2$-Lev), 163.9 (C=N), 170.4 (C=O), and 206.3 (C=O). MS (ESI$^+$, m/z): 172 [(M+H)$^+$, 30%] and 194 [(M+Na)$^+$, 100]. Anal. calcd (%) for $C_8H_{13}NO_3$: C, 56.13; H, 7.65; N, 8.18. Found: C, 56.3; H, 7.4; N, 8.3.

4.2. General procedure for the regioselective enzymatic acylation of 2′-deoxynucleosides

A suspension of **6** (0.2 mmol), the oxime ester (0.6 mmol), and the lipase in dry THF (2 mL) under nitrogen was stirred at 250 rpm for the time and at the temperature indicated in Table 1. The reactions were monitored by TLC (10% MeOH/CH_2Cl_2). The enzyme was filtered off and washed with CH_2Cl_2 and THF. The solvents were distilled under vacuum, and the residue was taken up in $NaHCO_3$ (aq) and extracted with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4 and evaporated. The residue was precipitated in diethyl ether to afford after filtration the monolevulinyl nucleosides **1** or **2** as solids. No further purification was necessary except in entries 8 and 9 (Flash chromatography, gradient eluent 5–20% ^{i}PrOH/CH_2Cl_2) to separate the traces of other acyl derivatives.

4.2.1. N^4-Benzoyl-3′-*O*-levulinyl-2′-deoxycytidine 1b. R_f (10% MeOH/CH_2Cl_2): 0.30; mp: 189–191°C; $[\alpha]_D^{20}$ = +20.5 (*c* 1.0, MeOH); IR (KBr): ν 3289, 2929, 1727, and 1709 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.20 (s, 3H, *Me*-Lev), 2.44 (m, 1H, $H_{2'}$), 2.59 (m, 2H, CH_2-Lev),

2.73 (m, 3H, $H_{2'}$+CH_2-Lev), 3.97 (m, 2H, $H_{5'}$), 4.20 (m, 1H, $H_{4'}$), 5.37 (m, 1H, $H_{3'}$), 6.26 (apparent t, 1H, $H_{1'}$, $^3J_{HH}$ 6.8 Hz), 7.58 (m, 4H, H_5+H_m+H_p), 7.90 (apparent d, 2H, H_o, $^3J_{HH}$ 7.3 Hz), and 8.30 (d, 1H, H_6, $^3J_{HH}$ 7.4 Hz); ^{13}C NMR (DMSO-d_6, 75.5 MHz): δ 27.8 (*C*H_2-Lev), 29.6 (*Me*-Lev), 37.5 (*C*H_2-Lev), 38.2 ($C_{2'}$), 61.2 ($C_{5'}$), 74.9, 85.6 ($C_{3'}$+$C_{4'}$), 86.4 ($C_{1'}$), 96.3 (C_5), 128.5 (C_o+C_m), 132.8 (C_i), 133.1 (C_p), 145.0 (C_6), 154.5 (Ph*C*=O), 163.3 (C_4), 167.4 (C_2), 172.1 (*C*=O), and 207.0 (*C*=O); MS (ESI$^+$, *m*/*z*): 430 [(M+H)$^+$, 30%], 452 [(M+Na)$^+$, 52], and 468 [(M+K)$^+$, 95]. Anal. calcd (%) for $C_{21}H_{23}N_3O_7$: C, 58.74; H, 5.40; N, 9.79. Found: C, 58.7; H, 5.5; N, 9.8.

4.2.2. N^6-Benzoyl-3′-*O*-levulinyl-2′-deoxyadenosine 1c. R_f (10% MeOH/CH_2Cl_2): 0.44; mp: 125–127°C; $[\alpha]_D^{20}$ = −26.7 (*c* 0.9, $CHCl_3$); IR (KBr): *v* 2360, 2341, 1733, 1715, and 1687 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 2.22 (s, 3H, *Me*-Lev), 2.61 (m, 3H, CH_2-Lev+$H_{2'}$), 2.82 (m, 2H, CH_2-Lev), 3.18 (m, 1H, $H_{2'}$), 3.97 (m, 2H, $H_{5'}$), 4.31 (s, 1H, $H_{4'}$), 5.57 (apparent d, 1H, $H_{3'}$, $^3J_{HH}$ 5.4 Hz), 6.38 (dd, 1H, $H_{1'}$, $^3J_{HH}$ 9.8, $^3J_{HH}$ 5.2 Hz), 7.57 (m, 3H, H_m+H_p), 8.05 (m, 2H, H_o), 8.17 (s, 1H, H_2 or H_8), and 8.79 (s, 1H, H_8 or H_2). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 27.7 (*C*H_2-Lev), 29.6 (*Me*-Lev), 37.6 (*C*H_2-Lev+$C_{2'}$), 62.8 ($C_{5'}$), 76.1, 86.8 ($C_{3'}$+$C_{4'}$), 87.0 ($C_{1'}$), 124.3 (C_5), 127.8, 128.6 (C_o+C_m), 132.7 (C_p), 133.2 (C_i), 142.3 (C_2 or C_8), 150.1 (C_4), 150.6 (C_6), 151.9 (C_8 or C_2), 164.8 (Ph*C*=O), 172.1 (*C*=O), and 206.4 (*C*=O); MS (ESI$^+$, *m*/*z*): 454 [(M+H)$^+$, 20%], 476 [(M+Na)$^+$, 33], and 492 [(M+K)$^+$, 5]. Anal. calcd (%) for $C_{22}H_{23}N_5O_6$: C, 58.27; H, 5.11; N, 15.44. Found: C, 58.2; H, 5.2; N, 15.5.

4.3. General procedure for the regioselective enzymatic acylation of 2′-alkylribonucleosides

The reactions were carried out at 0.2 M concentration following the same procedure as previously described for 2′-deoxynucleosides except the purification step. The monolevulinyl derivatives of 2′-alkylribonucleosides were soluble in Et_2O and separation from the remaining oxime ester by precipitation was not possible. Thus, after enzyme filtration the residue was purified by flash chromatography (gradient eluent 2–10% ^{i}PrOH/CH_2Cl_2, except 5–15% MeOH/CH_2Cl_2 for **4g**).

4.3.1. N^4-Benzoyl-3′-*O*-levulinyl-2′-*O*-(2-methoxyethyl)-5-methylcytidine 3c. R_f (10% MeOH/CH_2Cl_2): 0.56; mp: 102–104°C; $[\alpha]_D^{20}$ = +55.7 (*c* 0.4, MeOH); IR (KBr): *v* 3426, 3067, 2922, 1738, 1705, and 1645 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 2.09 (d, 3H, Me, $^3J_{HH}$ 1.0 Hz), 2.20 (s, 3H, *Me*-Lev), 2.66 (m, 2H, CH_2-Lev), 2.78 (m, 2H, CH_2-Lev), 3.28 (s, 3H, OMe), 3.46 (m, 2H, *CH*$_2$-MOE), 3.71–4.02 (m, 4H, *CH*$_2$-MOE+$H_{5'}$), 4.26 (m, 1H, $H_{4'}$), 4.51 (apparent t, 1H, $H_{2'}$, $^3J_{HH}$ 5.4 Hz), 5.32 (apparent t, 1H, $H_{3'}$, $^3J_{HH}$ 4.9 Hz) 5.70 (d, 1H, $H_{1'}$, $^3J_{HH}$ 5.4 Hz), 7.45 (m, 3H, H_m+H_p), 7.64 (s, 1H, H_6), and 8.30 (m, 2H, H_o); ^{13}C NMR ($CDCl_3$, 50.3 MHz): δ 13.5 (Me), 27.7 (*C*H_2-Lev), 29.8 (*Me*-Lev), 37.7 (*C*H_2-Lev), 58.9 (*Me*-MOE), 61.6 ($C_{5'}$), 70.4 (*C*H_2-MOE), 70.7 ($C_{3'}$), 71.8 (*C*H_2-MOE), 79.4, 83.3 ($C_{2'}$+$C_{4'}$), 91.7 ($C_{1'}$), 111.8 (C_5), 128.1, 129.9 (C_o+C_m), 132.5 (C_p), 136.8 (C_i), 139.2 (C_6), 148.0 (C_2), 159.5 (C_4), 172.2 (C=O), 179.4 (PhC=O), and 206.5 (C=O); MS (ESI$^+$, *m*/*z*): 518 [(M+H)$^+$, 20%], 540 [(M+Na)$^+$, 100], and 556 [(M+K)$^+$, 15]. Anal. calcd (%) for $C_{25}H_{31}N_3O_9$: C, 58.02; H, 6.04; N, 8.12. Found: C, 58.1; H, 6.2; N, 8.2.

4.3.2. N^6-Benzoyl-3′-*O*-levulinyl-2′-*O*-(2-methoxyethyl)-adenosine 3e. R_f (10% MeOH/CH_2Cl_2): 0.62; mp: 44–46°C; $[\alpha]_D^{20}$ = −30.6 (*c* 0.4, MeOH); IR (KBr): *v* 3019, 2849, 1734, 1716, and 1698 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.21 (s, 3H, *Me*-Lev), 2.71 (m, 2H, CH_2-Lev), 2.80 (m, 2H, CH_2-Lev), 3.04 (s, 3H, OMe), 3.27 (m, 2H, *CH*$_2$-MOE), 3.45 (m, 1H, *CH*-MOE), 3.59 (m, 1H, *CH*-MOE), 3.90 (m, 2H, $H_{5'}$), 4.37 (m, 1H, $H_{4'}$) 4.93 (dd, 1H, $H_{2'}$, $^3J_{HH}$ 7.8, $^3J_{HH}$ 5.1 Hz), 5.64 (apparent d, 1H, $H_{3'}$, $^3J_{HH}$ 4.9 Hz), 5.94 (d, 1H, $H_{1'}$, $^3J_{HH}$ 7.9 Hz), 7.56 (m, 3H, H_m+H_p), 8.02 (apparent d, 2H, H_o, $^3J_{HH}$ 7.2 Hz), 8.11 (s, 1H, H_2 or H_8), and 8.77 (s, 1H, H_8 or H_2); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 27.7 (*C*H_2-Lev), 29.8 (*Me*-Lev), 37.7 (*C*H_2-Lev), 58.6 (*Me*-MOE), 62.8 ($C_{5'}$), 70.4 (*C*H_2-MOE), 71.7 (*C*H_2-MOE), 72.3 ($C_{3'}$), 79.9, 86.1 ($C_{2'}$+$C_{4'}$), 89.6 ($C_{1'}$), 124.4 (C_5), 127.8, 128.8 (C_o+C_m), 132.9 (C_p), 133.2 (C_i) 143.2 (C_2 or C_8), 150.2 (C_4), 150.4 (C_6), 151.9 (C_8 or C_2), 164.5 (PhC=O), 171.9 (C=O), and 206.3 (C=O); MS (ESI$^+$, *m*/*z*): 528 [(M+H)$^+$, 100%] 550 [(M+Na)$^+$, 50], and 566 [(M+K)$^+$, 35]. Anal. calcd (%) for $C_{25}H_{29}N_5O_8$: C, 56.92; H, 5.54; N, 13.28. Found: C, 56.8; H, 5.4; N, 13.2.

4.3.3. 5′-*O*-Levulinyl-2′-*O*-(2-methoxyethyl)-5-methylcytidine 4b. R_f (10% MeOH/CH_2Cl_2): 0.32; mp: 135–137°C; $[\alpha]_D^{20}$ = +32.9 (*c* 0.4, MeOH); IR (KBr): *v* 3396, 3333, 3264, 3225, 2919, 1734, and 1662 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.89 (s, 3H, Me), 2.15 (s, 3H, *Me*-Lev), 2.58 (m, 2H, CH_2-Lev), 2.76 (m, 2H, CH_2-Lev), 3.32 (s, 3H, OMe), 3.52 (m, 2H, *CH*$_2$-MOE), 3.81 (m, 1H, *CH*-MOE), 4.05 (m, 4H, $H_{2'}$+$H_{3'}$+$H_{4'}$+*CH*-MOE), 4.38 (m, 2H, $H_{5'}$), 5.80 (m, 1H, $H_{1'}$), and 7.43 (s, 1H, H_6); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 13.1 (Me), 27.6 (*C*H_2-Lev), 29.6 (*Me*-Lev), 37.6 (*C*H_2-Lev), 58.6 (*Me*-MOE), 62.9 ($C_{5'}$), 68.7 ($C_{3'}$), 69.9 (*C*H_2-MOE), 71.4 (*C*H_2-MOE), 80.8, 82.2 ($C_{2'}$+$C_{4'}$), 89.5 ($C_{1'}$), 101.6 (C_5), 137.6 (C_6), 155.6 (C_2), 165.7 (C_4), 172.2 (C=O), and 206.4 (C=O); MS (ESI$^+$, *m*/*z*): 436 [(M+Na)$^+$, 10%] and 452 [(M+K)$^+$, 20]. Anal. calcd (%) for $C_{18}H_{27}N_3O_8$: C, 52.29; H, 6.58; N, 10.16. Found: C, 52.3; H, 6.5; N, 10.2.

4.3.4. 5′-*O*-Levulinyl-2′-*O*-(2-methoxyethyl)adenosine 4d. R_f (10% MeOH/CH_2Cl_2): 0.37; $[\alpha]_D^{20}$ = −40.0 (*c* 2.7, MeOH); IR (NaCl): *v* 3336, 3198, 2928, 1733, 1715, and 1651 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.12 (s, 3H, *Me*-Lev), 2.55 (m, 2H, CH_2-Lev), 2.72 (m, 2H, CH_2-Lev), 3.30 (s, 3H, OMe), 3.47 (m, 2H, *CH*$_2$-MOE), 3.74 (m, 1H, *CH*-MOE), 3.87 (m, 1H, *CH*-MOE), 4.26 (m, 1H, $H_{4'}$), 4.38 (m, 3H, $H_{3'}$+$H_{5'}$), 4.62 (m, 1H, $H_{2'}$), 6.06 (d, 1H, $H_{1'}$, $^3J_{HH}$ 4.3 Hz), 6.59 (br s, 2H, NH_2), 8.05 (s, 1H, H_2 or H_8), and 8.25 (s, 1H, H_8 or H_2); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 27.6 (*C*H_2-Lev), 29.6 (*Me*-Lev), 37.7 (*C*H_2-Lev), 58.7 (*Me*-MOE), 63.4 ($C_{5'}$), 69.6 ($C_{3'}$), 70.1 (*C*H_2-MOE), 71.5 (*C*H_2-MOE), 81.7, 82.0 ($C_{2'}$+

$C_{4'}$), 87.2 ($C_{1'}$), 119.7 (C_5), 139.1 (C_2 or C_8), 149.3 (C_4), 152.7 (C_8 or C_2), 155.6 (C_6), 172.3 (C=O), and 206.5 (C=O); MS (ESI$^+$, m/z): 424 [(M+H)$^+$, 70%], 446 [(M+Na)$^+$, 90], and 462 [(M+K)$^+$, 50]. Anal. calcd (%) for $C_{18}H_{25}N_5O_7$: C, 51.06; H, 5.95; N, 16.54. Found: C, 51.0; H, 5.8; N, 16.6.

4.3.5. 5′-*O*-Levulinyl-2′-*O*-methyladenosine 4f. R_f (10% MeOH/CH_2Cl_2): 0.30; mp: 124–126°C; $[\alpha]_D^{20}=-15.2$ (*c* 0.5, MeOH); IR (KBr): *v* 3146, 2968, 2927, 1731, 1713, and 1651 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.17 (s, 3H, *Me*-Lev), 2.60 (m, 2H, C*H*$_2$-Lev), 2.77 (m, 2H, C*H*$_2$-Lev), 3.57 (s, 3H, OMe), 4.24 (m, 1H, $H_{4'}$), 4.44 (m, 4H, $H_{2'}$+$H_{3'}$+$H_{5'}$), 6.12 (d, 1H, $H_{1'}$, $^3J_{HH}$ 3.1 Hz), 6.18 (br s, 2H, N*H*$_2$), 8.10 (s, 1H, H_2 or H_8), and 8.32 (s, 1H, H_8 or H_2); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 27.6 (*C*H$_2$-Lev), 29.6 (*Me*-Lev), 37.7 (*C*H$_2$-Lev), 58.7 (O*Me*), 63.2 ($C_{5'}$), 69.4 ($C_{3'}$), 81.9, 83.0 ($C_{2'}$+$C_{4'}$), 86.7 ($C_{1'}$), 119.7 (C_5), 138.8 (C_2 or C_8), 149.2 (C_4), 152.9 (C_8 or C_2), 155.6 (C_6), 172.4 (C=O), and 206.7 (C=O); MS (ESI$^+$, m/z): 380 [(M+H)$^+$, 10%], 402 [(M+Na)$^+$, 100], and 418 [(M+K)$^+$, 10]. Anal. calcd (%) for $C_{15}H_{21}N_5O_6$: C, 49.04; H, 5.76; N, 19.06. Found: C, 49.1; H, 5.6; N, 19.3.

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