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New Concept for the Separation of an Anomeric Mixture of α/*β*-D-Nucleosides through Regioselective Enzymatic Acylation or Hydrolysis Processes

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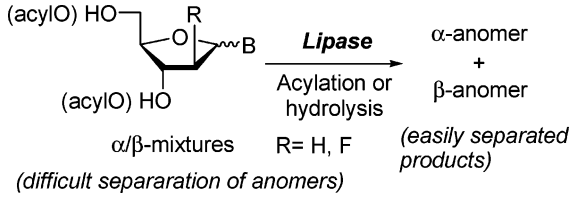

An efficient and high yield protocol for the synthesis and separation of 3′- and/or 5′-protected α-2′-deoxynucleosides has been developed through regioselective acylation/deacylation processes catalyzed by enzymes. *Pseudomonas cepacia* lipase (PSL-C) was found to be highly chemo- and regioselective toward the 3′-position of the *β*-2′-deoxynucleoside derivatives, whereas PSL-C displayed opposite selectivity toward the 5′-position for the corresponding α-anomer. The successful application of this protocol was demonstrated by a convenient separation of an α/*β*-mixture of thymidine derivatives from an industrial waste stream. Furthermore, this technique was also applied for the separation of an anomeric mixture of 2′-deoxy-2′-fluoro-α/*β*-arabinonucleosides that are useful building blocks for the antisense constructs.

Introduction

Therapeutic applications of modified oligonucleotides and nucleoside analogues is a promising area of research with numerous possibilities to treat human diseases.[1] A majority of these products are *β*-nucleoside analogues or oligonucleotides assembled from *β*-nucleosides. The principal reason for the use of *β*-nucleosides lies in their availability from the natural sources and well-established synthetic routes. On the contrary, the use of α-nucleosides in therapeutics has been limited because they are unnatural and difficult to synthesize in the pure state. Despite these limitations, α-nucleosides continue to be of general interest because of their unique properties. For example, the α-2′-deoxythioguanosine analogue has notable antitumor activity but exhibits lower toxicity than the corresponding *β*-anomer.[2] The 3′-hydroxymethyl branched α- and *β*-nucleosides were equally inhibitory to the growth of WI-L2 human lymphosbastoid cells.[3] The α-anomers of 2′-deoxy-5-fluoro-2-thiouridine and cytidine exert selective growth inhibitory properties against L1210 and L5178Y mouse leukemia cells.[4] The α-nucleosides have shown

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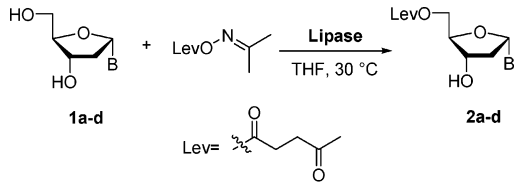
substrate/inhibitor properties toward various enzymatic systems.[5] Recently, α-thymidine analogues were shown to possess potent *M. tuberculosis* thymidylate kinase inhibitor actitivity.[6]

Furthermore, the α-oligonucleotides and their derivatives have been found as attractive agents for diagnostic applications via the antisense or antigene mode of action.[7] These oligonuclotides have shown good hybridization properties toward DNA or RNA[7] with stability against nuclease degradation.[8] Because of the ongoing interest in the utilization of α-nucleosides for therapeutic applications, we believe that development of efficient methods for the synthesis of α-nucleoside monomers is an important endeavor. Several methods have been reported in the literature for the synthesis of α-nucleosides.[9] For example, the C1′-epimerization of commercially available *β*-deoxynucleosides is a straightforward route to α-deoxynucleosides.[10] However, this method often results in a mixture of the blocked α- and *β*-anomers, which are tedious to separate. The separation of synthetically derived α/*β*-nucleosides remains an underexplored area of research.

Because of our[11] ongoing interest in the chemoenzymatic transformations, we elected to explore the possibilities of using lipases for the separation of α/*β*-anomeric nucleosides. Our prior experience with the selective acylation of α-thymidine using acetonoxime butyrate catalyzed by *Pseudomonas cepacia* lipase (PSL) and *Candida antarctica* lipase B (CAL-B) prompted us to further investigate this method.[12] Additional examples in the literature are the lipase-catalyzed diastereoselective deacetylation of an anomeric mixture of peracetylated α/*β*-thymidine,[13] and the separation of α/*β*-L-2′,3′-dideoxynucleosides using cytidine deaminase (CDA).[14] We have also described a general chemoenzymatic synthesis of monolevulinyl protected nucleosides using commercial lipases and its application in the resolution of D/L-mixtures.[15] Herein, we describe a new strategy for the separation of α/*β*-anomeric nucleosides using enzymatic acylation/hydrolysis protocols. We also provide two examples from the therapeutic industry where we applied the new separation technique for the isolation of α-nucleosides from a mixture of α/*β*-anomeric products.

SCHEME 1[a]



a, B= T; **b**, B= C^{Bz}; **c**, B= A^{Bz}; **d**, B= G^{Ibu}

[a] See Table 1 for details.

Results and Discussion

We chose to use the levulinyl group for our studies due to the following reasons. First, the levulinyl group[16] is very useful for orthogonal protection during the solution-phase synthesis of oligonucleotides.[11g] Second, we have had prior experience with the lipase-mediated levulinylation of *β*-nucleosides.[17] Third, the levulinyl group is small, atom-efficient, and inexpensive to use. To interpret the outcome of levulinylation reactions clearly, first we decided to perform the study on pure α-2′-deoxynucleosides **1a**−**d**.[18] These results were then applied to the acylation reaction of the mixture of α/*β*-anomeric nucleosides.

Based on our experience with acylation[11,15] of various *β*-nucleosides, the α-nucleosides **1a**−**d** were subjected to the enzymatic reaction with CAL-B at 30 °C in THF using *O*-levulinyl acetonoxime as an acylating agent (Scheme 1). Under these conditions, facile regioselective 5′-*O*-acylation was observed in 1−2 h furnishing 5′-*O*-levulinyl derivatives **2a**−**d** (B = T, C^{Bz}, A^{Bz}, and G^{Ibu}) in excellent yields (entries 1, 3, 5, and 7, Table 1). Importantly, the ^{1}H NMR data of **2a**−**d** were devoid of 3′-*O*-levulinyl and/or diacyl derivatives.

The exceptionally fast reaction rate observed with CAL-B may not be ideal for the separation of a mixture of α/*β*-anomeric products. Therefore, we investigated another alternative such

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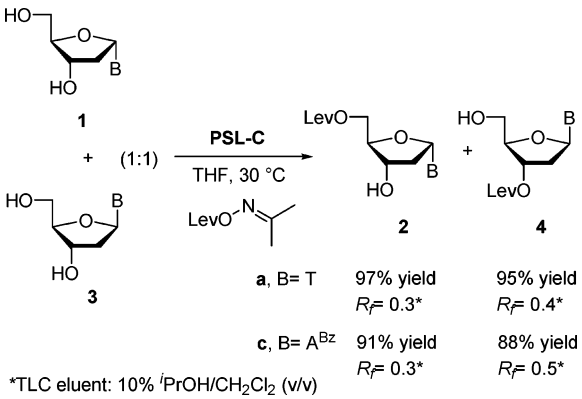
TABLE 1. Enzymatic Acylation of α-D-Nucleosides 1a−d[a]

entry	substrate	lipase	*t* (h)	**1** (%)[b]	product	yield (%)[b,c]
1	**1a**	CAL-B	1.5	nd	**2a**	>97 (92)
2	**1a**	PSL-C	4	nd	**2a**	>97 (90)
3	**1b**	CAL-B	1	nd	**2b**	>97 (94)
4	**1b**	PSL-C	3	nd	**2b**	>97 (94)
5	**1c**	CAL-B	2	nd	**2c**	>97 (92)
6	**1c**	PSL-C	9.5	nd	**2c**	>97 (94)
7	**1d**	CAL-B	1.5	nd	**2d**	>97 (86)
8	**1d**	PSL-C	72	10	**2d**	60[d]

[a] 3 equiv of *O*-levulinyl acetonoxime;[17] ratio **1**:CAL-B is 1:1 (w/w); ratio **1**:PSL-C is 1:3 (w/w); 0.1 M concentration. [b] Based on ^{1}H NMR signal integration. Percentages indicated as >97% (±3% by NMR analysis) signify that only one compound was detected even at high spectrum amplitude. [c] Percentages of isolated yields are given in parentheses. [d] Also, 30% of 3′,5′-di-*O*-levulinyl-dG^{Ibu} was isolated. nd = not detected by ^{1}H NMR.

SCHEME 2



as PSL-C, which is immobilized on ceramic beads, commercially available in bulk, and easy to recycle. Repeating the same process with PSL-C resulted in total selectivity toward the 5′-hydroxyl group of **1a**−**c** 2′-deoxynucleosides (entries 2, 4, and 6, Table 1). The acylation reaction catalyzed by PSL-C on N^2-isobutyryl-α-D-2′-deoxyguanosine (**1d**) was found to be significantly slower as compared to that on **1a**−**c**. A mixture of 5′-*O*-levulinyl **2d** and 3′,5′-di-*O*-levulinyl derivatives and unreacted nucleoside **1d** were obtained after 72 h (entry 8, Table 1). Nevertheless, we were able to make **2d** via acylation with CAL-B in good yield (entry 7, Table 1). We were pleased to note that the PSL-C exhibited the same regiospecificity as CAL-B at the 5′-hydroxyl group of α-nucleosides **1a**−**c** during the acylation reaction. In addition, the overall rate of the acylation reaction with PSL-C was slower as compared to the CAL-B process, which is desirable for the separation of α/β-nucleosides. In recent past, we observed that PSL-C catalyzes the acylation at the 3′-hydroxy group of β-nucleosides with high selectivity.[11f] We envisioned that this remarkable difference in the observed regioselectivity toward acylation of α- and β-nucleosides with PSL-C could become the cornerstone of the desired separation process for α/β-anomeric nucleosides.

Considering the opposite acylation preference exhibited by PSL-C in α- and β-nucleosides independently, the stage was set for the separation of a mixture of α/β-nucleosides (Scheme 2). We challenged the ability of PSL-C to recognize both α- and β-2′-deoxynucleosides present in one reaction at the same time and selectively acylate the appropriate hydroxyl groups of the two nucleosides. Therefore, treatment of a 1:1 mixture[19] of α/β-D-thymidine with *O*-levulinyl acetonoxime in the presence of PSL-C furnished a mixture of 5′-*O*-levulinyl-α-thymidine (**2a**) and 3′-*O*-levulinyl-β-D-thymidine (**4a**) after 4 h. As anticipated, the α-anomer **1** was acylated at the 5′-hydroxyl group, whereas the same lipase PSL-C exhibited an opposite selectivity for the β-anomer **3**, catalyzing the acylation of the 3′-hydroxyl group. It is noteworthy that the mixture of **1a** and **3a** showed almost identical R_f values on the TLC, whereas the two acylated products **2a** and **4a** show different R_f values. This marked difference in the R_f values enabled an easy chromatographic separation of the two products **2a** and **4a** in 97% and 95% yields (based on the amount of α- and β-starting materials), respectively. The structure of **2a** and **4a** was confirmed by analytical data.[20]

After the successful separation of a mixture of α/β-pyrimidine nucleoside, we decided to evaluate the same process for the separation of a mixture of α/β-purine nucleoside. A mixture[19] of *N*-benzoyl-α/β-D-2′-deoxyadenosine (**1c** and **3c**) was acylated in a similar manner using PSL-C. After 4 h, the reaction was complete, furnishing *N*-benzoyl-5′-*O*-levulinyl-α-D-2′-deoxyadenosine (**2c**) and *N*-benzoyl-3′-*O*-levulinyl-β-D-2′-deoxyadenosine (**4c**). Again, the separation of acylated products **2c** and **4c** was straightforward due to a large difference in their R_f values as compared to the starting materials **1c** and **3c**. Both products were isolated in high yield after silica gel column chromatography, and their structure was corroborated by analytical data.[20]

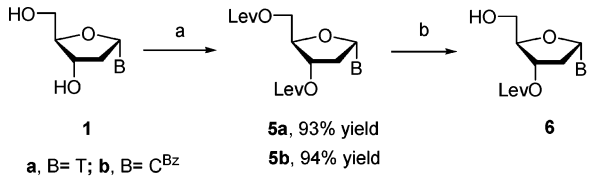
Next, we embarked on the synthesis of 3′-*O*-levulinyl protected α-nucleosides for two reasons. First, we wanted to prepare an authentic reference sample of 3′-*O*-levulinyl protected α-thymidine **6a** for comparison with its regio-isomer 5′-*O*-levulinyl-α-thymidine (**2a**). This exercise will enable us to conclusively demonstrate that the acylation of α-thymidine using PSL-C produced only **2a** and none of **6a** was formed. Second, 3′-*O*-levulinyl protected α-nucleosides are key building blocks for the synthesis of α-oligonucleotides.[7,8] Therefore, we proposed a two-step protocol for the unequivocal synthesis of 3′-*O*-levulinylated nucleosides **6** in the following manner (Scheme 3).

Treatment of α-2′-deoxynucleosides **1** with 5.2 equiv of levulinic acid (LevOH) and DCC in the presence of DMAP furnished 3′,5′-di-*O*-levulinyl-α-D-2′-deoxynucleosides **5** in >93% isolated yield. For the hydrolysis of **5**, we selected CAL-B and PSL-C as catalysts, due to its well-demonstrated selectivity with a variety of modified nucleosides.[17] When diesters **5a** and **5b** were treated individually with CAL-B at 30 °C in 0.15 M phosphate buffer (pH 7) containing 18% of 1,4-dioxane, total regioselectivity toward the hydrolysis of the 5′-

(19) The equimolar mixture of the two nucleosides was prepared by mixing the equal weight of α-D-thymidine and β-D-thymidine. A mixture of **1c** and **3c** was prepared in a similar manner.

(20) See Supporting Information for details.

SCHEME 3[a]



[a] See Table 2 for details.

O-levulinyl group was observed, furnishing 3′-*O*-levulinyl-α-D-thymidine (**6a**) and *N*-benzoyl-3′-*O*-levulinyl-α-D-2′-deoxycytidine (**6b**) in 91% and 96% yield, respectively (entries 1 and 3, Table 2). Similarly, PSL-C also exhibited high selectivity toward the hydrolysis of 5′-*O*-levulinyl group in α-nucleosides **5** (entries 2 and 4, Table 2) albeit with a significantly slower rate as compared to the hydrolysis with CAL-B. The reaction with PSL-C was incomplete even after 8 days with the formation of a small amount of **1** as completely hydrolyzed product detected by HPLC of the mixture. Nevertheless, we were able to synthesize **6a** and compared its analytical data with **2a**, confirming that these two compounds are indeed regio-isomers.

Application 1: Separation of α/*β*-Thymidine Nucleosides. With the foregoing insight into acylation and the hydrolysis reaction pattern of α-nucleosides, we were poised to apply this knowledge to a project of significant importance. One such project is the synthesis of *β*-thymidine that is performed in >100 metric tons/year quantities. The large demand for the production of *β*-thymidine is due to its use as the key raw material for the synthesis of widely prescribed anti-HIV drug Zidovudine (AZT). Currently several Asian companies are manufacturing *β*-thymidine following a well-established glycosylation route.[21] Although this protocol for the synthesis of *β*-thymidine is efficient, it produces a mixture of α- and *β*-thymidine analogues. Fortunately, a majority of the protected *β*-nucleoside **8** preferentially crystallizes out from the solution, but it leaves behind an inseparable mixture of α- and *β*-nucleosides **7** and **8**, respectively, as mother liquor. Because of the inseparable nature of these two products, Asian companies are discarding this mixture of nucleosides as an industrial waste. The traditional methods of separations such as crystallization or chromatography have failed to provide meaningful results. Our ongoing interest[22] in the development of "Green Chemistry" inspired us to apply our knowledge of lipase-mediated chemistry for harvesting the valuable[23] α-thymidine from the waste stream.

We were able to get a real sample of this waste stream from IDB, a Korean company.[24] The thymidine manufactured at IDB utilizes the gylcosylation protocol to produce a mixture of α- and *β*-anomers, from which *β*-thymidine is isolated by selective crystallization. The waste stream after removal of *β*-thymidine contained an α/*β* mixture of *p*-chlorobenzoyl protected nucleosides **7** and **8** in an 8:2 ratio determined by ^{1}H NMR. It is noteworthy that both anomers have identical R_f values by TLC and are inseparable by traditional chromatography.

To demonstrate the utility of our separation method, we carried out the enzymatic hydrolysis of the mother liquor collected from the commercial-scale synthesis of *β*-thymidine (Scheme 4). We chose PSL-C as biocatalyst because this lipase had shown desired opposite regioselectivity toward the hydrolysis of α- and *β*-nucleosides. We were pleased to see that the hydrolysis reaction took place with excellent selectivity toward the 5′-*p*-chlorobenzoate group of the α-anomer **7**, furnishing 3′-*O*-(*p*-chlorobenzoyl)-α-D-thymidine (**9**) as an exclusive product without a detectable amount of completely hydrolyzed α-thymidine **1a**. Despite the long reaction time, the *β*-anomer **8** was recovered unchanged. Interestingly, nucleosides **8** and **9** have a large difference in their R_f values and are easily separated by traditional silica-based column chromatography. The pure α-nucleoside **9** was isolated in 67% yield after chromatography. Next, we tried the hydrolysis reaction with CAL-B for two reasons. First, we demonstrated that CAL-B could be recycled several times to reduce the overall cost of the lipase. Second, CAL-B is available at a reasonable price in commercial quantity.[25] The hydrolysis of a mixture of **7** and **8** with CAL-B under similar conditions was found to be faster than PSL-C-mediated reaction and afforded us the same products. We observed a complete conversion of starting material **7** to nucleoside **9** with CAL-B in 104 h instead of the 164 h required for the reaction with PSL-C. The faster reaction rate with CAL-B was expected to reduce the overall cycle time in the plant for the hydrolysis process and assist us in meeting our lower cost objectives. After chromatographic separation, the conventional base hydrolysis of **8** and **9** furnished *β*-thymidine and α-thymidine, respectively, in excellent yields.

The waste mother liquor represents approximately 5% of the total *β*-thymidine production via the glycosylation procedure. Our protocol permits the environmentally benign biodegradation of industrial waste and transforms it into two valuable products: (i) α-thymidine, which is the key raw material for the synthesis of α-nucleosides and oligonucleotide analogues as therapeutics, and (ii) *β*-thymidine, which is a starting material for the synthesis of AZT.

Application 2: Separation of 2′-Deoxy-2′-fluoro-α/*β*-D-arabinofuranosylthymine Nucleosides. Recently, antisense oligonucleotides containing 2′-deoxy-2′-fluoro-*β*-D-arabinofuranosyl-residues (FANA) have shown high affinity for the target RNA and stability in various nucleases and plasma.[26b,c] Furthermore, FANA antisense oligonucleotides cause a reduction in target RNA levels in vivo via RNaseH mechanism.[26a] These facts combined have triggered testing of the FANA oligonucleotides as a clinical candidate for the treatment of asthma.[27]

The overall importance of FANA nucleosides motivated us to consider another challenging separation of α- and *β*-thymidine

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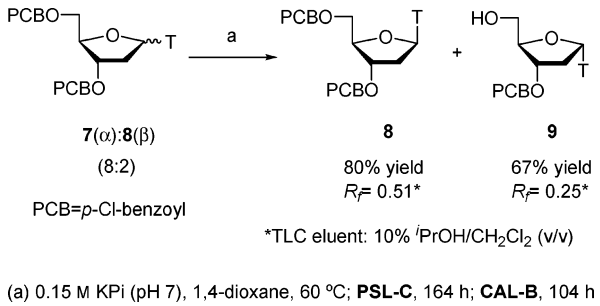
(23) The price of α-thymidine from ChemGenes Corp. is $645/gram.
(24) ID Biochem, Inc. Ulsan, Korea (www.idbio.co.kr).
(25) CAL-B is available from Novozyme (www.novozymes.com) for $2000/kg, and PSL-C is available from Amano (www.amano-enzyme.co.jp) for $3500/kg.
(26) (a) Kalota, A.; Karabon, L.; Swider, C.; Viazovkina, E.; Elzagheid, M.; Damha, M. J.; Gewirtz, A. *Nucleic Acids Res.* **2006**, *34*, 451−461. (b) Lok, C.-N.; Viazovkina, E.; Min, K.-L.; Nagy, E.; Wilds, C. J.; Damha, M. J.; Parniak, M. A. *Biochemistry* **2002**, *41*, 3457−3467. (c) Damha, M. J.; Wilds, C. J.; Noronha, A.; Brukner, I.; Borkow, G.; Parniak, M. A. *J. Am. Chem. Soc.* **1998**, *121*, 12976−12977.
(27) Topigen Pharmaceuticals Inc., Montreal, Canada (www.Topigen-.com).

TABLE 2. Enzymatic Hydrolysis of α-D-Di-levulinyl Esters 5 at 30 °C[a]

				products		
entry	substrate	enzyme	*t* (h)	**1** (%)[b]	**5** (%)[b]	**6** (%)[b,c]
1	**5a**	CAL-B	8	nd	**5a**: 1	**6a**: 99 (91)
2	**5a**	PSL-C	192	**1a**: 3	**5a**: 42	**6a**: 55
3	**5b**	CAL-B	23	nd	nd	**6b**: >97 (96)[d]
4	**5b**	PSL-C	187	**1b**: 6	**5b**: 7	**6b**: 87 (80)

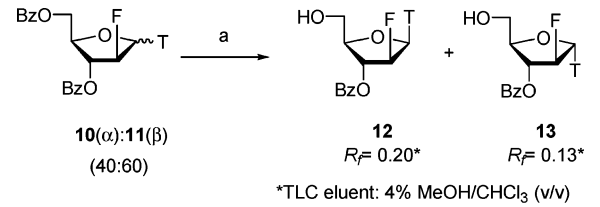
[a] Ratio **5**:CAL-B is 1:1 (w/w); ratio **5**:PSL-C is 1:3 (w/w); 0.1 M concentration. [b] Calculated by HPLC (method A) before workup. [c] Percentages of isolated yields are given in parentheses. [d] Calculated by ^{1}H NMR. Percentages indicated as >97% (±3% by NMR analysis) signify that only one compound was detected even at high spectrum amplitude.

SCHEME 4



(a) 0.15 M KPi (pH 7), 1,4-dioxane, 60 °C; **PSL-C**, 164 h; **CAL-B**, 104 h

SCHEME 5



(a) enzymatic hydrolysis, see Table 3

analogues **10** and **11**. The procedure[28] for the synthesis of 2′-deoxy-2′-fluoro-β-D-arabinofuranosylthymine results in the formation of desired β-anomer **11** as a major product and undesired α-anomer **10** as a minor product. A good portion of **11** is crystallized out from the solution, but some remains in the solution. We were able to obtain this mixture of **10** and **11** (Scheme 5) from Topigen, a Canadian company.[27] All attempts to harvest additional amounts of **11** or separation of the two products by chromatography resulted in failure. Therefore, the anomeric mixture of **10**/**11** was treated with CAL-B or PSL-C at 60 °C in 0.15 M phosphate buffer containing 20% of 1,4-dioxane. After several days, 10% and 60% of the starting material was hydrolyzed with the formation of two new products (entries 1 and 2, Table 3). The structures of the products were confirmed as 3′-*O*-benzoyl derivatives of the β- and α-anomeric nucleosides **12** and **13** by NMR studies. It is noteworthy that the enzymatic hydrolysis of the 5′-*O*-benzoyl ester of these arabinonucleosides takes place selectively at the 5′-position independently of the configuration at the anomeric carbon. This pattern of hydrolysis is clearly different from what we observed earlier with the mixture of α/β-thymidine. The use of acetonitrile instead of 1,4-dioxane led to lower conversion (entry 3, Table 3). For the hydrolysis of the mixture of **10**/**11**, we also screened other enzymes such as *Candida antarctica* lipase A (CAL-A), pig liver esterase (PLE), and esterase BS2, without much success (entries 4−7, Table 3). Among various enzymes tested, the PSL-C appeared to be the more effective catalyst for the hydrolysis process.

Next, we attempted to modulate the reaction conditions to improve the hydrolysis efficiency, keeping the PSL-C as a catalyst of choice. First, optimum ratios of buffer and cosolvent were studied. In these studies, use of less organic solvent (10% of 1,4-dioxane) furnished 77% conversion (entry 8, Table 3). In another experiment, the conversion was improved to 90% by adding the enzyme in two separate lots: first at the beginning of the reaction and second adding the enzyme after 48 h (entry 9, Table 3). Interestingly, when the reaction was carried out with additional enzyme where the ratio of the substrate:PSL-C was 1:4 (w/w), and the lipase was added in three portions (at the beginning, after 2 d, and after 5 d), 93% conversion was observed (entry 10, Table 3). The replacement of 1,4-dioxane with acetonitrile, THF, or acetone gave unsatisfactory results (entries 11−14, Table 3). Reduction in the volume of the buffer (50% less) led to a significant decrease in the rate of the reaction (entry 15, Table 3). When the hydrolysis was performed in 1 M phosphate buffer (pH 7) using 1,4-dioxane or acetonitrile as solvent (entries 16 and 17, Table 3), ≤5% conversion took place. Increasing the reaction temperature from 60 to 70 °C caused the inactivity of the catalyst (entries 18−21, Table 3).

After various attempts to drive the hydrolysis of **10**/**11** to completion, the best conditions are summarized in entries 9 and 10 of Table 3. Under these conditions, the inseparable mixture of the di-*O*-benzoyl anomeric compounds **10** and **11** was hydrolyzed to furnish the 3′-*O*-benzoyl derivatives **12** and **13**, respectively, which were separated by chromatography. To better understand the lipase behavior with FANA-nucleosides, we studied the enzymatic acylation of unprotected nucleosides **14**/**15**. These were conveniently prepared by the treatment of **10**/**11** with NaOMe in MeOH at rt to afford an α/β-mixture of **14**/**15** in 93% yield (Scheme 6).

The acylation at the primary hydroxyl group of **14**/**15** with *O*-levulinyl acetonoxime in the presence of PSL-C under standard conditions furnished the 2′-deoxy-2′-fluoro-5′-*O*-levulinyl-α-D-arabinofuranosylthymine (**16**) and 2′-deoxy-2′-fluoro-5′-*O*-levulinyl-β-D-arabinofuranosylthymine (**17**) after 6 days at 60 °C. Change of lipase to CAL-B also displayed total selectivity toward the acylation of the 5′-hydroxyl groups in the mixture of **14**/**15**. Interestingly, the two 5′-*O*-levulinyl protected nucleosides **16**/**17** have identical R_f values, and their separation by silica-based column chromatography was unsuccessful.

In view of these results, we decided to prepare benzoylated derivatives **18**/**19** expecting that the hydrophobic nature of the protecting group may enable the separation of these two products. Furthermore, these two products could be used as reference standards to ensure that the FANA-nucleosides **12**/

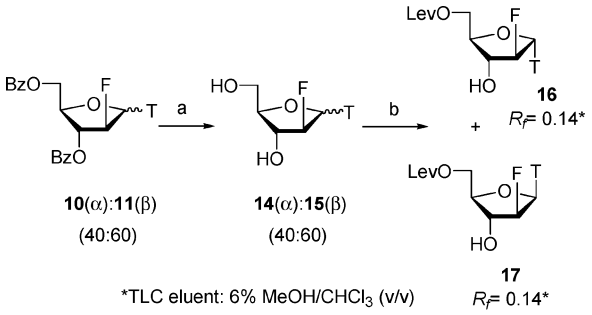
(28) Elzagheid, M.; Viazovkina, E.; Damha, M. J. In *Current Protocols in Nucleic Acid Chemistry*; Beaucage, S. L., Bergstrom, D. E., Glick, G. D., Jones, R. A., Eds.; John Wiley: New York, 2005; Chapters 1.7.1−1.7.10.

TABLE 3. Enzymatic Hydrolysis of 2′-Fluoro-α/β-di-benzoyl Esters (10:11)[a]

entry	enzyme	%% buffer (M)	solvent	*t* (h)	*T* (°C)	conv. (%)[b]
1	CAL-B	80 (0.15 M)	1,4-dioxane	119	60	10
2	PSL-C	80 (0.15 M)	1,4-dioxane	192	60	60
3	PSL-C	80 (0.15 M)	CH_3CN	192	60	41
4	CAL-A	80 (0.15 M)	CH_3CN	165	60	
5	PLE	80 (0.15 M)	1,4-dioxane	119	60	6
6	esterase BS2	80 (0.15 M)	1,4-dioxane	164	60	
7	esterase BS2	80 (0.15 M)	THF	118	60	
8	PSL-C	90 (0.15 M)	1,4-dioxane	161	60	77
9	PSL-C	90 (0.15 M)	1,4-dioxane	161	60	90[c]
10	PSL-C	90 (0.15 M)	1,4-dioxane	165	60	93[d,e]
11	PSL-C	90 (0.15 M)	CH_3CN	168	60	53
12	PSL-C	90 (0.15 M)	CH_3CN	164	60	65[d]
13	PSL-C	90 (0.15 M)	THF	168	60	32
14	PSL-C	90 (0.15 M)	acetone	168	60	48
15	PSL-C	50 (0.15 M)	1,4-dioxane	165	60	10
16	PSL-C	80 (1 M)	1,4-dioxane	160	60	5
17	PSL-C	80 (1 M)	CH_3CN	160	60	3
18	PSL-C	80 (0.15 M)	1,4-dioxane	160	70	2
19	PSL-C	90 (0.15 M)	1,4-dioxane	160	70	
20	PSL-C	80 (0.15 M)	CH_3CN	160	70	7
21	PSL-C	90 (0.15 M)	CH_3CN	160	70	1

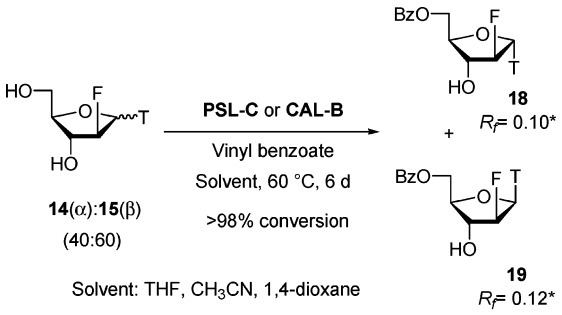
[a] Ratio substrate:CAL-B is 1:1 (w/w); ratio substrate:PSL-C is 1:3 (w/w); ratio substrate:esterase BS2 is 0.064 mmol:150 U; ratio substrate:PLE is 1:1 (w/w); ratio substrate:CAL-A is 1:1 (w/w); 0.1 M concentration. [b] Calculated by HPLC (method B). [c] The enzyme was added in two separated fractions. [d] Ratio substrate:PSL-C is 1:4 (w/w), and the enzyme was added in three separated fractions. [e] Isolated yields: 82% (**12**) and 87% (**13**).

SCHEME 6



(a) NaOMe, MeOH, rt, 1 h (93% yield); (b) **PSL-C** or **CAL-B**, *O*-levulinyl acetonoxime, 1,4-dioxane, 60 °C, 6 d, >98% conversion

SCHEME 7



13 were indeed 3′-*O*-benzoylated products. The benzoylation of **14**/**15** with vinyl benzoate in the presence of PSL-C or CAL-B took place with the predicted selectivity toward the 5′-OH groups (Scheme 7).[11e] The hydrophobic 5′-*O*-benzoate esters **18** and **19** showed increased difference in their R_f values as compared to the corresponding 5′-*O*-levulinate esters, and a clean separation of both products using chromatography was unavailing. Among the three methods (Schemes 5−7) tried for the separation of 2′-deoxy-2′-fluoro-α/β-D-arabinofuranosylthymine nucleosides, we recommend the first protocol (entries 9 and 10 in Table 3) where PSL-C was employed resulting in >90% conversion.

Conclusion

In this paper, chemoenzymatic syntheses have been employed, offering efficient separation techniques for the isolation of pure α-nucleosides from an inseparable mixture of α/β-nucleosides. Our results indicate that regioselective lipase-catalyzed hydrolysis is a powerful technique for the separation of α/β-nucleosides frequently obtained during the synthesis of nucleosides utilizing glycosylation protocols.[29] To the best of our knowledge, there are no formal studies on the separation techniques of α/β-nucleosides reported in the literature. The usefulness of our protocol is clearly demonstrated by the separation of α/β-nucleosides for two industrial projects. The discovery of opposite regioselectivity is a unique property exhibited by *Pseudomonas cepacia* lipase (PSL-C) when in contact with the mixtures of α- and β-anomeric nucleosides, thus providing a convenient method for the isolation of α/β nucleosides. Particularly, the isolation of α-thymidine from the industrial waste is of paramount importance considering the commercial value[23] of this product, now accessible in two simple steps. It was found that PSL-C had different substrate specificity for the FANA-nucleosides **10** and **11**, where both α- and β-nucleosides were hydrolyzed at the 5′-hydroxyl groups with comparable rates. The change in the recognition pattern of PSL-C for FANA-nucleosides may be attributed to the altered sugar pucker due to the electronegative fluorine atom present on the 2′-position. Further studies are in progress to find the correlation between the effects of sugar pucker on the hydrolysis and acylation reactions of various chemically modified nucleosides using lipases.

Our judicious selection of immobilized enzymes provides several advantages in organic syntheses. Notable traits for the immobilized lipases are enhanced stability, reusability, conve-

(29) *Handbook of Nucleoside Synthesis*; Vorbruggen, H., Ruh-Pohlenz, C., Eds.; John Wiley: New York, 2001 and references cited therein.

nient separation by filtration from the reaction mixture, preventing protein contamination in the product, and consistent performance of the commercial preparations.[30] It seems reasonable to speculate that this study will have implications for α/β-anomeric nucleoside separation processes ranging from large-scale manufacturing to small-scale synthesis of novel nucleosides.

Experimental Section

General Procedure for the Regioselective Enzymatic Acylation of α-D-2′-Deoxynucleosides 1. In a standard procedure, THF (2 mL) was added to an Erlenmeyer flask that contained **1** (0.2 mmol), *O*-levulinyl acetonoxime[17] (102.6 mg, 0.6 mmol), and the enzyme under nitrogen (the ratio of enzyme and time are indicated in Table 1). The reaction mixture was stirred at 30 °C and 250 rpm and monitored by TLC (10% MeOH/CH_2Cl_2). The enzyme was filtered off, washed with CH_2Cl_2 and MeOH, and the solvents evaporated under vacuum. Flash chromatography (EtOAc and then 20% MeOH/CH_2Cl_2 for **2a**, **2b**, and **2c**; gradient elution with 5−20% MeOH/CH_2Cl_2 for **2d**) of the residue afforded derivatives **2a**−**d** (yields are indicated in Table 1).

Separation of a 1:1 Anomeric Mixture of α/β-D-Thymidine. A procedure similar to that described for **2** was followed at 0.2 M concentration. The reaction was monitored by HPLC (method A).[20] Reaction time: 4 h. Flash chromatography (5% ^{i}PrOH/CH_2Cl_2) of the reaction crude afforded 97% yield of 5′-*O*-levulinyl-α-D-thymidine (**2a**) and 95% yield of 3′-*O*-levulinyl-β-D-thymidine (**4a**).

Separation of a 1:1 Anomeric Mixture of N^6-Benzoyl-α/β-D-2′-deoxyadenosine. A procedure similar to that described for **2** was followed at 0.2 M concentration. The reaction was monitored by HPLC (method A). Reaction time: 8 h. Flash chromatography (2% ^{i}PrOH/CH_2Cl_2) of the crude afforded 91% yield of N^6-benzoyl-5′-*O*-levulinyl-α-D-2′-deoxyadenosine (**2c**) and 88% yield of N^6-benzoyl-3′-*O*-levulinyl-β-D-2′-deoxyadenosine (**4c**).

3′,5′-Di-*O*-levulinyl-α-D-2′-deoxynucleosides 5. To a stirred mixture of **1** (0.75 mmol) and Et_3N (0.3 mL, 1.9 mmol) in 1,4-dioxane (5 mL) under nitrogen were added levulinic acid (0.40 mL, 3.9 mmol), DCC (803.4 mg, 3.9 mmol), and DMAP (7.3 mg, 0.06 mmol). The reaction was stirred at rt during 2.5 h for **1a** and 40 min for **1b**. The insoluble material was collected by filtration, and the filtrate was evaporated under vacuum. The residue was taken up in $NaHCO_3$ (aq) and extracted with CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4 and evaporated. The residue was subjected to flash chromatography (EtOAc and then 5% MeOH/CH_2Cl_2 for **5a**; gradient elution with 0−30% MeOH/EtOAc for **5b**) to afford **5a** in 93% yield and **5b** in 94% yield.

General Procedure for the Enzymatic Hydrolysis of 3′,5′-Di-*O*-levulinyl-α-D-2′-deoxynucleosides 5. To a solution of **5** (0.2 mmol) in 1,4-dioxane (0.35 mL) were added 0.15 M phosphate buffer pH 7 (1.65 mL) and the corresponding lipase (the ratio of enzyme and time are indicated in Table 2). The reaction mixture was stirred at 30 °C and 250 rpm and was monitored by TLC (10% MeOH/CH_2Cl_2) and HPLC (method B).[20] The enzyme was filtered off, washed with CH_2Cl_2 and MeOH, and the solvents evaporated under vacuum. For **6b**, 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 concentrated to give pure **6b** in 96% yield (entry 3, Table 2). For **6a** (entry 1, Table 2), the residue was subjected to flash chromatography (10% MeOH/CH_2Cl_2) to afford **6a** in 91% yield. For **6b** (entry 4, Table 2), the residue was subjected to flash chromatography (5% MeOH/CH_2Cl_2) to give pure **6b** in 80% yield.

Separation of an 8:2 Mixture of 3′,5′-Di(*p*-chlorobenzoyl)-α/β-D-thymidine from Industrial Synthesis of Thymidine. The waste stream from ID Biochem, which contains **7**/**8**, was evaporated until dryness, and then the residue was subjected to filtration on silica gel (3% MeOH/CH_2CL_2) before the enzymatic process. To a solution of **7**/**8** (52 mg, 0.1 mmol) in 1,4-dioxane (0.18 mL) were added 0.15 M phosphate buffer pH 7 (0.83 mL) and PSL-C (156 mg) or CAL-B (52 mg). The mixture was stirred at 250 rpm and 60 °C during 164 or 104 h, respectively. The enzyme was filtered off, washed with CH_2Cl_2 and MeOH, and the solvents were evaporated under vacuum. Flash chromatography (0.5% ^{i}PrOH/CH_2Cl_2) gave **8** in 80−84% yield and **9** in 67−71% yield.

General Procedure for the Enzymatic Hydrolysis of 1-(3′,5′-Di-*O*-benzoyl-2′-fluoro-2′-deoxy-α/β-D-arabinofuranosyl)thymine (10/11). To a solution of **10**/**11** (0.214 mmol) in an appropriate solvent were added phosphate buffer pH 7 and the corresponding enzyme. Reaction concentration: 0.1 M. The temperature, reaction time, and ratios of enzyme, buffer, and cosolvent are indicated in Table 3. The reaction mixture was stirred at 250 rpm and monitored by HPLC (method C).[20] The enzyme was filtered off, washed with CH_2Cl_2 and MeOH, and the solvents evaporated under vacuum. 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 crude was subjected to flash chromatography (3% ^{i}PrOH/$CHCl_3$) to give **12** in 82% yield and **13** in 86% yield (entry 10, Table 3).

1-(2′-Fluoro-2′-deoxy-α/β-D-arabinofuranosyl)thymine (14/15). Sodium methoxide (252 mg, 4.487 mmol) was added to a solution of **10**/**11** (350 mg, 0.048 mmol) in anhydrous MeOH (4.9 mL). The mixture was stirred at rt during 1 h. The reaction was neutralized with Dowex 50Wx4-400 ion-exchange resin. Next, the residue was filtered off, washed with MeOH, and the solvents evaporated under vacuum. The crude product was subjected to flash chromatography (50−100% EtOAc/hexane) to afford **14**/**15** (mixture α/β 40:60) in 93% yield.

Enzymatic Acylation of 1-(2′-Fluoro-2′-deoxy-α/β-D-arabinofuranosyl)thymine (14/15). In a standard procedure, solvent (1 mL) was added to an Erlenmeyer flask that contained **14**/**15** (0.1 mmol), *O*-levulinyl acetonoxime or vinyl benzoate (3 equiv), and the enzyme under nitrogen (ratio substrate:CAL-B is 1:1 w/w; ratio substrate:PSL-C is 1:3 w/w). The reaction mixture was stirred at 250 rpm and 60 °C and monitored by HPLC (method C).[20] The enzyme was filtered off, washed with CH_2Cl_2 and MeOH, and the solvents evaporated under vacuum to afford an α/β mixture of 5′-*O*-acyl derivatives. A successful separation by flash chromatography was not achieved.

Acknowledgment. We are grateful to ID Biochem and Topigen Pharmaceuticals Inc. for their generous gift of the mixture of protected α/β-thymidine nucleosides and FANA α/β-nucleosides, respectively, used for this study. Financial support of this work by the Spanish Ministerio de Educación y Ciencia (MEC, Project CTQ-2004-04185) and Principado de Asturias (FICYT, Project IB05-109) is gratefully acknowledged. S.F. thanks MEC for a personal grant (Ramón y Cajal Program). A.D.-R. thanks MEC for a pre-doctoral fellowship.

Supporting Information Available: ^{1}H, ^{13}C NMR spectral data and some 2D NMR experiments, in addition to mp, IR, optical rotation, microanalysis, HPLC, and MS data. The level of purity is indicated by the inclusion of copies of ^{1}H and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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(30) (a) *Biocatalysis in the Pharmaceutical and Biotechnology Industries*; Patel, R. N., Ed.; CRC Press: Florida, 2006. (b) García-Urdiales, E.; Alfonso, I.; Gotor, V. *Chem. Rev.* **2005**, *105*, 313−354. (c) Fernández, S.; Gotor-Fernández, V.; Ferrero, M.; Gotor, V. *Curr. Org. Chem.* **2002**, *6*, 453−469. (d) Ferrero, M.; Gotor, V. *Chem. Rev.* **2000**, *100*, 4319−4397.