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Enzymatic Parallel Kinetic Resolution of Mixtures of D/L 2′-Deoxy and Ribonucleosides: An Approach for the Isolation of β-L-Nucleosides†

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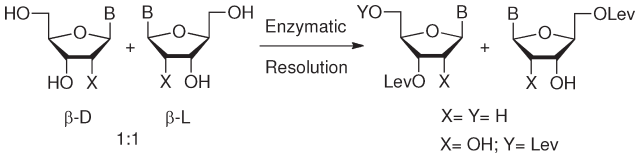

We have developed a lipase-catalyzed parallel kinetic resolution of mixtures of β-D/L-nucleosides. The opposite selectivity during acylation exhibited by *Pseudomonas cepacia* lipase (PSL-C) with β-D- and β-L-nucleosides furnished acylated compounds that have different R_f values. As a consequence, isolation of both products was achieved by simple column chromatography. Computer modeling of the transition-state analogues during acylation of β-D- and β-L-2′-deoxycytidine with PSL-C was carried out to explain the high selectivity. PSL-C favored the 3′-*O*-levulination of the β-D enantiomer, whereas the 5′-OH group was acylated in 2′-deoxy-β-L-cytidine. In both cases, the cytosine base was placed in the alternate hydrophobic pocket of PSL's substrate-binding site, where it can form extra hydrogen bonds (in addition to the five essential catalytically relevant hydrogen bonds) that stabilize these intermediates catalyzing the selective acylation of β-D/L-nucleosides.

Introduction

In recent years, unnatural β-L-nucleoside analogues have received much attention as a new class of antiviral and anticancer agents.[1] Since the discovery of lamivudine[2] (L-2′,3′-dideoxy-3′-thiacytidine, 3TC; **1**, Chart 1) for the treatment of human immunodeficiency virus (HIV) and hepatitis B virus (HBV) infections, various L-nucleoside derivatives have been synthesized and evaluated for their potential therapeutic applications.

Recently, telbivudine (LdT; **4**), the L-isomer of thymidine, was approved for the treatment of HBV infection. Clinical trials have shown LdT to be significantly more effective than lamivudine or adefovir and less likely to cause resistance.[3] Another anti-HBV L-nucleoside is valtorcitabine[4] (val-LdC; **5**), the 3′-*O*-valinyl ester of L-deoxycytidine, which has been developed as a fixed dose combination with LdT. In clinical trials, the combination of the two agents had demonstrated greater antiviral activity than either drug alone. One promising candidate for the treatment of chronic HBV is β-L-2′,3′-dideoxy-3′-thia-5-fluorocytidine (emtricitabine, FTC; **2**), the 5-fluorinated analogue of 3TC, available in combination with other antiretroviral agents as an anti-HIV therapeutic agent. This drug is currently being evaluated as a potential treatment for chronic HBV.[5]

† In memoriam to Professor José Manuel Concellón.

(1) See a mini-review: Mathé, C.; Gosselin, G. *Antiviral Res.* **2006**, *71*, 276–281.

(2) (a) Jarvis, B.; Faulds, D. *Drugs* **1999**, *58*, 101–141. (b) Cameron, J. M.; Collins, P.; Daniel, M.; Storer, R. *Drugs Future* **1993**, *18*, 319–323. (c) Chang, C.-N.; Doong, S.-L.; Zhou, J. H.; Beach, J. W.; Jeong, L. S.; Chu, C. K.; Tsai, C.-H.; Cheng, Y.-C. *J. Biol. Chem.* **1992**, *267*, 13938–13942. (d) Doong, S. L.; Tsai, C.-H.; Schinazi, R. F.; Liotta, D. C.; Cheng, Y.-C. *Proc. Natl. Acad. Sci. U. S. A.* **1991**, *88*, 8495–8499.

(3) Charuworn, P.; Keeffe, E. B. *Clin. Med. Ther.* **2009**, *1*, 157–166.

(4) (a) Gosselin, G.; Pierra, C.; Benzaria, S.; Dukhan, D.; Imbach, J.-L.; Loi, A. G.; La Colla, P.; Cretton-Scott, E.; Bridges, E. G.; Standring, D. N.; Sommadossi, J. P. β-L-2′-Deoxythymidine (L-dT) and β-L-2′-Deoxycytidine (LdC): How Simple Structures can be Potent, Selective and Specific Anti-HBV Drugs. In *Frontiers in Nucleosides and Nucleic Acids*; Schinazi, R. F., Liotta, D. C., Eds.; IHL Press: Tucker, GA, 2004; pp 309−317. (b) Licensed by Novartis. It has been discontinued in 2008 for reasons that were not disclosed: Ryder, N. S. *Expert Opin. Invest. Drugs* **2010**, *19*, 1–21.

(5) (a) Furman, P. A.; Davis, M.; Liotta, D. C.; Paff, M.; Frick, L. W.; Nelson, D. J.; Dornsife, R. E.; Wurster, J. A.; Wilson, L. J.; Fyfe, J. A.; Tuttle, J. V.; Miller, W. H.; Condreay, L.; Averett, D. R.; Schinazi, R. F.; Painter, G. R. *Antimicrob. Agents Chemother.* **1992**, *36*, 2686–2692. (b) Schinazi, R. F.; McMillan, A.; Cannon, D.; Mathis, R.; Lloyd, R. M.; Peck, A.; Sommadossi, J.-P.; Clair, M. St.; Wilson, J.; Furman, P. A.; Painter, G. R.; Choi, W. B.; Liotta, D. C. *Antimicrob. Agents Chemother.* **1992**, *36*, 2423–2431. (c) Jenh, A. M.; Thio, C. L.; Pham, P. A. *Pharmacotherapy* **2009**, *29*, 1212–1227. (d) Saag, M. S. *Clin. Infect. Dis.* **2006**, *42*, 126–131.

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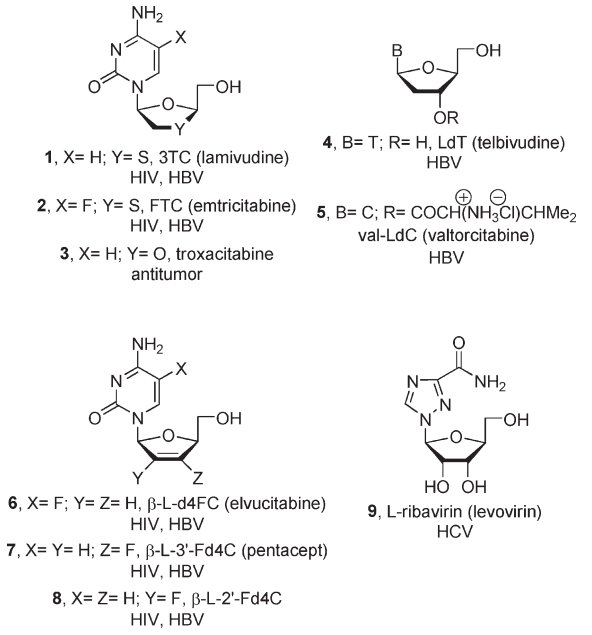
CHART 1. β-L-Nucleosides Used As Antiviral and Antitumor Agents



A β-L-nucleoside analogue that has received much attention as an HBV agent is clevudine [L-FMAU, 1-(2′-fluoro-5-methyl-β-L-arabinofuranosyl)uracil], but its phase III clinical trial was terminated due to some myopathy cases in patients.[6]

Additional β-L-nucleosides are under clinical investigation as potential antiviral agents. These include the unsaturated nucleoside analogues β-L-2′,3′-didehydro-2′,3′-dideoxy-5-fluorocytidine (elvucitabine, β-L-d4FC; **6**), β-L-2′,3′-didehydro-2′,3′-dideoxy-3′-fluorocytidine (pentacept, β-L-3′-Fd4C; **7**), β-L-2′,3′-didehydro-2′,3′-dideoxy-2′-fluorocytidine (β-L-2′-Fd4C; **8**),[7] and the L-enantiomer of ribavirin, 1-β-L-ribofuranosyl-1,2,4-triazole-3-carboxamide (levovirin; **9**), which was investigated as a potential anti-hepatitis C virus (HCV) agent.[8] However, the limited absorption of levovirin induced the development of the 5′-valinate monoester of levovirin, namely, R1518, a prodrug that results in improved bioavailability and significantly higher plasma concentrations of levovirin than oral administration of levovirin.[9]

β-L-Nucleoside analogues are also promising anticancer agents. Troxacitabine (**3**) is the first unnatural β-L-nucleoside analogue to show potent preclinical antitumor activity.[10] Use of L-nucleoside **3** is mainly being studied in patients with advanced pancreatic cancer or blast phase chronic myelogenous leukemia.

Interestingly, novel β-L-adenosine analogues have been synthesized and evaluated as cardioprotective agents. Thus, β-L-3′-amino-3′-deoxy-N^6-dimethyladenosine was found to enhance functional recovery from ischemia.[11] On the other hand, β-L-adenosine and β-L-thymidine (**4**) were selectively transported into malaria-infected cells through the induced transporter.[12]

General favorable features of β-L-nucleosides are lower cellular toxicity and more resistance to degrading enzymes than their D-counterparts.[13] Several routes have been reported for the synthesis of β-L-nucleoside derivatives. However, formation of a mixture of β-D- and β-L-nucleosides is a quite common occurrence during the preparation of these compounds. Due to the increased therapeutic applications of L-nucleosides, there is a need for a separation method that permits easy isolation of β-D-nucleosides from β-L-nucleosides. Enzymes are useful catalysts for the separation of racemic mixtures of nucleosides.[14] Enzymatic resolutions were performed for isolating the chiral forms with high enantiomeric excess. Relevant examples are the pig liver esterase-mediated kinetic resolution of β-D- and β-L-configured dideoxynucleosides (**10**, Chart 2),[15] the enantioselective synthesis of bicyclo[3.1.0]hexane carbocyclic nucleosides (**11**) via a lipase-catalyzed asymmetric acetylation,[16] or the preparative scale resolution of spiro[2.3]hexane carbocyclic nucleosides (**12**) by *Pseudomonas cepacia* lipase.[17] Recently, preparation of isoxazolidinyl nucleoside (**13**) enantiomers by double lipase-catalyzed resolution has been described.[18]

Taking into account the opposite preference shown for *Pseudomonas cepacia* lipase during the acylation or hydrolysis reactions of β-D- and β-L-2′-deoxynucleosides, our preliminary work described the resolution of β-D/L-thymidine.[19a] Herein, we report the validation and extension of this procedure to the resolution of other pyrimidine and purine 2′-deoxynucleoside derivatives. Also a study of the enzymatic acylation of ribonucleosides and its application in the resolution of racemic mixtures has been carried out. In addition, the results presented here have been rationalized using a computational approach.

Results and Discussion

The potential therapeutic application of val-LdC for the treatment of HBV disease motivated us to develop a successful

(6) (a) Discovered: Chu, C. K.; Ma, T. W.; Shanmuganathan, K.; Wang, C.-G.; Xiang, Y.-J.; Pai, S. B.; Yao, G.-Q.; Sommadossi, J.-P.; Cheng, Y.-C. *Antimicrob. Agents Chemother.* **1995**, *39*, 979–981. (b) Terminated: Drugs.com, Princeton, NJ, April 20, 2009.

(7) Komiotis, D.; Manta, S.; Tsoukala, E.; Tzioumaki, N. *Anti-Infective Agents Med. Chem.* **2008**, *7*, 219–244.

(8) Walker, M. P.; Appleby, T. C.; Zhong, W.; Lau, J. Y. N.; Hong, Z. *Antiviral Chem. Chemother.* **2003**, *14*, 1–21.

(9) Huang, Y.; Ostrowitzki, S.; Hill, G.; Navarro, M.; Berger, N.; Kopeck, P.; Mau, C. I.; Alfredson, T.; Lal, R. *J. Clin. Pharmacol.* **2005**, *45*, 578–588.

(10) (a) Damaraju, V. L.; Bouffard, D. Y.; Wong, C. K. W.; Clarke, M. L.; Mackey, J. R.; Leblond, L.; Cass, C. E.; Grey, M.; Gourdeau, H. *BMC Cancer* **2007**, *7*, 121. (b) Swords, R.; Giles, F. *Hematology* **2007**, *12*, 219–227. (c) Adema, A. D.; Radi, M.; Daft, J.; Narayanasamy, J.; Hoebe, E. K.; Alexander, L. E.; Chu, C. K.; Peters, G. J. *Nucleosides Nucleotides Nucleic Acids* **2007**, *26*, 1073–1077. (d) Gumina, G.; Chong, Y.; Chu, C. K. L-Nucleosides as Chemotherapy Agents. In *Cancer Drug Discovery and Development: Deoxynucleoside Analogs in Cancer Therapy*; Peters, G. J., Ed.; Humana Press: Totowa, NJ, 2006; pp 173−198. (e) Lapointe, R.; Letourneau, R.; Steward, W.; Hawkins, R. E.; Batist, G.; Vincent, M.; Whittom, R.; Eatock, M.; Jolivet, J.; Moore, M. *Ann. Oncol.* **2005**, *16*, 289–293.

(11) Kasiganesan, H.; Wright, G. L.; Chiacchio, M. A.; Gumina, G. *Bioorg. Med. Chem.* **2009**, *17*, 5347–5352.

(12) Gero, A. M.; Dunn, C. G.; Broen, D. M.; Pulenthiran, K.; Gorovits, E. L.; Bakos, T.; Weis, A. L. *Curr. Pharm. Des.* **2003**, *9*, 867–877.

(13) (a) Gumina, G.; Chong, Y.; Choo, H.; Song, G.-Y.; Chu, C. K. *Curr. Top. Med. Chem.* **2002**, *2*, 1065–1086. (b) Gumina, G.; Song, G.-Y.; Chu, C. K. *FEMS Microbiol. Lett.* **2001**, *202*, 9–15. (c) Jurovcik, M.; Holy, A.; Sorm, F. *FEBS Lett.* **1971**, *18*, 274–276.

(14) (a) Ferrero, M.; Gotor, V. *Chem. Rev.* **2000**, *100*, 4319–4347. (b) Ferrero, M.; Gotor, V. *Monatsh. Chem.* **2000**, *131*, 585–616.

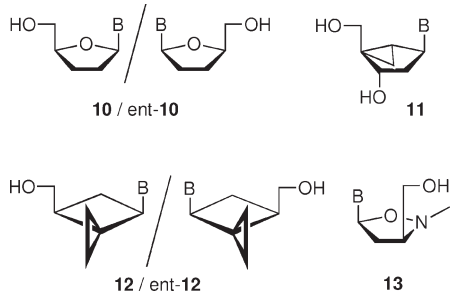
(15) Albert, M.; De Souza, D.; Feiertag, P.; Hönig, H. *Org. Lett.* **2002**, *4*, 3251–3254.

(16) Yoshimura, Y.; Moon, H. R.; Choi, Y.; Marquez, V. E. *J. Org. Chem.* **2002**, *67*, 5938–5945.

(17) Bondada, L.; Gurnina, G.; Nair, R.; Ning, X. H.; Schinazi, R. F.; Chu, C. K. *Org. Lett.* **2004**, *6*, 2531–2534.

(18) Carnovale, C.; Iannazzo, D.; Nicolosi, G.; Piperno, A.; Sanfilippo, C. *Tetrahedron: Asymmetry* **2009**, *20*, 425–429.

CHART 2. Enzymatic Resolutions for the Synthesis of β-L-Nucleosides



SCHEME 1

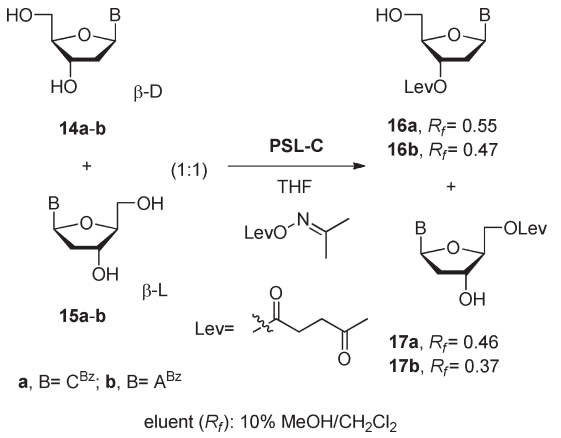


TABLE 1. Enzymatic Resolution of D/L-2′-Deoxynucleoside Racemic Mixtures with PSL-C[a]

					16		17	
entry	substrate	conc (M)	T (°C)	t (h)	yield (%)[b]	ee (%)[c]	yield (%)[b]	ee (%)[c]
1	**14a/15a**	0.1	30	24	93	>99	82	>99
2	**14b/15b**	0.05	45	20	80	>99	87	>99

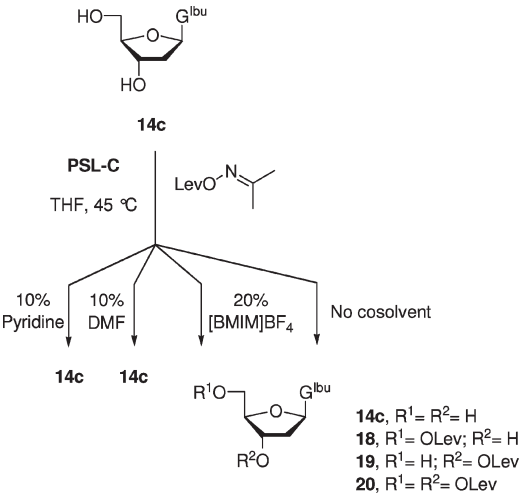
[a]Ratio of substrate:PSL-C, 1:3 (w/w); 3 equiv of acetonoxime levulinate. [b]Calculated after column chromatography. [c]Calculated by chiral HPLC analysis.

method for the separation of a β-D/L-dC mixture. For that, we selected *Pseudomonas cepacia* lipase (PSL-C) as the catalyst and acetonoxime levulinate as the acylating agent since these conditions have proven to be excellent during the acylation of β-D-dC^{Bz} and β-L-dC^{Bz}.[19] Treatment of *N*-benzoyl-β-D/L-dC (**14a**/**15a**)[20] with acetonoxime levulinate in the presence of PSL-C at 30 °C in THF afforded a mixture of two acylated products (Scheme 1). After workup, ^{1}H NMR spectra confirmed the formation of β-D-3′-*O*-levulinyl ester **16a** and β-L-5′-*O*-levulinyl derivative **17a** as the sole products. The formation of these compounds is attributed to the opposite acylation preference exhibited by PSL-C in β-D- and β-L-2′-deoxynucleosides. Thus, PSL-C catalyzes with total regioselectivity the acylation at the

(19) (a) García, J.; Fernández, S.; Ferrero, M.; Sanghvi, Y. S.; Gotor, V. *Org. Lett.* **2004**, *6*, 3759–3762. (b) García, J.; Fernández, S.; Ferrero, M.; Sanghvi, Y. S.; Gotor, V. *Tetrahedron: Asymmetry* **2003**, 3533–3540.

(20) The equimolar mixture of the two nucleosides was prepared by mixing the equal weight of β-D-dC^{Bz} (**14a**) and β-L-dC^{Bz} (**15a**). Mixtures of **14b** and **15b**, and **23a** and **25a** were prepared in a similar manner.

SCHEME 2



3′-hydroxyl group of β-D-nucleosides, whereas the same lipase PSL-C exhibited an opposite selectivity for the β-L-isomer, catalyzing exclusively the acylation of the 5′-hydroxyl group. The mixture of the two acylated products **16a** and **17a** showed different R_f values on the TLC and are easily separated by silica column chromatography (entry 1, Table 1). Enantiomeric excess were determined with chiral HPLC analysis by comparing the product from the enzymatic reaction and an artificial racemic sample. The data clearly supported the enantiomeric purity of the nucleosides (ee >99% by HPLC).

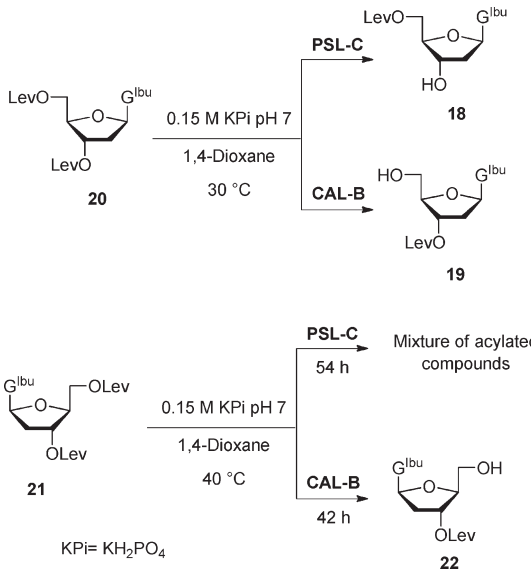
Next, we decided to evaluate the same process for the separation of a mixture of N^6-benzoyl-2′-deoxy-β-D/L-adenosine (**14b**/**15b**). After 24 h, the reaction was incomplete and a large amount of insoluble starting material was observed in the reaction mixture. In order to improve the solubility of the nucleosides **14b**/**15b** and reach total conversion, more dilute conditions (0.05 M instead of 0.1 M) were used. However, unreacted nucleoside still remained in the solution. Next, a higher reaction temperature was employed to drive the reaction to completion. We speculate that the higher temperature offered improved flexibility for binding to the active site on PSL-C. Entry 2 in Table 1 shows successful conversion under these conditions, furnishing **16b** and **17b** in high yields. We were pleased to note that the PSL-C exhibited the same regiospecificity with the deoxycytidine and deoxyadenosine derivatives. Again, this lipase catalyzes the acylation at the secondary hydroxyl group of β-D-dA^{Bz} with remarkable selectivity. Both compounds **16b** and **17b** were obtained with >99% ee determined by chiral HPLC.

The resolution of N^2-isobutyryl-2′-deoxy-β-D/L-guanosine resulted in incomplete acylation reaction catalyzed by PSL-C.[19a] The acylation on β-D-dG^{Ibu} (**14c**) afforded a mixture of mono- and di-*O*-levulinyl derivatives (**18**−**20**), in addition to unreacted starting nucleoside (Scheme 2). In order to reach 100% conversion, the process was carried out with a large excess of acylating agent (4 equiv). However, the reaction was incomplete even after 6 days probably due to the low solubility of the starting nucleoside in THF (see Table 2). The use of cosolvents such as pyridine or DMF inhibited the activity of the enzyme. In recent years, ionic liquids as a choice of solvent for biocatalysis have shown promising results.[21] Therefore, 1-butyl-3-methylimidazolium

TABLE 2. Solubility of Nucleosides in THF

entry	nucleoside	solubility (mg/mL)
1	T	4.4
2	dC^{Bz}	2.1
3	dA^{Bz}	5.4
4	dG^{Ibu}	0.4
5	U	3.0
6	A^{Bz}	0.5
7	C^{Bz}	≪0.1

SCHEME 3

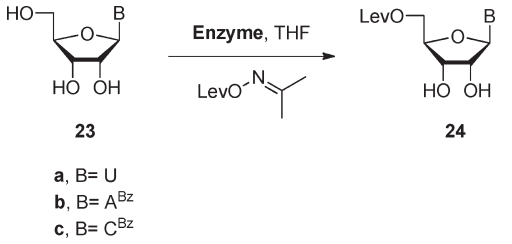


tetrafluoroborate [(BMIM)BF_4], an ionic liquid, was tried for acylation of **14c**. Surprisingly, the solubility of **14c** in (BMIM)BF_4 was insufficient to drive the reaction to completion. However, the acylation of β-L-dG^{Ibu} under very diluted conditions (0.025 M) and large excess of acylating agent (9 equiv) led to 100% conversion but furnished mixtures of acylated compounds.

Unsuccessful results were also observed in the hydrolysis reaction of corresponding 3′,5′-di-*O*-levulinyl D and L derivatives (Scheme 3). Hydrolysis reaction catalyzed by PSL-C on 3′,5′-di-*O*-Lev-β-D-dG^{Ibu} (**20**) afforded 5′-*O*-Lev-β-D-dG^{Ibu} (**18**), whereas CAL-B furnished the 3′-*O*-Lev compound **19**.[22] CAL-B exhibited the same selectivity with the L-isomer, and selective 5′-hydrolysis was accomplished by the reaction of 3′,5′-di-*O*-Lev-β-L-dG^{Ibu} (**21**) with phosphate buffer. However, PSL-C exhibited low selectivity, furnishing a mixture of acylated derivatives. Although several strategies were tried, we could not find an adequate enzyme for the desired separation of β-D/L-dG^{Ibu} mixtures.

We also became interested in the resolution of D/L-mixtures of ribonucleosides due to their applications in the synthesis of therapeutic aptamers.[23] Therefore, we decided to investigate the enzymatic acylation of β-D- and β-L-ribonucleosides with CAL-B and PSL-C. Enzymatic acylation of

SCHEME 4



β-D-uridine (**23a**) and N^6-benzoyl-β-D-adenosine (**23b**) with acetonoxime levulinate in the presence of CAL-B afforded the 5′-*O*-levulinyl esters **24a** and **24b** with excellent regioselectivity and high yields in short reaction times (Scheme 4, entries 1 and 2 of Table 3). Total selectivity was also observed when the same process was carried out with N^4-benzoyl-β-D-cytidine (**23c**). However, the reaction was slower and did not go to completion, despite the large excess of acylating agent (9 equiv vs 3 equiv). This is probably due to the poor solubility of the starting nucleoside in the reaction medium (see Table 2). More dilute conditions and a substrate:CAL-B ratio of 1:2 (w/w) drove the reaction to completion and furnished **24c** in 93% yield (entry 4, Table 3). The acylation of **23a** with PSL-C took place with poor selectivity, furnishing a mixture of 2′-*O*-Lev- and 3′-*O*-Lev-protected nucleosides as the major products (entry 5, Table 3). In the case of β-D-A^{Bz}, the unreacted starting material was observed after 24 h (entry 6, Table 3). The low solubility of β-D-A^{Bz} in THF and slow reactivity of ribonucleosides in the presence of PSL-C may have been the cause of failure to acylate. More dilute conditions increased the conversion rate to 22% but low selectivity persisted (entry 7, Table 3). This lipase also displayed low selectivity during acylation of β-D-C^{Bz} (entry 8, Table 3).

Acylation reaction catalyzed by CAL-B on β-L-uridine (**25a**) and N^6-benzoyl-β-L-adenosine (**25b**) afforded exclusively 5′-*O*-levulinyl-β-L-uridine (**26a**) and N^6-benzoyl-5′-*O*-levulinyl-β-L-adenosine (**26b**), respectively (Scheme 5, entries 1 and 3 of Table 4). Next, we performed the acylation catalyzed by PSL-C. The latter exhibited the same behavior as CAL-B in L-ribonucleosides, catalyzing selectively 5′-*O*-acylation of β-L-uridine (entry 2, Table 4) and N^6-benzoyl-β-L-adenosine (entry 4, Table 4). When the same process was carried out on β-L-C^{Bz}, PSL-C showed selective acylation of the 5′-hydroxyl group. However, the reaction stalled after 75% conversion (entry 6, Table 4). Prolonged reaction times did not increase the conversion, but the selectivity was lower. In the case of CAL-B, the 5′-*O*-Lev derivative was obtained as the major compound in addition to diacylated compounds (entry 5, Table 4).

On the basis of our experience, β-D/L-uridine was subjected to enzymatic resolution. The lack of selectivity observed with PSL-C on β-D-ribonucleosides makes the separation of a mixture of β-D/L-isomers difficult. Among the approaches tested, the route specified in Scheme 6 provided the most convenient strategy. Treatment of β-D/L-uridine (**23a**/**25a**) with acetonoxime levulinate in the presence of PSL-C at 30 °C in THF furnished multiple inseparable monoacylated products with the same R_f. ^{1}H NMR analysis showed the presence of 2′-, 3′-, and 5′-*O*-levulinyl derivatives. We attributed the 5′-*O*-levulinylation of the L-isomer to the expected selectivity

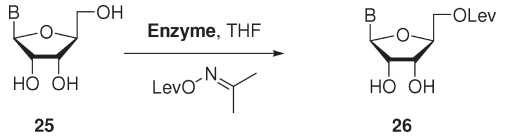
(21) van Rantwijk, F.; Sheldon, R. A. *Chem. Rev.* **2007**, *107*, 2757–2785.
(22) García, J.; Fernández, S.; Ferrero, M.; Sanghvi, Y. S.; Gotor, V. *J. Org. Chem.* **2002**, *67*, 4513–4519.
(23) Eulberg, D.; Jarosch, F.; Vonhoff, S.; Klussmann, S. Spiegelmers for Therapeutic Applications. In *The Aptamer Handbook*; Klussmann, S., Ed.; Wiley-VCH: Weinheim, 2006; Chapter 18, pp 417−439.

TABLE 3. Enzymatic Acylation of β-D-Ribonucleosides[a]

entry	substrate	enzyme	conc (M)	T (°C)	t (h)	**23** (%)[b]	**24** (%)[b,c]
1	**23a**	CAL-B	0.1	30	2		97 (80)
2	**23b**	CAL-B	0.05	45	2		>97 (83)
3	**23c**	CAL-B[d]	0.05	50	55	30	70 (56)
4	**23c**	CAL-B[d,e]	0.025	55	24		>97 (93)
5	**23a**	PSL-C	0.1	30	13		mixture of acylated compounds
6	**23b**	PSL-C	0.05	45	24	>97	
7	**23b**	PSL-C	0.025	45	27	78	mixture of acylated compounds
8	**23c**	PSL-C[d]	0.025	55	24		mixture of acylated compounds

[a]Ratio of **23**:CAL-B, 1:1 (w/w); ratio of **23**:PSL-C, 1:3 (w/w); 3 equiv of acetonoxime levulinate. [b]Percentage of compounds calculated by ^{1}H NMR. [c]Isolated yields are given in parentheses. [d]9 equiv of acetonoxime levulinate. [e]Ratio of **23**:CAL-B, 1:2 (w/w).

SCHEME 5



25 **26**

a, B= U
b, B= A^{Bz}
c, B= C^{Bz}

TABLE 4. Enzymatic Acylation of β-L-Ribonucleosides[a]

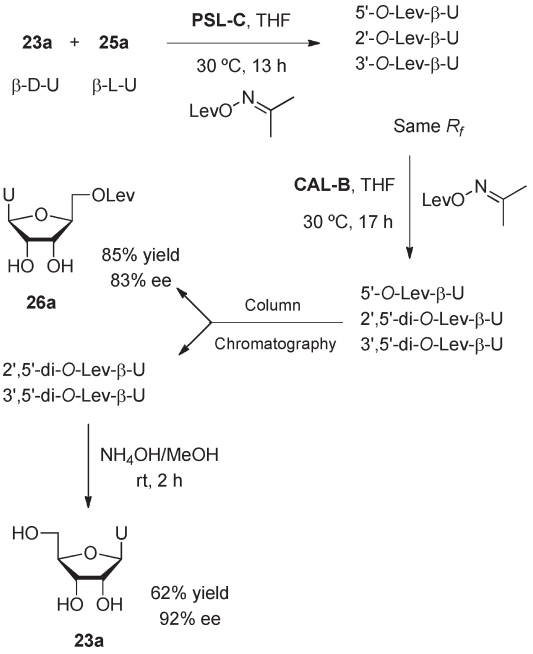
entry	substrate	enzyme	conc (M)	T (°C)	t (h)	**25** (%)[b]	**26** (%)[b,c]
1	**25a**	CAL-B	0.1	30	5.5		97 (81)
2	**25a**	PSL-C	0.1	30	10.5		>97 (87)
3	**25b**	CAL-B	0.05	45	3		>97 (80)
4	**25b**	PSL-C	0.025	45	26		>97(88)
5	**25c**	CAL-B[d,e]	0.025	55	41	18	63 (58)
6	**25c**	PSL-C[d,f]	0.025	55	46	25	75 (70)

[a]Ratio of **25**:CAL-B, 1:1 (w/w); ratio of **25**:PSL-C, 1:3 (w/w); 3 equiv of acetonoxime levulinate. [b]Percentage of compounds calculated by ^{1}H NMR. [c]Isolated yields are given in parentheses. [d]9 equiv of acetonoxime levulinate. [e]Ratio of **25**:CAL-B, 1:2 (w/w). [f]Ratio of **25**:PSL-C, 1:6 (w/w).

observed with PSL-C, whereas the secondary hydroxyl groups of the D-isomer were acylated in a nonselective manner with PSL-C. In order to accomplish the separation of these products, we elected to carry out a second acylation step catalyzed by CAL-B. Since this lipase is regioselective toward the 5′-hydroxyl group of the D-nucleosides, 2′- and 3′-*O*-acyl derivatives were transformed into 2′,5′- and 3′,5′-di-*O*-levulinyl nucleosides, while the 5′-*O*-levulinyl compound remained unreacted. The diacylated products and 5′-*O*-Lev-β-L-U are easily separated by column chromatography. Subsequently, the diacyl derivatives were treated with aqueous ammonium in MeOH to give the corresponding nucleoside β-D-U (**23a**), which was obtained with a satisfactory 92% ee, determined by transformation into 5′-*O*-Lev-β-D-U. The chiral HPLC analysis of 5′-*O*-Lev-β-L-U revealed 83% ee during acylation with PSL-C.

Molecular Modeling. The regio- and stereoselectivity obtained during enzymatic resolution of racemic mixtures of 2′-deoxy-β-D/L-nucleosides inspired us to undertake a modeling study of these processes to explain the observed results.

SCHEME 6



Thus, we obtained the X-ray structure of PSL[24] from the Protein Data Bank.[25] The X-ray crystal structure of the open form of PSL (3LIP) shows an active site with three subsites that bind to the substrate (Figure 1A).[24] Viewing the catalytic triad Asp264−His286−Ser87 from left to right, the three subsites are (a) the large hydrophobic pocket, where the acyl chain binds; (b) the medium-sized pocket, where the nucleophile is placed; and (c) the alternate hydrophobic pocket to the right of the medium pocket, which can also bind parts of the nucleophile. This alternate hydrophobic pocket lies below the catalytic triad in a narrow region.[26] Most other lipases, including CAL-B, lack this alternate hydrophobic pocket.[27] During the PSL-catalyzed acylation of N^4-benzoyl-2′-deoxycytidine, the nucleoside acts as the nucleophile and thus binds in the medium-sized pocket. Since N^4-benzoyl-2′-deoxycytidine is much larger than this pocket, it might extend into other pockets or into the solvent.

(24) Schrag, J. D.; Li, Y.; Cygler, M.; Lang, D.; Burgdorf, T.; Hecht, H. J.; Schmid, R.; Schomburg, D.; Rydel, T. J.; Oliver, J. D.; Strickland, L. C.; Dunaway, C. M.; Larson, S. B.; Day, J.; McPherson, A. *Structure* **1997**, *5*, 187–202.

(25) http://www.rcsb.org/pdb/explore/explore.do?structureId = 3LIP.

(26) Lavandera, I.; Fernández, S.; Magdalena, J.; Ferrero, M.; Grewal, H.; Savile, C. K.; Kazlauskas, R. J.; Gotor, V. *ChemBioChem* **2006**, *7*, 693–698.

(27) Lavandera, I.; Fernández, S.; Magdalena, J.; Ferrero, M.; Kazlauskas, R. J.; Gotor, V. *ChemBioChem* **2005**, *6*, 1381–1390.

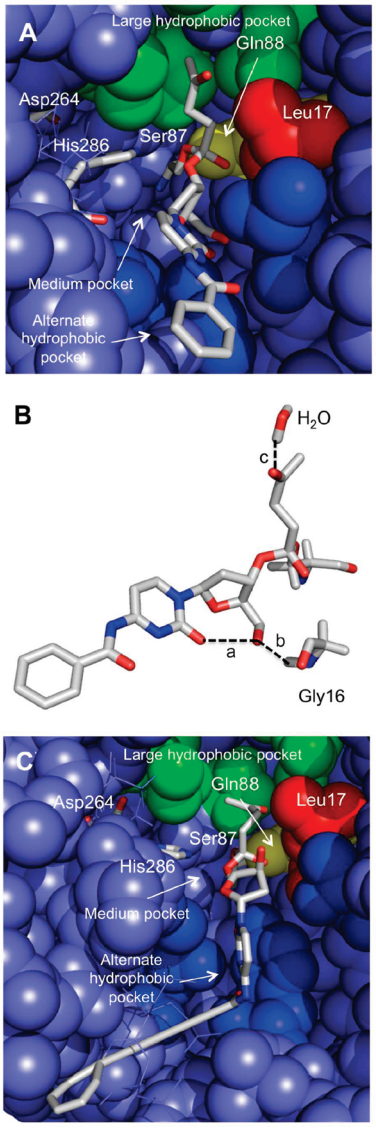
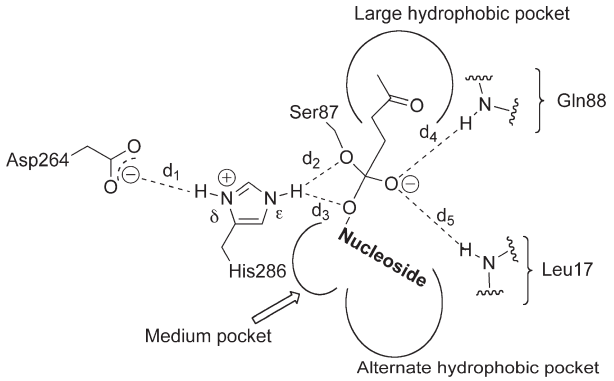


FIGURE 1. Best models of intermediates for PSL-catalyzed levulination of 2′-deoxy-β-D-cytidine. Residues surrounding the large hydrophobic pocket (Val266, Val267, Leu167, Phe119, and Pro113) are colored green, and residues surrounding the alternate hydrophobic pocket (Ile290, Leu287, Thr18, and Tyr29) are colored dark blue. The oxyanion hole residues Leu17 and Gln88 are colored red and yellow, respectively. (A) Conformation of the intermediate of levulination corresponding to the 3′-hydroxyl with the β-D-enantiomer. (B) When placed in this alternate pocket, three potential hydrogen-bond stabilizations appear (see text). (C) Conformation that mimics levulination of the 5′-hydroxyl with the β-D-enantiomer. To allow a better view of the active site, panel A displays Ala247 and Leu248 in a line representation, and panel C displays Ala247, Leu248, Gln292, Leu293, and Leu294 in a line representation.

To qualitatively explain the unusual regioselectivity of PSL-catalyzed acylations on each enantiomer of the racemic mixtures of thymidine,[19a] N^4-benzoyl-2′-deoxycytidine, and N^6-benzoyl-2′-deoxyadenosine, we focused on N^4-benzoyl-2′-deoxycytidine as a representative example of this study. Thus, we modeled the key intermediates for levulination of N^4-benzoylated 2′-deoxycytidine in both the 3′- and 5′-positions of each enantiomer, namely, β-D and β-L. This approach focused on how the substrate fits in the active site

SCHEME 7. Tetrahedral Intermediate for 3′- or 5′-Levulination of β-D- or β-L-Nucleosides[a]



[a]The five catalytically essential hydrogen bonds are as follows: one from the carboxylate of Asp264 to N_δ of His286 (bond d_1), two from N_ε of His286 to the oxygens of Ser87 (bond d_2) and the oxygen of the corresponding nucleoside (bond d_3), and two from the oxy anion to the main chain amides of Gln88 (bond d_4) and Leu17 (bond d_5). For computer modeling the different groups are introduced in a stepwise fashion as described in the text, forming both 3′- and 5′-regioisomers of each β-D- or β-L-enantiomer.

of the enzyme, but might omit subtle details about the transition state.

We started with a simplified intermediate that mimicked the tetrahedral intermediate for levulination of ethanol. Geometry optimization yielded a structure containing all five catalytically essential hydrogen bonds (d_1 to d_5 in Scheme 7). To model the nucleoside substrates, we replaced the ethoxy moiety with the 2′-deoxyribosyl group linked to the tetrahedral intermediate at either the 5′- or 3′-position with each of the nucleoside enantiomers (D or L) and then added the cytosine ring followed by the benzoyl protecting group. Since the structures contained only two (3′-acylation) or three (5′-acylation) rotatable bonds, a systematic search identified the catalytically productive conformations (see Supporting Information). The shape of the nucleophile-binding region below the catalytic triad (medium pocket and alternate hydrophobic pocket) restricted the nucleoside orientations, especially the cytosine-ring orientation, so that only a few conformations (a) contained all five catalytically essential hydrogen bonds (d_1-d_5)[28] shown in Scheme 7, (b) avoided steric hindrances between the nucleoside intermediate and the lipase, and (c) avoided internal steric clashes within the nucleoside intermediate. Acylation at the 5′-position allowed rotation of the three bonds in the nucleoside to adjust its position, while acylation at the 3′-position allowed only two bonds to adjust. For this reason, modeling the 5′-levulination yielded a larger number of productive conformations than the 3′-levulination.

When geometry optimization was carried out at 3′-hydroxyl of N^4-benzoyl-2′-deoxy-β-D-cytidine (Figure 1A), the tetrahedral intermediate for the levulination placed the cytosine base and its benzoyl protecting group in the alternate hydrophobic pocket and the 2′-deoxyribose moiety into the medium-sized pocket. Both moieties avoid steric hindrances in their respective pockets. Importantly, in addition to the five catalytically

(28) Hydrogen bonds must have a donor atom to acceptor atom distance of less than 3.30 Å and a donor atom−hydrogen−acceptor atom angle of 120° or greater.

key hydrogen bonds (d_1 = 3.04; d_2 = 3.03; d_3 = 2.84; d_4 = 3.17; d_5 = 3.23 Å), this intermediate formed three extra hydrogen bonds: one between the C2 carbonyl group of cytosine and the 5′-OH of the sugar (bond a, 2.95 Å, 171°, Figure 1B),[28] another between the later and Gly16 (bond b, 2.84 Å, 146°), and the third additional hydrogen bond (bond c, 2.71 Å, 171°) between the oxygen carbonyl group of the levulinyl substituent and a water molecule placed inside the large hydrophobic pocket. We propose that these stabilizing hydrogen bonds cause PSL to favor acylation at the 3′-hydroxyl group in the β-D-enantiomer of N^4-benzoyl-2′-deoxycytidine. However, when the tetrahedral intermediate for the levulination of 5′-hydroxyl of 2′-deoxy-β-D-cytidine was built and minimized, no single productive conformation was obtained. In all structures some of the key H-bond interactions were lost. In the best fit (Figure 1C; d_1 = 2.83; d_2 = 3.03; d_3 = 2.71; d_4 = 3.62; d_5 = 3.84 Å), the enzyme situates the cytosine ring in the alternate hydrophobic pocket but in such a way that the protecting benzoyl group cannot fit inside the active site of the enzyme. In another option the benzene ring is placed outside the active side, forming an improbable bond of 9.06 Å between the carbonylic carbon and the aromatic ring. The binding of the base and position of the protecting group appear to be the key factors in supporting the enzyme regioselectivity.

The geometry optimization of both regioisomers of N^4-benzoyl-2′-deoxy-β-L-cytidine, which come from levulination of the 3′- or 5′-hydroxyl group, yielded structures that are shown in Figure 2. The best model of the tetrahedral intermediate for the 3′-acylation (Figure 2A) shows the sugar in the medium-sized pocket and the cytosine and its protecting group pointing out to the solvent. Three of the five key hydrogen bonds were present while avoiding inter- and intramolecular steric interactions (d_1 = 3.10; d_2 = 4.07; d_3 = 3.15; d_4 = 3.57; d_5 = 3.10 Å). On the contrary, in the tetrahedral intermediate for the levulination of 5′-hydroxyl of N^4-benzoyl-2′-deoxy-β-L-cytidine (Figure 2B), the sugar was placed in the medium-sized pocket and the cytosine base, including its benzoyl protecting group, was inside the alternate hydrophobic pocket.

It is noteworthy that in addition to the five catalytically key H-bonds (d_1 = 2.86; d_2 = 2.86; d_3 = 2.82; d_4 = 3.30; d_5 = 3.15 Å), this intermediate formed four hydrogen bonds: between the 3′-hydroxyl group of the sugar and the 5′-*O* that is directly bound to the quaternary carbon of the tetrahedral intermediate (bond a, 3.29 Å, 124°, Figure 1C), from the water molecule placed inside the alternate hydrophobic pocket to the N-3 (bond b, 3.11 Å, 140°) and N-4 (bond c, 3.17 Å, 134°) of the cytosine base, respectively, and from that water molecule to the backbone hydroxyl group of Thr18 (bond d, 3.10 Å, 155°). As in the previous case for levulination of N^4-benzoyl-2′-deoxy-β-D-cytidine, the binding base and protecting group rings in 2′-deoxy-β-L-cytidine appear to be the key factors supporting the enzyme regioselectivity.

The overall result shows that when a racemic mixture of 2′-deoxy-β-D/L-cytidine reacts with PSL in the presence of acetonoxime levulinate, only the 3′-hydroxyl group of 2′-deoxy-β-D-cytidine and the 5′-hydroxyl group of 2′-deoxy-β-L-cytidine are selectively acylated. Thus, molecular modeling corroborates the experimental results.

In summary, we have described a parallel kinetic resolution approach to obtain chiral nonracemic β-D- and β-L-nucleosides. *Pseudomonas cepacia* lipase was found to be a suitable biocatalyst for the resolution of 2′-deoxy-β-D/L-nucleoside racemic mixtures via an acylation reaction. This lipase showed different selectivity with both isomers and furnished a mixture of easily separable compounds. *Pseudomonas cepacia* lipase also exhibited total selectivity toward the acylation of the 5′-hydroxyl group of β-L-uridine, whereas a mixture of acylated derivatives was obtained during bioacylation of β-D-uridine. Thus, a double sequential parallel kinetic resolution catalyzed by *Pseudomonas cepacia* lipase and *Candida antarctica* lipase B has been developed to separate the racemic mixture β-D/L-uridine. The methodology described here has proven to be very useful for the isolation of β-L-nucleosides, which are increasingly used especially as antiviral and anticancer therapeutic agents.

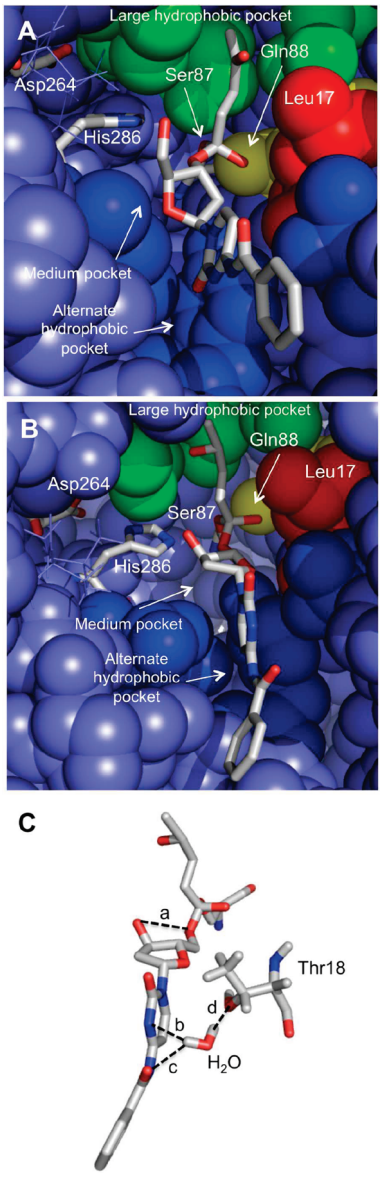


FIGURE 2. Best models of intermediates for PSL-catalyzed levulination of N^4-benzoyl-2′-deoxy-β-L-cytidine. The color code is the same as that used in Figure 1. (A) Conformation of the intermediate of levulination corresponding to the 3′-hydroxyl with the β-L-enantiomer. (B) Conformation that mimics the levulination of the 5′-hydroxyl with the β-L-enantiomer. (C) When placed in this alternate pocket, the intermediate is stabilized since four extra hydrogen bonds are formed (see text). To allow a better view of the active site, panels A and B display Ala247 and Leu248 in a line representation.

Results obtained from a molecular modeling study on the PSL-C-catalyzed levulination of mixtures of 2′-deoxy-β-D/L-nucleosides are in agreement with the observed experimental data. Thus, PSL-C showed that only the 3′-hydroxyl group of 2′-deoxy-β-D-cytidine and 5′-hydroxyl group of 2′-deoxy-β-L-cytidine selectively are acylated into the corresponding levulinate derivatives, allowing convenient and practical separation of the original racemic mixture. This study further reinforces the utilization of biocatalysis as an important tool for organic chemists working on the manufacturing of nucleoside-based therapeutic products.[29]

Experimental Section

General Comments. Enzyme Activities. *Candida antarctica* lipase B (CAL-B, Novozym 435, 7300 PLU/g) was a gift from Novo Nordisk Co., and *Pseudomonas cepacia* lipase (PSL-C, 1387 U/g) was purchased from Amano Phamaceuticals. The ee values of **16a** and **17a** were determined by HPLC using a Chiralcel OD column (250 × 46 mm), 0.8 mL/min, 20 °C, and hexane/isopropyl alcohol (1:1) as eluent. The ee values of **16b** and **17b** were determined using a Chiralcel OJ-H column (250 × 46 mm), 0.75 mL/min, 35 °C, and hexane/isopropyl alcohol (65:35) as eluent. The ee values of **24a** and **26a** were determined by HPLC using a Chiralcel OD column (250 × 46 mm), 0.8 mL/min, 30 °C, and hexane/isopropyl alcohol (45:55) as eluent. Optical rotations were recorded on a polarimeter, and values are reported as follows: $[\alpha]^{T}_{\lambda}$ (*c*: g/100 mL, solvent). Derivatives **16a**,[19b] **16b**,[19b] **17a**,[19a] **17b**,[19a] **18**,[22] **19**,[22] and **20**[22] have been previously described.

General Procedure for the Enzymatic Acylation of Nucleosides. In a standard procedure, THF was added to an Erlenmeyer flask that contained nucleoside derivative (0.2 mmol), acetonoxime levulinate, and lipase under nitrogen (ratio of acylating agent, ratio of enzyme, concentration, temperature, and time are indicated in Tables 1, 3, and 4). The reactions were stirred at 250 rpm and were monitored by TLC (10% MeOH/CH_2Cl_2). The enzyme was filtered off and washed with CH_2Cl_2 and MeOH, and the solvents were distilled under vacuum. Flash chromatography (gradient eluents 2−5% MeOH/CH_2Cl_2) afforded derivatives **16**, **17**, **24**, and **26** (yields and ee are indicated in Tables 1,3, and 4).

Synthesis of N^2-Isobutyryl-3′,5′-di-*O*-levulinyl-β-L-2′-deoxyguanosine (21). To a stirred mixture of β-L-dG^{Ibu} (0.40 mmol) and Et_3N (0.16 mL, 1.0 mmol) in 1,4-dioxane (2.7 mL) under nitrogen were added levulinic acid (0.21 mL, 2.08 mmol), DCC (428 mg, 2.08 mmol), and DMAP (3.9 mg, 0.03 mmol). The reaction was stirred at rt during 2 h. The insoluble material was collected by filtration, and the filtrate was evaporated under vacuum. The residue was subjected to flash chromatography with 2% MeOH/CH_2Cl_2 to afford **21** in 93% yield. The spectroscopic data of **21** are in full accordance with those described for its enantiomer.[22]

Enzymatic Hydrolysis of N^2-Isobutyryl-3′,5′-di-*O*-levulinyl-β-L-2′-deoxyguanosine (21). Synthesis of **22**. To a solution of **21** (0.1 mmol) in 1,4-dioxane (0.35 mL) was added 0.15 M phosphate buffer pH 7 (1.65 mL) and the corresponding lipase [ratio of **21**:CAL-B was 1:1 (w/w), ratio of **21**:PSL-C was 1:3 (w/w)]. The mixture was allowed to react at 250 rpm and at 40 °C for the time indicated in Scheme 3. The enzyme was filtered off and washed with CH_2Cl_2 and MeOH. The solvents were distilled under vacuum, and the residue was subjected to flash chromatography with 2% MeOH/CH_2Cl_2 to afford **22** (acylation reaction with CAL-B) in 80% yield. The spectroscopic data of **22** are in full accordance with those described for its enantiomer.[22]

Enzymatic Resolution of an Artificial 1:1 Mixture of β-D/L-Uridine (23a and 25a). A suspension of a mixture of β-D/L-uridine (60 mg, 0.25 mmol), acetonoxime levulinate (131 mg, 0.76 mmol), and PSL-C (180 mg) in anhydrous THF (2.5 mL) under N_2 atmosphere was stirred for 13 h at 250 rpm and 30 °C. Once the reaction was finished, the enzyme was filtered off and washed with CH_2Cl_2 and MeOH, and the solvents were distilled under vacuum. Flash chromatography of the residue (2−4% MeOH/CH_2Cl_2) afforded 2′-, 3′-, and 5′-levulinyl derivatives (same R_f). Subsequently, an Erlenmeyer flask containing this fraction, acetonoxime levulinate (113 mg, 0.66 mmol), CAL-B (75 mg), and anhydrous THF (2.2 mL) was stirred at 250 rpm and 30 °C for 17 h. The enzyme was filtered off, washed and solvents, and evaporated. The crude reaction was subjected to flash chromatography (2−4% MeOH/CH_2Cl_2). The first fraction contained diacyl derivatives, while 5′-*O*-levulinyl-β-D-uridine (**26a**) was obtained from the second fraction in 85% yield and 83% ee. Hydrolysis of the diacyl derivatives in a 1:1 mixture of aqueous ammonia and MeOH (3.2 mL) led after 2 h of reaction to β-D-uridine (**23a**) in 62% yield and 92% ee.

5′-*O*-Levulinyl-β-D-uridine (24a) or 5′-*O*-Levulinyl-β-L-uridine (26a). White solid; R_f (10% MeOH/CH_2Cl_2) 0.32; mp 60−62 °C; IR (KBr) ν 3172, 2920, 2850, 1650, 1460 cm^{-1}; **24a** $[\alpha]^{20}_{D} = +14$ (*c* 1, MeOH); **26a** $[\alpha]^{20}_{D} = -13$ (*c* 1, MeOH); ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ 2.11 (s, 3H, CH_3-Lev), 2.50 (t, 2H, CH_2-Lev, $^3J_{HH}$ 6.2 Hz), 2.70 (t, 2H, CH_2-Lev, $^3J_{HH}$ 6.0 Hz), 3.91−4.08 (m, 3H, $H_{2'}$+$H_{3'}$+$H_{4'}$), 4.14 (dd, 1H, $H_{5'}$, $^3J_{HH}$ 4.9 Hz, $^3J_{HH}$ 11.9 Hz), 4.26 (dd, 1H, $H_{5'}$, $^3J_{HH}$ 2.8 Hz, $^3J_{HH}$ 11.9 Hz), 5.28 (d, 1H, OH, $^3J_{HH}$ 4.5 Hz), 5.47 (d, 1H, OH, $^3J_{HH}$ 5.3 Hz), 5.67 (d, 1H, H_5, $^3J_{HH}$ 8.1 Hz), 5.74 (d, 1H, $H_{1'}$, $^3J_{HH}$ 4.9 Hz), 7.65 (d, 1H, H_6, $^3J_{HH}$ 8.1 Hz); ^{13}C NMR (DMSO-d_6, 75.5 MHz) δ 27.5 (CH_2-Lev), 29.5 (CH_3-Lev), 37.4 (CH_2-Lev), 63.7 ($C_{5'}$), 69.7, 72.8 ($C_{2'}$+$C_{3'}$), 81.2 ($C_{4'}$), 82.7 ($C_{1'}$), 102.0 (C_5), 140.7(C_6), 150.6 (C_2), 162.9 (C_4), 172.2 (C=O), 206.9 (C=O); MS (APCI$^+$, *m*/*z*) 343 [(M + H)$^+$, 90%], 366 [(M + Na)$^+$, 35]; HMRS (ESI$^+$) calcd for $C_{14}H_{19}N_2O_8$ [M + H]$^+$ 343.1141, found 343.1139. Anal. Calcd for $C_{14}H_{18}N_2O_8$: C, 49.12; H, 5.30; N, 8.18. Found: C, 49.1; H, 5.4; N, 8.0.

N^6-Benzoyl-5′-*O*-levulinyl-β-D-adenosine (24b) or N^6-Benzoyl-5′-*O*-levulinyl-β-L-adenosine (26b). White solid; R_f (10% MeOH/CH_2Cl_2) 0.38; mp 73−75 °C; IR (KBr) ν 3060, 2930, 1737, 1711 cm^{-1}; **24b** $[\alpha]^{20}_{D} = -15$ (*c* 2, MeOH); **26b** $[\alpha]^{20}_{D} = +17$ (*c* 2, MeOH); ^{1}H NMR (DMSO-d_6, 600.15 MHz) δ 2.09 (s, 3H, CH_3-Lev), 2.49 (t, 2H, CH_2-Lev, $^3J_{HH}$ 6.0 Hz), 2.71 (t, 2H, CH_2-Lev, $^3J_{HH}$ 6.4 Hz), 4.14 (c, 1H, $H_{4'}$, $^3J_{HH}$ 4.6 Hz), 4.22 (dd, 1H, $H_{5'}$, $^3J_{HH}$ 5.9 Hz, $^3J_{HH}$ 11.9 Hz), 4.28 (c, 1H, $H_{3'}$, $^3J_{HH}$ 4.9 Hz), 4.36 (dd, 1H, $H_{5'}$, $^3J_{HH}$ 3.6 Hz, $^3J_{HH}$ 11.9 Hz), 4.75 (c, 1H, $H_{2'}$, $^3J_{HH}$ 5.2 Hz), 5.44 (d, 1H, OH, $^3J_{HH}$ 5.4 Hz), 5.67 (d, 1H, OH, $^3J_{HH}$ 5.8 Hz), 6.14 (d, 1H, $H_{1'}$, $^3J_{HH}$ 5.2 Hz), 7.56 (t, 2H, H_m, $^3J_{HH}$ 7.7 Hz), 7.66 (t, 1H, H_p, $^3J_{HH}$ 7.4 Hz), 8.05 (d, 2H, Ho, $^3J_{HH}$ 7.5 Hz), 8.57 (s, 1H, H_8), 8.71 (s, 1H, H_2); ^{13}C NMR (DMSO-d_6, 90.14 MHz) 28.0 (CH_2-Lev), 30.0 (CH_3-Lev), 37.8 (CH_2-Lev), 64.4 (C5′), 70.7, 73.4 ($C_{2'}$+$C_{3'}$), 82.3 ($C_{4'}$), 88.3 ($C_{1'}$), 125.3 (C_5), 126.4 (C_o), 128.9 (C_m), 132.9 (C_P), 133.8 (C_i), 143.7 (C_8), 150.9−152.7 (C_2+C_6+C_4), 166.1 (Ph−C=O), 172.7 (C=O), 207.3 (C=O); MS (APCI$^+$, *m*/*z*) 469 [(M + H)$^+$ 75%]; HMRS (ESI$^+$) calcd for $C_{22}H_{24}N_5O_7$ [M + H]$^+$ 470.1670, found 470.1677. Anal. Calcd for $C_{22}H_{23}N_5O_7$: C, 56.29; H, 4.94; N, 14.92. Found: C, 56.2; H, 5.0; N, 14.8.

N^4-Benzoyl-5′-*O*-levulinyl-β-D-cytidine (24c) or N^4-Benzoyl-5′-*O*-levulinyl-β-L-cytidine (26c). White solid; R_f (10% MeOH/CH_2Cl_2) 0.47; mp 193−195 °C; IR (KBr) ν 3493, 3323, 3162, 1728, 1703, 1651 cm^{-1}; **24c** $[\alpha]^{20}_{D} = +35$ (*c* 1, DMSO); **26c** $[\alpha]^{20}_{D} = -36$ (*c* 1, DMSO); ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ 2.12 (s, 3H, CH_3-Lev), 2.54 (t, 2H, CH_2-Lev, $^3J_{HH}$ 6.3 Hz), 2.75 (t, 2H, CH_2-Lev, $^3J_{HH}$ 6.2.3 Hz), 3.97 (m, 1H, $H_{4'}$), 4.09 (m, 2H,

(29) Pollard, D.; Woodley, J. M. *Trends Biotechnol.* **2007**, *25*, 66–73.

$H_{2'}+H_{3'}$), 4.25 (dd, 1H, $H_{5'}$, $^3J_{HH}$ 5.1 Hz, $^3J_{HH}$ 12.2 Hz), 4.35 (dd, 1H, $H_{5'}$, $^3J_{HH}$ 2.3 Hz, $^3J_{HH}$ 12.1 Hz), 5.82 (d, 1H, $H_{1'}$, $^3J_{HH}$ 2.7 Hz), 7.38 (d, 1H, H_5, $^3J_{HH}$ 7.5 Hz), 7.51 (t, 2H, H_m, $^3J_{HH}$ 7.5 Hz), 7.63 (t, 1H, H_p, $^3J_{HH}$ 7.3 Hz), 8.01 (d, 2H, H_o, $^3J_{HH}$ 7.4 Hz), 8.16 (d, 1H, H_6, $^3J_{HH}$ 7.5 Hz); ^{13}C NMR (DMSO-d_6, 75.5 MHz) 28.0 (CH_2-Lev), 30.0 (CH_3-Lev), 37.9 (CH_2-Lev), 63.9 ($C_{5'}$), 69.7 ($C_{4'}$), 74.3 ($C_{2'}$), 81.3 ($C_{3'}$), 91.4 ($C_{1'}$), 96.9 (C_5), 128.9 (C_m+C_o), 133.2, 133.6 (C_p+C_i), 145.5 (C_6), 154.9 (C_2), 163.5 (C_4), 168.0 (Ph−C═O), 172.7 (C═O), 207.4 (C═O); MS (ESI$^+$, *m*/*z*) 468.1 [(M + Na)$^+$, 100%] and 446.1 [(M + H]$^+$, 35]; HMRS (ESI$^+$) calcd for $C_{21}H_{24}N_3O_8$ [M + H]$^+$ 446.1563, found 446.1564. Anal. Calcd for $C_{21}H_{23}N_3O_8$: C, 56.50; H, 5.42; N, 9.41. Found: C, 56.3; H, 5.2; N, 9.3.

Molecular Modeling. All minimizations were performed with an iMac by using the molecular modeling package Molecular Operating Environment (MOE) 2009.10 (Chemical Computing Group, Inc.).[30] The Amber99 force field[31] and the corresponding dictionary charges were used as implemented in MOE. Due to the lack of parameters for THF compatible with the force field used, we opted for a continuum solvation method, an approach that has already afforded good results in similar studies.[32] In particular, a distance-dependent relative dielectric constant of 2 was selected as a good compromise between the dielectric constant of THF[33] and that of a globular protein matrix.[34] The convergence criterion of the energy minimization was set to a rms gradient value of 0.000 01 kcal mol^{-1}. High-quality pictures of representative structures in Figures 1 and 2 were created by using PyMOL 0.99.[35]

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Supporting Information Available: ^{1}H, ^{13}C, and DEPT NMR spectral data and 2D NMR experiments, in addition to HPLC analysis. Molecular modeling information. This material is available free of charge via the Internet at http://pubs.acs.org.

(30) *Molecular Operating Environment* (*MOE*) *2009.10*; Chemical Computing Group, Inc.: Montréal, Québec, Canada; http://www.chemcomp.com.

(31) Wang, J.; Cieplak, P.; Kollman, P. A. *J. Comput. Chem.* **2000**, *21*, 1049–1074.

(32) (a) García-Urdiales, E.; Ríos-Lombardía, N.; Mangas-Sánchez, J.; Gotor-Fernández, V.; Gotor, V. *ChemBioChem* **2009**, *10*, 1830–1838. (b) Léonard, V.; Fransson, L.; Lamare, S.; Hult, K.; Graber, M. *ChemBioChem* **2007**, *8*, 662–667. (c) Cammenberg, M.; Hult, K.; Park, S. *ChemBioChem* **2006**, *7*, 1745–1749. (d) Raza, S.; Fransson, L.; Hult, K. *Protein Sci.* **2001**, *10*, 329–338.

(33) The dielectric constant of THF at 20° C is 7.58. In Speight, J. G. *Perry's Standard Tables and Formulas for Chemical Engineers*; McGraw-Hill: New York, 2003.

(34) Laberge, M. *Biochim. Biophys. Acta* **1998**, *1386*, 305–330.

(35) DeLano, W. L. *The PyMOL Molecular Graphics System*; DeLano Scientific LLC: San Carlos, CA; http://www.pymol.org.