April 17, 2015

Nucleosides and Nucleotides:
Synthetic and Biological Chemistry

Application of Green Technologies for Nucleic Acid Transformations

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Rasayan Inc.

Encinitas, CA, USA

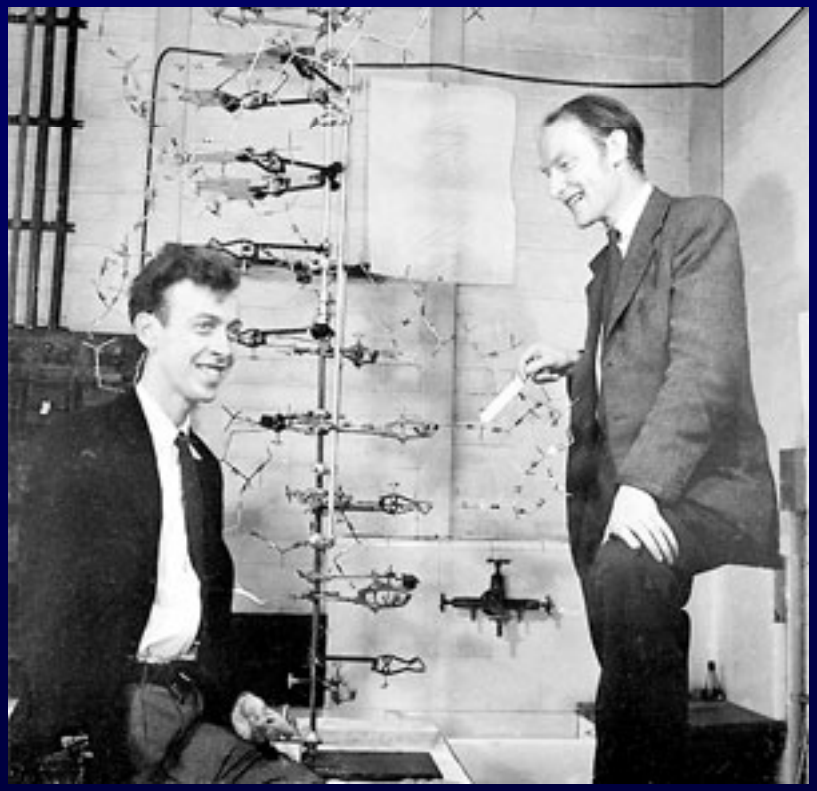
Celebrating >60 Years of the DNA Double Helix

original paper

April 25, 1953 NATURE

MOLECULAR STRUCTURE OF NUCLEIC ACIDS

A Structure for Deoxyribose Nucleic Acid



The discoverers of the DNA structure, James Watson, left, and Francis Crick, with their model of a DNA molecule. (A. Barrington Brown/Photo Researchers, Inc.)

James D. Watson and Francis H. Crick April 25, 1953 (2), *Nature* **(3),** 171, 737-738**.**

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Green Chemistry in Nucleosides and Oligonucleotide-Based Therapeutics

- Among oligonucleotides, Vitravene™, Macugen™ and Kynamro™ are the three FDA approved products on the market and >100 others are in various stages of human clinical trials.
- Among nucleoside analogs, >20 drugs have been approved by FDA and >25 are in various stages of human clinical trials.
- Successful commercial launch of therapeutic nucleosides and oligonucleotides may result in multi-ton scale demand for such molecules. As a result, very large amounts of various raw materials will be required posing serious challenges in the process development of nucleic acids chemistry.
- In 90s the EPA coined the phrase Green Chemistry "To promote innovative chemical technologies that reduce or eliminate the use or generation of hazardous substances in the design, manufacture and use of chemical products."
- An overview of various green processes and biocatalysis for nucleosides and oligonucleotide synthesis is presented and understanding the myriad effects on the environmental chemistry – the natural world in which we live.

Sanghvi *et al*. Applications of green chemistry in the manufacture of oligonucleotide drugs. *Pure Appl. Chem*. 2001, *73*, 175.

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Twelve Principles of Green Chemistry for Chemists

1. Waste prevention instead of remediation
2. Atom efficiency and atom economy (*E*-factor)
3. Less hazardous or toxic chemicals
4. Safe products by design
5. Innocuous solvents and auxiliaries
6. Energy efficient by design
7. Preferably renewable raw materials
8. Shorter synthesis – avoiding derivatization
9. Catalysis rather than stoichiometric reagents
10. Designing products for possible degradation/recycling
11. Analytical methodologies for pollution prevention
12. Inherently safer processes

Sustainable Process Chemistry:
Sanghvi *et al*. in *Org. Process Res. Dev.*, 2011, *15*, 898.

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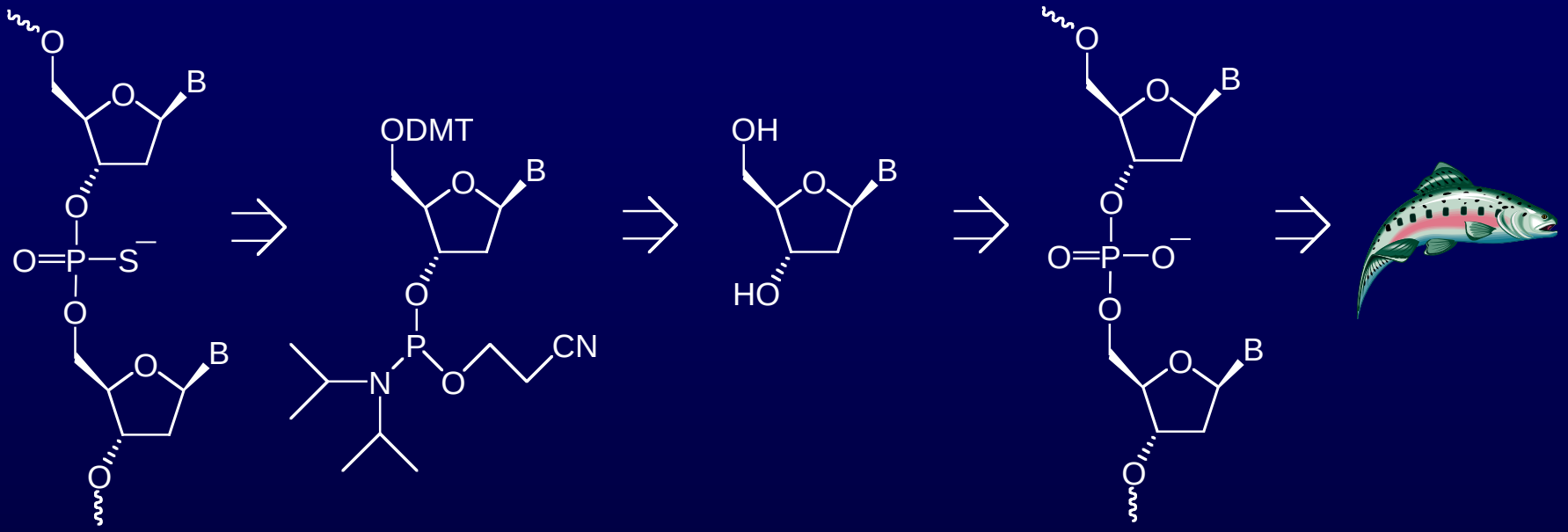
Natural Raw Material Pipeline for Oligo-Based Drugs



Oligos **Amidites** **dN's** **DNA Salt** **Fish**

1 Kg API **1.8 Tons**

Natural DNA ⇨ **Synthetic DNA**

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Inefficient Process for Oligonucleotide Synthesis

Process Steps	*Timelines*	*Energy*	*Solvents*
Salmon (Fish)	Nov. 2013	++++	
⇩ Salmon Milt		++	+
⇩ Cell Digest/DNA Solubilization		+	++
⇩ DNA Salt Precipitation		+	++
⇩ DNA Salt Digestion		+	++
⇩ IE Chromatography		++	+++
⇩ 2'-Deoxynucleosides	April 2014	+++	+++
⇩ Protected Nucleosides		+++	++++
⇩ Phosphoramidites		+++	++++
⇩ Oligos	April 2015	++	+++++

Unacceptable timelines, energy and solvent consumption for the development of therapeutic drugs!

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Limitations of the Fish-based 2′-Deoxy Nucleoside Pipeline

- **Very lengthy process with many steps (~1.5 years PT)**
- **Unfavorable *E*-factor: very inefficient process (Scale-up?)**
- **Depletion of natural resources: Save salmon fish?**
- **Produces all four nucleosides in equal volume (Can't use)**
- **Bulk price has hit the floor ~$1,000/Kg**
- **Small-scale production <1 metric ton/year**

Cost-effective Green alternatives?

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Green Synthesis of 2'-Deoxynucleosides

D-Glucose

2-Deoxy-D-ribose

5-Me dC

dC

α-Cl sugar

T

dG

(i) Glycosylation
(ii) Deamination

Glycosylation

dA

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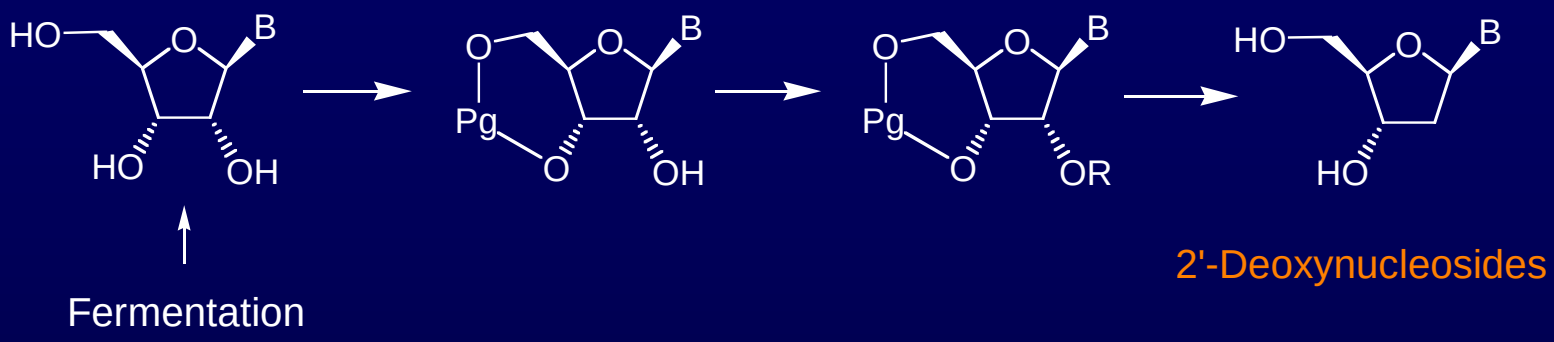
Green Alternatives For β-2'-deoxynucleosides

Chemical Deoxygenenation Method



Advantages of the protocol:

- **Recoverable and recyclable 3′ → 5′ protecting group: MDPSCl**
- **Use of significantly cheaper xanthate leaving group**
- **Replacement of explosive AIBN with safe activator**
- **Use of polymeric silane instead of toxic tin reagent**
- **Fermentation based RNA nucleosides as raw materials**

Wen, K.; Chow, S.; Sanghvi, Y. S. Theodorakis, E. A. *J. Org. Chem.* 2002, *67*, 7887.

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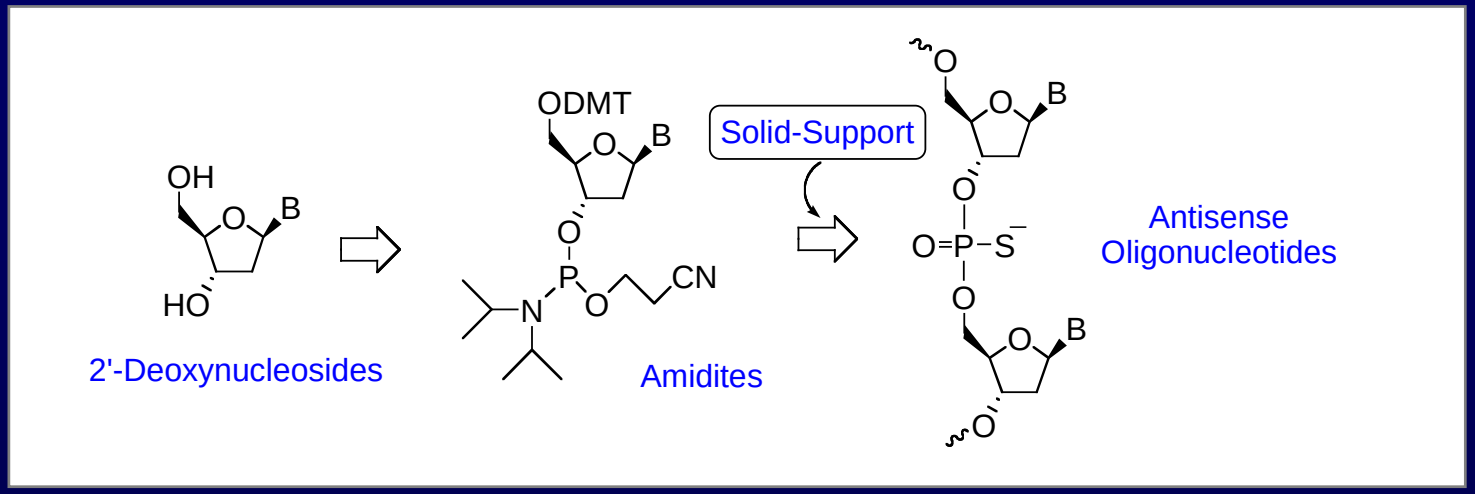
Solid-Phase Phosphoramidite Approach

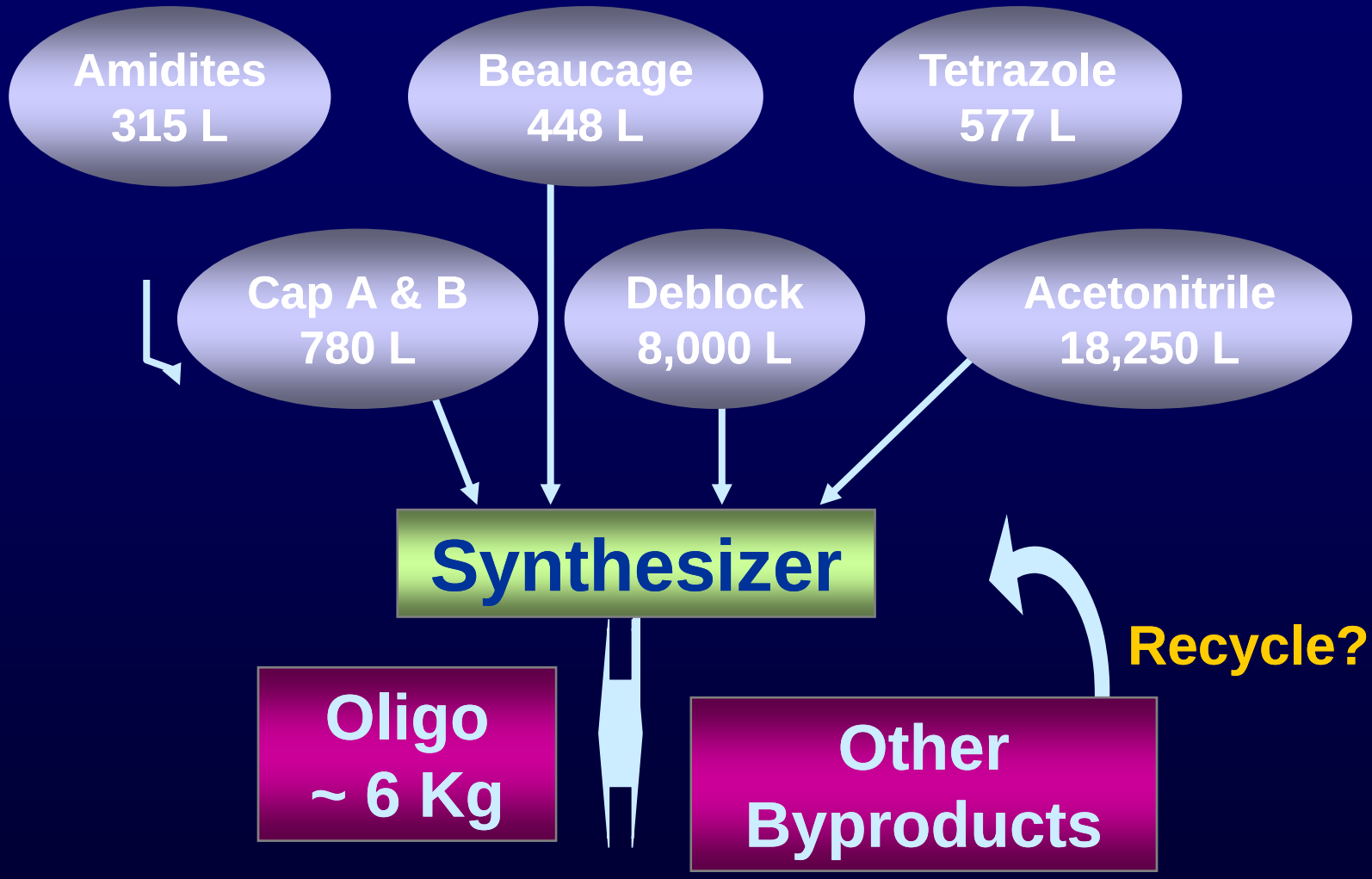


Limitations of the current protocol:

- **High cost of amidites and solid-support: ~80% of the raw material cost**
- **Significantly large excess of solvents and reagents are used and wasted**
- **Upper limit of production per campaign is under 3Kg/cycle**
- **Presence of n-1 mer as a key impurity in the product**

How do we plan to circumvent above limitations?

E-Factor in Oligonucleotide Manufacture



A lot of these reagents and solvents could be reused or recycled!

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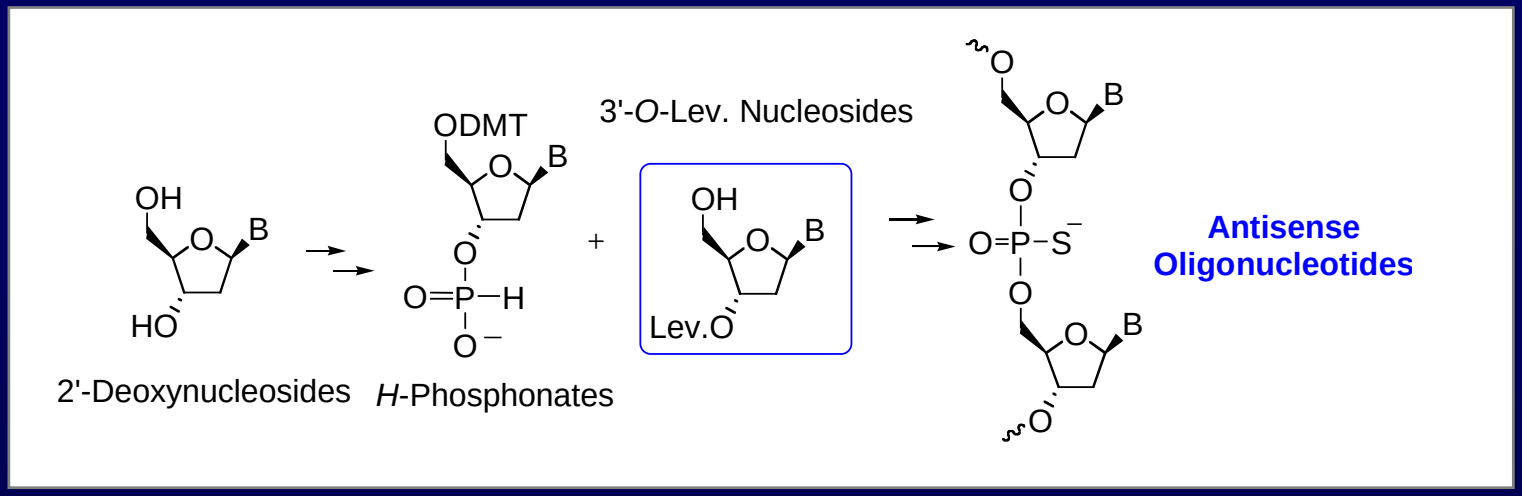
Solution-Phase *H*-Phosphonate Method



Advantages:

- **Very stable raw materials, such as *H*-phosphonate (P^{V}) *vs*. amidites (P^{III})**
- **Coupling efficiencies are excellent: Does not require large excess of reagents**
- **Use of expensive solid-support is completely avoided**
- **Blockmer approach results in product devoid of n-1 mer (Perkin 1, 1999, 1477)**

For the success of solution-phase approach, large-scale production of protected nucleoside is essential!

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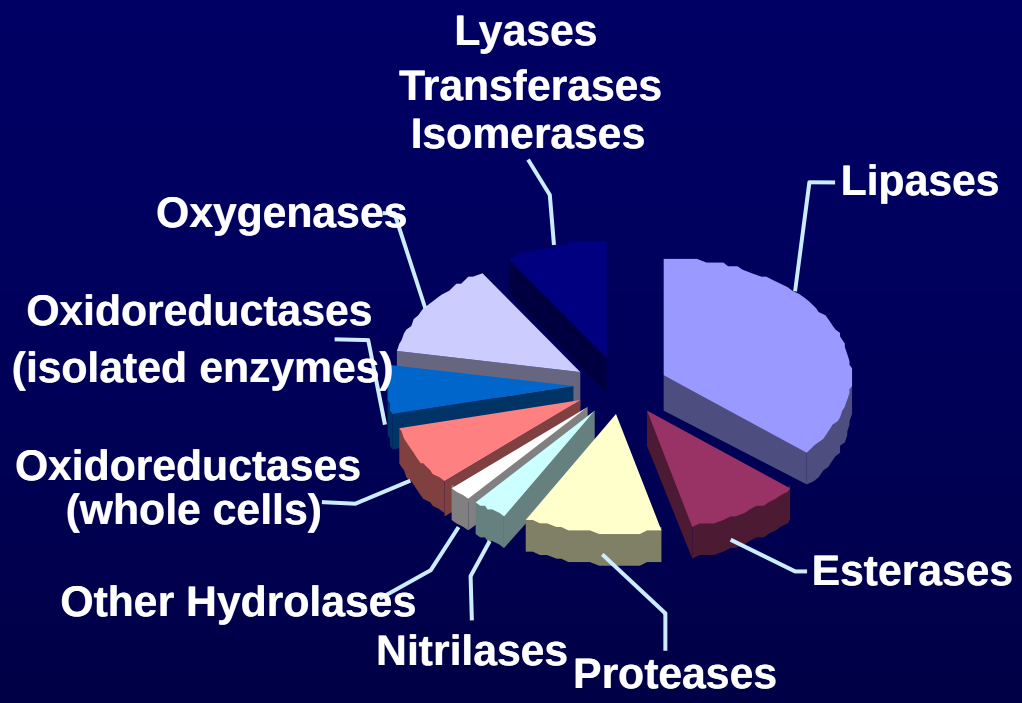
Part I
Chemo-, Regio- and Stereoselective Syntheses of Protected Nucleosides

Focus on Biocatalytic Acylation & Hydrolysis Reactions

- **Presence of multiple OH groups**
- **Exocyclic NH_2 groups**
- **Other reactive functional groups**
- **Anomeric center: α- and β-nucleosides**
- **Racemic mixture: D and L nucleosides**

Enzymatic Transformations Using Lipases



The large usage of lipases is due to:

- Availability from multiple commercial suppliers at **reasonable prices**
- Ease of handling and **reuse** when immobilized
- Do not need expensive cofactors
- **Stability** at high temperatures
- Retention of activity in organic solvents
- Ability to **accept diverse molecules**

Selected recent review articles:

- Carrea & Riva in *Angew. Chem. Int. Ed.,* 2000, 39, 2226
- Ferrero & Gotor in *Monatshefte fur Chemie* 2000, 131, 585
- Kaderit & Waldmann in *Chem. Rev.* 2001, 101, 3367

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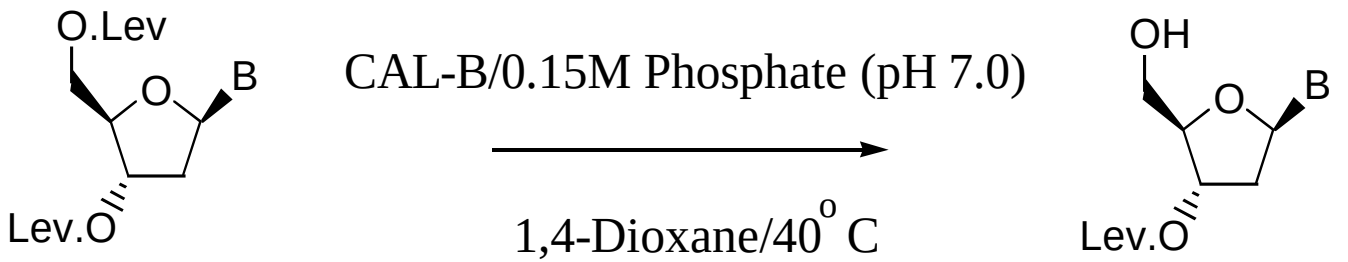
5′-Regioselective Hydrolysis of Bis-*O*-Levulinyl Protected Nucleosides Using Lipases



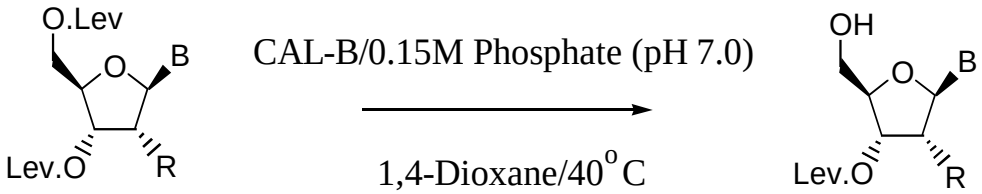
B = T (95%); C (100%); A (99%); G^{Ibu} (95%); C^{Bz} (NS); A^{Bz} (NS)

Results and Conclusions:

- ***Candida antarctica*** **lipase B (CAL-B, Novozym 435, 7300 U/g) regioselectively hydrolyzed 5′-*O*-Lev. Group**
- **The hydrolysis was complete in 18-62 hours with excellent isolated yields**
- **The hydrolysis of N-benzoyl protected A and C was non-selective (NS)**

I. Lavandera, J. Garcia, S. Fernández, M. Ferrero, V. Gotor and Y. Sanghvi
In *Current Protocols in Nucleic Acid Chemistry* 2005, 2.11.1-2.11.36.

5′-Regioselective Hydrolysis of 2′-Modified Bis-*O*-Levulinyl Protected Nucleosides Using Lipases



R = $O(CH_2)_2OCH_3$: B = T (97%); 5-Me-C (97%); 5-Me-C^{Bz} (NS); A (98%); A^{Bz} (NS)

R = $O(CH_2)_2OCH_3$: B = G^{Ibu} (98%); R = OCH_3: B = A (100%)

Results and Conclusions:

- Again, CAL-B was found to be regioselective with 2′-modified nucleosides
- Both, short OCH_3 and long $O(CH_2)_2OCH_3$ groups on 2′-position were tolerated
- Hydrolysis of small amide group in G-Ibu was not observed: Chemoselectivity?
- Presence of large *N*-benzoyl group resulted in non-selective hydrolysis in A and C
- Compared to dN's the 2′-modified Ns took longer time for hydrolysis (1-6 days)

J. Garcia, S. Fernandez, M. Ferrero, Y. Sanghvi & V. Gotor
J. Org. Chem. 2002, *67*, 4513.

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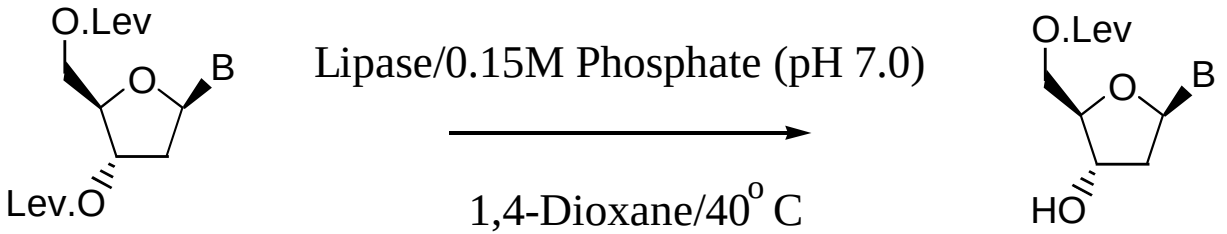
3′-Regioselective Hydrolysis of Bis-*O*-Levulinyl Protected Nucleosides Using Lipases



CAL-A: B = T (100%); C^{Bz} (97%); A^{Bz} (100%)

PSL-C: B = T (100%); G^{Ibu} (98%); **CAL-B**: B = G^{Ibu} (97%)

Results and Conclusions:

- **Hydrolysis in T was excellent with both *Candida antarctica* lipase A (CAL-A, Chirazyme L-5, 1000 U/g) and *Pseudomonas cepacia* lipase (PSL-C, Roche, 783 U/g)**
- **Interestingly, CAL-A was tolerant to the bulky benzoyl group in A and C**
- **Hydrolysis of G^{Ibu} was relatively slower (96 h) and consumed more lipase (x 3)**
- **Immobilized CAL-B was reused for hydrolysis of G^{Ibu} on large-scale without compromising the reaction rate or product yield**

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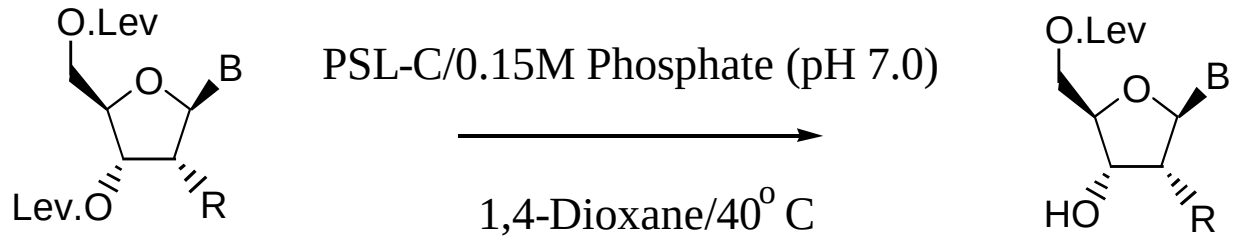
3′-Regioselective Hydrolysis of 2′-Modified Bis-*O*-Levulinyl Protected Nucleosides Using Lipases



R = $O(CH_2)_2OCH_3$: B = T (NS*); 5-Me-C (NS); 5-Me-C^{Bz} (99%); A (NS); A^{Bz} (97%); G^{Ibu} (97%)

Results and Conclusions:

- **PSL-C performed the best with all protected nucleosides**
- **T furnished 86% of the desired product with 6% of 3′-*O*-Lev. product**
- **Attempts with CAL-A and *Chromobacterium viscosum* lipase (CVL, Genzyme, 3800 U/g) were unsuccessful resulting in mixture of products**

J. Garcia, S. Fernadez, M. Ferrero, Y. Sanghvi & V. Gotor
***N N& NA* 2003, *22*, 1455.**

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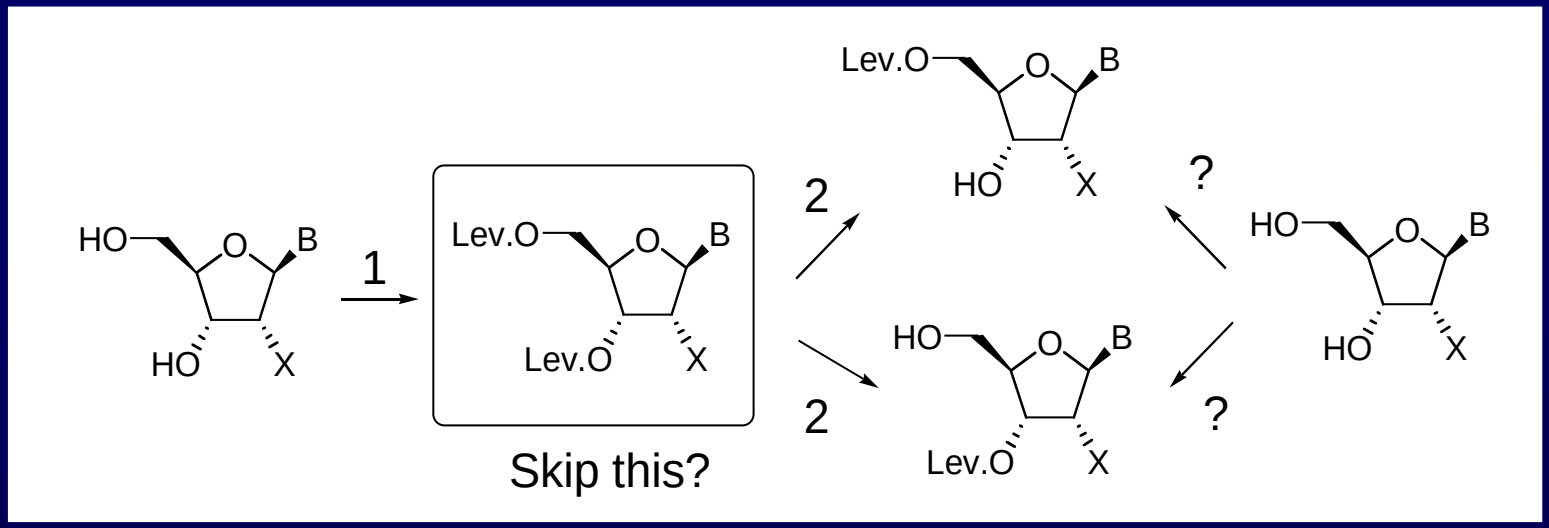
Why Two-Steps And Not One?



Background:

- Lipases have been reported to acylate nucleosides in a regioselective manner (Ferrero & Gotor in *Chem. Rev.* 2000, 4319)
- Nucleoside compatible mild reaction conditions: No detectable depurination
- Excellent regioselectivity with other natural products "Chemical Precision"
- Hydrolysis protocol required synthesis of bis-acylated nucleoside followed by regioselective hydrolysis: Over all two-steps
- Acylation could be carried out in a single step, if regioselective!

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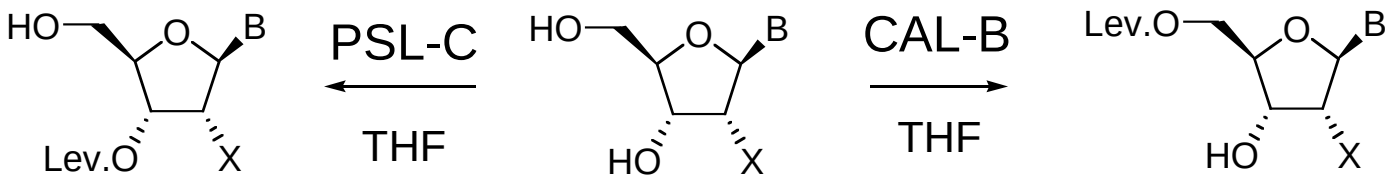
3´ or 5´-*O*-Regioselective Acylation of Nucleosides Using Lipases



B = T; C^{Bz}; A^{Bz}; G^{Ibu}; X = H, O-Me, $O(CH_2)_2OCH_3$

Results and Conclusions:

- **A direct one-step high yield protocol has been developed without CC**
- **Use of PSL-C gave excellent results furnishing 3´-*O*-Lev. nucleosides**
- **Faster acylation rates were accomplished by increasing the reaction temp.**
- **Oxime ester was synthesized in one step *via* $(Lev)_2O$ + hydroxylamine**
- **Both, base protected and 2´-modified nucleosides were good substrates**

J. Garcia, S. Fernandez, M. Ferrero, Y. Sanghvi & V. Gotor
***Tetrahedron Asymmetry* 2003, *14*, 3533.**

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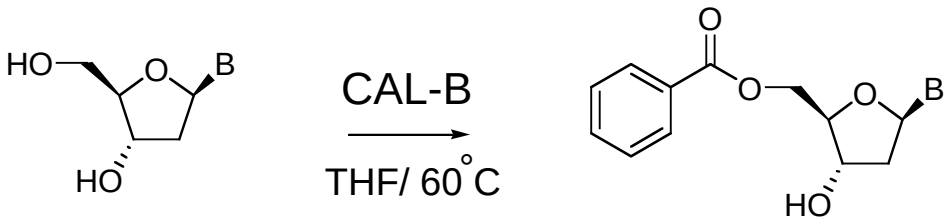
Regioselective 5′-*O*-Benzoylation of Nucleosides Using Lipases



B = T (98%); C^{Bz} (95%); A^{Bz} (95%); G^{Ibu} (93%)

Results and Conclusions:

- **Single-step protocol vs. multi-step chemical protection/deprotection procedure**
- **Excellent 5′-OH selectivity using commercial vinyl benzoate as acyl donor**
- **Best results were obtained with THF as solvent at 60 °C**
- **Both, enzyme and acyl donor were recycled (>5 times)**
- **These nucleosides are key building-blocks for the synthesis of amidate oligos**

J. Garcia, S. Fernández, M. Ferrero, Y. Sanghvi & V. Gotor
***Tetrahedron letters 2004*, 45, 1709-1712.**

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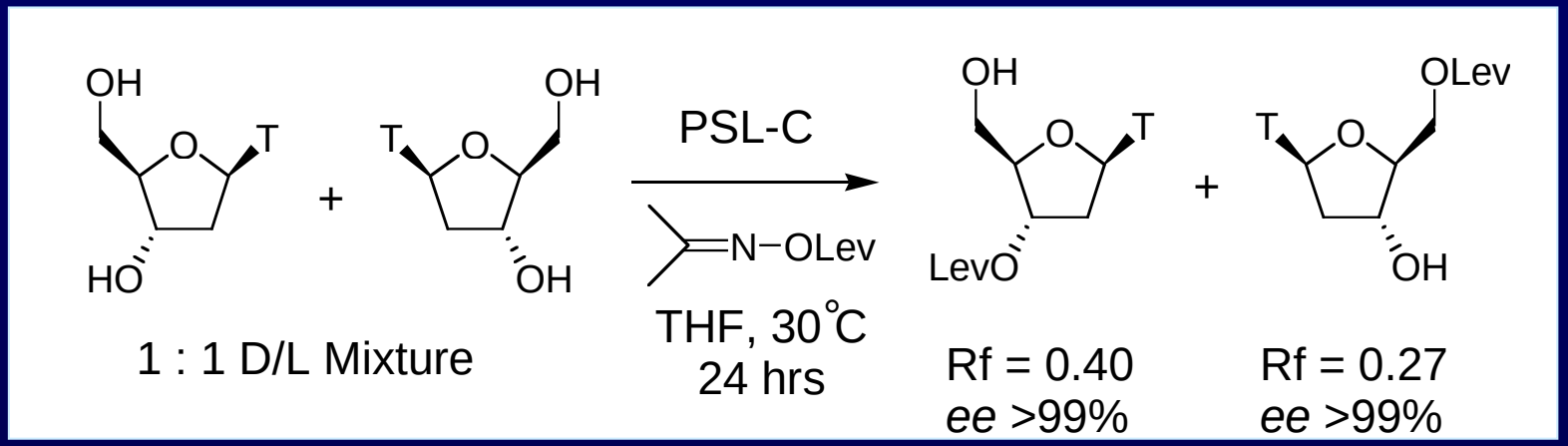
Enzymatic Separation of D/L Thymidine



Results and Conclusions:

- **First example of parallel kinetic resolution of D/L nucleosides**
- **Regioselective acylation of L-nucleosides is demonstrated**
- **Potential application in separation of racemic mixture of synthetic nucleosides of therapeutic value**
- **A Green alternative to the chemistry-based resolution methods**

J. García, S. Fernández, M. Ferrero, Y. Sanghvi & V. Gotor
***Org. Lett.* 2004, *6,* 3759.**

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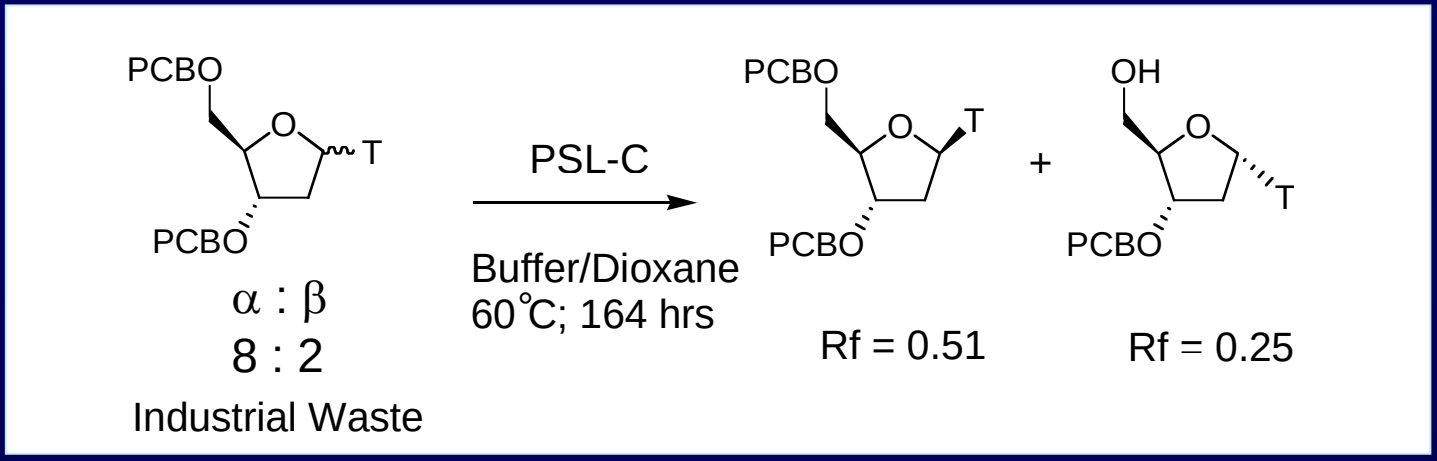
Enzymatic Separation of α/β Thymidine



Results and Conclusions:

- A sample of "Real Industrial Waste" containing α/β anomers was separated
- Hydrolysis of the 5'-OH in α-anomer was selectivity using PSL-C
- Best results were obtained with dioxane as solvent at 60 °C
- Both products were easily separated after chromatography
- A very attractive protocol for the isolation of α-thymidine from waste

J. García, S. Fernández, M. Ferrero, Y. Sanghvi & V. Gotor
J. Org. Chem. 2006, *71*, 9765.

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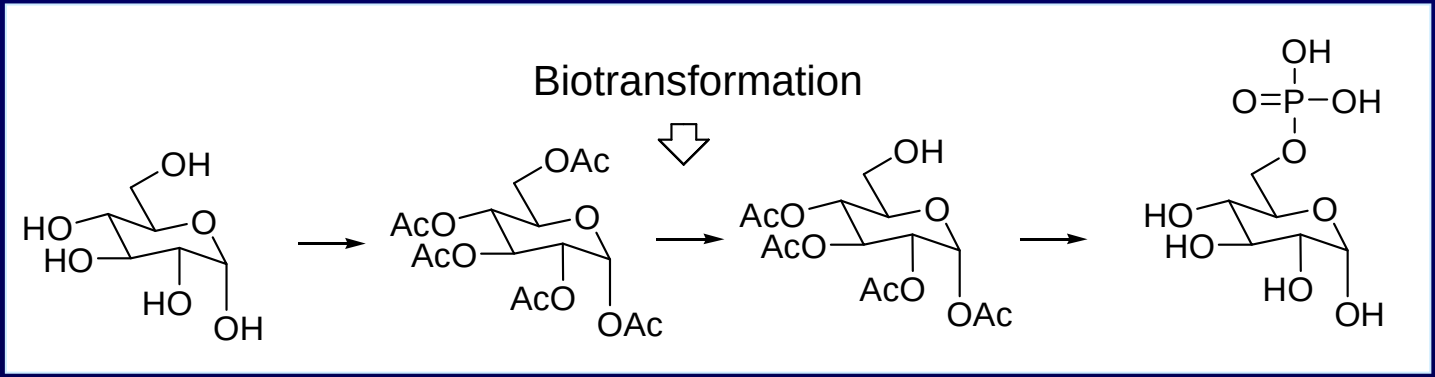
Novel Chemoenzymatic Synthesis of D-Glucose-6-Phosphate



Results and Conclusions:

- A concise synthesis of G-6-P has been developed on large-scale
- Hydrolysis of the 6-OAc group was selectivity using CRL
- >97% conversion was obtained with dioxane as solvent at 40 ˚C
- Anomerization at the C-1 was not observed during hydrolysis
- MM was used to support the observed selectivity with CRL

Rodríguez-Pérez, T.; Lavandera, I.; Fernández, S.; Sanghvi, Y.S.; Ferrero, M.; Gotor, V. *Euro J. Org. Chem*. 2007, 2769-2778.

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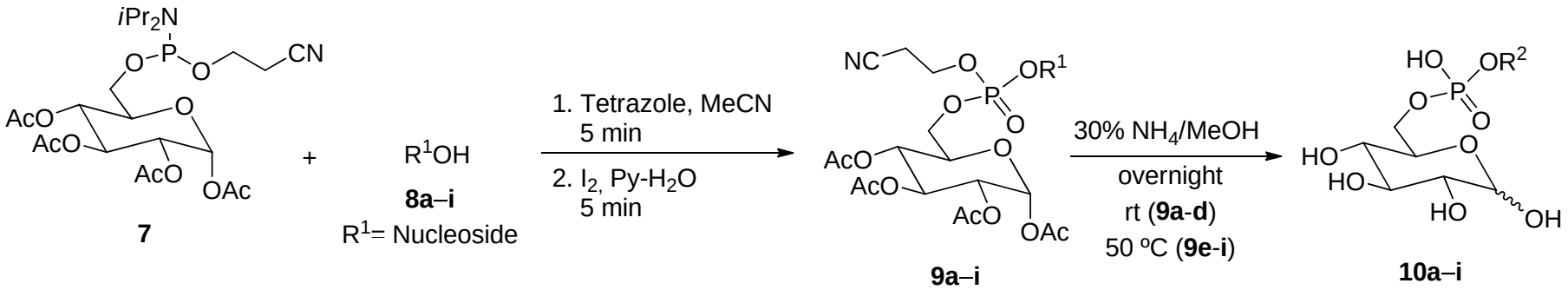
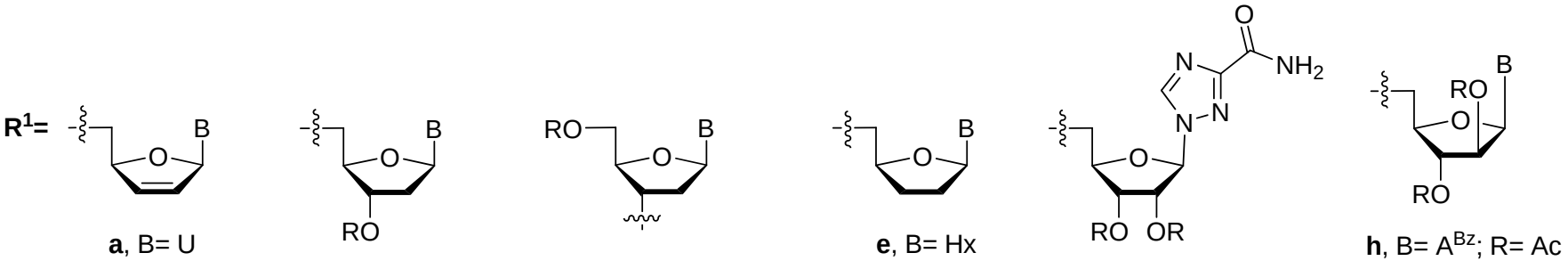
Syntheses of Glucose-Nucleoside Conjugates





a, B= U
b, B= T

c, B= 5-I-U; R= Lev

d, B= 5-I-U; R= Lev

e, B= Hx
f, B= A^{CHNMe2}

g, R= Ac

h, B= A^{Bz}; R= Ac
i, B= C^{Ac}; R= Ac

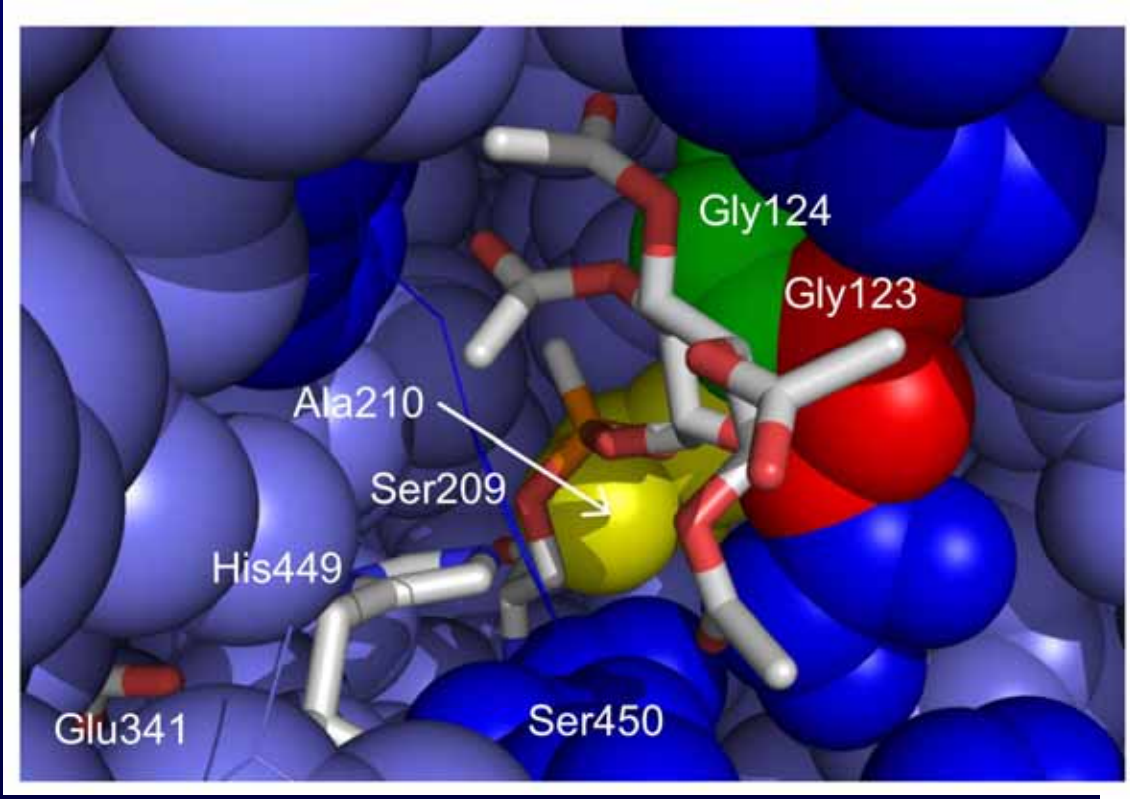
R^2= **a**, B= U; **b**, B= T; **c**, B= 5-I-U; R= H; **d**, B= 5-I-U; R= H; **e**, B= Hx; **f**, B= A; **g**, R= H; **h**, B= A; R= H; **i**, B= C; R= H

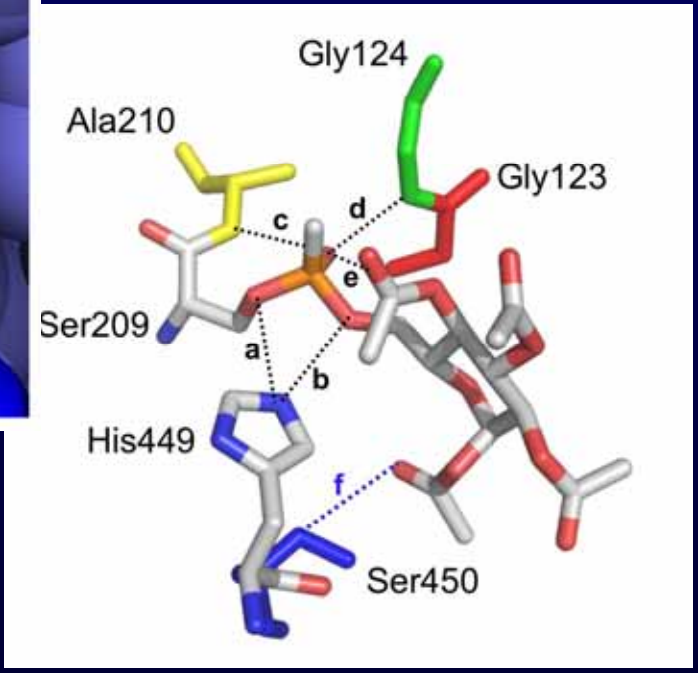
Lev= $COCH_2CH_2COCH_3$

Hx= Hypoxanthine

Rodríguez-Pérez, T.; Fernández, S.; Sanghvi, Y. S.; Detorio, M.; Schinazi, R. F.; Gotor, V.; Ferrero, M. *Bioconjug. Chem*. 2010, *21*, 2239-2249.

Molecular Modeling Studies on CRL Selectivity





In addition of the six key H-bonds, peracetylated α-glucose forms an additional H-bond between Ser450 and carbonyl group of anomeric acetyl group (bond f)

Peracetylated α-glucose conformation in the CRL binding site

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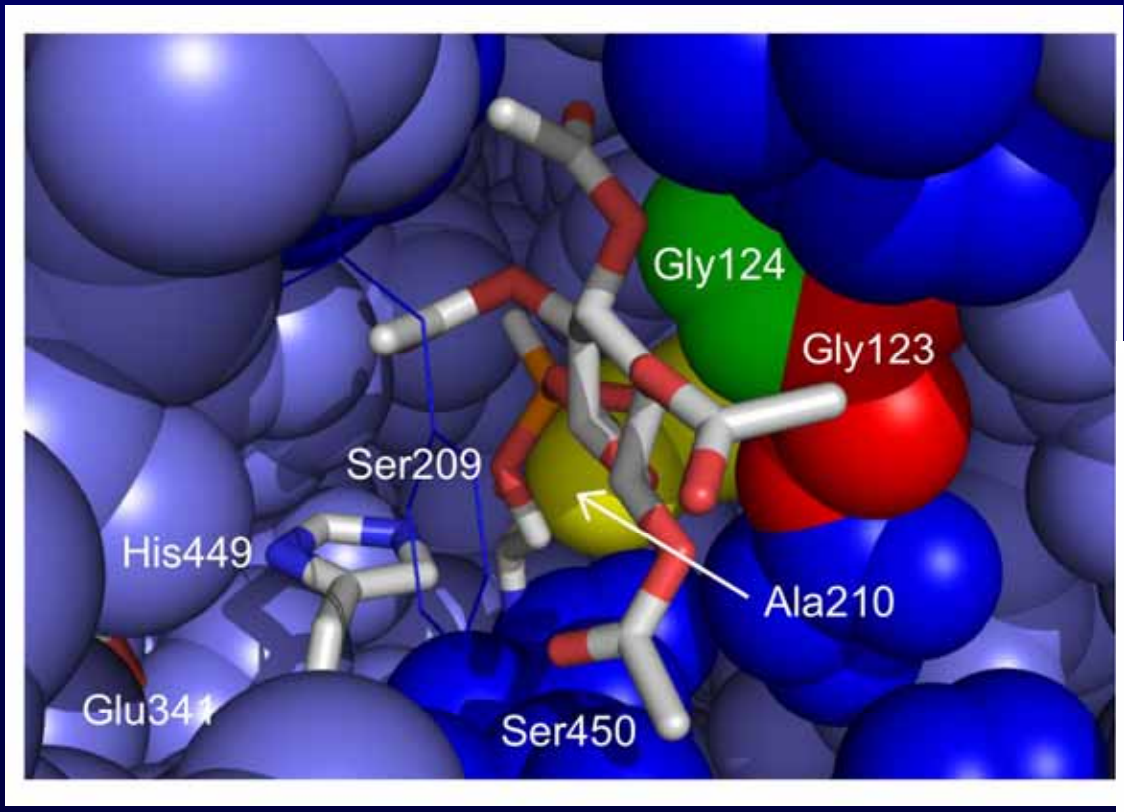
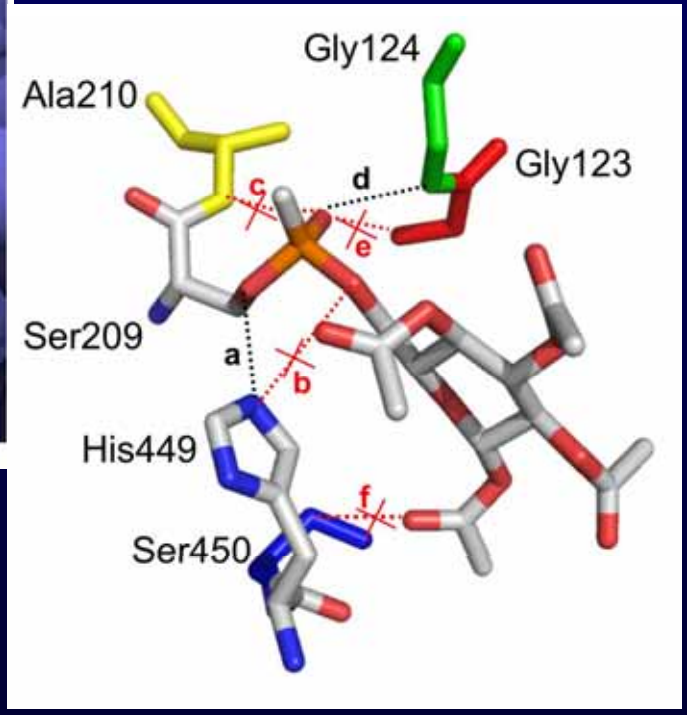
Molecular Modeling Studies on CRL Selectivity





Just three of the six key H-bonds remain in the transition state of peracetylated β-glucose

Peracetylated β-glucose conformation in the CRL binding site

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An Improved Route for the Synthesis of 2′-*O*-Me-A

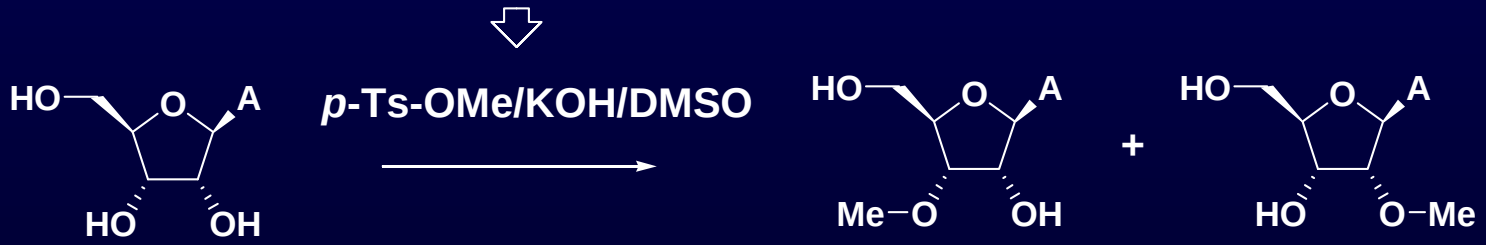
Old Route:

Hazardous Reagents

Difficult separation of 2'-*O*-Me-A

New Route:

Safer Reagents



Enzymatic separation of 2'-*O*-Me-A from 3'-*O*-Me-A

Martinez-Montero, S.; Fernández, S.; Rodríguez-Pérez, T.; Sanghvi, Y.S.; Wen, K.; Gotor, V.; Ferrero, M. *Euro J. Org. Chem*. 2009, 3265-3271.

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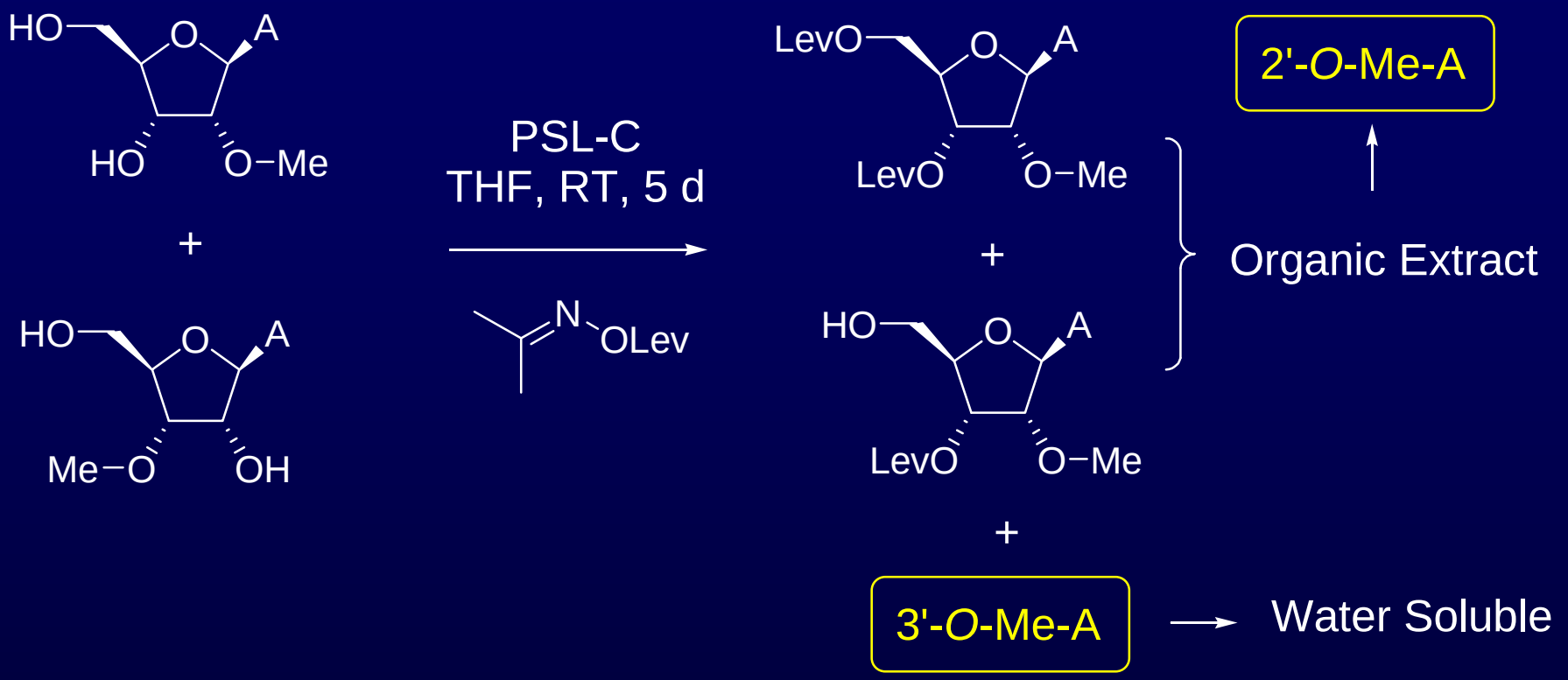
Enzymatic Separation of 2′-*O*-Me-A from 3′-*O*-Me-A



- **An efficient Green synthesis of 2′-*O*-Me-A and 3′-*O*-Me-A has been developed**
- **First report on the chemoselectivity demonstrated by PSL-C**
- **Offers a direct route to 3′-*O*-Lev-protected 2′-*O*-Me-A**
- **Demonstrated on large-scale without deactivation of lipase**

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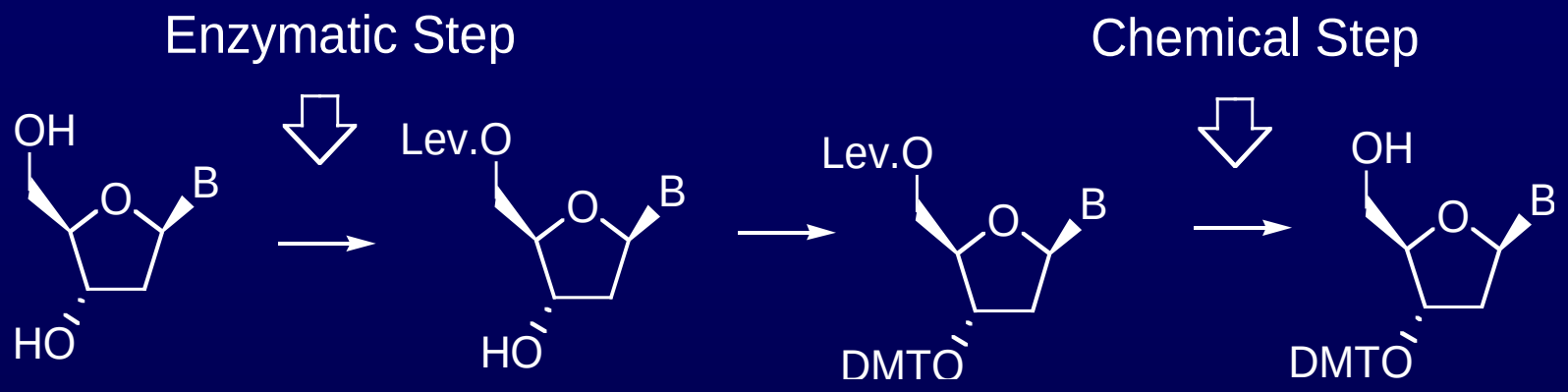
Improved Synthesis of 3´-*O*-DMT Nucleosides



Results:

- **Reaction conditions were optimized to furnish only 5´-*O*-levulinylated 2´-deoxynucleosides**
- **All products were easily isolated without column chromatography and no trace of 3´,5´-bis-*O*-levulinylated products**
- **Use of difficult to remove TBAF was avoided**
- **Use of neutral conditions for Lev. Deprotection furnished high yields of 3´-*O*-DMT protected nucleosides**

I. Lavandera, J. Garcia, S. Fernández, M. Ferrero, V. Gotor and Y. Sanghvi
***Org. Process Res. Dev. 2006, 10, 581*.**

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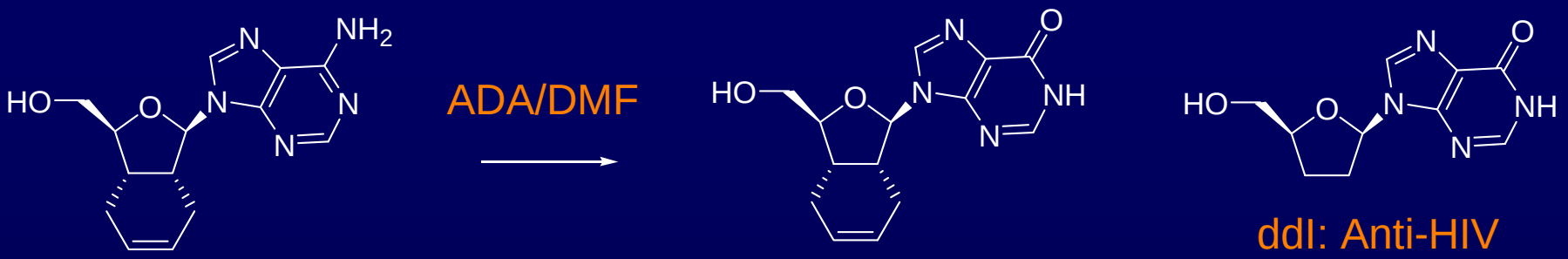
Synthesis of Anti-HIV Nucleosides



ddI: Anti-HIV

Results:

- **Over 24 novel bicyclic nucleosides were synthesized**
- **All nucleosides were screened for anti-HIV activity and Inosine analog was found to be most active**
- **These nucleosides exhibit *N*-conformation and are acid stable**
- **The adenosine analog was efficiently transformed into inosine analog using Adenosine deaminase (ADA)**

A. Diaz-Rodriguez, Y. Sanghvi, E. Theodorakis, S. Fernández, M. Ferrero and V. Gotor
***Org. Biomol. Chem. 2009, 7,* 1415-1423.**

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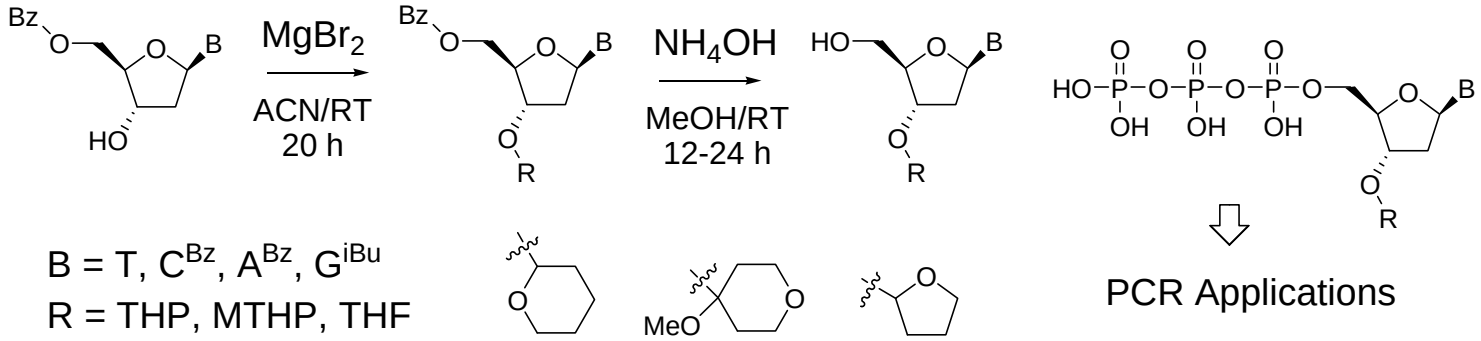
Synthesis of 3´-*O*-Acetal Protected Nucleosides as Building-Blocks for NTPs



Results and Conclusions:

- **Short protocol vs. multi-step chemical protection/deprotection procedure**
- **Excellent yield for 3´-*O*-acetal protection with use of $MgBr_2$ in acetonitrile**
- **Use of traditional PTSA and CSA also furnished the 3´-*O*-acetals**
- **Useful intermediate for orthogonal protection of nucleosides**
- **These nucleosides are key building-blocks for the synthesis of NTPs for PCR**

Rodríguez-Pérez, T.; Fernández, S.; Martinez-Montero, S.; González-Garcia, T.; Sanghvi, Y.S.; Gotor, V.; Ferrero, M. *Euro J. Org. Chem*. 2010, 1736-1744.

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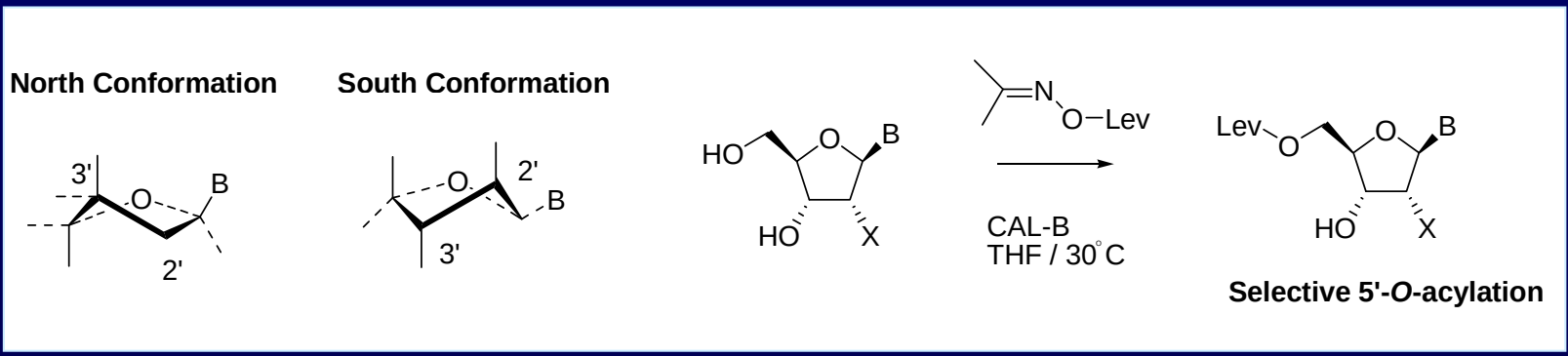
Role of Sugar Conformation in CAL-B Catalyzed Levulinylation of Nucleosides



Results and Conclusions:

- Among 15 nucleosides tested, analogues that presented sugar with *N*-conformation are acylated at higher ratios and with better selectivity
- MM confirmed the *N*-sugar puckering in the preferred binding site for CAL-B (ribo-, 2´-*O*-Me, and 2´-F have higher % of *N*-conformation in solution)
- The MM studies also confirmed that the base moiety (purine or pyrimidine) does not have any significant influence either on the rate or selectivity of the acylation reaction using CAL-B

Martinez-Montero, S.; Fernández, S.; Sanghvi, Y.S.; Gotor, V.; Ferrero, M.
Euro J. Org. Chem. 2012, 5483-5490.

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Batch *vs.* Continuous Flow Processes

Batch Process:

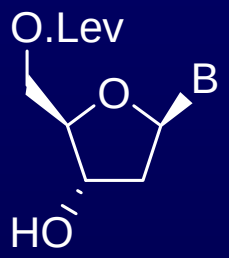
- Erlenmeyer flask
- Orbital shaker
- Reaction in suspension

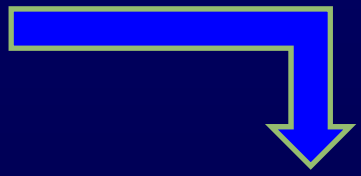
Continuous Flow Process:

- Pump
- Column filled with enzyme
- Reaction in solution

Continuous Flow Enzymatic Process

Scale-up: from 1 g to **10 g** and **25 g**





- **10 g** of T
- Ratio T:CAL-B **1:0.5**
- Reaction time: 6 h
- **Yield:**

93% (crude); 91% purity

75% (cryst.); >99% purity

- **25 g** of T
- Ratio T:CAL-B **1:0.5**
- Reaction time: 7 h
- **Yield:**

96% (crude); 93% purity

70% (cryst.); >99% purity

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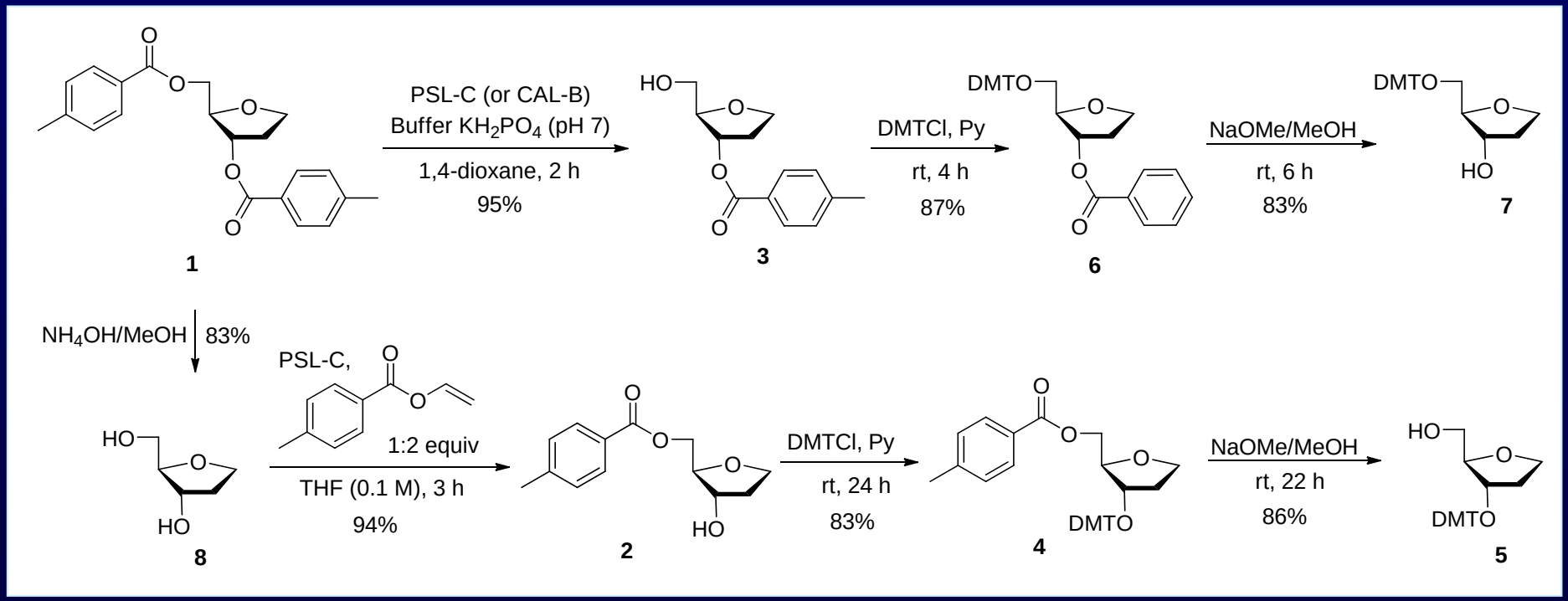
Chemoenzymatic Syntheses of 3′- or 5′-*O*-DMT Protected Abasic Nucleosides



Results and Conclusions:

High selectivity for both acylation and hydrolysis is retained for abasic moiety – participation of base is not necessary!

Rodríguez-Pérez, T.; Fernández, S.; Sanghvi, Y. S.; Gotor, V.; Ferrero, M.
***Org. Biomol. Chem*. 2011, *9*, 5960.**

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Biocatalysis is leading the way…

- Enzymes are a "Master Chemist" that rarely fails
- Enzymes are produced from renewable resources, such as fermentation on a very large-scale
- Enzymes are highly atom and energy efficient, ideal candidate for "Green Chemistry"
- Chemists and Biologists need to work together to make it happen and opportunities are endless…

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Final Thought…

"In an ideal chemical factory there is, strictly speaking, no waste but only products. The better a real factory makes use of its waste, the closer it gets to its ideal, the bigger is the profit."

A. W. von Hofmann (1884)

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Kudos to Collaborators

Enzymatic Nucleoside Transformations:

University of Oviedo (Spain): S. Montero, T. Rodríguez-Pérez,

Lavandera, J. Garcia, A. Diaz-Rodriguez, S. Fernandez

M. Ferrero and V. Gotor

Ionic Liquid and Enzymatic Chemistry:

University of Delh (India): A. K. Prasad, V. S. Parmar and V. Kumar

Nucleoside Synthesis:

University of California San Diego (USA): Emmanuel Theodorakis

2′-*O*-Me-A Synthesis:

University of Shangha (China): K. Wen