CHEMICAL SYNTHESIS OF BIOPOLYMERS

1.4 Synthesis of phosphate esters

In generally, **nucleoside phosphate ester synthesis** uses phosphory-lation reactions in which a **3'-nucleotide component** is converted into a **reactive phosphorylation species by a condensing agent**.

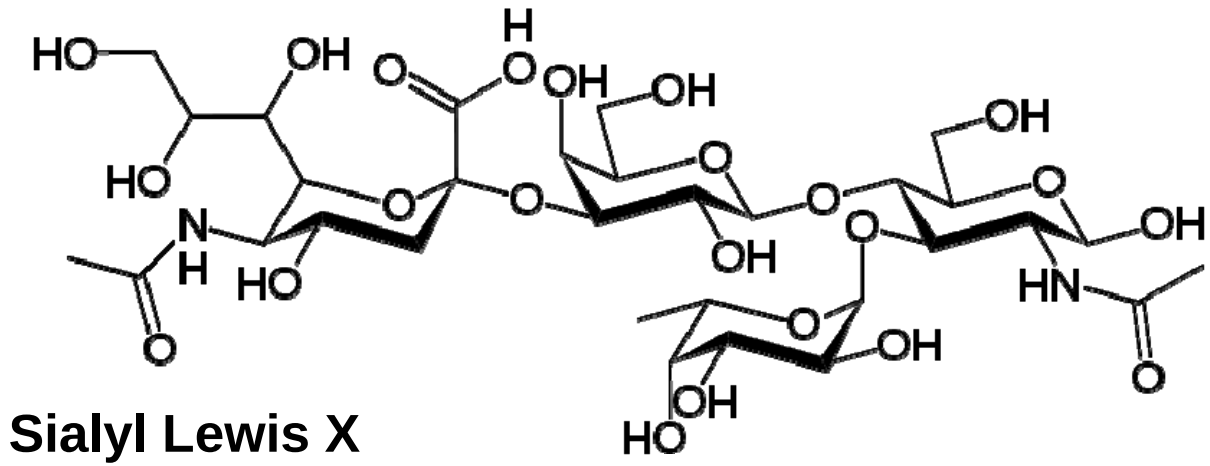
One of the major problems is that the **condensing agent not only activate the nucleotide, but may also react with the nucleoside bases**.

The most common approaches can be divided in **five types of phosphate ester formation:**

- **Via phosphate diesters**
- **Via phosphate triesters**
- **Via phosphite triesters**
- **Via H-phosphonate diesters**
- **Synthesis of phosphate monoesters**

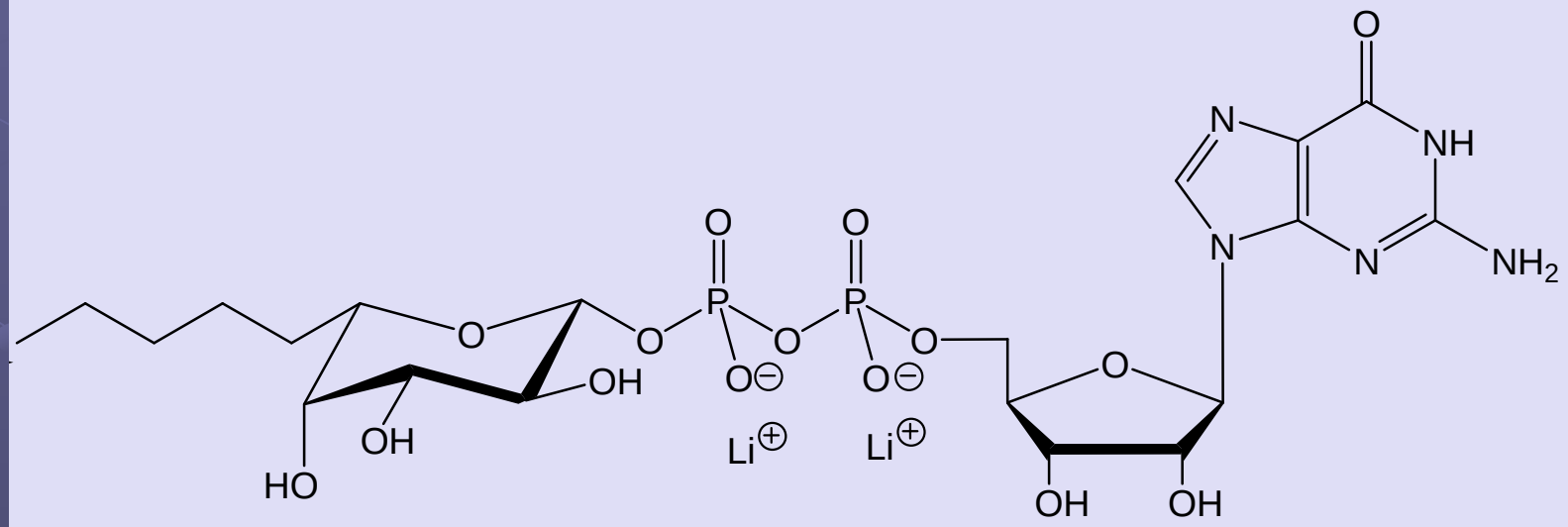
CHEMICAL SYNTHESIS OF BIOPOLYMERS

Synthesis of phosphate monoesters – why?



Sialyl Lewis X

C. Vogel, C. Bergemann, A.-J. Ott, T.K. Lindhorst, J. Thiem, W.V. Dahlhoff, C. Hällgren, M.M.Palcic, O. Hindsgaul, *Liebigs Ann./Recueil* **1997** 601-612.

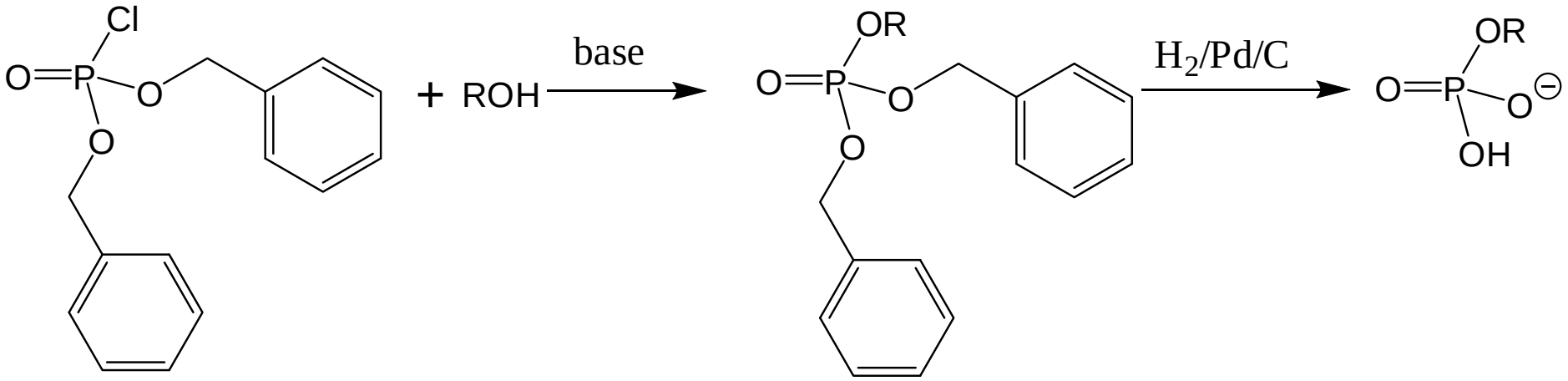


CHEMICAL SYNTHESIS OF BIOPOLYMERS

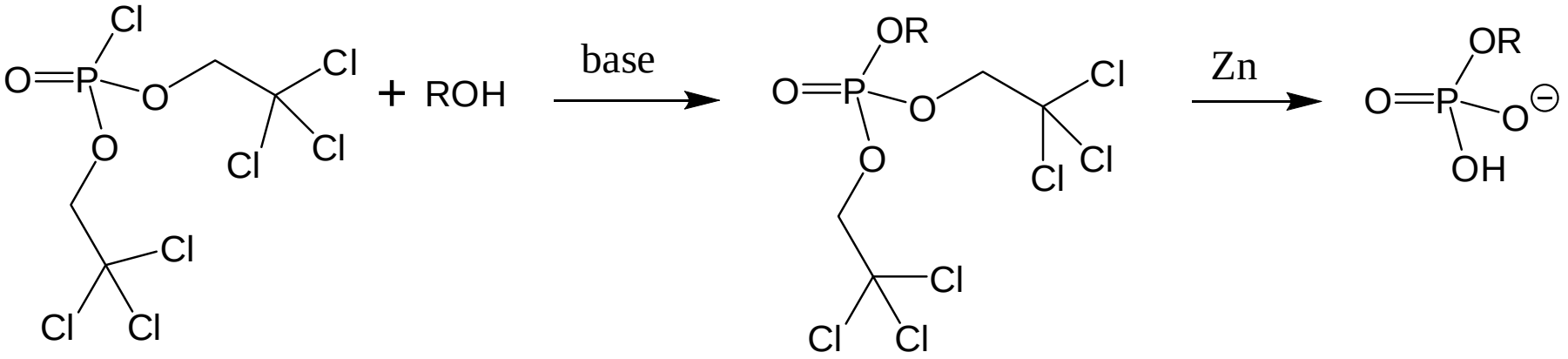
Synthesis of phosphate monoesters

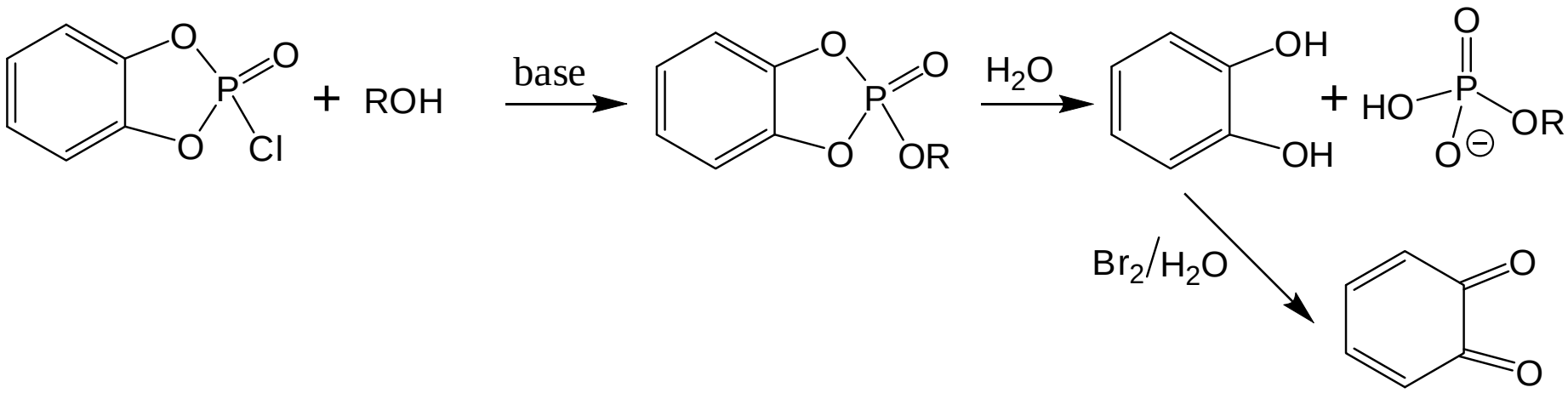
Monoesters usually made either **from triesters by selective deprotection** or by **direct condensation of an alcohol with $O{=}PCl_3$**.

In the case of the triester procedure reagents such as **dibenzyl phosphorochloridate**, **bis(2,2,2-trichloroethyl) phosphorochloridate**, or **1,2-phenylene phosphorochloridate** are used.



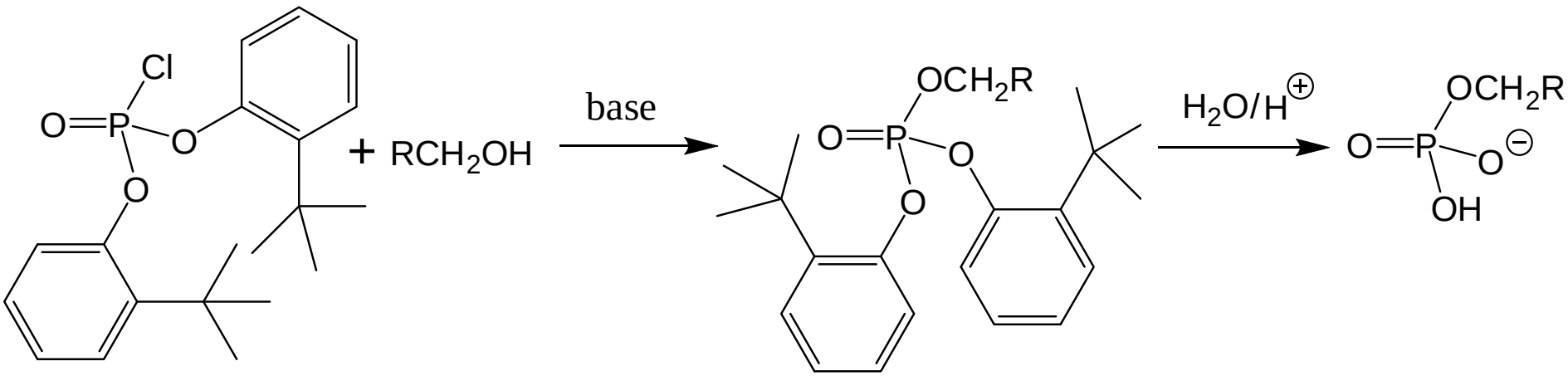
CHEMICAL SYNTHESIS OF BIOPOLYMERS

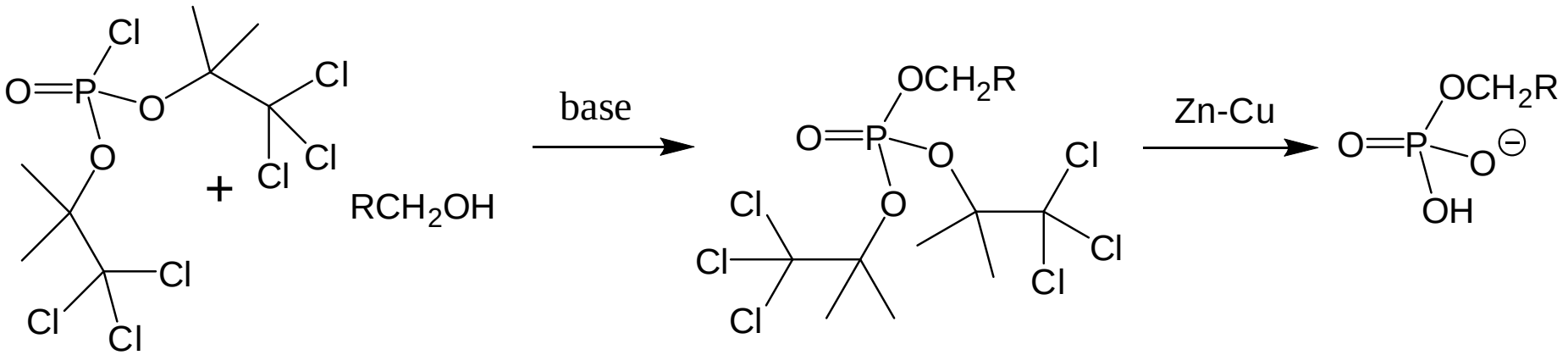




CHEMICAL SYNTHESIS OF BIOPOLYMERS

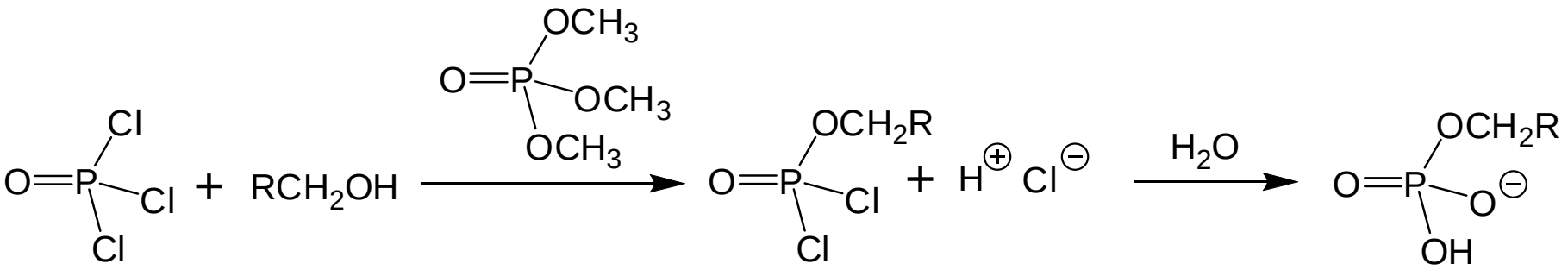
Regioselective phosphorylation of the 5'-hydroxyl function in unprotected ribo- and deoxyribonucleosides was achieved by **bis(2-*tert*-butylphenyl)** or **bis(2,2,2-trichloro-1,1-dimethylethyl) phosphorochloridate.**





CHEMICAL SYNTHESIS OF BIOPOLYMERS

Using **$O{=}PCl_3$** in the presence of trialkyl phosphate (**Yoshikawa method**) **in a special solvent system** (aq. pyridine in acetonitrile; **Sowa-Ouchi method**) direct phosphorylation at the 5'-position provides yields greater than 80% with over **90% regioselectivity**.

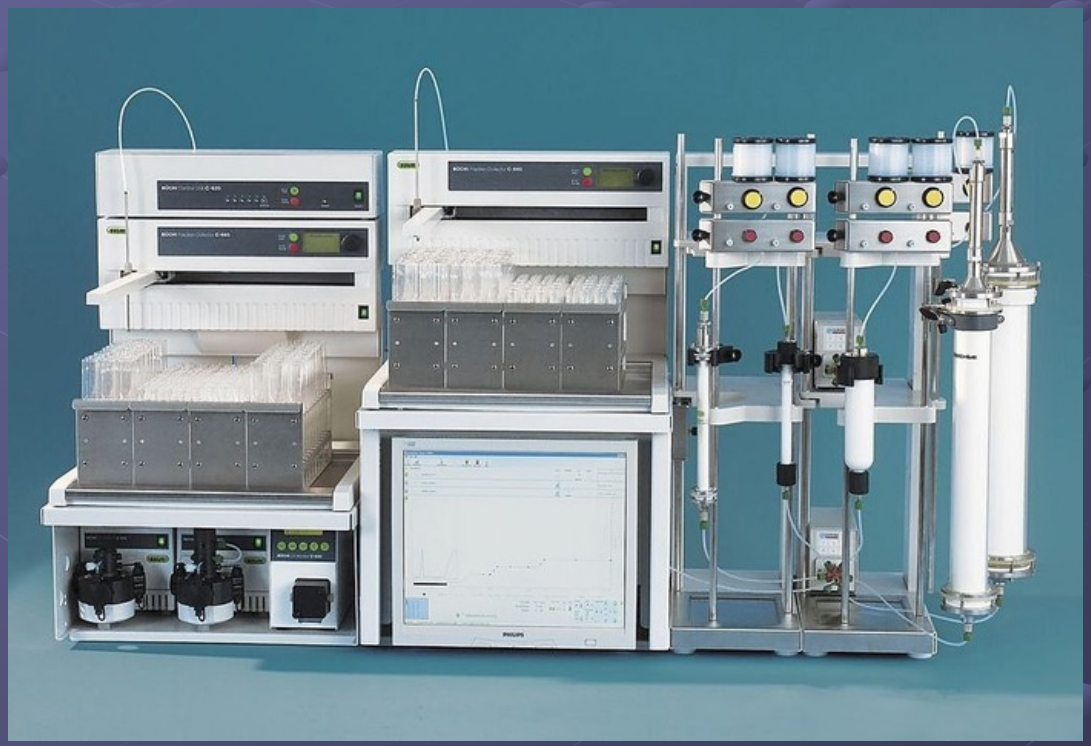


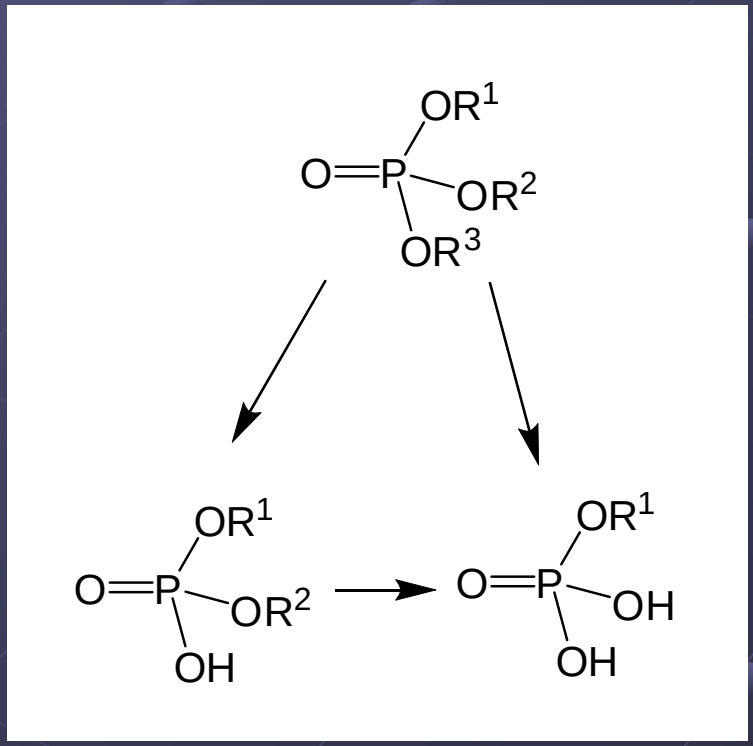
The **control mechanism of trialkyl phosphates** could be connected with solubility characteristics and intramolecular hydrogen bridge formation.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

Conclusions

Phosphate triesters have become the **preferred intermediates** for many syntheses. That is in part because they can be **purified by standard techniques** of organic chemistry and in part because methods have been devised for highly selective protection to give the desired **diesters** or **monoesters**.





CHEMICAL SYNTHESIS OF BIOPOLYMERS

1.5 Synthesis of oligodeoxyribonucleotides

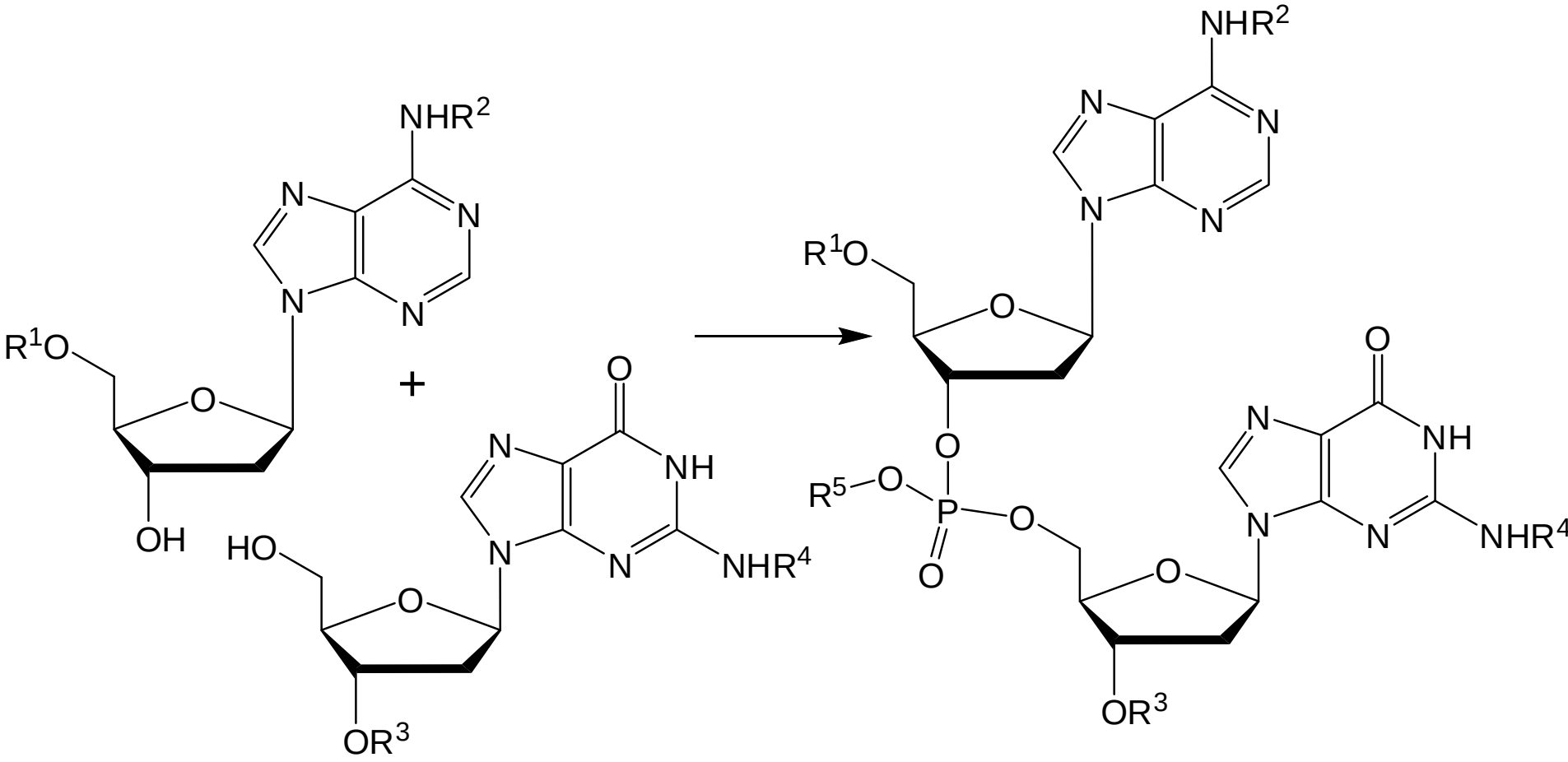
All **chemically synthesized single-stranded nucleic acid chains of defined length and sequence** are referred as ***oligonucleotides***, even if they are well **beyond 100 residues** in length.

The term ***polynucleotide*** is used to refer to **nucleic acid of less defined length and sequences obtained by a polymerization reaction**.

Nucleic acids are highly sensitive to a wide range of chemical reactions: **heterocyclic bases are prone to alkylation, oxidation, and reduction, phosphodiester** backbone is vulnerable to alkaline and acidic **hydrolysis**, and the **C-N-glycosylic bond** can be **attacked under acidic conditions**.

The **main nucleophilic centres** of a **deoxyribonucleoside** are the 5’-hydroxyl group, the 3’-hydroxyl group, and the base nitrogen atoms.

CHEMICAL SYNTHESIS OF BIOPOLYMERS



CHEMICAL SYNTHESIS OF BIOPOLYMERS

Protecting group manipulation

The exocyclic amino groups of **adenine**, **cytosine** and **guanine** must be protected.

The **5'-unit** must have a **protecting group on the 5'-hydroxyl** and the **3'-unit** must be **protected on the 3'-hydroxyl group**.

After **phosphorylation** (or **phosphitylation**) of one of the two units the other nucleoside is then connected by a **coupling reaction**.

Commonly the **phosphate carries a protecting group**, $\mathbf{R^5}$, such that the internucleoside phosphate becomes a **triester**.

In the **solution-phase synthesis** $\mathbf{R^1}$ and $\mathbf{R^3}$ are conventional protecting groups. Thus, the **extension of the chain** is possible **in either the 3'-to-5' (phosphate on 5') or 5'-to-3' (phosphate on 3') direction**.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

In the **solid-phase method R^1** or **R^3** are an insoluble polymeric or inorganic support. Therefore, the oligonucleotide can only be extended in one direction.

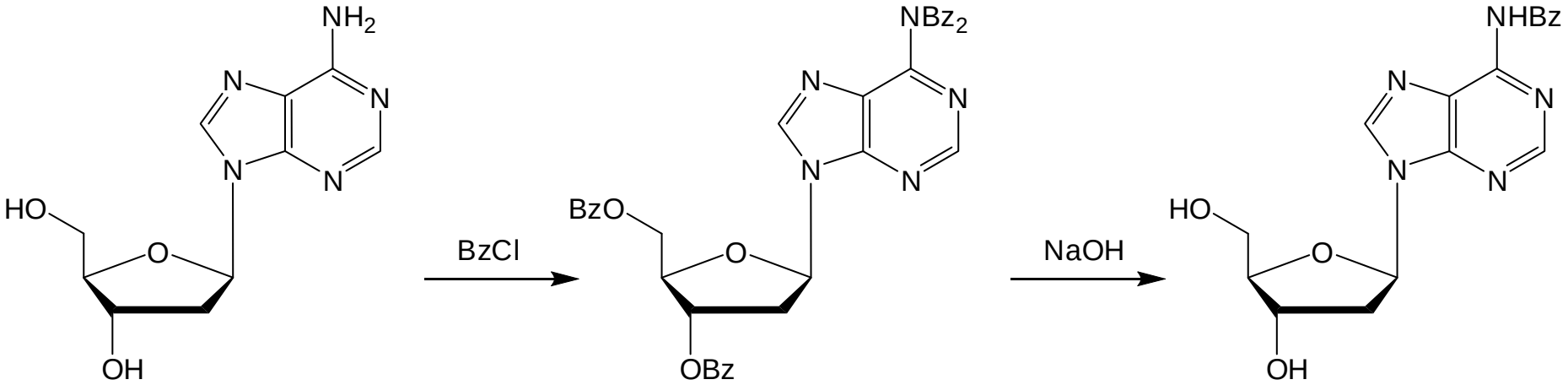
The **temporary protecting groups** are removed after each coupling step, whereas the **permanent protecting groups** are removed at the end of the synthesis in order to generate the final deprotected oligonucleotide.

The most convenient way to assemble an oligonucleotide is to utilize **pre-formed deoxynucleoside phosphates as basic building units**.

Since the **primary 5'-hydroxyl group is more effective nucleophile** than the secondary 3'-hydroxyl, the phosphate is best attached to a solid support on the 3'-position.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

Heterocyclic bases

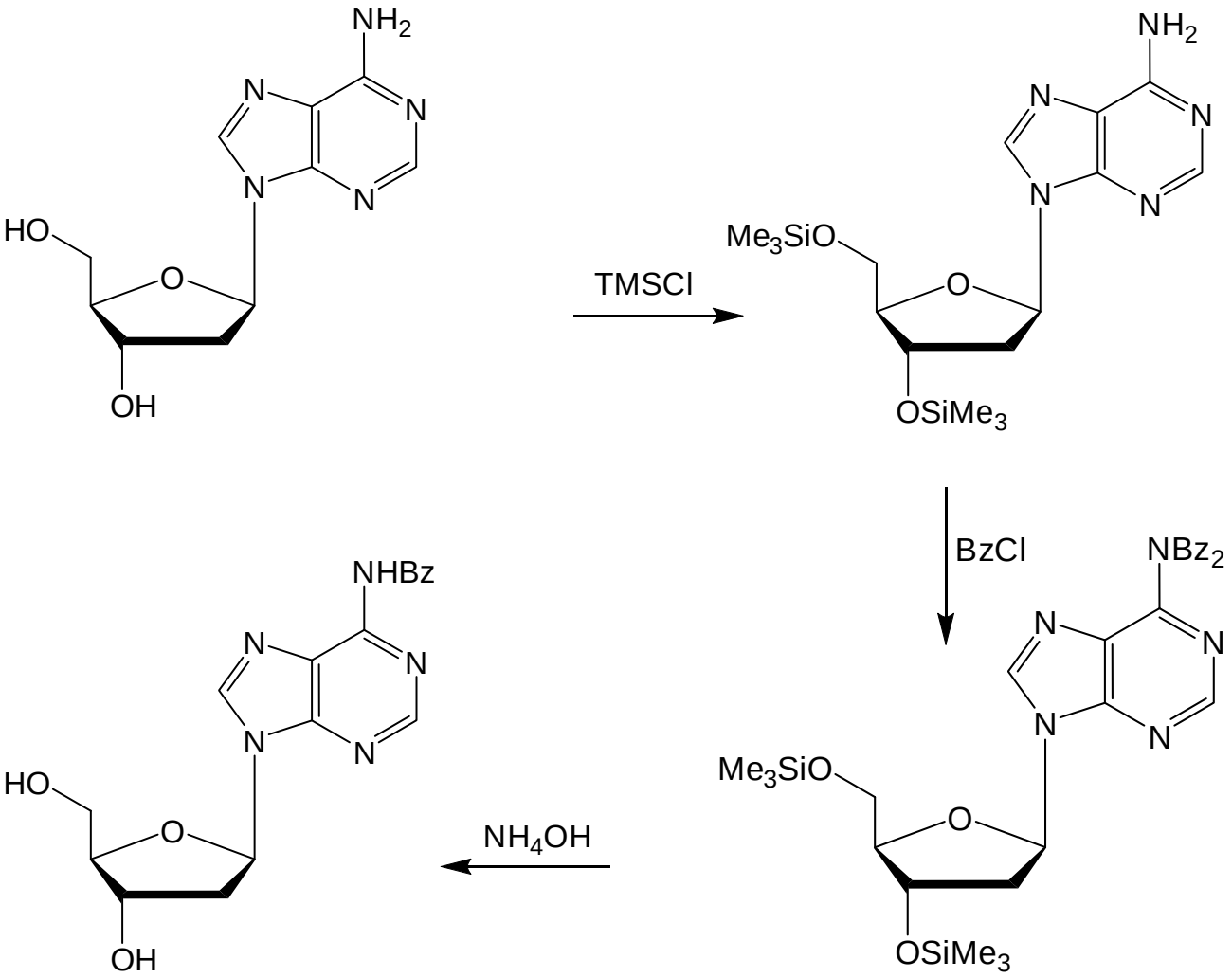


per-acylation route

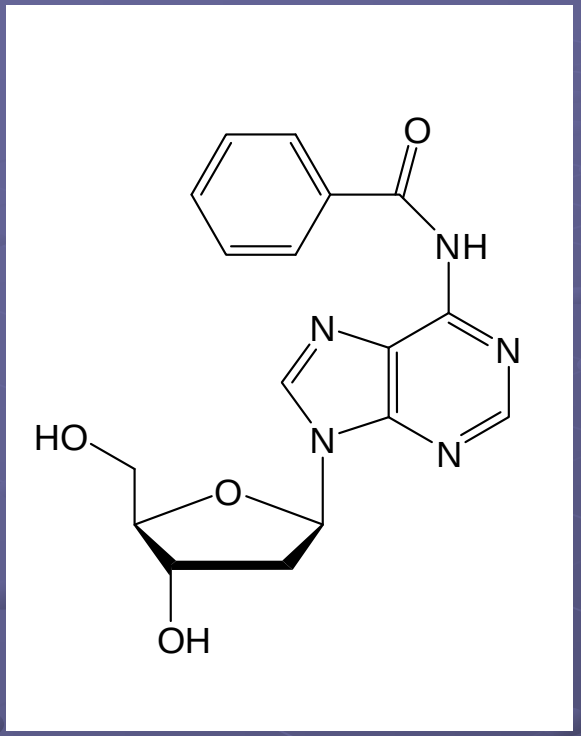
The **acyl protecting groups** (introduced by **Khorana** et al. over 50 years ago) remain stable for long periods under mild basic or acidic conditions and during chromatography. At the end of the synthesis they can be removed by treatment with concentrated ammonia.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

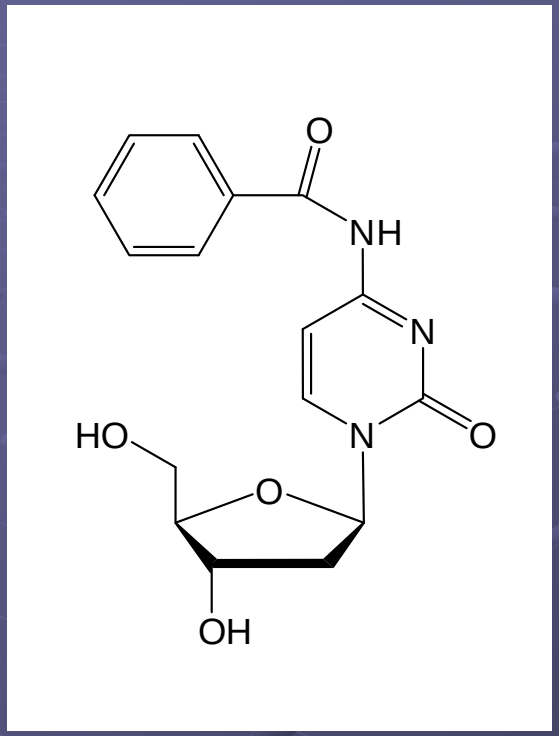
transient protection route



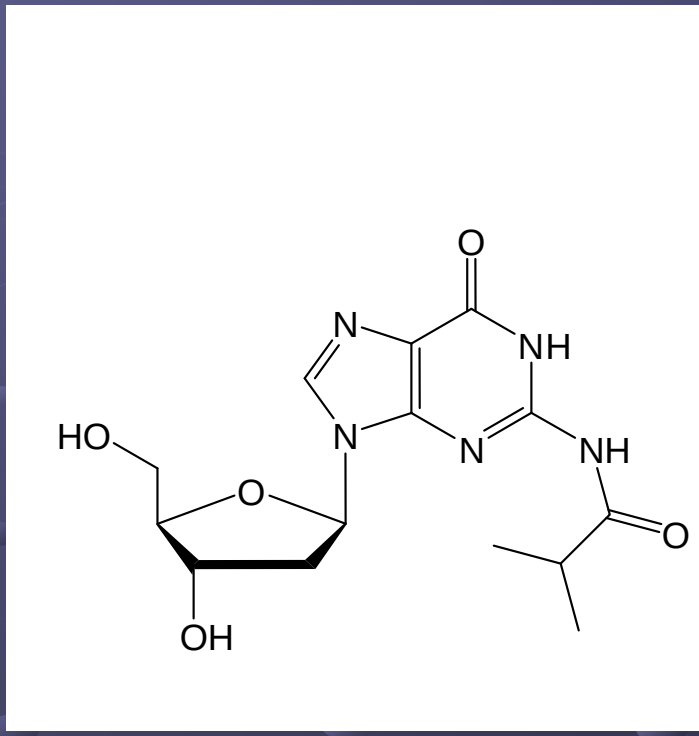
CHEMICAL SYNTHESIS OF BIOPOLYMERS



dA^{bz}



dC^{bz}

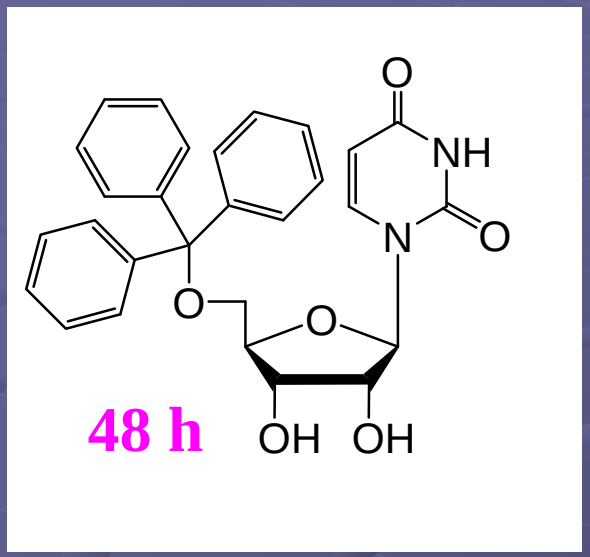


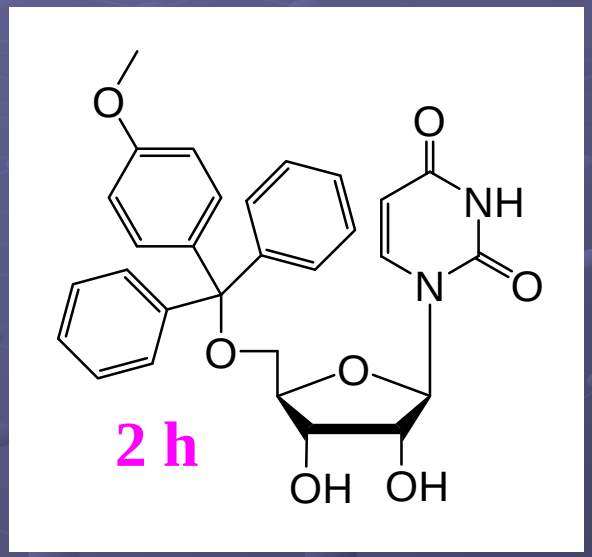
dG^{ib}

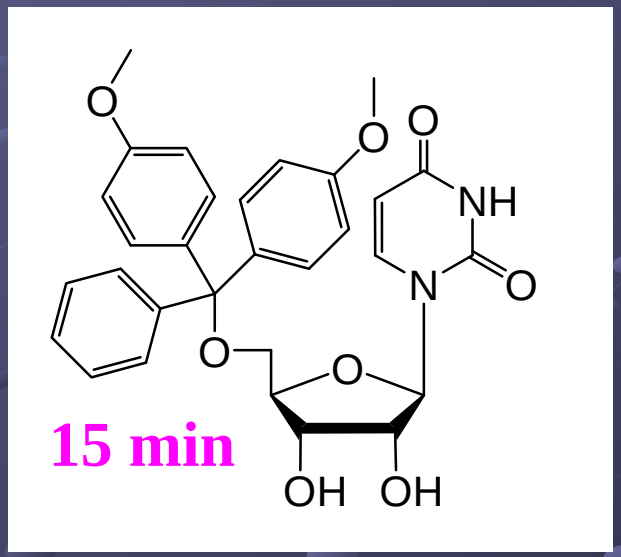
A **complication with deoxyguanosine** is that the **6-O-position is vulnerable** to reaction used in oligonucleotide synthesis, but in the case of the now widely applied **phosphoramidite method protection is not necessary**.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

Protection of the 5’-hydroxyl group

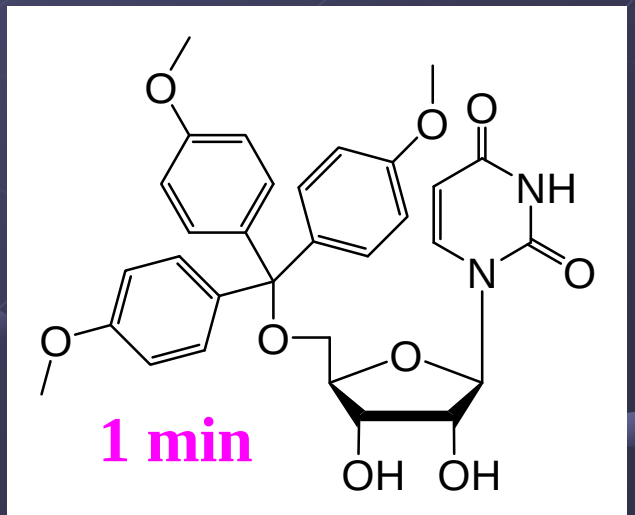






By far the most useful protecting groups for the 5’-position are the mono- and dimethoxytrityl group.

The DMTr group is introduced by reaction with DMTrCl in the presence of pyridine or 4-dimethyl-aminopyridine.



CHEMICAL SYNTHESIS OF BIOPOLYMERS

Formation of the internucleotide bond

No discussion of oligonucleotide synthesis would be complete without mention of the pioneering work of **Khorana** et al. who used the **phosphodiester method**.

The main **drawback** of this method is that the product **phosphodiester reacts** also **with** the **activated deoxynucleoside phosphomono-ester** to give a **trisubstituted pyrophosphate** derivative.

An **aqueous work-up is necessary** to regenerate the desired phosphodiester.

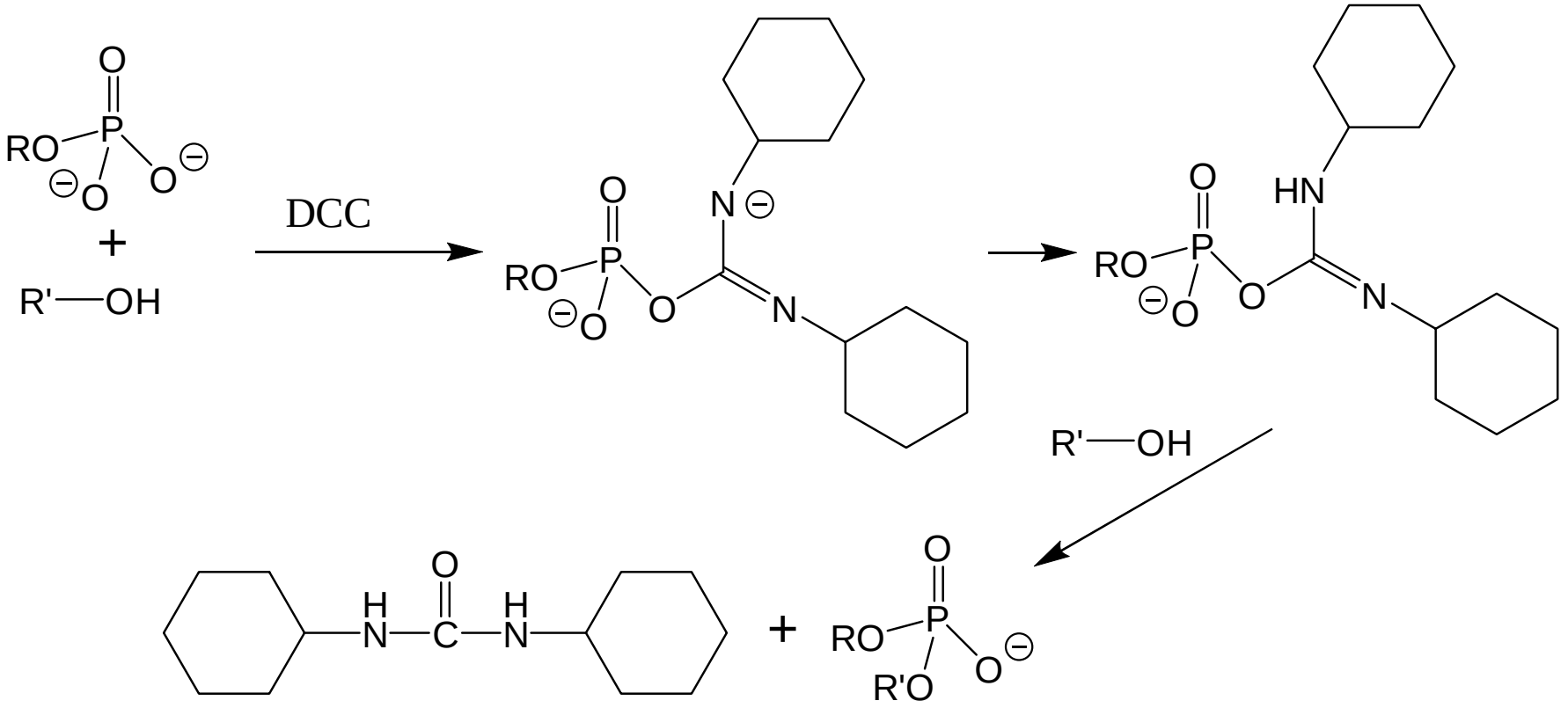
Har Gobind Khorana

Nobel Prize 1968
together with
M. W. Nirenberg & R. W. Holley

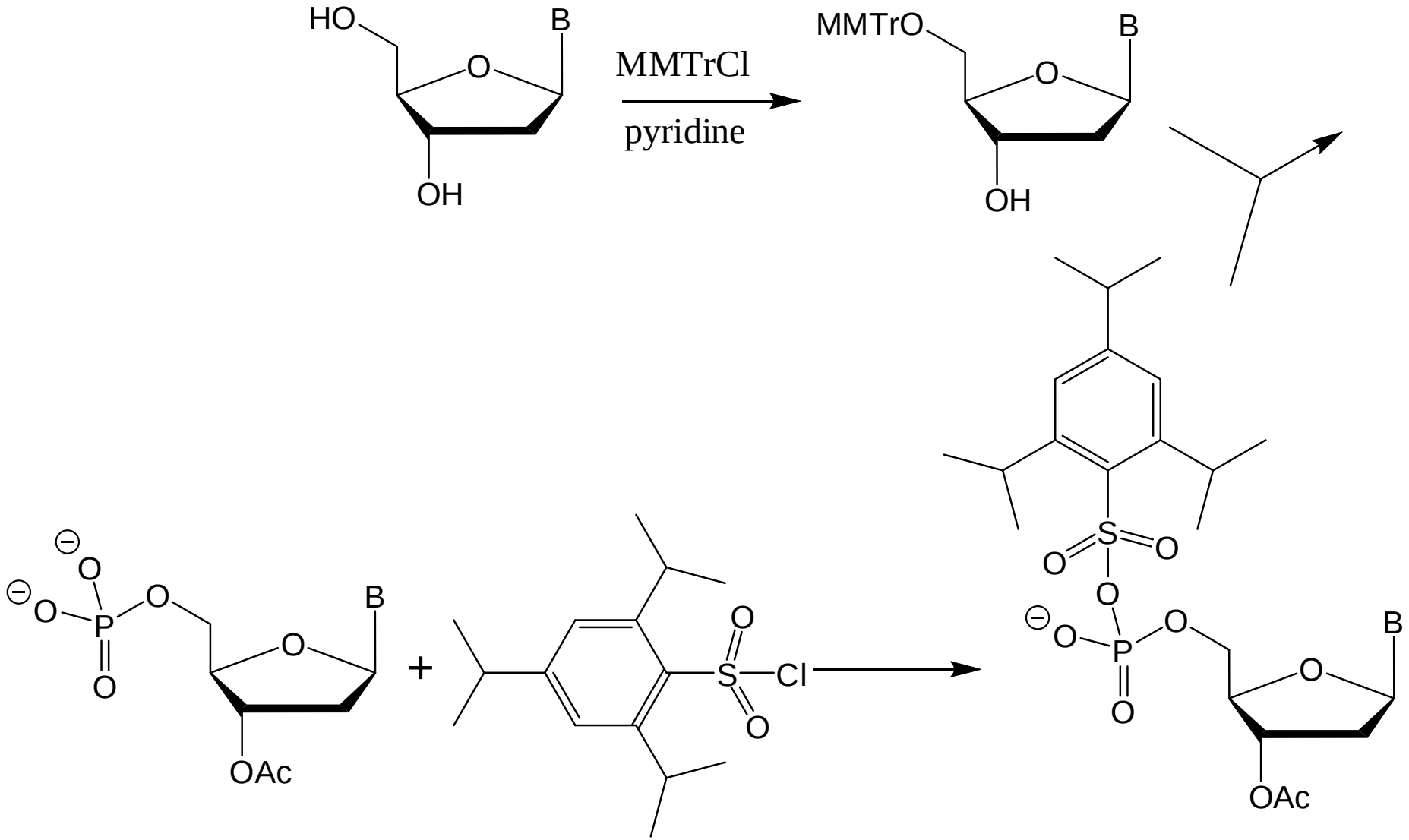
CHEMICAL SYNTHESIS OF BIOPOLYMERS

Syntheses via phosphate diesters

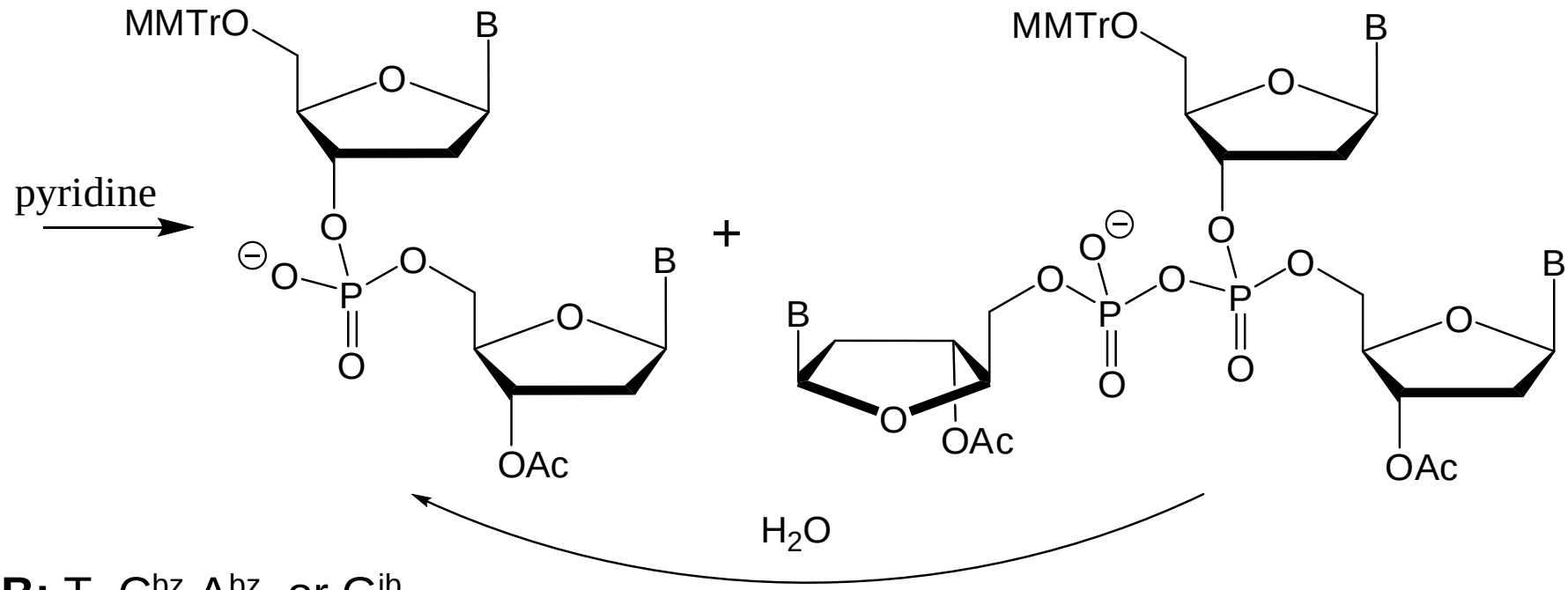
Early syntheses worked mainly with mild condensing agents such as **dicyclohexylcarbodiimide** (**DCC**).



CHEMICAL SYNTHESIS OF BIOPOLYMERS



CHEMICAL SYNTHESIS OF BIOPOLYMERS



B: T, C^{bz} A^{bz}, or G^{ib}

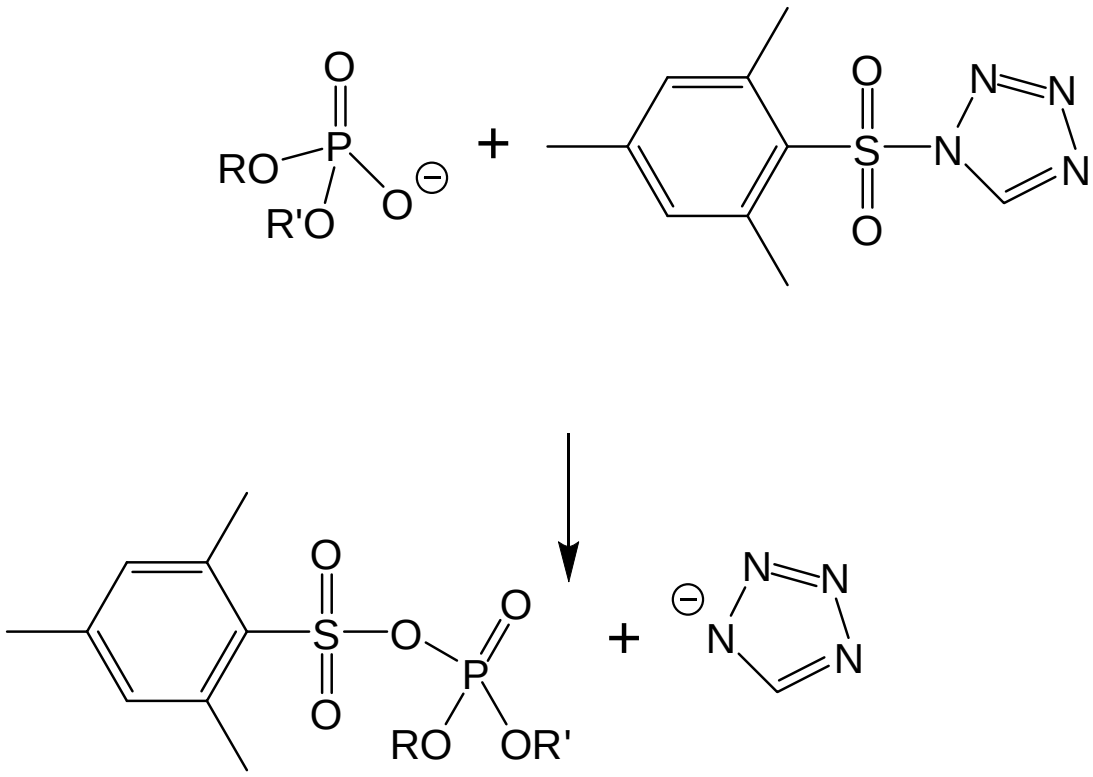
Synthesis of an oligonucleotide of 10 -15 residues takes upwards of 3 months.

In the **late1970s, the 5'MMTr group was replaced by linkage to a solid support**, that reduced the time for the same oligonucleotide to about two weeks, but the **low yields** intrinsic to phosphodiester chemistry remain.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

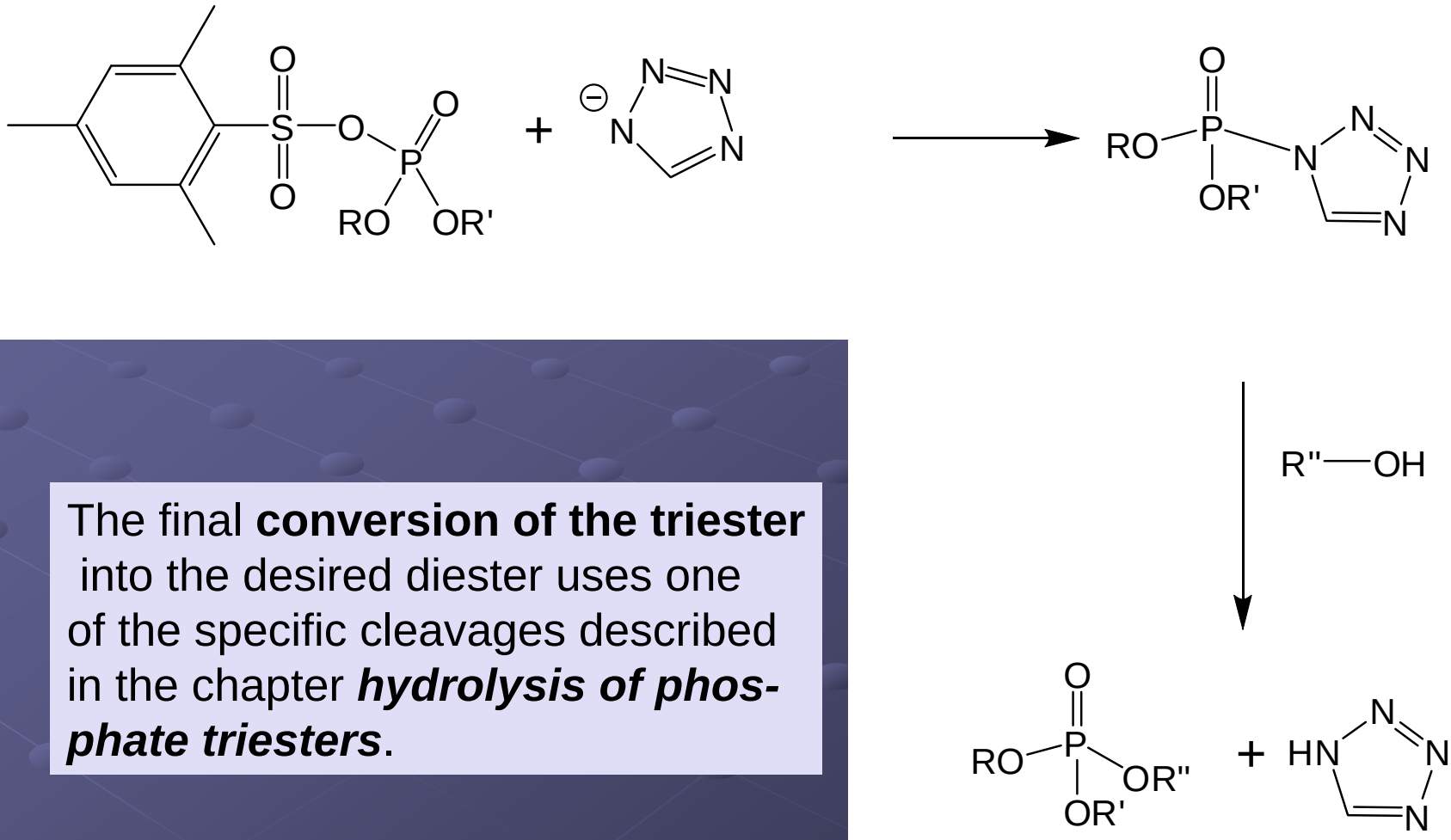
Syntheses via phosphate triesters

Mesitylenesulphonyl chloride is a faster and more efficient condensing agent and so it formed the **basis of the first triester synthesis** of oligonucleotides.



A **mixed anhydride** is produced in which the methyl groups of the mesitylene ring provide steric hindrance to reaction at sulfur and ensure reaction at phosphorus.

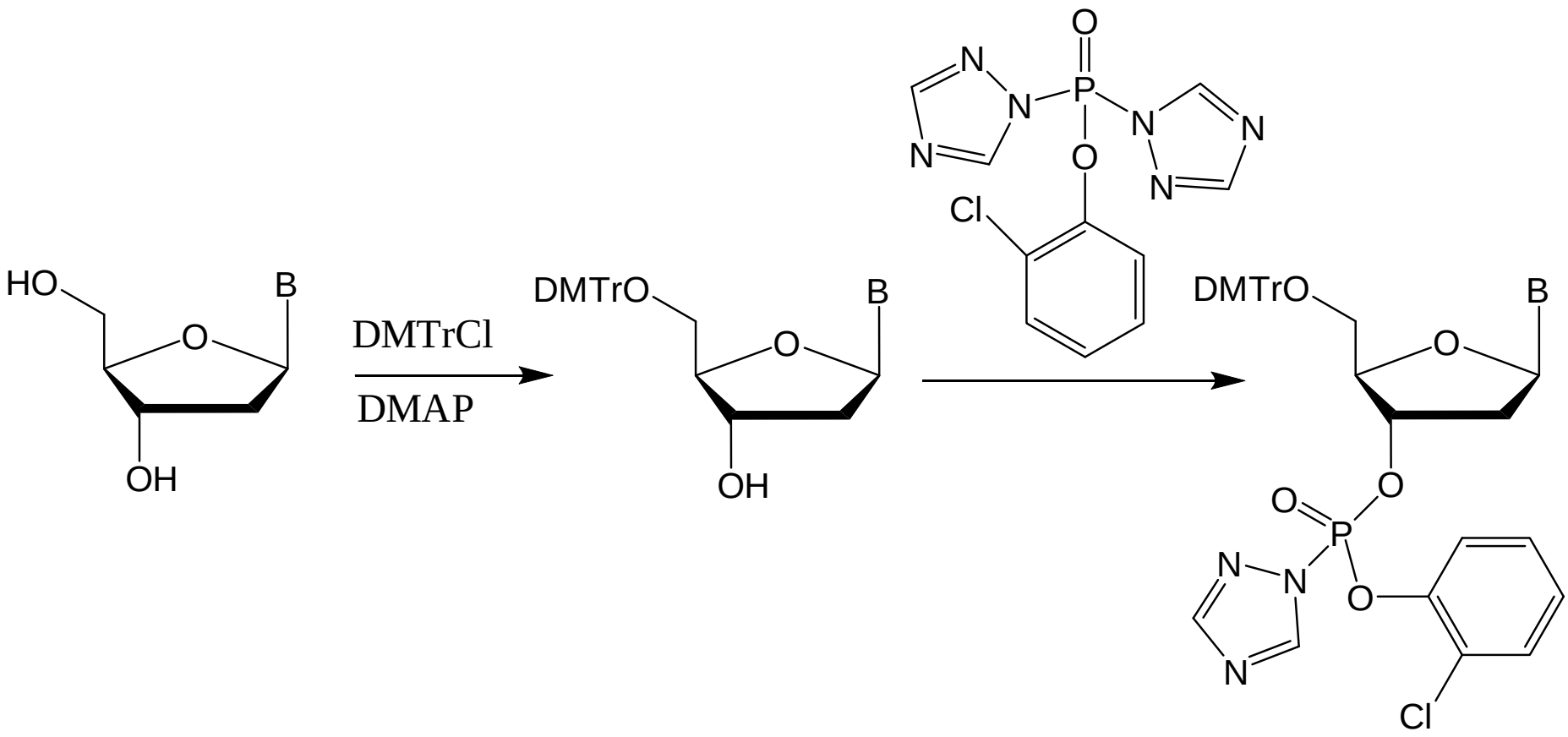
CHEMICAL SYNTHESIS OF BIOPOLYMERS



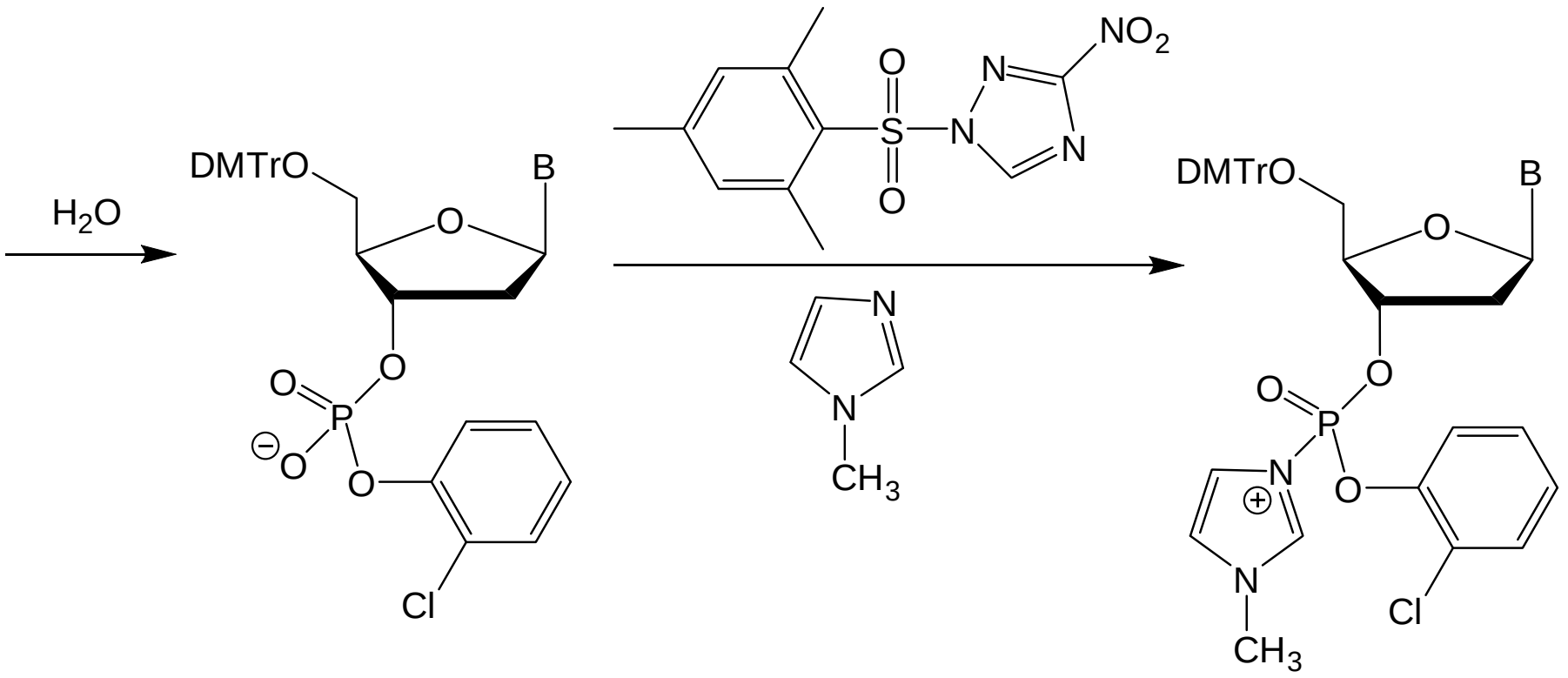
The final **conversion of the triester** into the desired diester uses one of the specific cleavages described in the chapter ***hydrolysis of phosphate triesters***.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

Today the **phosphotriester method** is particularly useful for **large-scale** (multi-gram) **synthesis** of **short oligonucleotides**.

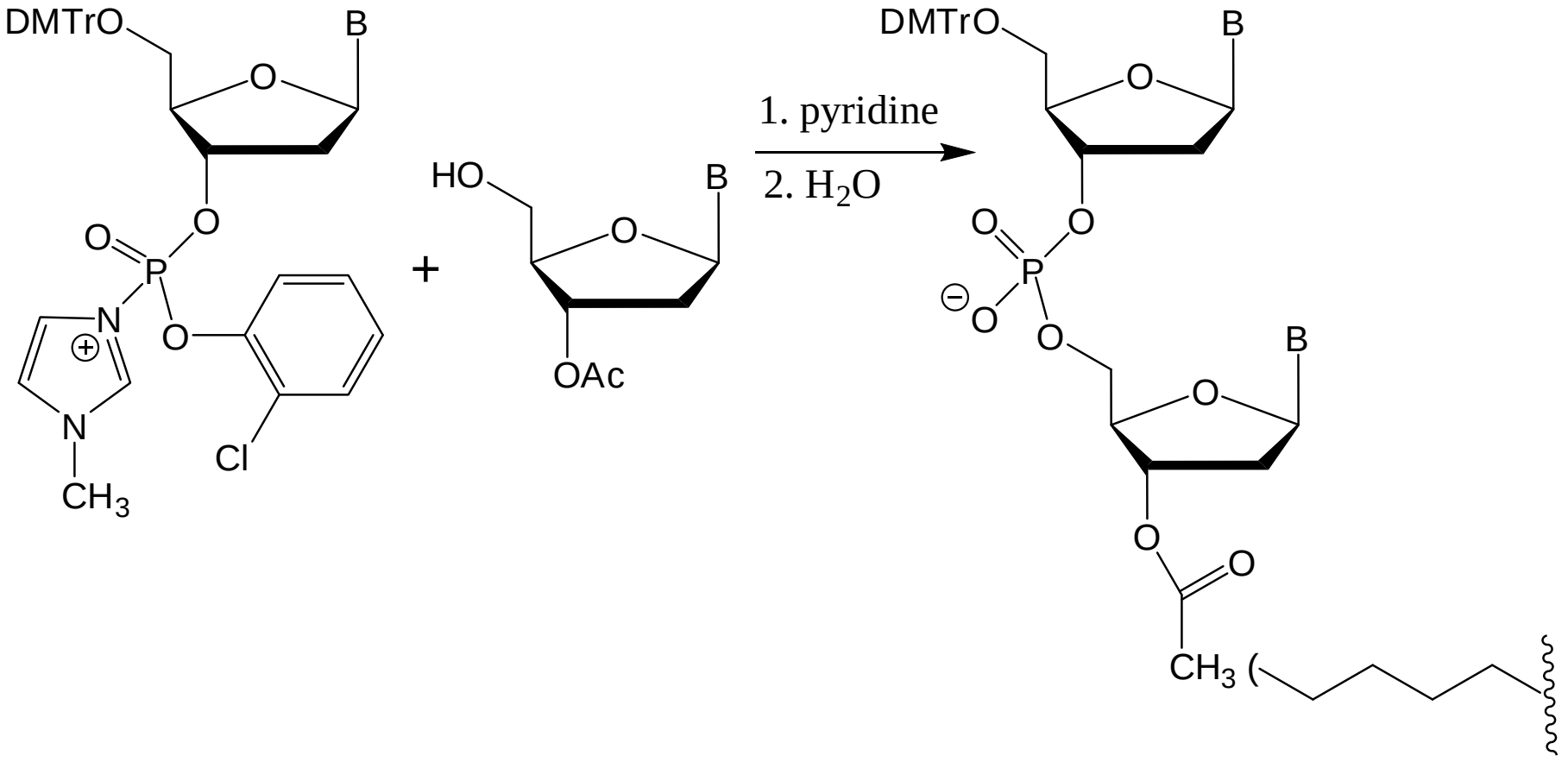


CHEMICAL SYNTHESIS OF BIOPOLYMERS



During coupling there is a **competitive reaction** (ca. 1%) of **sulphonylation of the 5'-hydroxyl group** by the coupling agent.

CHEMICAL SYNTHESIS OF BIOPOLYMERS



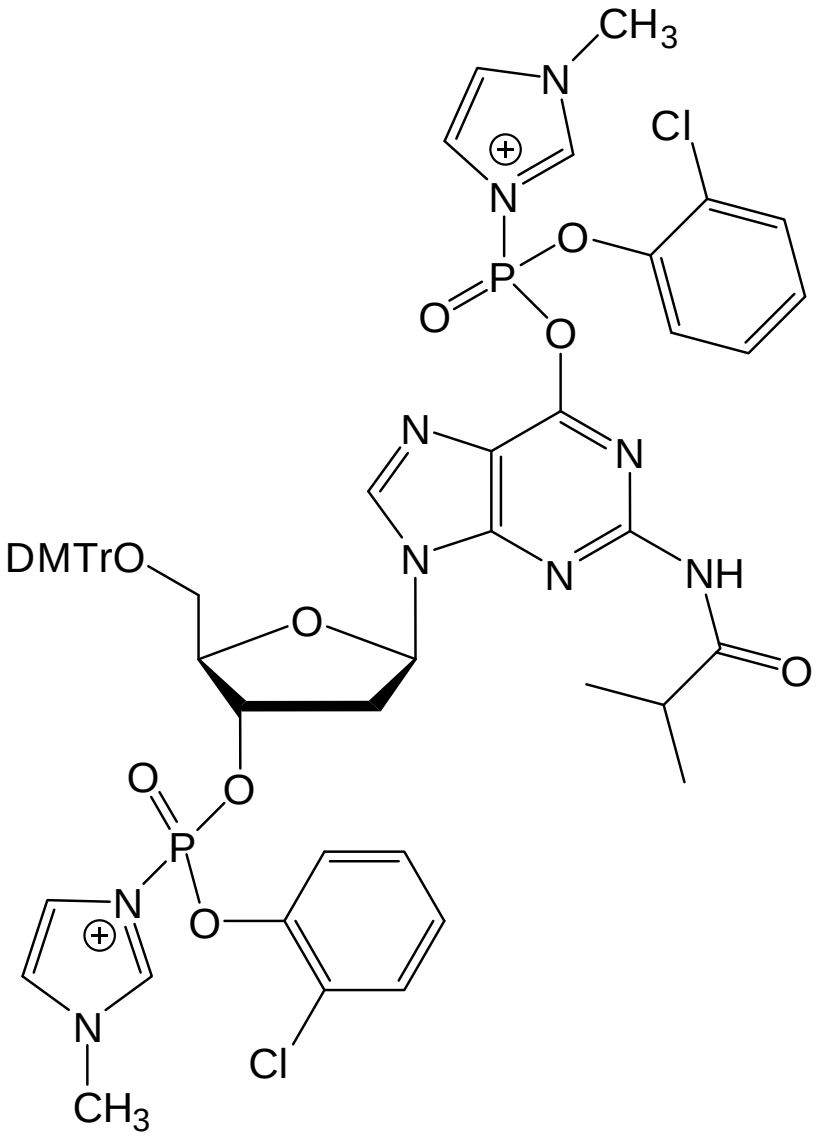
In the **early 1980s** this chemistry was applied to **solid-phase synthesis** (linkage to a solid support instead of the acetyl group).

CHEMICAL SYNTHESIS OF BIOPOLYMERS

A further **side-reaction** of this procedure is the **phosphorylation** at the 6-O-position of **guanine** residues unless an extra protecting group is used.

6-O-phosphorylation is particularly serious because it leads to **chain branching** and eventually **chain degradation.**

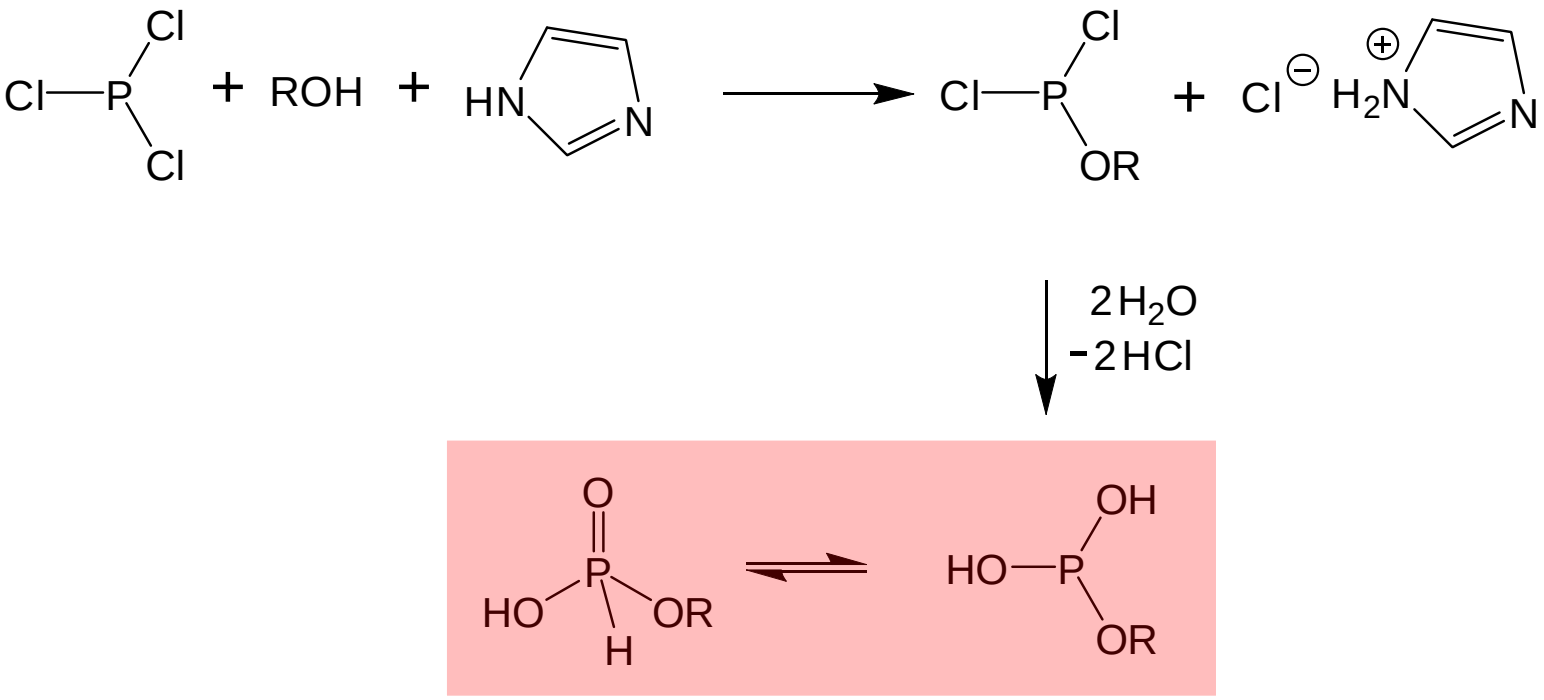
The two side-reaction limits the efficiency of phosphotriester coupling to **length of oligo-nucleotide of about 40 residues**.



CHEMICAL SYNTHESIS OF BIOPOLYMERS

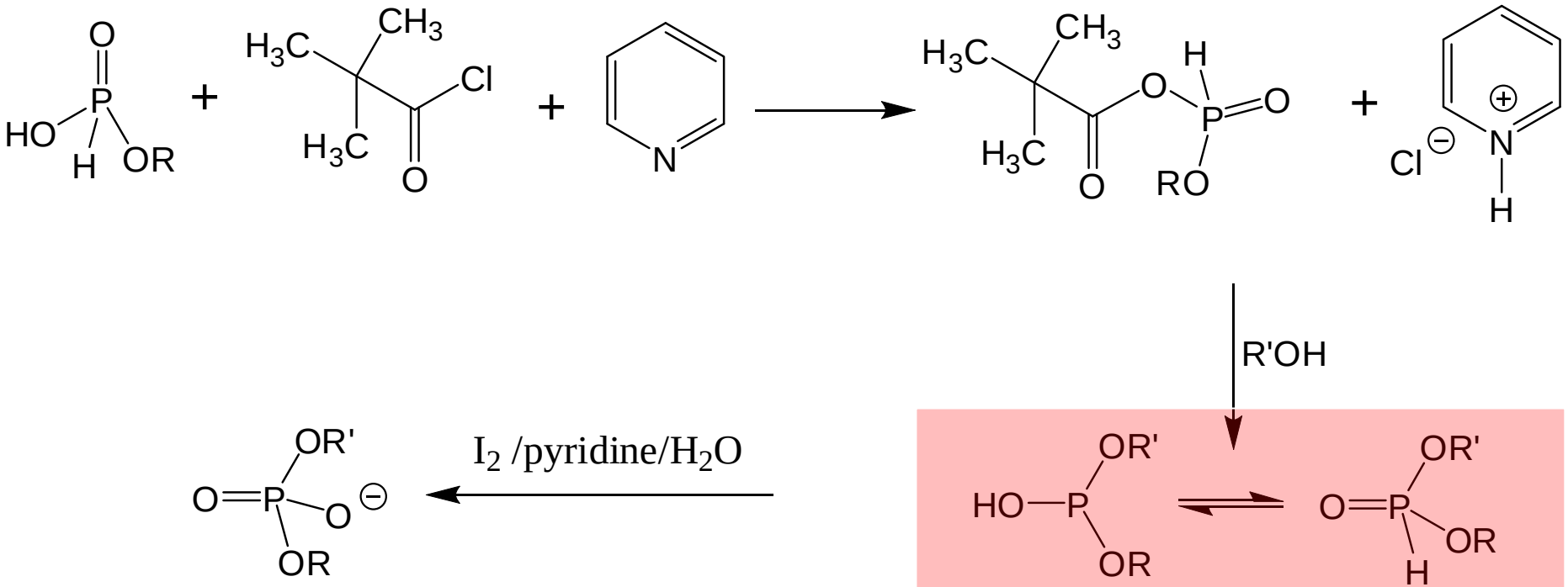
Syntheses via H-phosphonate diesters

The **H-phosphonate monoesters** of protected 3'-nucleosides are readily prepared using PCl_3 and excess imidazole followed by mild hydrolysis.



CHEMICAL SYNTHESIS OF BIOPOLYMERS

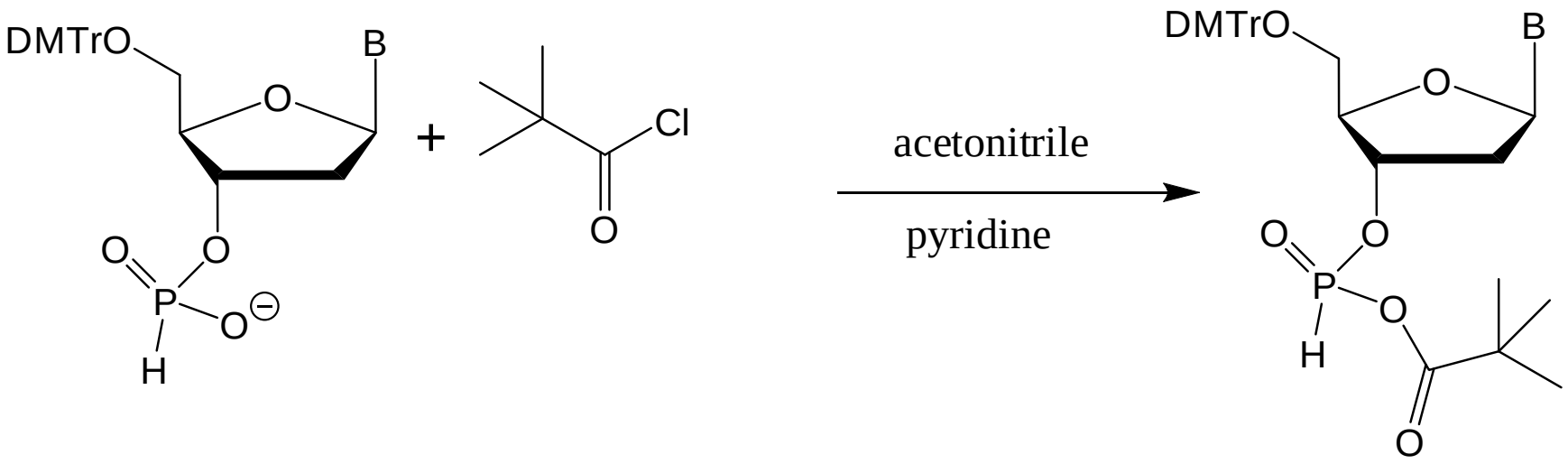
The **intermediate are rapidly and efficiently activated** by a range of condensing agents, such as **pivaloyl chloride** to form a mixed anhydride.



The **dinucleotide H-phosphonate** formation can be repeated many times before a **single oxidation step finally** provides the phosphate diester.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

Although the origins of this chemistry lie with TODD at all. in the 1950s, it has emerged with high potential in oligonucleotide synthesis only very recently.



The 3'-O-(H-phosphonate) is **essentially a tetracoordinated P(III) species**, preferring this structure to the tautomeric tricoordinated phosphite monoester.

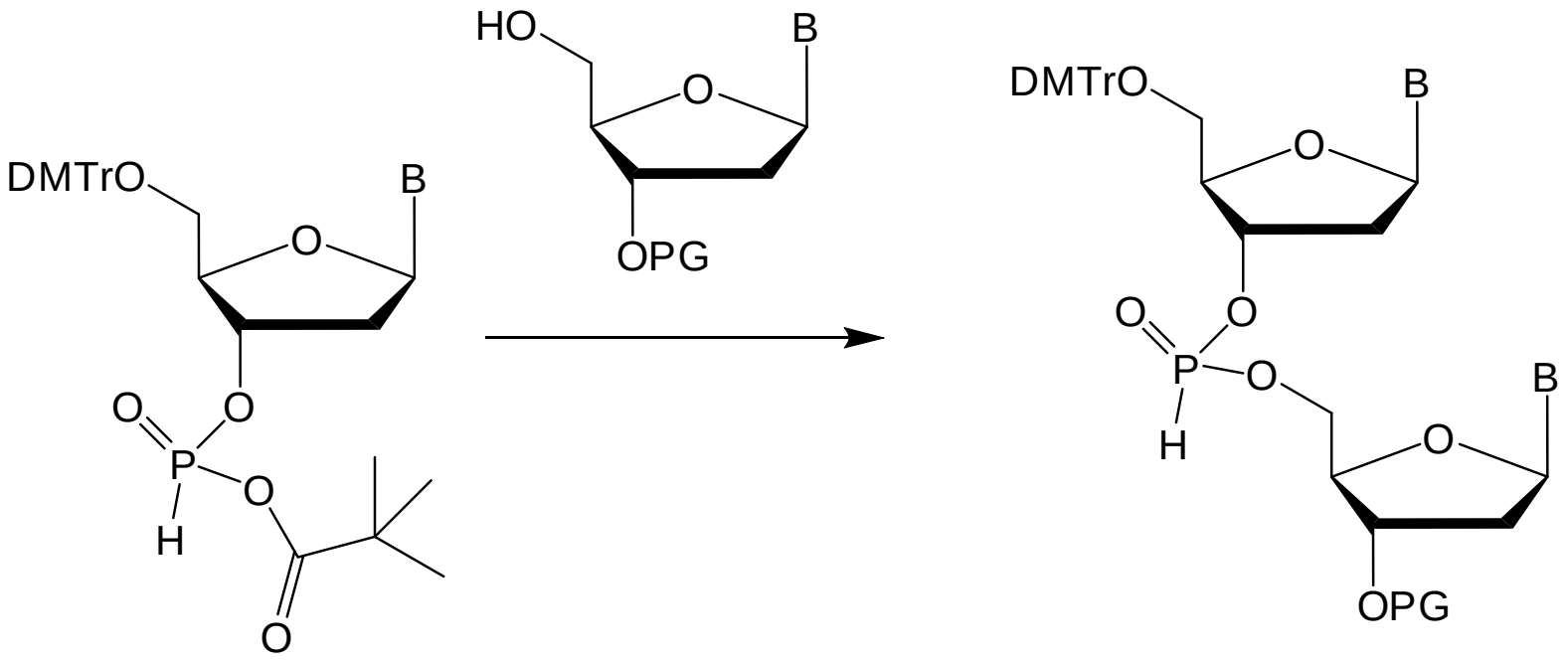
CHEMICAL SYNTHESIS OF BIOPOLYMERS

Lord Todd (he was knighted in 1954) was awarded the 1957 Nobel Prize in Chemistry for his work on the synthesis of nucleotides and nucleotide coenzymes. At the time that Todd began this work, no biologically significant purine or pyrimidine nucleoside had been synthesized. His major contribution in this area was the total synthesis of adenosine triphosphate (ATP).

Lord Alexander R. Todd

Nobel Prize 1957 in Chemistry

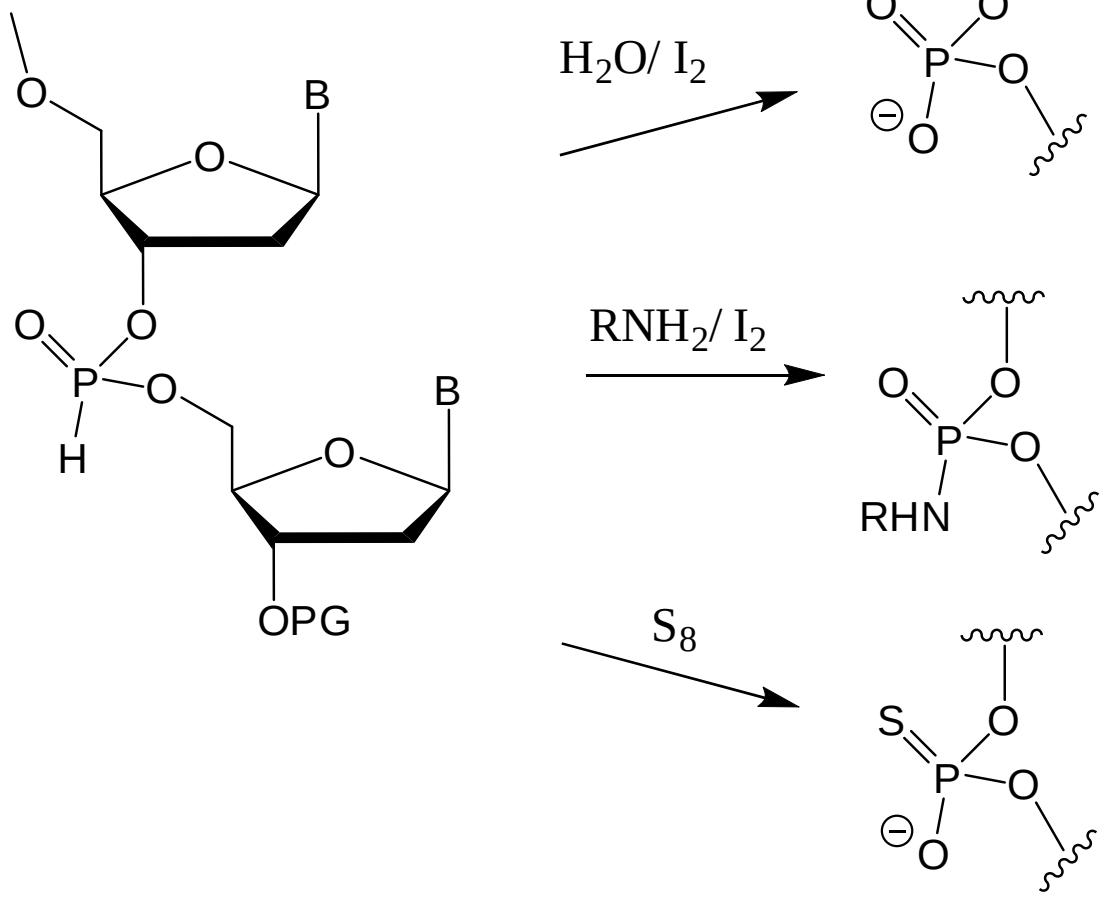
CHEMICAL SYNTHESIS OF BIOPOLYMERS



The resulting H-phosphonate diester is relatively inert to further phosphit-ylation such that the chain may be extended without prior oxidation.

Oxidation of all phosphorus centres is carried out simultaneously at the end of the synthesis.

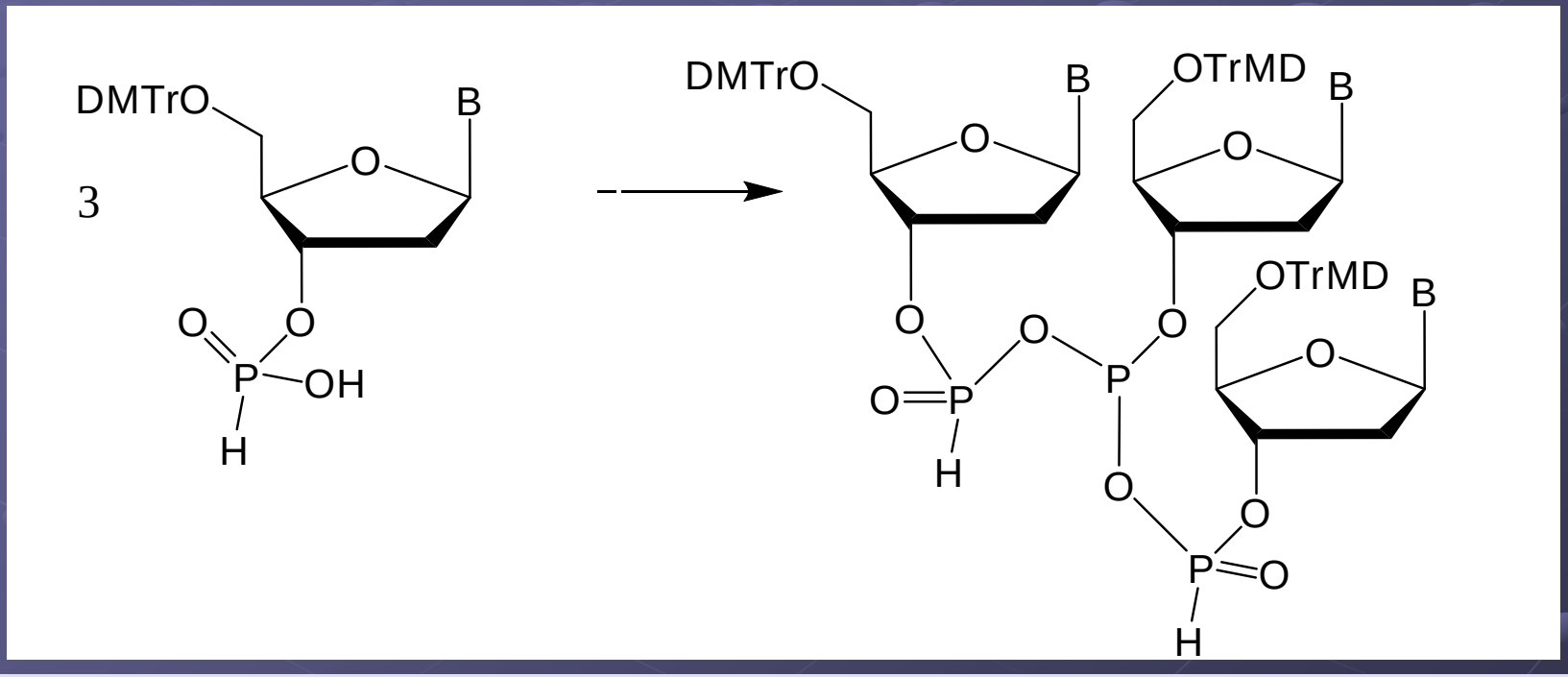
CHEMICAL SYNTHESIS OF BIOPOLYMERS



The general base catalysis of oxidation allows to introduce other nucleophils than water to give a range of oligonucleotide analogues.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

A **serious side-reaction** occurs if an H-phophonate is premixed with activating agent before coupling. The H-phosphonate rapidly dimerizes to form a symetrical phosphite anhydride.



The anhydride reacts then with a hydroxy group to form a branched trinucleoside derivative. The complete elimination of this side-reaction is probably impossible and may account for the marginally lower yields.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

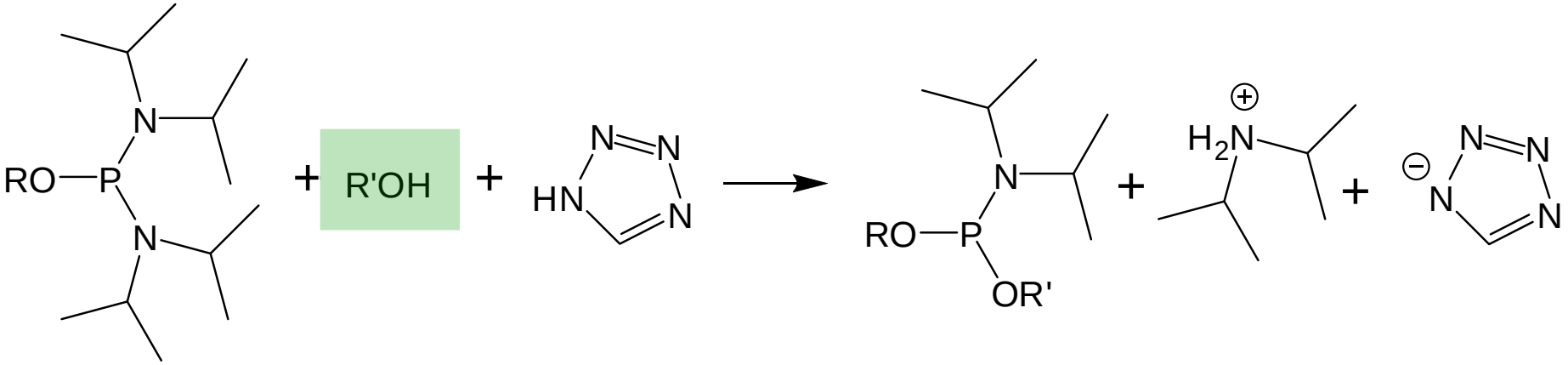
Phosphite triester coupling (phosphoamidite chemistry)

This type of procedure developed by **Caruthers** and co-workers in the early 1980s transformed oligonucleotide synthesis from a manual procedure into a commercialized process performed by machine.

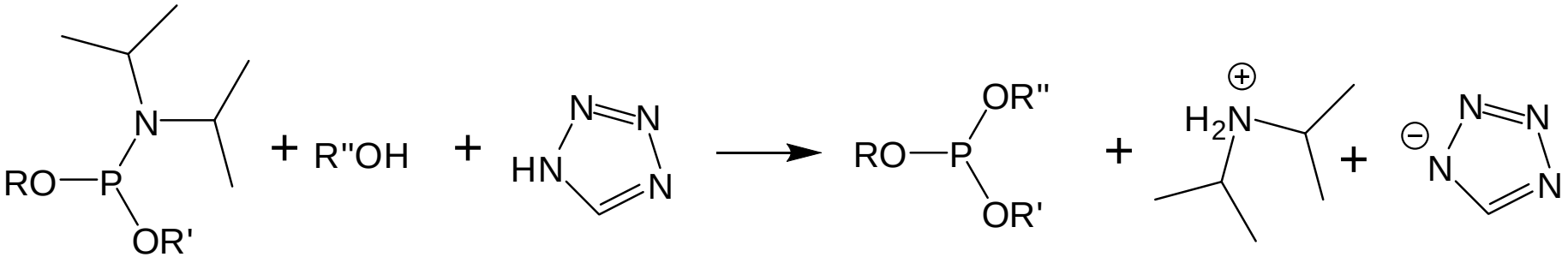
The **P(III)triester route** to oligonucleotides uses the intrinsically greater reactivity shown by **PCl_3** compared to **$O{=}PCl_3$**, to achieve faster coupling steps.

Prof. Dr. Marvin Caruthers

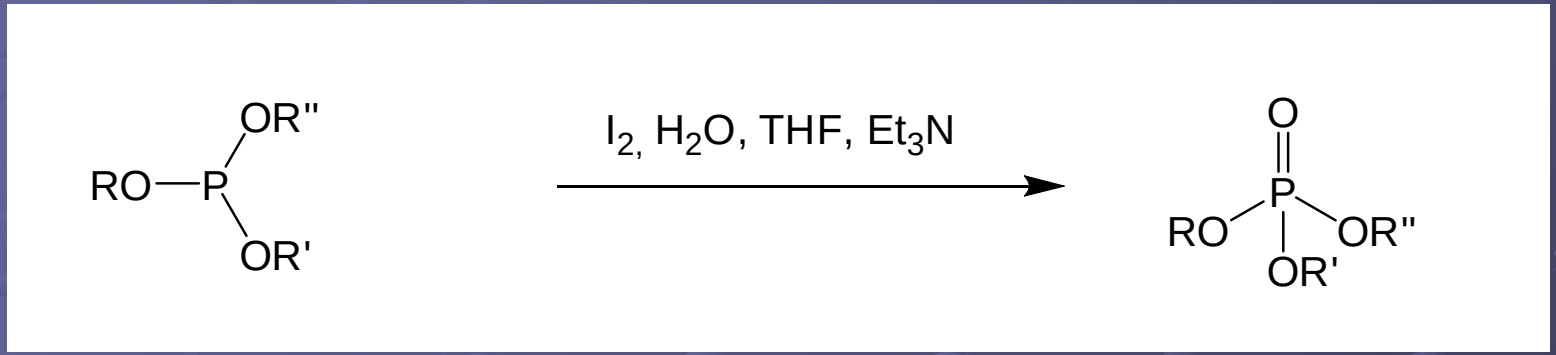
CHEMICAL SYNTHESIS OF BIOPOLYMERS



R = permanent protecting group; **R‘** = nucleoside



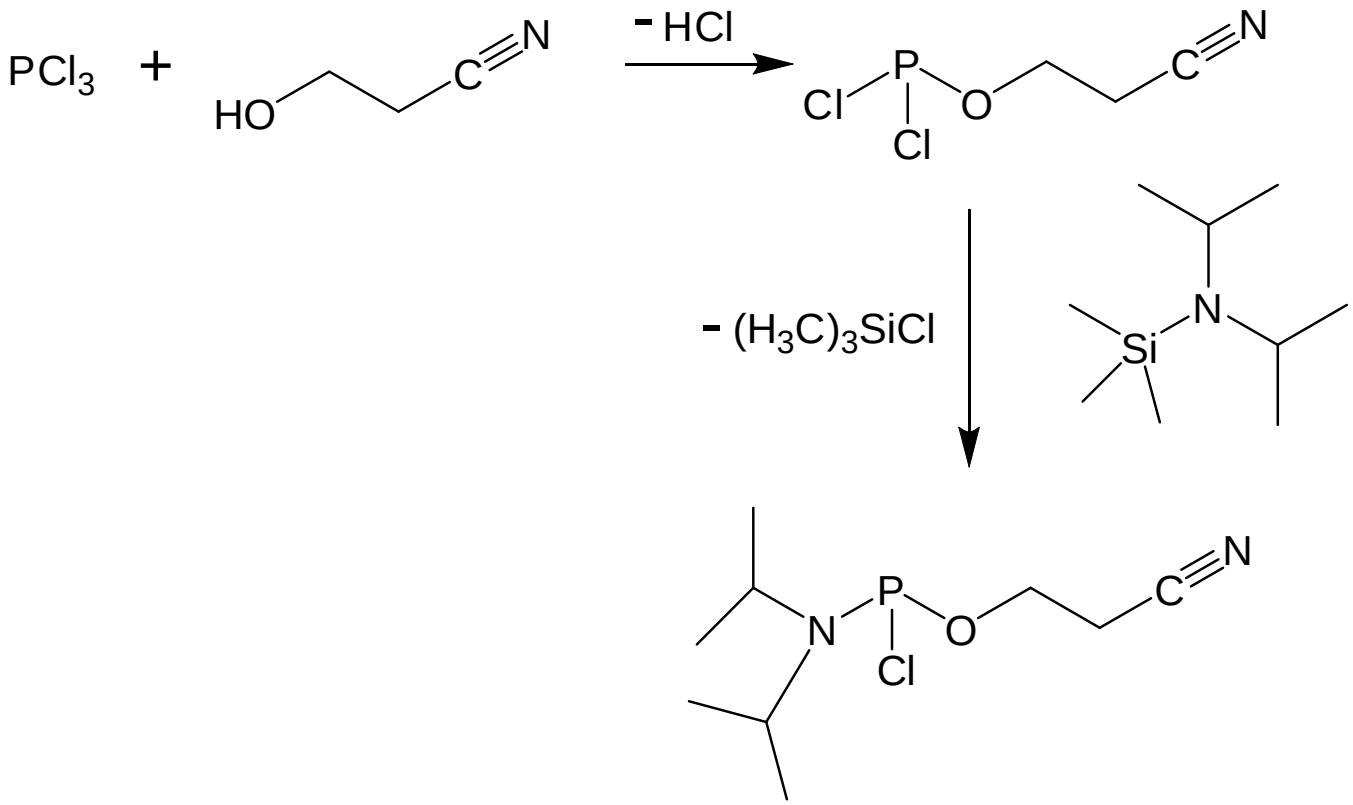
CHEMICAL SYNTHESIS OF BIOPOLYMERS



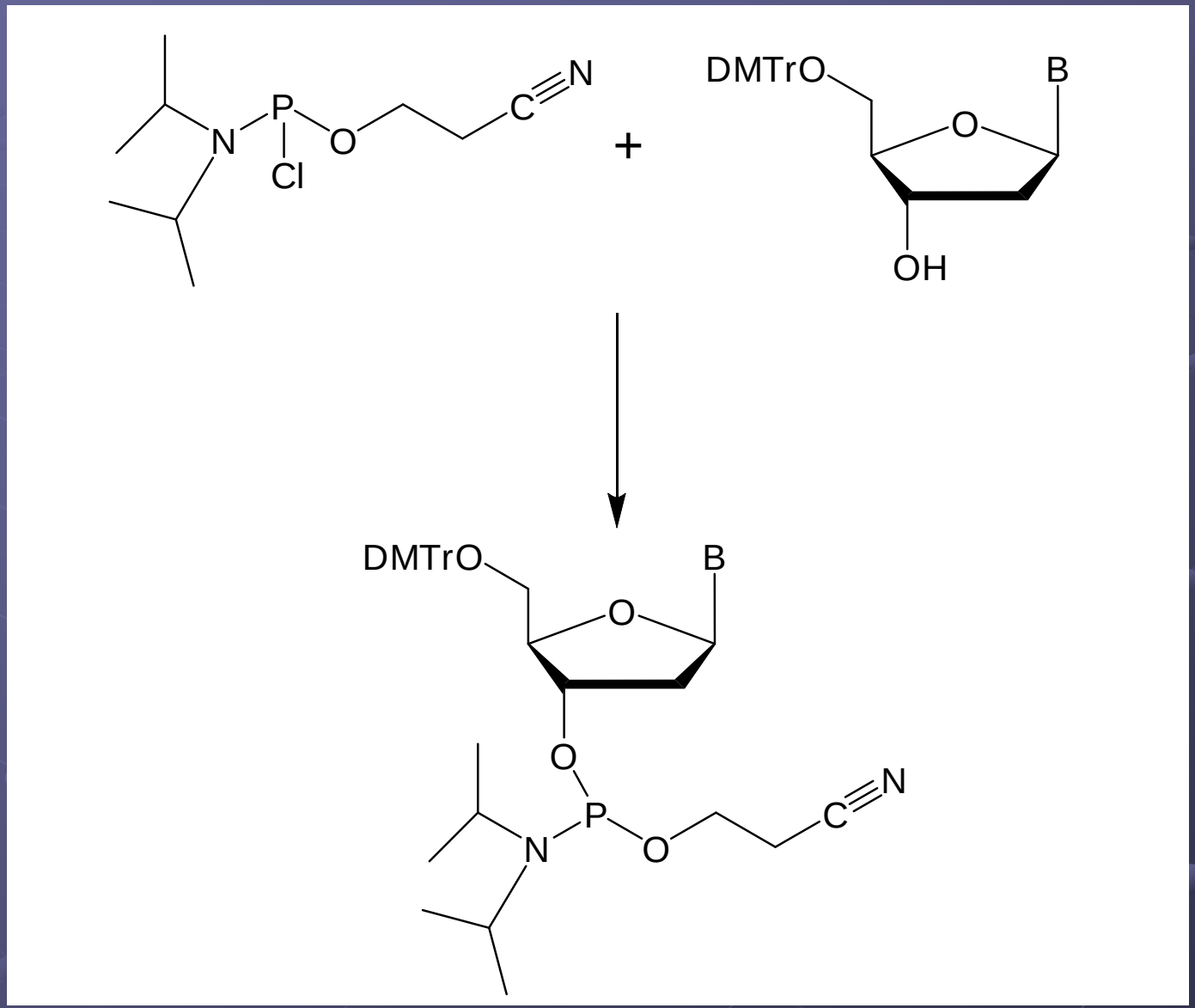
The resulting unstable phosphite triester must be oxidized immediately to give the stable phosphate triester in a process that can be cycled up to 100 times on a solid-phase support.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

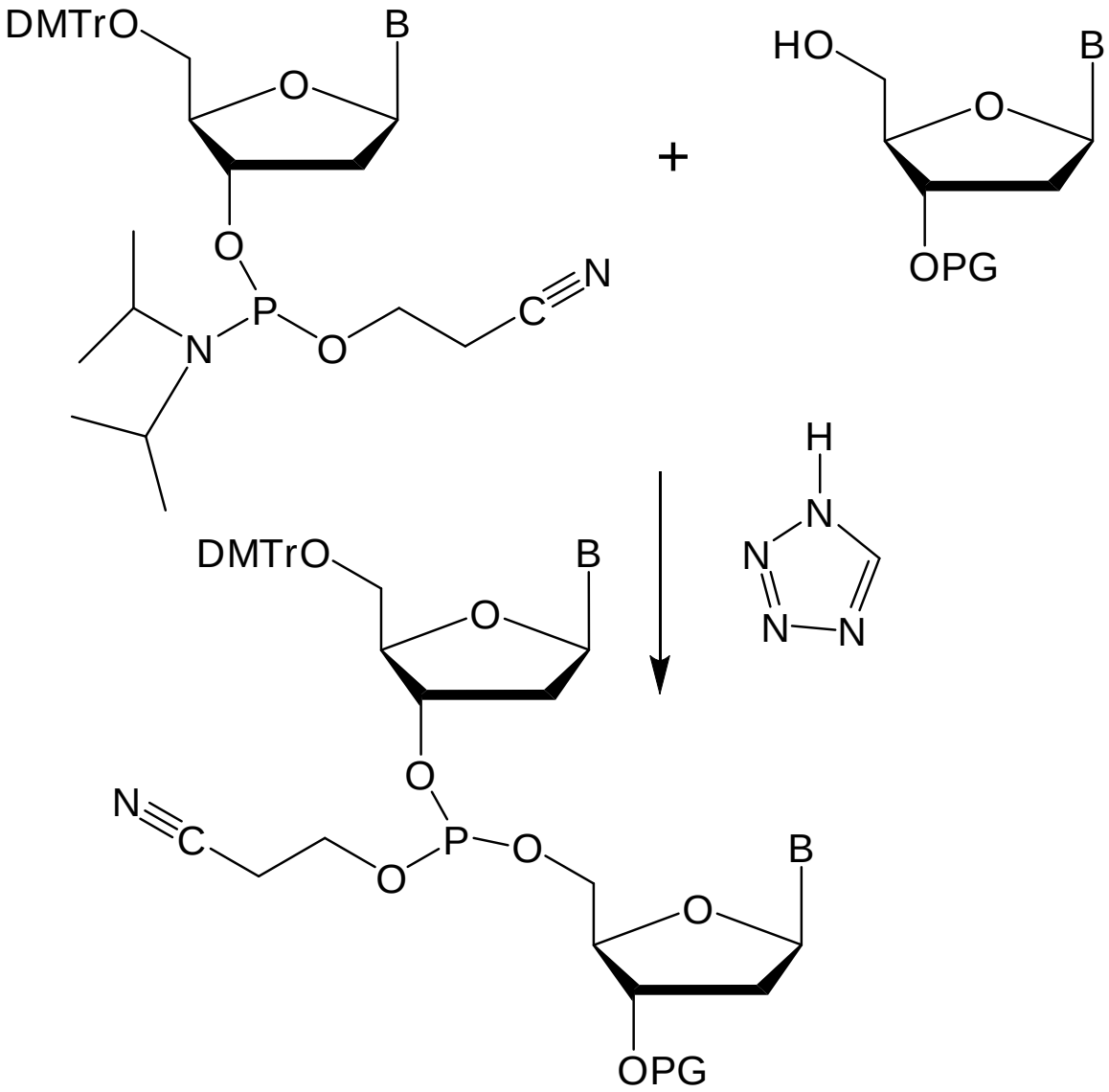
Synthesis of the phosphite component



CHEMICAL SYNTHESIS OF BIOPOLYMERS



CHEMICAL SYNTHESIS OF BIOPOLYMERS



CHEMICAL SYNTHESIS OF BIOPOLYMERS

A phosphoamidite requires **protonation at nitrogen** to transform it into a highly reactive phosphitylating agent.

Tetrazole is just sufficiently acidic to do this without causing loss of the DMTr group.

The product of coupling is a dinucleoside phosphite, which must be **oxidized with iodine** to the phosphotriester before proceeding with chain extension.

The efficiency of **coupling is extremely high (> 98%)** and the only major side reaction is phosphitylation of the *O*-6 position of guanosine.

The phosphoamidite method is the procedure of choice for **small-scale (microgram to milligram) synthesis** of **oligodeoxyribonucleotides** up to **150 residues** long.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

1.6 Solid-phase synthesis

The essence of solid-phase synthesis is the use of a **heterogeneous coupling reaction** between a deoxynucleotide derivative in solution and another residue bond to an insoluble support.

This has the advantage that a large excess of the soluble deoxynucleotide can be used to force the reaction to high yield.

The **support-bound product oligonucleotide can be removed** from the excess of reactant mononucleotide **simply by filtration and washings**.

Other reactions (**deprotection, capping**) can also be carried heterogeneously and reagents removed similarly.

This process is **faster than conventional separation technique** in solution and forms the **basic of mechanization**.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

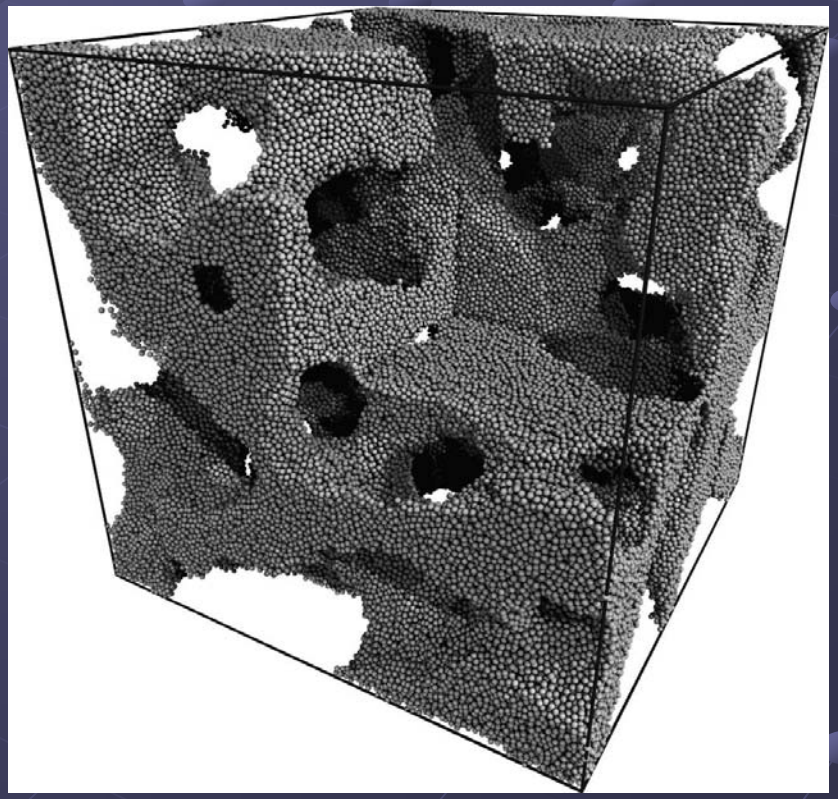
Properties of the support (solid-phase)

For solid-phase oligonucleotide synthesis, only **controlled pore glass (CPG)** and **polystyrene** have proved to be generally **useful**.

CPG-beads are ideal in being **rigid** and **non-swellable**.

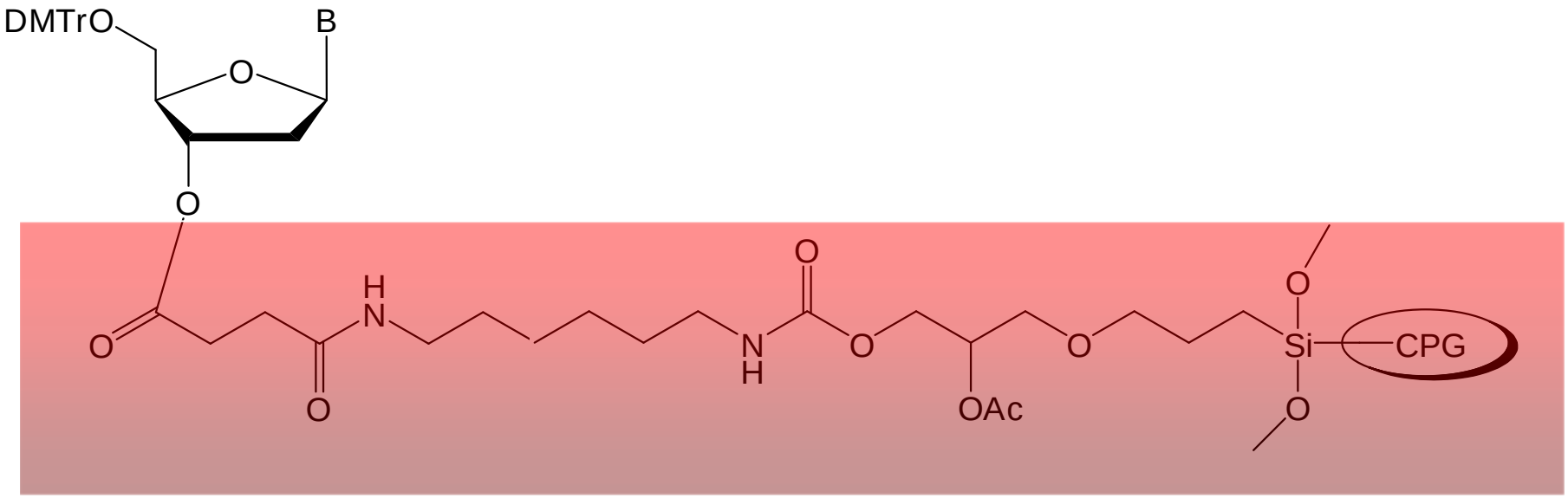
Porosity of CPG as well as the **loading density of spacer** has an influence on the outcome of the reaction.

The **spacer** is used to extend the sites away from the surface and **ensure accessibility** to all reagents.



CHEMICAL SYNTHESIS OF BIOPOLYMERS

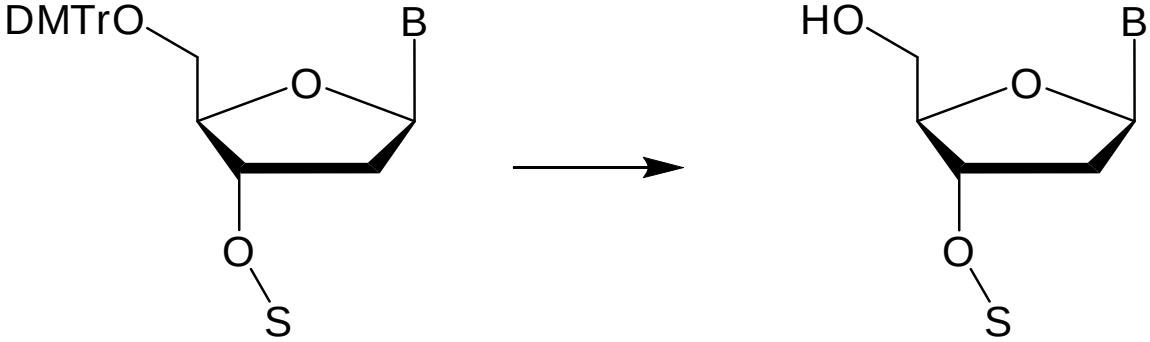
Attachment of the first deoxynucleoside to the support



The 5'-*O*-DMTr-derivative bearing a 3'-*O*-(4-nitrophenyl) succinate residue is attached to the support by reaction with the amino group.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

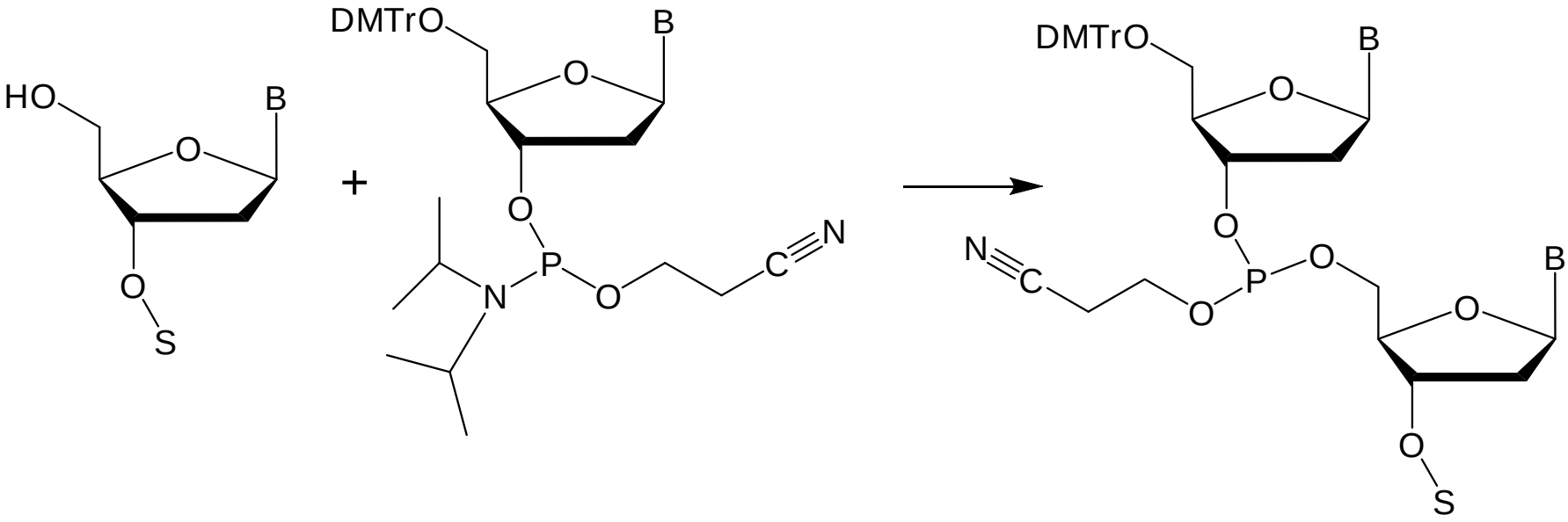
Step 1



Procedure:
Trichloroacetic acid (TCA), CH_2Cl_2

CHEMICAL SYNTHESIS OF BIOPOLYMERS

Step 2

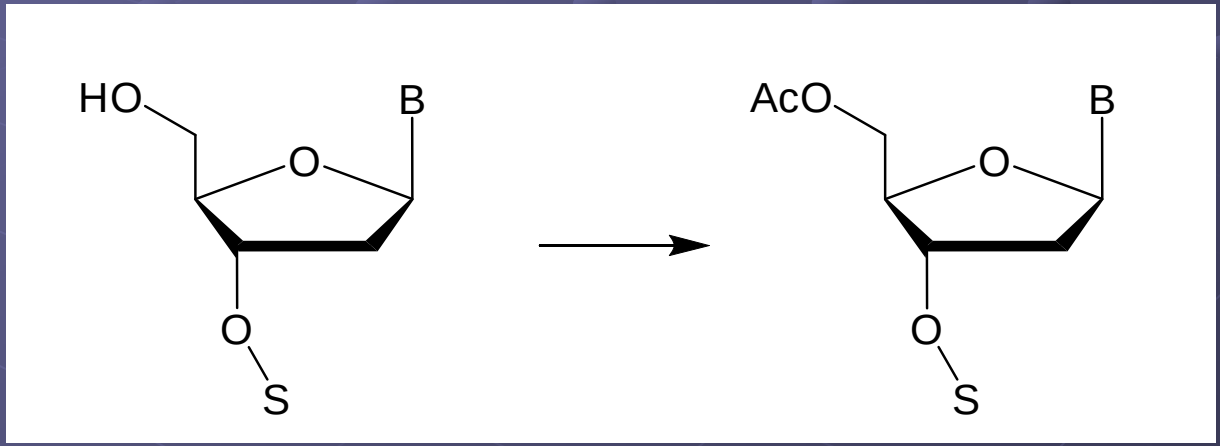


Procedure:
Tetrazole, CH_3CN

CHEMICAL SYNTHESIS OF BIOPOLYMERS

Step 3 (capping)

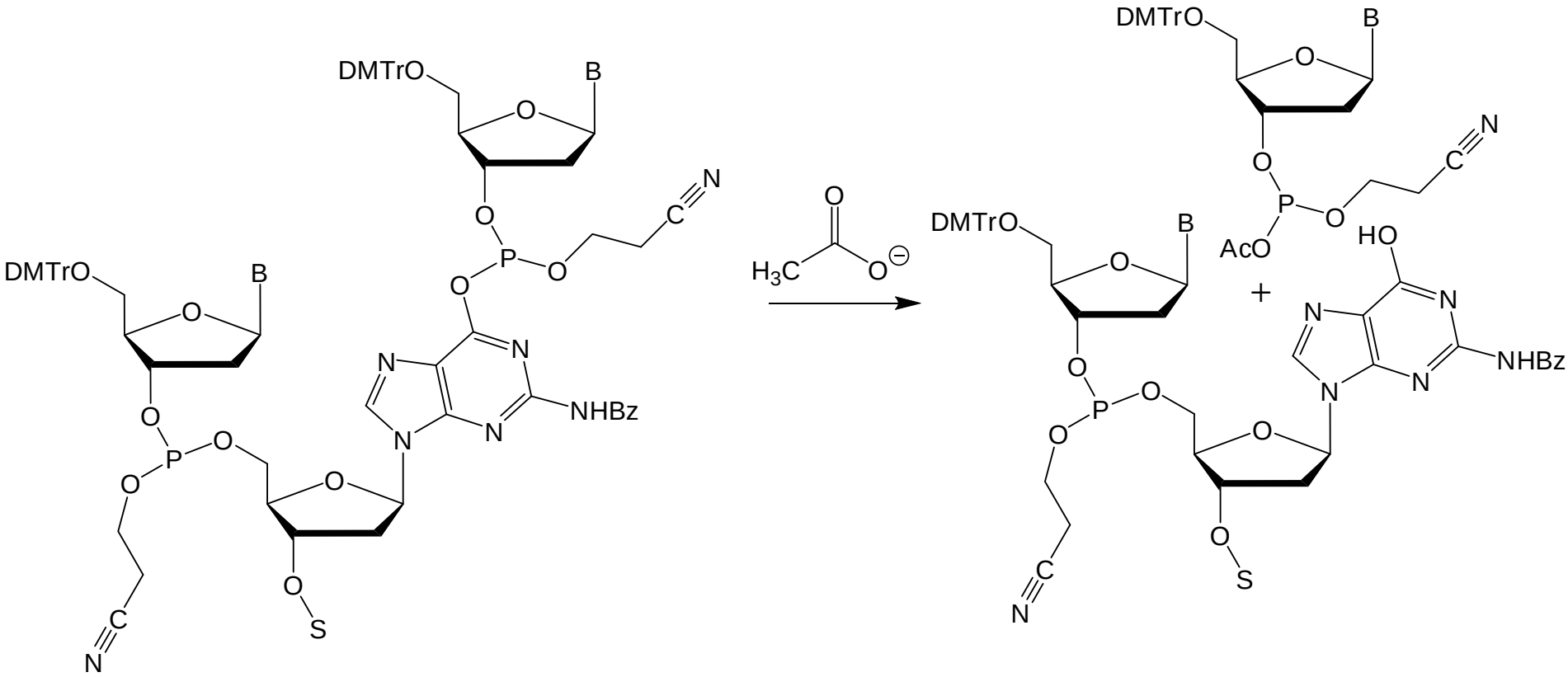
Capping is a safety step introduced to block chains which are not reacted during the coupling reaction in order to limit the number of failure sequences.



Procedure:
Acetic anhydride, *N*-methylimidazole

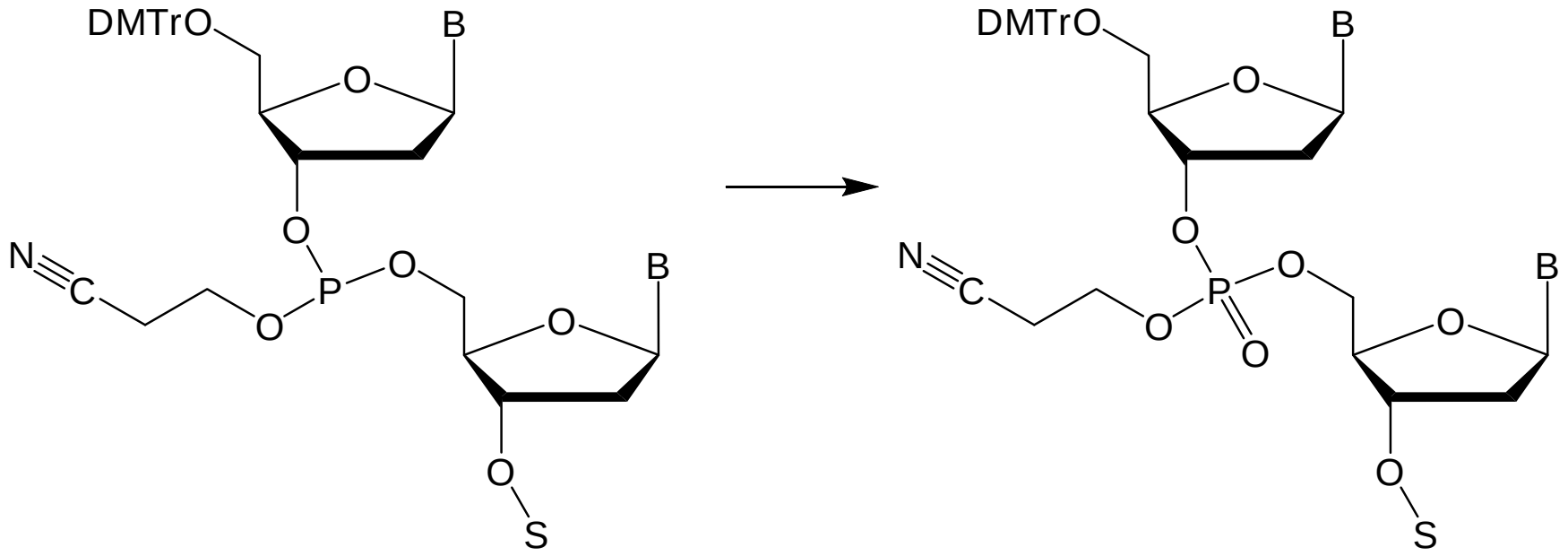
CHEMICAL SYNTHESIS OF BIOPOLYMERS

Fortunately, **capping** also **reverses the phosphitylation of the *O*-6 position of guanosine.**



CHEMICAL SYNTHESIS OF BIOPOLYMERS

Step 4 (oxidation)



Procedure:
I_2,H_2O, pyridine or 2,6-lutidine

Step 1 (next cycle)

CHEMICAL SYNTHESIS OF BIOPOLYMERS

Deprotection and removal of oligonucleotide from the support

The **5'DMTr group** is removed with trichloroacetic acid.

The **2-cyanoethyl group** is removed by β-elimination using aqueous trietylamine or ammonia.

All **heterocyclic base protecting groups** are removed with concentrated aqueous ammonia.

The **succinate linkage** is cleaved with mild aqueous base, that means, the last **three deprotecting steps are carried our simultaneously**.

Finally, the oligonucleotides are purified by **polyacrylamide gel electro-phoresis (**using unit charge differences: 50 – 150 residues but only for small scale up to 1 mg**)** and **high performance liquid chromatography ion exchange chromatography** and/or **reverse phase chromatography.**

CHEMICAL SYNTHESIS OF BIOPOLYMERS

1.7 Synthesis of oligoribonucleotides

All **principles and conclusions** mentioned before in this chapter hold for the synthesis of **DNA as well as RNA**.

What makes things different is the fact that **ribonucleotides have a 2'-OH group** that requires additional protection.

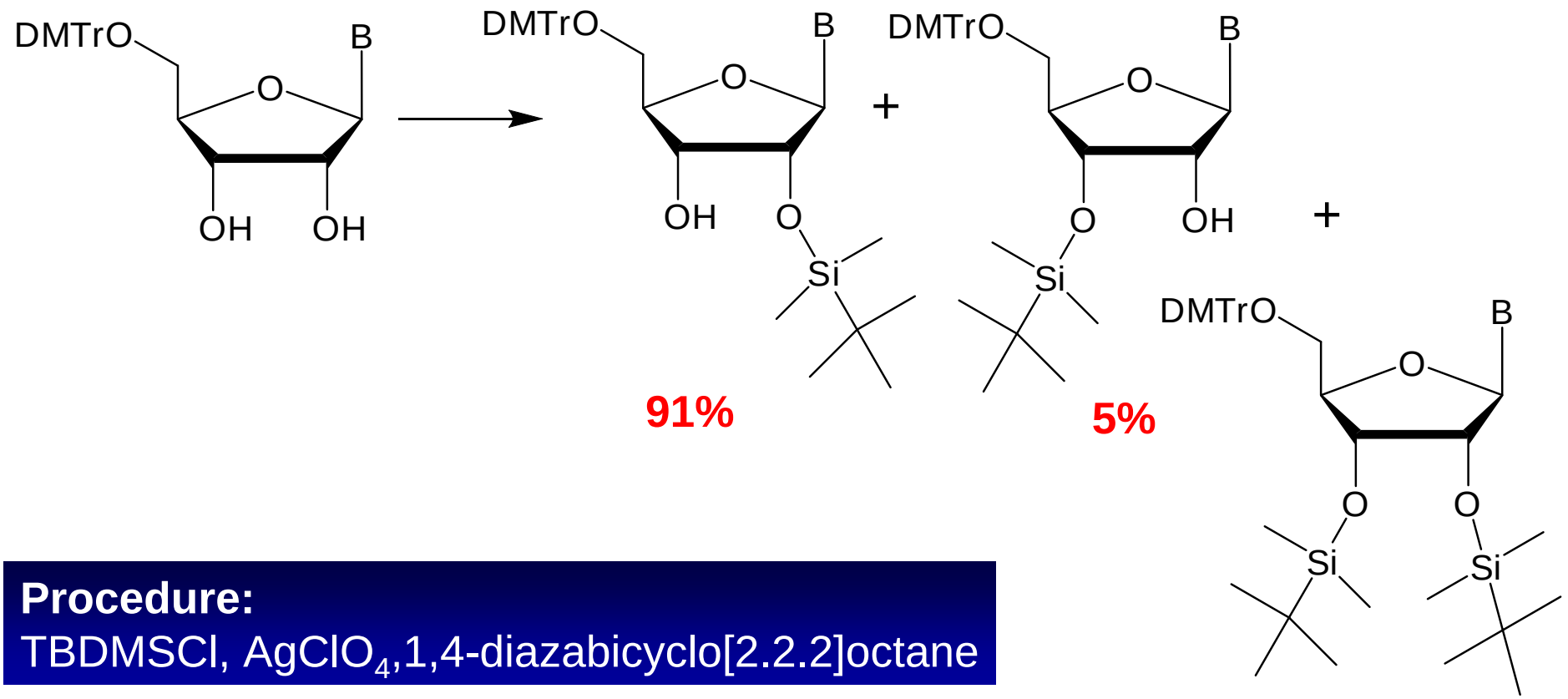
The choice of the 2'-protecting group is a crucial matter to **avoid base-** or **acid-catalyzed cleavage** of the RNA backbone or **migration** of the internucleotide linkages.

Nowadays, **2'-TBDMS** (2'-*O*-*tert*-butyldimethylsiliyl), **2'-TOM** (2'-O-(*tris* (isopropylsilyl)oxymethyl) and **2'-ACE** (2'-O bis(2-acetoxyethoxy)methyl) chemistry are used in parallel for the synthesis of oligoribonucleotides.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

2'O-TBDMS Method

The availability of the monomer building block is an important factor for a chosen synthesis strategy.

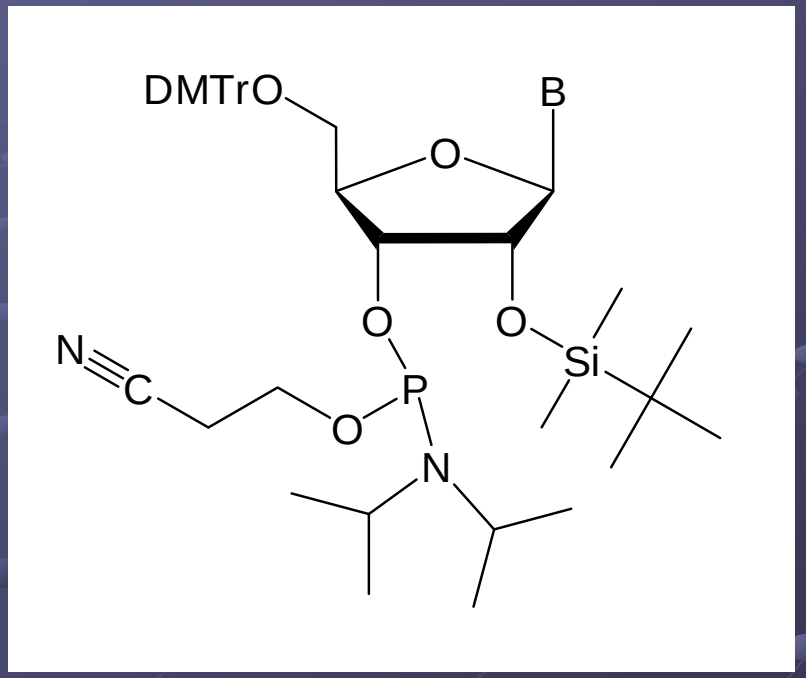


Procedure:
TBDMSCl, $AgClO_4$,1,4-diazabicyclo[2.2.2]octane

CHEMICAL SYNTHESIS OF BIOPOLYMERS

A problem consists in the ability of the **TBDMS group to migrate between 2'- and 3'-OH, particularly under alkaline conditions**.

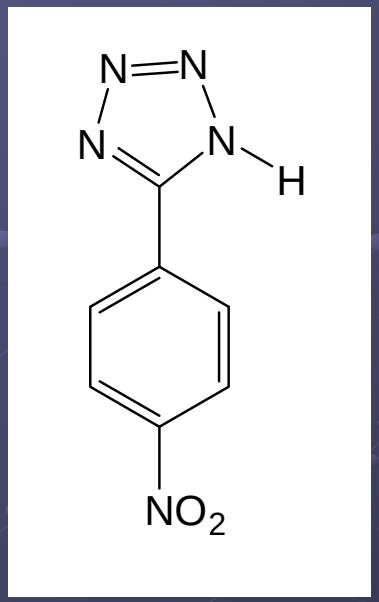
Another problem has been concerned with the **coupling efficiency** of 2'-*O*-TBDMS-ribonucleoside phosphoramidites.



Activation with 1*H*-tetrazole and **15 min coupling time** results in an average stepwise yield of about **98%** (compared to **>99% for deoxyoligonucleotides** using **2 min coupling time**).

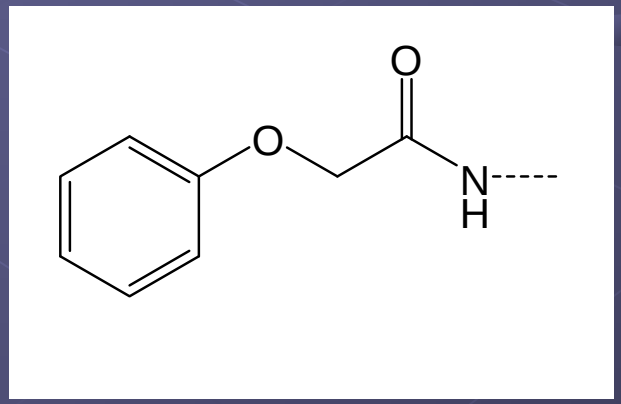
CHEMICAL SYNTHESIS OF BIOPOLYMERS

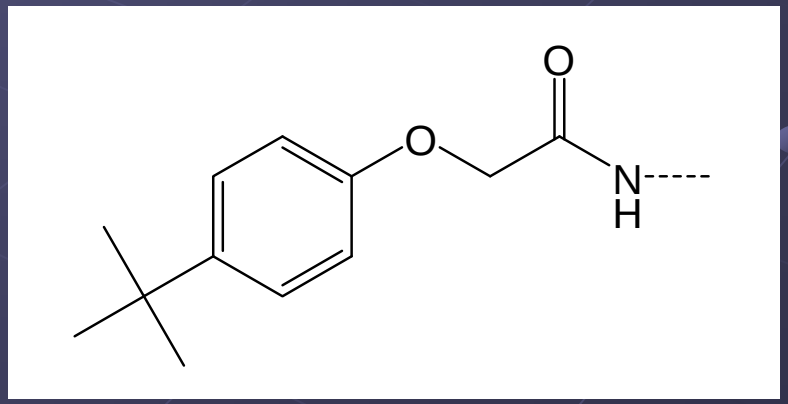
Using **5-*p*-nitrophenyl-1H-tetrazole as activator**, reaction with ribonucleotidephosphoramidites reached completion within 5 **min**.



After synthesis is completed, all protecting groups have to be removed.

In the presence of TBDMS group the more base-labile phenoxyacetyl- or *tert*-butylphenoxyacetyl groups are used for protection of adenine, guanine and cytosine.





CHEMICAL SYNTHESIS OF BIOPOLYMERS

The final deblocking step involves fluoride ion treatment to remove the TBDMS group. (water free tetrabutylammonium fluoride, 25 °C, 48 h)

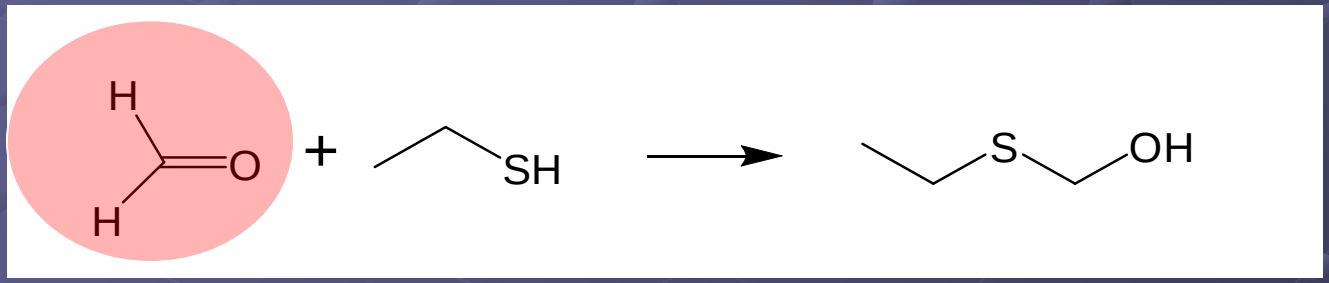
An improved method combines both the basic deprotection and desilylation reaction to a **one-pot procedure** by using a mixture of methylamine in ethanol followed by addition of triethylamine trihydrofluoride (Et_3N-3HF). After 3 h total deprotection is complete.

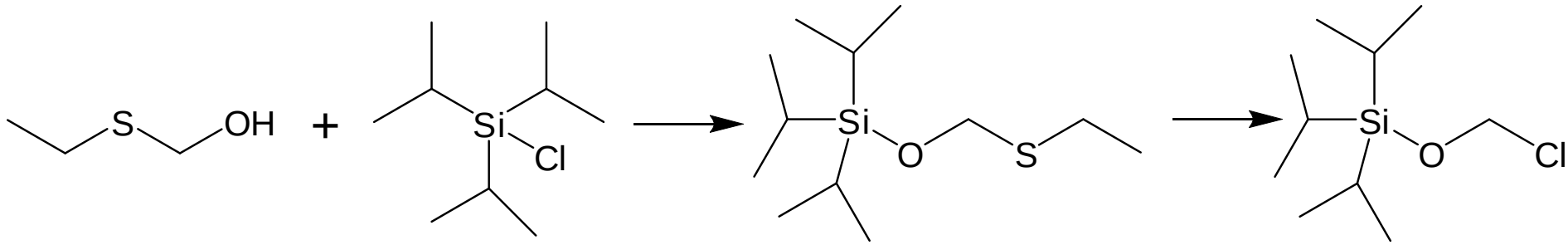
The RNA-pellet is dissolved in an appro-priate buffer and purified by ion exchange and/or reverse phase HPLC.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

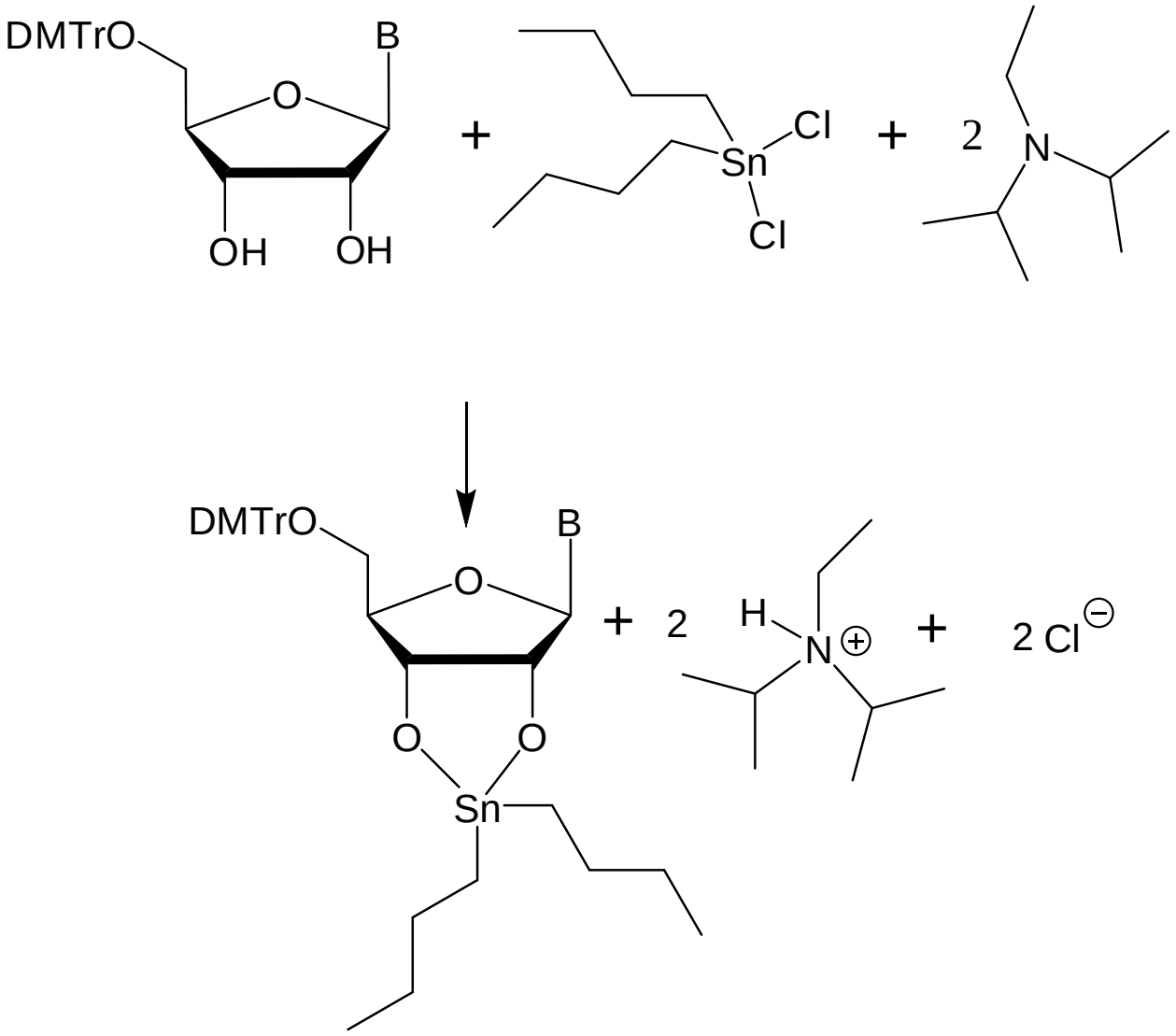
2'O-TOM Method

To overcome the relatively low coupling yield obtained with 2'-*O*-TBDMS groups and 1*H*-tetrazole, the 2'-*O*-[*tris*(isopropylsilyl)oxy]methyl group was introduced.

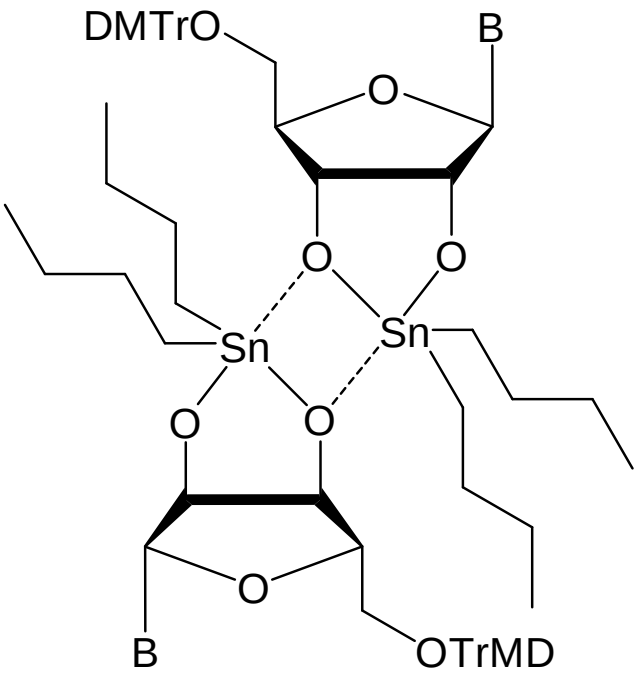




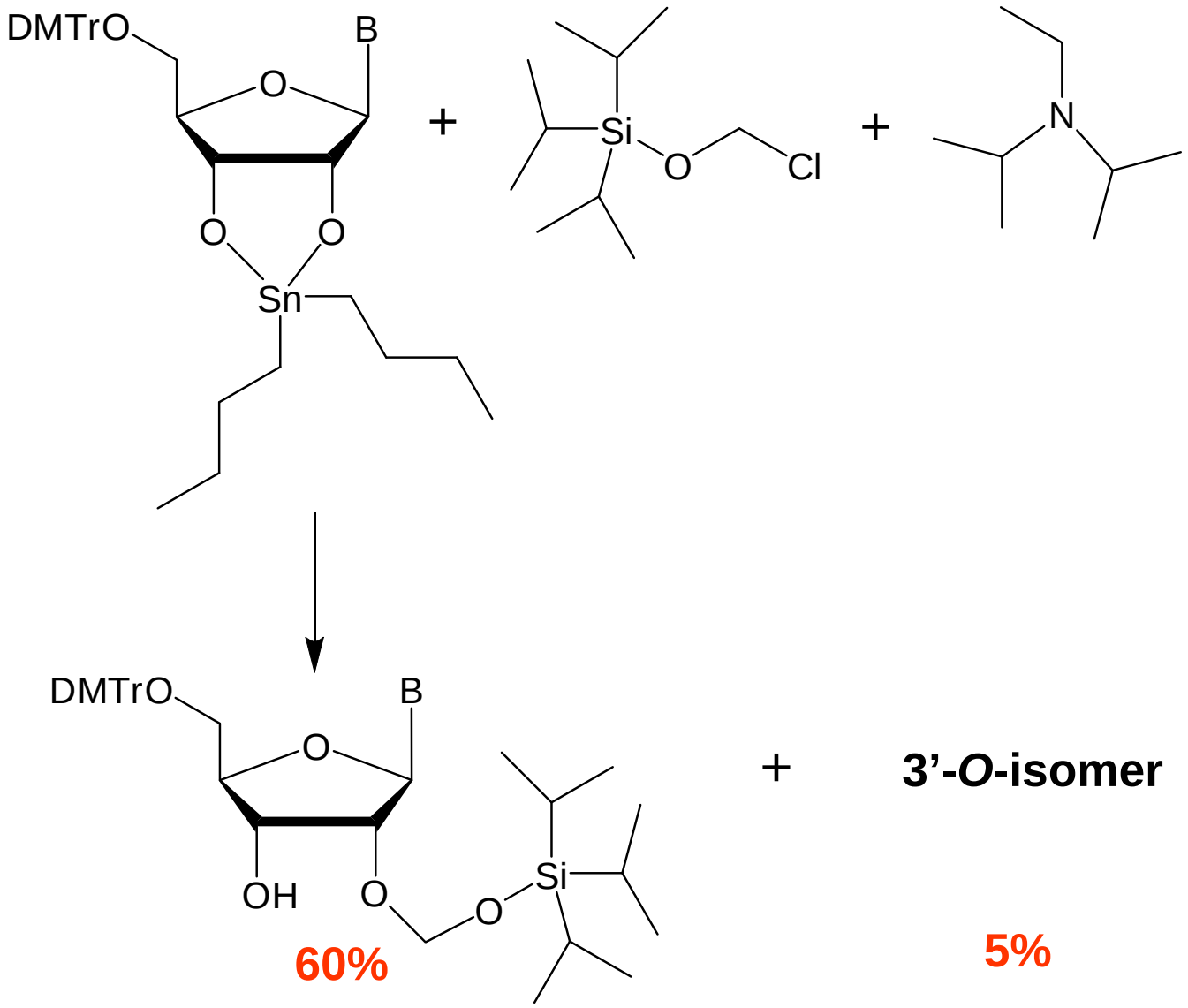
CHEMICAL SYNTHESIS OF BIOPOLYMERS



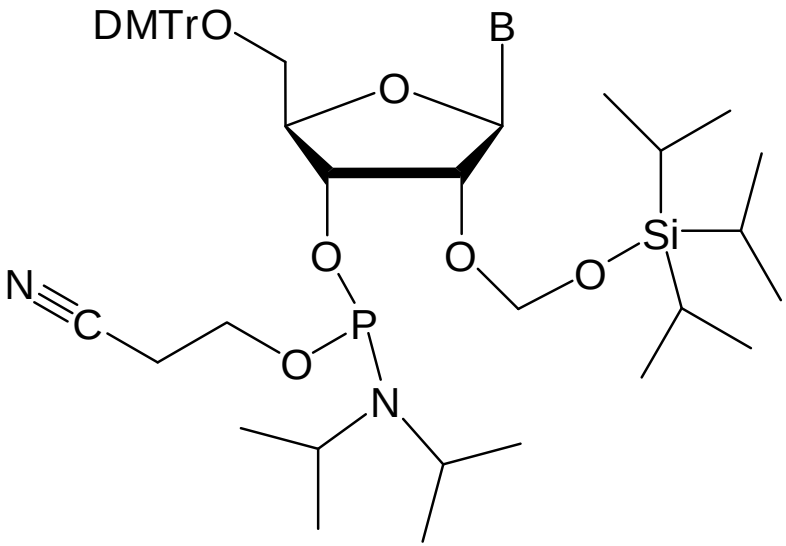
CHEMICAL SYNTHESIS OF BIOPOLYMERS



CHEMICAL SYNTHESIS OF BIOPOLYMERS



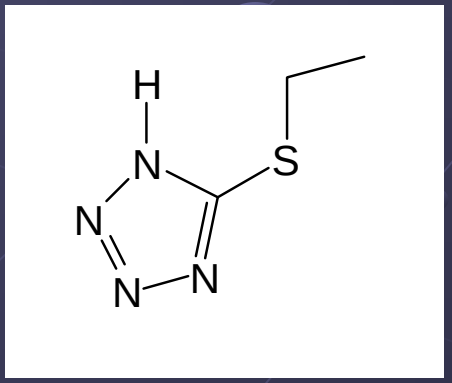
CHEMICAL SYNTHESIS OF BIOPOLYMERS



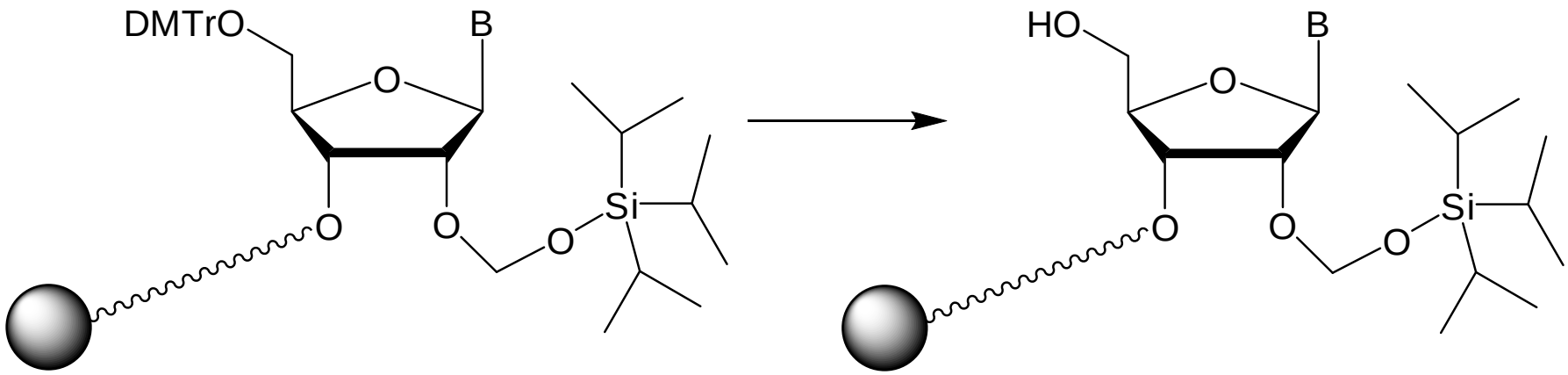
The preparation of the **TOM group** is relatively complex and **more costly**.

There is a considerable advantage: the **TOM group cannot migrate**.

For activation the more powerful 5-ethylthio- or 5-benzylthio-1*H*-tetrazole are used.



CHEMICAL SYNTHESIS OF BIOPOLYMERS



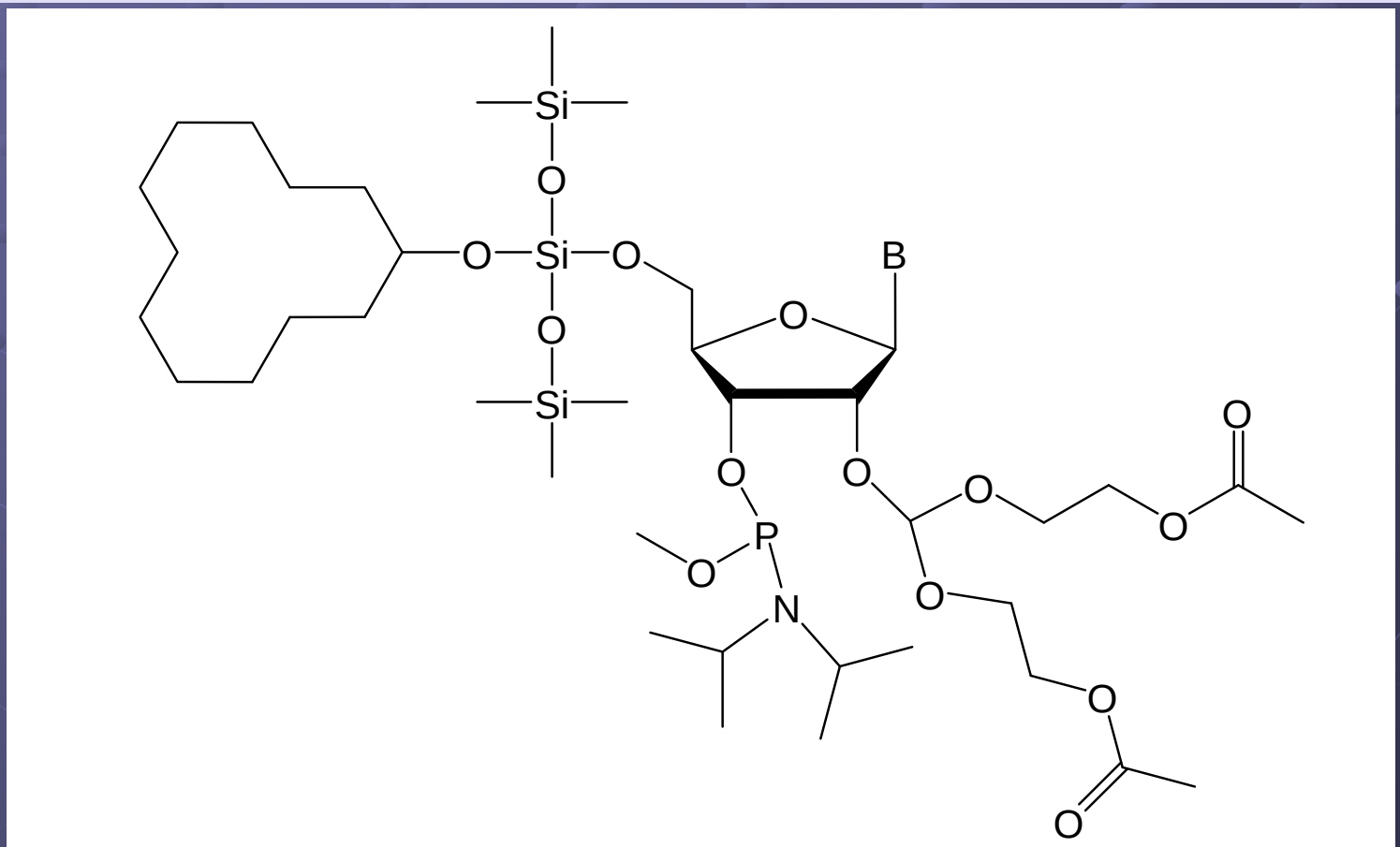
Methylamine in ethanol/water is used to **remove the acyl groups** followed by **TBAF in THF** to **remove the TOM group**.

TOM chemistry can be carried out at standard synthesizers and a **large pool of modified monomer building blocks**, **markers** and **labels** are available on the market.

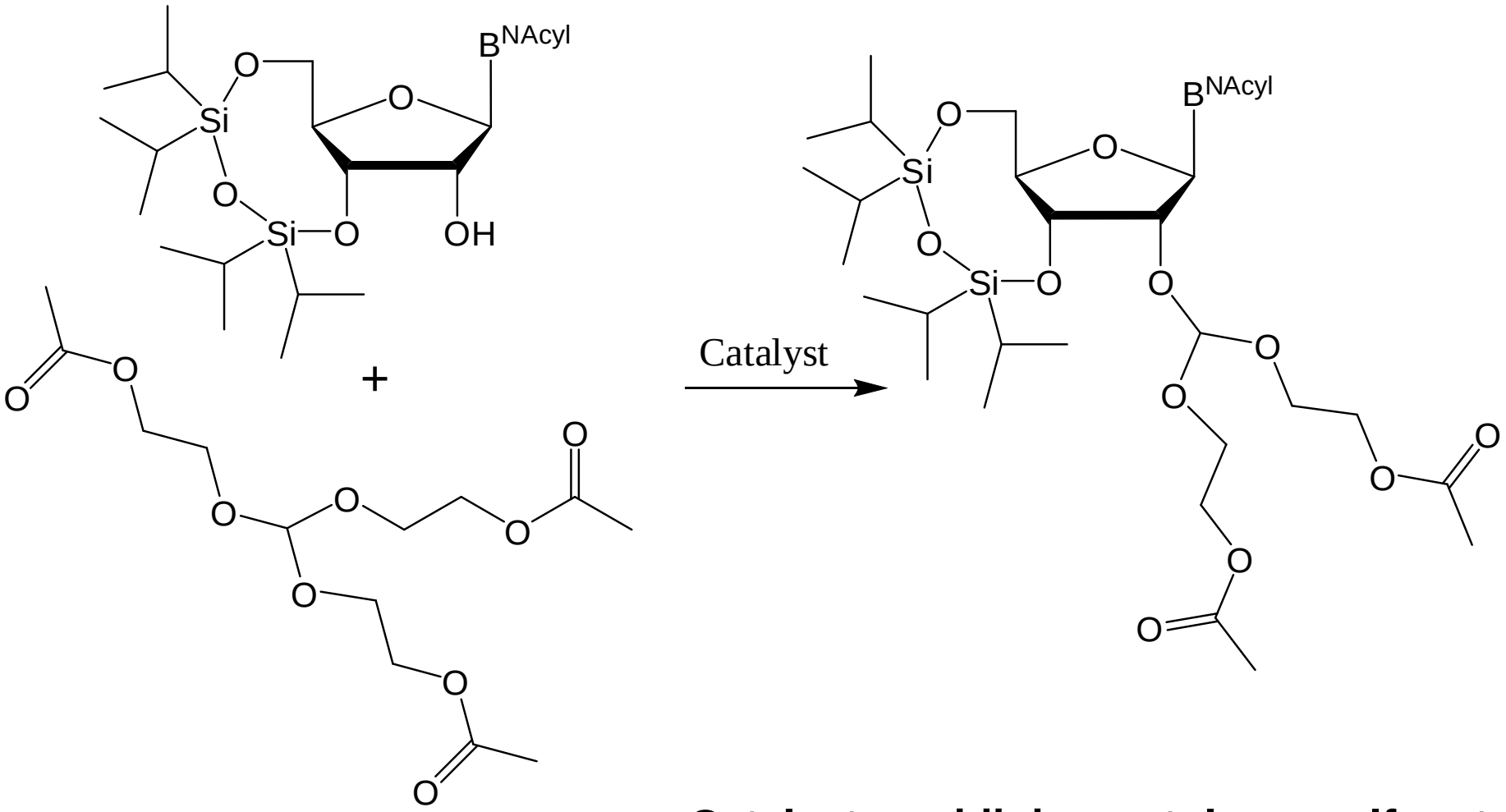
CHEMICAL SYNTHESIS OF BIOPOLYMERS

2’O-ACE Method

This impressive ***de novo* strategy** relies on the use of bis(2-acetoxyethoxy) methyl (ACE) **orthoester** to protect the 2’-hydroxyl group while the 5’-hydroxyl group is protected with a **silyl ether**.

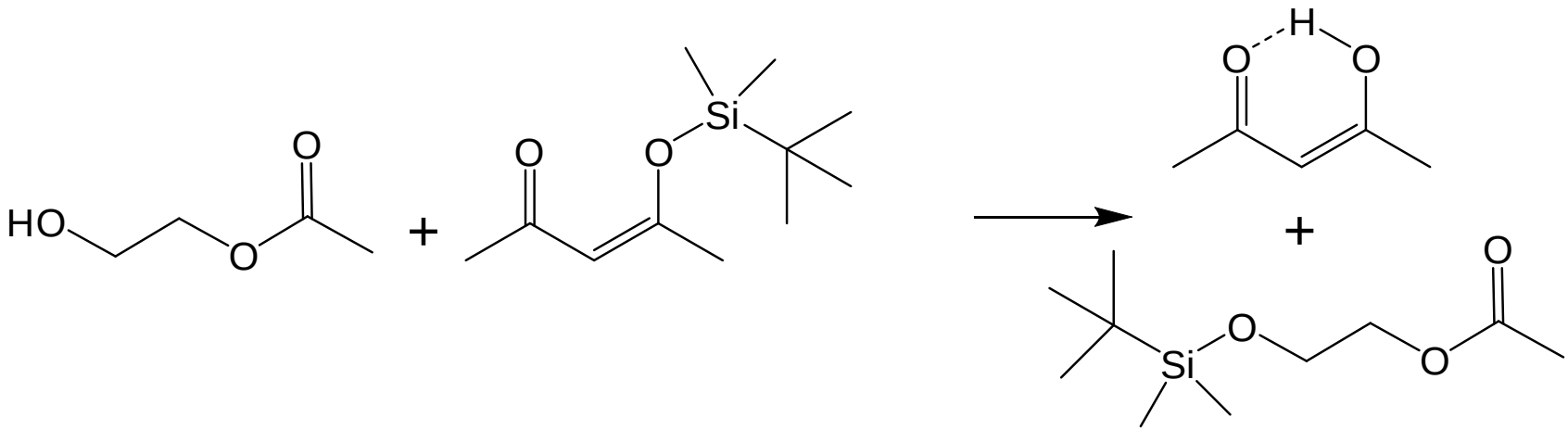


CHEMICAL SYNTHESIS OF BIOPOLYMERS



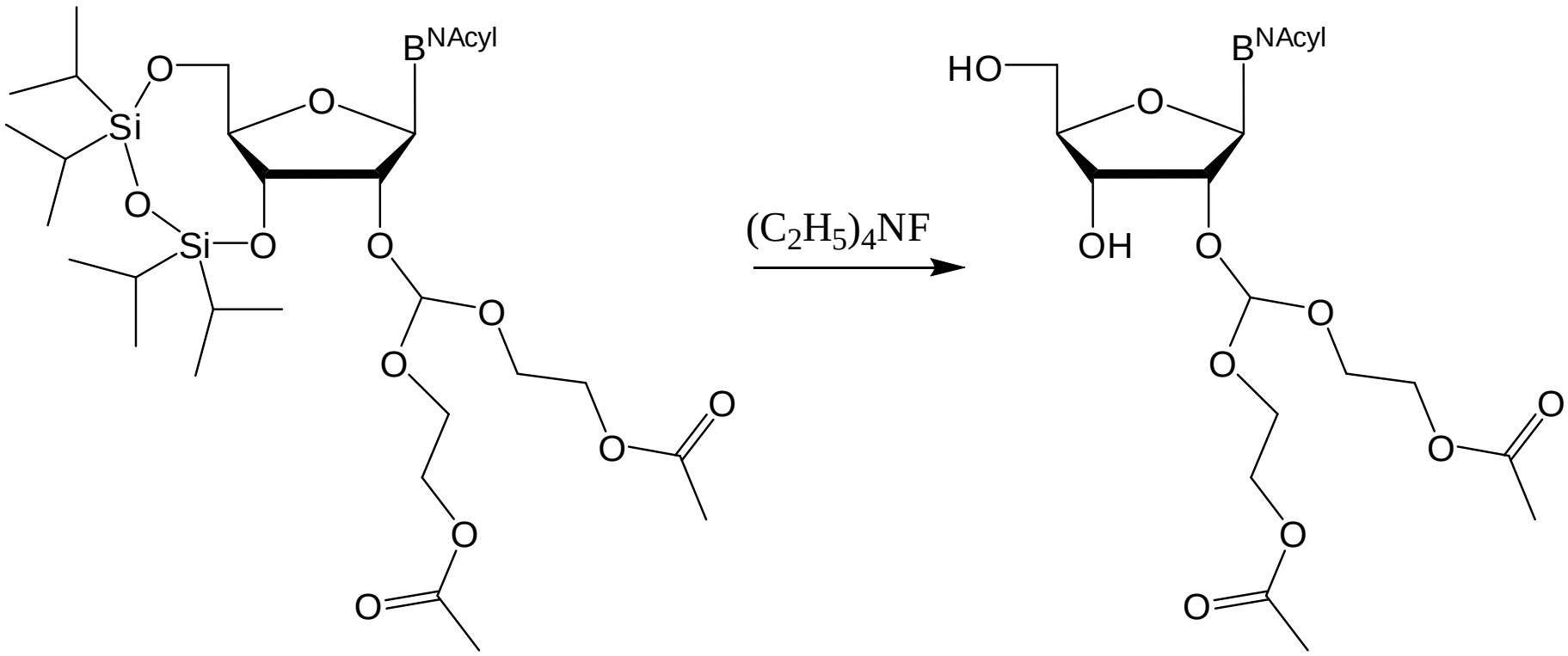
Catalyst: pyridinium *p*-toluenesulfonate

CHEMICAL SYNTHESIS OF BIOPOLYMERS

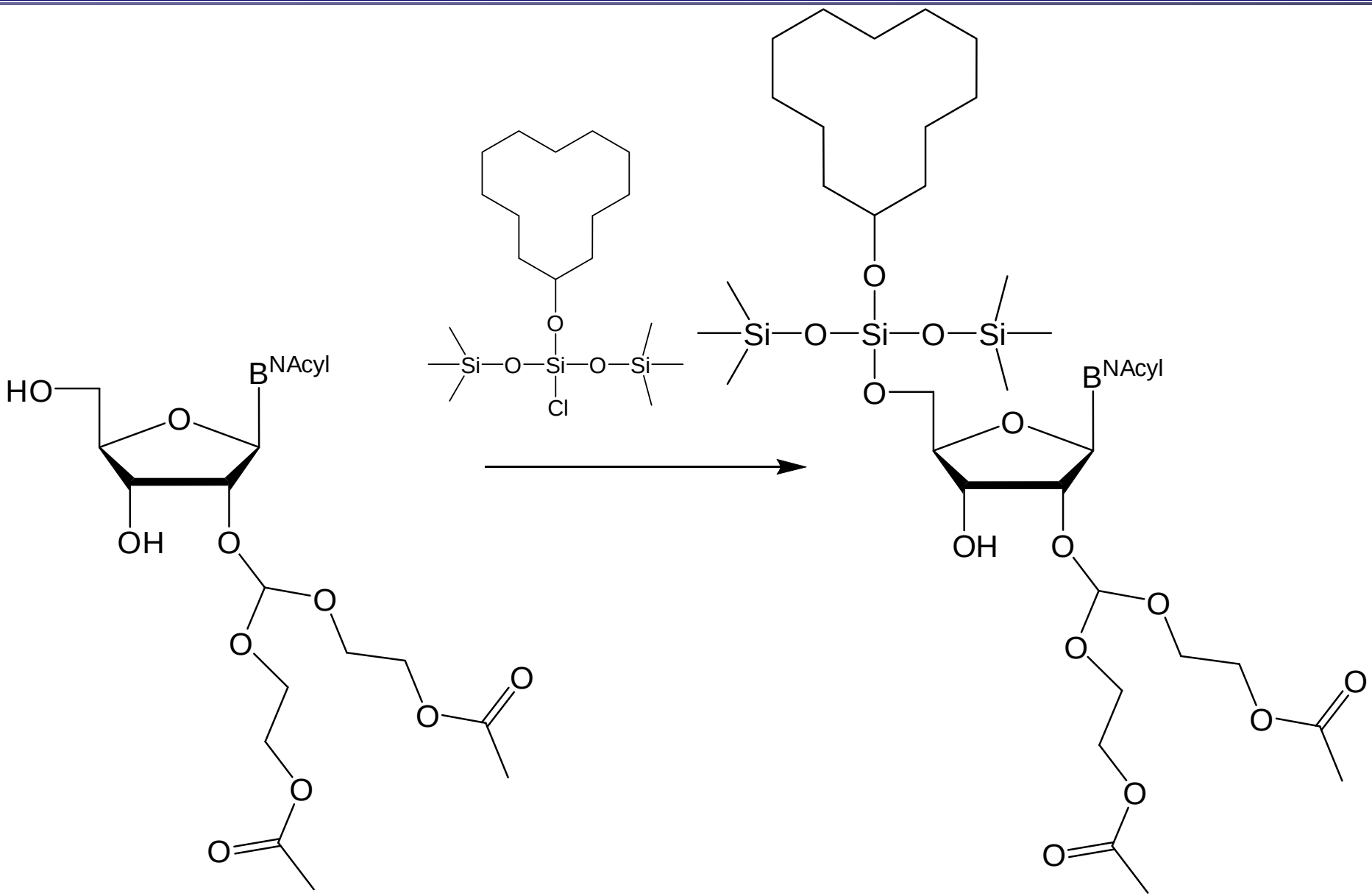


To drive the equilibrium of the reaction towards formation of ribose orthoester, the alcohol by-product is inactivated with ***tert*-butyldimethylsilylpentadione** which reacts selectively with the primary alcohol leaving the more hindered secondary 2'-hydroxyl of the ribosyl moiety intact.

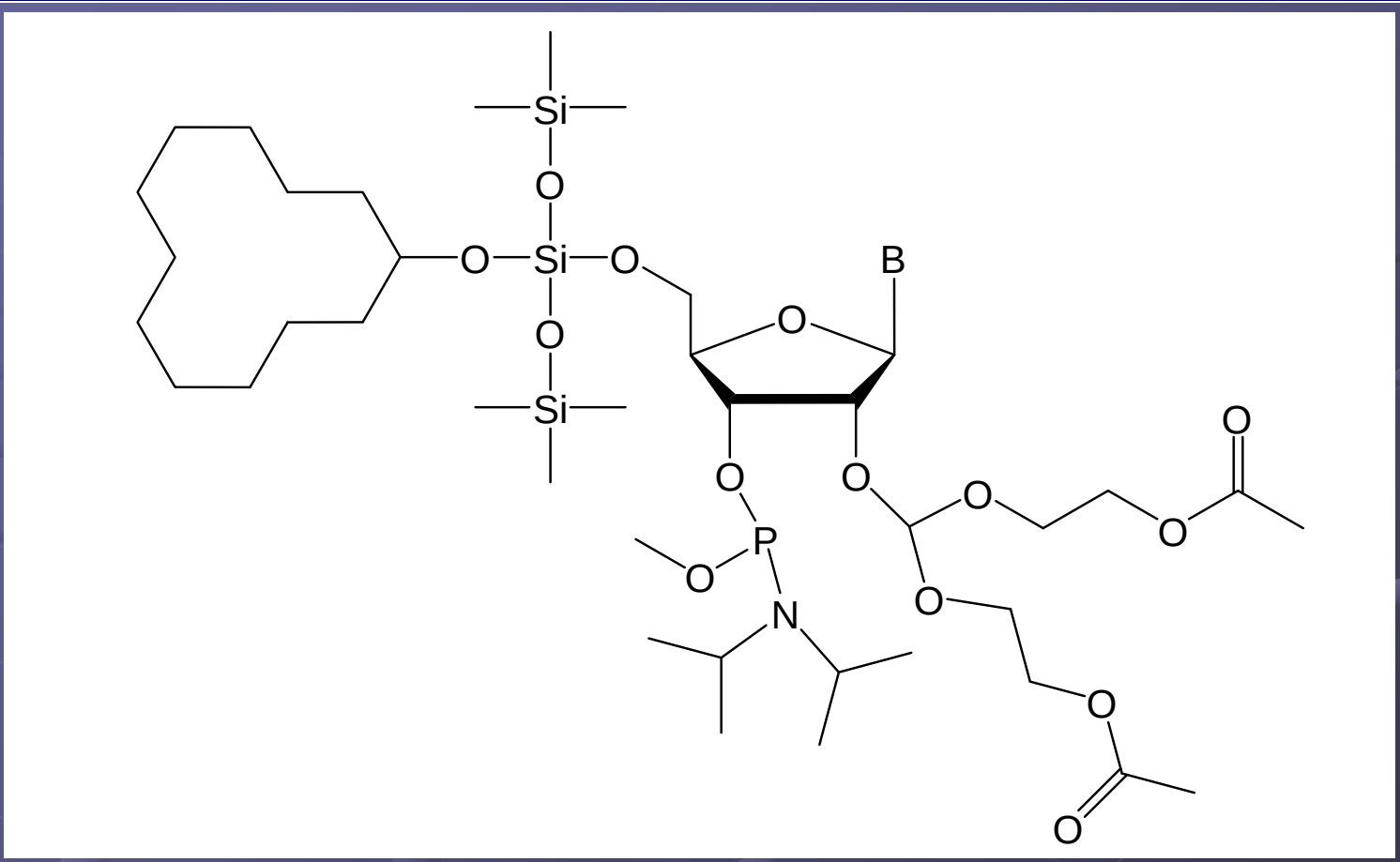
CHEMICAL SYNTHESIS OF BIOPOLYMERS



CHEMICAL SYNTHESIS OF BIOPOLYMERS



CHEMICAL SYNTHESIS OF BIOPOLYMERS



But now, the silyl ether used for protection of the 5'OH-group can be cleaved with fluoride ions under conditions that are compatible with the (ACE) orthoester.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

However, instead of β-cyanoethyl-*N,N*-diisopropylamino phosphoamidites, corresponding methyl derivatives need to be used since the β-cyano group proved to be unstable upon fluoride ion treatment.

The conditions for the final removal of the 2'-OH protecting group are mildly acidic treatment. Such a treatment is also necessary for the cleavage of the 5'-*O*-dimethoxytrityl group.

The 2'-*O*-ACE method allows for fast and highly efficient coupling (yield per step >99%) 5-Ethylthio-1*H*-tetrazole was used as promoter.

CHEMICAL SYNTHESIS OF BIOPOLYMERS

The ACE orthoester becomes deacetylated upon treatment with ammonia, the ultimate deprotection step at the end of the synthesis that is required to remove acyl protecting groups from the nucleobases and to detach the oligonucleotide from the solid phase.

Deacetylation of the orthoester makes it even more acid labile and the final deblocking of the 2'-OH can be carried out under very mild conditions (pH 3, 55 °C, 10 min.

A **drawback** still lies in the fact that 2'ACE chemistry is not compatible with established methods such that the large set of modified monomers, markers and labels that have been developed for DNA and TBDMS chemistry, cannot be integrated into this method.

We will see what will be happened in the near future!