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Scuola di Dottorato Bernardino Telesio - Scuola di Scienza e Tecnica

OMPI - Organic Materials of Pharmacological Interest

XXV CYCLE (CHIM/06)

PhD Thesis

Stereoselective synthesis of N,O-nucleosides via 1,3-dipolar cycloaddition of diacetone-D-glucose derived nitrone with vinylnucleobases

Determination of the diglyceride content in olive oils by employing ^{31}P NMR spectroscopy

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"A man should look for what is, and not for what he thinks should be."

Albert Einstein

"Measure what is measurable, and make measurable what is not so. "

Galileo Galilei

Abstract

Il lavoro svolto durante il periodo di dottorato di ricerca è stato suddiviso in due progetti: il primo incentrato sulla sintesi di analoghi nucleosidici di tipo isossazolidinici mediante l'impiego di cicloaddizioni 1,3-dipolari, il secondo progetto basato sulla determinazione del contenuto di digliceridi negli oli d'oliva mediante spettroscopia ^{31}P NMR, spostando la nostra attenzione nel campo dell'agroalimentare.

Nel primo progetto come primo step, si è costruito un glicosil nitrone opportunamente protetto, a partire da uno zucchero, il diacetone *D*-glucosio e, successivamente, si è passati alla sua cicloaddizione 1,3-dipolare con un set di vinilnucleobasi, preparate mediante vinilazione diretta delle seguenti nucleobasi: *N*-1-viniltimina, *N*-1-vinil-5-fluorouracile, *N*-1-viniluracile, *N*-1-vinilcitosina e *N*-9-viniladenina. Il metodo di sintesi utilizzato, si è dimostrato soddisfacente viste le buone rese, i tempi brevi di reazione, l'alta regiospecificità e un rapporto diastereoisomerico in favore prevalentemente dello stereoisomero *eso* rispetto a quello *endo*, riscontrando quindi una minore formazione di prodotti collaterali senza dover ricorrere ad alcun tipo di protezione delle vinilnucleobasi usate, per le cicloaddizione condotte in microonde ed in assenza di solvente.

Nello stesso tempo si è cercato di sintetizzare nitroni di zuccheri a catena aperta in modo da avere degli ossidrili facilmente disponibili per successive trasformazioni. La sintesi ha previsto l'iniziale formazione di *N*-benzil-*N*-glicosilidrossilammina a partire da *N*-benzilidrossilammina e 2,3,4,6-tetra-*O*-belzilglicopiranosio. L'idrossilammina ottenuta, in equlibrio con la forma nitronica, è stata fatta reagire con la *N*-1-viniltimina, la nucleobase generalmente più attiva verso questa tipologia di sintesi.

I composti ottenuti, i 4'-aza analoghi di dideossinucleosidi, già da tempo fanno parte di una classe di substrati che mostrano una potenziale applicazione nei trattamenti antitumorali e contro la diffusione di patologie di natura virale.

In tempi brevi i substrati isossazolidinici ottenuti saranno sottoposti a prove biologiche per verificarne la loro attività.

Questo progetto si aggiunge alla ricerca attualmente in atto presso il laboratorio di sintesi organica della Dott.ssa Loredana Maiuolo e il suo gruppo di lavoro, circa lo studio di cicloaddizioni 1,3-dipolari per la costruzione di eterocicli a cinque termini diastereoisomericamente puri.

Le conoscenze e la manualità acquisita, nel primo progetto, nel campo della sintesi organica sono state utili per la messa a punto del secondo progetto che prevede, inizialmente, la sintesi di standard per la determinazione di digliceridi in oli vergini d'oliva.

I digliceridi (DG), costituenti minor dell'olio vergine di oliva, possono essere utilizzati nella determinazione del processo di invecchiamento dell'olio, in base all'osservazione che un olio fresco presenta maggiori quantità di 1,2-DG e che la sua concentrazione nel tempo diminuisce per isomerizzazione nell'1,3-DG. Sebbene non esista una regolamentazione ufficiale che stabilisca il contenuto dei vari DG nei diversi oli di oliva, è tuttavia possibile considerare che oli di oliva freschi della stessa varietà di olive siano quelli che contengono un rapporto di 1,3-DG/1,2-DG basso.

La spettroscopia NMR può costituire un metodo di indagine alquanto valido nella determinazione dei digliceridi, anche se l'applicazione di ^{1}H e ^{13}C NMR risulta abbastanza complessa, considerando grosse sovrapposizione di segnali prevalentemente in alcune zone dello spettro. È possibile, invece, pensare di derivatizzare il gruppo funzionale alcolico presente sulla catena diacilglicerolica mediante reagenti al fosforo e utilizzare la spettroscopia ^{31}P NMR per identificare la presenza di 1,2-digliceridi in oli freschi e verificare

analiticamente la formazione di 1,3-digliceridi rispetto all'1,2-derivato in fase di invecchiamento dell'olio stesso.

In questo lavoro verranno illustrati le sintesi iniziali e i risultati ottenuti da uno screening con diversi composti al fosforo e la successiva applicazione del reagente che ha mostrato i migliori risultati con soluzioni standard di 1,2-DG e 1,3-DG e con alcuni campioni di olio d'oliva calabrese monitorati mediante ^{31}P NMR anche in diversi periodi dell'anno.

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Introduction

Heterocyclic compounds are compounds having a cyclic structure with at least two different kinds of atom in the ring, generally one is carbon and the most common heteroatoms present are nitrogen, oxygen and sulfur. The heterocyclic ring may contain one or more than one heteroatom which may be alike or unlike; can be classified into two categories aliphatic (saturated or unsaturated) and aromatic heterocycles. There is a rapid increase in the number, diversity as well as applications of heterocycles.[1] They are receiving more and more significance in recent years, particularly owing to their pharmacological as well as synthetic potential. In conjunction with nature's creation, chemists have artificially synthesized and tailored a number of heterocyclic compounds. The rationale behind nature's selection of heterocycles is perhaps due to the fact that they are chemically more flexible and able to respond to the demands of biochemical system. The ability of many heterocycles to produce stable metalloheterocycles has immense biochemical significance (for *e.g.*, hemoglobin, chlorophyll, vitamins, enzymes, *etc*.).

Introduction of heteroatoms into a carbocyclic compound makes a spectacular change in its chemistry and render it synthetically much more attractive. For instance, depending on pH of the medium, heterocycles may behave as acids or bases, forming anions or cations. Some interact readily with electrophilic reagents, others with nucleophiles, yet others with both. Some are easily oxidized, but resist reduction, while others can be readily hydrogenated but are stable towards the action of oxidizing agents. Certain amphoteric heterocyclic systems simultaneously demonstrate all the above mentioned properties. In addition to this, presence of heteroatoms brings tautomerism in

heterocyclic series.[2] Such a versatile reactivity is associated with the electron distribution within heterocyclic systems. In view of this, it is of significant interest to synthesize heterocyclic compounds and their derivatives.

The most important scientific event of the twentieth century, in the medical field, has been the discovery of molecules that can prevent the proliferation of bacteria and viruses in an infected organism, without causing serious consequences in the same organism.

The accumulated knowledge about viral and cellular replication have made possible the identification of some substances able to act selectively with the viral functions. Among these, modified nucleosides play an important role, just think that most of approved drugs for the treatment of viral infections are nucleoside analogues. The substantial characteristic of this class of compounds is the replacement of one or more atoms, or functional groups compared to natural metabolites that are the essential constituents of DNA and RNA. Have been developed different synthetic strategies for the formation of nucleoside analogues and for this reason is useful to subdivide these species, on the base of different structural characteristics, into three broad categories:

- nucleosides containing modified nucleobase;
- nucleosides containing modified sugar moiety;
- highly modified nucleosides.

This work is focused on the study of 1,3-dipolar cycloaddition reactions, nowadays one of the best and more general methods for the construction of five-membered rings in a convergent and stereocontrolled manner. In a classical 1,3-dipolar cycloaddition reaction a cyclic or acyclic dipolarophile reacts with a 1,3-dipole (nitrone) in order to give a wide variety of heterocyclic compounds. The importance of these reactions stems from the utility of the obtained isoxazolidines as useful precursors in the total synthesis of complex natural products.

In particular our interest is directed toward the synthesis of nucleoside analogues (4'-aza analogues of dideoxynucleosides) using the 1,3-DC reaction between sugar-derived nitrones with vinylated nucleobases. Nucleoside analogues are pharmacologically diverse family that includes cytotoxic compounds, antiviral agents and immunosuppressive molecules. These agents behave as antimetabolites that interact with a number of intracellular targets blocking the DNA chain elongation or interfering with biosynthesis of nucleosides and nucleotides, a limiting process in cell proliferation.

1. 1,3-Dipolar cycloaddition reactions of nitrone with alkene

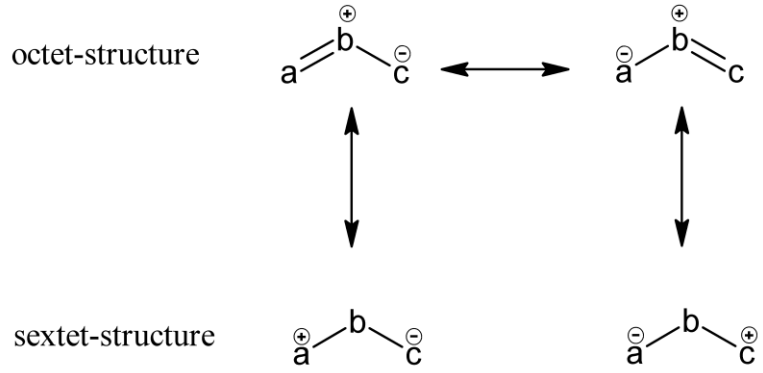
1.1 Mechanism and reactivity

1,3-Dipolar cycloaddition (1,3-DC) reaction, where two organic molecules, a 1,3-dipole and a dipolarophile combine to give a five-membered heterocycle , is one of the typical reactions in synthetic organic chemistry. Starting from relatively simple and easily accessible molecules, 1,3-DC reaction can offer a wide variety of simple as well as complex heterocyclic compounds of primary importance for both academia and industy.

The most common dipolarophiles are reactive molecules with a double bond or a triple bond alkenes and alkynes. α, β-Unsatured aldehydes, ketones, and esters, allylic alcohols, allylic halides, vinylic ethers *etc*. are examples of dipolarophiles that can readily react with 1,3-dipoles. In addition to these compounds, molecules with a double bond such as carbonyls, thiocarbonyls, imines can also undergo cycloaddition.

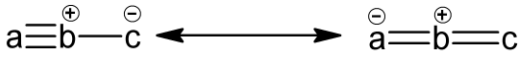
A 1,3-dipole is defined as an a-b-c structure, with a positive and negative charge distributed over three atoms and has four π electrons, that undergoes 1,3-DC reactions. Basically, 1,3-dipoles can be divided into two different types: the allyl anion type and the propargyl/allenyl anion type. They are occasionally referred to as sp^2 and sp hybridized 1,3-dipoles respectively.[3]

The allyl anion type is characterized by four electrons in three parallel p_z orbitals perpendicular to the plane of the dipole and that the 1,3-dipole is bent. Two resonance structures in which the three centers have an electron octet, and two structures in which a or c has an electron sextet, can be drawn (Scheme 1.1). The central atom b can be nitrogen, oxygen, or sulphur.



Scheme 1.1. Allyl anion type.

The propargyl/allenyl anion type has an extra π orbital located in the plane orthogonal to the allenyl anion type molecular orbital (MO), and the former orbital is therefore not directly involved in the resonance structures and reactions of the dipole. The propargyl/allenyl anion type is linear and the central atom b is limited to nitrogen (Scheme 1.2).



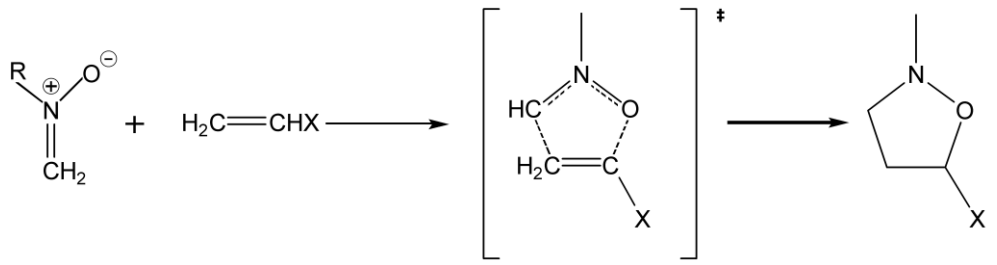
Scheme 1.2. Propargyl/allenyl anione type.

The 1,3-dipoles are occasionally presented as hypervalent structures (Scheme1.3).

Sheme 1.3. Hypervalent representations.

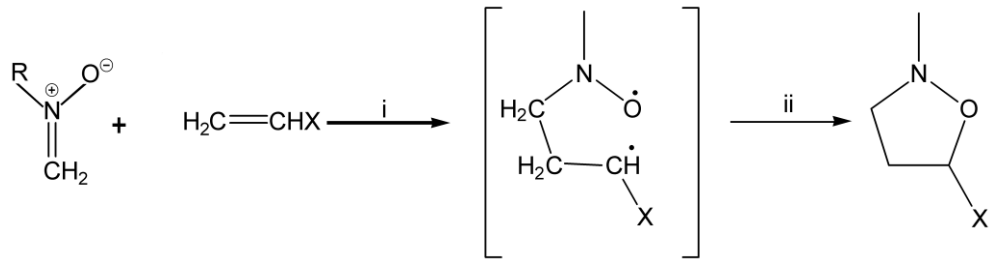
The 1,3-dipoles consist mainly of elements from main group IV, V and VI. Since the parent 1,3-dipoles consist of elements from the second row, and considering the above limitations on the central atom of the dipole, a limited number of structures can be formed by permutations of nitrogen, carbon and oxygen. Higher row elements such as sulphur and phosphorus can also be incorporated in 1,3-dipoles, but only few asymmetric reactions involving these types of dipoles have been published.[4]

The 1,3-DC reaction of the parent 1,3-dipoles, with alkenes, and alkynes involves 4π electrons from the dipole and 2π electrons from the alkene. If the 1,3-DC reaction proceeds via a concerted mechanism it is thermally allowed with the description $[_{\pi}4_{s} + _{\pi}2_{s}]$ according to the Woodward-Hoffmann rules. This means that the three p_z orbitals of the 1,3-dipole and the two p_z orbitals of the alkene both combine suprafacially. However , in the 1960s the reaction mechanism was subject to a great deal of debate.[5] On the basis of an extraordinary series of investigations with lead to a monumental collection of data Huisgen et al. developed a detailed rationale for a concerted mechanism for the 1,3-DC reaction (Scheme 1.4).[6]



Scheme 1.4. Huisgen's mechanism.

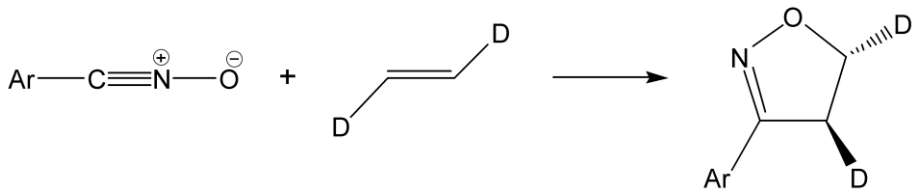
Firestone considered the 1,3-DC reaction to proceed via a singlet diradical intermediate (Scheme 1.5).



Sheme 1.5. Firestone's mechanism.

Both sides in the debate based their arguments on a series of experimental facts. Mechanistic investigations have shown that cycloadditions of 1,3-dipoles to alkenes are stereospecifically suprafacial, solvent polarity has little effect on reaction rates, and small activation entalpies and large negative activation entropies are generally found. These facts, along with reactivity and regioselectivity phenomena, have been considered totally compatible only with a concerted four-center mechanism. For example the 1,3-DC reaction of

benzonitrile oxide with *trans*-dideuterated ethylene gave exclusively the *trans*-isoxazoline (Scheme 1.6).



Scheme 1.6. Concerted mechanism.

A diradical intermediate would allow for a 180° rotation of the terminal bond and would thus be expected to yield a mixture of the *cis* and *trans* isomers. Huisgen et al. have later shown that the 1,3-DC reaction can take place by a stepwise reaction involving an intermediate and in these cases the stereospecificity of the reaction may be destroyed.

Molecular orbital theory has achieved a solid position in cycloaddition chemistry. According to MO theory when two molecules approach each other, the mutual "perturbation" consists of three terms:[7,8]

1. The closed shell repulsion stems from the interaction of filled orbitals of the reaction.
2. Coulombic forces can be repulsive or attractive depending on the polarities of the reactant pair.
3. The "second order perturbation term" consists of attractive interactions between all the occupied and unoccupied MOs of the reactants as long as these orbitals are of correct symmetry.

According to FMO theory the course of reaction is directed by most favourable interaction between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) of two reactants. Overlapping and mixing of two orbitals having smallest energy separation result in the formation of a bonding orbital. The activated complex of 1,3-dipolar cycloaddition is assumed to consist of an arrangement of 1,3-dipole and dipolarophile in two parallel planes. The complex has a symmetry plane σ according to which HOMOs and LUMOs can be classified as symmetric or antisymmetric. Both HOMO-LUMO pairs possess the correct symmetry for interaction.[9] The course of concerted 1,3-dipolar cycloaddiction reaction is controlled by frontier molecular orbitals of the substrates. The $LUMO_{dipole}$ can interact with the $HOMO_{alkene}$ and the $HOMO_{dipole}$ with the $LUMO_{alkene}$. Sustmann has classified 1,3-DC reactions into three types (Figure 1.1), on the basis of the relative FMO energies between the dipole and the alkene.[10]

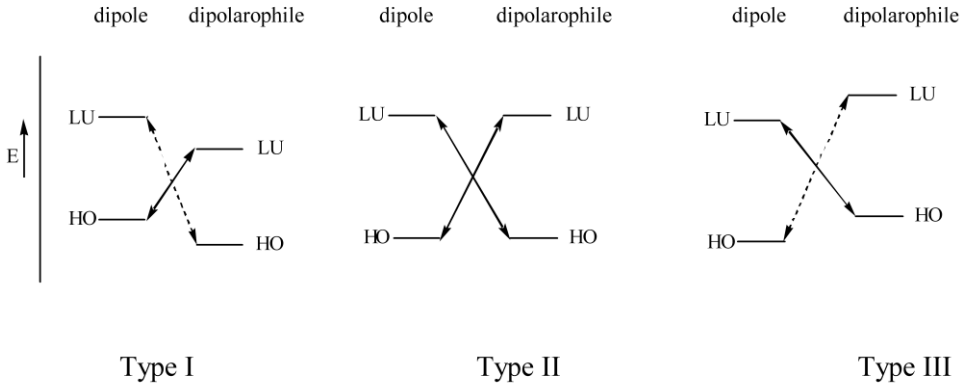


Figure 1.1. The classification of 1,3-DC reactions on the basis of the FMOs.

In type I 1,3-DC reaction, the dominant FMO interaction is that of $HOMO_{dipole}$ with $LUMO_{dipolarophile}$. Generally this type of 1,3-DC reaction is referred to as "normal electron demand" or "HOMO controlled" reactions. Cycloadditions of 1,3-dipoles of type-I are accelerated by the presence of *edg* in 1,3-dipole. The reason here is that as electrons are donated into the HOMO of the dipole, it becomes less stable and rises in energy towards the LUMO of dipolarophile. On the other hand, *ewg* in the dipolarophile will lower the energy of the LUMO towards the HOMO of the dipole. In both cases HOMO-LUMO separation of the predominant interaction is diminished.

For type II 1,3-DC reactions the similarity of the dipole and alkene FMO energies implies that both HOMO-LUMO interactions are important. Adding either an *edg* or *ewg* to the dipole or dipolarophile can accelerate these reactions.

1,3-DC reactions of type III are dominated by the interaction between the $LUMO_{dipole}$ and the $HOMO_{dipolarophile}$. The term "inverse electron demand" is used to refer to this type of 1,3-DC, this is also know "LUMO controlled" reactions. Since the dominant interaction is between $LUMO_{dipole}$ and $HOMO_{dipolarophile}$, *edgs* on dipolarophile and *ewgs* on dipole will accelerate the reaction. Type-III dipoles are referred to as electrophilic because they tend to react more efficiently with electron rich dipolarophile. The reactions of nitrones are normally classified as type-II. However, the introduction of electron-donating or electron-withdrawing substituents on the dipole or alkene can alter the relative FMO energies, and therefore the reaction type dramatically.

Factors which stabilize/destabilize the MOs, of reactants affect the reactivity. Thus, electron withdrawing substituents stabilize the MOs, electron donating substituents raise the MO energy and aromatic substituents raise the HOMO and lower the LUMO. A reduction of the MO energy of an electron-poor dipolarophile leads to a decrease of MO energy.[11] The presence of

metals, such as a Lewis acid, in 1,3-DC reactions, can alter both the orbital coefficients of the reacting atoms and the energy of the frontier orbitals of both the 1,3-dipole or the alkene depending on the electronic properties of these reagents or the Lewis acid.[12] The coordination of a Lewis acid to the 1,3-dipole, or the alkene, is of fundamental importance for asymmetric 1,3-DC reactions since the metal can catalyze the reaction. Furthermore, the Lewis acid may also have influence on the selectivity of the 1,3-DC reaction, since both regio-, diastereo-, and enantioselectivity can be controlled by the presence of a metal-ligand complex. The catalytic effect of a Lewis acid on the 1,3-DC reaction can be accounted for by the FMOs of either the 1,3-dipole, or the alkene, when coordinated to the metal. This principle of activation can be applied to the 1,3-dipolar cycloaddition of nitrones in two different ways.

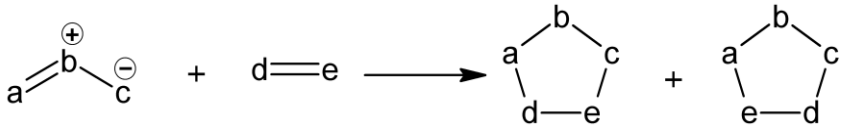
In the case of normal electron demand reaction, for example reaction between nitrone and an electron deficient alkene such as α,β-unsatured carbonyl compound, dominant FMO interaction is that of $HOMO_{dipole}$-$LUMO_{alkene}$. Coordination of a Lewis acid to the alkene will lower the energy of FMOs of alkene, relative to uncoordinated alkene. Lowering in energy of $LUMO_{alkene}$ will lead to a decrease in the energy difference between E_{HOMO} of dipole and E_{LUMO} of alkene coordinated to the Lewis acid, compared to the interaction in the absence of Lewis acid. Decreased energy gap between the interacting FMOs leads to faster reaction rates.[13]

In the case of inverse electron demand reaction , for example, reaction between nitrone and an electron rich alkene like vinyl ether, FMO interaction that governs the course of reaction is $HOMO_{alkene}$-$LUMO_{dipole}$ interaction. Here the frontier molecular orbitals of alkene have higher energies than frontier molecular orbitals of nitrone. Coordination of a Lewis acid to the nitrone will lower the energy of FMOs of nitrone relative to uncoordinated nitrone. Lowering in energy of $LUMO_{dipole}$ will lead to a decrease in the energy difference between E_{HOMO} of alkene and E_{LUMO} of dipole coordinated to the

Lewis acid, compared to the interaction in the absence of Lewis acid. The decreased energy gap between FMOs is responsible for the dominating interaction which leads to an enhanced rate of 1,3-dipolar cycloaddition. Hence the increase in reactivity of 1,3-dipole and alkene in presence of metal catalysis is due to a change in the FMO energy of substrate interacting with the catalyst.[14]

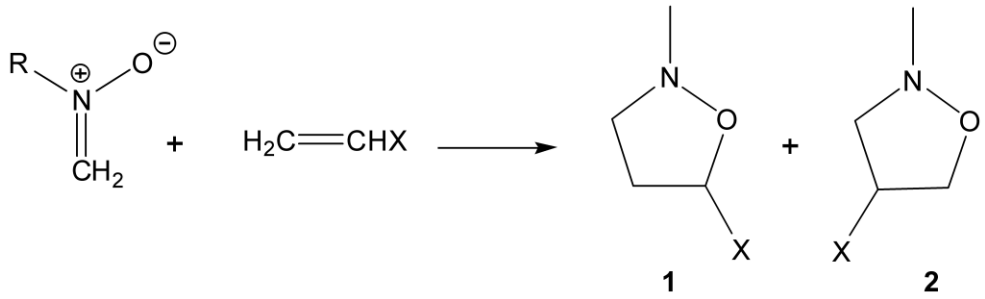
1.2 Regioselectivity

1,3-Dipolar cycloaddition can be regioselective (Scheme 1.7).



Scheme 1.7. 1,3-Dipolar cycloaddition: regioselectivity.

The observed regioselectivity is controlled by steric as well as electronic factors. Sometimes pure cycloadducts are isolated and occasionally a mixture of isomers is obtained.



Scheme 1.8. Cycloadduct 5-substituted **1**, cycloadduct 4-substituted **2**.

Addition of terminal alkenes to the sterically crowded 1,3-dipoles generally leads to the formation of 5-substituted isomers. Electronic effects sometimes preponderate over steric effects. For example, the cycloaddition reaction between nitrone and terminal alkenes, with *edg* in the dipolarophiles, leads to the formation of 5-substituted regio-isomer. On the other hand, terminal alkene with *ewg* leads to the formation of 4-substituted isomer.[15] The former reaction is mostly controlled by $LUMO_{dipole}$-$HOMO_{dipolarophile}$ interaction. The $LUMO_{dipole}$ has largest coefficient at the carbon atom an the $HOMO_{dipolarophile}$ has largest coefficient at the terminal carbon atom. Thus, the nitrone and alkene combine in a regioselective manner to give the 5-isoxazolidine.

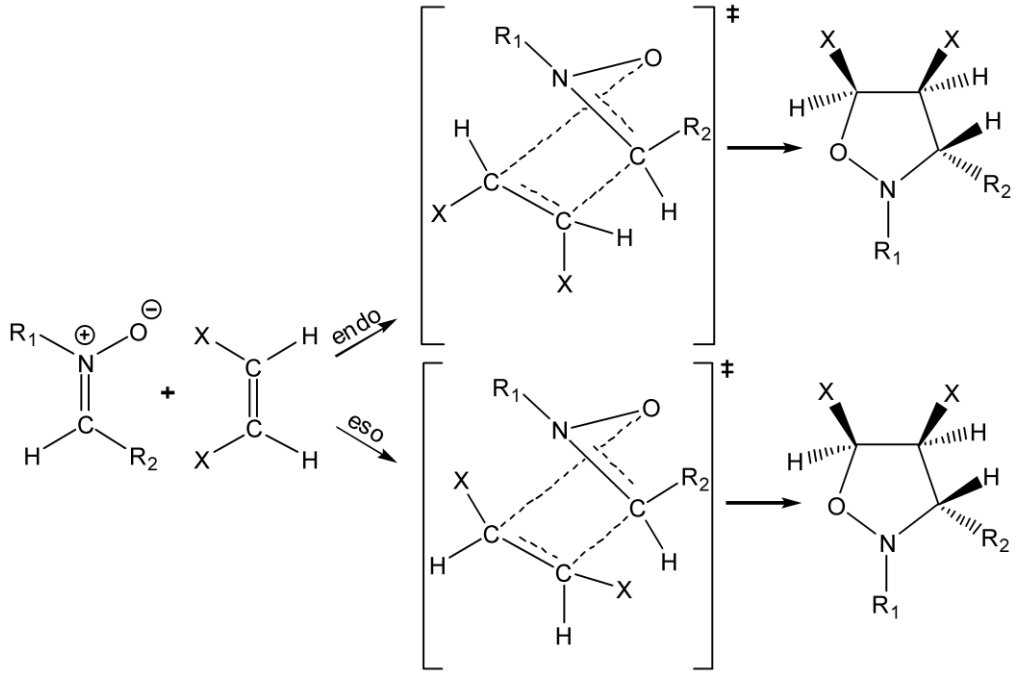
When the reaction is mainly controlled by the $HOMO_{dipole}$-$LUMO_{dipolarophile}$ interaction, the $HOMO_{dipole}$ has largest coefficient at the oxygen atom whereas $LUMO_{dipolarophile}$ has largest coefficient at the terminal carbon atom. This favours formation of the 4-isomer, but when steric effects preponderate over electronic effects a mixture of regioisomers is often obtained.[16] In the case of the reaction between nitrone and 1,2-disubstituted alkene with *ewg*, steric factor is eliminated, leading to the formation of 4-*ewg*-substituted isomer as the sole product.

1.3 Diastereoselectivity

Preferential formation of one stereoisomer over another in a chemical reaction is know as stereoselectivity. When the stereoisomers are enantiomers, the phenomenon is called enantioselectivity and is quantitatively expressed by the enantiomer excess (*ee*); when they are diastereoisomers, it is called diastereoselectivity and is quantitatively expressed by the diastereoisomer excess (*de*). Stereospecificity is an important criterion for the concertedness of cycloaddition.

As long as the 1,3-dipole and dipolarophile are configurationally stable compounds, no rotation about the crucial bonds is conceivable during the concerted formation of new σ-bonds. That is, stereospecificity of the cycloaddition has been cited as evidence supporting the concerted reaction: *cis*-1,2-disubstituted dipolarophiles give *cis*-substituted pentacycles, and *trans*-1,2-disubstituted dipolarophiles give *trans*-substituted pentacycles. This stereospecific nature of 1,3-dipolar cycloaddition rules out the diradical mechanism proposed by Firestone.[17]

When 1,2-disubstituted alkenes are involved in 1,3-dipolar cycloaddition reaction with 1,3-dipoles, two new chiral centers can be formed in a stereospecific manner due to the *syn* attack of the dipole on the double bond. If the alkene and 1,3-dipole, containing a chiral center approach is an *endo* or *exo* fashion, they give rise to a pair of diastereoisomers; each of them exist as a mixture of two enantiomers. The *endo* isomer arises from the reaction in which nitrogen atom of the dipole points in the same direction as the substituent of alkene, whereas, the *exo* isomer arises from the reaction in which the nitrogen atom of the dipole points in opposite direction as the substituent of alkene (Scheme 1.9).



Scheme 1.9. Diastereoisomers of the two different possible transition states *endo* and *exo*.

Since the secondary orbital interactions are very weak the *endo*/*exo* selectivity (or occasionally *cis*/*trans* selectivity) in 1,3-dipolar cycloaddition reaction is mainly controlled by the structure of substrates and the presence of catalysts.

It is know that nitrones having an electron-withdrawing group at the α position are configurationally unstable and they can be found as a mixture of E/Z isomers. The equilibrium between these isomers has been studies in solution and a dependence on the polarity of the solvent has been found. As a consequence of the interconversion between E/Z isomers, parallel models are always proposed for cycloaddition reactions of nitrone. In all cases it is possible to explain the obtention of the *trans* isomer by invoking either an *endo* approach to the Z-isomer an *exo* approach to the E-isomer. Similarly, the

obtention of *cis* isomers can be explained through an *exo* approach to the Z-isomer or an *endo* approach to the E-isomer. The reaction of nitrones having an electron-withdrawing group in the α position with electron-deficient alkenes usually give *trans* adduct preferentially. It has been invoked that these reactions take place through an E-*exo* approach due to the higher stability of the E-isomer.[18,19] However, it is also possible to propose that the reaction undergoes through an *endo* approach (preferred in all cycloaddition reactions with electron-deficient alkenes) to the more reactive Z-isomer.[20] The corresponding parallel can be done with electron-rich alkenes, too. The stereochemistry of cycloaddition reactions is in function of different factors, such as: steric effects, secondary orbital interactions, formation of hydrogen bonds and electrostatic interactions.

1.4 Biological evalutation of modified nucleosides

Newly emerging viral infections represent the major threat to human health. Apart of the three more extended global virus infections namely immunodeficiency virus (HIV), hepatitis C virus (HCV), and influenza A and B viruses, ebola, Marburg, dengue, yellow fever, nipah, enterovirus 71 and oncoviruses are the most dangerous viruses causing many human fatalities. An additional drawback of these microorganisms is their fast mutation rates because a small genetic variation in an inoffensive virus can originate a new strand with increased capacity to cause disease (virus with increased virulence).

The vaccine development is an extremely effective strategy to protect people from specific virus infections. However, an appropriate vaccine cannot be developed before a virus can be replicated in sufficient quantities to

manufacture it. In fact, vaccines are very effective on stable virus but difficult to apply to rapidly mutagenic viruses such as influenza, HCV, *etc*. In this last situation, and obviously when the patient has already been infected, is when antiviral drugs became crucial. An antiviral drug does not kill the virus, but acts by interfering one step of the viral replication process. This lowering in the replication frequency allows the body's immune system to destroy the virus using many natural defences.

Researchers working on strategies for developing antivirals have tried to attack viruses at every stage of their life cycles, namely attachment to a host cell, replication of viral components, assembly of viral components into complete viral particles and release of viral particles able to infect new hosts cells. Genomics helps scientists to find targets at every viral stage and also provides crucial data for understanding the drug-virus effective interaction. Closely, the organic synthetic chemists are dedicating many efforts to design and prepare potential drugs once targets have been identified. From all of the resources, methodologies, reaction, *etc*., put into service of the preparation of antiviral agents, 1,3-dipolar cycloaddition constitute a powerful classical synthetic tool and one of the most productive fields in modern organic chemistry.[21]

Inter- and intramolecular cycloaddition reactions play a key role in organic chemistry for the straightforward construction of cyclic scaffolds. The 1,3-dipolar cycloaddition reaction of nitrones with alkenes in particular has received considerable attention. One of the reasons for the success of the synthetic application of nitrones is that, contrary to the majority of other 1,3-dipoles, most nitrones are stable compounds that do not require *in situ* formation. The [3+2] cycloaddition reaction of nitrones with alkenes is a powerful synthetic method that is applied for the synthesis of isoxazolidines derivatives, which are valuable intermediates for the synthesis of chiral nitrogen heterocycles. Isoxazolidine intermediates have been used in the total

synthesis of natural products, because of their versatility to be transformed to aminosugars, alkaloids, β-lactams and amino acids; these heterocycles exhibit antibacterial and antifungal activities. Regioselective nitrone cycloaddition, followed by reduction of the N-O bond to produce both amino and hydroxyl functions, allows the synthesis of compounds of many potential interests.[22,23]

Nucleosides are generally defined as DNA or RNA subunits and consist of both a base moiety such as adenine, thymine, guanine, cytosine and uracil, and a sugar moiety such as *D*-ribose or 2-deoxy-*D*-ribose.[24] Many nucleoside analogues have been synthesized with modification of the base, sugar, and phosphate moieties. In particular, nucleoside analogues in which the furanose ring has been replaced by different carbon or heterocyclic systems, have attracted special interest by virtue of their biological action as antiviral and/or anti-cancer agents. Among these, the uracil-, thymine-, cytosine-, and adenine-nucleosides possessing an isoxazolidinyl moiety (carbocyclic-2'-oxo-3'-azanucleosides) are emerging as an interesting class of dideoxynucleoside analogues with potential pharmacological activity (Figure 1.2).[25]

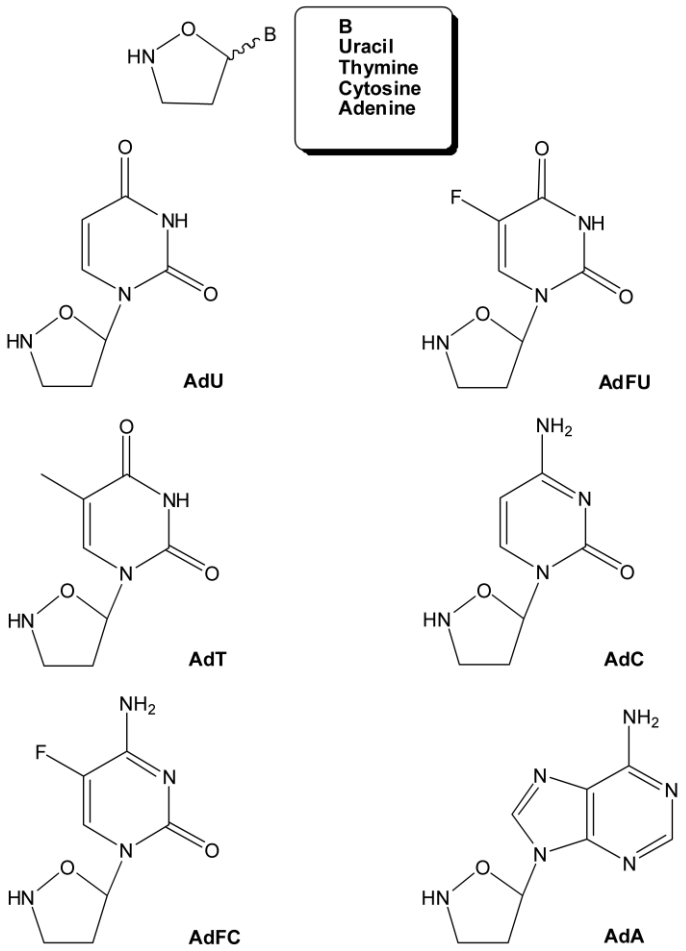
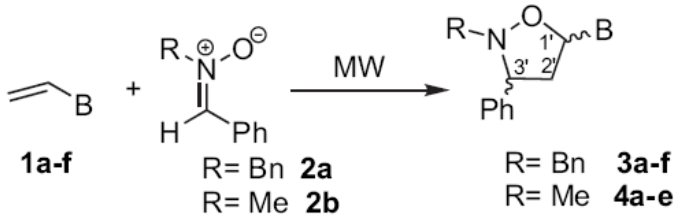


Figure 1.2. Isoxazolidinyl nucleoside

Some examples of modified nucleosides that have been synthesized in the past in our laboratory are reported below.

The first one describes the direct 1,3-dipolar cycloadditions between the model *N*-benzyl- or *N*-methyl-*C*-phenyl nitrone **2a,b** and a set of vinylnucleobases **1a-f**, acting as dipolarophiles, in the absence of solvent and under microwave irradiation conditions were carried out according to Scheme 1.10, and the pertinet results are collected in Table 1.1.[26]



B= thymine (**1a**); uracil (**1b**); cytosine (**1c**); 5-fluoro cytosine (**1d**); adenine (**1e**); 2-(*N*-trityl) guanine (**1f**)

Scheme 1.10. Direct 1,3-dipolar cycloabbitions between **2a,b** and **1a-f**.

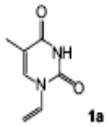
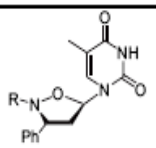
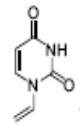
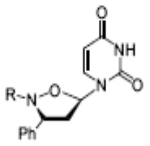
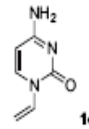
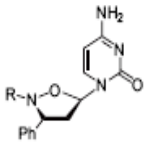
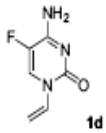
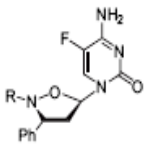
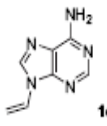
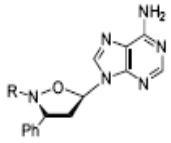
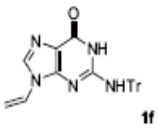
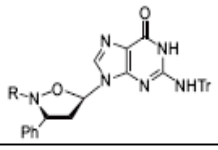
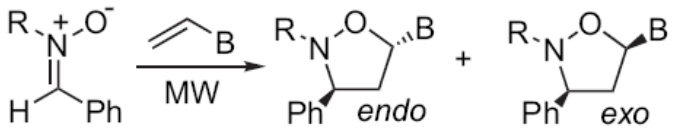
Entry	*N*-Vinyl nucleobases	Nitrones	Time (min)	Products		*endo*/*exo* ratio	Yield %[b]
1	 **1a**	**2a**	10		**3a**	80:20	80
2		**2b**	15		**4a**	80:20	80
3	 **1b**	**2a**	12		**3b**	78:22	80
4		**2b**	20		**4b**	70:30	80
5[c]	 **1c**	**2a**	25		**3c**	75:25	50
6[c]		**2b**	25		**4c**	65:35	60
7[c]	 **1d**	**2a**	25		**3d**	84:16	90
8[c]		**2b**	25		**4d**	71:29	90
9	 **1e**	**2a**	20		**3e**	70:30	70
10		**2b**	20		**4e**	72:28	72
11	 **1f**	**2a**	50		**3f**	75:25	20

Table 1.1. Final results where the exponents indicate: a) **1a-f**/**2a,b** ratio 1:2, b) isolted yields after column chromatography, c) **1a-f**/**2a,b** ratio 2:1.

In the majority of the entries, the cycloaddition products are formed fast and with final yields rangin from acceptable to very good. Worthy of notes is the fact that the cycloadditions proceed directly on unprotected vinylnucleobases, with the only exception being *N*-vinylguanine. No product is detected using this vinyl derivative, and only modest amounts of cycloadduct are found when employing the protected tritylated derivative **1f**, entry 11. The unnecessary protection of the vinylnucleobase, and consequent deprotection of the resulting *N*,*O*-nucleoside, represent an interesting improvement characterizing the overall synthetic protocol. The stereochemical outcome of the reaction showed a certain degree of control that appeared to be very highly regioselective with formation of the 1'-substituted isoxazolidines only.[27] On the other hand, the *endo*/*exo* ratio is satisfactory, in the range 70:30 with a maximum value of 84:16 for the 5-fluorocytosine derivative, entry 7 of Table 1.1 and Scheme 1.11.



Scheme 1.11. Formation of *endo* and *exo* isomers.

The assigned *endo*/*exo* stereochemical output, as established by accurate ^{1}H NMR spectroscopic analysis, is based on the J values measured for the coupling of proton H_a with protons at *C*2' and proton H_d with protons at *C*2', in the substantiated assumption, applicable to isoxazolidines, that *cis* vicinal ^{1}H couplings are always higher than the *trans*. As an example, in the cytosine

derivative **3c** the values of coupling constants J_{Ha-Hc}=4.4 Hz and J_{Ha-Hb}=7.5 Hz indicate that H_a is *trans* to H_c and *cis* to H_b. Furthermore the values of coupling constants J_{Hd-Hb}=7.6 Hz and J_{Hd-Hc}=9.9 Hz indicate that H_d is *trans* to H_b and *cis* to H_c, thereby establishing that H_a and Hd are *trans* each other (*endo* isomer) Figure 1.3.

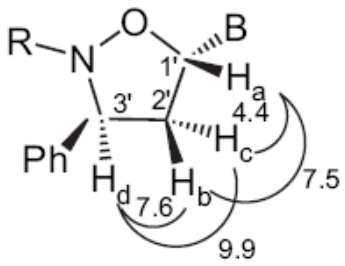
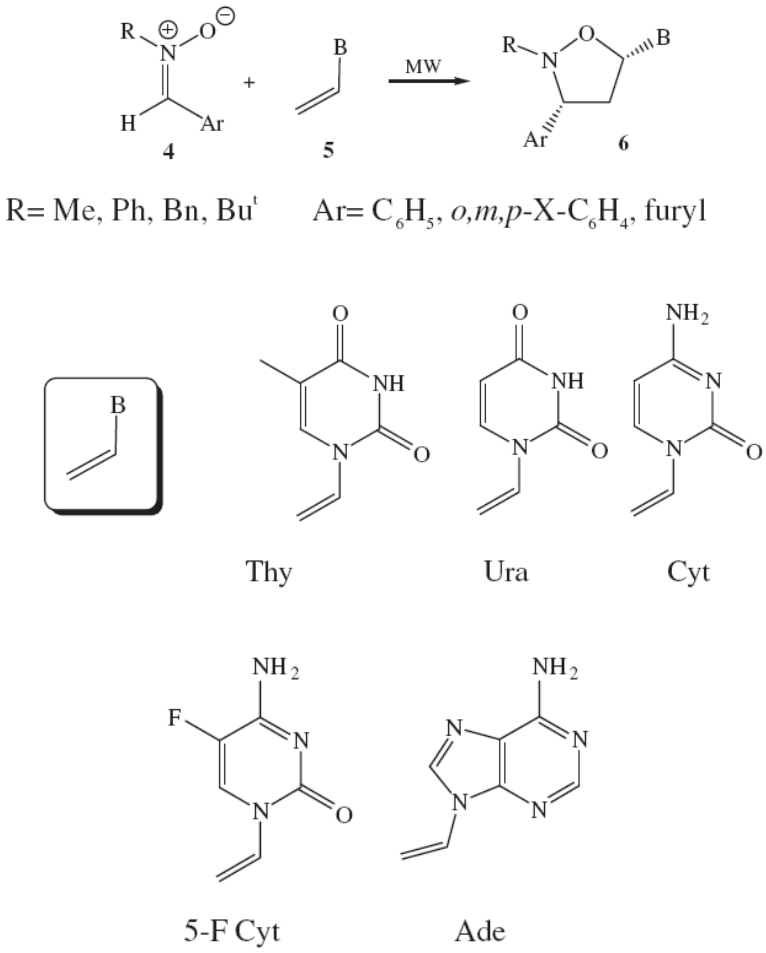


Figure 1.3. ^{1}H NMR coupling constants expressed in Hz.

The ESI-MS analysis of **3a-f** and **4a-e** nucleobases showed the presence of $[MH]^+$ ions and little fragmentation. The protonated molecular ions, submitted to MS/MS experiments, decomposed via cleavage of the nucleosidic bond with preferential formation of the isoxazolidine ion at m/z 238 (R=Bn) and 162 (R=Me), respectively. As a final consideration, it should be noted that 1,3-dipolar cycloaddition protocol presented here is one of the few examples of microwave mediated cycloadditions that proceeded in the absence of solvent, taking into account that from an environmental point of view ‘ *the best solvent is no solvent’*.[28]

The other one is focused toward the synthesis of modified nucleosides based on the *N*,*O*-heterecyclic ring **6**, obtained through microwave irradiated

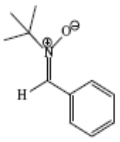
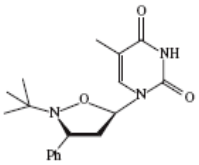
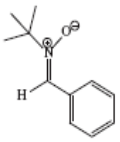
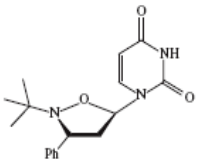
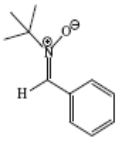
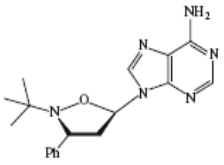
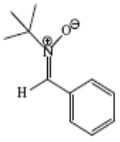
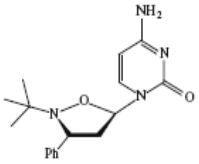
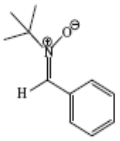
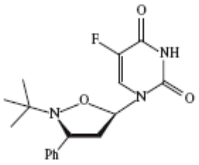
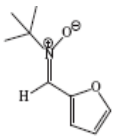
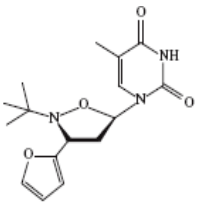
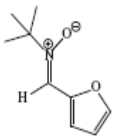
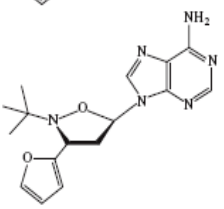
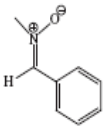
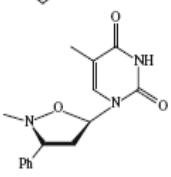
direct cyclization of suitable nitrones **4** and unprotected vinylated nucleobases **5** (Scheme 1.12).[29]



Scheme 1.12. Direct uncatalyzed synthesis of *N*-*O*-nucleosides under microwave conditions.

These class of nucleosides are obtained in high diastereoisomeric *cis*-*trans* excess, by tuning the substituents on the nitrone moiety. The diastereoisomeric excess is in many cases of 98% in favour of the *cis* isomer,

expecially when a bulky alkyl group is present on the nitrone moiety (Table 1.2).

Entry	*N*-Vinyl nucleobase	Nitrone	*t* (min)	Product		de[b] (%)	Yield[c] (%)
1[a]	Thy		10		**6a**	98	95
2[a]	Ura		13		**6b**	98	75
3[a]	Ade		10		**6c**	92	79
4[a]	Cyt		10		**6d**	92	70
5	F-Ura		10		**6e**	98	70
6	Thy		11		**6f**	98	81
7	Ade		35		**6g**	94	78
8	Thy		15		**6h**	60	80

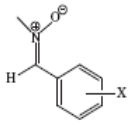
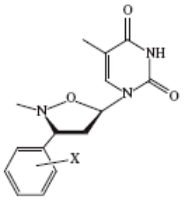
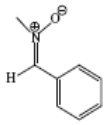
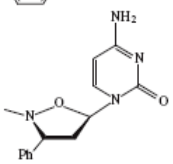
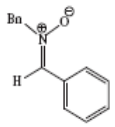
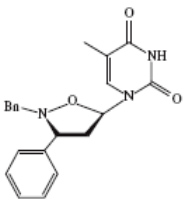
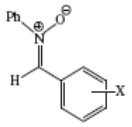
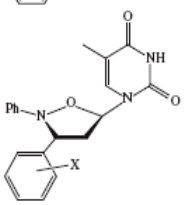
Entry	*N*-Vinyl nucleobase	Nitrone	*t* (min)	Product		de[b] (%)	Yield[c] (%)
9	Thy		20		**6i** X = *o*-OH	60	62
10			22		**6l** X = *p*-OH	62	60
11			20		**6m** X = *o*-Cl	80	80
12	Cyt		25		**6n**	50	50
13	Thy		10		**6o**	60	80
14	Thy		30		**6p** X = *p*-NO_2	60	90

Table 1.2. Final results where the exponents indicate: a) MW power 850 W other cases 750 W, b) de = (*cis*-*trans*) x 100, c) isolated yields.

The nitrones used in this investigation, in particular those possessing the *C*-aryl *N*-tert-butyl structures, were proved to posses neuroprotective properties. The obtained *N*,*O*-nucleosides have been evaluated for cytotoxic activity against lymphoblastoid cell lines (LCL), *in vitro* EBV-transformed B lymphocytes, JiJoye cells and Jurkat cells. Some of the tested compounds have proven to be potential antiproliferative drugs. In particular the Thy-phenyl-substituted compound **6o** and the *o*-chloro analog **6m** were able to prevent the proliferative activity at a relatively low concentration.

Nucleoside analogues can be directly used or can be incorporated into antisense oligonucleotide strands. The screening of the biological activity of a number of nucleosides derivatives has shown that modification of the 2' and 3' positions of the ribose ring do not change extensively the molecular recognition properties of the drugs. Many of the nucleoside analogues proposed against viruses present similar mechanism of action as inhibitors of viral replication: following intracellular phosphorylation to their 5'-triphosphate forms, they serve as chain terminators, thus acting as inhibitors in the viral reverse transcription reaction.

However, several problems associated with this kind of nucleoside analogues, due to their high toxicity and the appearance of cross-resistance, have led to the search for different structural solutions which have afforded new prodrugs comparable in their antiviral activity to the clinically used nucleoside analogues, but without their drawbacks. The biological activity of nucleoside analogues (ddNs) showing antiviral properties is strictly linked to their conversion, through cellular enzymes, to the corresponding 5'-mono-, di and triphosphates (ddNMP, ddNDP, ddNTP), which interact with viral reverse transcriptase (RT) or interfere with cell growth, slowing the cell cycle progression.[30] In many cases, among the three successive phosphorylation steps, the first is rate-limiting, and further conversions to di- and triphosphates are catalyzed by less specific kinases.

The known antiviral nucleoside analogues have often shown inefficiency in the first intracellular phosphorylation step by nucleoside kinases.[31] A possibility to improve the uptake of ddNs might be to bypass the phosphorylating step; unfortunately, nucleosides, due to their polar nature, are not able to cross the cell membrane efficiently. Moreover, they are readily dephosphorylated in extracellular fluids and on cell surfaces by nonspecific phosphohydrolases. Several strategies to overcome the problem of the initial selective and regulated phosphorylation step could be foreseen. A good

delivery system for a nucleoside across membranes might be represented by phosphotriester molecules, which should ensure the absorption and the transport of the active molecule and should then be able to deliver intracellular monophosphate forms. On this basis the use of nucleotide prodrugs (pronucleotides) incorporating enzyme-labile transient phosphate protecting groups has emerged, and a large number of prodrugs derivatives of nucleoside monophosphates have been prepared. In this respect, an alternative approach to the discovery of new and potent RT inhibitors involves the design of phosphate analogues where the phosphate moiety is changed to isosteric and isoelectronic phosphonates. Those enzimatically and chemically stable phosphonate analogues, which mimic the nucleoside monophosphates, are able to overcome the instability of nucleotides toward phosphodiesterases and to enhance their cellular uptake by bypassing the initial enzymatic phosphorylation and could potentially be effective antiviral agents.

The concept of nucleoside phosphonate has been applied to design chain terminators for anti-HIV chemotherapy and proved to be valid.[32,33]

For example: 9-(2-phosphonylmethoxypropyl) adenine (PMPA) and 9-(2-phosphonylmethoxyethyl) adenine (PMEA) are two effective and potent nucleoside phosphonate chain terminators for HIV reverse transcriptase.[34]

Compounds of type **2** show low levels of cytotoxicity and exert, on RT from almost two different retroviruses, a specific inhibitory activity, which is comparable with that of AZT. These compounds represent the lower phosphonate homologous of monophosphate compounds **1** where the oxygen atom in *C*-5' is eliminated. It is interesting to speculate that the biological effects exhibited by nucleoside analogues depend importantly on the relative disposition of the phosphate or phosphonate moiety and the heterocyclic base. In particular, it seems to be important to preserve the distance generated from the sugar moiety (spacer unit) between these two key elements. Phosphonates **3** preserve the five atom chain length between the heterocyclic base and the

phosphonate moiety of natural nucleosides. As a consequence, this modification could improve the anti-viral activity of **2** (Figure 1.4).

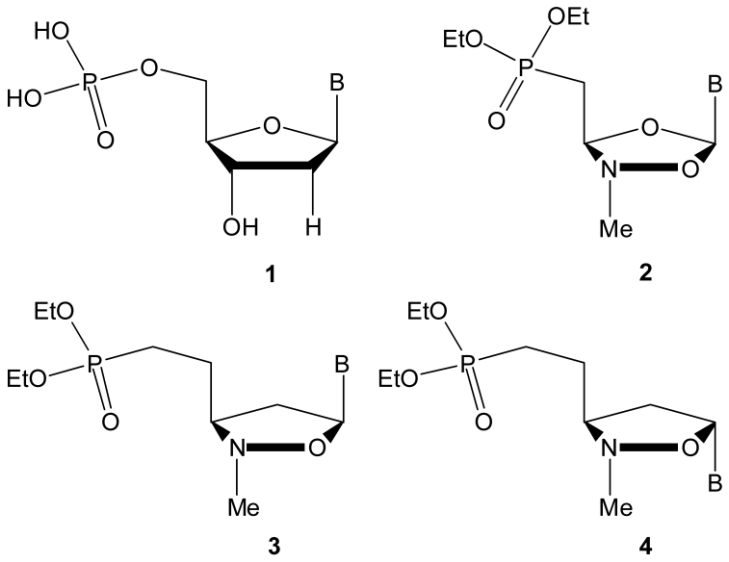


Figure 1.4. Reverse transcriptase (RT) inhibitors.

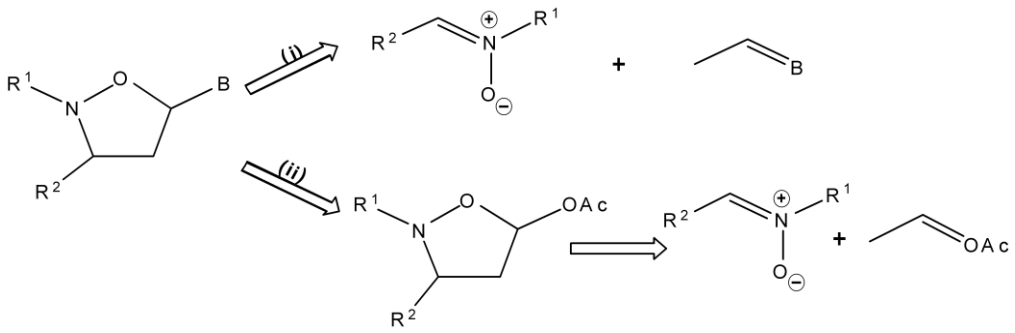
Glycosides are important as enzyme inhibitors, and as a chiral synthons for the synthesis of many natural product. Since the 1,3-dipolar cycloaddition has a nearly singular capability of establishing large numbers of stereogenic centers in one synthetic step in recent years attention has been focused on the preparation of chiral sugar-derived nitrones.

It should be mentionated that the stereoselectivity of cycloaddition of chiral sugar-derived nitrone to an alkene is difficult to predict, and would appear to be dependent on minor structural changes in either component. Three structural features can influence the stereochimical outcome of nitrone/alkene cycloadditions: E/Z nitrone isomerization about the C=N bond, alkene or/and nitrone facial selectivity, and *endo*/*exo* preferences. In many

cases the stereoselectivity of cycloadditions was dependent on the steric hindrance of the nitrone. The selectivity increases as the size of *C*-"chiral" group and *N*-alkyl group attached to the nitrone increases.

The use of *C*-chiral nitrones allows diastereoselective synthetic access to homochiral isoxazolidinyl nucleosides.

For the synthesis of modified isoxazolidinyl nucleosides, two strategies can be used: (i) a one-step approach based on the 1,3-dipolar cycloaddition of nitrone with vinylnucleobases, (ii) and a two-step methodology based on the Vorbrüggen nucleosidation of 5-acetoxyisoxazolidines (Scheme 1.13).[35]



Scheme 1.13. (i) 1,3-Dipolar cycloaddition of nitrone with vinylnucleobases, (ii) Vorbrüggen nucleosidation.

Among nitrones, the sugar-derived nitrones represent versatile substrates as they provide a polyhydroxylated carbon framework with multiple avenues of chirality, as well as an access for the amino group transformation required for the synthesis of polyhydroxylated piperidine, pyrrolidine, pyrrolizidine, indolizidine, and quinolizidine alkaloids. This class of compounds, commonly know as iminosugars, has attracted considerable attention because of promising glycosidase inhibitory activity, and, therefore, possible therapeutic

applications as immunosuppressive, antimalarial, antiviral, anticancer, and antidiabetic agents.

Glycosidases and glycosyltransferases act on the glycosidic linkage of oligosaccharides and glycopeptides by stabilizing an intermediate oxonium ion, thus facilitating the lysis and modification of the anomeric center.

It is now well established that the replacement of the intracyclic oxygen atom by a nitrogen atom in sugar moieties leads to a protonated species at physiological pH which mimics the oxocarbenium intermediate formed during enzymatic catalysis. 5-Membered cyclic amino-sugars like 4-amino-4-deoxypentoses, which mimic the furanose form of carbohydrates, prove to be importantant glycosidase inhibitors. In addition, iminosugars also exhibit immunomodulatory property this is an emerging area in drug development, however, it has received limited attention.[36]

Among iminosugars nojirimycin **1** and castanospermine **2** (Figure1.5), have attracted considerable attention because of their promising glycosidase inhibitory activity. In the search for a structure activity relationship, a number of natural and unnatural derivatives of castanospermine have been synthesized and evaluated for glycosidase inhibition in the treatment of various diseases such as diabetes, cancer, and viral infections, including AIDS.[37,38]

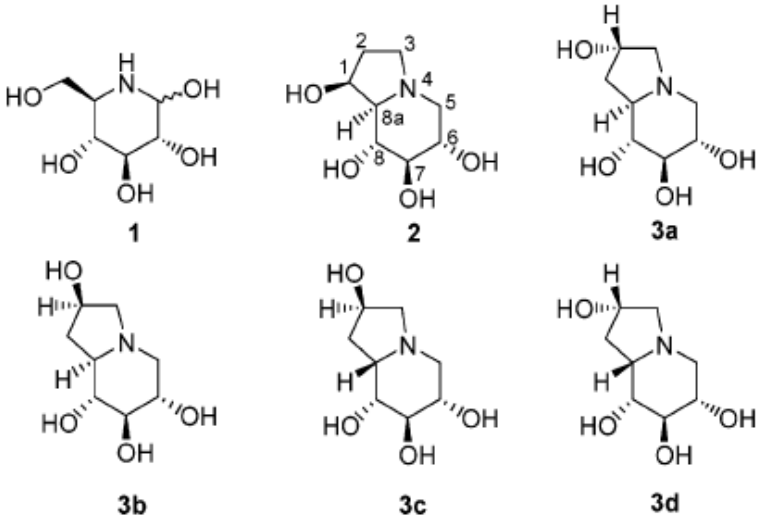
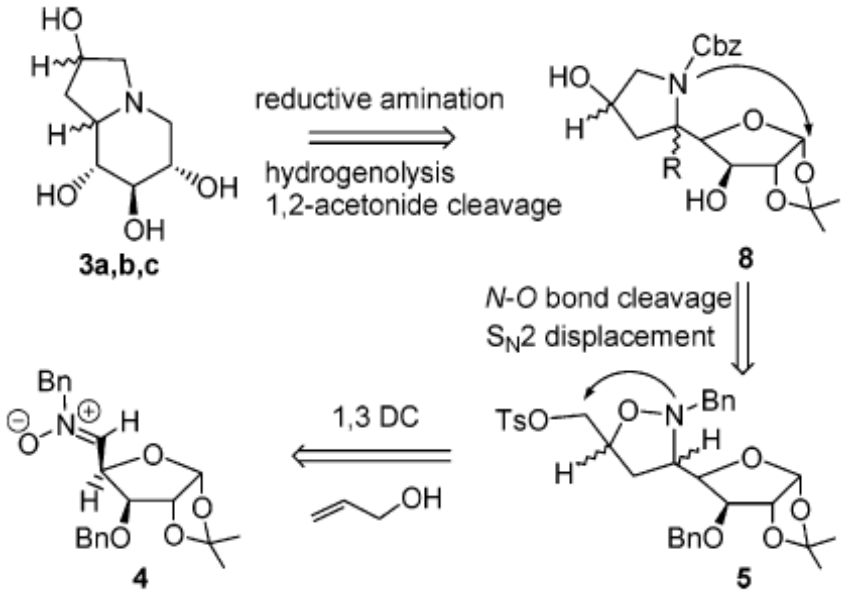


Figure 1.5. Azasugars and analogues

Here is reported the intermolecular 1,3-DC reaction of *D*-glucose-derived nitrone **4** with allyl alcohol, as a key step, in the formation of sugar-substituted isoxazolidines that are elaborated in the synthesis of 2-hydroxy-1-deoxycastanospermine analogues **3a,d**.[39]

As shown in the retrosynthetic analysis (Scheme 1.14), the requisite bicyclic ring skeleton of the 2-hydroxy-1-deoxycastanospermine could be built up by 1,2-acetonoide cleavage of **8** followed by hydrogenation (one-pot hydrogenolysis and reductive aminocyclization). The N-O bond reductive cleavage of tosyloxylated isoxazolidine **5** and concomitant nucleophilic displacement of the –*O*-tosyl group, by in situ generated secondary amino functionality, will give an access to **8**. Thus, the isoxazolidine **5** is the key intermediate that could be derived from the 1,3-DC of *D*-glucose-derived nitrone **4** with the allyl alcohol followed by tosylation. The 1,3-DC of nitrones with the allyl alcohol occur with perfect regioselectivity, wherein the oxygen of the 1,3-dipole attacks the more highly substituted carbon of the double bond

to produce the corresponding cycloadduct. The same regioselectivity would be obtained with the sugar nitrone **4** and as far as the regioselectivity is perfect, the π-facial stereoselectivity will not be a serious problem as the *Re* face cycloaddition will provide *D-gluco*-configurated isoxazolidine while the *Si* facial selectivity will afford *L-ido*-configurated isoxazolidines and all the stereoisomers, if obtained, could be converted to 2-hydroxy-1-deoxycastanospermine **3a,b** and 2-hydroxy-1-deoxy-8a-epi-castanospermine **3c,d**, respectively.



Scheme 1.14. Retrosynthetic analysis.

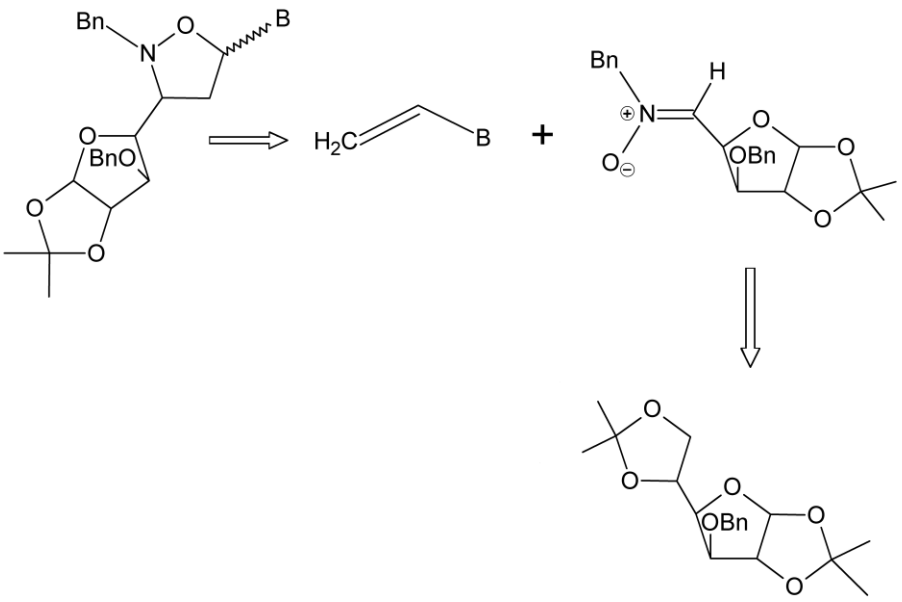
The 1,3-DC of **4** with the allyl alchol in acetone at 30°C for 7 days was sluggish; however, refluxing for 48 h afforded an inseparable mixture of isoxazolidines in 95% yield which on further treatment with p-toluenesulfonyl chloride in pyridine followed by careful separation of the crude mixture by flash chromatography afforded tosylated isoxazolidine (crystalline solids) in 49%, 7%, 17% and 14% yield respectively, with complete regioselectivity. Treatment of *O*-tosylated isoxazolidine with ammonium formate and 10% Pd/C followed by selective amine protection, with benzyl chloroformate, afforded *N*-Cbz protected diol. This one-pot three-step hydrogenation reaction resulted in N-O bond cleavage, intramolecular aminocyclization to form the pyrrolidine ring skeleton, and the removal of *N*- and *O*-benzyl groups. Subsequently, deprotection of 1,2-acetenoide functionality in the diol with TFA-water followed by hydrogenation afforded **3a,d**.

2. Results and discussion

Many strategies for the preparation of modified nucleosides have been reported in last decades in response to the pressing need of new treatments against virus infections. In a recent approach to these derivatives the ribose unit has been replaced by either carbo- or heterocyclic rings, being *N,O*-containing moieties, that is, isoxazolidine and isoxazoline nucleosides, particularly promising.[40]

The synthesis of isoxazolidinyl nucleosides is usually carried out using a classical 1,3-dipolar cycloaddition that represents the most successful protocol for the construction of biologically active derivatives. Our contribution to this field has been devoted toward the synthesis of modified nucleosides based on the *N,O*-heterocyclic ring, obtained through microwave irradiated direct cyclization of suitale sugar-derived nitrones and unprotected vinylated nucleobases. Notably, no protection is required for the vinylnucleobases during cycloaddition.

Microwave irradiation (MW) has been used for the rapid synthesis of a variety of compounds and this technique, under solvent-free conditions, is regarded as an environmentally acceptable practice for a number of reasons, including the fact that the reactions are quite often cleaner, faster, and higher yielding than conventional synthesis. The aim of this project is to synthesize a set of isoxazolydinil nucleosides with a carbohydrate structure on *C*-3 with a free –OH in order to obtain phosphorylated or phosphonate nucleosides increasing in this manner their biological activity; thus become possible to construct short polymers (Scheme 2.1).



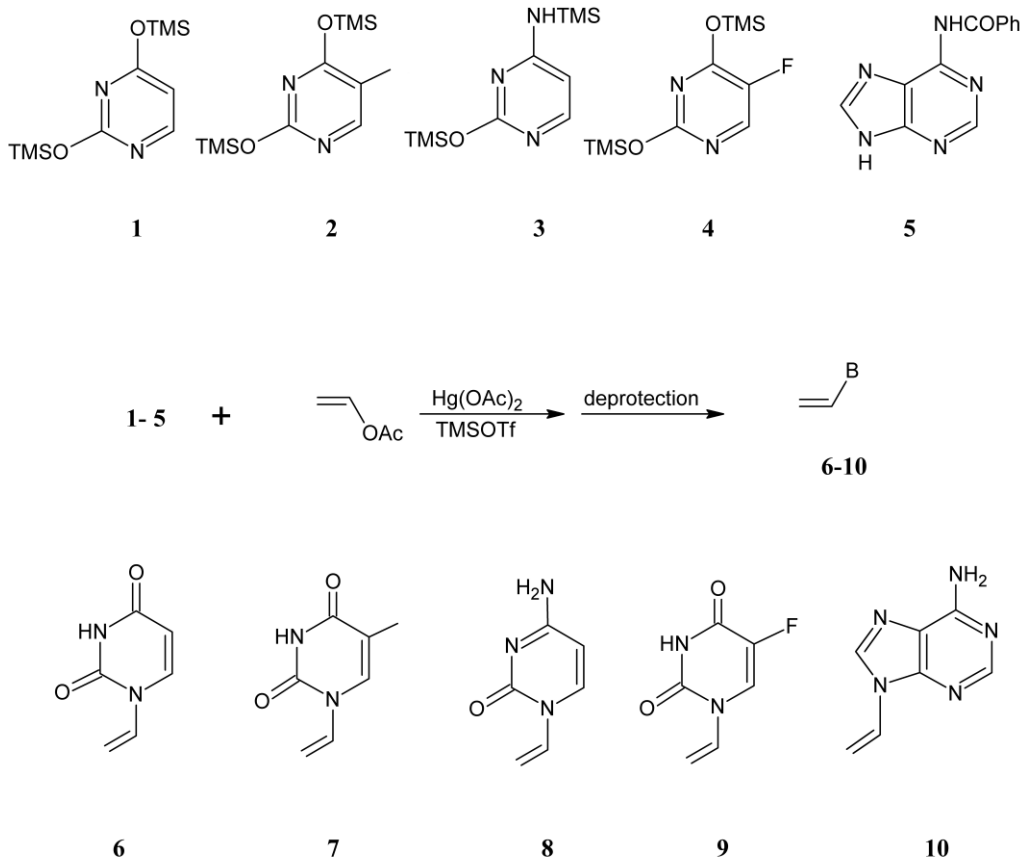
Scheme 2.1. Retrosynthetic steps.

Among the different strategies for the preparation of nucleosides using this protocol, the insertion of the nucleobase via nucleophilic substitution of a suitable leaving group on the isoxazolidinyl cycloadduct or, alternatively, the cycloaddition reaction on an appropriate vinylnucleobase acting as dipolarophile is the most used. This latter strategy suffers, however, of two major drawbacks: (i) the difficulty in obtaining *N*-vinyl derivatives of all nucleic acid bases and (ii) the drastic experimental conditions necessary to obtain cycloaddition products in satisfactory yields. In recent years these limitations have been successfully overcome, and the whole set of N-vinylnucleobases may now be prepared in a convenient, efficient, and simple way, using trimethylsilyl trifluoromethane sulfonate as catalyst.[41]

N-Vinyl derivatives of nucleobases are important starting materials for the synthesis of polymeric analogues of nucleic acids, useful tools in biomimetic studies on the interaction between purine and pyrimidine

nucleobases. Several methods have been developed for the preparation of vinyl derivatives of nucleobases, some consisting of relatively low yielding multistep procedures, and some others involving the direct exchange of the acetyl group of vinyl acetate with pyrimidine and purine bases or their trimethylsilyl derivatives.

In Scheme 2.2 is reported a simple one-pot procedure to prepare 1-vinyluracil (**6**), 1-vinylthymine (**7**), 1-vinylcytosine (**8**), 1-vinyl-5-fluorouracil (**9**), and 9-vinyladenine (**10**) using as catalyst in direct exchange of the acetate group of vinyl acetate with pyrimidine and purine bases trimethylsilyl trifluoromethanesulfonate (TMSOTf).



Scheme 2.2. Synthesis of *N*-vinyl derivatives of nucleobases.

There are two different experimental procedure, one for the pyrimidine nucleobases: uracil, thymine, 5-fluorouracil and cytosine and the other one for the purine nucleobase adenine. A transient in situ trimethylsilyl protection of the pyrimidine nucleobases, before the vinyl exchange reaction, was sufficient to ensure good results. For the adenine, used as protected 6-*N*-benzoyladenine instead a final deprotection step was necessary.

The low acidity of trimethylsylil triflate increases the selectivity of vinylation toward the more basic nitrogen and performs generally cleaner reactions. Finally, mercuric acetate is necessary to vinyl activation, in fact no reaction was obtained in the attempts to perform the vinyl exchange without it. Yields and reaction times obtained for the synthesis of vinylnucleobases are reported in Table 2.1.

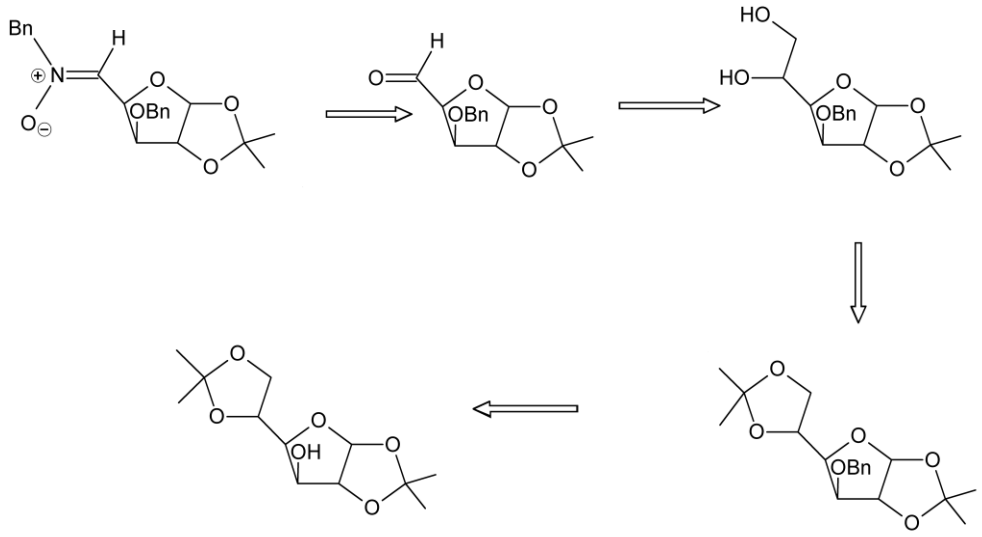
Vinylnucleobases	**6**	**7**	**8**	**9**	**10**
t (h)	4	3.5	6	5	6
yield (%)	81	79	70	65	78

Table 2.1. Yields and reaction times for the different vinylnucleobases.

The first step of this work was the synthesis of the nitrone and considering the complexity of carbohydrates was necessary to start with appropriately protected precursors.

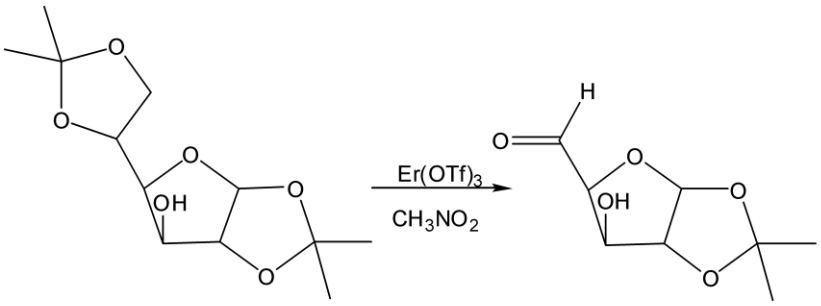
We used diacetone-*D*-glucose (1,2:5,6-di-*O*-isopropylidene-α-*D*-allose) as starting material which has four hydroxyls on *C*-1, *C*-2, *C*-5 and *C*-6 protected with two acetal groups and a free –OH on *C*-3 in order to obtain an aldehydic system, -CHO, in the *C*-5 position for selective deprotection of an

acetal group because one of the mehods used in literature to obtain nitrones is the reaction between aldehydes with *N*-substituted hydroxylamines; the last step was the formation of the 1,3-dipole system on the same carbon atom. In Schema 2.3 is reported the retrosynthetic analysis for the preparation of the nitrone.



Scheme 2.3. Retrosynthetic analysis.

In first instance we decided to proceed directly, starting from diacetone-*D*-glucose, with the deprotection of the acetal group present on *C*-5 and *C*-6 through an oxidation process that is know in literature (Scheme 2.4).[42]

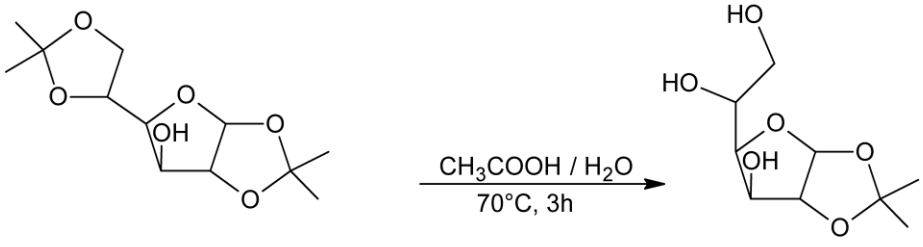


No formation of product is observed

Scheme 2.4. Oxidation of diacetone-*D*-glucose using $Er(OTf)_3$.

The reaction was carried out on the substrate having a single deprotected hydroxyl group on *C*-3 using $Er(OTf)_3$ as catalyst, this latter shows efficient and selective activities of deprotection in presence of polar solvent (CH_3NO_2). This procedure did not give formation of the product due at the reactivity of the free –OH on *C*-3, in fact is observed by TLC the presence of different by-products.

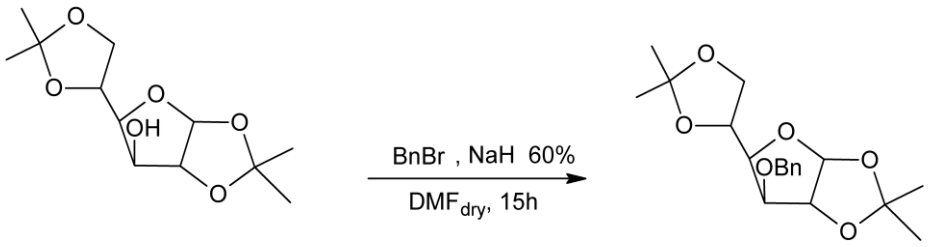
Subsequently, following the reaction conditions reported in literature on similar substrates protected on *C*-3, the deprotection step was carried out using CH_3COOH/ H_2O at 70°C (Scheme 2.5).



Scheme 2.5. Deprotection of diacetone-*D*-glucose using CH_3COOH/H_2O.

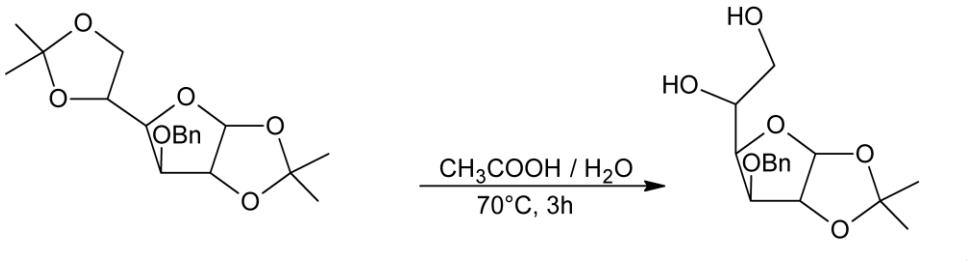
The crude was purified by silica gel flash column chromatography to give the product with a yield of 23%.

Preliminary evidence for the formation of the nitrone through the use of diacetone-*D*-glucose without further protection of the –OH group lead to a reduced reactivity of the substrate, therefore we started with the protection of the single free –OH with a benzyl group (a transient protective group used in carbohydrates chemistry) using NaH (60%), BnBr and DMF dry as a solvent.[43] BnBr and DMF were dried over standard drying agent and freshly distilled prior to use. The reaction was followed by TLC and after 15 h at room temperature was judged to be complete. The product, a tick yellow oil, was obtained with a yield of 86% (Scheme 2.6). The choice of the benzyl group was determined by the need to introduce a resistant group in basic and acid conditions, that can be removed at the appropriate time.



Scheme 2.6. Protection of the free –OH with a benzyl group.

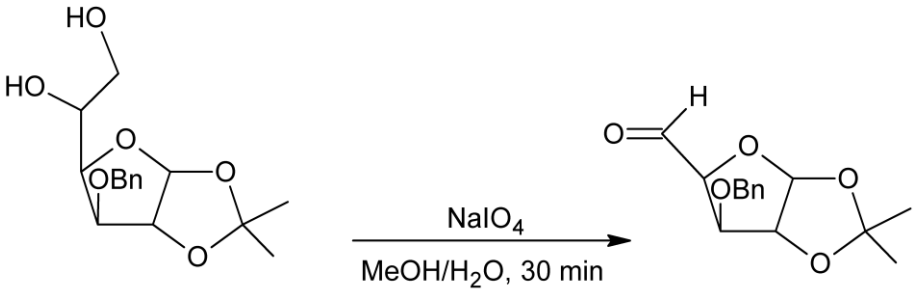
The procedure that leads to the aldehyde, precursor of the nitrone, includes an initial deprotection of the acetal group to obtain a diol compound (Scheme 2.7).[44,45]



Scheme 2.7. Deprotection step using CH_3COOH/H_2O.

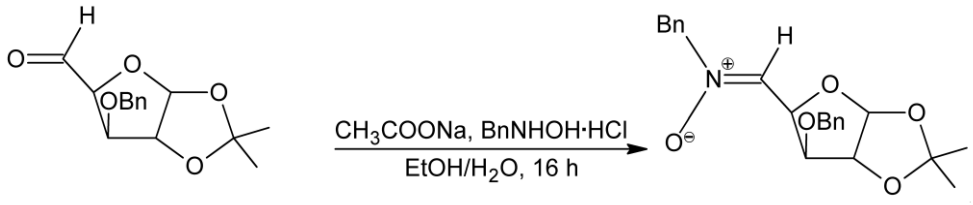
The above benzyl derivative was dissolved in CH_3COOH/ H_2O at 70°C for (3 h), after extraction with solvent the diol was obtained with a yield of 55%, the crude was used directly in the next step.

Subsequent oxidation of the diol with sodium metaperiodate gave the corresponding aldehyde 3-*O*-benzyl-1,2-*O*-isopropylidene-α-*D*-ribo-pento-dialdofuranose with a yield of 92% (Scheme 2.8).[46]



Scheme 2.8. Oxidation of the diol with $NaIO_4$.

Finally was synthesized the nitrone, the crude was purified by silica gel flash column chromatography to give the product as a white solid with a yield of 83% (Scheme 2.9).[47]

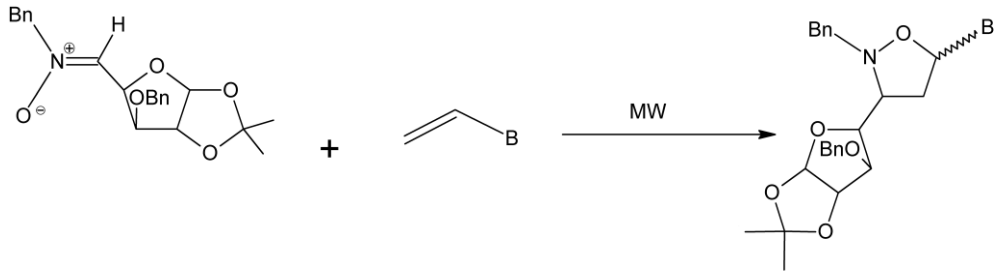


Sheme 2.9. Synthesis of *N*-benzyl-1,2-*O*-isopropylidene-α-*D*-ribo-pento--dialdofuranosyl nitrone.

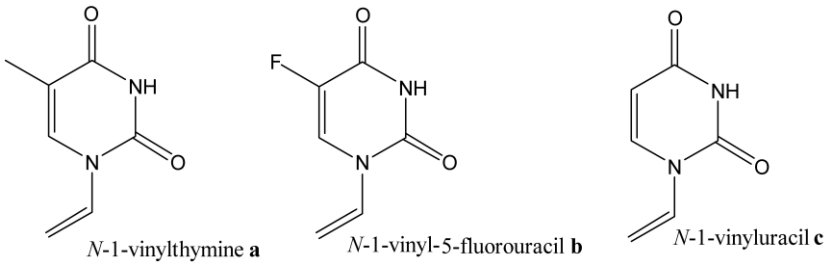
The nitrone was obtained between the reaction of the aldehydic compound with *N*-benzylhydroxylamine hydrochloride, the selection of the latter was dictated by the possibility of being able to remove, in the final step, the same protecting benzyl group in order to obtain free –NH and –OH on the cycloadduct.

Subsequent was the direct 1,3-dipolar cycloaddition reaction between the nitrone and a set of vinylnucleobases, acting as dipolarophiles, in the absence of solvent and under microwave irradiation conditions.

We tested the 1,3-dipolar cycloaddition reaction of the nitrone with these vinylnucleobases: *N*-1-vinylthymine **(a)**, *N*-1-vinyl-5-fluorouracil **(b)**, *N*-1-vinyluracil **(c)**, *N*-1-vinylcytosine **(d)**, and N-9-vinyladenine **(e)** (Scheme 2.10).



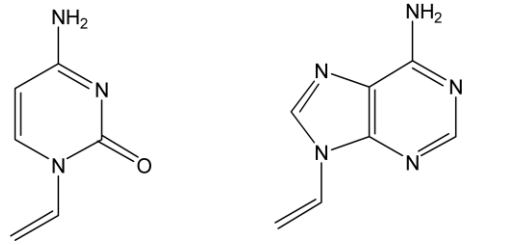
B = a, b, c, d, e



N-1-vinylthymine **a**

N-1-vinyl-5-fluorouracil **b**

N-1-vinyluracil **c**



N-1-vinylcytosine **d**

N-9-vinyladenine **e**

Sheme 2.10. 1,3-DC between the nitrone with vinylnucleobases.

The results obtained are reported in the following table (Table 2.2).

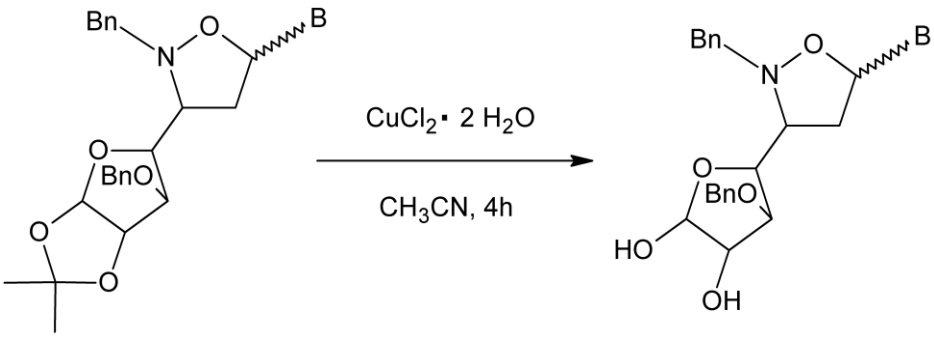
Row	Nitrone	Vinylnucleobase	t(min)	Yield (%)	Ratio *exo*:*endo*
1	*N*-benzyl glicosyl nitrone	*N*-1-vinylthymine **a**	6	75	72:28
2	*N*-benzyl glicosyl nitrone	*N*-1-vinyl-5-fluorouracil **b**	6	65	68: 32
3	*N*-benzyl glycosil nitrone	*N*-1-vinyluracil **c**	7	70	77:23
4	*N*-benzyl glycosil nitrone	*N*-1-vinylcytosine **d**	10	70	71:29
5	*N*-benzyl glycosil nitrone	*N*-9-vinyladenine **e**	6	78	75:25

Table 2.2. Experimental results of the 1,3-DC.

As demonstrated by the results reported in the table, in all cases reactions have led to the isolation of the required products in high enough yields and in short times (few minutes). The regiochemistry of these reactions showed a certain degree of control that appeared to be very highly regioselective with formation of the 1'-substituted isoxazolidines only, infact the formation of a single regioisomer is confirmed by accurate TLC and ^{1}H-NMR analysis. On the other hand, the *exo*//*endo* ratio is quite satisfactory. The regioisomeric and stereoisomeric assignment was done in analogy to other similar cycloadducts

obtained in the past in the same laboratory where this project was conducted as well as on the basis of ^{1}H-NMR spectroscopic data.

This work is continuing with the deprotection[48] of the acetal group present on isoxazolydinil nucleosides in order to obtain free –OH that could be phosphorylated or phosphonated to amplify the biological activity (Scheme 2.11).



Scheme 2.11. Deprotection of the acetal group

The deprotection step was carried out under reflux for 4 h using $CuCl_2 \cdot 2H_2O$ in CH_3CN, but the major problem with this reaction was inconsistency in the yield (30%) due to partial conversion into the desired product. In future we will try to increase the yield changing the reaction conditions or using another procedure to obtain the desiderate product. Similar tests, for removing the benzyl groups, are still in progress in order to obtain free –OH (2') and/or –N(4') that could be phosphorylated or phosphonated.

After further purification by HPLC all the cycloadducts , deprotected and protected (also these compounds may exhibit biological activity), will be sent

at the University of Ferrara in order to perform on them biological activity studies.

At the end of this project our attention was focused toward the synthesis of the sugar-derived nitrone with an open chain in order to have free –OH groups that can be used for further subsequently transformations. In literature is reported that *N*-benzyl and *N*-metil-*N*-glycosylhydroxylamines can be obtained by reaction of the corresponding monosaccharides with *N*-substituted hydroxylamines.[49,50] These compounds are either precursors of chiral nitrones with the glycosil moiety working as a chiral auxiliary or highly functionalized hidden chiral nitrones by themselves, which are able to undergo highly stereoselective 1,3-dipolar cycloaddictions. We decided to synthesize the *N*-benzyl-*N*-glycosylhydroxylamine using the commercially available sugar 2,3,4,6-tetra-*O*-benzyl-*D*-glucopyranose (Figure 2.1).

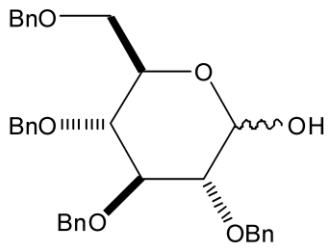
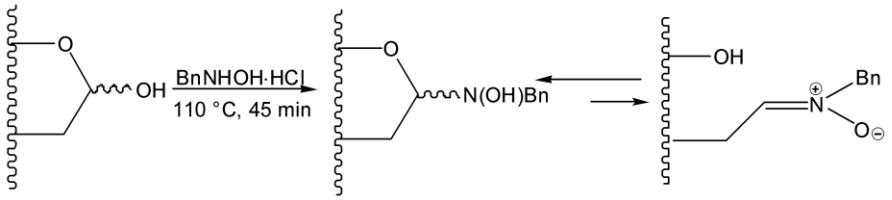


Figure 2.1. 2,3,4,6-Tetra-*O*-benzyl-*D*-glucopyranose.

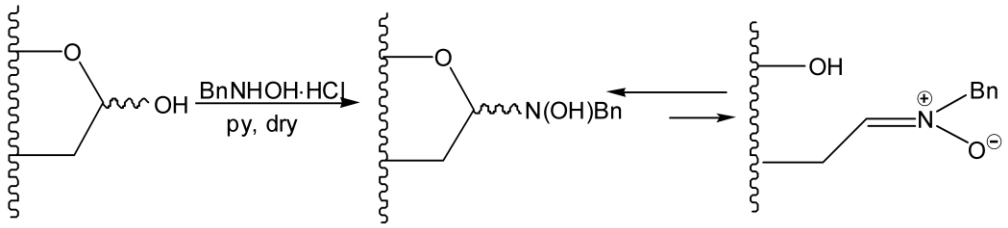
The reaction was carried out using the reaction between the sugar with BnNHOH·HCl at 110°C for 30 min in the absence of solvent (Scheme 2.12).



No formation of product is observed

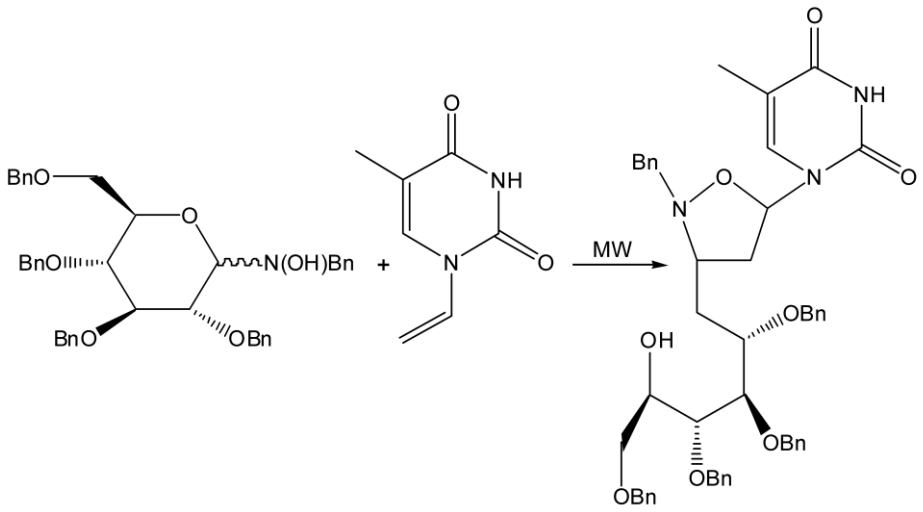
Scheme 2.12. Synthesis of N-benzyl-N-glycosylhydroxylamine without solvent.

The reaction even after the addition of sulphuric acid as catalyst did not give the product. Subsequently was decided to change the reaction conditions using anhydrous pyridine at room temperature. In this case was observed the formation of the product, 1-(*N*-benzylhydroxylamine)-2,3,4,6-*O*-tetrabenzyl-1-deoxy-β-*D*-glucose in equilibrium with the open chain nitrone tautomer. The crude reaction mixture was purified by column chromatography to give the product with a yield of 60% and then characterized by ^{1}H-NMR that confirmed the presence of an anomeric mixture where the thermodynamically most stable isomer prevals (Scheme 2.13).



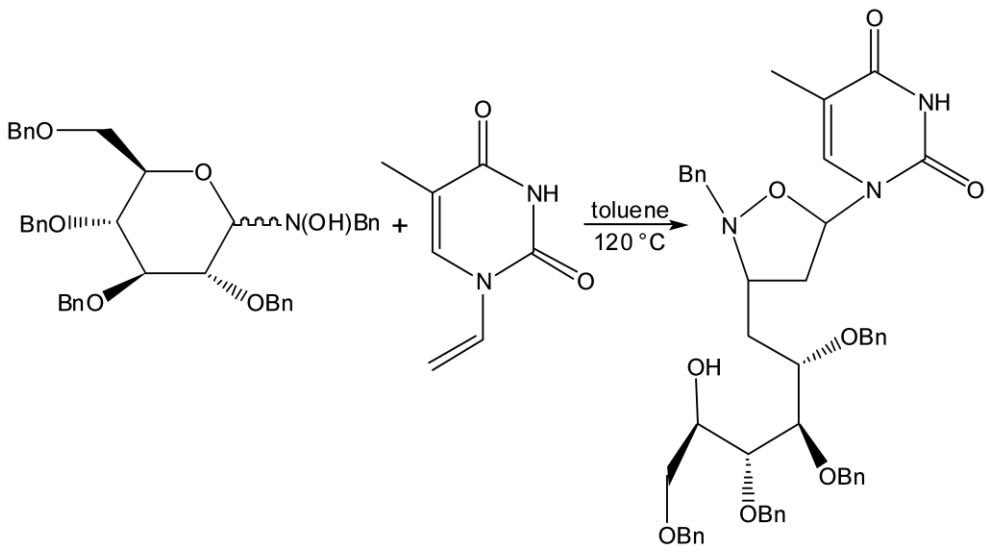
Scheme 2.13. Oxidation step

Subsequent was the direct 1,3-dipolar cycloaddition reaction between the obtained compound and *N*-1-vinylthymine, the more active vinylnucleobase used for these type of reactions, in the absence of solvent and under microwave irradiation conditions but in this case is not observed formation of the product (Scheme 2.14).



Scheme 2.14. 1,3-DC under microwave irradiation.

For this reason the reaction was carried out in classic conditions under reflux at 120°C for 74 h and using toluene as solvent, the crude was obtained with a yield of 79% (Scheme 2.15).



Scheme 2.15. 1,3-DC under classical conditions.

The product with a free -OH will be sent at the University of Ferrara in order to perform on them biological activity studies.

Conclusions

There is an increasing interest in the synthesis of modified nucleosides in connection with their potential applications in antiviral and anticancer terapies. In particular, modified nucleosides have been proved to efficiently inhibit *in vitro* and *in vivo* virus infections caused by HIV, HBV and HTLV-1.

In this work has been performed the synthesis of isoxazolidinyl nucleosides (4'-aza analogues of 2'-3'-dideoxynucleosides) using a classical 1,3-dipolar cycloaddition. The strategy of the synthetic approach is fast and simple consisting in the direct reaction of the selected nitrone (dipole) and the unprotected vinylnucleobase (dipolarophile) under microwave. The 1,3-dipolar cycloadditions were conveniently carried out in environmentally acceptable conditions as the absence of solvent and the quantitative recover/recycle of the unreacted nitrone present in stoichiometric excess.

With this method all the *N*,*O*-nucleosides possessing different nucleobases (Thy, Ura, F-Ura, Ade, Cyt) in position 1' have been synthetized in good chemical yields and remarkable stereoselectivity with a diastereoisomeric excess in favour of the *exo* isomer. We also tried to synthesize, starting from a sugar, isoxazolidinyl nucleosides with an open chain in order to give at the nucleoside analogues a greater ability to adapt in the receptive site active.

In summary, we have shown an expeditious, easy-to-handle, and environmentally friendlier approach to the synthesis of a variety of non-easily-available 4'-aza-2'-3'-dideoxy nucleosides prepared from unprotected vinylnucleobases. Studies aimed to establish the biological activity of these compounds are currently under way.

3. Experimental section

3.1 Reagents and instrumentation

Solid reagents, commercially available, were used without further preliminary purification; liquid reagents, instead, before being used were purified by distillation.

All solvents were dried and distilled according with the normal procedures reported in the literature.

Reactions were monitored by TLC on silica gel 60 F_{254} (Merck) with detection by charring with ethanolic solution of sulfuric acid; flash column chromatography was performed on silica gel 60 (Merck Kieselg 60H).

Vinylnucleobases were synthesized according to published procedures. ^{1}H NMR spectra were recorded at 300 MHz in $CDCl_3$ or DMSO-d_6 using tetramethylsilane (TMS) as internal standard (Bruker WM 300). Chemical shifts (δ) are given in part per million (ppm) from TMS and coupling costants (J) in hertz.

All the cycloaddition reactions were carried out in a household microwave oven (Whirpool AVM119/1/WP/WH) at 750 W irradiation power.

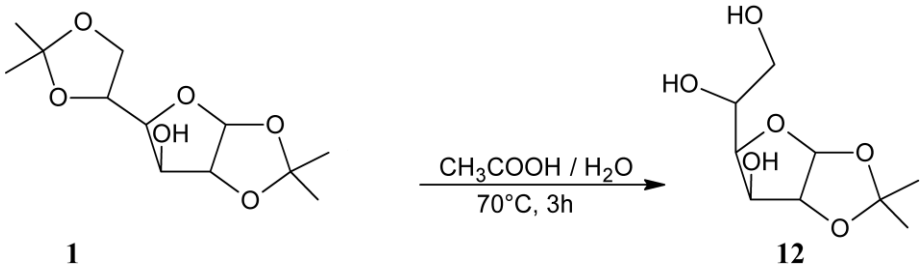
3.2 N-Vinylation of nucleobases: general procedures

Method A for pyridine nucleobases 1-4: the appropriate pyrimidine nucleobase (4.4 mmol) was heated at 140-150°C with hexamethyldisilazane (2.62 g, 16.2 mmol), trimethylsylil chloride (217 mg, 2.0 mmol) and trace of $(NH_4)_2SO_4$ until a clear solution was formed. Then, the solution was concentrated in vacuo. The residue was suspended in vinyl acetate (25.0 ml) and $Hg(OAc)_2$ (96 mg, 0.3 mmol), trimethylsilyl trifluoromethanesulfonate (245 mg, 1.1 mmol), and hydroquinone (0.1 g), the latter as a polymerisation inhibitor, were added under N_2. The mixture was refluxed for the appropriate time when the reaction was finished, the mixture was filtered through neutral activated alumina and was washed with EtOAc. The solvents were removed at reduce pressure and the crude product was purified by flash chromatography with $CHCl_3$-MeOH (92.5:7.5) as eluent.

Method B for purine nucleobases 5: the appropriate protected purine nucleobase (4.4 mmol) was added to a suspension of $Hg(OAc)_2$ trifluoromethane sulfonate (245 mg, 1.1 mmol) and hydroquinone (0.1 g), the latter as a polimerisation inhibitor, were added. The mixture was refluxed for the appropriate time. When the reaction was over, the mixture was filtered through neutral activated alumina and was washed wit EtOAc. The solvents were removed at reduced pressure. The crude product was purified by flash chromatography ($CHCl_3$-MeOH, 97.5:2.5 v/v) after suitable deprotection with gaseous ammonia.

All the NMR data obtained are comparable with those of Reference 41.

Synthesis of 1,2-O-isopropylidene-α-D-allofuranose



1 **12**

Reactive	**1**	**CH_3COOH**	**H_2O**
FW	260.29	60.05	18.0158
Quantity	2 g	30 ml	7.5 ml
Moles	0.0077	0.5259	0.4173
Density	-	1.05 g/ml	1 g/ml
Molar ratio	1	68.3	54.2

Diacetone-*D*-glucose **1** was dissolved in CH_3COOH (30 ml) and H_2O (7.5 ml). The solution was refluxed at 70°C until TLC showed the disappearance of the starting material (3 h), and then neutralised with satured aqueous Na_2CO_3 solution. The mixture was extracted with CH_2Cl_2 (3 x 15 ml), the combined organic layers were dried with Na_2SO_4 and the solvent removed under reduce pressure.

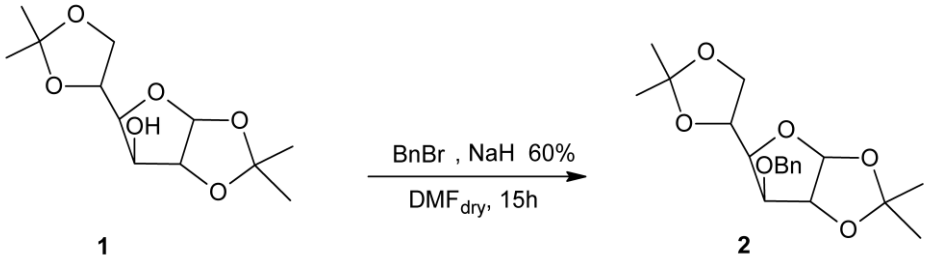
Product	FW	Quantity	Yield
12	220.29	0.40	23%

The product, a pale yellow oil, was characterized by ^{1}H-NMR.

^{1}H-NMR (300 MHz, $CDCl_3$)

δ (ppm): 1.35 (s, 3H, CH_3); 1.53 (s, 3H, CH_3); 2.38-2.67 (m, 2H, OH); 2.86 (s, 1H, OH); 3.75 (dd, 1H, J=5.10, 11.22 Hz, H_6); 3.77 (dd, 1H, J=3.55, 11.20 Hz, H_6'); 3.90-4.20 (m, 1H, H_5); 4.06-4.22 (m, 2H, H_3 + H_4); 4.77 (d, 1H, J=3.86 Hz, H_2).

Synthesis of 3-O-benzyl-1,2:5,6-di-O-isopropylidene-α-D-allofuranose



1 2

Reactive	**1**	**NaH 60%**	**BnBr**	**DMF dry**
FW	260.29	23.90	171.04	73.095
Quantity	20 g	7.3764 g	9.6 ml	100 ml
Moles	0.0768	0.1537	0.08064	-
Density	-	-	1.44 g/ml	-
Molar ratio	1	2	1.05	-

Diacetone-*D*-glucose **1** was added to a suspension of sodium hydride (7.3764 g of a 60% suspension in mineral oil, before use was washed with hexane and after with DMF) in DMF. The reaction was stirred for 2 h at room temperature. Benzyl bromide, freshly distilled, (9.6 ml) was added slowly, under nitrogen atmosphere, and the reaction mixture stirred for 15 h. Satured aqueous ammonium chloride was added and the mixture extracted with Et_2O (3 x 20 ml). The combined organic extracts were dried with Na_2SO_4, filtered

and concentrated under vacuum to yield the crude, a thick yellow oil, that was used directly in the next step without any further purification.

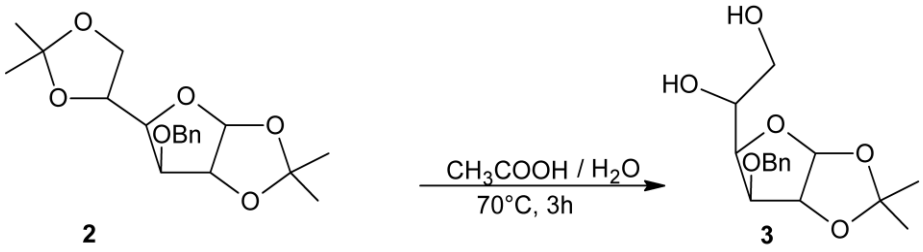
Product	FW	Quantity	Yield
2	349.4029	22.9841	86%

The product was characterized by ^{1}H-NMR.

^{1}H-NMR (300 MHz, CDCl3)

δ (ppm): 1.35 (s, 3H, CH_3); 1.42 (s, 3H, CH_3); 1.47 (s, 3H, CH_3); 1.54 (s, 3H, CH_3); 4.03-4.09 (m, 2H, H_6 + H_6'); 4.13-4.23 (m, 2H, H_3 + H_5); 4.37-4.46 (m, 1H, H_4); 4.63 (d, 1H, J=3.79 Hz, H_2); 4.71 (m, 2H, H_{Bn}); 5.95 (d, 1H, J=3.79 Hz, H_1), 7.30-7.50 (m, 5H, Ar).

Synthesis of 3-O-benzyl-1,2-O-isopropylidene-α-D-allofuranose



Reactive	**2**	**CH_3COOH**	**H_2O**
FW	349.4029	60.05	18.0158
Quantity	16.98 g	195 ml	52.3 ml
Moles	0.0486	3.4018	2.9030
Density	-	1.05 g/ml	1 g/ml
Molar ratio	1	70	59.73

The above benzyl derivative **2** was dissolved in CH_3COOH (195 ml) and H_2O (52.3 ml). The solution was refluxed at 70°C until TLC showed the disappearance of the starting material (3 h), and then neutralised with satured aqueous Na_2CO_3 solution and KOH. The mixture was extracted with EtOAc (3 x 15 ml), the combined organic layers were dried with Na_2SO_4 and the solvent removed under reduce pressure.

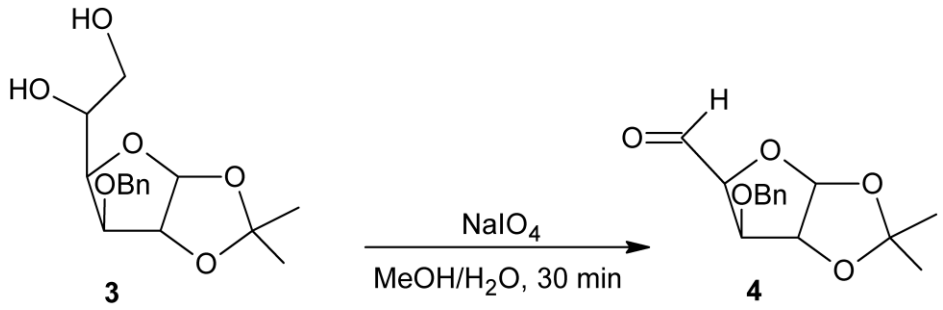
Product	**FW**	**Quantity**	**Yield**
3	310.3498	8.95	55%

The product, a pale yellow oil, was characterized by ^{1}H-NMR and used directly in the next step.

^{1}H-NMR (300 MHz, $CDCl_3$)

δ (ppm): 1.32 (s, 3H, CH_3); 1.49 (s, 3H, CH_3); 2.40-2.82 (m, 2H, OH); 3.69 (dd, 1H, J=5.42, 11.51 Hz, H_6); 3.81 (dd, 1H, J=3.45, 11.51 Hz, H_6'); 3.98-4.08 (m, 1H, H_5); 4.09-4.16 (m, 2H, H_3 + H_4); 4.55 (d, 1H, J=11.75 Hz, H_{Bn}); 4.63 (d, 1H, J=3.95 Hz, H_2).

Synthesis of 3-O-benzyl-1,2-O-isopropylidene-α-D-ribo-pento-dialdofuranose



Reactive	**3**	**$NaIO_4$**	**MeOH**	**H_2O**
FW	310.3498	213.89	32.039	18.0158
Quantity	8.95 g	11.4645 g	115 ml	61 ml
Moles	0.0288	0.0536	2.8392	3.3859
Density	-	-	0.791 g/ml	1 g/ml
Molar ratio	1	1.86	98.58	117.57

A solution of sodium metaperiodate (11.4645 g) in water (61 ml) was added dropewise, with stirring, to a solution of the diol **3** (8.95 g) in methanol (115 ml). After 30 min at room temperature, the solvent was removed by evaporation and the solid residue was extracted with chloroform (3 x 20 ml). The combined extracts were dried ($MgSO_4$), filtered and the filtrate was concentrated under reduced pressure to yield the crude **4** which was used directly in the next stage without further purification.

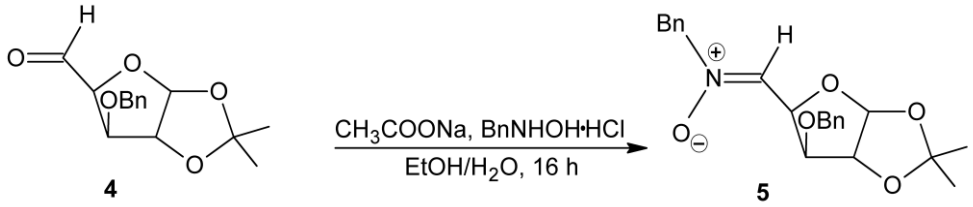
Product	FW	Quantity	Yield
4	278.3072	7.3946	92%

The product was characterized by ^{1}H-NMR.

^{1}H-NMR (300 MHz, $CDCl_3$)

δ (ppm): 1.33 (s, 3H, CH_3); 1.47 (s, 3H, CH_3); 4.15-4.81 (m, 5H, H_1 + H_2 + H_3 + H_{Bn}); 6.13 (d, 1H, J=3.46 Hz, H_4); 7.32-7.55 (m, 5H, Ar); 9.67 (s, 1H, --CHO).

Synthesis of N-benzyl-1,2-O-isopropylidene-α-D-ribopento-dialdofuranosyl nitrone



Reactive	**4**	**CH_3COONa**	**BnNHOH·HCl**	**$EtOH/H_2O$**
FW	278.3072	82.035	159.62	46.0694/18.0158
Quantity	7.3946g	2.6284 g	5.1078 g	(31.7/21.1) ml
Moles	0.0267	0.0320	0.0320	0.5505/1.1712
Density	-	-	-	(0.80/1) g/ml
Molar ratio	1	1.2	1.2	21 ml:44 ml

A mixture of **4** (7.3946 g), anhydrous sodium acetate (2.6284 g) and *N*-benzylhydroxylamine hydrochloride (5.1078 g) in ethanol/water (21 ml:44 ml) was stirred at room temperature for 16 h. The reaction mixture was extracted with chloroform (3 x 15 ml) and the collected organic layers were washed with satured aqueous $NaHCO_3$, dried over anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude reaction mixture was purified by column chromatography (eluent $CHCl_3$/MeOH 9.5:0.5) to give the nitrone **5** as a white solid.

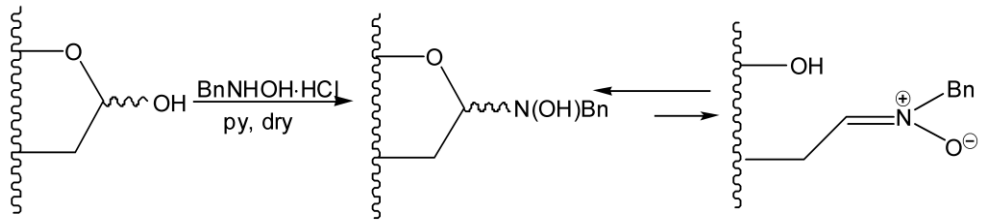
Product	FW	Quantity	Yield
5	383.4395	8.5614	83%

The product was characterized by ^{1}H-NMR.

^{1}H-NMR (300 MHz, $CDCl_3$)

δ (ppm): 1.30 (s, 3H, CH_3); 1.48 (s, 3H, CH_3); 4.11 (d, 1H, J=11.52 Hz, H_{Bn}); 4.58 (d, 1H, 11.52 Hz, H_{Bn}); 4.53-4.64 (m, 2H, H_3 + H_4); 5.22 (t, 1H, J=3.56 Hz, H_2); 5.95 (d, 1H, J=3.56 Hz, H_1); 6.85 (d, 1H, J=4.39 Hz, -CH=N); 7.10-7.48 (m, 10H, Ar).

Synthesis of 1-(N-benzylhydroxylamine)-2,3,4,6-O-tetrabenzyl-1-deoxy-β-D-glucose



13 **14**

Reactive	**13**	**BnNHOH·HCl**	**Py dry**
FW	540.65	159.62	79.045
Quantity	3.2439 g	1.1493 g	25 ml
Moles	0.006	0.072	0.3091
Density	-	-	0.978 g/ml
Molar ratio	1	1.2	51.52

To a stirred solution of sugar **13** (3.2439 g) in pyridine (25 ml) and in presence of activated powdered molecular sieves was added BnNHOH·HCl (1.1493 g) under nitrogen atmosphere. The solution was stirred overnight at room temperature. At the end of the reaction, TLC monitoring indicate the transformation of the starting material into the product, the mixture was filtered and the residue was washed with AcOEt. The solvent was removed at reduce pressure and the crude was purified by flash chromatography.

Product	FW	Quantity	Yield
14	646.7902	2.34	60%

The product, a white solid, was characterized by ^{1}H-NMR.

^{1}H-NMR (300 MHz, $CDCl_3$)

δ (ppm): 3.39-3.50 (m, 1H, H_5); 3.56 (t, 1H, J=9.67 Hz, H_4); 3.66(t, 1H, J=8.93 Hz, H_3); 3.70-3.82 (m, 2H, H_6+$H_{6'}$), 3.89 (t, 1H, J=8.66 Hz, H_2); 4.05 (d, 1H, J=8.66 Hz, H_1); 4.07 and 4.24 (2d, 2H, J=13.26 Hz, $PhCh_2N$); 4.47 (s, 1H, OH); 4.55 and 4.81 (2d, 2H, J=11.09 Hz, $PhCh_2$); 4.60 (s, 2H, $PhCh_2$); 4.82 and 4.94 (2d, 2H, J=11.10 Hz, $PhCH_2$); 4.77 and 5.03 (2d, 2H, J=11.10 Hz, $PhCH_2$); 6.99-7.60 (m, H, Ar).

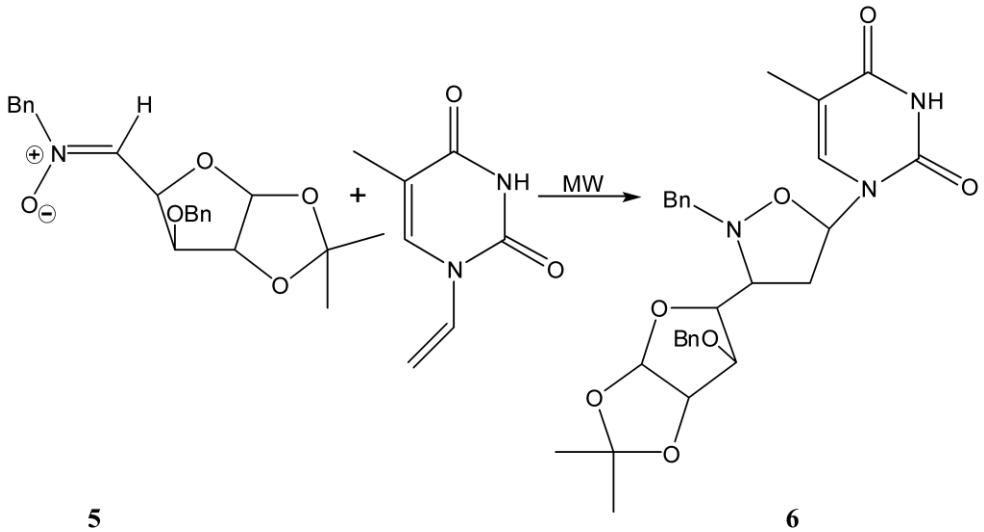
3.3 1,3-Dipolar cycloadditions: general procedure

The procedure is simple and straightforward consisting of the co-grinding of the two components, the nitrone **5** and the vinylnucleobase, in a mortar, further mixing of the solids in a vortex, followed by transfer of the mixture in an apposite open vessel, that is placed within an household microwave oven, at 750 W irradiation power.

After the appropriate time the reaction mixture, that appeared as a syrup, is dissolved in $CHCl_3$ or MeOH and analyzed by TLC. The product is purified by flash column chromatography and the isolated cycloadducts are subjected to ^{1}H-NMR spectroscopy.

The unreacted nitrone is equally recovered from the chromatographic separation and may be reused without loss of efficiency or selectivity.

Synthesis of 4'-aza-4'-(N-benzyl)-3'-(3-O-benzyl-1,2-O-isopropylidene)-α-D-allofuranosyl-2'-3'-dideoxythymidine



5 **6**

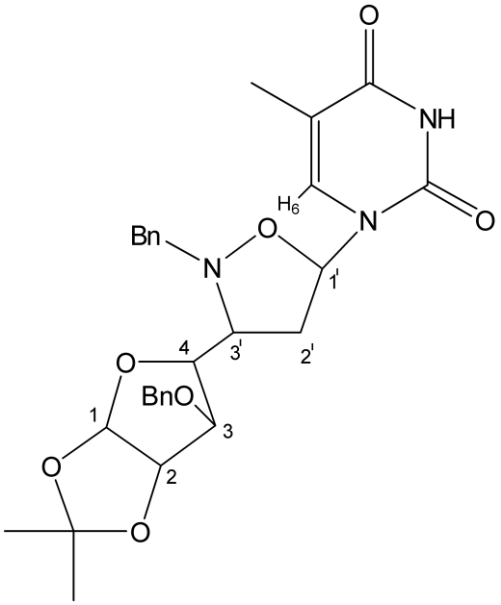
Reactive	**5**	***N*-1-vinylthymine**	**MW**	**t (min)**
FW	383.4395	152	750	6
Quantity	0.5 g	0.0991 g		
Moles	0.00130	0.000652		
Molar Ratio	2	1		

Product	**FW**	**Quantity**	**Yield**
6	535.5797	0.2618	75%

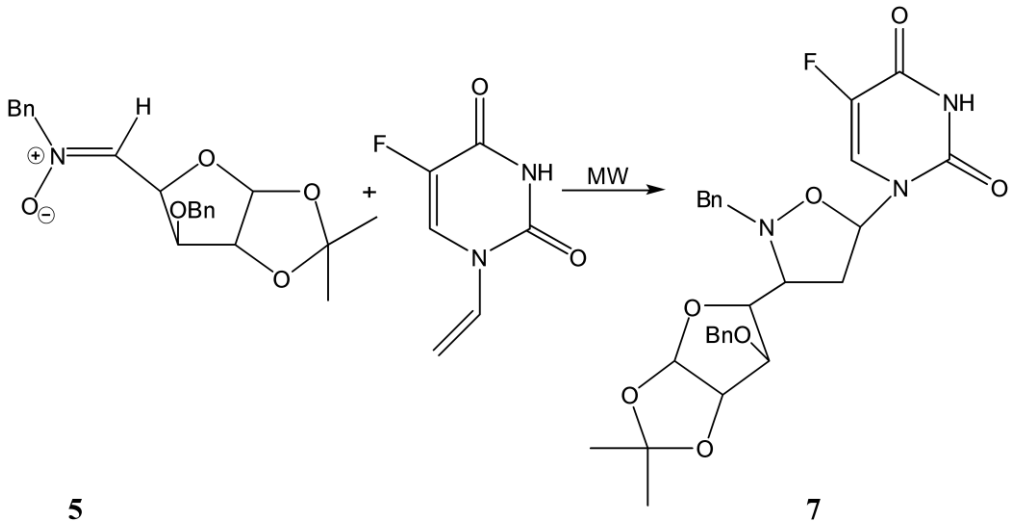
The product **6**, a brown solid, was characterized by ^{1}H-NMR.

^{1}H-NMR (300 MHz, CDCl3)

δ (ppm): 1.36 (s, 3H, CH_3); 1.50 (s, 3H, CH_3); 1.95 (m, 3H, CH_3-Thy); 2.46-2.73 (m, 1H, $H_{2a'}$); 3.10-3.27 (m, 1H, $H_{2b'}$); 3.29-3.38 (m, 1H, $H_{3'}$); 3.76-4.00 (m, 2H, H_3 + H_4); 4.23-4.38 (m, 3H, NCH_2Ph + H_2); 4.62-4.80 (m, 3H, OCH_2Ph + H_1); 6.24 (dd, 1H, J=3.22, 7.90 Hz, $H_{1'}$); 7.18-7.65 (m, 10H, Ar); 8.01 (m, 1H, H_6-Thy); 9.54 (s_b, 1H, NH).



Synthesis of 4'-aza-4'-(N-benzyl)-3'-(3-O-benzyl-1,2-O-isopropylidene)-α-D-allofuranosyl-2'-3'-dideoxy-5-fluorouridine



5 **7**

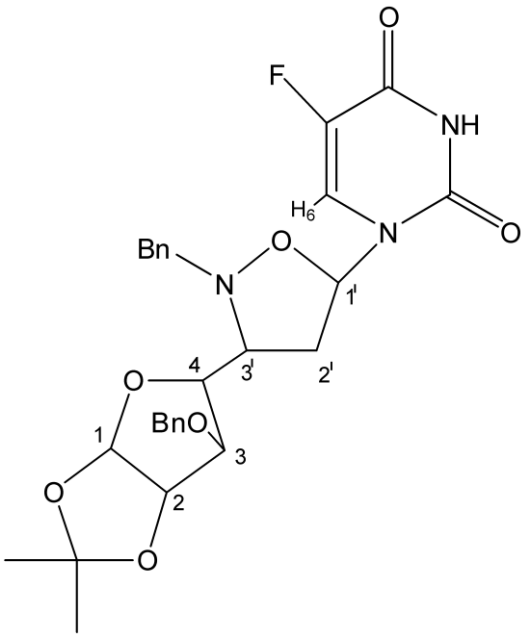
Reactive	**5**	***N*-1-vinyl-5-fluorouracil**	**MW**	**t (min)**
FW	383.4395	156.1035	750	6
Quantity	0.5 g	0.1018 g		
Moles	0.00130	0.000652		
Molar Ratio	2	1		

Product	**FW**	**Quantity**	**Yield**
6	539.543	0.2287	65%

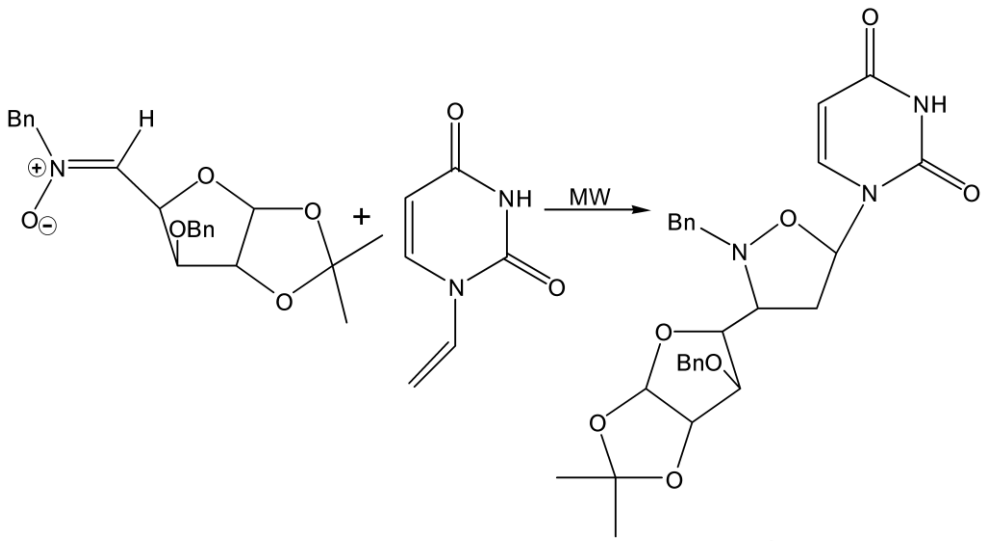
The product **7**, a brown solid, was characterized by ^{1}H-NMR.

^{1}H-NMR (300 MHz, CDCl3)

δ (ppm): 1.36 (s, 3H, CH_3); 1.57 (s, 3H, CH_3); 2.60-2.75 (m, 1H, $H_{2a'}$); 3.18-3.33 (m, 1H, $H_{2b'}$); 3.35-3.46 (m, 1H, $H_{3'}$); 3.90-4.16 (m, 2H, H_3 + H_4); 4.35-4.52 (m, 3H, NCH_2Ph + H_2); 4.63-4.78 (m, 3H, OCH_2Ph + H_1); 6.26 (dd, 1H, J=3.24, 7.87 Hz, $H_{1'}$); 7.25-7.81 (m, 10H, Ar); 7.95 (m, 1H, H_6-5F-Ura); 9.40 (s_b, 1H, NH).



Synthesis of 4'-aza-4'-(N-benzyl)-3'-(3-O-benzyl-1,2-O-isopropylidene)-α-D-allofuranosyl-2'-3'-dideoxyuridine



5 **8**

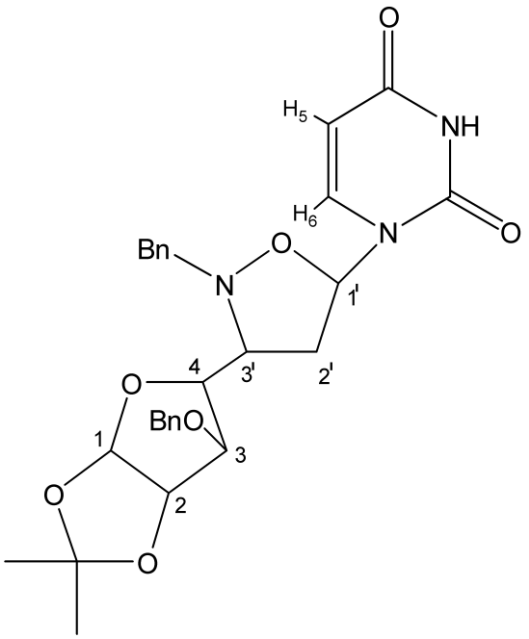
Reactive	**5**	***N*-1-vinyluracil**	**MW**	**t (min)**
FW	383.4395	138	750	7
Quantity	0.5 g	0.0899 g		
Moles	0.00130	0.000652		
Molar Ratio	2	1		

Product	**FW**	**Quantity**	**Yield**
8	521.5529	0.2381	70%

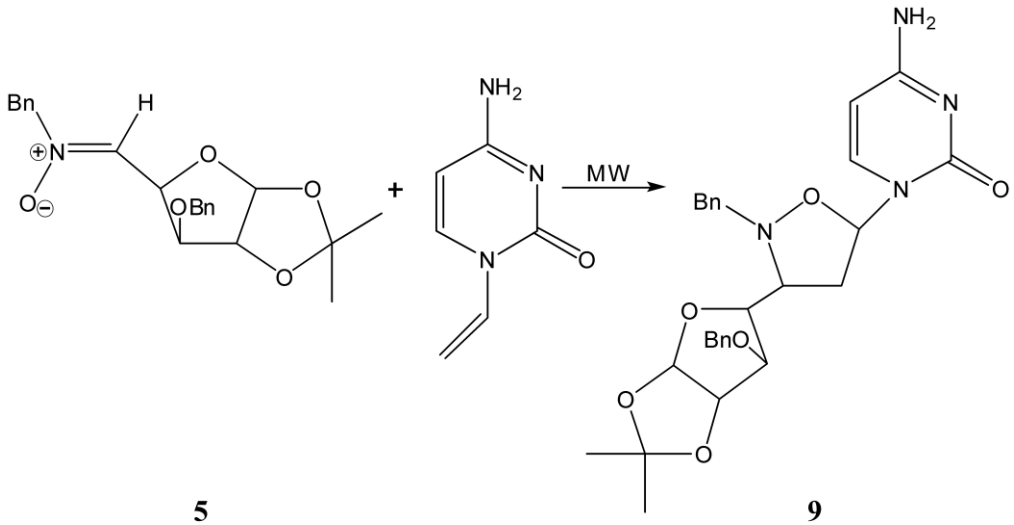
The product **8**, a brown solid, was characterized by ^{1}H-NMR.

^{1}H-NMR (300 MHz, CDCl3)

δ (ppm): 1.34 (s, 3H, CH_3); 1.53 (s, 3H, CH_3); 2.58-2.72 (m, 1H, $H_{2a'}$); 3.15-3.24 (m, 1H, $H_{2b'}$); 3.32-3.41 (m, 1H, $H_{3'}$); 3.88-4.10 (m, 2H, H_3 + H_4); 4.29-4.44 (m, 3H, NCH_2Ph + H_2); 4.60-4.74 (m, 3H, OCH_2Ph + H_1); 5.58 (d, 1H, J=8.15 Hz, H_5-Ura); 6.22 (dd, 1H, J=3.18, 7.94 Hz, $H_{1'}$); 7.20-7.70 (m, 10H, Ar); 7.90 (d, 1H, J=8.15 Hz, H_6-Ura); 9.35 (s_b, 1H, NH).



Synthesis of 4'-aza-4'-(N-benzyl)-3'-(3-O-benzyl-1,2-O-isopropylidene)-α-D-allofuranosyl-2'-3'-dideoxycytidine



5 **9**

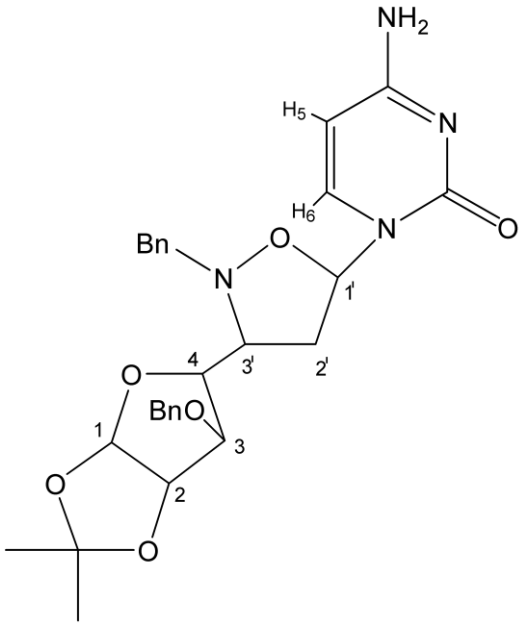
Reactive	**5**	***N*-1-vinylcytosine**	**MW**	**t (min)**
FW	383.4395	137	750	10
Quantity	0.5 g	0.0893 g		
Moles	0.00130	0.000652		
Molar Ratio	2	1		

Product	**FW**	**Quantity**	**Yield**
9	520.5608	0.2376	70%

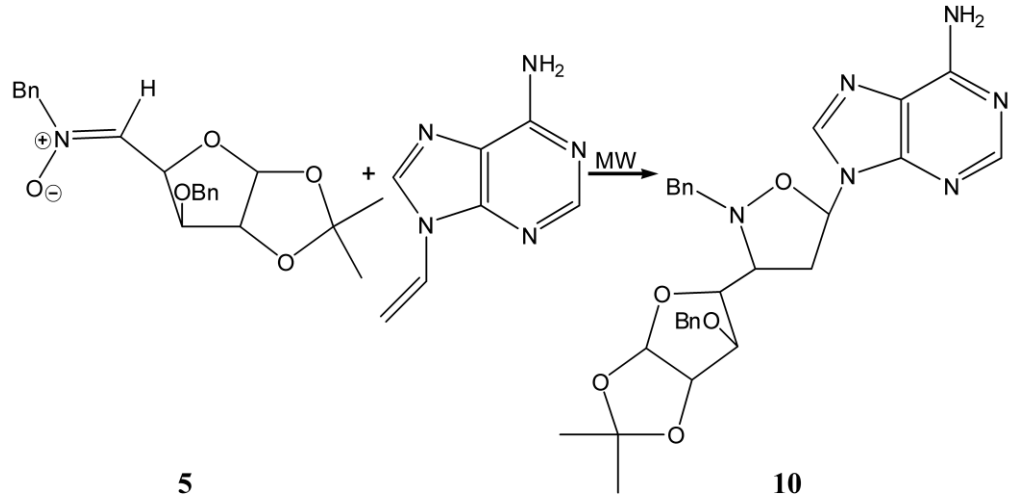
The product **9**, a brown solid, was characterized by ^{1}H-NMR.

^{1}H-NMR (300 MHz, CDCl3)

δ (ppm): 1.30 (s, 3H, CH_3); 1.49 (s, 3H, CH_3); 2.56-2.78 (m, 1H, $H_{2a'}$); 3.18-3.29 (m, 1H, $H_{2b'}$); 3.35-3.48 (m, 1H, $H_{3'}$); 3.79-4.06 (m, 2H, H_3 + H_4); 4.36-4.42 (m, 3H, NCH_2Ph + H_2); 4.58-4.71 (m, 3H, OCH_2Ph + H_1); 5.88 (d, 1H, J=7.32 Hz, H_5-Cyt); 6.32 (dd, 1H, J=3.25, 7.88 Hz, $H_{1'}$); 7.34-7.77 (m, 10H, Ar); 8.16 (d, 1H, J=7.32 Hz, H_6-Cyt); 10.03 (s_b, 1H, NH_2).



Synthesis of 4'-aza-4'-(N-benzyl)-3'-(3-O-benzyl-1,2-O-isopropylidene)-α-D-allofuranosyl-2'-3'-dideoxyadenosine



5 **10**

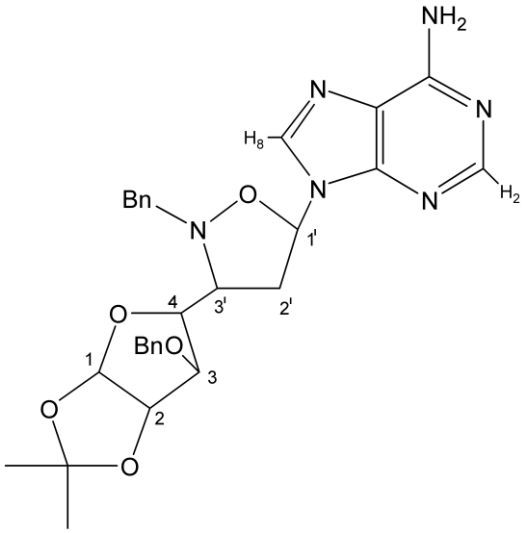
Reactive	**5**	***N*-1-vinyladenine**	**MW**	**t (min)**
FW	383.4395	161	750	6
Quantity	0.5 g	0.1051 g		
Moles	0.00130	0.000652		
Molar Ratio	2	1		

Product	**FW**	**Quantity**	**Yield**
10	544.5695	0.2769	78%

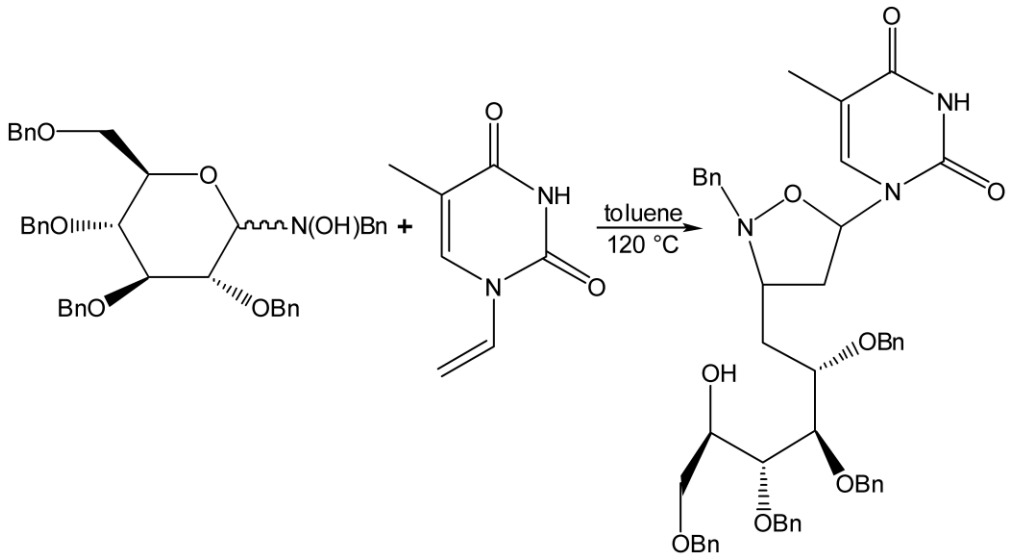
The product **10**, a brown solid, was characterized by ^{1}H-NMR.

^{1}H-NMR (300 MHz, CDCl3)

δ (ppm): 1.38 (s, 3H, CH_3); 1.56 (s, 3H, CH_3); 2.53-2.74 (m, 1H, $H_{2a'}$); 3.20-3.27 (m, 1H, $H_{2b'}$); 3.29-3.46 (m, 1H, $H_{3'}$); 3.85-4.15 (m, 2H, H_3 + H_4); 4.36-4.42 (m, 3H, NCH_2Ph + H_2); 4.58-4.71 (m, 3H, OCH_2Ph + H_1); 6.28 (dd, 1H, J=3.25, 7.98 Hz, $H_{1'}$); 7.24-7.77 (m, 10H, Ar); 8.30 (s, 1H, H_2-Ade); 8.55(s, 1H, H_8-Ade);10.00 (s_b, 1H, NH_2).



Synthesis of 4'-aza-4'-(N-benzyl)-3'-(2,3,4,6-O-tetrabenzyl glucosyl)-2',3'-dideoxythymidine



14 **15**

Reactive	**14**	***N*-1-vinylthymine**	**t (h)**
FW	645.7907	152	74
Quantity	0.250 g	0.0293 g	
Moles	0.000387	0.000193	
Molar Ratio	2	1	

A mixture of **14** (0.250g), *N*-1-vinylthymine (0.0293 g), hydroquinone, this latter was added as polymerisation inhibitor, in toluene(15 ml) was heated at 120°C for 74 h. The reaction was controlled by TLC (Et_2O-Hexane 8:2 v/v).

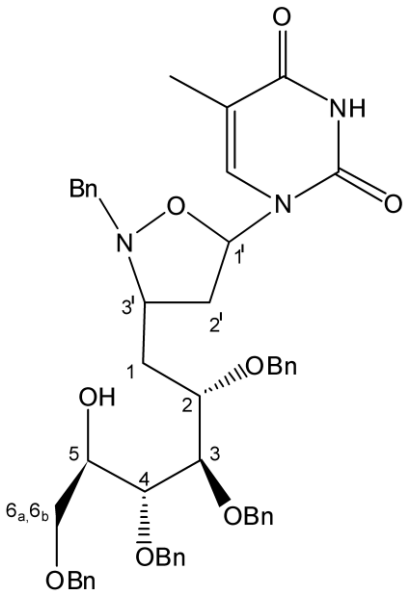
When the reaction was finished the solvent was removed at reduce pressure and the crude purified by flash chromatography.

Product	FW	Quantity	Yield
15	813.7907	0.1241	79%

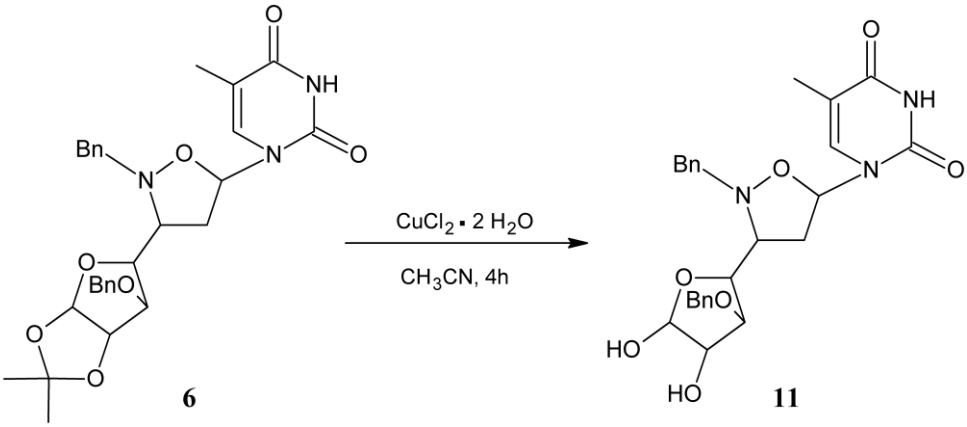
The product **15**, a brown solid, was characterized by ^{1}H-NMR.

^{1}H-NMR (300 MHz, CDCl3)

δ (ppm): 5.04-4.76 (m, 3H, H_1 + CH2Ph); 4.73-5.59 (m, 3H, H_2 + CH_2Ph); 4.49-4.21 (m, 5H, H_5 + $2CH_2Ph$); 4.05-3.82 (m, 1H, H_3); 3.90-3.78 (m, 1H, H_4); 3.59-3.42 (m, 2H, H_{6a} + H_{6b}); 2.85 (s, 1H, OH).



Deprotection of 4'-aza-4'-(N-benzyl)-3'-(3-O-benzyl-1,2-O-isopropylidene)-α-D-allofuranosyl-2'-3'-dideoxythymidine



Reactive	**6**	**$CuCl_2 \cdot H_2O$**	**CH_3CN**
FW	535.5797	170.48	41.05
Quantity	0.2618 g	0.1667 g	5 ml
Moles	0.00049	0.00098	-
Density	-	-	-
Molar ratio	1	2	-

To the cycloadduct **6** was added CH_3CN (5 ml) and $CuCl_2 \cdot 2H_2O$ (0.1667 g) and the mixture was stirred under reflux for 4 h. When the reaction is finished the solvent was removed under reduce pressure. The residue was washed with satured aqueous $NaHCO_3$ solution and then extracted with Et_2O

(3 x 15 ml), the combined organic layers were dried with Na_2SO_4 and the solvent removed under reduce pressure. The crude reaction mixture was purified by column chromatography (eluent $CHCl_3$/MeOH 9.5:0.5) to give the product **11** as a brown solid.

Product	**FW**	**Quantity**	**Yield**
11	495.5797	0.0728	30%

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Introduction

The botanical name of the olive tree is *Olea europaea*, the *genus Olea* is a member of the family *Oleaceae* which also contains several other well know genera.[1]

Since 1950s medical and nutritional investigations have confirmed the importance of olive oil, with a high content of monounsaturated fatty acids, in reducing mortality from cardiovascular disease.[2] It has also been suggested that a high intake of olive oil may offer protection against a number of cancers. Olive oil is promoted as part of the "Mediterranean diet", which is currently viewed as making a favorable dietary contribution and has a positive image in terms of consumer appeal.[3] The olive oil and table olive industries play an important role in the agricultural and processing sectors of the major olive producing countries. The main exporter countries, decreasing respectively, are Spain, Italy, Greece, Turkey, Tunisia, Portugal and Morocco, and other countries near the Mediterranean Sea as well as from Argentina and California.[4]

Several typical olive oils produced in the European Union's olive growing countries have received a European protected origin denomination (POD) trademark or a European protected geographical indication (PGI) trademark; therefore, reliable multivariate statistical procedures should be studies to classify them in order to disclose commercial frauds.[5] Therefore, attempts to adulterate this commodity with less expensive materials, such as seed oils and/or olive oils of lower quality (refined olive oil) are by no means rare. These typical oils are generally mixtures made up of a major oil variety and fixed proportions of some minor ones; thus, the studies to classify them are approached with the same procedures followed for monovarietal oils.[6]

Among all the existing olive oils, obtained from the grinding of olives, the extra virgin oil must be obtained simply by crushing and centrifugation procedures conducted at low temperature without any chemical treatment. Besides, extra virgin olive oils have to comply with a maximum acid content (up to 0.8% free fatty acids, calculated as oleic acid) and are submitted to a panel test to evaluate the peculiar flavourings and tastes of the finest products.

These oils are complex mixtures containing a wide variety of substances and their composition is linked to cultivar, region, altitude, time of harvest and extraction process. The main fat components, that represent more than 98% of the total substances, are the triglycerides (TAGs) (consisting of three fatty acids linked to a glycerol backbone). The minor components are free fatty acids, vitamins, polyphenols, phytosterols, chlorophyll, carotenoids, mono and diacylglycerides.[7]

Diacylglycerols (DGs) are found in edible vegetable oils in low amounts (between 1 and 10%). They are formed as intermediate products in the biosynthesis of triacylglycerols (TGs), as well as by acidic and enzymatic hydrolysis of TGs during oil extraction, refining and storage. Simultaneously, isomerisation processes produce changes in the composition of the DGs. Knowledge of the quantity and composition of DGs is, therefore, of great interest for the evaluation of the quality of the oil and of the treatments to which the oil is subjected. Finally, as the freshness of olive oils is strictly connected with their peculiar organoleptic and nutritional properties, it seemed of interest to study the aging effects on these products.

Determination of DGs can be performed using two different methodologies: (i) isolation of the DGs and further analysis; (ii) direct analysis of the sample using instrumental techniques.[8]

1. ^{31}P NMR spectroscopy in the quality control and authentication of extra-virgin olive oil

1.1 The minor constituents in vegetable oils

Vegetable oils are mainly constituted by triacylglycerols (95-98%) and complex mixtures of minor compounds (2-5%) of a wide range of chemical nature. These minor constituents show a broad qualitative and quantitative composition, depending on the vegetal species from they were obtained. Moreover, in the same species, content and composition of these components can vary due to the agronomic and climatic conditions, fruit or seed quality, oil extraction system and refining procedures. Finally, during storage of the oil, the hydrolysis, esterification and oxidation also originate changes in the minor constituents. Accordingly, the determination of the minor constituents is essential for the analytical assessment of the quality, origin, extraction method, refining procedure and possible adulteration of the vegetable oils.

The main groups of minor constituents present in vegetable oil are: fatty alcohols, wax esters, hydrocarbons, tocopherols and tocotrienols, phenolic compounds, volatiles, pigments, minor glyceridic compounds, phospholipids and triterpenic acids.

Alcoholic compounds: long-chain aliphatic and triterpenic alcohols, methylsterols, sterols and triterpenic dialcohols are important for the characterization of vegetable oils. They vary more widely between different oils than the fatty acid composition, and their content and composition in the oil are a rich source of information about the oil origin, providing information on the detection of mixtures. These compounds are present as free alcohols and fatty acid esters. The saponification allows a detailed separation of these

constituents, but does not provide information about their original structure, whether or not the component was esterified in the oil. The compositions of the free and esterified sterols, alcohols and triterpenic alcohols are not identical, and different extraction procedures or refining methods have different effects on free and esterified constituents. In addition, the ratio of free/esterified alcohols is related to the oil quality.[9]

Wax esters: formed by the reaction of alcohols (aliphatic, triterpenic, methylsterols and sterols) and free fatty acids are present in seed and fruits. During the oil extraction process, a fraction of these esters is transferred into the oil, depending on the oil extraction system. So solvent extracted oils contain higher concentration of wax esters compared with cold-pressed and centrifuged ones. C_{40}, C_{42}, C_{44} and C_{46} waxes, deriving from straight chain alcohols are very abundant in olive pomace oils while low concentrations are found in olive oils. In addition, great concentration of waxes yields turbidness during refining or storage. Consequently, the determination of waxes is important to evaluate the quality and genuineness of some vegetable oils.[10]

Hydrocarbons: in virgin olive oil, the major hydrocarbon is the squalene a terpenoid hydrocarbon occurring in high concentrations (800-12000 mg/kg). It is accompanied by n-alkanes in the range of C_8-C_{35}, being the more abundant the comprehended between C_{21} and C_{35}, in which alkanes with an odd number of carbon atoms predominated over those of even numbers.[11] Besides, also low amounts of unsaturated aliphatic, sesquiterpenic, low-molecular-mass aromatic (from benzene to tetra-methylbenzene, including styrene), and polycyclic aromatic (mainly low-molecular-mass ones) hydrocarbons have been detected. In other crude vegetable oils the hydrocarbons series is similar to those encountered in olive oil, although the concentration of squalene is significantly lower. The origin of the volatile aromatic hydrocarbons in virgin olive oils has been attributed to the metabolism inside the fruits and the equilibrium of olives and oils with the air

environmental pollution. During the refining process, the squalene isomerize yielding a number of components with molecular mass 410. In the same way, isoprenoid alkenes of molecular mass 408 have been characterized attributed to dehydration products of oxidized squalene. Significant amounts of hydrocarbons with steroidal skeleton are formed in vegetable oils because of thermal treatments during the refining process. The composition of steroidal hydrocarbon fraction enable to identify the oil origin, since their composition reflects that of the sterols.

Tocopherols and tocotrienols: there are four natural tocopherols and four tocotrienols, all R-configuration, which form the vitamin E group. The two classes different by the presence of three double bonds in the side-chain of the tocotrienol series. The vitamin E group is lipid-soluble and the member are abundant in most vegetable oils in varying amounts.[12] Tocopherols in crude vegetable oils are partially destroyed by refining treatments. A further decomposition during storage is possible; an additional effect of light was of greater influence (higher decrease of the content) than the effect of the oxygen.

Phenolic compounds: are a group of polar components, which contain one or more aromatic hydroxylated rings. Some of them show antioxidant activity and are responsible of the bitter taste of the vegetable oils. They are present in significant amounts (<350ppm) in virgin olive oils and crude grape seed oils, but they are destroyed in the various refining stages. In olive oil, the main phenols are secoiridoid compounds originated by degradation of the glucosides oleuropein and ligstroside. The subsequent hydrolysis of these compounds leads to the formation of simple phenols such as tyrosol and hydroxytirosol.[13] Minor amounts of phenolic acid as caffeic, p-coumaric and syringic, and flavonoids as luteolin and apigenin are also detected.

Volatile compounds: flavour and aroma of the oils are generated by a number of volatile constituents that are present at extremely low

concentrations. The volatile fraction of virgin olive oils has been profusely studied, and includes saturated, unsaturated, aromatic and terpenic hydrocarbons, as well as, alcohols, aldehydes, esters and ethers.[14] During oxidation of crude and refined vegetable oils various compounds responsible of undesirable flavour are formed. The term volatile fraction is confusing and there is not an agreement about its exactly definition, mainly due to the experimental conditions applied by each research group for their obtention. Thus, depending on the temperature and time used in the obtention of the volatile fraction, different results can be found.

Pigments: Chlorophylls and carotenoids are the main pigments in vegetable oils, being pheophytin the principal component of the chlorophyll group. Carotenoids are divided into two groups: carotenes and xantophylls. While carotenes are purely hydrocarbons, xantophylls are oxygenated at the end groups and hence polar. The type and amount of pigment in vegetable oils depend fundamentally on the species, cultivars, state of ripeness, agronomic conditions, and in general undergo a considerable variation during storage and preparation as edible oils. Both chlorophylls and carotenoids are considered to have an important role in keeping the quality of edible oils, mainly due to their action as photo-sensitizers or singlet oxygen quenchers respectively.[15]

Minor glyceridic polar compounds: water, temperature and oxygen are considered as the main factors affecting oil degradation. Thus, water present in food is the cause of the hydrolysis which produce free fatty acids, partial glycerides and glycerol capable of generate from dehydration acrolein.[16] In the presence of oxygen, glyceride autoxidation leads to formation of hydroperoxide derivatives. The primary oxidation product produces other oxidized triacylglycerols monomers (alcohols, epoxi, ketones) and polymers. In addition, the high temperatures used in the deodorization step during the oil refining and during frying or cooking uses, lead to the cycling of the unsaturated fatty acids and polymerization of triacylglycerols (TGs). The

above-mentioned factors alter in more or less extension all the oil constituents depending on the unsaturation and on their initial quality in the original oil. Consequently, altered products coming from TGs will be more important quantitatively than the minor constituents, as TGs are the major constituents. They are called genetically minor glyceridic polar compounds and have been more extensively studied than those coming from non-glyceridic constituents. The intensity of the changes is narrowly related to the duration of the processes and to the oil composition. Besides, degradations are not independent, and the compounds interact once and toward to a more advance-altered stage, increasing the complexity of the process and the compounds obtained. Many chemical and physicochemical parameters has been proposed for measuring the oil alteration, even, countless compounds have been isolated and identified. Actually, it is well accepted that analytical index widely used in the past, supply only partial information about oil degradation, being advisable the evaluation of polar glyceridic compounds.

Phospholipids (PLs): are important constituents of crude oil seeds. The measurement of PLs is important in determining the stability and quality of vegetable oils. Phospholipids are undesirable in oil since they are responsible for oil discoloration during deodorization and steam distillation and losses of neutral lipids during neutralization. They affect the stability of the oil by chelating metals and increasing the amount of metal ions. The removal of PLs results in elimination of iron and copper, which increases the oxidative stability and facilitates the refining process.[17]

Triterpenic acids: virgin olive oils contains minor amounts of oleanolic and maslinic acids, but significant amounts (0.1% and 0.4% respectively) were isolated from crude olive pomace oils.[18] These compounds seem to be responsible for the gelatinous precipitates in crude olive-pomace oil and the turbidity observed in some physically refined olive oils.[19]

1.2 The natural content of diacylglycerols in virgin olive oils

Diacylglycerols, usually termed diglycerides (DGs), are minor constituents of virgin olive oils accompanying the major triacylglycerol or triglyceride (TG) components. They are found as 1,2-diglycerides (1,2-DGs) and 1,3-diglycerides (1,3-DGs). Originally, 1,2-DGs arise from the incomplete biosynthesis of triacylglycerols, whereas a second source of 1,2-DGs formation is the limited enzymatic hydrolysis (lipolysis) of TGs. On the other hand, 1,3-DGs are considered to be secondary products resulting from the isomerization of 1,2-DGs during the extraction process and continued during the storage of the olive oil.[20,21,22]

Therefore, freshly made virgin olive oil from healthy olive fruits are expected to contain almost solely 1,2-DGs, the concentration of which decreases during storage while the 1,3-DGs content and the total DG concentration increase. In this respect, the concentration levels of both 1,2-DGs and 1,3-DGs may be indicative of the olive oil freshness. From these facts, it has been suggested that the ratio of 1,3-DGs to 1,2-DGs and the ratio of 1,2-DGs to the total amount of diglycerides [D = 1,2-DGs/(1,2-DGs + 1,3-DGs)] are useful indices to assess the age and quality of olive oils. Although no official regulations have been established regarding the DG content of the various olive oils grades, fresh extra virgin olive oils of the same olive variety are expected to have the lowest ratio 1,3-DGs/1,2-DGs and the highest value for the parameter D.

The natural content of DGs in fresh virgin olive oils does not exceed 1-3%, in refined olive oils the level of diglycerides (mainly 1,3-DGs) is higher (4-5%), and it goes up to 15-20% in pomace oils.[23,24] Also, larger amounts of DGs are obtained in neutralized oils produced from starting materials with high levels of free fatty acids or when they are extracted with solvents from olive husks and then refined through industrial process. In this respect, the

content of 1,2-DGs and 1,3-DGs can provide a good discrimination between virgin olive oils and low-quality olive oils.

For virgin olive oils, the amount of DGs and the ratio D depend on several factors, such as the olive variety, the ripeness of the olive fruit, the environment, and the storage life of the product. The effect of the olive variety on the amount of diglycerides and the ratio D cannot be distinguished easily from the effect of the environment. The effect of the degree of olive ripeness is reflected on the diminution of the amount of 1,2-DGs and the ratio D as the olive fruit becomes ripe. Recent studies have show that unripe olives are characterized by larger amounts of 1,2-DGs than overripe olive fruits, whereas normal ripeness results in an intermediate amount of 1,2-DGs. Storage seems to have an effect on commercial olive oils, which in addition are of unknown origin. [25]

Evaluation of the storage time is important for the olive oils suppliers, who deal with tons of olive oil, and, of course, for the consumers. The concentration changes of DGs upon storage do not have an immediate effect on the organoleptic properties of olive oil. However, they do reflect in a quantitative manner the aging of olive oil. This is quite important since aging is accompanied by degradation of the natural antioxidants of olive oil, such as α-tocopherol and phenolic compounds, thereby downgrading the quality of olive oil. After long storage (one year or more), olive oil becomes rancid with poor nutritional and organoleptic characteristics.

1.3 Some different analytical methods used to characterize a vegetable oil

The ability of an analytical method to characterize a vegetable oil is based on the identification and quantification of those compounds that are expected to be in connection with their origin and quality attributes. This is, however, a difficult task because these groups contain numerous species with a wide range of polarities, concentrations and chemical structures. Therefore, the methods require usually the isolation and analysis of minor constituents by means of several procedures of separation, identification and quantification. An enrichment of the components of interest is usually necessary, but also a high separation efficiency and selectivity. Those characteristics are normally achieved by chromatographic techniques.

To analyze the minor constituents of vegetable oils, a preliminary qualitative and quantitative isolation step from the triacylglycerol matrix is required. Three basic procedures are normally used: saponification, liquid-liquid partition and chromatographic techniques. The saponification (heating with alcoholic solution of potassium hydroxide) transforms the glyceridic compounds in polar soaps allowing the extraction of the unsaponifiable matter with hexane or diethyl ether. Nevertheless, this procedure is not appropriate for wax esters, sterol, esters, phenols, pigments, minor glyceridic compounds and phospholipids, since they are altered during the saponification.[26]

Liquid-liquid partition with polar solvents (methanol, methanol-water, etc.) is suitable for the isolation of phenols, polycyclic aromatic hydrocarbons and chlorophylls. Recently supercritical fluid extraction has gained importance in the separation techniques, due to the possibility of modifying product solubilities through alteration of pressure and/or temperature, or adding modifiers, substituting a wide variety of liquid solvents.

Column chromatography is widely used to separate fractions having constituents of similar polarities. Actually, the latter its being substituted by solid-phase extraction (SPE), as it is a quicker technique and saves solvent volumes. Finally, the isolation of volatile compounds is achieved by gas stripping or distillation. The isolated fractions are still complex and require further fractioning, usually by means of thin-layer chromatography (TLC) or preparative high-performance liquid chromatography (HPLC).[27]

The qualitative and quantitative determination of the constituents is often done by capillary gas chromatography (GC) of the compounds or their derivatives. GC in general assumes that the compounds injected are volatile at the temperature of analysis and that they do not decompose at either the temperature of injection or analysis. In standardized analytical methods, flame ionization detection (FID) is the most widely used. Mass spectrometry (MS) allows obtaining molecular mass data, structural information and identification of compounds.[28]

HPLC is used normally for separating non-volatile, high-molecular-mass constituents employing either adsorption or partition chromatography. Adsorption chromatography, namely normal phase, is widely used to separate classes of constituents according to the nature and number of polar functional groups. In normal-phase HPLC the adsorbent is silica gel and the eluent is a non-polar solvent. Reversed-phase HPLC, which is based on partition chromatography, is used to separate individual components that belongs to one constituent class. In this case, the stationary phase usually consist of a non-polar octadecylsilane (C_{18}) bonded phase, while the mobile phase is a polar solvent. Several detection methods can be used in conjunction with HPLC, the ultraviolet-visible (UV-Vis) being the most commonly used. Other detection methods, such as refractive index (RI), FID, MS, evaporative light scattering (ELSD), fluorescence (FD) and electrochemical detection are also used.[29]

A new technique have been developed and widely used in recent years, that combines HPLC and capillary GC achieving in one process the isolation of the fraction, its transfer, through an interface to the gas chromatograph and the GC analysis, allowing high separation efficiency and high sensitivity. Online coupling provides a very interesting approach to integrate sample preparation into chromatographic procedure and, consequently, offers a new and practical alternative to traditional methods of sample preparation. Coupled chromatographic techniques (HPLC-GC) are being increasingly used, since they allow the isolation and determination of the compounds avoiding the sample preparation and clean up.

Supercritical fluid chromatography (SFC) is a relative new separation technique that has features from both GC and HPLC, using supercritical CO_2 (SC-CO_2), as the mobile phase and both HPLC and GC detectors.

1.4 Determination of diglycerides in olive oils

The quantitative determination of diglycerides in olive oils has been carried out by using several chromatographic techniques, such as gas chromatography, high-performance size exclusion chromatography, and high-performance liquid chromatography.[30,31] In recent years, high-resolution ^{1}H and ^{13}C NMR spectroscopy have been applied effectively for the characterization of mono- and diglycerides in olive oil samples.[32] The amount of information contained in an NMR spectrum obtained fairly rapidly, combined with easy sample preparation, renders this spectroscopic technique very attractive for the determination of the composition of olive oil. ^{1}H NMR spectroscopy has provided valuable information about lipid classes, fatty acid composition, unsaturation levels and several minor compounds (sterols,

squalene, terpenes, volatile compounds, etc.), whereas ^{13}C NMR, among others, has given unique information about the positional distribution of fatty acids on the glycerol moiety and the stereochemistry of unsaturation.[33]

Although these magnetic resonance methods are quantitative and do not require any sample pretreatment, they are not so effective as ^{31}P NMR spectroscopy under certain circumstances. For instance, the unambiguous identification and quantification of certain minor constituents of olive oil, such as mono- and diacylglycerols, demand ^{1}H NMR spectrometers operating at rather high magnetic field strengths (>14.1 T or 600 MHz in terms of the proton Larmor frequency). At lower magnetic field strengths, the diacylglycerol resonances are overlapped by the strong resonances of the triacylglycerols, questioning therefore the ability of this magnetic resonance method for reliable quantitative determination of these minor components at lower magnetic field strengths.[34] On the other hand, the large range of chemical shifts (~1000ppm) reported for the ^{31}P nucleus ensures a good separation of the diacylglycerol signals. The study of diacylglycerols (but not monoacylglycerols due to their low concentration in olive oil, <0.3%) in olive oil by ^{31}P NMR spectroscopy was feasible at even lower magnetic field strengths.

Apart from the wide range of ^{31}P chemical shifts, the 100% natural abundance of the ^{31}P nucleus and its high sensitivity, which is only ~15 times less than that of the proton nucleus, make the ^{31}P NMR experiments a reliable analytical tool to determine amounts of the order of micromolar, or lower, depending on the available instrumentation. These properties of the ^{31}P nucleus should be contrasted with the low natural abundance and sensitivity of the ^{13}C nucleus, which, in addition, is characterized by long relaxation times. Thus, quantitative ^{13}C NMR experiments require lengthy accumulations and long relaxation delays to achieve a satisfactory signal to noise (S/N) ratio.

This method is much faster than the corresponding classical methods of titration and gas chromatography (GC) because it determines several constituents (e.g. monoacylglycerols, diacylglycerols, total free sterols and free acidity) in a single spectrum. Moreover, it avoids several problems, such as lipid oxidation, involved in the traditional GC analysis. Finally, the quantification of phenolic compounds and, in particular, total tyrosol and total hydroxytyrosol that contribute to the stability of EVOO against oxidation involves one more step, i.e. extraction, which lengthens further the duration of this NMR method. Nevertheless, it can be considered as a valuable alternative to the conventional high-performance liquid chromatography (HPLC). The HPLC method, although accurate with low detection and quantification limits, when applied to olive oil extracts requires calibration with standards that may not be available commercially. In addition, analytical results depend on the mobile phase used, whereas difficulties arise in the interpretation of the chromatograms whenever unknown substances with the same retention time as those of EVOO constituents are present.[35]

An advantage of the ^{31}P NMR method is the introduction of an IS of know amount (usually cyclohexanol) in the reaction mixture, which allows the determination of the absolute concentration of the phosphitylated product, avoiding thereby normalization conditions. The magnetic field strength of the NMR spectrometer to be used depends on the type of study being made. For the detection and quantification of the diacylglycerols, total free sterols and acidity, which give well-separated signals, relatively low magnetic field strengths from 7.05 to 9.4 T (Larmor frequencies 121.5 and 162.0 MHz for the ^{31}P nucleus) are adequate.

To obtain reliable quantitative data by using one dimensional (1D) ^{31}P NMR spectra, several criteria must be considered: (i) complete derivatization with the tagging phosphorus reagent must be achieved; (ii) thermal equilibrium must be reached by the phosphorus nuclei before the pulse

sequence repetition. This was guaranteed by using repetition time at least 5 times greater than the longest spin-lattice relaxation time (^{31}P-T_1). The measured ^{31}P spin-lattice relaxation times for several phosphitylated model compounds in the mixture of pyridine and chloroform solvents, including the phosphitylated IS (cyclohexanol) were very long (5-10 s), lengthening considerably the duration of the experiment; (iii) addition of paramagnetic $Cr(acac)_3$ lowers the spin-lattice relaxation times of the phosphorus nuclei, shortening thus the duration of the measurements significantly. The S/N ratio of the ^{31}P NMR experiments depends on the concentration of the constituent in EVOO, the number of the functional groups in the molecule to be derivatized and the chemical nature of the functional group and its environment.

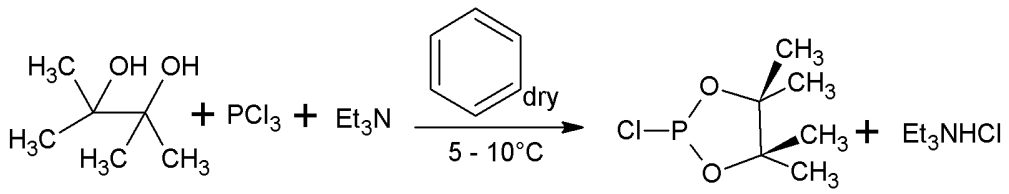
^{31}P NMR spectra are often obtained by proton decoupling, this means that the multiplicity of the ^{31}P NMR signals is lost and by implication all inherent structural information. Nevertheless, proton decoupling results in single resonance lines for each magnetic non-equivalent phosphorus nucleus, facilitating thereby spectroscopic assignment and quantitative measurements. Spectroscopic parameters, such as signal intensities, chemical shifts δ and ^{31}P-^{1}H and ^{31}P-^{31}P coupling constants are readily measurable from the decoupled and coupled spectra, respectively, although assignment of the chemical shifts is not always an easy task.

The ^{31}P chemical shifts assignment for model compounds bearing a single functional group, such as free fatty acids, and diacylglycerols present no problem. Also compounds with one, two or even three different functional groups can be easily detected.[36,37]

2. Results and discussion

This work is based on the derivatization of the hydroxyl group of 1,2-DGs and 1,3-DGs present in various olive oil samples by some phosphorus reagents, and the use of the ^{31}P chemical shifts.[38]

Initially was developed the synthesis of the cyclic chlorophosphite 2-chloro-4,4,5,5-tetramethyldioxaphospholane using the following procedure: a solution of the corresponding glycol (pinacol) and triethylamine in benzene was added dropwise, with efficient stirring and cooling, to a solution of phosphorus trichloride in benzene at 5-10°C (Scheme 2.1).[39]



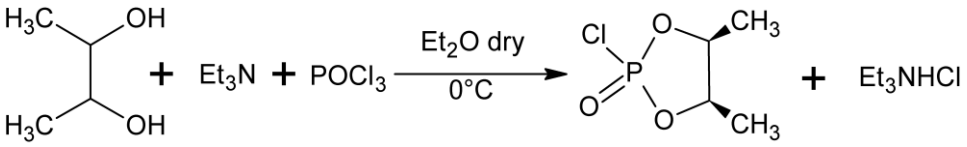
Scheme 2.1. Synthesis of cyclic chlorophosphites.

The method was improved by changing, from time to time, solvent, glycol and reaction conditions.

The product obtained after purification by distillation was a colorless, mobile liquid that showed the usual chemical characteristics of acyl halides, such as fuming in the air and reacting rapidly with water, alcohols and amines (primary and secondary). However the high reactivity of this compound, even in presence of small percentages of water, makes it difficult to treat; for this reason can be used only in particular conditions and when is freshly made.

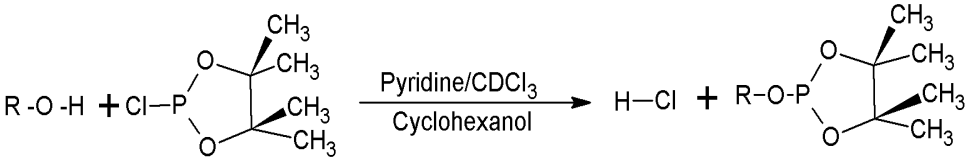
Another disadvantage is the main side reaction of polymer formation in which PCl forms a link between glycol molecules.

Parallel attempts to synthesize cyclic chlorophosphates[40] (Scheme 2.2), easier to isolate but much slower to react, did not lead to expected results due to the lower yields and the presence of some by-products, although their future use has not been completely excluded.



Scheme 2.2. Synthesis of cyclic chlorophosphates.

The cyclic chlorophosphite, freshly made, was used in preliminary spectroscopic tests using some standards (cyclohexanol, 1,2-DG and 1,3-DG) dissolved in a stock solution composed of pyridine, $CDCl_3$ and $Cr(acac)_3$ (Scheme 2.3). The mixture was left to react at room temperature, upon completion of the reaction the solution was used to obtain the ^{31}P NMR spectra.



Scheme 2.3. Reaction of hydroxyl groups of olive oil constituents with the phosphorus reagent.

Pyridine was selected as the second component of the solvent mixture because it captures immediately the hydrogen chloride gas liberated during the phosphitylation reaction forming the pyridine hydrochloride salt. This drives the overall phosphitylation reaction to total conversion. Pyridine solvent should be in excess relative to the phosphorus reagent, since the latter is still a strong derivatizing reagent and the HCl liberated in further derivatization is capable of inducing decomposition of the derivatized compounds.

The role of the chloroform solvent is twofold: first it ensures the dissolution of olive oil and second it does not allow the precipitation of the pyridine-HCl salt. All the innumerable ^{31}P-NMR spectra recorded using the chlorophosphite (2-chloro-4,4,5,5-tetramethyldioxaphospholane) as reagent *in situ* have shown an incomplete reproducibility.

To avoid all the problems connected with the use of these compounds, such as the high instability which increase the formation of some by-products that make the interpretation of the NMR spectra a difficult task, we decided to use some phosphorus reagents: chloro(diisopropylamino) methoxy-phosphin **(1)**, diphenylphosphinic chloride[41] **(2)**, bis(2-oxo-3-oxazolidinyl)phosphinic chloride **(3)**, bis(2-furyl)phosphine chloride **(4)** all commercial available.

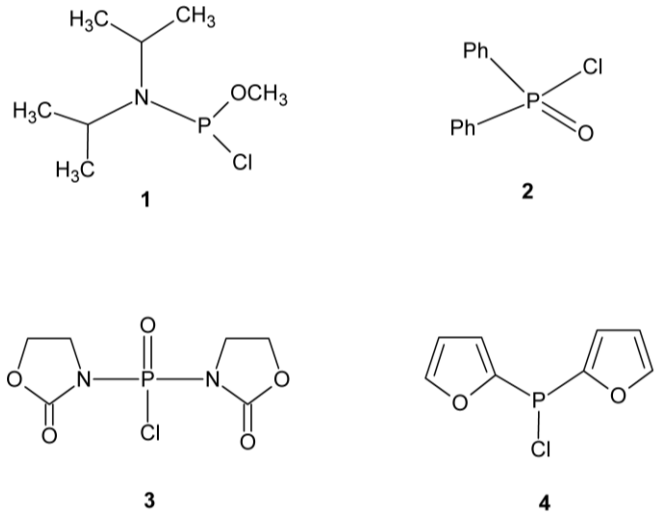
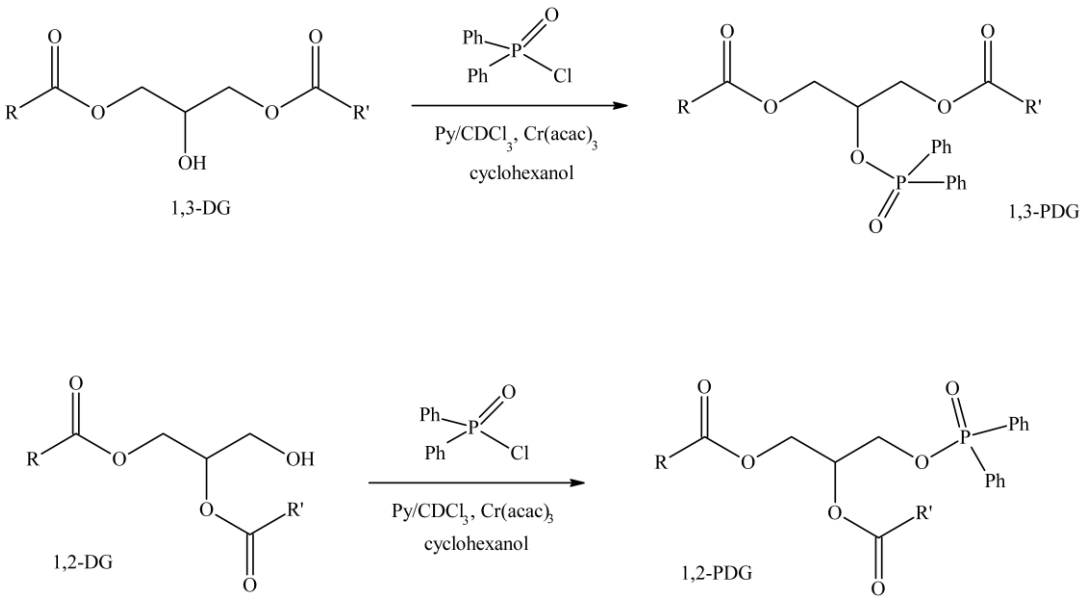


Figure 2.1. Some different phosphorus reagents.

These reagents were used in preliminary spectroscopic tests using some standards (cyclohexanol, 1,2-DG, 1,3-DG) dissolved in a stock solution composed of pyridine, $CDCl_3$ and $Cr(acac)_3$. While the reagents **1** and **4** have led to the formation of many different by-products and then to a great difficulty in the interpretation of spectra, good results were obtained with reagents **2** and **3**, not only with standards but also with three olive oil samples: Cassanese n.124 (30.10.2010), Carolea (15.10.2009) and old commercial olive oil. The same stock solution was prepared and protected from moisture with 5Å molecular sieves.

One hundred and fifty milligrams of the olive oil samples, the required volume of the stock solution and the phosphorus reagent were placed in a NMR tube to react a room temperature. In particularly for the compound **2** is reported the scheme of the reaction (Scheme 2.4).



Scheme2.4. Reaction of hydroxyl groups of olive oil constituents with the phosphorus reagent diphenilphosphinic chloride **(2)**.

Upon completion of the reaction, the solution was used to obtain the ^{31}P NMR spectra. The comparison between the signals obtained by ^{31}P-NMR spectroscopy using three olive oil samples, showed us a significant presence of 1,3-DG in aged oil while in fresh olive oil, like Cassanese, appears almost exclusively 1,2-DG (Figure 2.2).

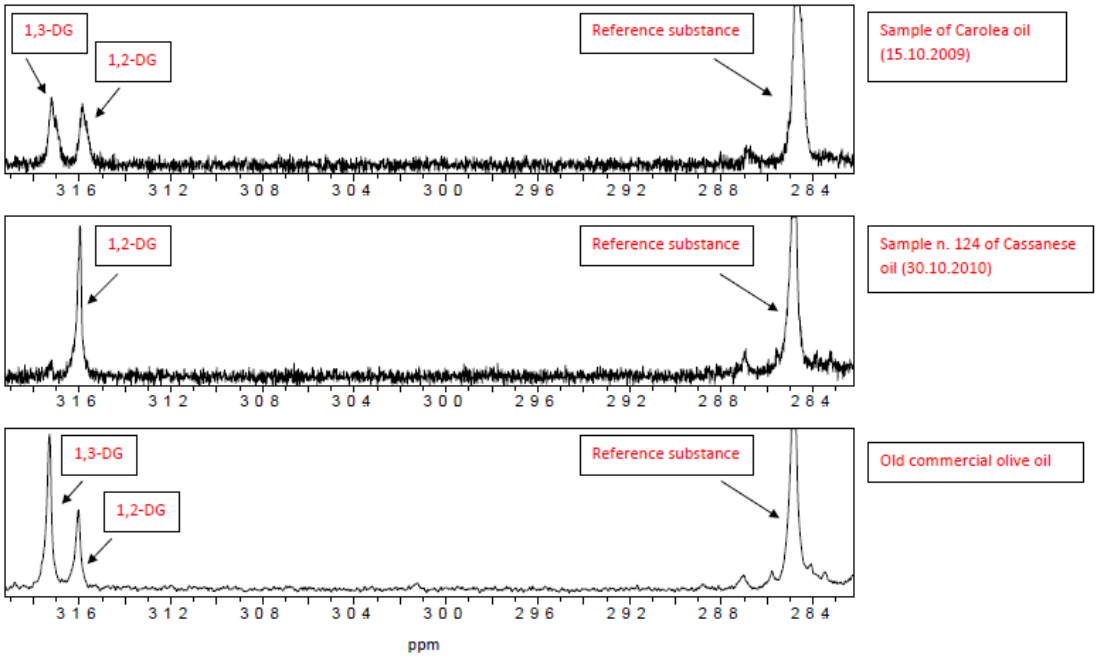


Figure 2.2. ^{31}P NMR decoupled spectra: comparison between three olive oil samples.

These three olive oil samples were analyzed with the reagent **2** also after one year. In this case is observed that the percentage of 1,3-DG increase with the age of the oil (Figure 2.3).

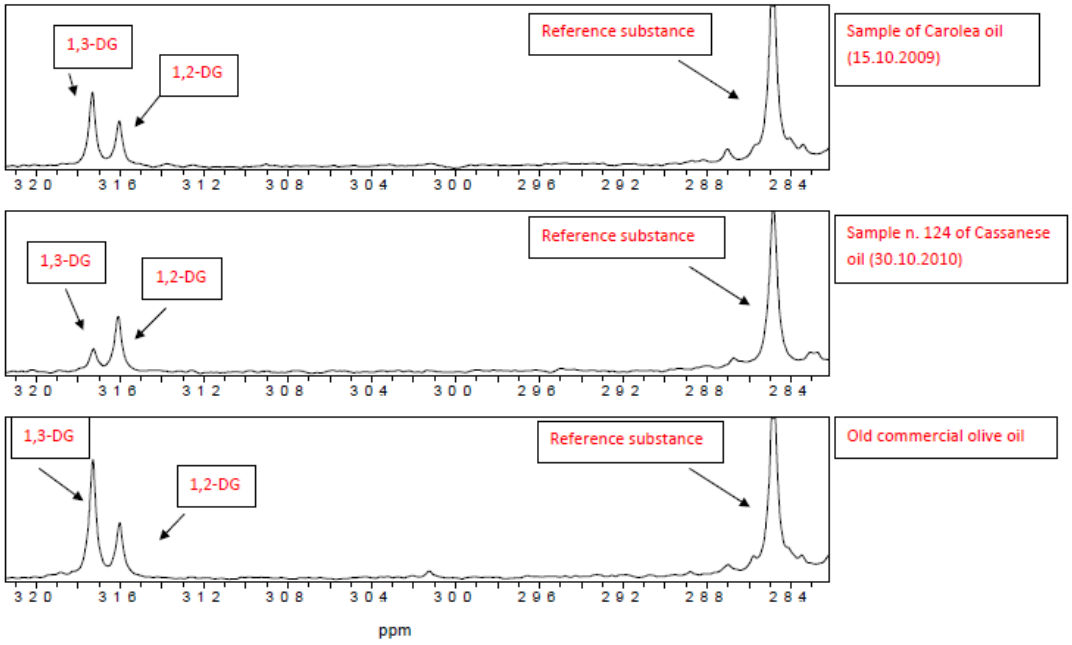


Figure 2.3. ^{31}P NMR decoupled spectra: the same olive oils after one year.

Finally, the reagent diphenylphosphinic chloride was used to analyze two samples of fresh oil, after four months from extraction: Carolea and Cassanese.

The comparison between the two olive oils shows a small and variable amount of 1,3-DG, probably depending from the different cultivar (Figure 2.4).

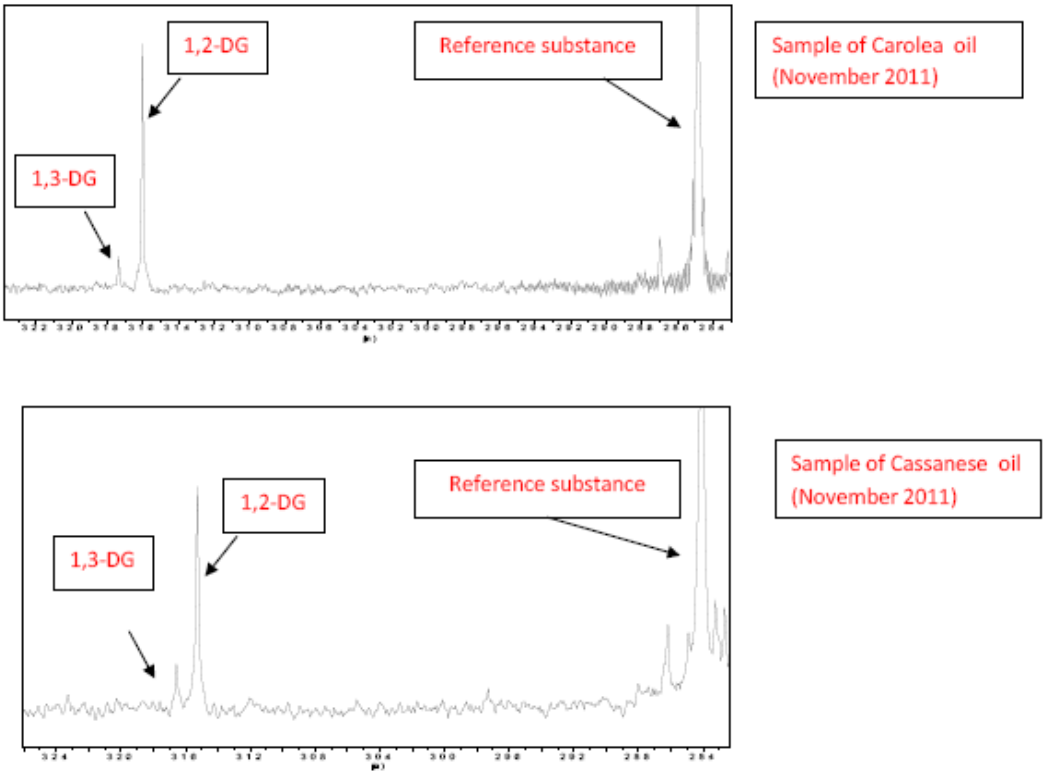


Figure 2.4. ^{31}P NMR decoupled spectra of two fresh olive oil samples: Carolea and Cassanese.

All results obtained are reported in Table 2.1

Olive cultivar	Year of extraction	Year of experiments	% 1,2-DGs	% 1,3-DGs	1,3-DGs/1,2DGs	D
Carolea	November 2009	January 2011 (after 1 year)	45	55	1.22	0.45
		October 2011 (after 2 year)	35	65	1.85	0.35
	November 2011	March 2012 (after 4 months)	91	9	0.098	0.91
Cassanese	November 2010	January 2011 (after 2 months)	98	2	0.020	0.98
		October 2011 (after 1 year)	70	30	0.42	0.70
	November 2011	March 2012 (after 4 months)	84	16	0.19	0.84

Table 2.1. Final results obtained with the different olive oil samples.

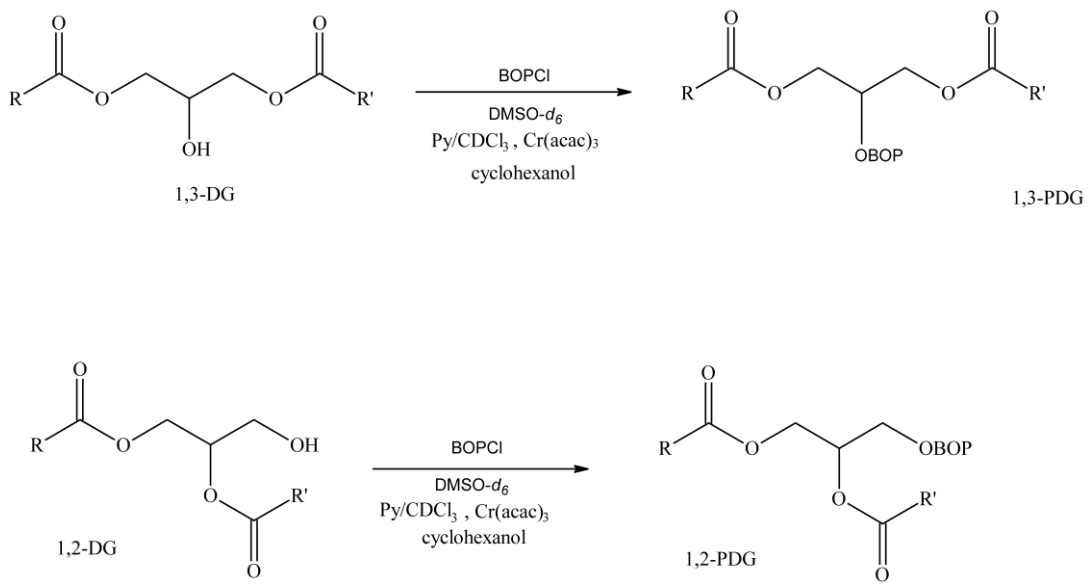
In the Table are not reported the results obtained with the old commercial olive oil, because the percentage of 1,3-DG did not change in the years.

So surely the identification, by ^{31}P NMR spectroscopy, of the signals relative to phosphorus and standards is important in the presence of olive oil samples because it is a reference system in the determination of the ratio 1,3-DGs/1,2-DGs used to assess the age and quality of olive oils.

The repeatability was measured on the basis of several consecutive ^{31}P NMR spectra that were recorded with the same model compound **2** and the same olive oil samples after one, two and three hours and for five times, for this reason the data reported in the table are an average of all results obtained.

The observed repeatability demonstrate that this ^{31}P-NMR methodology is accurate and precise.

The same reaction conditions and the same procedure were used for the reagent **3** which was dissolved in DMSO-d_6 before being added to the stock solution (Scheme 2.5).



Scheme2.5. Reaction of hydroxyl groups of olive oil constituents with the phosphorus reagent BOPCl **(3)**.

In this case the BOPCl gives very slow reactions and also the ^{31}P NMR spectra are less sensitive than those with the reagent **2**. Preliminary tests carried out only with $CDCl_3$ did not lead to complete dissolution of the reagent **3**. The addition of DMSO-d_6 resulted in an increase of the solubility, but during the time in the NMR tube was observed the formation of a precipitate (products and by-products).

Conclusions

The study, presented briefly in this work, demonstrates the efficiency of the ^{31}P NMR technique to detect the diglyceride content in olive oils. This methodology is based on the derivatization of the labile hydrogens of functional groups, such as hydroxyl and carboxyl groups, of olive oil constituents with some phosphorus reagents and the use of the ^{31}P chemical shifts to identify the phosphitylated compounds. The excellent resolution of the ^{31}P chemical shifts permits a reliable detection of the phosphitylated 1,2-diglycerides and 1,3-diglycerides. The ratio of these components was determined upon integration of their corresponding signals with respect to the integral of the signal owing to the phosphitylated internal standard (cyclohexanol).

Initially we started with the synthesis of cyclic chlorophosphite and cyclic chlorophosphates but did not lead to expected results due to the lower yields and the presence of some by-products that make the interpretation of the spectra a difficult task. To avoid all the problems connected with the use of these compounds we decided to use some commercial available phosphorus reagents. Good results were obtained with the reagent diphenylphosphinic chloride not only with standards (cyclohexanol, 1,2-DG, 1,3-DG) but also with some different olive oil samples.

Although this technique is considered to be more expensive than conventional methods of analysis, it has a number of advantages that compensate the rather high cost of an NMR spectrometer. A single run detects in a rapid way all the phosphitylated minor compounds present the olive oil sample and provides signals, the intensities of which reflect the number of magnetically equivalent phosphorus nuclei. The assignment of the shifts of the

various functional groups is well documented, making thus this technique very appropriate for the screening of a large number of samples, and a valuable tool for the quality control and authentication of EVOO.

In future this new methodology will be used, for the quantitative determination of diglycerides, in different Calabrian olive oils in order to have more information about the different factors that influence the quality and the age of an olive oil.

3. Experimental section

3.1 Reagents and instrumentation

Solid reagents, commercially available, were used without further preliminary purification; liquid reagents, instead, before being used were purified by distillation.

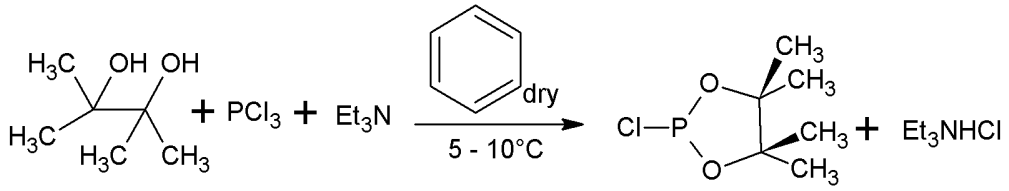
All solvents were dried and distilled according with the normal procedures reported in the literature.

The standard of 1,2-DG, 1,3-DG and some reagents were purchased from Sigma-Aldrich. All reaction were run under nitrogen atmosphere.

^{31}P NMR spectra were recorded at 500 MHz in $CDCl_3$ and DMSO-d_6 using cyclohexanol as internal standard (Bruker Avance 500 MHz). Chemical shifts (δ) are given in parts per million (ppm) from cyclohexanol and coupling costants (J) in hertz.

Olive oil samples were extracted from the olive varieties Carolea and Cassanese and were provided by the local cooperatives (Gabro, and CRA-oil), the other one is a commercial olive oil.

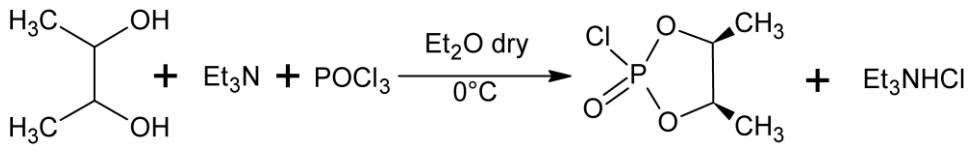
Synthesis of 2-chloro-4,4,5,5-tetramethyldioxaphospholane



Reactive	**Pinacol**	**Et_3N**	**PCl_3**	**Benzene dry**
FW	118.17	101.19	137.33	78.11
Quantity	4.0 g	9.5 ml	2.9 ml	55 ml
Moles	0.034	0.068	0.034	-
Density	-	0.73 g/ml	1.57 g/ml	0.87 g/ml
Molar ratio	1	2	1	-

A solution of the corresponding glycol (2,3-dimethyl-2,3-butanediol) and triethylamine in benzene was added dropewise, with efficient stirring and cooling, to a solution of phosphorus trichloride freshly distilled in benzene at 5-10°C. The mixture was kept for 1 h at room temperature and then filtered under nitrogen. The triethylamine hydrochloride was washed with benzene. Evaporation of the filtrate and flash distillation of the residue *in vacuo* yielded the corresponding cyclic chlorophosphite. All the NMR data obtained are comparable with those of Reference 39.

General protocol for the preparation of phospholanes



Reactive	**2,3-butanediol**	**Et_3N**	**$POCl_3$**	**Et_2Odry**
FW	90.12	101.19	153.33	74.12
Quantity	3.0 g	10.1 ml	3.3 ml	100 ml
Moles	0.033	0.073	0.037	-
Density	0.99 g/ml	0.73 g/ml	1.64 g/ml	0.71 g/ml
Molar ratio	1	2.2	1.1	-

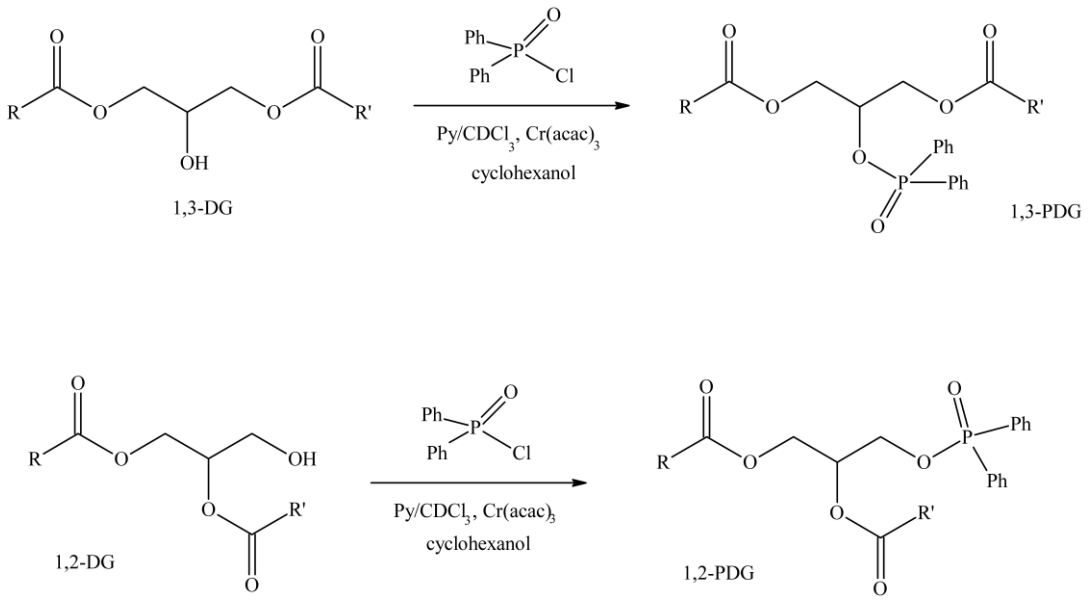
To a solution of 2,3-butanediol and Et_3N in dry Et_2O was added dropewise $POCl_3$ freshly distilled at 0°C under nitrogen atmosphere. After stirring at this temperature for 30 min, triethylammonium chloride was removed by filtration and the filtrate concentrated *in vacuo.* All the NMR data obtained are comparable with those of Reference 40.

Sample preparation

A stock solution was prepared by dissolving 0.6 mg of chromium acetylacetonate $Cr(acac)_3$ and 14 µl cyclohexanol in 10 ml of a mixture of pyridine and $CDCl_3$ solvents (1.6 : 1.0 volume ratio) and protected from moisture with 5 Å molecular sieves freshly activated.

One hundred and fifty milligrams of the olive oil samples was placed in a NMR tube. The required volume of the stock solution (0.5 ml) and the reagent diphenylphosphinic chloride (29 µl) were added.

The reaction mixture was left to react for ~0.5 h at room temperature.



Upon completion of the reaction, the solution was used to record the ^{31}P NMR spectra.

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