Università degli Studi di Siena

Department of Biotechnology, Chemistry and Pharmacy

PhD in Chemical and Pharmaceutical Sciences

Cycle XXXIV

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"Ruthenium nanomicellar catalysis under MW irradiation" and "Development of an alternative synthetic strategy for Sepiapterin"

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CHIM/06 – Organic Chemistry

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Academic Year 2021/2022

List of abbreviations

A	Pre-exponential factor
Ac_2O	Acetic anhydride
AE	Atom Economy
API	Active Pharmaceutical Ingredients
BH	Borrowing Hydrogen
BH2	Dihydrobiopterin
q-BH2	quinoid-dihydrobiopterin
BH4	Tetrahydrobiopterin
BIODEF	International Database of Tetrahydrobiopterin Defiencies
BnBr	Benzyl Bromide
Boc_2O	Tert-butyl decarbonate
Boc-On	[2-(tert-butoxycarbonyloxyimino)-2-phenylacetonitrile]
Bs	broad singlet
CbZCl	Benzyl chloroformate
CDI	Carbonyl diimidazole
$CDCl_3$	Deuterated chloroform
CTAB	Cetyltrimethylammonium bromide
δ	Chemical shift
ddd	Doublet of doublet of doublets
DCM	Dichloromethane
DHPR	Dihydropteridine reductase
DMAP	Dimethyl-amino-pyridine
DMF	Dimethyl formamide
DMSO-d_6	Deuterated dimethyl sulphoxyde
Ea	Activation energy
EC	European Commission
EDS	Energy-dispersive Xray spectroscopy
E-Factor	Environmental impact factor
EPA	Environmental Protection Agency
ES-MS	Electron spray – Mass spectroscopy
Et_2O	Diethyl ether
EtOAc	Ethyl acetate

EtOH	Ethanol
FDA	Food and Drug Administration
GC	Gas chromatography
Glu	Glutamine
GTP	Guanosine triphosphate
GTPCH	Guanosine triphosphate cyclohydrolase
$HCOONH_4$	Ammonium formate
HCl	Hydrochloric acid
His	Hystidine
IBX	2-Iodoxybenzoic acid
***i*PrOH**	Isopropyl alcohol
IR	Infrared spectroscopy
$KMnO_4$	Potassium permanganate
LDA	Lithium diisopropyl amide
$LiAlH_4$	Lithium aluminium hydride
M	Multiplet
MAOS	Microwave assisted organic synthesis
MeOH	Methanol
MeONa	Sodium methoxide
MS	Mass spectroscopy
MW	Microwave
m/z	Mass to charge ratio
$NaBH_4$	Sodium borohydride
$NaBH_3CN$	Sodium cyanoborohydride
$NaNO_2$	Sodium nitrite
$Na_2S_2O_4$	Sodium dithionite
^{1}H-NMR	Proton nuclear magnetic resonance
^{13}C NMR	Carbon-13 nuclear magnetic resonance
ODD	Orphan Drug Designation
PAH	Phenylalanine hydroxylase
PAL	Phenylalanine-ammonia lyase
PCD	Protocatechuate 3,4-Dioxygenase
PEG-600	Polyethylenglycole-600
Phe	Phenylalanine
PKU	Phenylketonuria

$POCl_3$	Phosphoryl chloride
PTPS	6-pyruvoyl-tetrahydropterin synthase
PTSA	Para-toluene sulphonic acid
q	Quartet
$Ru_3(CO)_{12}$	Triruthenium dodecacarbonyl
s	Singlet
SDS	Sodium dodecyl phosphate
SP	Sepiapterin
SR	Sepiapterin Reductase
STEM	Scanning transmission Electron Microscopy
t	triplet
TBDMSCl	*tert*-butyl dimethyl-silyl chloride
TEA	Triethyl amine
TEM	Transmission Electron Microscopy
TFA	Trifluoroacetic acid
TFE	Trifluoro ethanol
THF	Tetrahydrofuran
Tyr	Tyrosine

Abstract

During my Ph.D. I had the opportunity to work on two projects, thanks to the collaboration with Dipharma Francis s.r.l., a chemical company in which I also spent an internship of one year.

The first project regards the academic research and have the aim to develop new sustainable synthesis through nanomicellar catalytic system assisted with MW irradiation. The second project is based on the investigation for alternative synthesis of Sepiapterin, an orphan drug used for the treatment of PKU, without infringing its Patent.

The first part of my thesis, as already mentioned before, will describe the development of a new synthetical method for the synthesis of organic compounds combining two technologies already known in green chemistry: Microwave dielectric heating and nanomicellar catalyst.

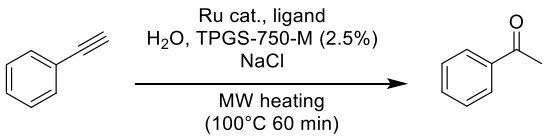
In the last century the exploration of new eco-friendly procedures is a main purpose in pharmaceutical industry because beyond the reduction of waste production, the application of "green method" could lead to a reduction in production costs together with the synthetic steps

Microwaves application in organic synthesis are a useful technology for a more sustainable process because, compared with conventional methods, produce higher yields with less side products and shorter reaction time resulting in cleaner reactions with high energy savings.

Another emergent "green method" is represented by micellar system which allow to perform organic rection in water media instead of toxic organic solvents.

With the combination of the two reported technologies and following the indications of" green chemistry principles" we developed two sustainable process for the synthesis of organic compounds.

Following our interest in ruthenium-based catalyst we become interested in alkyne hydration, so we start by screening all the Ru catalyst present in our laboratories independently from their oxidation states and tested on a model reaction using phenylacetylene as substrate for hydration and the best result was achieved with Shvo's catalyst, a ruthenium complex with cyclopentadienone, with catalytic amount of aniline as ligand (Scheme 1).



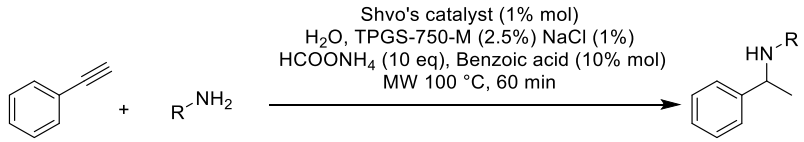
Scheme 1

After optimization of reaction parameters, it was possible to obtain selective Markovnikov hydration product on different substituted aromatic terminal alkyne, only hydration of internal or aliphatic alkynes was not possible with this procedure.

Further we become interested to study the catalyst nature, for this reason we analyse the organic matrix at TEM, observing the formation of Ru nanoparticles embedded into micellar environment, suggesting us the possibility to develop one-pot reductive amination of alkynes.

We thought that after the formation of the ketone, a primary amine already present in reaction environment could form an intermediate imine subsequently reduced to secondary amine in the presence of a hydrogen donor by the same ruthenium catalyst.

The possibility to perform this reaction was first studied on a model reaction using phenylacetylene and aniline as substrate and tested with several hydrogen donor together with an organic acid that could improve amine formation from the intermediate imine, achieving the best result with an excess of $HCOONH_4$ as a hydrogen source and a catalytic amount of benzoic acid and tested on different amines (scheme 2).



Scheme 2

In the last part of this study, we attempt to generate in situ Shvo's catalyst by mixing and heating together under MW irradiation $Ru_3(CO)_{12}$ and tetraphenyl cyclopentadienone, before adding the reactants for reductive amination and after we test its residual activity for catalyst recycling.

The second chapter of this thesis was done during my internship in Dipharma Francis laboratories and will look to the development of a new synthetic route for the orphan drug Sepiapterin) (Figure 1), a precursor of the cofactor tetrahydrobiopterin for the enzyme DHPR (dihydropterin reductase) for the treatment of a rare metabolic disorder known as Phenylketonuria (PKU.

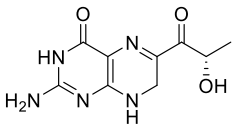
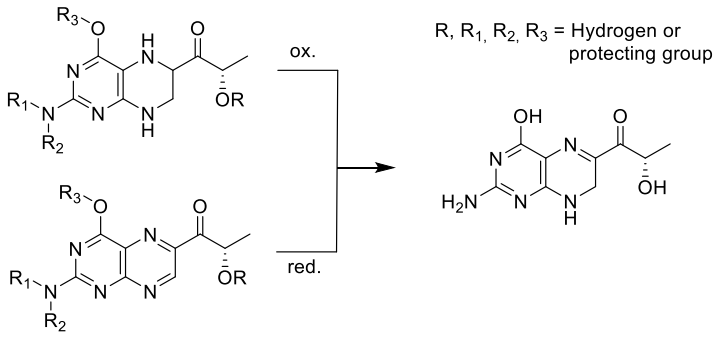


Figure 1 Structure of Sepiapterin

As showed in figure 1, Sepiapterin is a heterocycle compound characterized by a pyrimidine ring fused with a pyrazine ring and a 2-hydroxy propanone side chain.

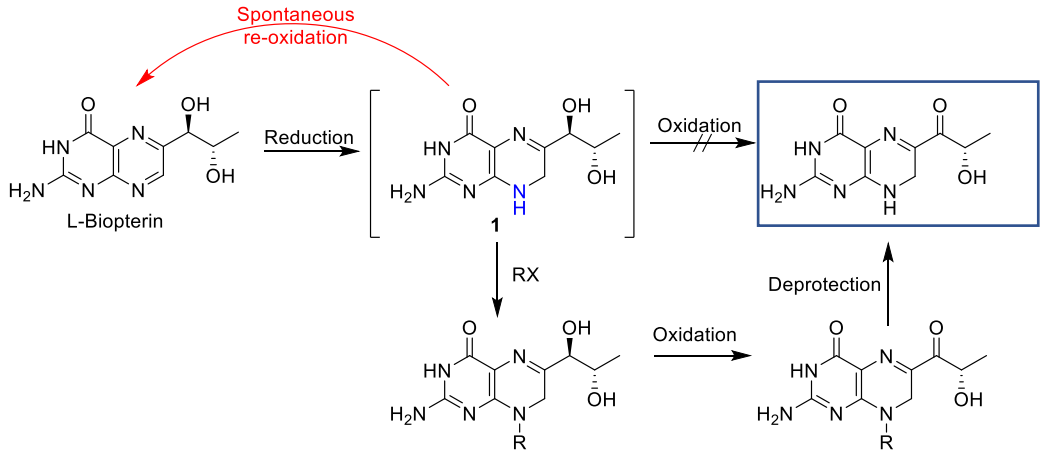
Looking at its patented route of synthesis (Scheme 3) is clear that we have to develop a synthetic strategy avoiding the alcohol oxidation as last.



Scheme 3 Patented synthetic routes to Sepiapterin

To bypass this route different approaches has been tried.

The main challenge was to prevent the re-oxidation of the partially reduced pyrazine ring because we found that the ketone moiety has a stabilizing effect while in the semi oxidized state, in fact without the protection of the generated secondary amine, compound **1** spontaneously re-oxidize to Biopterin (Scheme 4).



Scheme 4

Summary

Chapter 1. Sustainable Catalysed Reactions under Microwave Dielectric Heating

1.1 Introduction

In the last century with the alarming increasing of pollution, scientists focused on the development of new sustainable processes in many fields for the conservation of our planet.
In organic chemistry a lot of research has been done for the development of less hazardous synthesis based on the 12 principles of Green Chemistry introduced in 1998 by Paul T. Anastas[1].
During a part of my Ph.D. I had the opportunity to study, in the laboratories of prof. Maurizio Taddei, new sustainable process mainly focused on the development of micellar catalyst reactions assisted by microwave dielectric heating for new sustainable protocols for the synthesis of substituted amines.

1.1.2 Green Chemistry

Paul T. Anastas is an American chemist that in the early 90s define Green Chemistry as "design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances".
Nowadays many progresses have been made in green chemistry embracing many more aspects of the synthetic process of new products, for example, through the development of new technologies that minimize the use of materials harmful to human health and the environment.
Microwave, ball milling reactors or continuous flow synthesis are some of these technologies that reduces energy and solvents consumption and increase the efficiency of a chemical process[3,4,5].
The development of a new green process should follow a rigorous evaluation in terms of co-efficiency, risk minimization and socioenvironmental impact quantifying the costs and benefits of the new process.
To achieve this goal, in 1998, Anastas drawn up a list of 12 principles as a guideline[6,7] (Figure1):

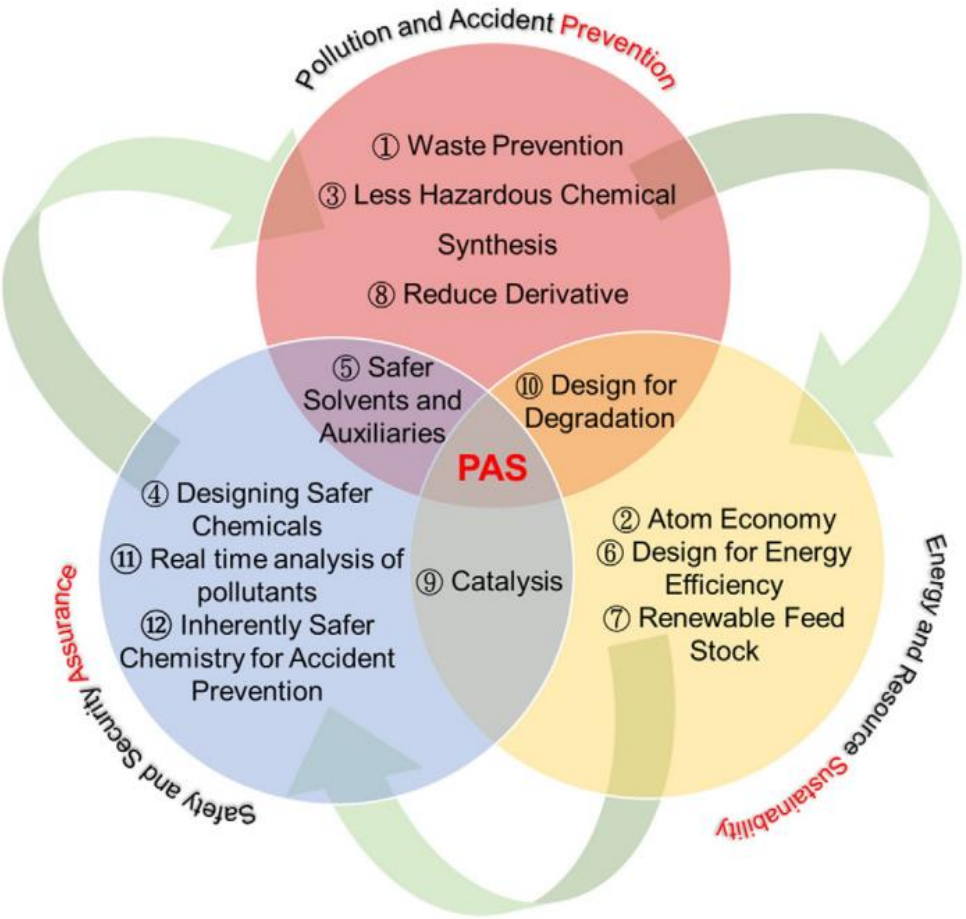


Figure 1 12 Green chemistry principles[8]

1) Prevent waste: Waste could be calculated through the E-Factor (or Environmental Impact Factor), introduced in 1992 by Roger Sheldon, an important parameter that quantifies the amount of waste generated per kilogram of product. This parameter is calculated dividing the total mass of waste by the mass of final product and is expressed as the actual amount of waste produced during the process and consider waste from all auxiliary components used for the process (Figure 2)[9].

$$\text{E-factor} = \frac{\text{mass of total waste}}{\text{mass of product}}$$

Figure 2

If a process has high E factor entails more waste and therefore negative environmental impact, on the other hand a lower E factor are related with the reduced manufacturing costs.

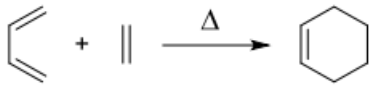
2) Maximize atom economy: This concept was introduced in 1991 by Barry Trost and express the ratio between the molecular weight of the product and the

molecular weight of all components involved in the reaction, which take part in the stoichiometric equation[10].

Furthermore, this parameter is a theoretic number that supposes the use of exact stoichiometric quantities of starting material and a chemical yield of 100%, and excludes substances, such as solvents and chemicals involved in the work-up of the reaction mixture, which are not present in the stoichiometric equation.

Theoretically to have atom economy almost near 100%, most of the atoms of the reactant are incorporated in the desired products and only small amounts of unwanted by products are formed.

A perfect example is represented in the Diels-Alder reaction (figure 3), where most of the reactant are incorporated in the product.



$$AE = \frac{\text{MW of Product}}{\text{MW of Reactants}} = 100\%$$

Figure 3 Diels-Alder reaction and its Atom Economy

3) Less Hazardous chemical synthesis: This principle means that, when it's possible, new methodologies that use safer and less hazardous process should replace traditional synthesis that use dangerous and toxic reactants Safer chemical and products.
4) Safer solvents and reaction conditions: Solvents are often used in large amount in a chemical process as in reaction preparation, extraction of product from reaction environment and purification from impurities through chromatography or crystallizations. Because it's almost impossible to completely avoid the use of solvents in a chemical process, the objective is to choose solvents that make sense chemically, reduce the energy requirements, have low toxicity and have the fewest life cycle environmental[11] (figure 4).

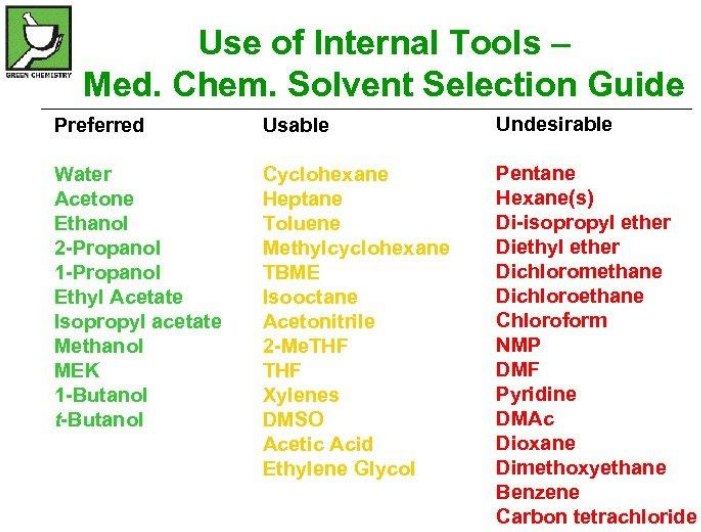


Figure 4 Pfizer's solvent guide[12]

5) Increase energy efficiency: The energy requirements should consider environmental and economic impact and should be kept as low as possible. This could be achieved using renewable energies, such as solar or wind power, or with alternative energy sources like microwaves. Moreover, to reduce energy waste, when is possible, synthetic processes should be conducted at room temperature and ambient pressure.
6) Use renewable feedstocks: Because most industrial production derives from resources such as oil and natural gas that is getting less accessible to use, it is then important to shift our consumption on renewable feedstock such as biomass, the material available from living organisms[13].
7) Avoid chemical derivatives: Sometimes the use of protective group for a functional group sensitive to a certain reaction condition is needed but increase reaction steps of synthesis and consequently the use of materials, time, power and waste.
8) Use of catalysts: The use of catalytic amount of reactant over stoichiometric reagents is a primary goal to achieve during the design of a chemical process. Catalyst in fact offer several advantages, first in terms of selectivity, which means that there is no need for protecting groups and less purification of the final product, secondly in terms of minimization of the activation energy of a reaction and at least of course the reduce of waste.
9) Design chemicals and products to degrade after use: Understanding the mechanisms of degradation and persistence are required to design a chemical process that promote degradation and eliminate persistence. Chemicals should be designed with the aim that at the end of their function they are transformed into harmless degradation products.

10) Analyse in real time to prevent pollution: Analytical methodologies are fundamental for real-time in-process monitoring in order to prevent the formation of hazardous substances.
11) Minimize potential for accidents: Of course, when it is possible, substances used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.

Since 1996 the US Environmental Protection Agency (EPA) established a "Green Chemistry Challenge", an annual award that recognize the application of the green chemistry principles into chemical design, manufacture, and use for both industry and academia[14]. In fact, in the last two decades, a lot of research was done also on an industrial level, because a green process can conduct to a risk reduction for the operators and the environment, but also lead to a significant reduction in costs of production and waste disposal.

1.1.3 Microwave dielectric heating

The convectional thermal heating requires an external heat source using an oil-bath to perform reactions and need higher external temperature to achieve the desired temperature inside the reaction mixture, a possible solution to this limitation could be achieved with Microwave dielectric heating, resulting in a more efficient and homogeneous thermal internal heating (figure 5)[15].

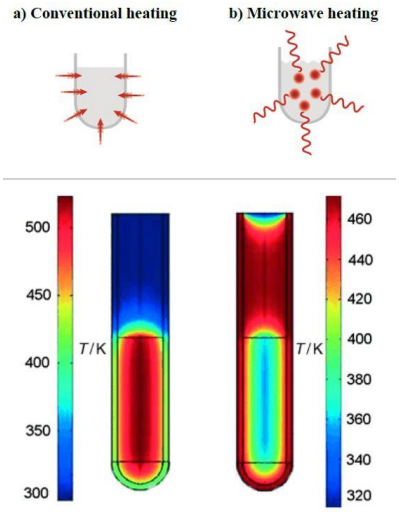


Figure 5 Differences between conventional and Microwave heating[16]

In the last thirty years microwave assisted organic synthesis (MAOS) has been received interest from the chemists in order to accelerate organic chemical transformations.

Between 1980 and 1990 due to lack knowledge of their functioning in terms of reproducibility and controllability of different parameters the utilization was less frequent.[17]

Other problems encountered were related to the flammability of organic solvents when domestic kitchen microwave ovens were used, for the absence of safety equipment for the use in a chemical laboratory, resulting in many explosions occurred because of the absence of control technology.

Fortunately, domestic microwave oven was replaced with innovative instruments because its use resulted advantageous due to the possibility to reduce chemical reaction times and its reproducibility was improved.

In addition to these aspects also the formation of by-product was reduced, yield was increased and sustainable ways and new transformations were investigated. Nowadays MAOS has been applied for the heterocycle's synthesis, in homogeneous transition-metal catalysis and in medicinal chemistry**.**

Microwave irradiation is an electromagnetic irradiation in the frequency range of 0.3 to 300 GHz (Figure 6). All microwave ovens operate at a frequency of 2.45 GHz to avoid interference with telecommunication and cellular phone frequencies. In this frequency region, the energy of a microwave photon (0.0016 eV) is lower than the energy of Brownian motion and it is too weak to break a chemical bond[18].

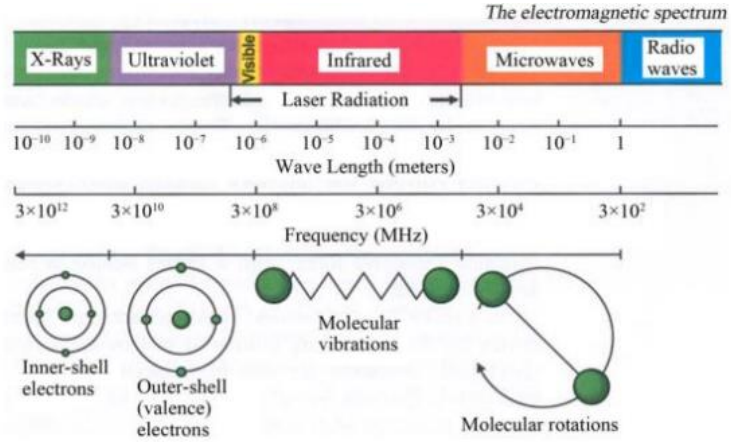


Figure 6

Dielectric heating is caused by the electric component of the electromagnetic field by two main mechanisms: dipolar polarization and ionic conduction effects and rely on the ability of a specific material to absorb microwave energy and convert it into heating based on its dielectric properties[19].

The loss factor tan δ is a value that determines the ability of a specific substance to convert electromagnetic energy into heat at a specific temperature and frequency, high tan δ value is required, in the reaction medium, for an efficient absorption and, consequently, rapid heating. This means that polar solvents like methanol and water,

having a high tan δ value, will give more interaction and therefore better heating, while solvents with low tan δ, such as dichloromethane or toluene, are "transparent" to microwaves, giving a minimum or no heating[20,21] (table 1).

Solvent	tan δ	Solvent	tan δ
Ethylene glycol	1,35	DMF	0,161
Ethanol	0,941	1,2-dichloroethane	0,127
DMSO	0,825	Water	0,123
2-propanol	0,799	Chlorobenzene	0,101
Formic acid	0,722	Chloroform	0,091
Methanol	0,659	Acetonitrile	0,062
Nitrobenzene	0,589	Ethyl acetate	0,059
1-butanol	0,571	Acetone	0,054
2-butanol	0,447	Tetrahydrofuran	0,047
1,2-dichlorobenzene	0,28	Dichloromethane	0,042
NMP	0,275	Toluene	0,04
Acetic acid	0,174	Hexane	0,02

Table 1 Solvents tan δ calculated at 2.45 GHz, 20 °C[22]

Of course, solvents with low tan δ could be used in MAOS, because the presence in the reaction environment of a component that can absorb the electromagnetic energy can provide the necessary heating.

Another interesting aspect of microwaves (MW) is that reactions performed with these technologies are cleaner, meaning that produce lower amount of waste, which can be further decreased in solvent free reactions[23,24].

Considering the twelve principles of the green chemistry previously reported, the energy efficiency, heating with microwave radiation is a highly resourceful process, resulting in substantial energy savings because microwaves heat up solely the sample and not the apparatus faster when compared to conventional heating.

Besides the well-established thermal effect of the MW heating that cannot be achieved with conventional heating, like the “superheating” of solvents[25], the selective heating of some components in a less polar reaction medium, some authors suggested the existence of the so-called non-thermal effects[26].

These effects boost of the reaction rates by a direct interaction between the microwave electromagnetic field and the reactant molecules, in a way that cannot be rationalized by either thermal or kinetic effects, substantially, MW irradiation can increase the rate of

chemical reactions by increasing the pre-exponential factor (A) or decreasing the activation energy (Ea) in the Arrhenius equation (figure 7), due to orientation effects on dipolar molecules, or by direct reduction of the apparent activation energy, changing the interior energy level of the molecules[27,28].

$$k = Ae^{-Ea/(RT)}$$

Figure 7

1.1.4 Micellar Catalysis

In conventional organic chemistry, water is manly used as workup medium and very little as reaction solvent, even if the impact on pollution and its E factor is close to zero.
Other interesting properties of water are the large heating capacity and heat of evaporation, high polarity, the ability to form hydrogen bond as donor and acceptor and high tan δ value that gives water the perfect characteristic for MAOS reactions.
Although these properties, water is not the best choice for the solubilization of nonpolar molecules, which represent an important part of organic compounds.
Nowadays, this limitation could be avoided using surfactant.
Surfactant are organic compounds characterized by amphiphilic properties which mean that are composed by both hydrophobic (tails) and hydrophilic groups (head).
In aqueous media, surfactants form aggregate where the core is composed by the hydrophobic tails in which organic reactions are performed and the surface in contact with the liquid by the hydrophilic head (Figure 8)[29,30,31].

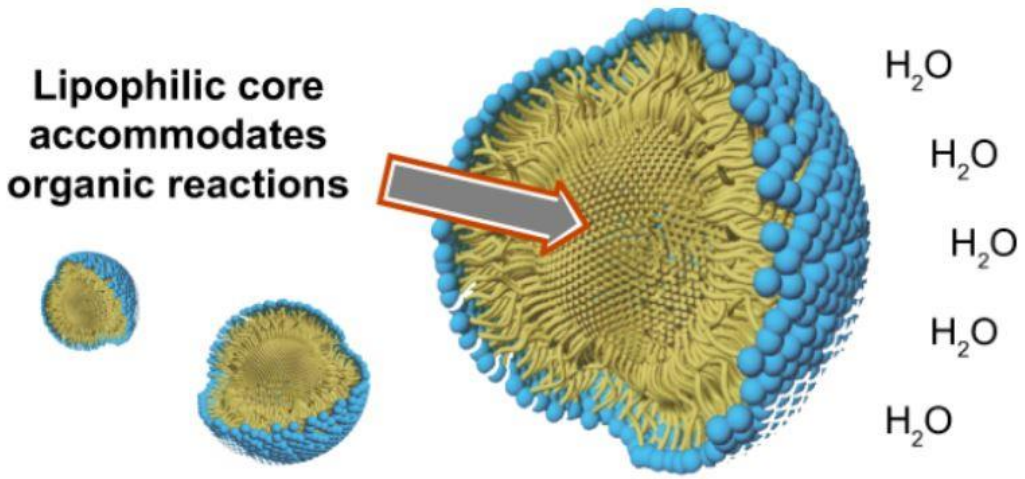


Figure 8 Example of micelles structure in water[32]

The nature of the micelles are dependent on the ratio between the hydrophilic and hydrophobic portion, the solvent characteristic (pH, temperature and ionic strength) and could be classified as: anionic, cationic, non-ionic, gemini and zwitterionic.

In chemical synthesis to because anionic and cationic surfactants could act as nucleophile or ligand, due to positive or negative charge on their structures, non-ionic surfactant are preferred[33].

Lipshutz's research group developed many types of non-ionic surfactants used in organic chemistry and are divided in three generations (Figure 9).

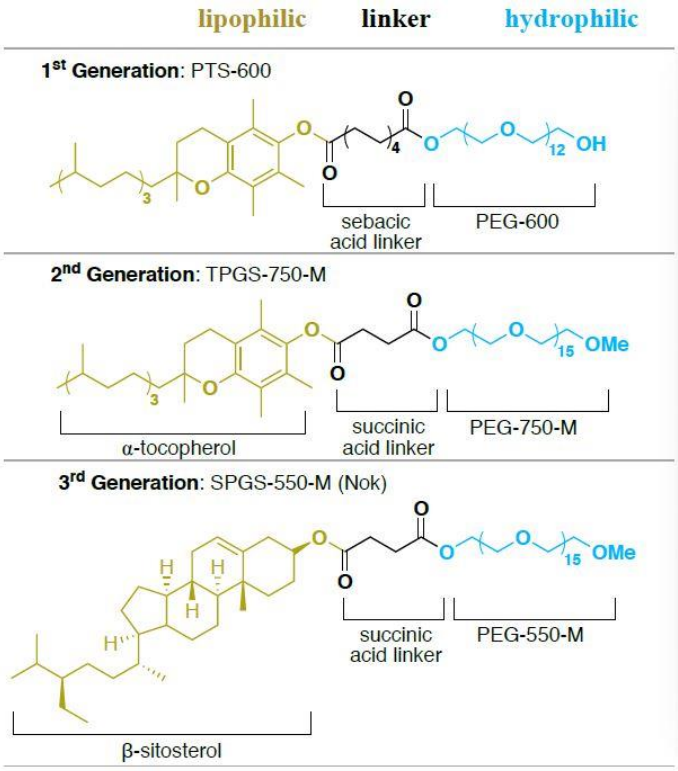


Figure 9 Surfactants structures

In the first generation, racemic vitamin E compose the lipophilic part, PEG-600 as the hydrophilic portion bounded through sebacic acid linker.

The second generation of surfactant are obtained from the evolution of PTS as polyoxyethanyl-α-tocopherol succinate (TPGS-750-M)[34].

The third generation differs from the previous one from the substitution of a-tocopherol with β-sitosterol for the lipophilic moiety[35].

With these surfactants a large variety of traditional and well-known chemical reactions are performed, but under mild conditions and in water medium, in particularly cross coupling reactions like Heck[36], Suzuki-Miyaura[37], Sonogashira[38] and Buchwald-Hartwing amination[39], metathesis[40], click[41], and amidation reactions[42] are also reported (figure 10).

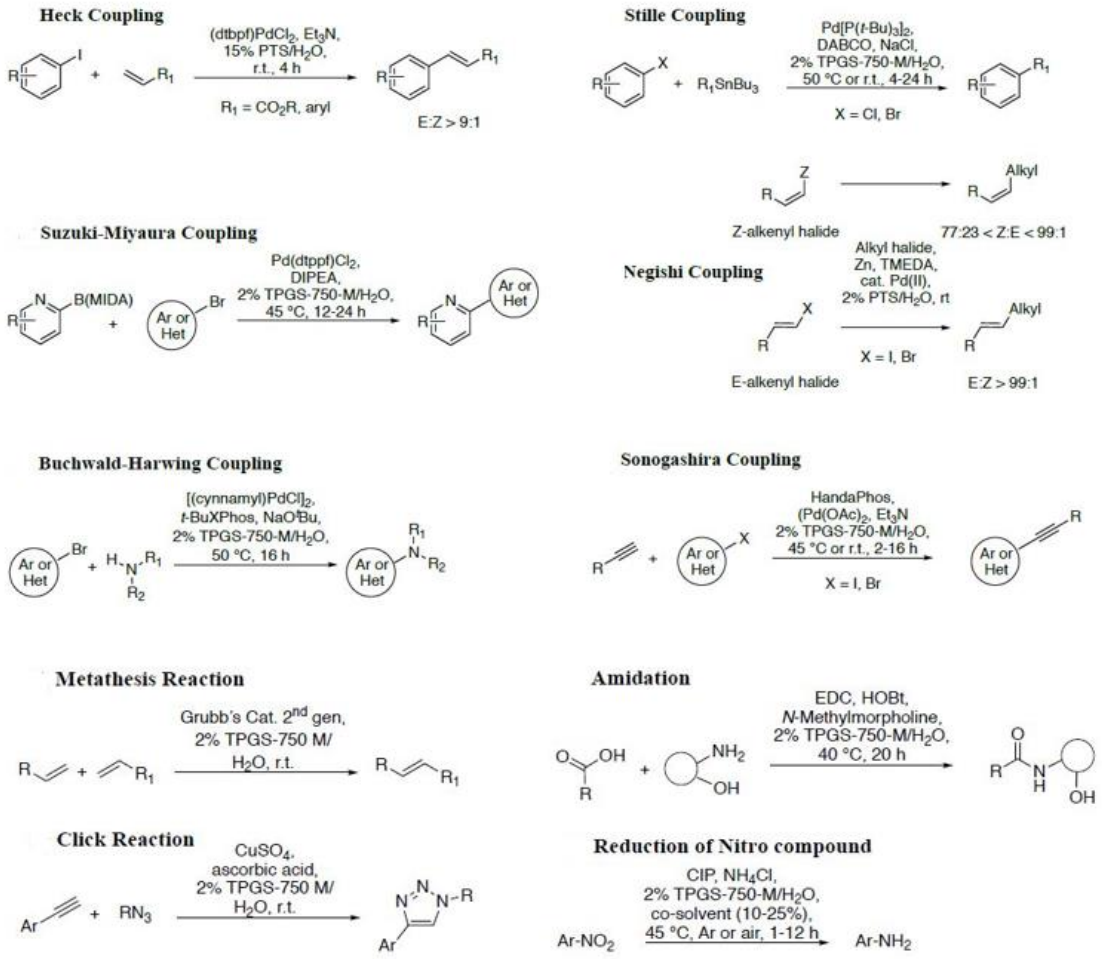


Figure 10 Micellar catalysts examples

1.1.5 Borrowing hydrogen in reductive amination

Amines are predominant functional groups in drugs and natural products, being present in the 40% of active pharmaceutical ingredients (APIs). Amongst the different methodologies developed for their preparation, such as alkylation, hydro-aminomethylation, reduction of amides, nitriles and azides, reductive amination of carbonyl compounds is the best way of making secondary amines.

This should be the first choice for amine synthesis, especially for secondary and tertiary amines.

This process is based on the formation of an imine or iminium ion through reaction of the carbonyl with the appropriate amine.

Afterwards the reduction is usually carried out:

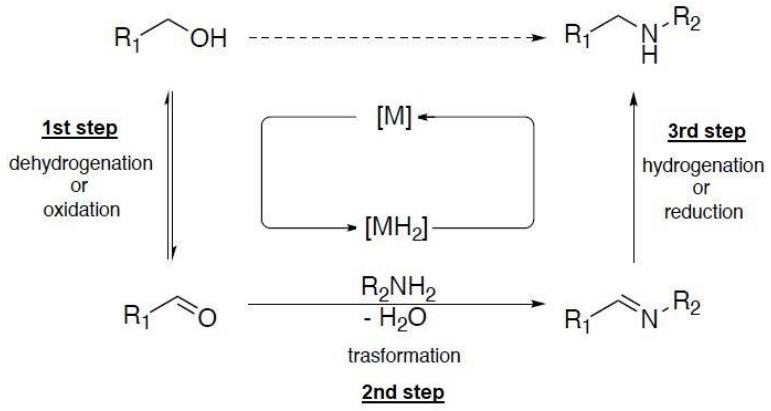
- In the presence of an over stoichiometric amount of a metal hydride ($NaBH_4$, $LiAlH_4$, $NaBH_3CN$)[43]
- Under H_2 gas at high pressure with a homogeneous or heterogeneous noble metal catalyst (Pd, Pt, Rh, Ru)[44]

- Through hydrogen transfer process metal-catalysed (formates, *i*PrOH, cyclohexadiene)[45]

The last cited method is also known as Borrowing hydrogen (BH), which use hydrogen source from the intermediate of the reaction, avoiding the direct use of molecular hydrogen.

From a green point of view this catalytic reaction is a promising atom economical approach for the selective preparation of substituted amines using an alcohol as alkylating reagents, and generate water as only by-product[46].

As shown in **scheme 2** to achieve this, the alcohol is oxidized into the corresponding carbonyl compound and a molecule of hydrogen is transferred to the catalyst (1st step). The reaction between carbonyl compound and the amine generates the corresponding imine (2nd step), which subsequently is reduced into the alkylated amine by the hydrogenated catalyst (3rd step)[47].



Scheme 1 BH mechanism

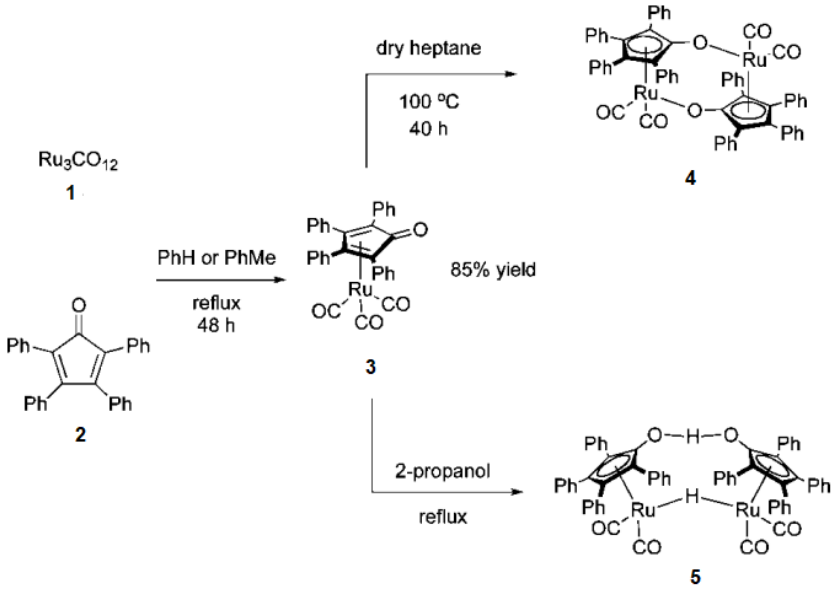
The first step is thermodynamically favoured leading to the completed formation of the products and giving a very low E-factor. In other words, the irreversibility of the third step makes possible that the reactant is completely consumed.

Iridium and rhodium complexes are mostly used in homogeneous catalysed reaction but require harsh conditions, using organic solvents (*e.g.* toluene) and high temperatures (110-120°C) to obtain reasonable results[48,49].

1.1.6 Shvo's catalyst

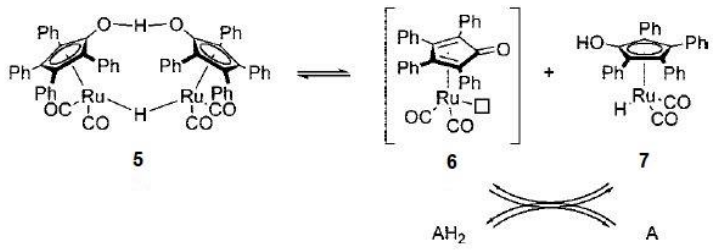
In 1981 Youval Shvo and his team observed that triruthenium dodecacarbonyl, $Ru_3(CO)_{12}$ catalyses the transfer dehydrogenation of alcohols in the presence of hydrogen acceptors such as diphenylacetylene, supposing that a cyclopentadienone-ligated ruthenium complex could be involved[50].

After several experiments, they isolate a water-stable crystalline solid composed by a cyclopentadienone-ligated ruthenium complex and develop its synthesis (scheme 2)[51].



Scheme 2 Shvo's catalyst synthesis

Extensively studies have been done to understand catalyst structure and reactivity. It was observed that compound **5** undergo upon dissociation in solution in two monomers, respectively **6** in the zero oxidation state, responsible of the oxidizing properties and **7** is in the 2^+ oxidation state governed by equilibrium effect through the gain or loss of H_2 from hydrogen donors (scheme 3)[52].

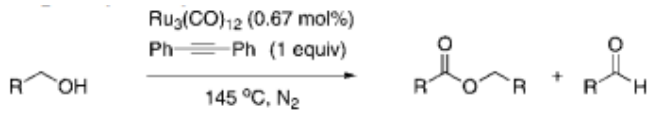


Scheme 3 Shvo's catalyst equilibrium[52]

The mechanism of hydrogenation and dehydrogenation reactions catalyzed by Shvo's catalyst involves simultaneous transfer of separate hydrogen atoms from or to the

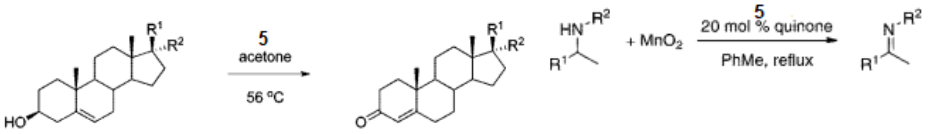
metal center and the ligand[53] which make it very useful for many types of reactions such as:

- Esterification of alcohols[54]



Scheme 4

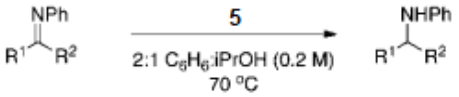
- Oxidation of alcohols and amines[55,56]



,

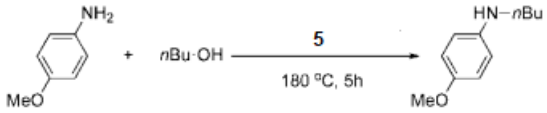
Scheme 5

- Hydrogenation of ketones alkenes and imine[57]



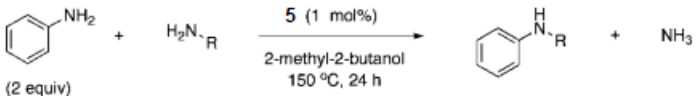
Scheme 6

- Alkylation of amines with alcohols[58]



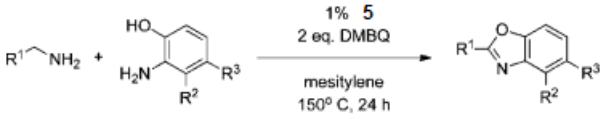
Scheme 7

- Alkylation of amines with amines[59]



Scheme 8

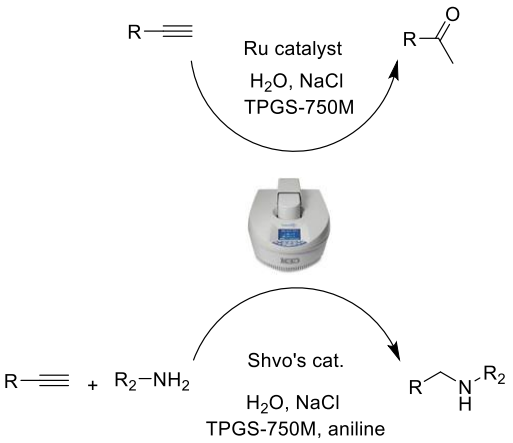
- Condensation of amines[60]



Scheme 9

1.2 Project Aim

Considering the importance to develop greener ways to synthesize organic molecules that in future could be useful for large scale synthesis, the experience and knowledge in microwave assisted reactions in the laboratories of professor Maurizio Taddei where I spent part of my PhD, and the potential application of Shvo's catalyst in borrowing hydrogen reaction, we became interested to study the possibility to perform alkyne hydration, and reductive amination trough micellar catalyst in water environment assisted by microwave dielectric irradiation.



Scheme 10

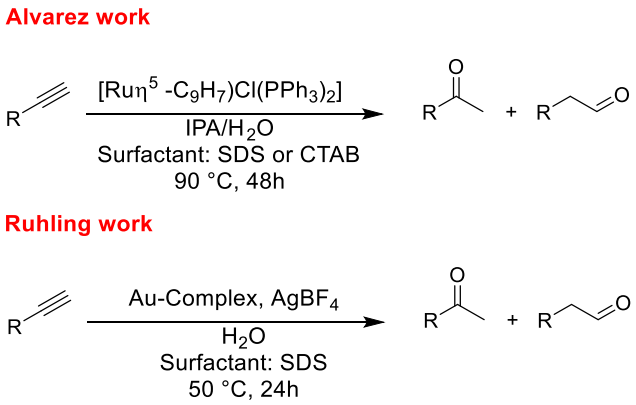
1.3 Result and discussion

The design of this study was driven by our interest in ruthenium-based catalyst and its possible application in organic transformations.

Our aim was to perform alkyne hydration under ruthenium catalyst combined with MWs assisted synthesis in water media with the use of surfactants.[61,62,63]

In literature it's possible to find different example of alkyne hydration with different metal catalyst, in particular as it's described in **scheme 11**, Patricia Alvarez and her team develop this chemical transformation using [Ru(η^5-C_9H_7)Cl(PPh_3)$_2$] in water and SDS (sodium dodecyl phosphate) or CTAB (Cetyltrimethylammonium bromide) used as surfactant.[64]

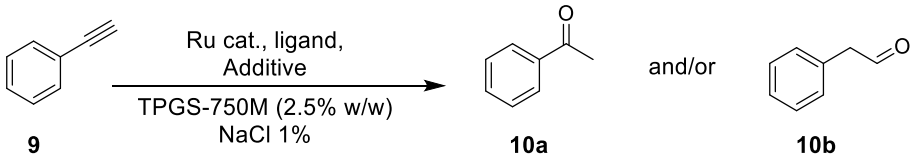
Also, other metal catalysts were used for hydration reaction such as Pt, Au and Ag in the presence of surfactants[65] (scheme 11).



Scheme 11

First, we start to test all the ruthenium complexes available in our laboratory in order to find the best Ru-catalysts for Markovnikov or anti-Markovnikov hydration.

We selected phenylacetylene as the simplest aromatic terminal alkyne substrate and TPGS-750-M as surfactant for its successful application in micellar catalyst, and perform the reaction with MW irradiation for at least 1 hour at 100 °C



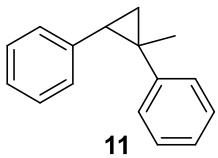
Scheme 12

Entry	Catalyst	Additive	T (°C), t (min)	Results
1	$RuCl_3.H_2O$	-	100, 60	**9** (7%), **10a**/**10b** (38%), **11** (35%)
2	$RuCl_3.H_2O$	PPh3	100, 60	**9** (13%), **10a**/**10b** (50%), **11** (35%)
3	$Ru(acac)_3$	-	110, 120	sm
4	$(Ph_3P)_3Ru(CO)(Cl)H$	-	100, 60	sm
5	$(Ph_3P)_3Ru(CO)(Cl)H$	-	150, 60	sm
6	$(Ph_3P)_3Ru(CO)(Cl)H$	Xphos 3%	100, 60	sm
7	$[ClCp^*Ru]_4$	-	100, 60	sm
8	$[Ru(COD)Cl_2]_n$	-	100, 60	sm
9	Shvo's catalyst	-	100, 60	sm, by-products
10	Shvo's catalyst	Xphos 3%	100, 60	sm, by-products
11	Shvo's catalyst	Aniline 5%	100, 60	**10a** (95%)
12	Shvo's catalyst	Aniline 5%	100, 480	**10a** (93%)
13	$Ru(acac)_3$	Aniline 5%	100, 60	sm
14	$(Ph_3P)_3Ru(CO)(Cl)H$	Aniline 5%	100, 60	sm
15	$[ClCp^*Ru]_4$	Aniline 5%	100, 60	sm

Table 2

Unfortunately, with the exception of $RuCl_3$, and Shvo's catalyst, the other Ru-complexes, independently from its oxidation state, the ligands used, or the microwave heating conditions, gave only the starting compound **1**.

With $RuCl_3$ we observe the formation of a mixture of hydration compounds **10a** and **10b** together with (1-methyl-2-phenyl-cyclopropyl)benzene (**11**) (figure 11) obtained probably through dimerization of the Ru(II) acetylene (or vinylidene) complex, but it was not possible to optimize the reaction to selectively synthesize hydration product.



11

Figure 11

In entry **9-12**, was explored the reaction conditions for hydration under Shvo's catalyst, we observed almost quantitative conversion into

acetophenone (Markovnikov addition), only when 5 % of the Bronsted acid aniline was used.

Even if it's possible to hydrate phenylacetylene under conventional heating on oil bath, harsh condition such as longer time and higher temperature are needed as described in entry **12**.

We perform the reaction under MW dielectric heating at 100 °C for 60 min (6 cycles of 10 min each), mixing phenylacetylene (1 eq.), the catalysts (1% mol), aniline (5 % mol) in water containing NaCl (1% mol) and the surfactant TPGS-750-M (2.5% w/w).

After 60 min a black organic phase was formed on the water surface and extracted with EtOAc, after filtration on a short path of silica and evaporation of the solvent under vacuum, acetophenone was isolated in 85% yield.

We investigate the catalyst nature, analysing the composition of the organic phase after the extraction at the ^{1}H-NMR spectrum, that clearly show that during the reaction Shvo's catalyst has changed.

It was confirmed by TEM analysis (Transmission Electron Microscopy analysis) of the matrix that highlighted the formation of Ru nanoparticles embedded into micellar environment (figure 12).

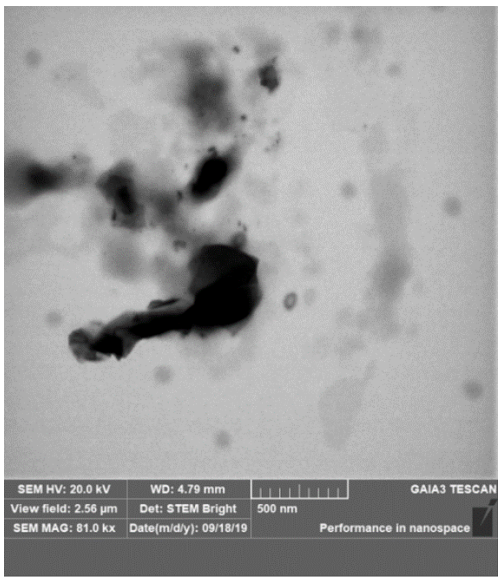


Figure 12 STEM analysis of organic matrix showing the presence of particles with high electron density

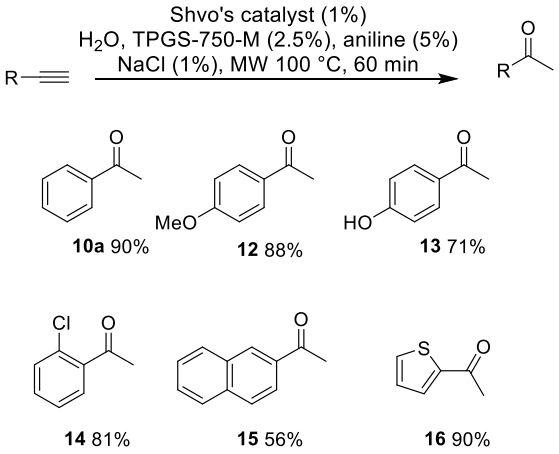
It seems that the formation of nanoparticles already happens while heating a mixture of Shvo's catalysts, aniline and TPGS-750-M under MW heating at 100 °C for 30 min and could explain the selectively formation of Markovnikov orientation while other Ru (II) catalysed hydration in the presence of sodium

dodecyl sulphate (SDS) have been reported to give the anti-Markovnikov orientation product.

When the hydration was performed with SDS as surfactant and heated under MWs at 100°C, despite the result of Patricia Alvarez work, only acetophenone was obtained.

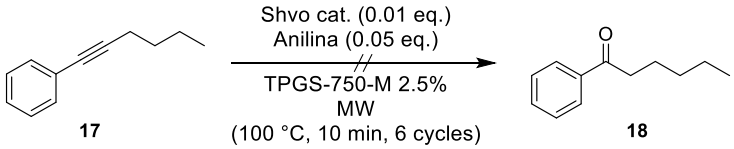
This result could demonstrate that the properties to obtain only Markovnikov hydration, rely on the catalyst nature and is independent of the surfactant nature.

After that we found the optimal condition, we attempt hydration on different substrates (scheme 13)



Scheme 13

As it' s shown in **scheme 13**, with the exception of 2-acetylnaphthalene (compound **15**) and internal alkyne (compound **17**) that did not undergo hydration at all (scheme 4) substituted aryl acetylenes always gave good conversions.



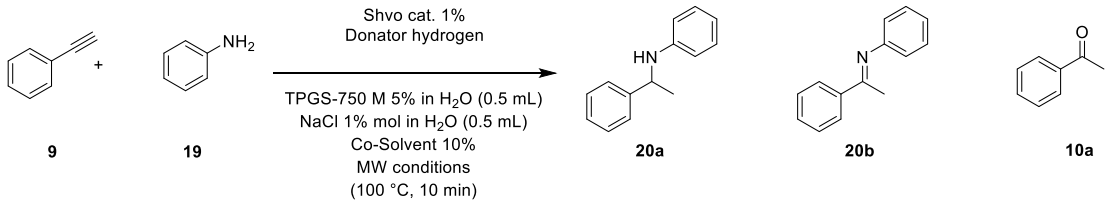
Scheme 14

Encouraged by the promising results and according to the literature about the possibility to perform reductive amination with Shvo's catalyst, the potential formation of Ru nanoparticles suggested us the possibility to try a one pot reductive hydroamination of alkynes.

We supposed that after hydration of alkyne, the resulting ketone could be intercepted by the amine present in reaction environment with the formation of the imine intermediate.

The imine undergoes to reduction by transfer hydrogen from a hydrogen donor catalysed by Ruthenium nanoparticles with the formation of the secondary amine.

As for the hydration reaction we start from two simple substrate like phenylacetylene and aniline and performed the reaction with different hydrogen donor.



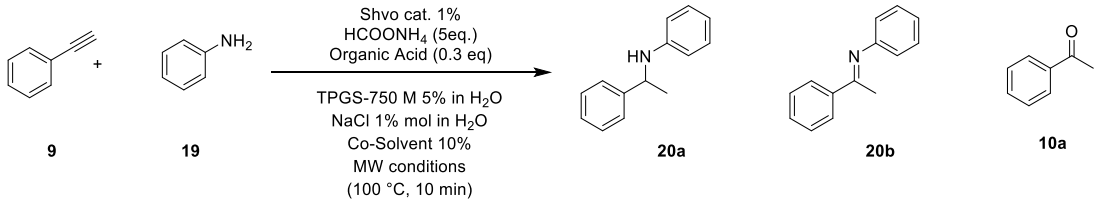
Scheme 15

Entry	Hydrogen Donor	Results
1	HCOONa (1.1 eq)	**19** (38 %), **20a** (25 %), **20b** (5 %), **10a** (32 %)
2	HCOOH (1.1 eq)	**19** (24 %), **20a** (45 %), **20b** (5 %), **10a** (25 %)
3	$HCOONH_4$ (1.1 eq)	**19** (29 %), **20a** (50 %), **20b** (4 %), **10a** (27%)
4	$HCOONH_4$ (5 eq)	**19** (26 %), **20a** (61 %), **20b** (2 %), **10a** (10 %)
5	$HCOONH_4$ (10 eq)	**19** (36 %), **20a** (56 %), **20b** (2 %), **10a** (7 %)
6	$HCOONH_4$ (15 eq)	**19** (35 %), **20a** (50 %), **20b** (1 %), **10a** (12 %)

Table 3

As it is described in **table 3** the best result was obtained with an excess of 5 equivalents of ammonium formate, while increasing to 10 or 15 equivalent the amount of the hydrogen donor, was observed a decrease in its conversion probably for the variation in the pH of the solution that could affect surfactant structure.

We then thought that a catalytic amount of an organic acid could improve the reduction of the imine through the formation of the iminium salt that easily undergo to reduction to the corresponding amine.



Scheme 16

Entry	Organic Acid (0.3 eq)	Conversion
1	PTSA	**10a** (20 %), **20a** (80 %),
2	Benzoic acid	**10a** (16 %), **20a** (83 %)
3	p-nitro Benzoic acid	**10a** (30 %), **20a** (60 %), **12b** (8 %),
4	Phenylacetic acid	**10a** (39 %), **20a** (58 %), **20b** (6 %), **19** (5 %)
5	Lauric Acid	**10a** (24 %), **20a** (65 %), **20b** (11 %)
6	Camphorsulphonic acid	**10a** (39 %), **20a** (60 %), **20b** (1 %)

Table 4

Surprisingly, when a catalytic amount of benzoic acid or lauric acid were used, phenylacetylene was completely converted to compound **20a** and no side products were detected.

We also attempt to generate, under MW dielectric heating at 100 °C for 60 min, the Shvo's catalyst in-situ by mixing in a ratio of 1:3, $Ru_3(CO)_{12}$ together with and tetraphenylcyclopentadienone in H2O/TPGS-750- M (2.5 %), NaCl (1%).

After catalyst formation, phenylacetylene, aniline, ammonium formate and benzoic acid were added, and the mixture heated at 100°C (MWs, 6 cycles of 10 min) giving 85% conversion into compound **20a**, confirming the possibility to synthesize the Ru catalyst in shorter time and greener way if compared with its conventional synthesis (figure 13).

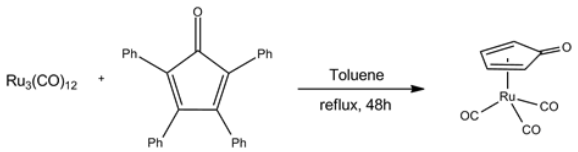
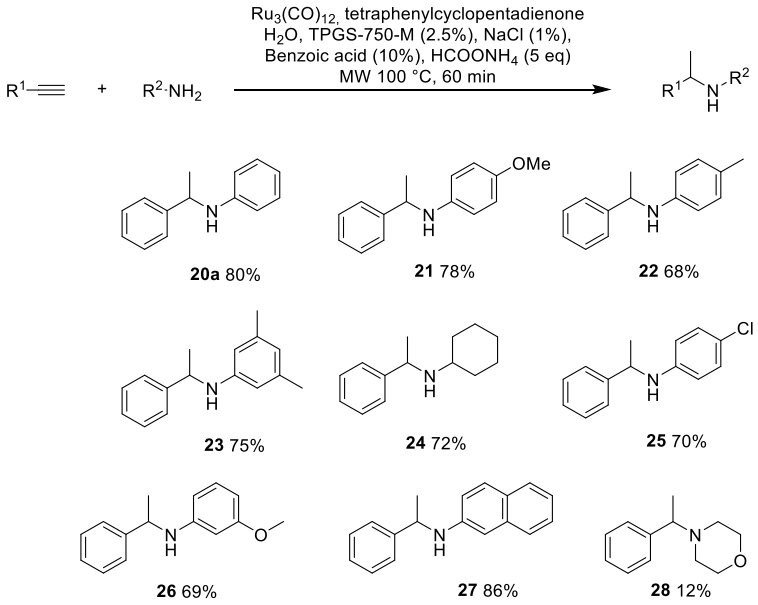


Figure 13 Traditional synthesis of Shvo's catalyst[66]

We set the optimal condition for one pot reductive hydroamination of phenylacetylene with 10 % of benzoic acid, 5 equivalents of ammonium formate, heating the reaction vessel with 6 cycle of 10 minutes each at 100 °C after catalyst formation and tested on different substituted aniline and aliphatic amines.

The reaction worked well with most of the amines as shown in **scheme 17** with the only exception of morpholine, where only a 12 % of compound **27** was formed. A possible explanation could be its higher solubility in aqueous

media and consequently low concentration in the micellar core where the reaction take place.



Scheme 17

We decided to test the residual activity of the catalyst for the possibility to recycle it in order to perform other reductive hydroamination and prevent waste of the precious metal used.

We extract the organic compounds with EtOAc and washed the organic phase with aqueous HCl in order to remove both product and starting material from the organic phase as quaternary ammonium salts.

After the evaporation of the organic solvent, the residue was suspended in water together with phenylacetylene and aniline amine and heated at MW for the next reaction.

As it's show in **figure 4** the catalyst was recycled up to 4 times without any significant decrease in its activity.

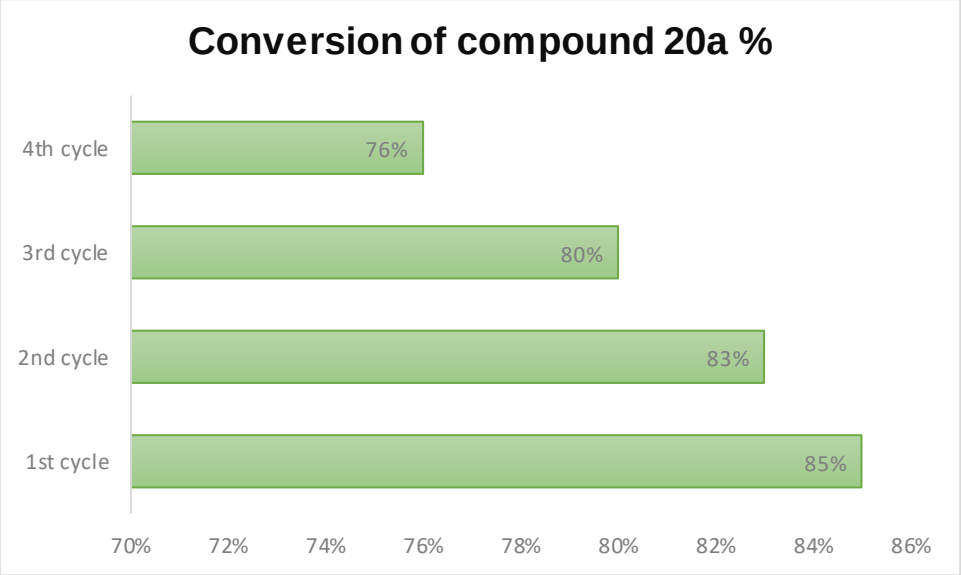


Figure 14

1.5 Conclusion

In conclusion, we have developed a new catalyst based on Ru nanoparticles assisted with MW dielectric heating suitable for hydration of alkynes in water containing 2.5% of TPGS-750-M surfactant.

With the same micellar nano catalytic system, we discover the possibility to perform the one-pot single step reductive hydroamination of alkynes in water. The process produces ketones and secondary amines with low environmental impact as surfactant is required in low quantity, the use of organic solvents is limited to product separation and the catalyst could be recycled for several times.

The potential application of this MW assisted Ru nanoparticle nano-micelle catalysis allow the possibility to study its properties on other organic reactions.

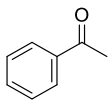
1.6 Experimental part

1.6.1 General Methods

All reagents were used as purchased from commercial suppliers without further purification. Merck aluminium backed plates pre-coated with silica gel 60 (UV254) were used for analytical and preparative thin layer chromatography and were visualized by staining with a solution of ninhydrin in EtOH or a $KMnO_4$ solution. Proton nuclear magnetic resonance ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on NMR spectrometer. Deuterated chloroform was used as the solvent, and chemical shift values (δ) are reported in parts per million referred to the residual signals of this solvent (δ 7.26 for 1 H and δ 77.6 for ^{13}C). Data are represented as follows: chemical shift, multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, m = multiplet and/or multiple resonances, bs=broad singlet), coupling constant (J) in Hertz and the integration. Mass spectroscopy data of the products were collected on GC/MS spectrometer. GC conditions: ion trap detector equipped with a 30 m OV-101 capillary column, splitting injector at 300 °C, method: 80 °C 5 min, 80-280 °C 10 °/min, 280 °C 5 min. Scanning transmission electron microscopy (STEM) and Energy-dispersive Xray spectroscopy (EDS) analysis was done using a FIB/SEM TESCAN GAIA 3 installed at the Microscopy Center (Ce.me.) at ICCOM-CNR (Florence). Reactions carried out under MW dielectric heating were performed with a microwave oven (Discover from CEM) under monomode irradiation in a 10 mL or 50 mL sealed vial. The internal temperature was monitored through an internal IR sensor and the maximal internal pressure monitored and maintained under the value of 250 psi.

1.6.2 General procedure for alkynes hydration

Acetophenone [10a]



10a

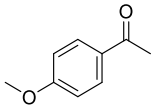
A 50 mL MW tube equipped with a magnetic stir bar was charged with phenylacetylene (306 mg, 3 mmol) and aniline (14 mg 0.15 mmol). A solution of TPGS-750-M (5 wt. % in H_2O, 1.5 mL) was added and diluted with a solution of NaCl (0.02 M in H_2O, 1.5 mL). Then, Shvo's catalyst (33 mg, 0.03 mmol) was added and the tube sealed. The mixture

was submitted to MW irradiation for 6 cycles of 10 min at 100 °C (internal max temperature). The dark organic layer was dissolved in EtOAc (5 mL) and washed with water and brine in a separatory funnel, dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum. The crude of the reaction was filtered through a silica pad to afford, after evaporation, pure acetophenone as a colourless viscous liquid (324 mg, 90% yield).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.98- 7.96 (m, 2H), 7.59-7.55 (m, 1H), 7.49-7.45 (m, 2H), 2.61 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 198.2, 137.1, 133.1, 128.6, 128.3, 26.6.

p-Methoxyacetophenone [12]



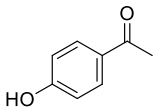
12

The product was isolated as a crude compound following the general procedure. Obtained 396 mg, 88% yield.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78 (d, J=8.9 Hz, 2H), 6.78 (d, J=8.9 Hz, 2H), 3.71 (s, 3H), 2.39 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 196.9, 163.8, 130.9, 130.8, 114.0, 55.7, 26.5.

p-Hydroxyacetophenone [13]



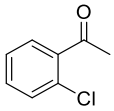
13

The product was isolated by column chromatography on silica gel (eluent EtOAc / petrol ether (40-60) 1 / 1). Obtained 290 mg, as a colourless oil 71 % yield.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78 (d, J=8.9 Hz, 2H), 6.78 (d, J=8.9 Hz, 2H), 2.39 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 196.9, 163.8, 130.9, 130.8, 114.0, 55.7, 26.5.

o-Chloroacetophenone [14]



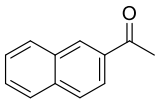
14

The product was isolated by column chromatography on silica gel (eluent EtOAc / petrol ether (40-60) 1 / 1). Obtained 290 mg as a colourless oil, 71 % yield.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.93 (t, J = 2.0 Hz, 1H) 7.83 (ddd, J = 8.0, 2.0, 1.2 Hz, 1H), 7.54 (ddd, J = 8.0, 2.0, 1.2 Hz, 1H), 7.41 (t, J = 8.0 Hz, 1H), 2.60 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 196.7, 138.5, 134.9, 133.0, 129.9, 128.4, 126.4, 26.6.

1-Naphtyl-1-ethanone [15]



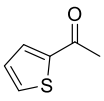
15

The product was isolated by column chromatography on silica gel (eluent EtOAc / petrol ether (40-60) 3 / 7). Obtained 285 mg, 56 % yield.

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.76–8.74 (m, 1H), 7.90–7.87 (m, 1H), 7.83–7.77 (m, 2H), 7.56–7.52 (m, 1H), 7.47–7.43 (m, 1H), 7.40–7.36 (m, 1H), 2.65 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 202.2, 136.0, 134.4, 133.4, 130.6, 129.0, 128.8, 128.4, 126.8, 126.2, 124.7, 30.3.

1-(2-Thienyl)-1-ethanone [16]



16

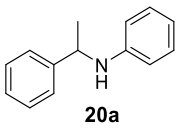
The product was isolated as a crude following the general procedure. Obtained 340 mg, 90% yield.

^{1}H NMR (400 MHz, $CDCl_3$): d = 7.71 (m, 1H), 7.64 (m, 1H), 7.14 (m, 1H), 2.57 (s, 3H).

^{13}C NMR (400 MHz, $CDCl_3$): d = 191.2, 145.0, 134.2, 132.9, 128.5, 27.3.

1.6.2 General procedure for alkynes reductive hydroamination

(1-phenylethyl)-aniline [20a]



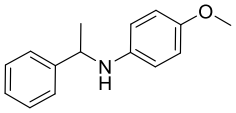
20a

In a 50 ml MW tube, a solution of TPGS-750-M (5 wt. % in H_2O 500 µL) was added and was diluted with a solution of NaCl (0.02 M in H2O, 500 µL). $Ru_3(CO)_{12}$ (6.4 mg, 0.01 mmol) and tetraphenylcyclopentadienone (12 mg. 0.03 mmol) were added. The mixture was submitted to MWs dielectric heating for 6 cycles of 10 min at 100 °C (max internal pressure reached 150 psi). Then the tube was opened and charged with phenylacetylene (306 mg, 3 mmol) and aniline (418 mg, 4.5 mmol). A solution of TPGS-750-M (5 wt. % in H_2O, 1 mL) was added followed by a solution of NaCl (0.02 M in H_2O, 1 mL). Then solid HCOONH4 (945 mg, 15 mmol) and benzoic acid (36 mg, 0.3 mmol) were added and the tube sealed. The mixture was submitted to MW dielectric heating for 6 cycles of 10 min at 100 °C. The organic layer was dissolved in EtOAc (5 mL) and washed with water and brine in a separatory funnel, dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum. The crude of the reaction was dissolved in Et_2O and HCl 2M in Et_2O was added, observing the formation of a brown precipitate. The solid was filtered and re-crystallized from EtOAc to give a pale-brown solid (m.p.183-184 °C). Free amine was obtained by selective extraction with a solution of Na_2CO_3 (5 mL 10% wt) and EtOAc (10 mL). After separation of the organic layer, drying over Na_2SO_4 and evaporation of the solvent, amine 10 was isolated as a dense oil (472 mg, 80%).

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.34-7.30 (m, 4H), 7.22-7.20 (m, 1H), 7.08-7.06 (m, 2H), 6.61 (t, J = 7.8 Hz, 1H), 6.50 (d, J = 8.0 Hz, 2H), 4.22 (t, J = 6.6 Hz, 1H), 4.04 (bs, NH), 1.86-1.78 (m, 2H), 0.95 (t, J = 7.8 Hz, 3H).

^{13}C NMR (150 MHZ, $CDCl_3$) δ 147.7, 144.1, 129.2, 128.7, 127.0, 126.7, 117.3, 113.5, 59.9, 31.9, 11.0.

(1-Phenylethyl)(p-methoxyphenyl)amine [21]



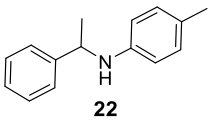
21

The product was isolated as a solid following the general procedure, obtained 531 mg as a yellow oil, 78% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36-7.34 (d, J = 8.4 Hz, 2H), 7.31 (t, J = 18 Hz, 1H), 7.21-7.20 (m, 2H), 6.67(d, J = 6.6 Hz, 2H), 6.46(d, J = 8.4 Hz, 2H), 4.39 (q, J = 13.2, 1H), 3.67(s, 3H), 1.48 (d, J = 6.6 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 151.9, 144.4, 141.5, 128.6, 126.8, 125.9, 114.7, 114.5, 55.7, 54.2, 25.0.

(1-Phenylethyl)(p-tolyl)amine [22]



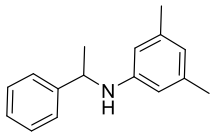
22

The product was isolated as a solid following the general procedure, obtained 430 mg as a yellow oil, 68% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34-7.35 (m, 1H), 7.28-7.33 (m, 2H), 7.16-7.27 (m, 2H), 6.87 (d, J = 10.8 Hz, 2H), 6.42 (d, J = 10.8 Hz, 1H), 4.42 (m, 1H), 3.98 (bs, NH), 2.17 (s, 3H), 1.47 (d, J = 6.6 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 145.4, 144.9, 129.5, 128.5, 126.7, 126.3, 125.8, 113.4, 53.6, 24.9, 20.8.

(1-Phenylethyl)(3,5-xylyl)amine [23]



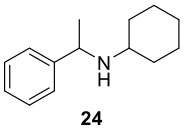
23

The product was isolated as a solid following the general procedure, obtained 506 mg, 75% yield.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39- 7.29 (m, 4H), 7.22 (t, J = 7.2 Hz, 1H,), 6.32 (s, 1H), 6.17 (s, 2H), 4.48 (q, J = 6.7 Hz, 1H,), 3.9 (br s, 1H), 2.17 (s, 6H), 1.50 (d, J = 6.7 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 147.9, 145.8, 139.2, 129.0, 127.2, 126.3, 119.7, S34 111.5, 53.7, 25.3, 21.9.

(1-Phenylethyl)cyclohexylamine [24]



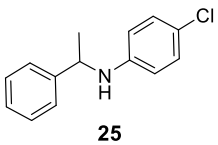
24

The product was isolated as a dense oil following the general procedure, obtained 438 mg, 72% yield.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.34–7.16 (m, 5H), 3.96 (q, J = 7.0 Hz, 1H), 2.26 (m, 1H), 1.72 (m, 11H), 1.33 (d, J = 7.0 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 146.4, 128.5, 126.5, 54.6, 53.8, 34.6, 33.3, 26.3, 25.4, 25.1, 14.2.

(1-Phenylethyl)(p-chlorophenyl)amine [25]



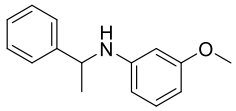
25

The product was isolated as a solid following the general procedure, obtained 485 mg, 70% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30-7.06 (m, 4H), 7.05 (t, J = 7.84 Hz, 2H), 6.63 (t, J = 7.30 Hz, 1H), 6.43 (d, J = 8.53 Hz, 2H), 4.39 (q, J = 6.69 Hz, 1H), 3.83 (br s, 1H), 1.43 (d, J = 6.88 Hz, 3H),

^{13}C NMR (100 MHz, $CDCl_3$) δ 147.2, 144.1, 132.6, 129.4, 129.1, 127.5, 117.8, 113.6, 53.4, 25.6.

(1-Phenylethyl)(m-methoxyphenyl)amine [26]



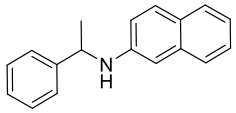
26

The product was isolated as a waxy material, obtained 469 mg, 69% yield

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36-7.26 (m, 4H), 7.23-7.17 (m, 1H), 6.98 (t, J = 8.1 Hz, 1H), 6.20 (ddd, J = 8.3, 2.4, 0.6 Hz, 1H), 6.13 (ddd, J = 8.0, 2.2, 0.7 Hz, 1H), 6.05 (t, J = 2.3 Hz, 1H), 4.45 (q, J = 6.6 Hz, 1H), 4.04 (br s, 1H), 3.65 (s, 3H), 1.48 (d, J = 6.7 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 160.6, 148.6, 145.1, 129.7, 128.6, 126.8, 125.7, 106.4, 102.3, 99.3, 54.9, 53.4, 24.9.

(1-Phenylethyl)-2-naphthylamine [27]



27

The product was isolated as a solid following the general procedure, obtained 485 mg, 70% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97-7.94 (m, 1H), 7.82e7.80 (m, 1H), 7.50 (m, 2H), 7.45 (d, J = 7.16 Hz, 2H), 7.36-7.33 (m, 2H), 7.28-7.24 (m, 1H), 7.21 (d, J = 4.9 Hz, 2H) 6.43-6.40 (m, 1H), 4.76 (br s, 1H), 4.70 (q, J = 6.7 Hz, 1H,), 1.69 (d, J= 6.7 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 144.9, 142.1, 128.7, 128.7, 126.9, 126.5, 125.8, 125.6, 124.6, 123.2, 119.7, 117.2, 106.0, 53.6, 25.2.

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Chapter 2. New synthetic route for Sepiapterin

2.1 Introduction

During my Ph.D., my research group worked in collaboration with Dipharma Francis S.r.l., an Italian chemical company producing Active Pharmaceutical Ingredients (APIs). This collaboration led us to look for a new synthetic way to produce the drug Sepiapterin, known as a precursor of tetrahydrobiopterin (Saproteprin), cofactor of phenylalanine hydroxylase, for the treatment of phenylketonuria, a rare metabolic inborn disorder.

2.1.1 Phenylketonuria

Phenylketonuria (PKU) is a rare inherited autosomal recessive metabolic inborn disorder caused by a deficiency or completely loss of function of phenylalanine hydroxylase (PAH) which catalyse the hydroxylation of phenylalanine (Phe) to generate tyrosine (Tyr), a non-essential amino acid, precursor of important molecules such as tyrosine, dopamine, nor-adrenaline, adrenaline and melatonin, which result in the toxic accumulation of phenylalanine and incapacity to synthesize tyrosine and its derivative.[1,2,3]
When PKU is caused by loss of functionality of Dihydropteridine reductase (DHPR) which catalyse the biosynthesis and regeneration of tetrahydrobiopterin (BH4), cofactor of PAH enzyme, is known as mild hyperphenylalaninemia.[4]
It was classified as one of the most frequent inherited disorders with global prevalence of 1:23.930 live births.[5]
PKU can be classified based the severity of the disease in:

- Classic PKU (62%)
- Mild PKU (22%)
- Mild hyperphenylalaninemia (16%)

Phenylketonuric infants are often asymptomatic until the consumption of food containing Phe, that explain the difficulties of the identification by new-born screening.
Untreated patients often develop severe mental, neurological and physical symptoms caused by toxic accumulation of Phe and lack of Tyr such as: mental retardation, microencephaly, hyperactivity Parkinsonian and pyramidal signs, growth reduction and iris and skin pigmentation[6,7,8].
Early screening identification and treatment could improve quality of life of most of patients, giving them the opportunity to live independent as adults even if their neurocognitive level is always below a control group from the general population.[9]

2.1.2 Phenilalanine hydroxylase (PAH)

Phenylalanine hydroxylase is a homo-tetrameric enzyme of 50 kDa subunits each one composed by 3 domain: regulatory, oligomerization and catalytic (figure 1).[10]

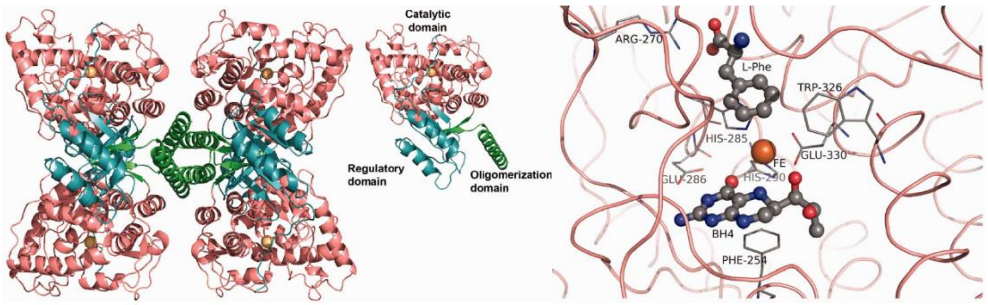


Figure 1[10] a) Phenylalanine hydroxylase structure b) catalytic domain

PAH is primary present in the liver and its role is the removal of Phe catalysing the para-hydroxylation and generate Tyr that could be used as a precursor for the synthesis of proteins and neurotransmitters or degraded by the citric acid cycle.[11]

The catalytic domain core as described in figure 1b is composed by an iron atom bound with two histidine (His) and a glutamine (Glu) which mediate the formation of Tyr trough a mechanism that could be divided in 3 main step[12,13] (figure 2):

1. Oxidation of BH4 cofactor to 4a-hydroxypterin by forming the reactive intermediate
2. Insertion of oxygen into Phe
3. Dehydration of pterin cofactor to its quinoid form and generation of tyrosine

BH4 is an important cofactor for PAH activity and modulation that could be synthesize *de novo* starting from guanosine triphosphate or recycled starting with its quinoid form through DHPR.

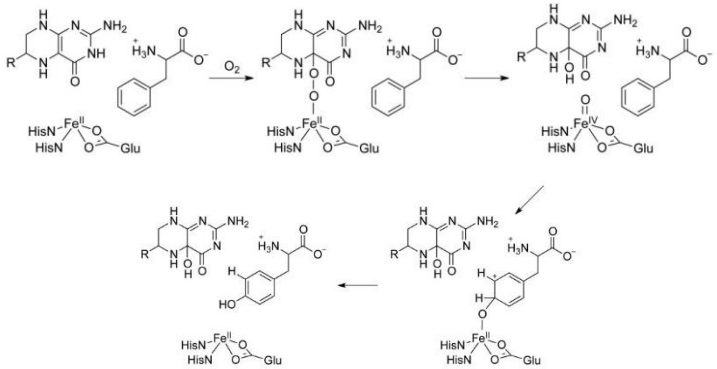


Figure 2 Biosynthesis of tyrosine[13]

2.1.3 Biosynthesis of tetrahydrobiopterin

Human organism can synthesize BH4 in two different ways, via *de novo* synthesis or recycling it from BH4-4a-carbinolamine, which is the by-product of tyrosine hydroxylation.

As described in **figure 3** the first route starts from guanosine triphosphate (GTP) to generate 7,8-dihydroneopterin triphosphate catalysed by guanosine triphosphate cyclohydrolase (GTPCH) an homodecamer enzyme, consisting in two dimers of pentamers containing a Mg^{2+} eme[14,15].

The described enzyme mediates the imidazole ring opening through water addition to the molecule with formic acid elimination, followed by Amadori rearrangement of the side chain with condensation to a 6-member ring, forming compound **5**.[16]

The second intermediate of BH4 synthesis is catalysed by 6-pyruvoyl-tetrahydropterin synthase (PTPS), characterized by 2 trimers containing Zn^{2+} forming an hexamer which generate by hydrogen transfer 6-Pyruvoyl-tetrahydropterin (compound **8**) from compound **5**. [17,18,19]

The metal ion is foundamental for this step, because stabilizes and activate the proton of the pteridine and allow the C=N reduction, alcohol oxidation and tautomerization after triphosphate elimination.

Last step is the reduction of compound **8** catalysed by Sepiapterin Reductase (SR), a homodimer enzyme stabilized by a four-helix bundle, carried out by two NADPH-dependent reduction with the formation of the PAH cofactor, tratrahydrobiopterin (compound **11**).[20]

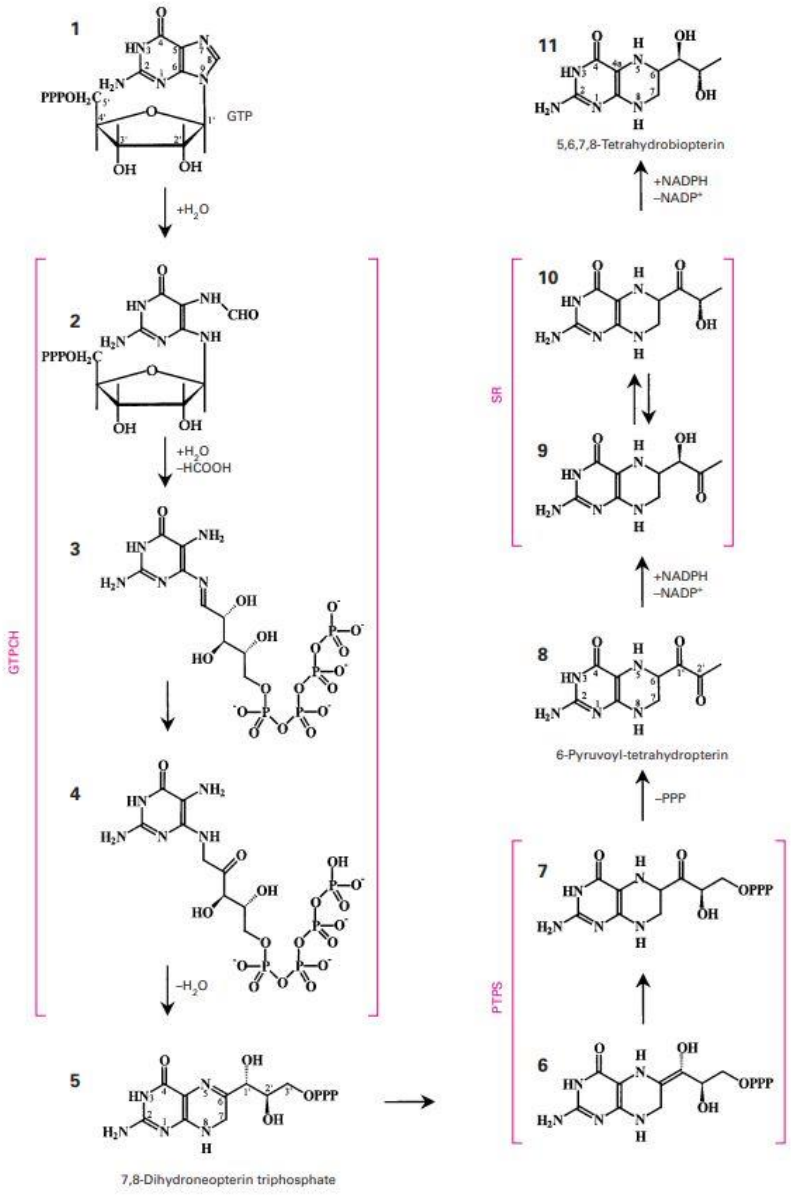


Figure 3 *De novo* synthesis of tetrahydrobiopterin[21]

The second important route of synthesis as described in **figure 4** is by recycling BH4 from Tetrahydrobiopterin-4a-carbinolamine (compound **12**), the oxidated form of BH4 generated after hydroxylation of phenylalanine by PAH.

Protocatechuate 3,4-Dioxygenase (PCD) is an homotetrameric enzyme that catalyse the dehydrataion to the quinoid form of dihydrobiobterin (compound **14**). [22,23]

At this point q-BH2 can undergo in two different pathways to regenerate BH4:

- First one is catalysed by DHPR, an α/β enzyme with a central twisted β-sheet flanked on each side by a layer of α-helices with two bounds for NADH each dimer and widely distributed in human body, reduction of compound **14** directly proceed through hydride transfer from NADH ring to q-BH2.[24,25]
- The second way is a non enzymatic rearrangement of q-BH2 followed by SR reduction to BH4.[26]

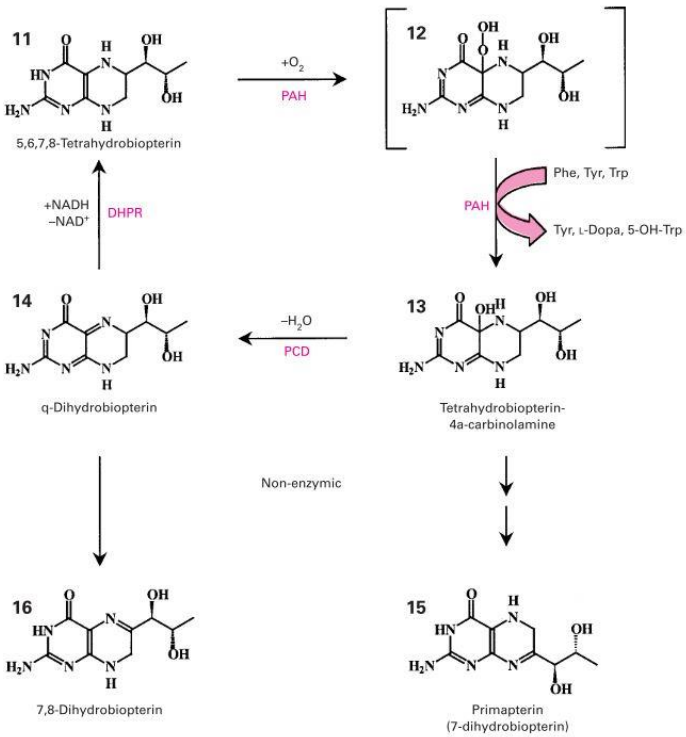


Figure 4 Recycling pathway of tetrahydrobiopterin[21]

It was found by different studies that several mutations located on gene coding DHPR may lead to partially or completely loss of function of the enzyme, decreasing of BH4 level necessary for PAH activity, characterized by hyperphenylalaninemia and classic symptoms of PKU patients.[27]

2.1.4 Current therapies in PKU

1) Dietary therapy

Reduce the Phe income from food with a restricted diet to prevent its accumulation in human body is one of the oldest approaches to prevent PKU severe neurological damage.

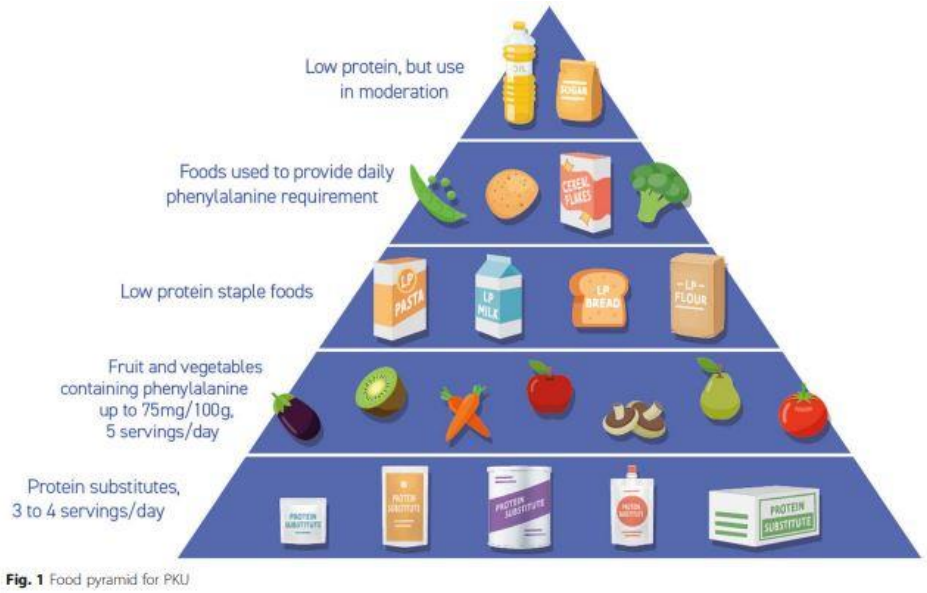


Figure 5 Food pyramid for PKU[28]

To achieve this goal, Phe assumption should be replaced with synthetic protein that are Phe-free or at very low concentration, less than 250 mg/day for severe PKU and no more than 400 mg/day in moderate/mild PKU. In order to assure the normal growth of the patients, the intake of all the nutrients that will be avoided with this diet, vitamins and minerals, should be added separately or to the protein-substitute.

The main problem associated with dietary therapy is of course the risk of malnutrition and related issues, such as growth retardation and osteoporosis, followed by huge socioeconomical burdens.

However, recent neuropsychological studies shows that even with a strict adherence to the diet, early treated PKU patients generally have a lower mean IQ than their unaffected siblings or the normal population.[29,30]

2) Enzyme therapy

Because PKU is caused by a loss of function or decrease activity of PAH, enzyme replacement is another approach to reduce income from food.

Only two enzymes have been developed: non immunogenic PAH and PAL (phenylalanine- ammonia lyase).

PAL is extracted and purified from *Rhodotorula glutinis* and convert Phe to Trans-cinnamic acid without the need of cofactor for its activity, metabolized in benzoic acid by the liver and excreted with urine.

The main problem of this enzyme is the pH inactivation by the stomach and duodenum, that could be avoided through parenteral administration, but immunological response should be considered.

PAH has been used for other metabolic disease and the immunological reaction was retarded by PEGylation of the enzyme, increasing its half-life from 6 h to 20h, but it requires coadministration with cofactor (BH_2, BH_4).[31]

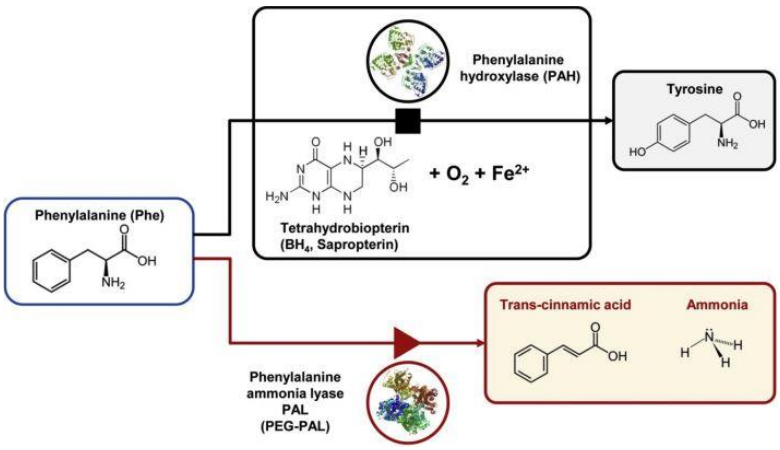


Figure 6 Alternative degradation of phenylalanine[31]

3) Gene therapy

In the last 20 years several studies have been developed using viral and non-viral vectors for DNA deliver to cells in murine models.

In a first phase, liver PAH gene transfer in vitro or in vivo was promising, but failed due to poor efficiency of gene delivery and lack of gene expression, in addition to the problem of combining PAH with BH4-cofactor gene expression in heterologous tissues.[32]

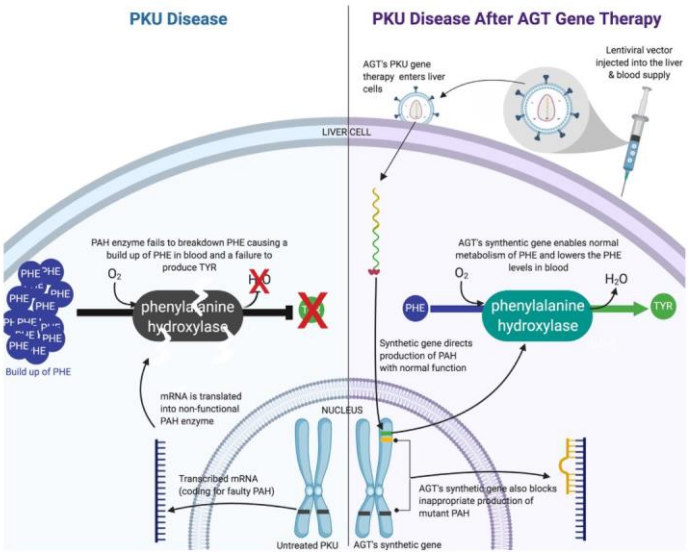


Figure 7 Gene therapy for PKU[33]

4) Sapropterin (BH_4) and Sepiapterin (BH_2)

Tetrahydrobiopterin (BH4) is the natural co-factor of PAH and is fundamental for the activity of PAH enzyme.

Sapropterin dihydrochloride, synthesized by Biomarin Pharmaceutical, is the synthetic formulation of the natural PAH cofactor, approved in EU for oral administration as treatment for children over 4 years old affected with hyperphenylalaninemia with PKU-BH_4 responsive usually caused by mutations in genes encoding biosynthesis and regeneration of BH_4.[34]

Because is also cofactor of other several enzymes, such as tyrosine hydroxylase, tryptophan hydrolase and nitric oxide synthase, the administration of BH_4 may also reduce oxidative stress and damage caused by Phe accumulation.

After the administration, Sapropterin act as a chemical chaperon in different ways:

- Stabilization of PAH
- Protecting the enzyme from ubiquitination or proteolytic cleavage
- Up-regulation of BH_4 biosynthesis and PAH expression
- Stabilization of PAH mRNA

After 20 years of treatment, no side effect was reported in BIODEF (International Database of Tetrahydrobiopterin Deficiencies) only several adverse reactions in a trial of 318 patients such as: psychoneurotic symptoms (13.8%), urological (9.1%) and gastrointestinal (2.8%).[35,36]

The inventor of WO 2011132435 has found that a precursor of BH4, Sepiapterin, after peripheral administration, passes through the blood-brain barrier and is taken up into neurons 10 times more efficiently than Sapropterin.

The reason is that after the administration, as shown in **figure 8a**, the prodrug easily passes the blood-brain barrier and is taken up by monoaminergic neurons via facilitated transport and converted to BH4 in two step enzymatic reactions made by SR and dihydrofolate reductase (DHFR).[37]

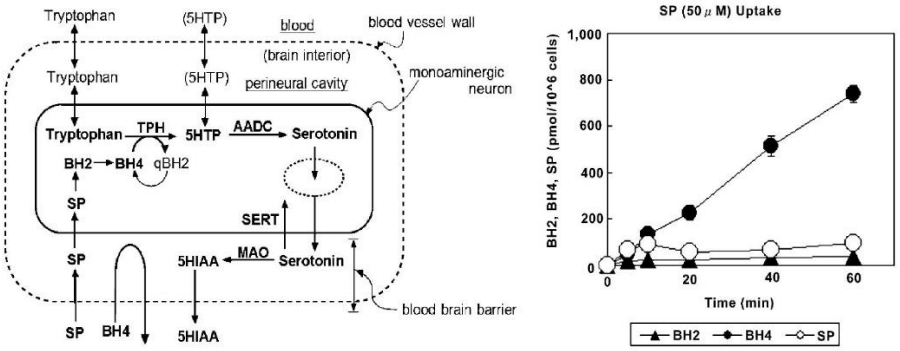


Figure 8 a) Conversion of Sepiapterin (SP) to tetrahydrobiopterin (BH4) in monoaminergic neuron b) BH2, BH4 and SP concentration after SP administration[37]

Moreover, BH4 is present at a constant level in neuron cells, reason why after administration, Sapropterin hydrochloride is taken up very little even when increasing its dosage, on the contrary, Sepiapterin is kept at relatively low concentration inside the cell, that explain why is taken in higher amount comparing with Sapropterin administration (**Figure 9**).

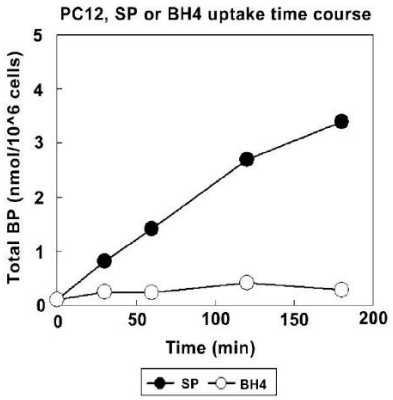


Figure 9[37]

A recent clinical study conducted by double blind randomization, placebo controlled, confirm that Sepiapterin (CNSA-001) could have a better profile for oral administration compared to Sapropterin.

The study was divided in two part as described in **table 1**, to evaluate its pharmacokinetic and safety administration.

Details of the study design.

Study Part A		Study Part B	
Cohort	Study treatments	Cohort	Study treatments
1	Single dose of: CNSA-001 2.5 mg/kg (n = 6) Sapropterin dihydrochloride 2.5 mg/kg (n = 3) Placebo (n = 2)	1	7 days once-daily: CNSA-001 5 mg/kg (n = 6) Placebo (n = 2)
2	Single dose of: CNSA-001 7.5 mg/kg (n = 6) Sapropterin dihydrochloride 7.5 mg/kg (n = 3) Placebo (n = 1)	2	7 days once-daily: CNSA-001 20 mg/kg (n = 6) Placebo (n = 2)
3	Single dose of: CNSA-001 20 mg/kg (n = 6) Sapropterin dihydrochloride 20 mg/kg (n = 3) Placebo (n = 1)	3	7 days once-daily: CNSA-001 60 mg/kg (n = 6) Placebo (n = 2)
4	Single dose of: CNSA-001 40 mg/kg (n = 6) Placebo (n = 2)		
5	Single dose of: CNSA-001 80 mg/kg (n = 6) Placebo (n = 2)		
6	CNSA-001 10 mg/kg (2 single doses, under fasting and fed conditions, respectively) (n = 12)		

Table 1 Study organization[38]

As described in **figure 10**, CNSA-001 shows higher plasma concentration compared to Sapropterin reaching a peak in about 4 h dose-dependent, followed by increasing BH4 plasma concentration, demonstrating that its rapidly metabolized to Tetrahydrobiopterin.

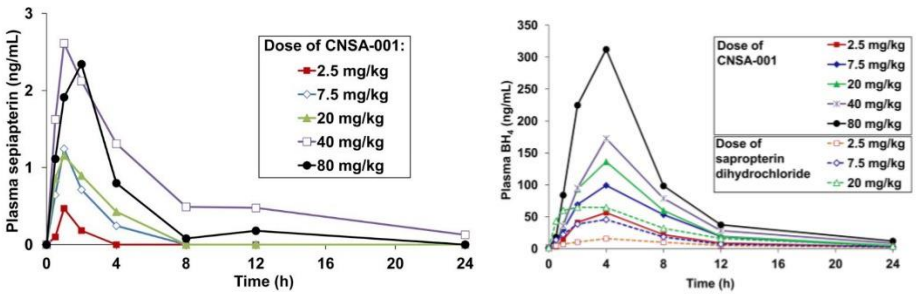


Figure 10[38]

After 7 days of administration no changes from day one was observed and no adverse reaction that could be correlated to Sepiapterin because its incidence was comparable with placebo group.[38]

On 26/05/2021 American Food and Drug Administration (FDA) and European Commission (EC) has granted Orphan Drug Designation (ODD) to Sepiapterin for the treatment of patient with hyperphenylalaninemia.[39]

2.1.5 Chemical structure and synthesis of Pteridines

Pteridines are a group of heterocyclic compounds characterized a pyrimidine ring fused with a pyrazine ring.

There are three main classes of natural occurring pteridines namely, lumazines, isoalloxazine and pterins as shown in **figure 11**.

Isoalloxazines differ from Lumazines for the ring in the 6- and 7- position, although having the same oxo-substituents at the 2- and 4- positions, while pterins, the most common class of naturally occurring pteridines, have an amino group at the 2-position and an oxo-group at the 4-position.[40]

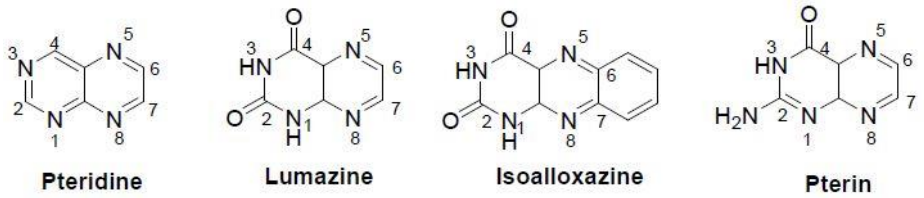


Figure 11[40]

Pterin exists in three main redox states: the fully oxidized, the semi-reduced or dihydro and the fully reduced or tetrahydro state, interconverted by $2e^-$,$2H^+$ reactions.

The redox chemistry of pterin show its complexity when all the tautomers of the semi reduced state are considered, in fact on the type of reduction method chosen only one of the tautomers could be initially produced, regardless, unless highly substituted, will eventually rearrange to the most thermodynamically stable form, the 7, 8-dihydropterin (Figure 12 compound **21**).[41]

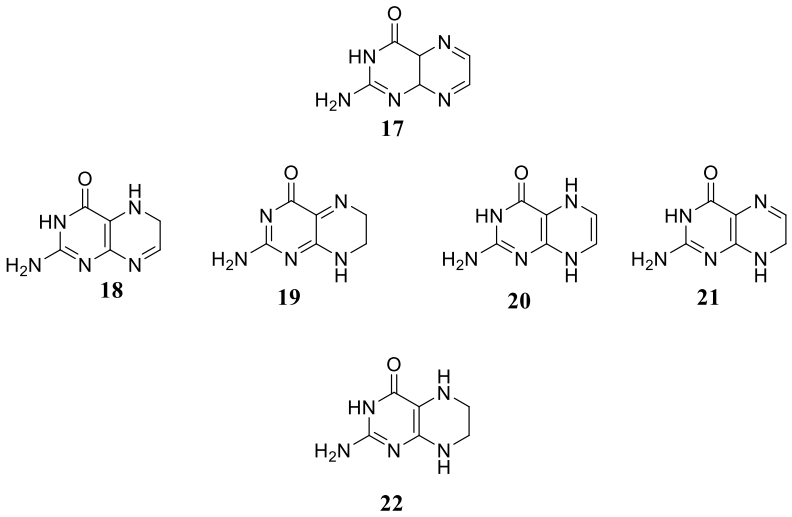
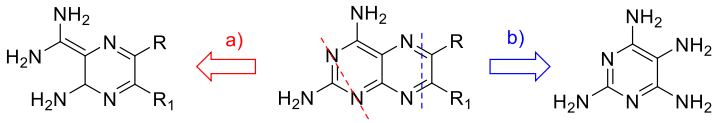


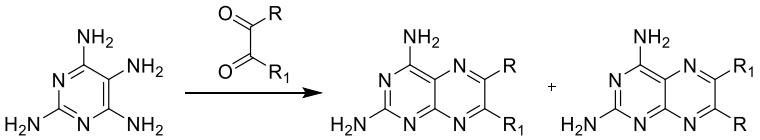
Figure 12

During the XIX century, many ways were developed for the synthesis of pteridines, chemical compounds characterized by a pyrazine ring fused with a pyrimidine ring.
Synthesis of the pteridine ring system can be accomplished in different ways as it's shown in **scheme 1**.



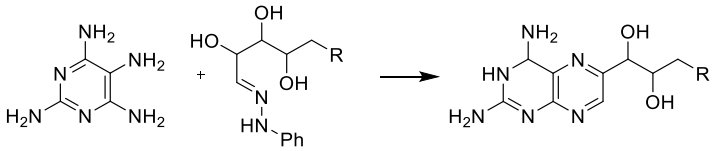
Scheme 1

A first disconnection approach starts with a pyrazine ring on which the pyrimidine ring is build up (**scheme 1a**). Second approach is the synthesis of the pyrazine ring through condensation on the pyrimidine ring (**scheme 1b**)
The Gabriel-Isay synthesis is a condensation reaction that start with pyrimidine ring and the synthesis of pyrazine ring was done by reacting the heterocyclic compound with a dialdehyde, glyoxal, aldehydoketone or diketone, to obtain in one step a pteridine already functionalized in position 6 or 7.
The limit of this synthesis is that the reaction is not selective and produce both 6 and 7 substituted pterins as a mixture.[42,43]



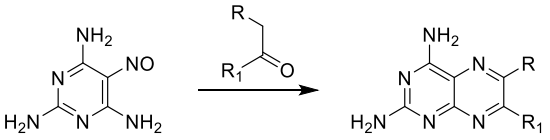
Scheme 2 Grabriel-Isay synthesis

A similar methodology that allows the introduction of sugar moiety is the Viscontini reaction which makes use of the phenylhydrazone derivative of a sugar and 2,5,6-triaminopyrimidine-4(3H)-one that undergo to condensation after Amadori rearrangement in mildly acidic solution to the 6- substituted 2-aminopteridin-4(3H)-one.[44]



Scheme 3 Viscontini reaction

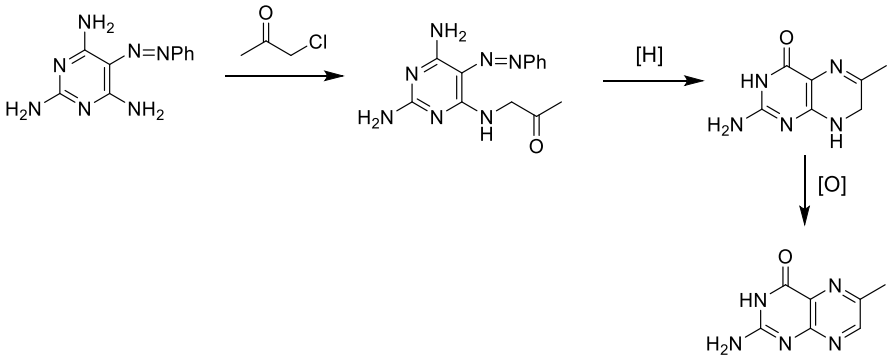
Timmis synthesis is a type of reaction that can avoid the problem of 6, 7 substituted mixture of pterins by using 5-nitroso-2,4,6-pyrimidine-triamine and an α-carbonylmethylene for condensation, with the formation of a pterin carrying the substituent in α position to the carbonyl of the ketone into 6 position.
The limitation in this case is the nitroso group preparation.[45]



Scheme 4 Timmis synthesis

Boon synthesis is a twostep cyclization and involves chloroacetone and 2,6 diamino-5-phenylazo-4(3H)-pyrimidinone.

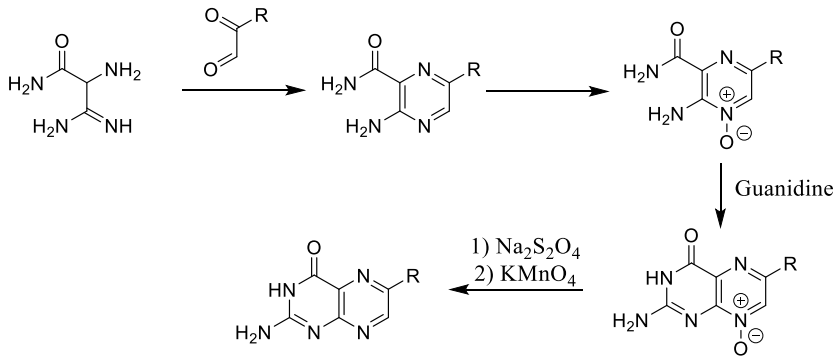
After acylation to the amine in 6th position of the pyrimidinone, the reduction of the azo moiety allows the spontaneous cyclization and formation of the pyrazine ring leading to 7-8 dihydropterin oxidized to 6-methylpterin.[46]



Scheme 5 Boon synthesis

As previously said pterin can be synthesized starting with pyrazine ring.

Taylor *et al* developed a route by reacting a substituted pyrazine with guanidine with the formation of pterin substituted only at 6 position with substituents such as aryl, alkyl and different heterocycles.[47]



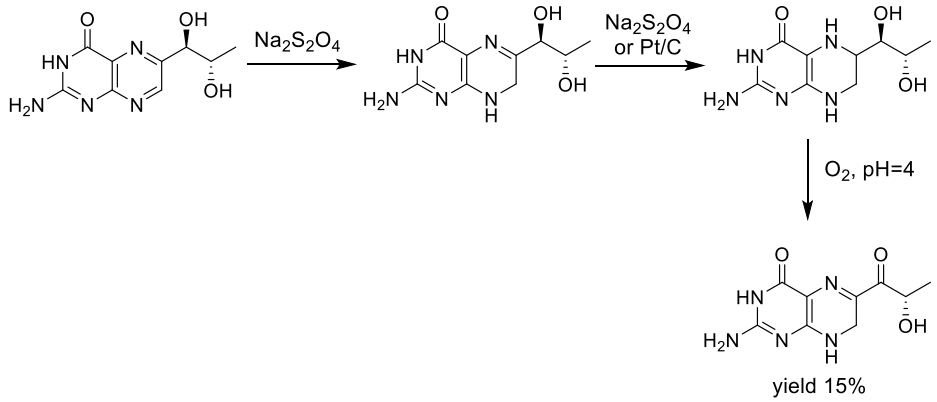
Scheme 6 Taylor synthesis

2.1.6 Synthesis of Sepiapterin

Sepiapterin, as previously said, is a precursor of the cofactor tetrahydrobiopterin, biosynthesized in *vivo*.

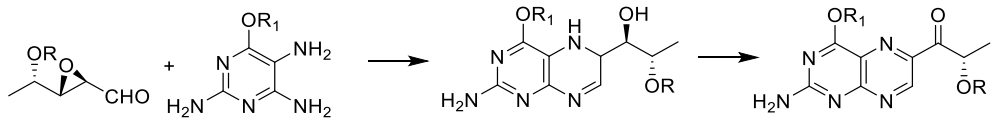
Its first chemical synthesis was reported in in 1978 by Max Viscontini *et al.* using L-Biopterin as starting compound, by reduction of the pyrazine ring using sodium dithionite or hydrogenation with Platinum on carbon with the formation of 5,6,7,8-Tetrahydrobiopterin.

Sepiapterin was obtained with a yield of 15% through oxidation with molecular oxygen in 6 days.[48]



Scheme 7 Viscontini's Sepiapterin synthesis

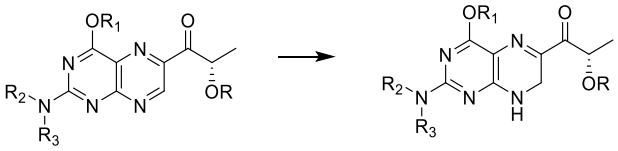
In 2012 Yoshino Hiroshi *et al* patented a synthetic route using two different approaches to obtain Sepiapterin. The pterin ring is constructed from the aldehyde and 2,4,5-triamminopyrimidin-6-one in a polar solvent or using an acid with pKa of 4.5, and subjected to oxidation with peroxide or iodine to produce L-biopterin. The alcohol is then oxidised with tetrapropylammonium perruthenate to lactoylpterin (scheme 8).



Scheme 8

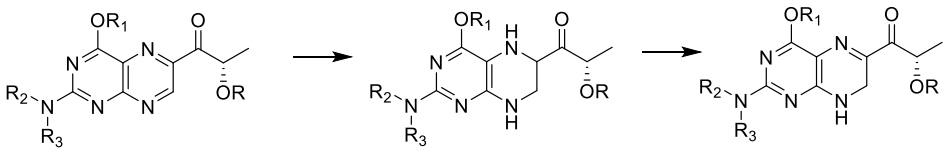
The first approach starts with the partial reduction of the pyrazine ring of lactoylpterin, using sodium dithionite or catalytic reduction with palladium or platinum.

This synthesis was achieved using a protected derivative to enhance the poor solubility of the compound.



Scheme 9

The second approach uses tetrahydrobiopterin as starting compound, obtained by the total reduction of the pyrazine ring with Pt/C and subjecting to partial oxidation the pyrazine ring using peracid or air oxidation. In this synthesis a protected derivative was used for the same reason as written above.[49]

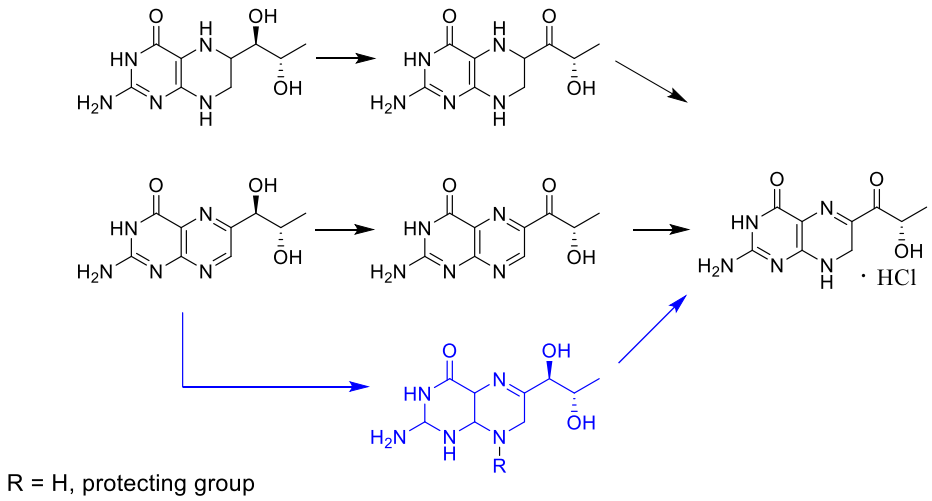


Scheme 10

2.2 Project aim

During the collaboration of our research group with Dipharma Francis S.r.l, we were asked to find a new, non-infringing synthetic route to produce sepiapterin.

As in patent EP 2 848 619, Sepiapterin is always synthesized after alcohol oxidation to ketone before oxidation or reduction of the pyrazine ring, we attempted to synthesize it first by reducing the pyrazine ring to 7,8-dihydropterin as free or protected amine, followed by the oxidation of the alcohol to ketone and its isolation as hydrochloride.



Scheme 11

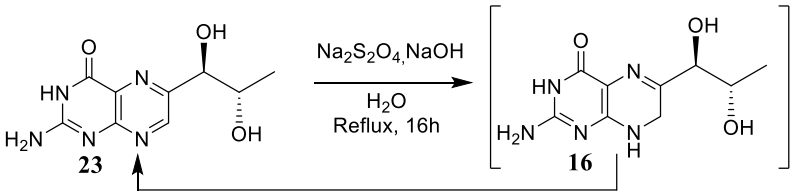
2.3 Result and Discussion

2.3.1 Study on the stability of BH2 and BH4 for one pot oxidation to Sepiapterin

The first aim was to understand if it was possible to synthesize 7,8-dihydropterin without the pre-formed ketone moiety. Our initial strategy was based on the method described by Max Viscontini, using sodium dithionite in waterusing sodium dithionite in acqueous NaOH (pH around 10) in order to prevent $Na_2S_2O_4$ degradation and solubilize L-Biopterin. The reaction was conducted at reflux temperature for 16 hours during which a pH fall to around 6-7 was observed.

The reaction was monitored with ES-MS every hour, observing the formation of compound **16** together with biopterin.

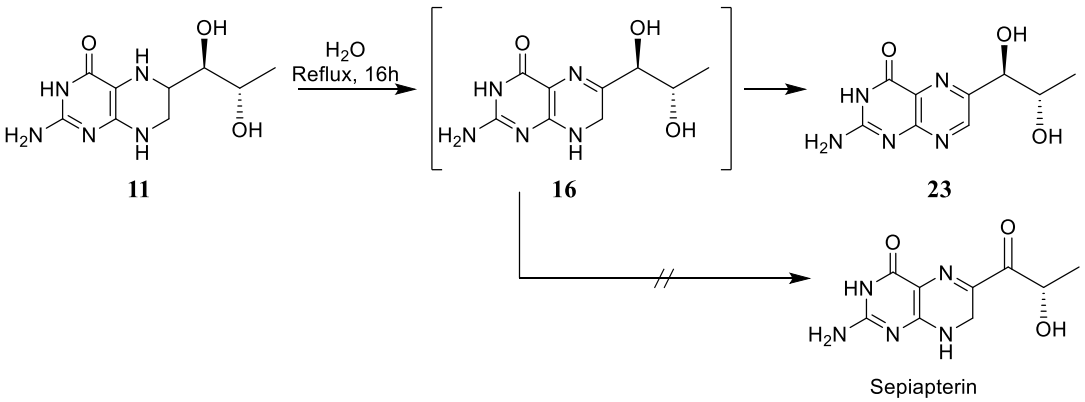
A part of the solution was concentrated to residue under vacuum and analysed at ^{1}H-NMR showing only the presence of compound **23.**



Scheme 11

We decided to study the long-term stability of tetrahydrobiopterin hydrochloride, for 6 days in water at 60 °C, following known procedures for the oxidation of compound **11** to Sepiapterin.

However, the results showed the formation of L-Biopterin as a major product, along with unreacted BH4 and other unclassified impurities.



Scheme 12

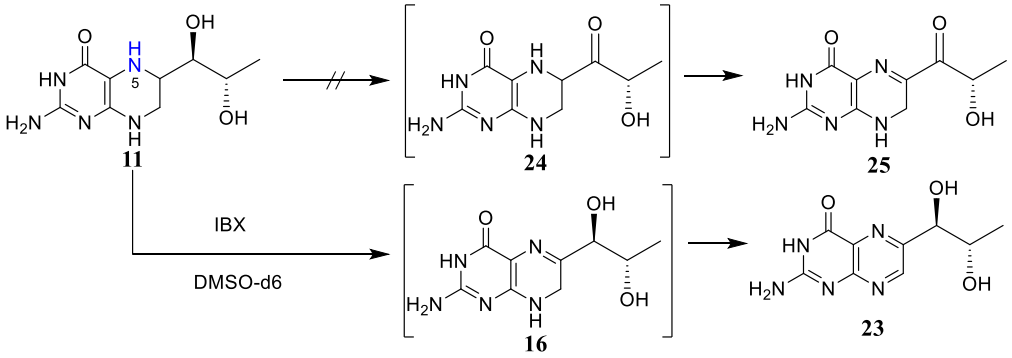
Assuming that the driving force for Sepiapterin formation could be the presence of the keto group through stabilization for conjugation, we attempted to oxidize compound **11**

to Sepiapterin in one step using 2-Iodoxybenzoic acid (IBX), often employed for very specific and regioselective alcohol oxidation. (Scheme 13)

The reaction was conducted in deuterated solvent, DMSO-d_6, to monitor directly the reaction over time by means of 1H-NMR avoiding any work-up. Also in this case, the formation of only compound **23** together with the starting material was observed.

Despite our hypothesis, it's likely that the first oxidation step is carried on N5, and not on the oxygen atom as previously supposed, with the formation of **16** that undergo aromatization leading once again to L-Biopterin.

A possible explanation of this behaviour can be that, after the formation of 7,8-dihydrobiopterin, the oxidation to the aromatic system is favoured over the oxidation of the alcohol to ketone. On the contrary, to obtain the conjugation to a stable 7,8-double bond, the presence of the ketone seems to be necessary to drive the oxidation to Sepiapterin. In such a situation the simple approach of a two-step, polar solvent mediated oxidation resulted unviable.

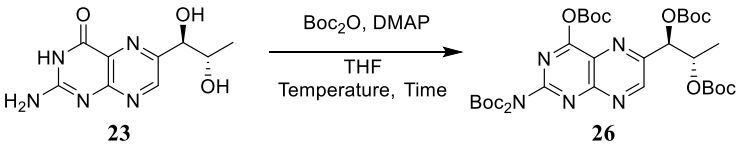


Scheme 13

2.3.2 Synthesis of L-Biopterin as protected derivative

Due to the poor solubility of Biopterin in most of the organic solvents, except water, DMF and DMSO, we attempted the synthesis of a more soluble derivative through protection of both amine and hydroxyl moiety using tert-butyl dicarbonate (Boc_2O) as protecting group and dimethyl-amino-pirydine (DMAP) as base (Scheme 14).

The best result was obtained with high temperature and excess of Boc_2O as shown in entry **5** of **table 1** because of the poor solubility in THF of compound **23**



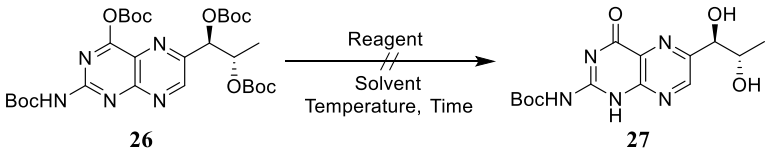
23 26

Scheme 14

Entry	Eq. Boc_2O	Temperature (°C)	Time	Yield (%)
1	6	r.t.	16	-
2	6	Reflux	16	6
3	12	Reflux	16	48
4	12	Reflux	24	55
5	15	Reflux	16	75

Table 1

After isolation from the previous reaction, compound **26** was then subjected to selective deprotection of the hydroxyl moiety in different conditions to understand if it was possible to free the alcoholic groups without affecting the carbamate moiety. Our intention was to identify a suitable substrate with a hydroxyl group available to perform the oxidation of the alcohol to ketone as the last step of Sepiapterin synthesis.



26 27

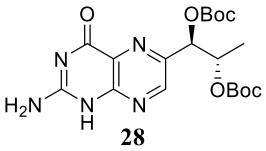
Scheme 15

Entry	Reagent (eq.)	Solvent	Temperature (°C)	Time (h)	Yield (%)
1	TFA (2)	DCM	t.a	12	-
2	MeONa (2)	MeOH	t.a	12	-
3	-	TFE	Reflux	6	-
4	-	H_2O	Reflux	6	-

Table 2

Unfortunately, none of the attempts showed in **table 2** afforded the desired product, only Biopterin was recovered in most of the experiments.

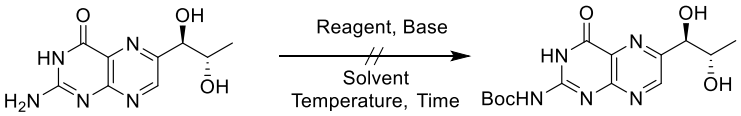
In entry **1** a protected derivative identified as compound **28** was isolated with 6 % of yield together with Biopterin, even if it is not the desired product, this derivative suggested that it might be possible to introduce other protective groups that could resist in an acidic environment.



28

Scheme 16

In a last attempt to prepare the compound **27**, we decided to study the selective protection of the primary amine.



23 **27**

Scheme 17

Entry	Reagent	Base	Solvent	Temp. (°C)	Time (h)	Yield (%)
1	Boc_2O (1.2)	$NaHCO_3$	H_2O/DCM	t.a.	16	-
2	Boc_2O (1.2)	$NaHCO_3$	H_2O/THF	t.a.	16	-
3	Boc_2O (1.2)	TEA	H_2O/THF	t.a.	16	-
4	Boc_2O (2)	TEA	THF anid.	t.a.	16	-
5	Boc_2O (1)	I_2 (10% mol)	THF anid.	t.a.	16	-
6	Boc_2O (12)	I_2 (10% mol)	THF anid.	50	16	-
7	Boc_2O (12)	I_2 (10% mol)	THF anid.	50	24	-
8	Boc_2O (12)	I_2 (10% mol)	THF anid.	70	16	-
9	Boc-On (1.1)	TEA	H_2O/Dioxane	t.a.	16	-
10	Boc-On (2.2)	TEA	THF anid.	65	16	-
11	Boc-Succinimide (1.3)	NaOH	H_2O/CH_3CN	t.a.	16	-

12	Boc-Succinimide (3)	NaOH	H_2O/EtOAc	t.a.	16	-
13	Boc-Succinimide (4)	NaOH	H_2O/DCM	t.a.	16	-

Table 3

In most of the experiments described in **table 3** we didn't observe the formation of compound **27** but only unreacted Biopterin was recovered. In entries **5-8,** where molecular iodine was used as catalyst for selective protection of amine, the recovered precipitate was also identified as Biopterin (95% of starting weight). Nonetheless, the solution was concentrated under vacuum and analysed with ES-MS, detecting a compound with mass corresponding to compound **27** together with mono and di-protected compounds mixture. The isolation and full characterization of the mixture was impossible.

2.3.3 Selective protection of hydroxyl moiety, partial reduction of the pyrazine ring and amine protection

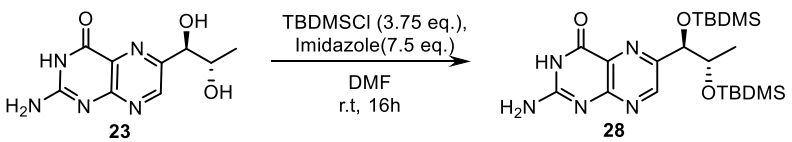
After unsuccessfully attempting to selectively protect the amine moiety in presence of the hydroxyl group, we chose to protect the two alcohols to enhance the solubility of Biopterin and introduce another protecting group on the amine.

Silyl ethers are known to selectively protect alcohols in presence of other functional groups, so we started our investigation using tert-butyl-dimethyl-silyl chloride (TBDMSCl) instead of other less substituted silyl ethers to obtain a more lipophile compound.

The reaction, conducted in DMF using 2.5 equivalents of silyl chloride and 5 equivalents of imidazole, after 16h showed the presence of unreacted Biopterin by 1H-NMR analysis of the suspension.

After the addition of a further excess of reagents, 1.25 equivalent of TBDMSCl and 2.5 of imidazole, the suspension turned into an orange solution, and then the reaction reached the completion after 2 more hours.

The reaction was quenched with water and the solid crystallised from toluene, yielding compound **28** as an orange crystalline solid.

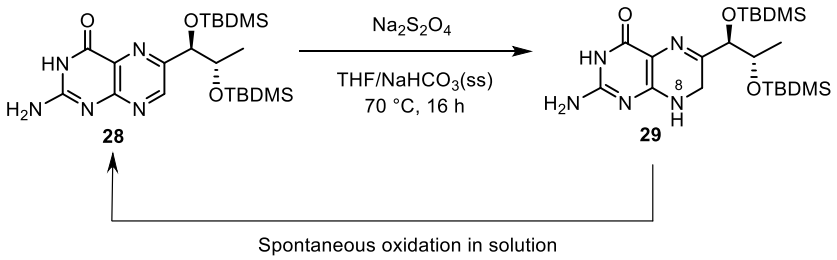


Scheme 18

The protection with silyl ether gave access to the solubilisation of compound **28** in solvents like THF, MeOH and EtOH.

For the reduction step of the pyrazine ring, we ran the reaction in a biphasic system using THF as solvent for compound **28**, and sodium dithionite ($Na_2S_2O_4$) as reducing agent added in an oversaturated solution of $NaHCO_3$.

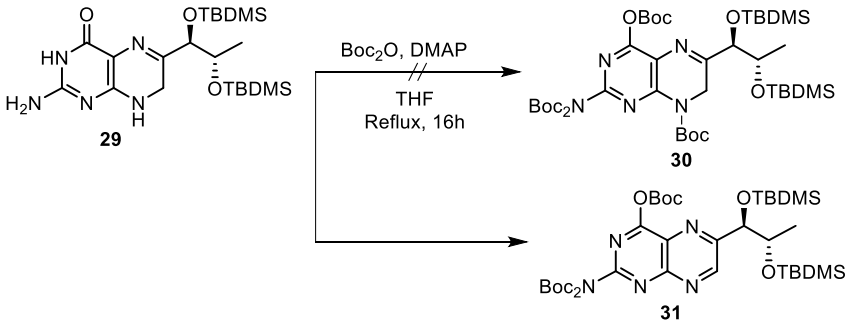
As we expected from the stability study we did before, after concentration of the organic phase at vacuum, the analysis of the solid at ^{1}H-NMR showed only the presence of the desired product (**29**). The observation that an air-exposed product (**29**) solution rapidly converts back to the starting material (**28**) over-time, pointed out that, in order to prevent the re-oxidation, protection of N8 is necessary.



Scheme 19

We attempted the protection of all the amine groups of compound (**29**) using an excess of Boc_2O, but unfortunately after 16h at reflux temperature and excess of the anhydride, we didn't observe the formation of the carbamate at N8.

The isolated product, analysed via ^{1}H-NMR, has the proposed structure of compound **31**. Because of an unexpected poor nucleophilicity of N8, we observed that after the protection of the primary amine and oxygen moiety of compound **29**, the molecule rapidly undergoes spontaneous re-oxidation in the reaction matrix.



Scheme 20

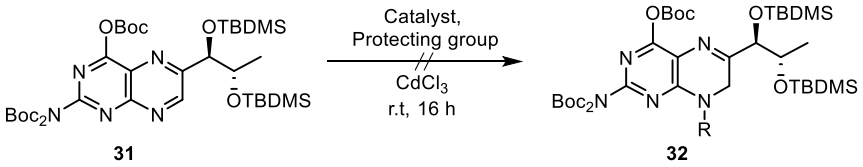
Despite this setback, we then decided to use the alternative product obtained from the last reaction as starting material for different reduction and protection experiments.

After the optimization of amine protection using 6 equivalents of Boc_2O and 0.6 of DMAP at reflux temperature in THF for 16h, compound **31** was isolated as a yellowish solid after crystallization from a mixture of Acetone/water with a yield of 75%.

2.3.4 Catalytic reduction and protection in one step

Our first attempt was the one-pot reduction and protection using catalysts such as Ruthenium, Rhodium and Platinum as reducing agents with the protecting group already charged in the reaction media.

We selected electrophiles like BnBr, chloroformate or CDI/EtOH as protecting group because we assumed that the reason of the unsuccessful protection of N8 lies in its poor nucleophilicity caused by the presence of other electron-rich nitrogen.



31 32

Scheme 21

Entry	Catalyst	Protecting Group	Yield
1	Ru/Al_2O_3	BnBr	-
2	Rh/C	CDI/EtOH	-
3	Pt/C reduced	CDI/EtOH	-
4	Pt/C unreduced	BnBr	-
5	Pt/C unreduced	BnBr/TBAI	-
6	Pt/C unreduced	Ethyl Chloroformate	-
7	Pt/C unreduced	Vinyl Chloroformate	-
8	Pt/C unreduced	Benzyl Chloroformate	-
9	Pt/C unreduced	CDI/EtOH	-

Table 4

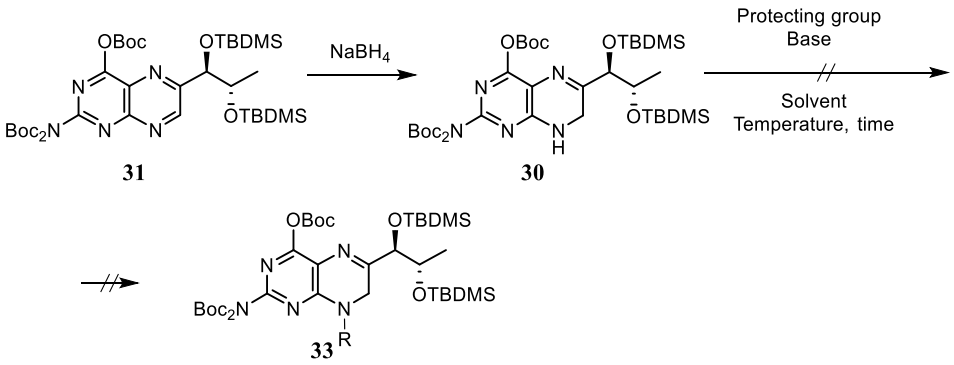
As shown in **Table 4**, only starting compound **31** was recovered with ruthenium or rhodium. Conversely, when the hydrogenation was conducted with platinum on carbon as in entries **4** to **9,** we initially observed the disappearance of biopterin and the formation of semi-reduced pyrazine ring in the monitoring of the ongoing reaction.

However, after the reaction was observed to be complete, the catalyst filtered off and the solvent removed under vacuum, ^{1}H-NMR of the isolated product showed the re-oxidated compound **31.**

In these experiments we have tested several electrophiles as protecting group but the desired product was never obtained. For this reason, we attempted the protection reaction separately from the reduction step in order to confirm that the problem lies in the poor reactivity of the amine when in the semi-reduced state.

2.3.5 Reduction with sodium borohydride and protection in different conditions

As described in patent EP 2 848 619 it's also possible to partially reduce Biopterin using $NaBH_4$, so we attempted the reaction in the conditions described in the literature yielding only compound **30** that was used directly for the protection step (scheme 22)



Scheme 22

Entry	Protecting Group	Base	Solvent	T (°C)	T (h)
1	AcCl (1. 2eq)	$NaHCO_3$ ss	DCM/H_2O	t.a	16
2	AcCl (10 eq)	NaOH 30%	DCM/H_2O	t.a	16
3	BnBr (1.2 eq)	NaH 80%	Dioxane	t.a	16
4	BnBr (1.2 eq)	t-BuOK	?	t.a	16
5	BnBr (1 eq)	Esil lithium (0.8 eq)	DMSO	t.a	16
6	BnBr (1 eq)	Esil lithium (0.8 eq)	THF	-10	16
7	BnBr (2 eq)	Esil lithium (1 eq)	THF	-10	16
8	BnBr (2 eq)	Esil lithium (1 eq)	THF	-10	16

9	-	Esil lithium 2 (0.8 eq)	THF	-10	16
10	BnBr (1.2 eq)	LDA (1.1 eq)	THF	-10	16

Table 5

In entry **1** and **2**, we attempted the protection in Schotten-Baumann conditions, using DCM as organic solvent for solubilization of compound **30** and alkaline water in order to remove the boron salt of the reduction step and for amine deprotonation for its protection as acetyl amide.

Unfortunately, in these conditions it wasn't possible to obtain the desired product, as the basic conditions deprotected the primary amine and therefore, after concentration of the solution, only oxidated species were recovered.

For entry **3** and **4** we attempt deprotonation of N8 with stronger bases such as NaH and t-BuOK but again we recovered compound **31**.

In the next experiment we choose esil-lithium in DMSO and BnBr as alkylating agent, with the objective to reproduce conditions similar to those described in **figure 12**, where a similar pteridine was protected.[50]

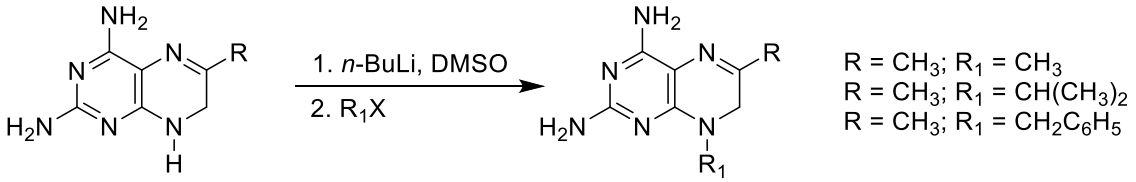


Figure 12

The reason for the use of DMSO is not only for the solubilization of the pteridine, but mainly to generate the methylsulfinyl carbanion through reaction with esil-lithium which, in this case, acts as nucleophile for N8 deprotonation, allowing alkylation by benzyl bromide.

In our case, the analysis of the crude of the reaction by ^{1}H-NMR showed only the presence of the benzylic CH_2 of benzyl bromide, together with compound **31** and an unidentified subproduct, so we investigated the use of esil-lithium in different conditions.

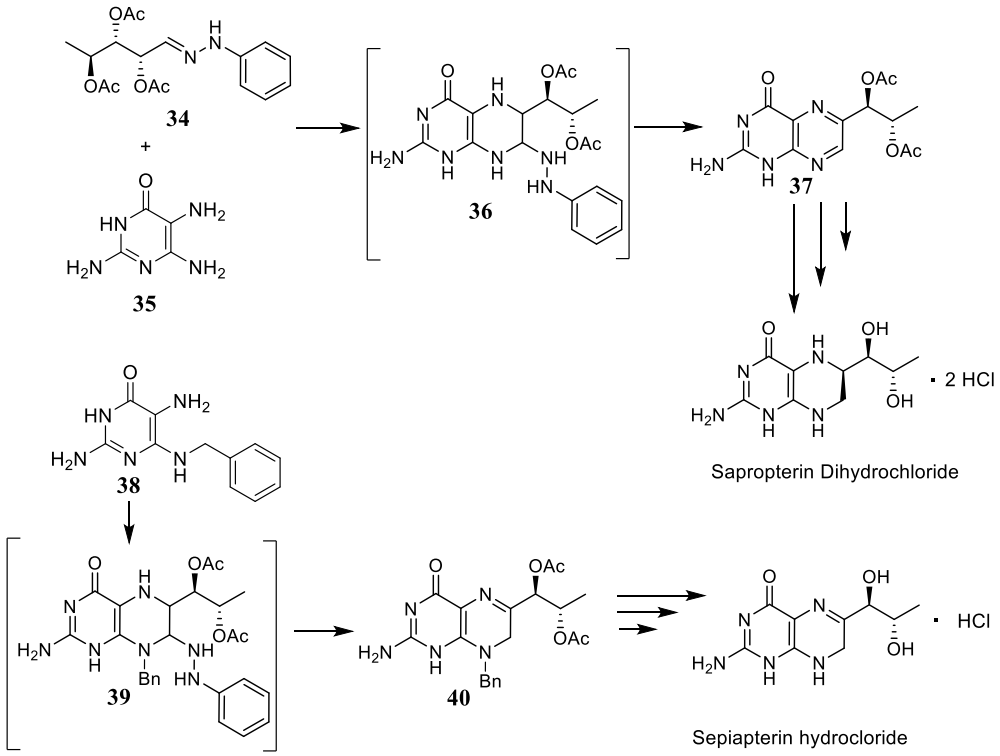
Firstly, we used THF as solvent and ran the reaction at low temperature in order to understand if the problem was the competition between the nucleophile generated in DMSO with the amine, but the result was the same.

In entry **7** and **8** we first increased the equivalents of BnBr used and subsequently changed the order of the addition of the base and electrophile, obtaining similar NMR spectra as in entry **5** and **6**.

We then ran the reaction only with base to better understand if the side product formed were independent from BnBr presence, and as we suspected the result was the same, confirming that esil-lithium was not compatible with the silyl moiety of compound **30**.

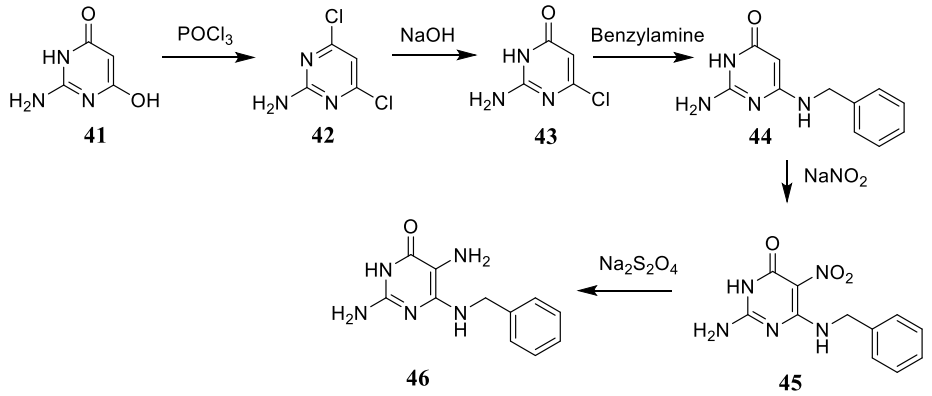
2.3.6 Ring synthesis with N8 already protected as benzylamine

Because it was not possible to protect N8 due to its poor reactivity, we started from an intermediate already used in Dipharma laboratories for Sapropterin hydrochloride synthesis as described in scheme 22, but in our case, our aim was to introduce a benzyl moiety at N8 before the formation of the pyrazine ring in order to synthesize compound **40**.



Scheme 23

Because compound **38** was not commercially available, we synthetized it from 2-amino-4,6-hydroxy pyrimidine.

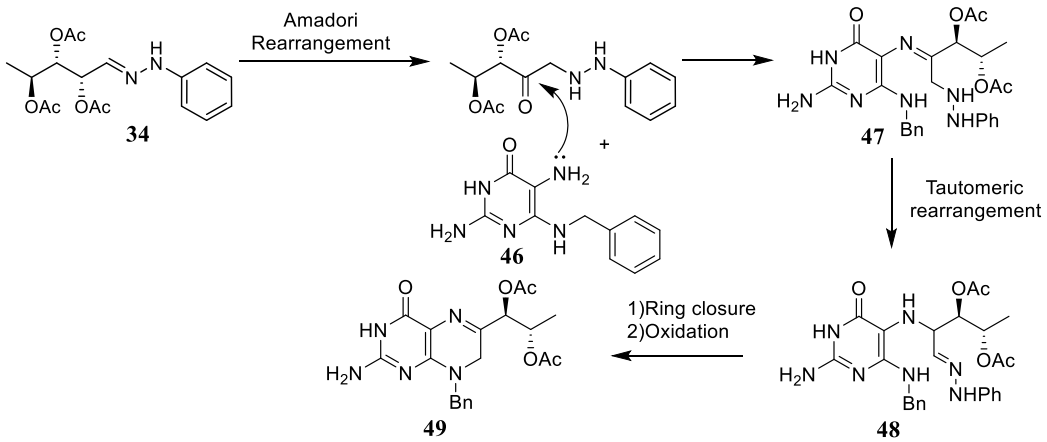


Scheme 24

To introduce the benzylic moiety, the first step was the chlorination of hydroxyl groups using $POCl_3$, forming compound **42** with a quantitative yield, which was subsequently subjected to hydroxylation in position 2 by refluxing the suspension in a 1M solution of NaOH.

Compound **44** was obtained by reaction with benzylamine in n-butanol at reflux temperature and isolated as a yellow precipitate that was immediately used for nitration with sodium nitrite ($NaNO_2$) and reduced to amine in one step with sodium dithionite ($Na_2S_2O_4$), with the formation of compound **46** as a yellow precipitate.

The next step should have been the pyrazine ring synthesis by Amadori rearrangement to α-amino ketone of compound **34** with the formation of compound **47**, followed by tautomeric rearrangement with ring closure and oxidation with a peroxide to yield compound **49**.

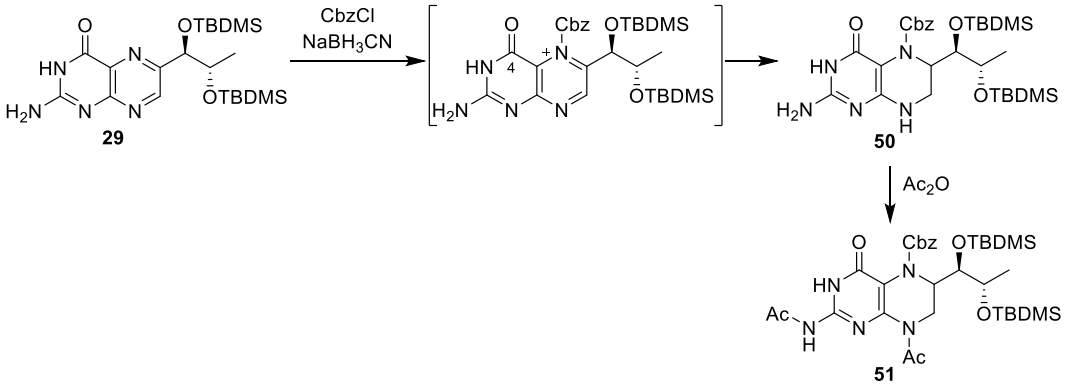


Scheme 25

In our case, however, the analysis of the crude by NMR and HPLC-MS didn't show the formation of compound **49** or the starting materials, which were probably degradated.

2.3.7 Protection/reduction of compound 29 and Acetylation of N8

Our last attempt for the synthesis of Sepiapterin was through the synthesis of compound **51**.



Scheme 26

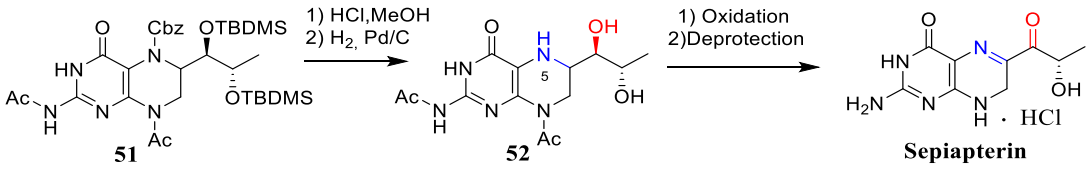
We managed to selectively introduce Cbz at N5 through the formation of an ammonium quaternary salt followed by complete reduction of the pyrazine ring with $NaBH_3CN$ without further acylation at N8 position.

It seems that, due to the stabilization of carbonyl group in 4' position, the protection at N5 is favoured (scheme 26) while no side reaction was observed, such as di-alkylated pteridine or mono protected derivative at N8 position, even when the equivalents of Cbz-Cl were increased.

For the next step, we acetylated compound **50** with Ac_2O in order to have different protecting groups that could be cleaved selectively from the others.

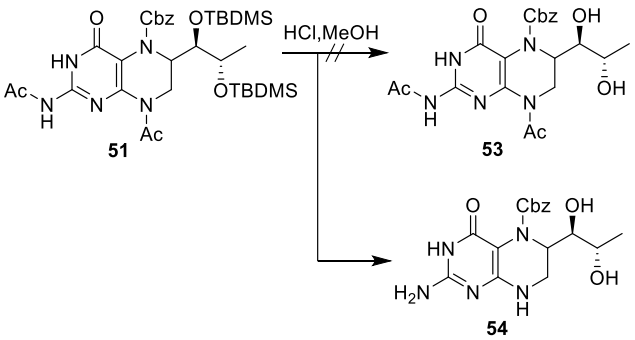
In this case we obtained compound **51**, confirming our supposition that N8 of the pteridine is poorly reactive in the semi reduced state (BH2), while in BH4 form the N8 protection is possible.

As described in **scheme 27** to obtain Sepiapterin our last strategy was to subject compound **51** to selective deprotection of TBDMS through acid cleavage followed by reductive deprotection of Cbz to oxidize the N5 and alcohol to compound **52** and subsequently deprotect the acetyl groups to yield Sepiaptein.



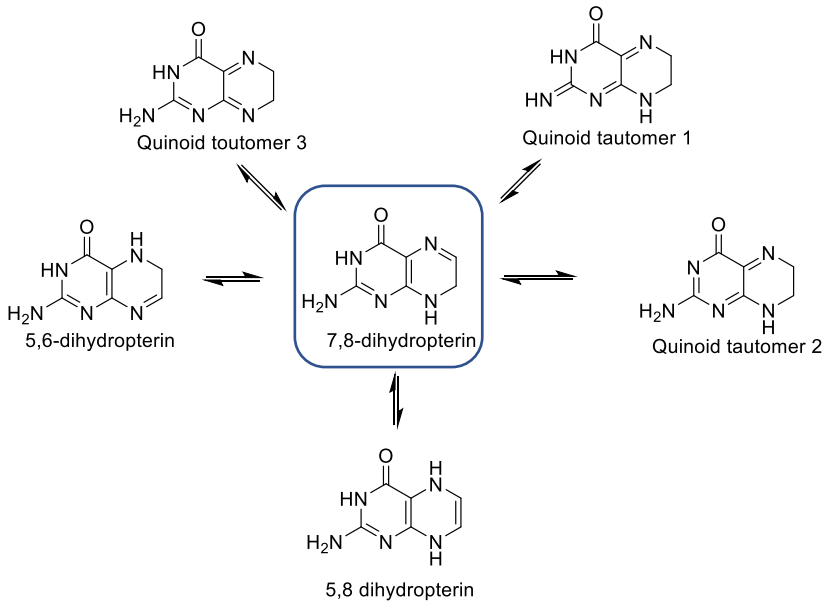
Scheme 27

Despite our expectations, the acid deprotection with 5M HCl in MeOH also cleaved the two acetyl amide moieties (**scheme 28**), suggesting that other strategies to selectively remove TBDMS or alternative alcohol protection should be evaluated.



Scheme 28

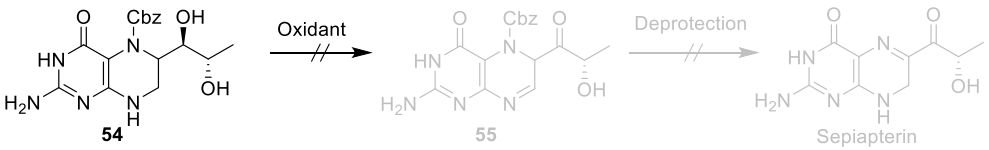
In literature, several tautomers of pterins are described and is reported that, with the exception of high substituted derivatives, they will rearrange thermodynamically to the most stable one: 7,8-dihydropterinine (Scheme 29).[51]



Scheme 29 Tautomers of dihydropterin

With this information we tried to synthesize 5,6 dihydropterin of compound **54** with the idea to rearrange to Sepiapterin after Cbz deprotection (scheme 30).

We test different oxidant such as: H_2O_2, peroxybenzoic acid and IBX, unfortunately, we couldn't oxidize **54** to **55**, confirming the low reactivity of the amine at 8' position.



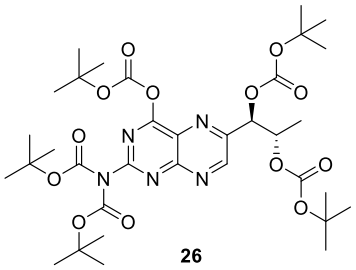
Scheme 29

2.4 Experimental part

All reagents were used as purchased from commercial suppliers without further purification. The solvents used were purified according to Perrin et al.29 Merck aluminium backed plates pre-coated with silica gel 60 (UV254) were used for analytical thin layer chromatography and were visualized by exposure to the UV lamp (254 nm) and/or staining with a solution of $KMnO_4$ or a solution of ninhydrin in EtOH. Flash chromatographic separations were performed using methodologies and instruments described by W. C. Still et al.30 using solid phase silica gel 60 (Merck, 230-400 mesh). Proton nuclear magnetic resonance 1H NMR (400 MHz) and 13C NMR (100 MHz) spectra were recorded on NMR spectrometer. Chemical shift values (δ) are reported in parts per million (ppm) of tetramethylsilane (TMS) used as internal standard. Data are represented as follows: chemical shift, multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet and/or multiple resonances, bs=broad singlet), coupling constant (J) in Hertz and the integration. Mass spectroscopy data of the products were collected on LC/MS ESI mass spectrometer. LC/MS conditions: ES ionization after passage through a C-18, 35 x 5 mm, 3 µ column, elution: mixture A (99.9% H_2O, 0.1% HCOOH); mixture B (99.9% CH_3CN, 0.1% HCOOH): 0-20 min, 20-60% mixture B; 20-25 min 60% mixture B; 25-30 min 60-20% mixture B, flow 0.6 mL/min, at rt.

2.4.1 Procedure

Synthesis of [2-[bis(tert-butoxycarbonyl)amino]-6-[(1R,2S)-1,2-bis(tert-butoxycarbonyloxy)propyl]pteridin-4-yl] tert-butyl carbonate



26

In a three necked round flask of 500 mL equipped with a thermometer, a condenser and a dropping funnel, Biopterin (10 g, 42.16 mmol, 1 eq) and DMAP (4.12 g, 33.73 mmol, 0.8 eq) were suspended in 150 mL of THF and a solution of BOC_2O (94.38 g, 632.48 mmol, 15 eq) in 100 mL of THF was slowly added and refluxed for 16 h.

After the end of the reaction monitored with TLC (Tol/EtOAc 9:1), the solution was concentrated under vacuum and the resulting oil was dissolved in 100 mL of Toluene and washed with 0.1 M of of HCl (3x50 mL) using separatory funnel.

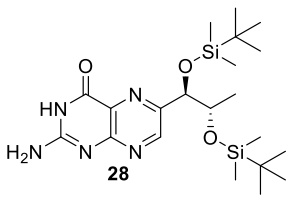
The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum.

The residue was purified with a medium pressure chromatographic system Sepacore® Buchi (eluent: Tol/EtOAc with an elution gradient ranging from 100% Tol to a mixture Tol/EtOAc 8:2 in 10 minutes).

Compound **26** was isolated as a brown oil (23.33 g, 31.62 mmol, 75%)

^{1}H NMR (300 MHz, $CDCl_3$) δ 9.02 (s, 1H), 6.44 (d, *J* = 4.0 Hz, 1H), 5.74 (qd, *J* = 5.8, 4.0 Hz, 1H), 1.54 – 1.42 (m, 45H).

Synthesis of 2-amino-6-[(1R,2S)-1,2-bis[[tert-butyl(dimethyl)silyl]oxy]propyl]-3H-pteridin-4-one



28

In a three necked round flask of 250 mL equipped with a thermometer, a condenser and a dropping funnel, compound **26** (10 g, 42.16 mmol, 1 eq), imidazole (21.53 g, 316.2 mmol, 7.5 eq) were suspended in 100 mL of DMF and a solution of TBDMSCl (23.83 g, 158.1 mmol, 3.75 eq) in 25 mL of DMF was slowly added and stirred under nitrogen atmosphere for 16 h.

After the end of the reaction monitored with TLC (DCM/EtOAc 5:5), 150 mL of H_2O was slowly added to the solution with the formation of a sticky orange precipitate which was filtered and washed with 50 mL of H_2O.

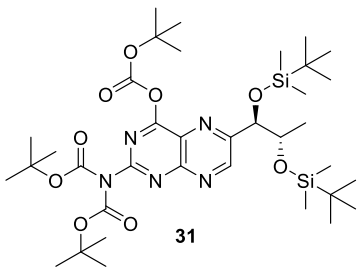
The solid was recrystallized from Toluene affording compound **28** as an orange crystalline solid (18 g, 38.64 mmol, 85%)

^{1}H NMR (300 MHz, DMSO-d_6) δ (s, 1H), 8.68 (s, 1H), 7.50 (s, 1H), 6.94 (s, 2H), 4.58 (d, *J* = 9 Hz, 1H), 4.13 – 4.00 (m, 1H), 1.10 (d, *J* = 6 Hz, 3H), 0.82 (s, 9H), 0.70 (s, 9H), 0.04 (s, 3H), -0.04 (s, 3H), -0.17 (s, 3H), -0.21 (s, 3H).

^{13}C NMR (150 MHz, DMSO-d_6) δ 162.15, 154.05, 151.98, 150.38, 149.44, 122.59, 74.90, 72.52, 25.66, 25.66, 25.66, 25.66, 25.66, 25.66, 18.59, 18.59, 17.92, -3.09, -3.09, -3.09, -3.09.

[ES/MS]: 467 $[M+H]^+$, 489 $[M+Na]^+$

Synthesis [2-[bis(tert-butoxycarbonyl)amino]-6-[(1R,2S)-1,2-bis[[tert-butyl(dimethyl)silyl]oxy]propyl]pteridin-4-yl] tert-butyl carbonate



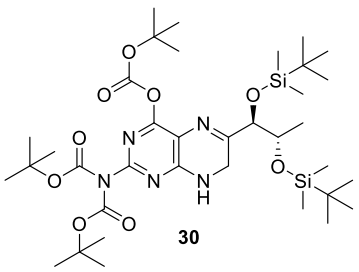
31

In a three necked round flask of 500 mL equipped with a thermometer, a condenser and a dropping funnel, Compound **28** (10 g, 21.47 mmol, 1 eq) and DMAP (1.6 g, 12.88 mmol, 0.6 eq) were suspended in 100 mL of THF and a solution of BOC_2O (28.11 g, 128.8 mmol, 6 eq) in 100 mL of THF was slowly added and refluxed for 16 h.
After the end of the reaction monitored with TLC (Tol/EtOAc 9:1), the solution was concentrated under vacuum and the residue was crystallized in Acetone/H2O 8:2 affording compound **31** as a white crystalline solid (12.3 g, 16.05 mmol, 75%)

^{1}H NMR (300 MHz, $CDCl_3$) δ 9.21 (s, 1H), 4.83 (d, *J* = 6.0 Hz, 1H), 4.20 – 4.02 (m, 1H), 1.75 (s, 9H), 1.46 (s, 18H), 1.20 (d, *J* = 6 Hz, 3H), 0.90 (s, 9H), 0.75 (s, 9H), 0.11 (s, 3H), -0.02 (s, 3H), -0.10 (s, 4H), -0.22 (s, 3H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 167.86, 154.96, 152.27, 151.49, 151.49, 150.24, 149.13, 148.08, 137.02, 81.86, 80.96, 80.96, 74.71, 72.52, 28.41, 28.41, 28.41, 28.41, 28.41, 28.41, 28.41, 28.41, 28.41, 25.66, 25.66, 25.66, 25.66, 25.66, 25.66, 18.59, 18.59, 17.92, -3.09, -3.09, -3.09, -3.09.

Synthesis of [2-[bis(tert-butoxycarbonyl)amino]-6-[(1R,2S)-1,2-bis[[tert-butyl(dimethyl)silyl]oxy]propyl]-7,8-dihydropteridin-4-yl] tert-butyl carbonate



30

In a three necked round flask of 250 mL equipped with a thermometer, a condenser and a dropping funnel, under nitrogen atmosphere Compound **31** (10 g, 13.05 mmol, 1 eq) was suspended in 100 mL of THF and refluxed until complete solubilization.

A solution of $Na_2S_2O_4$ (22.7 g, 130.5 mmol, 10 eq) in 100 mL of an oversaturated solution of $NaHCO_3$ was slowly added to the reaction environment and heated until pH of the solution fall off to 6.

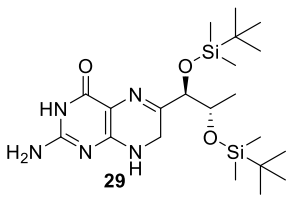
The organic phase was separated and the aqueous phase was extracted with DCM (3x100 mL).

All the collected organic phases were dried over anhydrous Na_2SO_4, filtered and dried under vacuum yielding compound **30** as a brown solid (9.5 g, 12.37 mmol, 95%)

^{1}H NMR (300 MHz, DMSO-d_6) δ 4.16 - 3.97 (m, 3H), 3.94 – 3.87 (m, 1H), 1.47 (s, 9H), 1.38 (s, 18H), 1.12 (d, *J* = 6 Hz, 3H), 0.85 (s, 9H), 0.79 (s, 9H), 0.07 – 0.00 (m, 12H)

[ES/MS]: 769 $[M+H]^+$, 791 $[M+Na]^+$

Synthesis of 2-amino-6-[(1R,2S)-1,2-bis[[tert-butyl(dimethyl)silyl]oxy]propyl]-7,8-dihydro-3H-pteridin-4-one



29

In a three necked round flask of 500 mL equipped with a thermometer, a condenser and a dropping funnel, under nitrogen atmosphere Compound **28** (10 g, 21.47 mmol, 1 eq) was suspended in 100 mL of THF and refluxed until complete solubilization.

A solution of $Na_2S_2O_4$ (37.38 g, 214.7 mmol, 10 eq) in 100 mL of an oversaturated solution of $NaHCO_3$ was slowly added to the reaction environment and heated until pH of the solution falls off to 6.

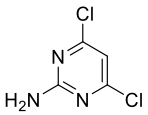
The organic phase was separated and the aqueous phase was extracted with THF (3x100 mL).

All the collected organic phases were dried over anhydrous Na_2SO_4, filtered and dried under vacuum yielding compound **29** as an orange solid (9 g, 19.24 mmol, 90%)

^{1}H NMR (300 MHz, dmso) δ 4.56 (d, *J* = 5.3 Hz, 1H), 4.08 – 3.96 (m, 1H), 3.98 (d, *J* = 3, 2H) 1.04 (d, *J* = 6.2 Hz, 3H), 0.84 (s, 9H), 0.80 (s, 9H), 0.04 – -0.01 (m, 12H).

[ES/MS]: 468 $[M+H]^+$, 490 $[M+Na]^+$

Synthesis of 4,6-dichloropyrimidin-2-amine



42

In a three necked round flask of mL equipped with a thermometer, a condenser and a dropping funnel, under nitrogen atmosphere, 2-amno-4,6-dihydroxy-pirimidine (5 g, 39.33 mmol, 1eq) was suspended in a solution of $POCl_3$ (7.24 g, 47.22 mmol, 3 eq) and heated at 69 °C.

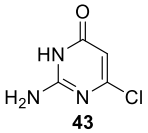
After 15 min DIPEA (5.09 g, 39.35 mmol, 2.5 eq), was slowly added using a dropping funnel and stirred under nitrogen atmosphere for 16 h.

Excess of $POCl_3$ was quenched by the careful addition of 25 mL of H_2O, being sure not to allow the internal temperature to exceed 60 °C. The resultant yellow powder was collected via vacuum filtration and washed with 30 mL of H_2O to afford 5.5 g (85%) of compound **42**.

^1H NMR (400 MHz, DMSO-d_6) δ 7.61 (br s, 2H), 6.87 (s, 1H)

^{13}C NMR (100 MHz, DMSO-d_6) δ 162.9, 161.0, 107.6

Synthesis of 2-amino-4-chloro-1H-pyrimidin-6-one



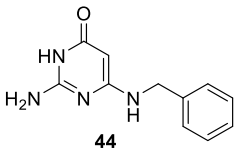
43

In a three necked round flask of mL equipped with a thermometer a condenser and a dropping funnel, a suspension of **42** (5.0 g, 30.5 mmol) in 75 mL of 1 M NaOH was heated to reflux. After the solution became homogeneous, was allowed to cool to rt and adjusted to pH 4 via addition of glacial acetic acid. The resultant, white precipitate was collected via vacuum filtration, washed with 100 mL of H_2O on the filter paper and dried in vacuo to afford 4.12 g (93%) of **43**.

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.14 (br s, 1H), 6.99 (br s, 2H), 5.59 (s, 1H)

^{13}C NMR (100 MHz, DMSO-d_6) δ 162.3, 159.5, 155.5, 99.4

Synthesis of 2-amino-4-(benzylamino)-1H-pyrimidin-6-one



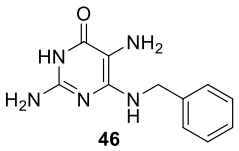
44

To a suspension of 4.0 g (27.48 mmol) of **43** in 40 mL of n-butanol was added TEA (5.56 g, 54.96 mmol, 2 eq) and benzylamine (4.71 g, 43.97 mmol, 1.6 eq). The suspension was heated to 120 °C for 16 h.

The resulting solution was allowed to cool at room temperature and washed with 3 × 20 mL portions of water in a separatory funnel. The organic layers were combined, dried over Na_2SO_4, filtered and concentrated in vacuo. The resultant yellow solid was suspended in 15 mL of n-pentane and vacuum filtered to afford compound **44**, 5.10 g (86%) as a white powder.

^{1}H NMR (300 MHz, Dmso-d_6) δ 7.44 – 7.12 (m, 5H), 6.91 (s, 1H), 6.14 (s, 2H), 4.39 (s, 1H), 4.28 (d, 2H).

Synthesis of 2,5-diamino-4-(benzylamino)-1H-pyrimidin-6-one



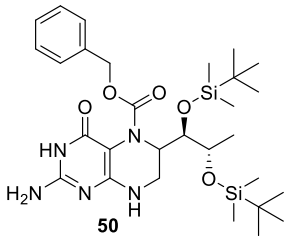
46

In a three necked round flask of 100 mL equipped with a thermometer a condenser and a dropping funnel a solution of $NaNO_2$ (4.88 g, 70.76 mmol, 3 eq) in water (15 mL) was added to a mixture of compound **44** (5.1 g, 23.58 mmol, 1 eq), glacial acetic acid (15 mL), and water (70 mL). The mixture was stirred at room temperature for 2 h, and the resulting red suspension was then filtrated under vacuum and washed with water (30 mL). The solid was then suspended in water (100 mL) and heated at 60 °C. Sodium dithionite (7.5 g) was added to this hot stirring mixture in small portions over a period of 1 h until the reaction turn yellow.

After cooling to room temperature, the suspension was filtrated under vacuum to yield compound **46** as a yellow solid (4.6g, 85%).

^{1}H NMR (300 MHz, DMSO-d_6) δ 7.29 (s, 5H), 6.07 (t, *J* = 6.2 Hz, 1H), 5.84 (s, 2H), 4.49 (d, J = 6, 2H).

Synthesis of benzyl 2-amino-6-[(1R,2S)-1,2-bis[[tert-butyl(dimethyl)silyl]oxy]propyl]-4-oxo-3,6,7,8-tetrahydropteridine-5-carboxylate



50

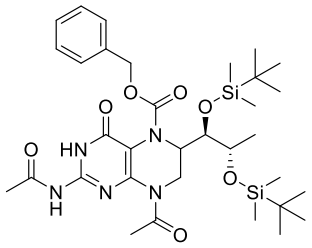
In a round necked flask, under nitrogen atmosphere, Benzyl chloroformate (16.15 g, 94.66 mmol, 3.8 eq), was added to a suspension of compound **28** (11.6 g, 24.91 mmol, 1 eq) in methanol (300 mL). After 0.5 h, $NaBH_3CN$ (6.26 g, 99.64 mmol, 4 eq) was added, and the suspension stirred for 16 h.

The solvent was evaporated and the residue washed with hexane (3 x 50 mL), filtered under vacuum yielding compound **50** as an orange solid (14.7 g, 98%).

^{1}H NMR (300 MHz, DMSO-d_6) δ 7.38 (d, *J* = 25.6, 5.8 Hz, 5H), 6.98 (s, 1H), 6.24 (s, 2H), 5.10 (s, 2H), 4.02-3.96 (m, 2H), 3.55-3.49 (m, *J* = 12.4, 3.0 Hz, 2H), 3.10 (dd, *J* = 12.6, 4.3 Hz, 1H), 1.07 (d, *J* = 6.0 Hz, 3H), 0.91 (s, 9H), 0.83 (s, 9H), 0.13 (s, 3H), 0.08 (s, 3H), 0.01 (s, 3H), -0.05 (s, 3H).

[ES/MS]: 605 $[M+H]^+$, 627 $[M+Na]^+$

Synthesis of benzyl 2-acetamido-8-acetyl-6-[(1R,2S)-1,2-bis[[tert-butyl(dimethyl)silyl]oxy]propyl]-4-oxo-6,7-dihydro-3H-pteridine-5-carboxylate



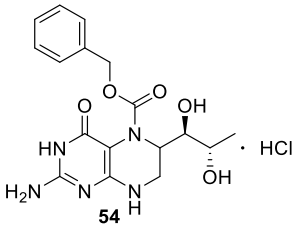
51

In a two necked round flask, under nitrogen atmosphere, compound **50** (1 g, 1.656 mmol, 1 eq) was suspended in Ac_2O (30 mL) and refluxed for 3h.
After the reaction was considered finished by HPLC, it was dried under vacuum and and the residue was solubilized in hot IPA (15 mL) and stirred for 1 h.
After was cooled to room temperature, the yellow precipitate filtered and dried to yield compound **51** as a yellow solid (0.790 mg, 70%)

^{1}H NMR (300 MHz, DMSO-d_6) δ 7.34 (s, 5H), 5.09 (s, 2H), 4.32 (d, *J* = 13.6 Hz, 1H), 3.98-3.92 (m, 1H), 3.51 (dd, *J* = 13.4, 5.0 Hz, 1H), 3.42 (d, *J* = 9.1 Hz, 2H), 2.57 (s, 3H), 2.12 (s, 3H), 1.04 (d, *J* = 2.7 Hz, 3H), 0.84 (s, 9H), 0.76 (s, 9H), 0.03 (s, 3H), -0.04 (s, 3H), -0.10 (s, 6H).

[ES/MS]: 689 $[M+H]^+$, 711 $[M+Na]^+$

Synthesis of benzyl 2-amino-6-((1R,2S)-1,2-dihydroxypropyl)-4-oxo-4,6,7,8-tetrahydropteridine-5(3H)-carboxylate



54

In a bottom round necked flask of 100 mL, compound **51** (500 mg, 0.73) was suspended in IPA (10 mL) of HCl 37% (2 mL) for 16 h at room temperature.
The precipitate was filtered and dried under vacuum, yielding compound **54** as a white-off precipitate (150 mg, 60%).

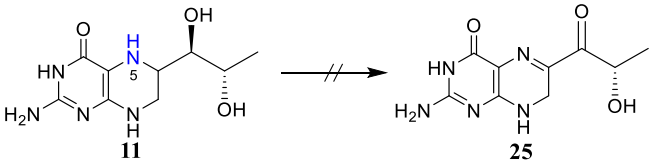
^{1}H NMR (300 MHz, D_2O) δ 7.31 (s, 5H), 5.10 (s, 2H), 4.16 (dd, *J* = 10.5, 2.9, 0.5 Hz, 1H), 3.73 (qd, *J* = 6.5, 2.7 Hz, 1H), 3.64 (d, *J* = 13.1 Hz, 1H), 3.39 (dd, *J* = 10.3, 2.5 Hz, 1H), 3.21 (dd, *J* = 13.3, 4.5 Hz, 1H), 1.05 (d, *J* = 6.5 Hz, 3H).

[ES/MS]: 376 $[M+H]^+$, 398 $[M+Na]^+$

2.5 Conclusion

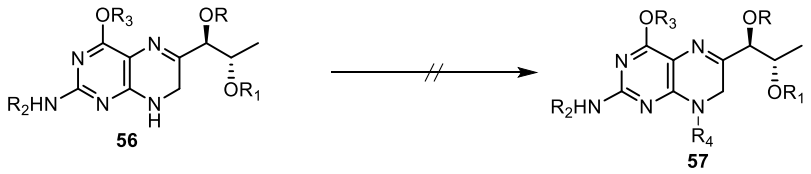
Based on the result obtained during my internship in Dipharma laboratories, we could understand that it's not possible to:

- Oxidize directly compound **11** to **25**



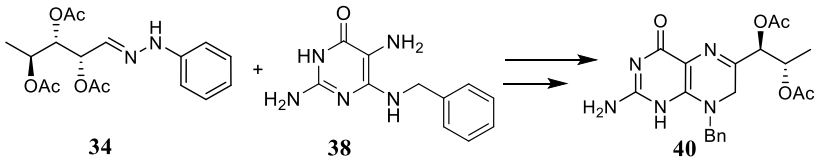
Scheme 30

- Protect amine at 8' position of the protected derivative **56**



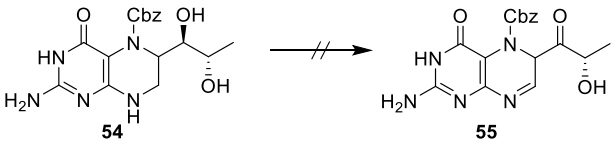
Scheme 31

- Perform Viscontini reaction with compound **38** to form compound **40**



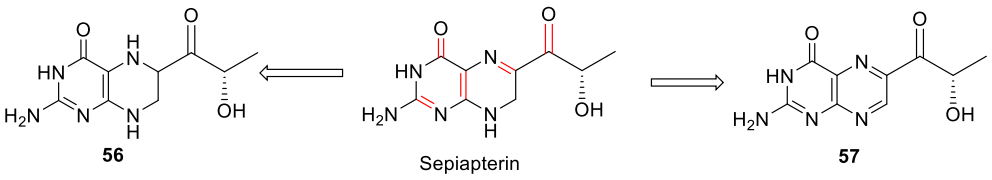
Scheme 32

- Oxidize compound 54 to 55 in order to obtain Sepiapterin for tautomeric re-arrangement



Scheme 33

It seems that the ketone moiety stabilizes the 5,6 imine for conjugation effect and allow the oxidation of compound **56** or reduction of compound **57** to Sepiapterin (scheme 34) and could explain why it wasn't possible its synthesis in any alternative ways before the formation of the ketone.



Scheme 34

In conclusion, to synthesize Sepiapterin without infringing its current patent, alternative oxidant or reductant should be evaluated on compounds **56** and **57**, in order to preserve its semi-reduced state and prevent the spontaneous re-oxidation.

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