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Ph.D. Thesis in Chemical Sciences
XXIX Course

**Synthesis and characterization of new modified metabolites,
molecules with strong pharmacological activities**

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Summary

Polyphenols are the most widely distributed class of plant secondary metabolites and several thousand different compounds have been identified. They play many different roles in plant biology and human life, as UV protective agents, defensive compounds against herbivores and pathogens, they contribute to plant colors and to the taste of food and drink. Moreover it is widely known that natural metabolites have played a crucial role in the identification of many of the drugs that are on the market today. Nowadays natural products used pure in pharmacological preparations represent only 5% of the total, but their semi-synthesis derivatives represent more than 50% of the drugs in use.¹

My PhD project is focused on the chemistry of natural substances, and I developed some strategies to synthesize new silibinin conjugates. Silibinin is the major biologically active component of the seeds extract of the milk thistle (*Silybum marianum*) also known as silymarin.² Structurally, silibinin is a diastereoisomeric mixture of two flavonolignans, silybin A and silybin B in a ratio of approximately 1:1 (Figure 1).³

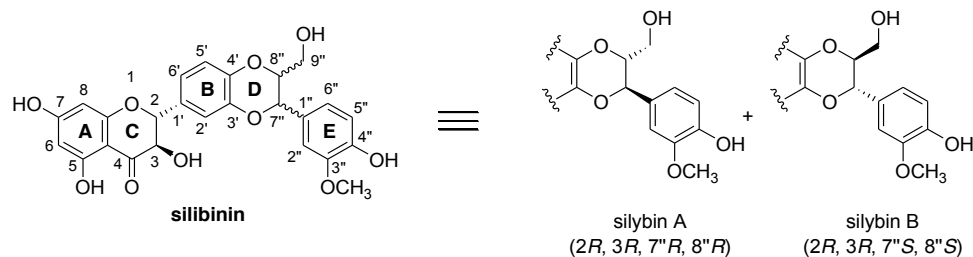


Figure 1

Silibinin is a metabolite with multiple biological activities operating at various cell levels.⁴ Unfortunately, its therapeutic efficiency is rather limited by its low bioavailability due to its very low water solubility.

Aiming to improve the solubility, during the first year of my PhD project, we have developed an efficient synthetic procedure to obtain new 9''-phosphodiester silibinin conjugates with different mono- and di-saccharide labels through the anomeric hydroxyl group (Figure 2).⁵

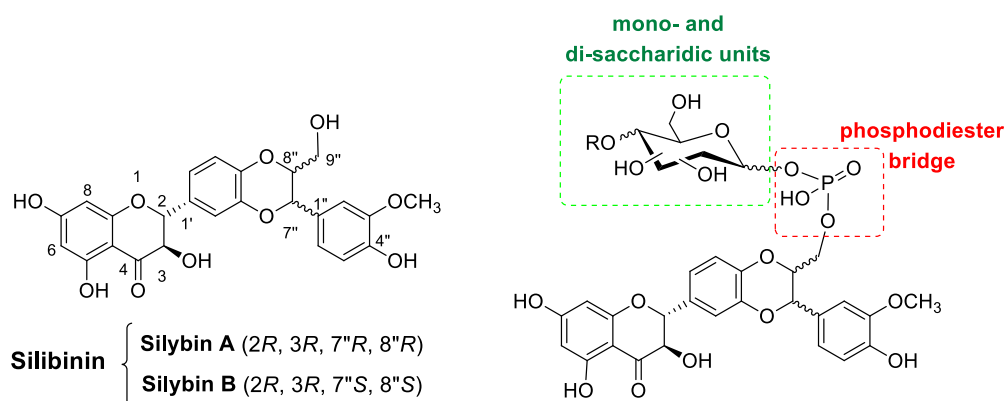


Figure 2

In our approach 9''-phosphoramidite has been used as silibinin substrate and 1-OH full protected mono- and disaccharide derivatives as sugar starting materials. We initially converted full acetylated mono and di-saccharides into 1-OH derivatives and these compounds were coupled with 9''-phosphoramidite silibinin. The oxidation and the deprotection treatments led to the desired phosphodiester derivatives in good yields. The crude materials were then subjected to the purification by reverse phase analysis (RP-18 HPLC), using a variety of columns and elution conditions, but unfortunately it was too difficult to purify the mixture of diastereoisomers. In the end new silibinin analogues were obtained as a mixture of diastereoisomers, observed by ^{31}P NMR analysis. The NMR analysis has proved to be very complex, in fact ^1H and ^{31}P NMR spectra of all compounds showed a dramatic complexity, due to the presence of a lot of diastereoisomers. This drawback has not allowed a complete and detailed NMR characterization of the new derivatives. The structures were confirmed by ^{31}P NMR and ESI-MS mass spectra signals. In preliminary study, new derivatives were subjected to DPPH free radical scavenging and Xanthine Oxidase inhibition assays to evaluate their antioxidant activities. Independently of the sugar moiety present, all compounds exhibited a radical scavenging activities slightly higher than that of the silibinin, and Xanthine Oxidase inhibition at least as that of the silibinin. On the other hand the new derivatives showed a water solubility well above that of silibinin, in fact it was possible to prepare solutions of about 70 mg/mL in water.

These two data encouraged our studies during my second year of PhD to improve this synthetic strategy and to realize libraries of optically pure glyco-conjugated silibinins.

The 9''-phosphoramidite has been used as silibinin substrate and fully protected 6-OH mono- and di-saccharide derivatives as sugar starting materials (Figure 3).⁶

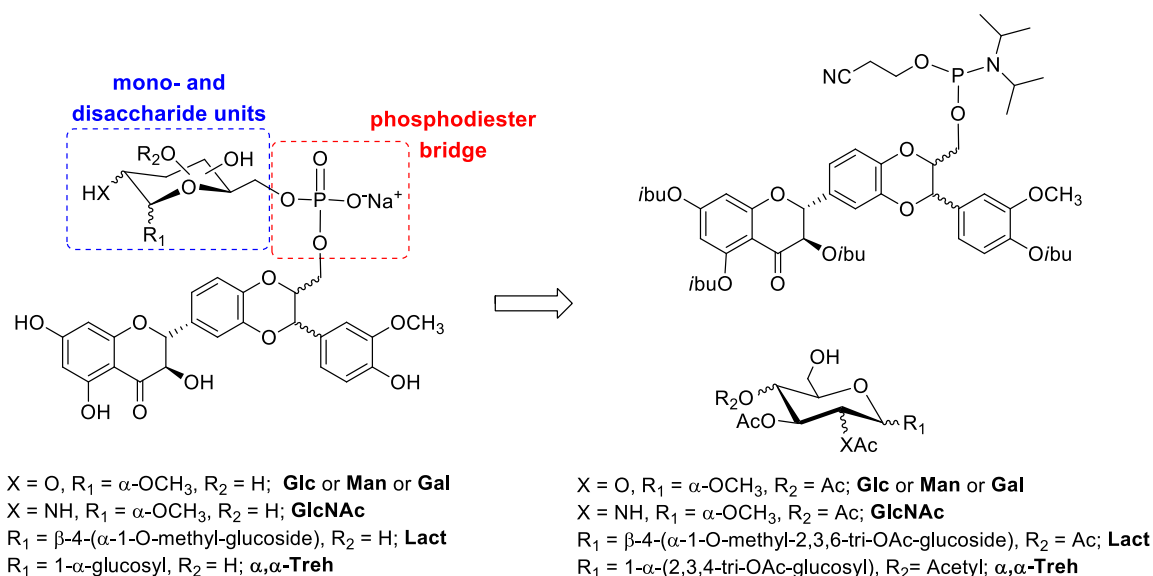


Figure 3

We initially converted opportunely protected mono- and di-saccharides (Glucose, Mannose, Galactose, N- Acetylglucosamine, Trehalose and Lactose) into 6-OH derivatives and then they were coupled with 9'-phosphoramidite silibinin following typical phosphoramidite chemistry procedure. The crude materials were purified by reverse phase chromatography (RP-18 HPLC) and characterized by NMR and MALDI- TOF/TOF-MS analyses. All compounds were obtained in good yields and as a mixture of two silibinin diastereoisomers (A and B). Finally new phosphodiester derivatives were converted into the corresponding sodium salt by cation exchange resin carrying crystalline samples. New derivatives showed water solubility well above that of silibinin, with the possibility to prepare solutions of about 70 mg/mL in water. The stability of new glyco-conjugates was investigated in human serum by HPLC analyses and the 50% disappearance of the peak corresponding to the intact glyco-conjugate was observed after ca. 40-68 hours. All derivatives were subjected to DPPH free radical scavenging assay and they exhibited radical scavenging activities slightly higher than that of the silibinin. In order to verify the potential biological properties of these derivatives, a biological assay was used to evaluate the cytotoxicity of the new glyco-conjugates, compared with that of the silibinin, on human liver cancer cell line (Hep G2). No significant changes in the viability of treated cells were observed when they were subjected to the action of glyco-conjugates, also considering the longest incubation (72 hours) at the maximum dose taken (30 μM). Also, in order to investigate the role of the different OH groups in the antioxidant activity of new glyco-conjugates in comparison with silibinin, their acidity and redox capacity have been evaluated. A redox-deep characterization was carried out in collaboration with Prof. Mauro Iuliano and Dr Gaetano De Tommaso of our Department, using potentiometric and voltammetric techniques. The determination of the acidity constants was conducted by

potentiometric titrations measuring the concentration of hydrogen ions with a glass electrode. By processing data, it was possible to define three equilibrium constants. Analysis of the data shows that the conjugation in position 9" does not affect the redox behaviour of the silibinin scaffold.

As a part of our continuing research effort towards the synthesis of new natural product analogues exploiting the phosphoramidite chemistry, in the last year of my PhD project the attention was focused on the development of synthetic methods to obtain oligoflavonoids based on silibinin, and to investigate their anti-radical activity. Exploiting the selective protection to hydroxyl groups of the silibinin we have developed an efficient strategy for the synthesis of new 3-9", 3-3 and 9"-9" dimers of silibinin in good yields (Figure 4).⁷

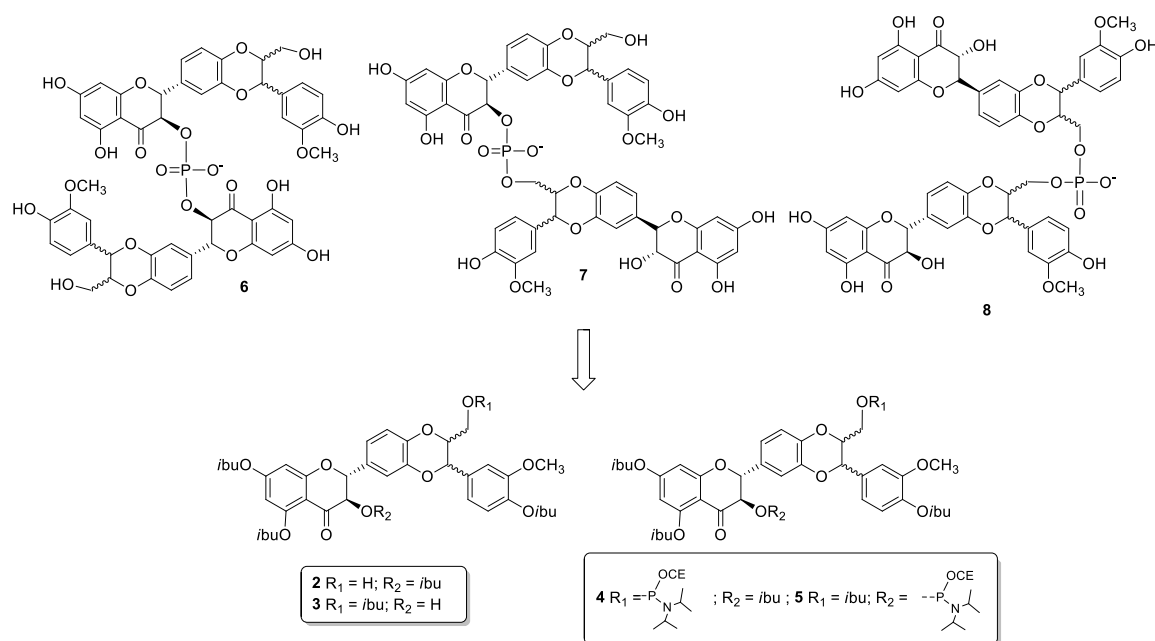


Figure 4

In order to obtain suitable building blocks for the dimers synthesis, we have developed a selective protective reaction for the different hydroxyl groups with isobutyric anhydride. The building blocks obtained were coupled in the three different provisions with 3- and 9"-silibinin phosphoramidite using the well known phosphoramidite chemistry. After oxidation, deprotection and RP-18 HPLC purification the products were converted into the corresponding sodium salts by cation exchange on a DOWEX (Na^+ form) resin, leading to the desired phosphodiester dimers derivatives in good yields. The structures of new analogues were confirmed by ^{31}P NMR and MALDI-TOF/TOF-MS analyses. The stability of new silibinin dimers was investigated in human serum by HPLC analysis and the 50% disappearance of the peak corresponding to the intact molecule ($t_{1/2}$) was observed after ca. 80 hours. All derivatives were subjected to DPPH free radical scavenging assay and they exhibited activities quite higher than silibinin.

Moreover, the solubility in water of silibinin and its dimers as well as their ability to react with

reactive oxygen species (ROS) were determined by estimating their second order rate constant with singlet oxygen ($^1\text{O}_2$) and hydroxyl radical ($\text{HO}\cdot$) in solution. This data were obtained in the laboratories of Prof. Marcello Brigante during my experience work in his research team at the University Blaise Pascal, Institute of Chemistry of Clermont-Ferrand (France). Solubility experiments indicate that dimers were completely dissolved (0.1 mg in 10 mL) in water and solubility can be estimated to be $\geq 19.5 \mu\text{M}$ corresponding to $> 20 \text{ mg/L}$.

Reactivity between silibinin and dimers with $^1\text{O}_2$ and $\text{HO}\cdot$ was determined by use of Rose Bengal (RB) and hydrogen peroxide as respective ROS sources. For this purpose, 545 nm centered excitation of RB and laser flash photolysis (LFP) experiments were coupled with a kinetic competition approach. Dimers reactivity toward singlet oxygen results to be close to the value determined for silibinin, or about 35% lower. Second order rate constant are in the same order of magnitude reported in literature for molecules with similar structure. Morales and co-workers⁸ reported a reactivity ranging from 2.4 to $13.4 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for flavonoid derivative such as quercetin and morin. Estimation of second order rate constant reactivity with $\text{HO}\cdot$ indicates that some dimers showed a second order rate constant $\geq 1.5 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$. Wang and co-workers⁹ investigated the reactivity of hydroxyl radical with phenolic compounds in order to estimate their anti-oxidative ability using aqueous pulse radiolysis. The value of $1.5 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$ was found for quercetin that is close to those estimates for green tea polyphenols. Interestingly, Husain et al.¹⁰ reported that reactivity of flavonoids toward photo-generated hydroxyl radical increases with the number of hydroxyl groups in the aromatic ring.

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Chapter 1: Introduction

1.1 Natural products and secondary metabolism

For million years, humankind is completely dependent on plants as source of carbohydrates, proteins and fats for food and shelter. In addition, plants are a valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavours, fragrances, colours, biopesticides and food additives.

The secondary metabolites, as the name suggests, are so-called because they are produced by the plant secondary metabolism. Primary metabolism refers to the processes producing the carboxylic acids of the Krebs cycle, α -amino acids, carbohydrates, fats, proteins and nucleic acids, all essential for the survival and well-being of the organism.¹ All organisms possess the same metabolic pathways by which these compounds are synthesized and utilized. Secondary metabolites, on the other hand, are non-essential to life but contribute to the species fitness for survival.¹ Secondary metabolites are also produced using other metabolic pathways than primary metabolites. These pathways are more characteristic for the particular family or genus and are related to the mechanism of evolution of species. In fact, the specific constituents in a certain species have been used to help with systematic determination, groups of secondary metabolites being used as markers for botanical classification.¹

The division between primary and secondary metabolism is not clear, but the two types are linked together because primary metabolism provides the small molecules that are the starting materials of the secondary metabolic pathways (Figure 1.1).

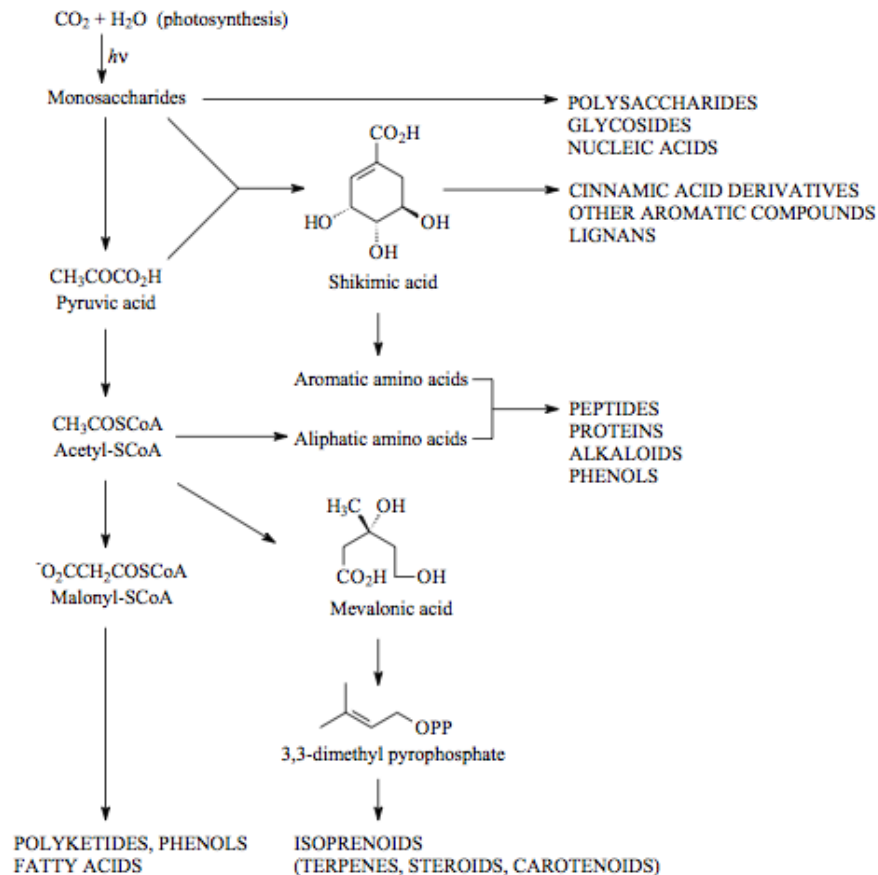


Figure 1.1 Primary metabolites and their links to secondary metabolism.

One of the most important families of secondary metabolites is that of *polyphenols*. Polyphenols are the most widely distributed class of plant secondary metabolites and several thousand different compounds have been identified. They play many different roles in plant biology and human life, including UV protection, defense against herbivores and pathogens, and they contribute to plant colors, taste of food and drink, and are used as pharmaceuticals.

1.2 Plant polyphenols: molecules with a high degree of molecular diversity

“Eating five servings of fruits and vegetables per day”

This is what is highly recommended and heavily advertised nowadays to the general public to stay fit and healthy. *“Drinking green tea on a regular basis”*, *“eating chocolate from time to time”*, as well as *“savoring a couple of glasses of red wine per day”* have been claimed to increase life expectancy even further. Why? The answer is in fact still under scientific scrutiny, but a particular class of compounds,

naturally occurring in fruits and vegetables, is considered to be crucial for the expression of such human health benefits: the *polyphenols*.

1.2.1 A bit of history

Before being called polyphenols, these plant-derived natural products were globally referred to as “vegetable tannins” as a consequence of the use of various plant extracts containing them in the conversion of animal skins into leather. The first mentions of vegetable tanning in the classical literature are accredited to the founder of the science of botany, Theophrastus of Eressus (371–286 BC), in his *Historia Plantarum* encyclopedia and the origins of this leather-making process get lost in the depths of the most ancient records of the history of human civilizations.²

Over the centuries, “vegetable tannins” have never ceased to garner general (and commercial) interest, as well as scientific curiosity,³ and from the beginning of the last century an increasingly numerous of chemists got involved in this *affair*, mainly concerned with finding an accurate method for analyzing tanning extracts used in the leather industry. This was indeed a valuable and quite honorable objective, but far from trivial given the means of chemical analysis available at the time. Fortunately, over the years, botanists, plant physiologists, phytochemists and biochemists, as well as a few obstinate organic chemists, kept on studying polyphenols and underlying their significance not only as major and ubiquitous plant secondary metabolites, but also as compounds that express properties with numerous implications and potential exploitations in various domains of general public and commercial interests. So polyphenols gradually became a topic of intensive investigation in various plant-related scientific domains, including applied research areas such as agriculture, ecology, food science and nutrition, as well as medicine.^{4,5} The development of more and more advanced analytical techniques that paralleled this gradual expansion of interest in polyphenol research during the second half of the past century clearly had a major positive impact on both the development of the field and its appreciation by the scientific community at large.

Nowadays, plant polyphenols enjoy an ever-increasing recognition not only by the scientific community but also, and most remarkably, by the general public because of their presence and abundance in fruits, seeds, vegetables and derived foodstuffs and beverages, whose regular consumption has been claimed to be beneficial for human health. It is their capacity to scavenge oxidatively generated free radicals, such as those derived from lipids and nucleic acids, that has often been highlighted as the fundamental chemical event that underlies their utility in reducing the risk of

certain age-related degenerations and diseases. Although this so-called antioxidation property is not listed among the qualifying factors that make a plant phenolic a “true” polyphenol, it has become the trademark of “polyphenols” in recent exploitations by the agro-food, cosmetic, and parapharmaceutic industries. However, antioxidation is not a property limited to polyphenols, as numerous simple plant phenols are strong antioxidants, with many of them being in fact used as the active principles present in some industrial formulations. The use of the term “plant phenols” by industry would definitely be more appropriate, but the term “polyphenols” is preferred for commercial communications. As in the case of earlier confusions surrounding the use of the term “tannins” in the scientific literature, the term “polyphenols” has been and is still often misused by scientists from industry as well as academia.

1.2.2 What are plant polyphenols really?

According to a common classification, the polyphenols are divided into several sub-classes, based on the number of phenolic rings present in their structure, the structural elements that bind these rings between them and to the substituents bound on the rings. On this basis, they can be identified two major groups: **flavonoids** and **non-flavonoids** polyphenol.⁶

- The **flavonoids** (Figure 1.2), share a structure formed by two aromatic rings, indicated as A and B, linked together by three carbon atoms that form an oxygenated heterocycle, the ring C. They can be further divided into several subclasses, depending on the type of involved heterocycle : flavones, flavonols, flavanones, flavanonols, flavanols or flavan-3-ols or catechins, anthocyanins and anthocyanidins, isoflavones, neoflavonoids, chalcones.

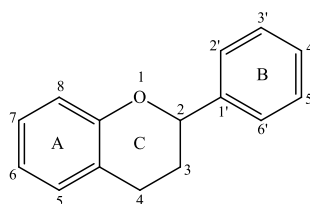


Figure 1.2 Basic structure of flavonoids.

- Among the **non-flavonoid polyphenols** are identified: simple phenol, phenolic acids, benzoic aldehydes, hydrolysable tannins, acetophenones and phenylacetic acid, hydroxycinnamic acids, coumarin, benzophenones, xanthenes, stilbene, lignans, secoiridoids.

The above assortment of structure types is admittedly far from providing a clear picture of the family of plant polyphenols. Of course, the presence of more than one hydroxy group on a benzene ring or other arene ring does not make them polyphenolic. Catechol, resorcinol, pyrogallol, and phloroglucinol (all di- and trihydroxylated benzene derivatives) are still defined as “phenols” according to the IUPAC official nomenclature rules of chemical compounds⁷ but many such plant-derived monophenolics (Figure 1.3) are often quoted as “polyphenols”, not only in cosmetic, parapharmaceutic, or nutraceutic commercial advertisements, but also in the scientific literature, which has succumbed to today’s fashionable use of the term.

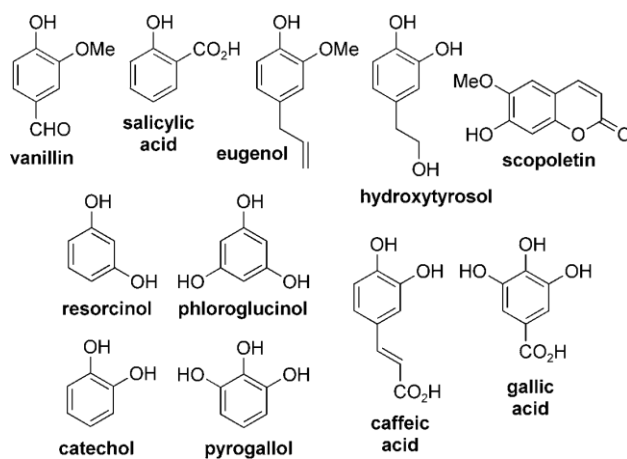


Figure 1.3 Examples of simple plant-derived “monophenolics”.

The meaning of the chemical term “phenol” includes both the arene ring and its hydroxy substituent(s). Hence, even if we agree to include polyphenolic compounds with no tanning action in a definition, the term “polyphenol” should be restricted in a strict chemical sense to structures bearing at least two phenolic moieties, irrespective of the number of hydroxy groups they each bear. However, as judiciously pointed out earlier by Jeffrey B. Harborne,⁸ such a purely chemically based definition of (poly)phenols needs additional restrictions, since many natural products of various biosynthetic origins contain more than one phenolic unit. The existence of such alkaloids still gives us another problem when attempting to define plant polyphenols in an as simple and yet comprehensive manner as possible, since the tyrosine amino acid from which they are derived is itself a (primary) metabolite of the phenylpropanoid pathway. It isn’t totally surprising by the difficult qualification of these molecules, but

the interest that the (poly)phenols plant arouse in various scientific fields requires greater clarity. This raises the need to arrive at a revised definition of "real" plant polyphenols, purely chemical mold, referring to the biosynthetic process by which they are formed. This definition may be as follows: *the term "polyphenol" should be used to define plant secondary metabolites derived exclusively from the shikimate-derived phenylpropanoid and/or the polyketide pathway(s), featuring more than one phenolic ring and being devoid of any nitrogen-based functional group in their most basic structural expression.*

This definition leaves out all monophenolic structures, which include di- and trihydroxyphenyl variants, but contains three main categories of compounds, that can be identified as "true" polyphenols:

1. the **proanthocyanidins** (condensed tannins) such as procyanidins, prodelphinidins, and profisetinidins (Figure 1.4), which are derived from the oligomerization of flavan-3-ol units such as (epi)catechin, epigallocatechin, and fisetinidol (Figure 1.5).⁹

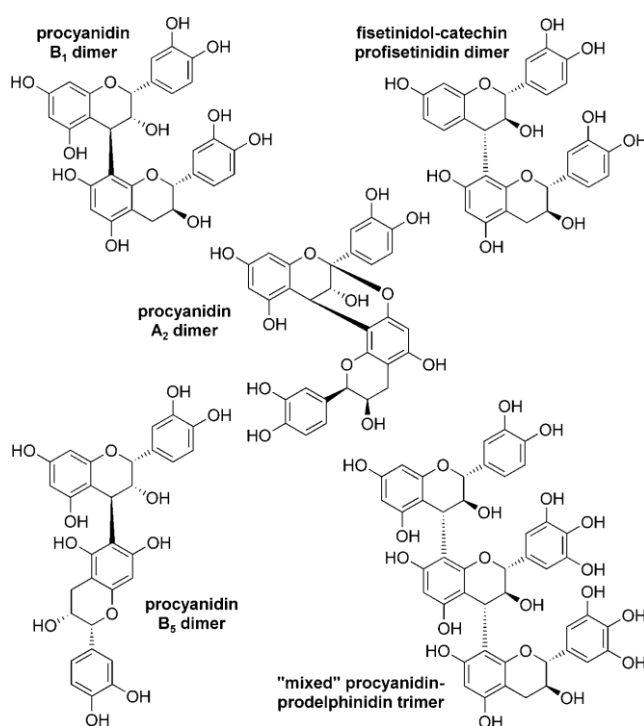


Figure 1.4 Representative examples of condensed tannins.

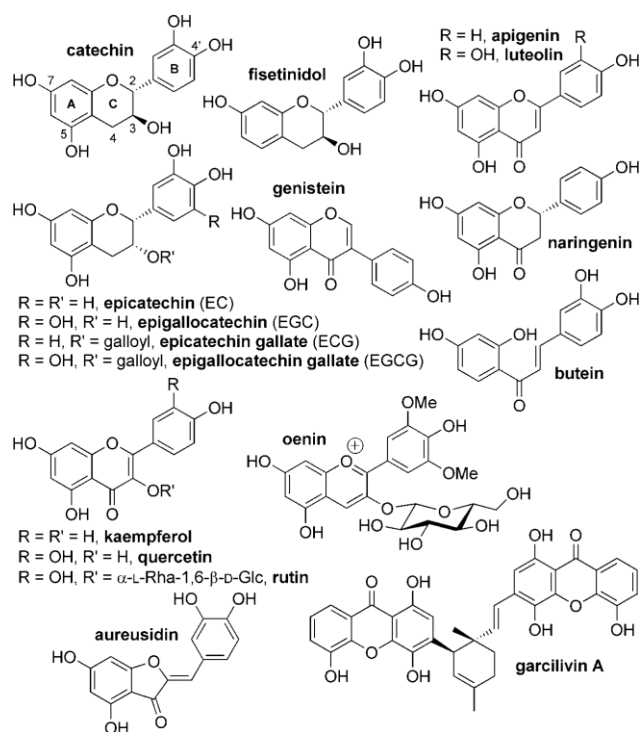


Figure 1.5 Representative examples of flava/flavonoids.

- the **gallo- and ellagitannins** (hydrolyzable tannins), which are derived from the metabolism of the shikimate-derived gallic acid (3,4,5-trihydroxybenzoic acid) that leads through esterification and phenolic oxidative coupling reactions to numerous (near 1000) monomeric and oligomeric polyphenolic galloyl ester derivatives of sugar-type polyols, mainly D-glucose (Figure 1.6),^{10,11}

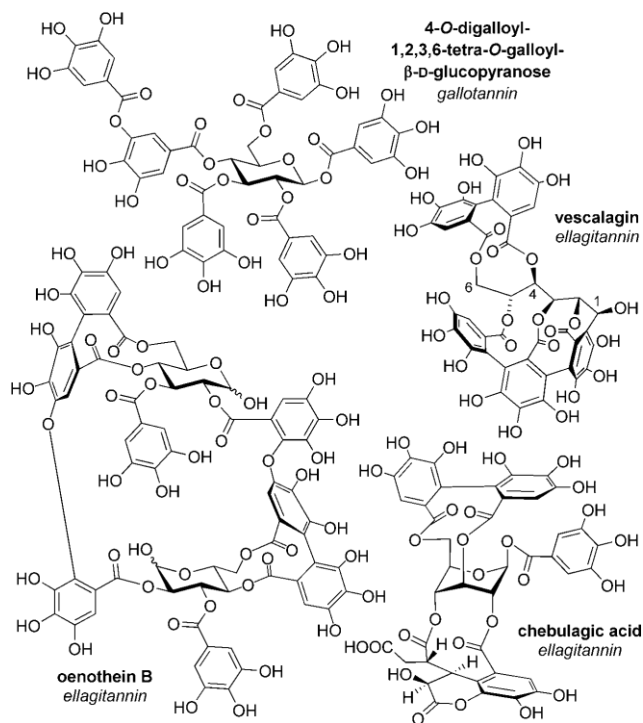


Figure 1.6. Representative examples of hydrolyzable tannins.

- the **phlorotannins** that are found in red-brown algae (Figure 1.7) and essentially derived from the oligomerizing dehydrogenative coupling of phloroglucinol (1,3,5-trihydroxybenzene).¹²

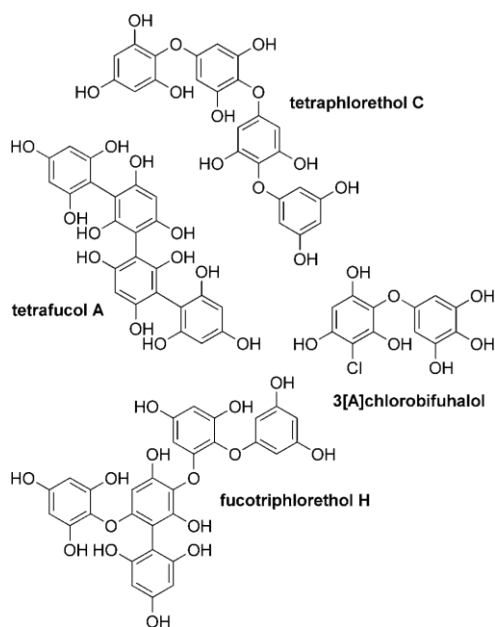
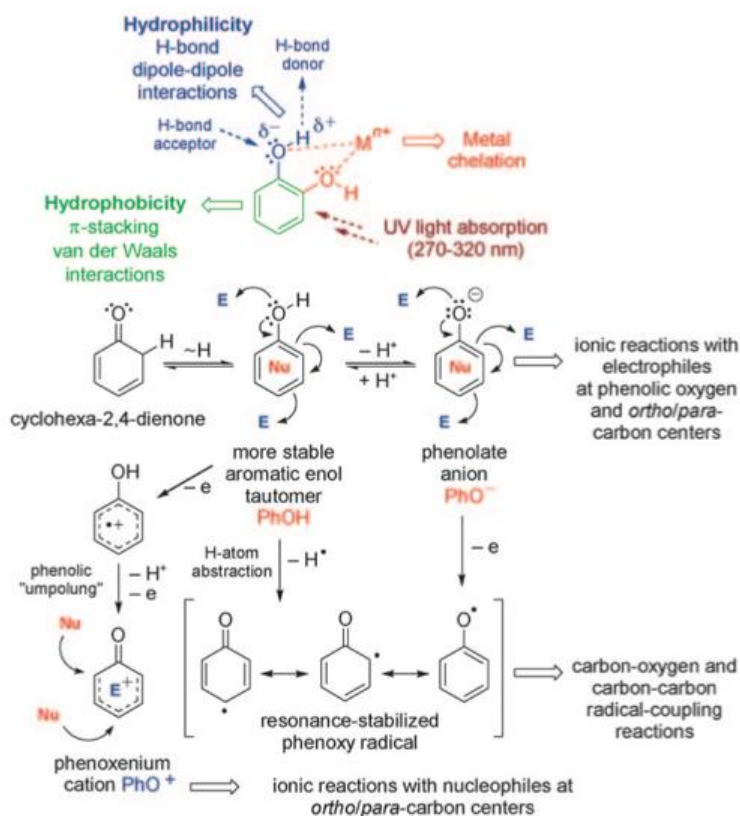


Figure 1.7 Representative examples of phlorotannins.

1.3 Polyphenols: focus on the antioxidant activity

There are numerous reasons to investigate plant polyphenols. From their most basic structural expressions to their elaboration into further chemically transformed and complex oligo/polymeric assemblies, plant polyphenols exhibit a remarkably diverse range of bio-physicochemical properties that makes them rather unique and intriguing natural products. The first question that comes to mind is why did plants choose to rely so heavily on the production of metabolites with multiple phenolic moieties. The answer to this question is still a subject of debate and speculation, and possibly differs for the different types of polyphenols.¹³ Generally speaking, plant polyphenols, as defined above, have been implicated in diverse functional roles, including plant resistance against microbial pathogens and animal herbivores such as insects (antibiotic and antifeeding actions), protection against solar radiation (screens against DNA-damaging UV-B light), which probably was a determining factor in early terrestrial plant evolution, as well as reproduction, nutrition, and growth, notably through interactions with other organisms above and below ground (insects, symbiotic fungi, and bacteria).¹³ Over the course of long-term evolution, as well as compulsory quick seasonal adjustments, plants have learnt to cope with changing environmental conditions and pressures by relying on the formidable chemical arsenal available to them through their remarkably dynamic secondary metabolisms, endless sources of structural diversity, and variation.¹³ Of course, among the main groups of secondary metabolites, others such as alkaloids and terpenoids have also demonstrated their value in protecting plants during their evolution, while contributing by chemical means to maintain a fair ecological balance between plants and other living organisms, many of which feeding on them, including humans. However, plant phenolics arguably deserve a special mention if we consider the wide-ranging benefits that they offer to plants and hence to other living organisms are essentially all a result of their inherent physicochemical properties bundled within the phenol functional group (Scheme 1.1).



Scheme 1.1 Basic physicochemical properties and reactivities of the phenol functional group. E = Electrophile, Nu = Nucleophile.

A tremendous increase in the number of scientific publications on “polyphenols” has appeared over the course of the last 20 years. Such reports include numerous epidemiological studies that have confirmed the potential value of these natural products for the prevention of age-related diseases. These studies show that polyphenols act as scavengers of free radicals and reactive oxygen species, which are overproduced under oxidative stress conditions and unable to be subdued by the regular action of endogenous cellular antioxidants such as glutathione (GSH), glutathione peroxidase, or superoxide dismutase, or by dietary antioxidant vitamins (for example, vitamins E and C, carotenoids).

It did not take long for the cosmetic industry to exploit polyphenols extracted from various plant parts, including diverse fruits, herbs, nuts, grape seeds, and tree barks, in their development of new lines of products that aimed to better protect the skin from damages caused by solar radiation and aging. The food industry did not stand still and initiated the development of functional foods or “nutraceuticals” based on the use of selected natural polyphenolic molecules as additives.¹⁴

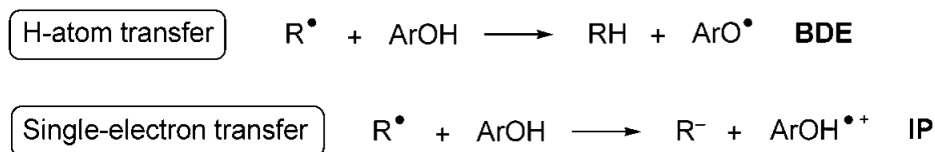
The most talked about characteristic of polyphenols, and plant phenolics in general, is without doubt

their acclaimed capability to scavenge reactive oxygen species (ROS), which include radical and non-radical oxygen species such as $O_2^{\cdot-}$, HO^{\cdot} , NO^{\cdot} , H_2O_2 , 1O_2 , $HOCl$, as well as oxidatively generated free radicals RO^{\cdot} and ROO^{\cdot} such as those derived from biomolecules such as low-density lipoproteins (LDLs),¹⁵ proteins, and oligonucleic acids (DNA and RNA).¹⁶ All these species can have deleterious effects on human health.^{17,18,19}

This so-called antioxidation ability is frequently cited to be the key property underlying the prevention and/or reduction of oxidative stress-related chronic diseases and age-related disorders such as cardiovascular diseases (for example, atherosclerosis), carcinogenesis, neurodegeneration (for example, Alzheimer's disease), as well as skin deterioration, by dietary plant (poly)phenolics and other plant polyphenol-containing commodities.

In view of the overwhelming emphasis that has been rightly or wrongly placed on plant polyphenols as “super” antioxidants, it's important to describe the fundamental aspects of the chemistry behind it. Plant (poly)phenolic compounds can act as antioxidants by chelating metal ions such as iron(II)/copper(I) and iron(III)/copper(II) ions that are involved in the conversion of $O_2^{\cdot-}$ and H_2O_2 into highly aggressive HO^{\cdot} through Haber-Weiss/Fenton-type reactions.^{20,21} They can also block the action of some enzymes responsible for the generation of $O_2^{\cdot-}$, such as xanthine oxidase and protein kinase C.²² However, it is through the direct quenching of radical ROS and/or free radicals in general that (poly)phenols appear to best exhibit their protective role. A synergistic antioxidant action through the regeneration of other potent antioxidants such as α -tocopherol (α -TOH; α -TO $^{\cdot}$ + ArOH \rightarrow α -TOH + ArO $^{\cdot}$) is another conceivable option that has also been examined.^{22,23}

Two main antioxidation mechanisms have been proposed.²⁴ The first is based on the aforementioned capacity of the phenol functional group to donate a hydrogen atom to a free radical R^{\cdot} , such as peroxy radicals LOO^{\cdot} generated during lipid (LH) autoxidation (peroxidation; LH \rightarrow L $^{\cdot}$, then L $^{\cdot}$ + $^3O_2 \rightarrow$ LOO^{\cdot}). In this case, the (poly)phenols act as chain-breaking antioxidants. Through this so-called hydrogen-atom transfer (HAT) mechanism, the phenolic antioxidant (ArOH) itself becomes a free radical (ArO $^{\cdot}$; Scheme 1.2).



Scheme 1.2 Hydrogen-atom transfer and single-electron transfer are the main mechanisms through which plant (poly)phenols express their radical-scavenging-based antioxidant action. The dissociation energy (BDE) and the ionization potential (IP) of the phenol are the two basic physicochemical parameters that can be used to determine the potential efficacy of each process, respectively.

The efficiency of the antioxidant action essentially relies on the rapidity of the H-atom transfer to LOO^\bullet ($\text{ArOH} + \text{LOO}^\bullet \rightarrow \text{ArO}^\bullet + \text{LOOH}$) and on the stability of the resulting phenoxy radical ArO^\bullet , which should neither react back with LOOH nor react with the substrate LH , hence terminating the propagating radical chain reaction ($\text{LOO}^\bullet + \text{LH} \rightarrow \text{LOOH} + \text{L}^\bullet$). The ease of formation and stability of ArO^\bullet is strongly dependent upon the structural features of the ArOH parent compound. The most important determining factors are the presence, number, and relative position of additional phenolic hydroxy groups, their implication in the formation of intramolecular hydrogen bonds,²⁵ and the conformationally dependent possibility of allowing electronic delocalization throughout the largest part of the molecule. All of these factors affect the dissociation energy of the phenolic O-H bond: the weaker the O-H bond, the easier the H-atom transfer will be.

The second mechanism is the single-electron transfer (SET) from ArOH to a free radical R^\bullet with formation of a stable radical cation $\text{ArOH}^{\bullet+}$ (Scheme 1.2). The ionization potential (IP) of ArOH is thus another important physico-chemical parameter for assessing the antioxidant efficacy of plant (poly)phenols: the lower the ionization potential, the easier the one- electron transfer is.

Numerous techniques have been developed to evaluate the antioxidant capacity of plant (poly)phenols, essentially all based on monitoring directly or indirectly the decay of radical species and on determining the rate constants for radical scavenging.^{15,22,26,27} For example, Jovanovic et al., then Bors and Michel, relied on pulse radiolysis to evaluate the reactivity of various polyphenols with HO^\bullet , $\text{O}_2^{\bullet-}$, and N_3^\bullet .^{28,29} Bors and Michel suggested that flavanols such as (epi)catechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate (EGCG), and oligomers thereof (proanthocyanidins) are better radical scavengers than many monomeric flavones and even flavonols. The reason for this is their (multiple) expression of catecholic and pyrogallolic moieties as privileged radical-scavenging sites. The increasing rates of reactions with the highly reactive HO^\bullet species ($t_{1/2} \approx 10^{-9}$ s) nicely

correlated with the number of phenolic units bearing adjacent hydroxy groups.²⁸ Extensive structure–activity relationship studies have been carried out on large numbers of plant polyphenols by relying on numerous antioxidation activity assays, notably based on the ability of an antioxidant to scavenge the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS⁺) in comparison to that of the water-soluble vitamin E analogue Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) or to scavenge the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH[•]). Determining the ability of an antioxidant to inhibit copper(II) -or 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH)-induced LDL peroxidation is another option. All of the assays unveiled more or less the same trends.^{15,21,26}

1.4 Polyphenol-protein interactions: pharmacological properties of natural polyphenols

For a long time, the biological activities of plant polyphenols in plant, as well as in humans, have arguably been attributed to their capacity to exert antioxidant actions (as discussed above) and/or to their propensity to form precipitating complexes with proteins in a rather nonspecific manner.^{31,32} Today, there is compelling evidence that strongly suggests that the mechanisms by which plant polyphenols exert their protective actions against cardiovascular and neurodegenerative diseases, as well as cancer and diabetes, are not simply due to their redox properties, but rather to their ability to directly bind to target proteins (or peptides). Such a mode of action would induce the inhibition of key enzymes, the modulation of cell receptors or transcription factors, as well as the perturbation of protein (or peptide) aggregates, which can regulate cell functions related to, for example, growth and proliferation, inflammation, apoptosis, angiogenesis, metastasis, and immune responses, in various ways by affecting signal transduction pathways.^{33,34,35,36} Numerous reports describe the significant inhibition of various enzymes by various polyphenols.^{33,34,37,38} Among the most therapeutically relevant enzymes are inflammatory ones such as COXs and LOXs, CYPs, signal transduction kinases (generally inhibited more strongly by simple flavonoids, ellagitannins, and ellagic acid than by gallotannins and condensed tannins³⁹), xanthine oxidase, NADH-oxidase, thioredoxin reductase,⁴⁰ adenosine deaminase, matrix metalloproteinases,⁴¹ telomerase, DNA polymerases,⁴² topoisomerases and methyl transferases, ATPase/ATP synthase,⁴³ ornithine decarboxylase, as well as urokinase, an enzyme required by human tumors to form metastases and notably inhibited by EGCG.⁴⁴

The current appreciation of the capacity of several plant polyphenols to modulate cellular signaling cascades by binding to specific target proteins has certainly refreshed opinions on polyphenol-protein

interactions, and should provide a new impetus for (re)considering polyphenolic compounds in pharmacological drug developments. It is important to emphasize, again, that the structural diversity of plant polyphenols is huge, and that the manner with which they can interact (specifically or not) with proteins strongly (and mutually) depends on both their physicochemical characteristics and those of their protein partners.

In the previous years, the ability of polyphenols to strongly associate with proteins with a high proline content was clearly established⁴⁵ and the molecular interactions of polyphenols with proline-rich proteins (PRPs) in saliva were examined in detail, notably in relation to the phenomenon of astringency. NMR spectroscopic analyses of complexes formed between different polyphenols and model peptides mimicking extended polyproline helices of PRPs were performed, and some details of the association between the 1,2,3,4,6-penta-O-galloyl- β -D-glucopyranose (β -PGG) and the mouse salivary proline-rich peptides were then revealed.⁴⁶ A preference for an interaction between the pyrrolidine ring of prolyl groups and the aromatic ring of galloyl units (s-p attraction) was thus discerned, in tandem with the deployment of hydrogen bonds between the carbonyl group of the peptide residue preceding the proline unit and one of the meta-hydroxy groups of the β -PGG moieties (Figure 1.8).^{47,48}

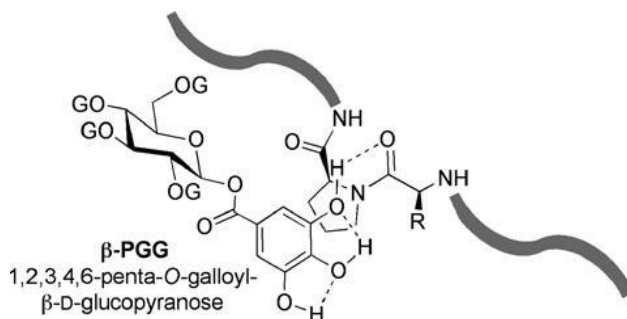


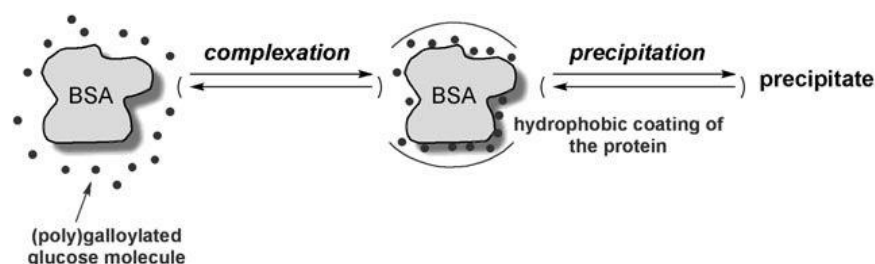
Figure 1.8 Proposed interaction between the 1,2,3,4,6-penta-O-galloyl- β -D-glucopyranose (β -PGG) and a prolyne residue with formation of a hydrogen bond with its preceding amide bond; G = galloyl (3,4,5-trihydroxybenzoyl).^{47,48}

This selectivity for proline residues was, however, challenged in the case of the complex formed between Gly-Pro-Gly-Gly and the procyanidin B3 catechin-(4 α \rightarrow 8)-catechin, for which no preferential interaction with the proline residue was observed.⁴⁹

Numerous other studies, using either peptides or full-length proteins and various polyphenolic

molecules, have been carried out over the years to provide further insight into the physicochemical basics that govern polyphenol–protein complexation (and precipitation). The aim of these studies was not just understanding the astringent effect of dietary polyphenols, but also how their binding to proteins could affect (not necessarily negatively) their biological activities, including their antioxidant action, and their bioavailability.^{37,50}

In the case of PRPs, hydrophobic stacking of phenolic rings against proline rings would constitute the primary associative driving force, followed by the formation of hydrogen bonds between phenolic hydroxy groups and carbonyl groups linked to proline amino groups, hence stitching up the resulting complex (Figure 1.8).³² However, some researchers have suggested that the principal driving forces towards association are instead governed by hydrogen bonding between the carbonyl groups of proline residues and the phenolic hydroxy groups.^{45,51,52} It is from these observations that Haslam proposed that the less hydrophilic the polyphenol, the better is its ability to complex with proteins,⁵³ at least with extended random-coil type proteins such as salivary PRPs, collagen (or gelatin), casein, as well as peptides such as bradykinin, or loosely structured globular proteins such as BSA. Kawamoto et al. proposed a follow-up two-stage process for the precipitation of BSA by galloylated glucose derivatives (Scheme 1.3).⁵⁴



Scheme 1.3 Two-stage process for the precipitating complexation of BSA by gallotannin-like “hydrophobic” galloylglucopyranoses: For complexation, more than 3 galloyl units are required per galloylglucose, for precipitation, a total of more than 30 galloyl units per BSA is required.

The first stage in this process is a complexation between the protein and those polyphenols bearing a minimum number of three available-for-binding galloyl groups, until a “hydrophobic” coat is formed around the protein. Precipitation would commence during the second stage, once the total number of galloyl units bound to BSA reaches 30 units. It certainly does not entirely apply to all types of polyphenols and proteins, and probably also depends on the experimental conditions used that may or may not be relevant to the conditions encountered in natural systems. One example is the relative

concentrations of the protein and the polyphenol involved.^{55,56} In this regard, most of the literature data converge to elect water-soluble higher proanthocyanidin oligomers -especially those harboring regularly (4 α →8) linked sequence- as being the best precipitators of PRPs. The multiple phenolic moieties (catechol and/or pyrogallol Brings) made available for binding by virtue of their helical threadlike shape⁵⁷ would be particularly well suited to interact in a cooperative manner with multiple sites on conformationally extended proteins,^{58,59,60,61} notably at pH values near their isoelectric points.⁴⁵ In contrast, tightly coiled globular proteins have much lower affinities for proanthocyanidins. Recent studies have clearly established that not only resveratrol but also the tea 3-O-galloylated flavanol EGCG exerts antifibrillogenic properties, which are of value for the fight against human protein misfolding disorders involved in neurodegenerative pathologies. For example, Wanker and co-workers showed that EGCG binds directly to natively unfolded amyloid- β (A β) and α -synuclein (α S) polypeptides, and hence prevents their aggregation into toxic β -sheet-rich fibrillar A β and α S oligomers that are implicated in the development of Alzheimer's and Parkinson's diseases, respectively.⁶² These authors proposed interesting mechanisms of the inhibitory action of EGCG against β -sheet formation (and aggregation) of α S. By preferentially binding to a highly flexible region of this peptide, EGCG would promote the rapid self-assembly of EGCG-bearing monomers into highly stable unstructured α S oligomers, thus redirecting β -sheet-forming and aggregation-prone molecules toward a different and nontoxic assembly pathway (Figure 1.9 A). Furthermore, EGCG-stabilized monomers and lower oligomers would not be incorporated into preformed amyloidogenic β -sheet intermediates, hence interfering with the seeded aggregation pathway to amyloidogenesis, and might thus even be able to antagonize the fibrillogenic process even after fibrils had started to accumulate (Figure 1.9 B).⁶²

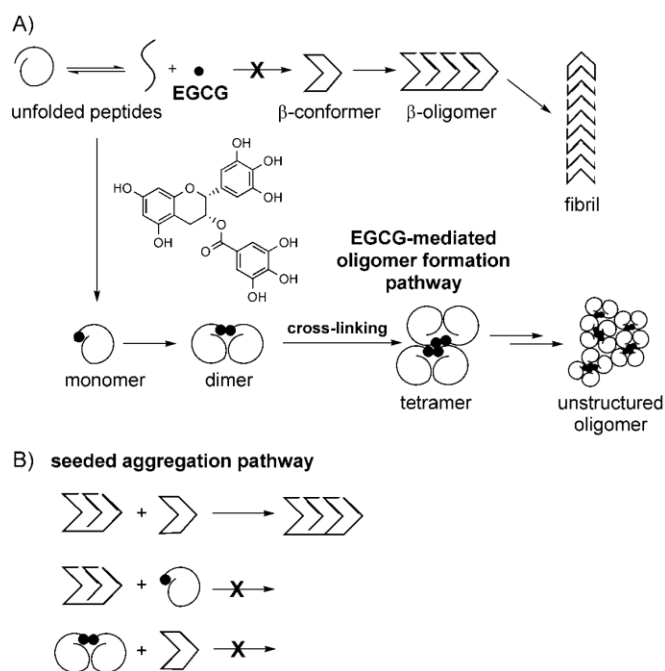


Figure 1.9 Models to explain the effects of EGCG on A β fibrillogenesis: A) Amyloidogenesis of monomeric polypeptides, which exist in equilibrium between unfolded and partially folded conformations, proceeds via oligomeric states to amyloid fibrils. EGCG preferentially binds to unfolded polypeptide chains and prevents amyloidogenesis by inducing the formation of unstructured, seeding-incompetent, and nontoxic oligomers. B) EGCG prevents monomer and lower oligomer addition to amyloid β -sheet intermediates, thus interfering with the seeded aggregation pathway to amyloidogenesis.

All of these investigations on the interactions between polyphenols and amyloidogenic or prionogenic polypeptides show great promise for the design of polyphenol-inspired fibrillogenesis inhibitors as therapeutic agents for the treatment of neurodegenerative diseases.⁶³

However, all of the structure types mentioned, including monomeric flava/flavonoids and hydroxystilbenes such as resveratrol and even its glucoside piceid, are “true” polyphenols according to the proposed definition. All of the lignan/neolignan dimers displaying two free phenolic moieties and lignin polymers also fit this definition. Among other plant-derived phenolic compounds that have been the subject of intensive investigations on account of their remarkable biological activities, the ellagitannin metabolite ellagic acid, which is naturally present in many red fruits and berries, the phenylpropanoid-derived pigment curcumin, isolated from *Curcuma* spp. such as turmeric (*Curcuma longa*), and the flavonolignan **silibinin**,⁶⁴ isolated from *Silybum marianum* seeds, are also “true” polyphenols (Figure 1.10).

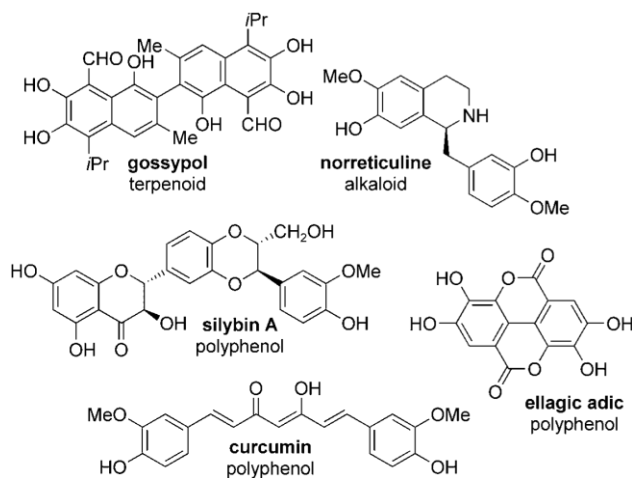
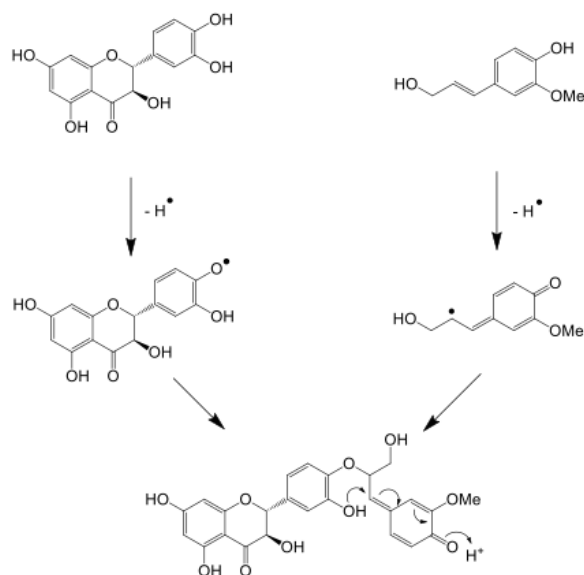


Figure 1.10 Plant polyphenolic: to be or not to be!

1.5 Silibinin: a flavonolignan extracted from the milk thistle

Silibinin is a flavonolignan extracted from milk thistle (*Silibum marianum* L.). As the flavonolignan name suggests, this class is derived from the oxidative condensation, through a radical reaction, between a flavonoid and a lignan (phenyl-propanoid). In the specific case, the flavonolignan extracted from milk thistle are derived from the non-stereoselective reaction between the taxifolin (flavonoid) and coniferyl alcohol (lignan).

One-electron oxidation of taxifolin generates a free radical, a highly unstable species with a strong reactivity, which combines with the free radical generated by the coniferyl alcohol. It has, therefore, the formation of a specific product, which can be structured in different ways, generating a mixture of metabolites known as Silymarin (Scheme 1.4). The latter consists of several flavonolignan present, in their turn, in the form of diastereomeric mixtures.



Scheme 1.4 Reaction between taxifolin and coniferyl alcohol to form the silibinin precursor.

The milk thistle is a biennial spontaneous plant of the Asteraceae family (Figure 1.11), very abundant in the entire Mediterranean basin, in southern Russia, North Africa and the eastern part of the United States of America. Its fruits are harvested in late summer to be subjected to beating and drying; what is obtained from the extract is a complex of flavonolignan named by Wagner "Silymarin", in which the main component (about 50-70% of the entire complex) appears to be the silibinin (Figure 1.11), mixture of two diastereoisomers, silybin A and silybin B, present in a ratio of 45:55 respectively.⁶⁵

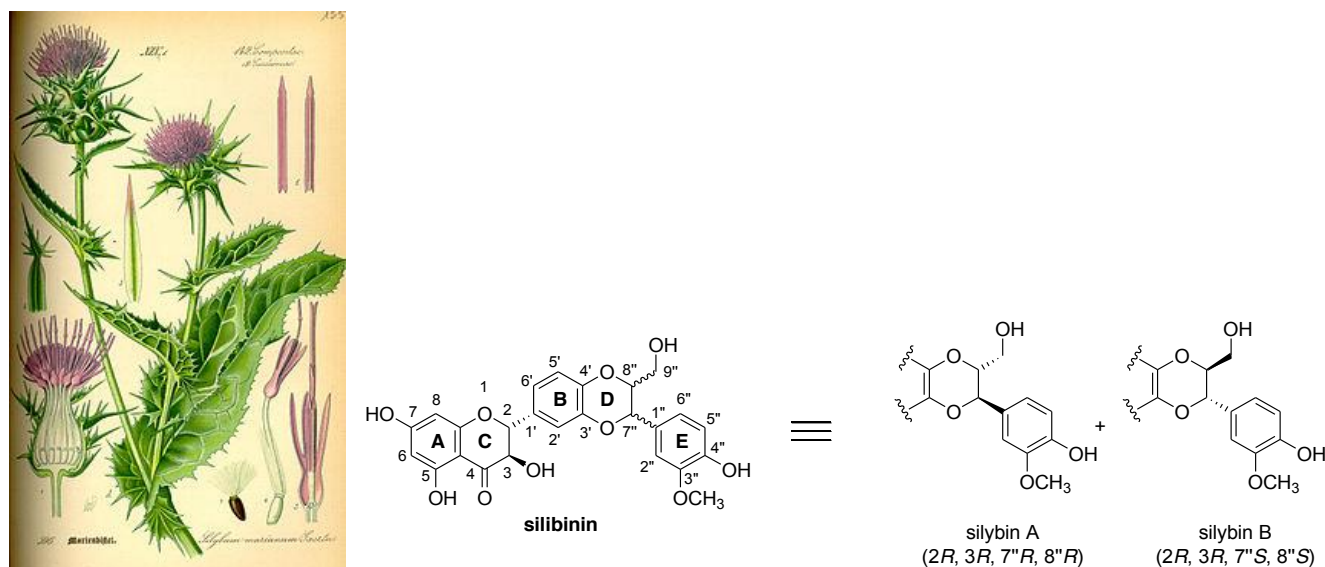


Figure 1.11 *Silybum marianum* (*Carduus marianus* L., Asteraceae; milk thistle); structure of **silibinin** and its diastereoisomers: **silybin A** and **silybin B**.

Over the years different modes of numbering for silibinin have been proposed.⁶⁶ In this thesis we used a systematic numbering as shown in Figure 1.11.

1.5.1 Biological activities of silibinin

Over the last two decades silymarin, and so it's most abundant component silibinin, returned to the attention of the scientific community for reasons not directly related to its antioxidant capacity, but for it's numerous biological activities (Figure 1.12).

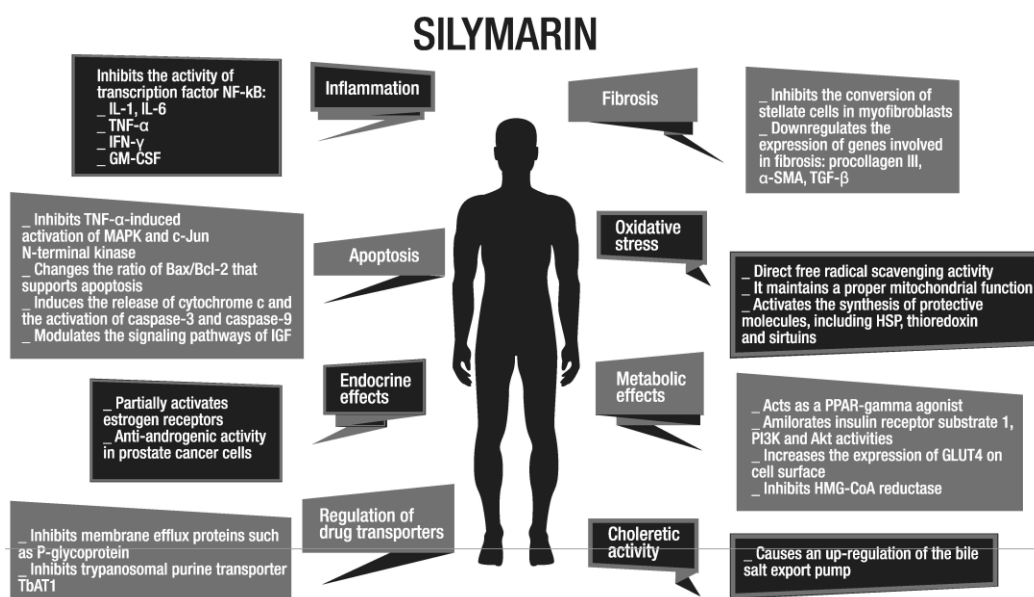


Figure 1.12 Biological properties recognized to the silymarin.

For example, a great number of studies conducted on silibinin to assess the **anti-cancer activity**, have shown that it is capable of:

- stop the cell cycle in G1 with a cyclin-dependent mechanism;⁶⁷
- reduce tumor growth following the downregulation of EGFR;⁶⁸

- reduce the expression of HER2 in breast cancer;
- induce apoptosis in p53 wild-type cells;⁶⁹
- inhibit the activation of ERK1/2 and Akt, overexpressed in aberrant phenotypes of some solid tumors;
- inhibit angiogenesis by preventing the expression of VEGF, iNOS, COX-2 and NOS3;⁶⁹
- modulate the activity of P-glycoprotein by binding competitively to the intracellular domain that binds ATP and inhibits the extrusion of cytotoxic chemotherapy drugs;⁷⁰
- inhibit the expression of telomerase.⁷¹

In view of these activities, silibinin can fill the role of adjuvant antineoplastic therapy.

Also, some researchers have investigated the **anti-inflammatory activity** of the silibinin, due to its ability to inhibit the nuclear translocation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), preventing the cascade of transduction that is under the control of this important transcription factor (Figure 1.13).⁷²

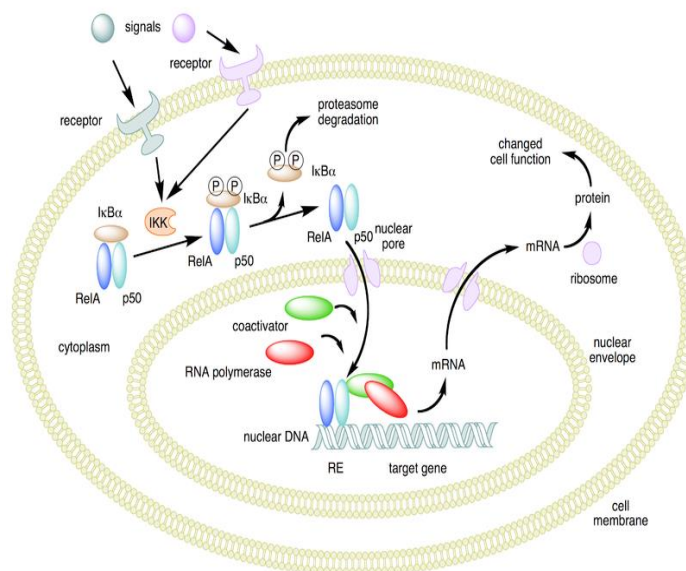


Figure 1.13 Mechanism of NF- κ B activation.

NF- κ B is sequestered in the cytosol by its inhibitor I κ B- α which is degraded when phosphorylated by I κ K, and the transcription factor can move into nucleus increasing the transcription of mRNA encoding cytokines (IL-1 β , IL-6, IL-11, TNF- α), chemokines (IL-8, CCL5), proinflammatory enzymes (iNOS, COX-2, LA-2, 5-LOX), adhesion molecules (CD-54, CD106, CD62E), receptors (CD25, β chain of the TCR) and proteins involved in apoptosis (bcl-2, IAPs, cCD95).

The **neuroprotective action** of silibinin, instead, is explicated through antioxidant activity and inhibition of NF- κ B: it is known that oxidative stress is one of the major causes of diseases such as Alzheimer or multiple sclerosis. The silibinin was tested as in vitro neuroprotectant, proving to increase the cell survival in cultures dispossessed of NGF (nerve growth factor).⁷³

Some researcher proved that silibinin has, also, **antiviral properties**. Hepatitis C is an infectious disease caused by the hepatitis C virus (HCV, Hepatitis C Virus). In most cases the infection is asymptomatic, but in severe cases it may progress to cirrhosis, fibrosis and hepatic cancer. It is known that fibrosis is accompanied by loss of tissue function and is manifested as a result of damage to stromal or parenchymal cells, which are then replaced by connective tissue. In this context it can placed the most important and recognized biological activity of silibinin and, more general of silymarin, against chronic liver diseases, cirrhosis and hepatocellular carcinoma, because of antioxidant, anti-inflammatory and antifibrotic power. Indeed, the anti-oxidant and anti-inflammatory effect of silymarin is oriented towards the reduction of virus-related liver damages through inflammatory cascade softening and immune system modulation. It also has a direct antiviral effect associated with its intravenous administration in hepatitis C virus infection. With respect to alcohol abuse, silymarin is able to increase cellular vitality and to reduce both lipid peroxidation and cellular necrosis. Furthermore, silymarin/silybin use has important biological effects in non-alcoholic fatty liver disease. These substances antagonize the progression of non-alcoholic fatty liver disease, by intervening in various therapeutic targets: oxidative stress, insulin resistance, liver fat accumulation and mitochondrial dysfunction. Silymarin is also used in liver cirrhosis and hepatocellular carcinoma that represent common end stages of different hepatopathies by modulating different molecular patterns.⁷⁴ In vivo studies have demonstrated that silibinin is capable of reducing the deposition of collagen, lipid peroxidation and the proliferation of hepatic cells in rats with induced fibrosis,⁷⁵ thanks to the inhibition of NF- κ B factor. The latter controls the synthesis of factors involved in inflammatory states

such as IL-1, which, in turn, induces the synthesis of MCP-1, protein responsible for the chemo-attraction of monocytes 1 and tissue remodeling of fibrotic phenotype.

It also has been shown that silibinin acts on the **glucose metabolism** as it is capable of modulating the up-take of glucose in adipocytes, blocking GLUT 4 (between the conveyor-insulin-dependent glucose).⁷⁶ In addition, studies in rat hepatocytes have shown that it, in a concentration range between 25 and 100 mmol/L, can reduce gluconeogenesis and glycogenolysis, block the hydrolysis of glucose-6-phosphate, inhibit pyruvate kinase and glucose-6-phosphatase.

From the many biological data available, it is possible to observe that the silibinin may be used in the field of pharmacology for treatment of numerous diseases. Pharmacokinetic studies have shown that the silibinin, taken orally (in the form of silymarin), is not toxic, even at high doses. However, in vivo studies have shown that its bioavailability is very poor not only because it is susceptible to metabolism in liver enzymes of phase II, but also because its low solubility in water limits the absorption (which takes place at the stomach level).

Both the free form of the silibinin and its conjugates show a rapid distribution tissue that occurs thanks to the presence of endogenous lipoproteins, which act as carriers for transport to the extra-hepatic compartments. The bile concentration of silibinin is two orders of magnitude higher than plasma: in fact, its elimination is mainly through the biliary excretion (although a discrete portion enters the enterohepatic recirculation), while a small percentage (about 5% of the total dose) is excreted in the urine.⁷⁷

1.6 Synthesis of silibinin modified: state of art

The growing number of experimental data for pharmacological studies to understand silibinin action mechanisms have directed research towards the synthesis of new analogues, molecules with greater solubility and bioavailability. In this context, however, the synthetic efforts to date are still limited and the number of new analogues synthesized and subjected to biological assays is very small. The first and, currently, only drug that present silibinin as active ingredient came into therapy is Legalon® SIL, produced by Rottapharm Madaus, and used in the treatment of hepatic acute intoxication by mycotoxins.⁷⁸ Its active ingredient is the sodium salt of silibinin bis-succinate (Figure 1.14) that has a greater solubility in water; in fact, the preparation can be administered intravenously but presents

considerable limitations, because it is an extremely expensive drug that requires patient hospitalization. Over the years the poiesis of analogues has not stopped and between all the available examples (ethers, esters, glycoside, product of oxidation and isomerization),⁷⁹ worthy of note emerges the silibinin phosphate (Figure 1.14), synthesized for the first time in 1997 that appears to be a pro-drug activated by endogenous phosphatase, and exhibits greater solubility in water.⁸⁰

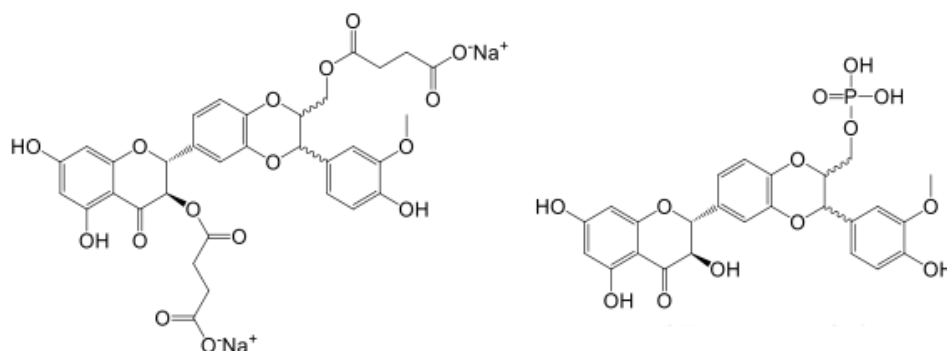


Figure 1.14 Structures of silibinin bis-succinate and silibinin phosphate.

Moreover, another drug based on silibinin is commercially available. It comes to a lipophilic preparations not obtained by chemically modifying the molecule, but creating a non-covalent complex with phosphatidyl choline (Silipide, IdB 1016, Indena, IT).^{81,82} This complex possess not only better bioavailability than silibinin but also exhibited higher antioxidant activities.^{83,84} Silipide, also, exhibits promising anticancer activities as was demonstrated by its significant inhibition of the growth of human ovarian cancer xenografts.⁸⁵

Some researchers, over the years, have focused their work on the development of new analogues based on silibinin. For example, Křen and his collaborators have developed two selective acylation methods for silibinin esterification with long-chain fatty acids were, yielding a series of silibinin 7-O- and 9''-O-acyl-derivatives of varying acyl chain lengths (Figure 1.15). These compounds were tested for their antioxidant (inhibition of lipid peroxidation and DPPH-scavenging) and anti-influenza virus activities. The acyl chain length is an important prerequisite for both biological activities, as they improved with increasing length of the acyl moiety.

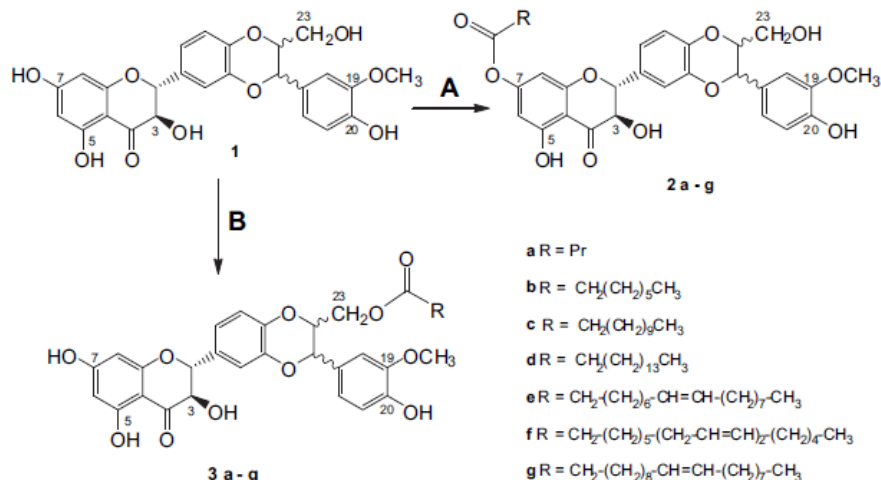


Figure 1.15 Silibinin 7-O- and 9''-O-acyl-derivatives.

At the same way, in the research group of Křen have been prepared silibinin dimers and glycosides. Silibinin glycosides (9''-O-β-glucoside, β-galactoside, β-lactoside and β-maltoside, Figure 1.16) have been synthesized by different methods (Helferich glycosylation, Lewis acid catalysis) and their cytoprotective effects of were studied in isolated rat hepatocytes intoxicated by CCl₄ (10 mM).⁸⁶ Reduction in the lactate dehydrogenase (LDH) leakage was higher for the new synthesized compounds than the silibinin. Similar results were obtained in hepatocytes intoxicated with tert-butyl hydroperoxide and allyl alcohol.⁸⁷

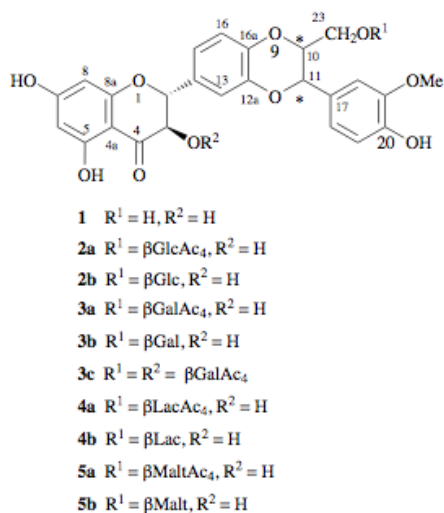


Figure 1.16. Silibinin glycosides.

The same researchers have proposed, also, the chemical synthesis of silibinin dimers (Figure 1.17), in which the monomer units are linked to each other by an ether spacer. Various biological in vitro tests have been used to investigate the behavior of the products, and, as expected, DPPH-scavenging and inhibition of microsomal lipid peroxidation was slightly improved by dimerization.

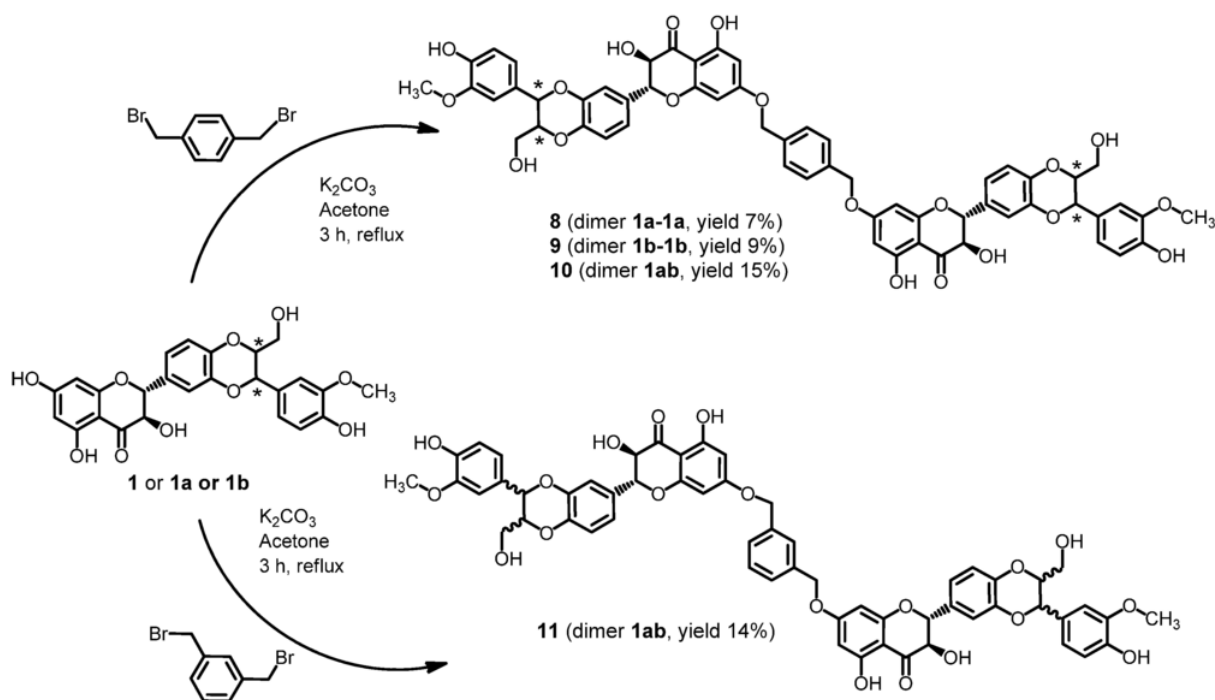


Figure 1.17 Silibinin dimers.

Our research team, in recent years, is engaged in the synthesis of new silibinin analogues with chemical changes for the improvement of the water solubility of this metabolite, without affecting the properties of radical scavenger.⁸⁸ For this purpose has been developed a synthetic strategy for obtaining analogues modified in position 9" of the silibinin and the 2,3-dehydro-silibinin, with functional groups able to confer to the molecule a greater solubility. From a structural analysis of silibinin, we can see that five hydroxyl groups of different nature are present; in fact, the groups present in positions 5, 7 and 4' are phenolic type, while in position 3 and in position 9" there are a primary and a secondary hydroxyl group, respectively. The choice of insert conjugates on position 9" it has also been dictated by the fact that changes in this position did not seem to alter the antioxidant capacity of silibinin.⁸⁹

The different hydroxyl groups of the silibinin were protected with orthogonal functional groups. This

strategy has enabled the obtaining of a key intermediate useful for insertion of various chemical changes in position 9". For this purpose, new analogs were obtained by introducing in position 9" a sulphate function, a phosphodiester function and an amino function (Figure 1.18). Similarly new analogues were obtained for the 2,3-dehydro-silibinin.

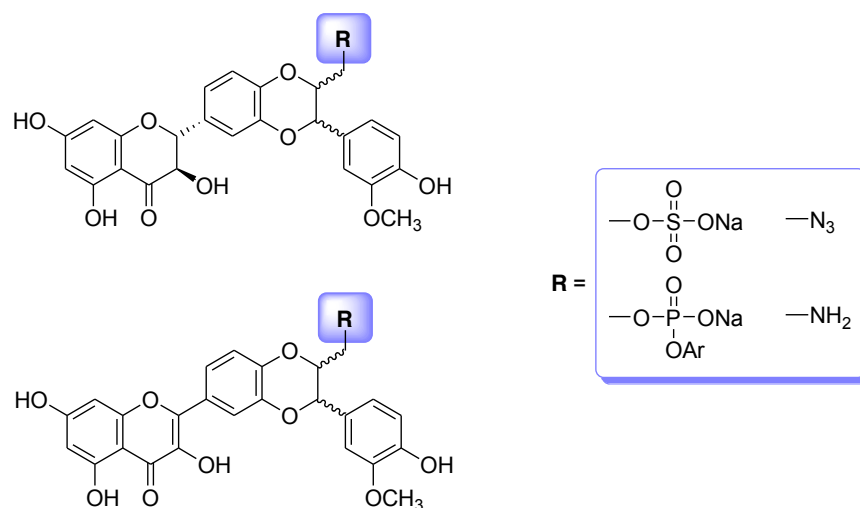


Figure 1.18 Silibinin analogs obtained by introducing in position 9" a sulphate function, a phosphodiester function and an amino function.

The antioxidant capacity of the analogues obtained was evaluated in vitro with the DCFH-DA (2', 7'-dichlorofluorescein diacetate), using rat fibroblasts, allowing to measure the levels of ROS converted into more stable species. The results showed that all analogues have reduced levels of ROS present in basal conditions and were able to prevent the formation of H_2O_2 more efficiently than the silibinin. The analysis of the data led to the conclusion that the modifications made to increase the hydrophilicity of silibinin, and do not change, as expected, the antioxidant capacity but, instead, in most cases the antioxidant activity was greater than the silibinin. Were also performed cytotoxicity assays using a viability test conducted with MMT in which the rat fibroblasts were pre-incubated with increasing concentrations of analogues (0-800 μM) and their viability was assessed spectrophotometrically after 48 hours. A reduction in cell viability was observed in all compounds with IC_{50} range of between 124 and 178 μM (the silibinin showed IC_{50} of 162 μM) and no toxic effects were observed for 30 μM values (used to evaluate the activity antioxidant).

Moreover, as a part of the continuing research effort towards the synthesis of new natural product analogues of my research group^{90,91} an efficient synthetic procedure to obtain 9"-phosphodiester

silibinin conjugates with different labels, aiming to improve the bioavailability, the delivery as well as the biological activity, was carried out. Particularly, some molecules known for their ability as molecular carriers (steroids, bile acids),^{92,93} radical scavengers (nucleosides),^{94,95} and capable to improve water solubility (as polyether)^{96,97} have been selected (Figure 1.19). The introduction of a phosphate group may bring pharmaceutical and pharmacokinetic benefits⁹⁰ and conjugation is usually considered as an efficient route in drug discovery to improve the biological properties of a large number of drugs and can improve the bioavailability and delivery as well as the biological activity. Also in this case the different hydroxyl groups of the silibinin were protected with orthogonal functional groups, leading to obtain the 9''-phosphoramidite building block **3** (Figure 19) and, starting from it, a series of conjugates were used to obtain new analogues by a solution-phase parallel array protocol, exploiting standard and reliable phosphoramidite chemistry.⁹⁰

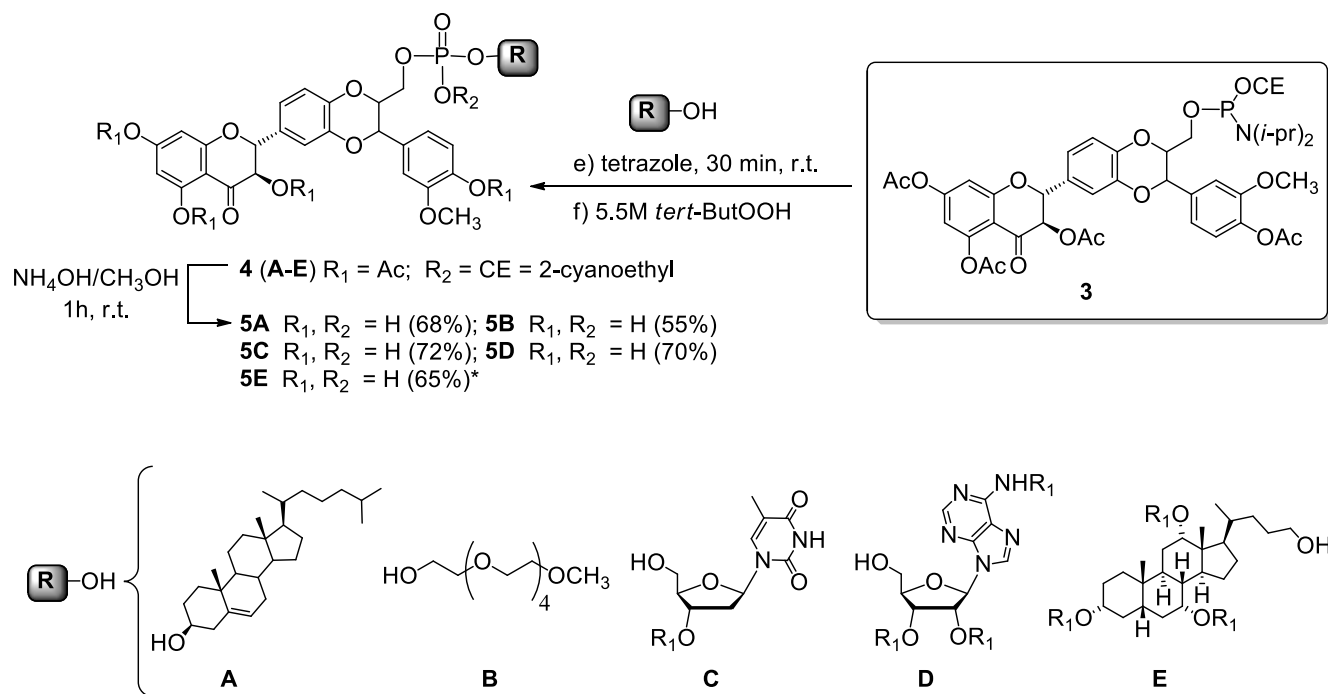


Figure 1.19 Synthetic procedure to obtain 9'' phosphodiester silybin conjugates with different labels.

As described up to now demonstrates that the application of synthetic strategies to improve the pharmacological and pharmacodynamic characteristics of the molecule leads to obtain biologically active molecules. Therefore, the future challenge lies in the application of new synthetic strategies to increase the number of new analogues. This is, in particular, the objective presented in this PhD thesis,

which also aims to identify new molecules that exhibit improved anti-radical and biological activity, and therefore possible candidates for the identification of new drugs.

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Chapter 2: Results and discussion

2.1 Aim of the research work

It is known that natural metabolites have played a crucial role in the identification of many of the drugs that are on the market today. The natural products used as such as pharmacological preparations represent only 5% of the total, but their semi-synthesis derivatives represent more than 50% of the drugs in use.¹ In the context of the chemistry of natural substances is focused this PhD thesis, in which the attention is mainly aimed on the development of strategies for the realization of new modified metabolites based on silibinin. In particular, in the research group where I worked during my PhD period, we focused at first on the synthesis, characterization and evaluation of biological activity of new glycosylated silibinin derivatives, through a phosphate bridge. At a later stage, the attention was aimed at the development of synthetic methods to obtaining oligoflavonoids based on silibinin, and investigates their anti-radical activity.

2.1.1 Synthesis, characterization, evaluation of antioxidant and biological properties and investigation of electrochemical behavior of new silibinin glyco-conjugates

Silibinin is a diastereoisomeric mixture of two flavonolignans, namely silybin A and silybin B in a approximately 1:1 ratio. Silibinin is one of the flavonoid antioxidants with multiple biological activities, mostly related to its radical-scavenging activity. Recently, this metabolite has received attention due to its anticancer and chemopreventive actions,² as well as hypocholesterolemic, cardioprotective, and neuroprotective activities.³ Unfortunately, the pharmacological use *in vivo* of silibinin is dramatically limited for its very low water solubility.^{4,5} To remedy this problem, various procedures are known in literature that improve the solubility and bioavailability, and therefore the pharmacokinetic and pharmacological properties of the molecule. An example could be the coniugation with phosphorous or PEG groups. The presence of phosphate group increase the water solubility and brings the possibility of *prodrug* approach making possible the improvement of its pharmaceutical, pharmacokinetic and/or pharmacodynamic properties. In recent years, many efforts have been made to overcome these limitations leading to the production of two water soluble derivatives of silibinin, the FlavobionTM and LegalonTM, endowed with high hepatoprotective activities are used in chronic liver diseases caused by oxidative stress (such as alcoholic and non-alcoholic fatty liver diseases or drug- and chemically-induced hepatic toxicity).⁶

Another family of molecules suitable to improve the solubility, and therefore the bioavailability, is sure that of carbohydrates. In this contest, it has been reported, for the quercetin, natural product endowed with potent antioxidant activity, that the therapeutic absorption of its modified quercetin glycoside, is more efficient than the quercetin aglycon, ascribing this benefit to the hydrophilic character of saccharide moieties that increase the water solubility of the aglycon, and enhance its bioavailability.^{7,8} Moreover, carbohydrate-based conjugates allow targeting of a certain class of cell membrane receptors that recognize a specific carbohydrate motif and internalize their ligands by endocytosis. The presence of hydroxyl groups brings the possibility of *prodrug* approach making possible the improvement of its pharmaceutical, pharmacokinetic and/or pharmacodynamic properties.⁹

While remaining within of chemistry of silibinin, we dealt with the low water solubility of silibinin that dramatically limits its pharmacological use.⁵ In this frame, we developed a synthetic strategy to obtain new silibinin derivatives linking suitable chemical conjugations, in particular glico-conjugation, able to improve its applications in biomedicine and biochemistry.^{10,11}

2.1.2 Synthesis, spectroscopic properties, solubility and anti-oxidative ability of silibinin dimers

As described before, it is shown from the literature that plant polyphenol exhibit an important and remarkable antioxidant activity. This activity is mainly performed through two mechanisms: the first regards the capacity of the phenol functional group to donate a hydrogen atom to a free radical, acting as chain-breaking antioxidants (HAT mechanism) and the second mechanism is the single-electron transfer (SET) from the polyphenol to a free radical with formation of a stable radical cation. The most important determining factors in this activity are the presence, number, and relative position of additional phenolic hydroxy groups, their implication in the formation of intramolecular hydrogen bonds,¹² and the conformationally dependent possibility of allowing electronic delocalization throughout the largest part of the molecule.

In this context, divalent or multivalent molecules often exhibit enhanced biological and antioxidant activity relative to the simple monovalent units.¹³ It is not only the multivalency effect that modifies their biological activity (multi-ligand binding) but also their pharmacokinetic parameters, as shown recently in artemisinin dimers used as antimalarial drugs.¹⁴ The dimerization of flavonoids, aiming at the preparation of more effective P-glycoprotein inhibitors, has been accomplished by the synthesis of apigenin homodimers linked with polyethylene glycol spacers.¹⁵ This study also demonstrated that the length of the spacer is a critical parameter for the binding of dimers to the target protein(s). Křen and

his collaborators have prepared dimers of silibinin, wherein the monomers are linked to each other by short aliphatic chain, using an enzyme-catalyzed and also chemical approach and they show a DPPH-scavenging and inhibition of microsomal lipid peroxidation slightly improved by dimerization.

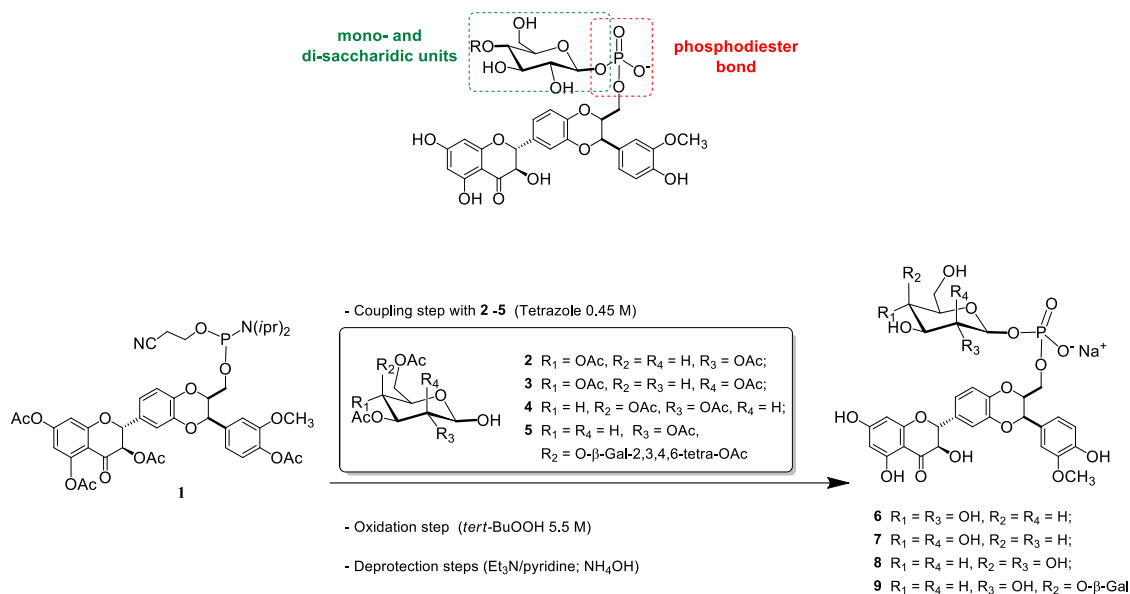
Due to this information, as a part of our continuing research effort towards the synthesis of new natural product analogues by a *prodrug* approach, we have developed an efficient synthesis of dimers linked-phosphate silibinin.

2.2 New Silibinin glyco-conjugates: synthesis and evaluation of anti-oxidant properties

As previously described, therapeutic efficiency of silibinin is rather limited by its low bioavailability that limited its affectivity.⁶ In this contest, it has been reported, for the quercetin, natural product endowed with potent antioxidant activity, that the therapeutic absorption of its modified quercetin glycoside, is more efficient than the quercetin aglycon, ascribing this benefit to the hydrophilic character of saccharide moieties that increase the water solubility of the aglycon, and enhance its bioavailability.^{7,8} Moreover, the presence of hydroxyl groups brings the possibility of prodrug approach making possible the improvement of its pharmaceutical, pharmacokinetic and/or pharmacodynamic properties.⁹

Starting from these considerations, we synthesized and biologically characterized, new 9"-silibinin conjugates connecting the mono- and di-saccharide residues to hydroxyl anomeric position through phosphodiester bond (Scheme 2.1). In our approach 9"-phosphoramidite **1** has been used as silibinin substrate and 1-OH full protected mono- and di-saccharide derivatives (**2–5**, Scheme 2.1) chosen as sugar starting materials. We initially converted full acetylated mono and di-saccharides into 1-OH derivatives by a reaction with benzylamine in THF at room temperature.^{16,17}

Thus compounds **2–5** (Scheme 2.1) were coupled with derivative **1** using 0.45 M tetrazole in CH₃CN/CH₂Cl₂ (1:1, v:v). After the treatment with 5.5 M tert-butyl hydroperoxide solution in decane and subsequently with Et₃N/pyridine (1:1, v:v) at 50 °C and conc. aq. Ammonia/MeOH (1:1, v:v) at room temperature, the phosphodiester derivatives **6–9** were obtained in good yields.



Scheme 2.1. General structures of new silibinin glycoconjugates (top); synthesis of new 9''-glyco-silibinin analogues (bottom).

The crude materials were then subjected to the purification step, by reverse phase analysis (RP-18 HPLC), using a variety of columns and elution conditions, but unfortunately the mixture of diastereoisomers was too much complicated to be purified. In the end new silibinin analogues (**6–9**) were eluted onto Sep-Pak C18 cartridge and were obtained as a mixture of diastereoisomers, as observed ^{31}P NMR analysis. All compounds were then converted into the corresponding sodium salts by cation exchange on a DOWEX (Na^+ form) resin to have crystalline samples. The NMR analysis has proved very complex, in fact ^1H and ^{31}P NMR spectra of all compound showed a dramatic complexity, due to the presence of a complex mixture of diastereoisomers. This drawback has not allowed a complete and detailed NMR characterization of the new derivatives. In this preliminary study we show the values of the ^{31}P NMR and ESI-MS mass spectra signals. In order to evaluate their antioxidant activity, all silibinin derivatives were subjected to 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay¹⁸ and the Xanthine Oxidase (XO) inhibition models assay (Table 2.1).

Table 2.1. Free Radical Scavenging capacity (DPPH) and Xanthine Oxidase Inhibition (X/XO)

Samples	IC ₅₀ (μM) ^a	Cell viability (%) ^b	
	DPPH scavenging		<i>after induction of oxidative stress</i>
Silibinin-9"-phosphoryl-D-glucopyranoside (6)	301.7 ± 15.1	89.2 ± 3.1	42.7 ± 3.2
Silibinin-9"-phosphoryl-D-mannopyranoside (7)	108.0 ± 3.2	89.5 ± 2.3	42.3 ± 4.2
Silibinin-9"-phosphoryl-D-galactopyranoside (8)	233.0 ± 14.0	84.1 ± 2.4	42.8 ± 3.6
Silibinin-9"-phosphoryl-D-lactopyranoside (9)	154.7 ± 6.2	91.8 ± 2.1	48.4 ± 3.8
Silibinin	392.2 ± 7.8	45.3 ± 2.5	42.4 ± 2.9
2,3-Dehydrosilybin	27.0 ± 0.8	-	-
Quercetin	0.31 ± 0.01	-	-

^a IC₅₀ values (defined as concentrations that inhibits activity by 50%) were calculated using data obtained from at least three independent experiments.

^b (Mean ± SD from three separate experiments run in duplicate, at 10, 25, 50, 100, and 200 μM).

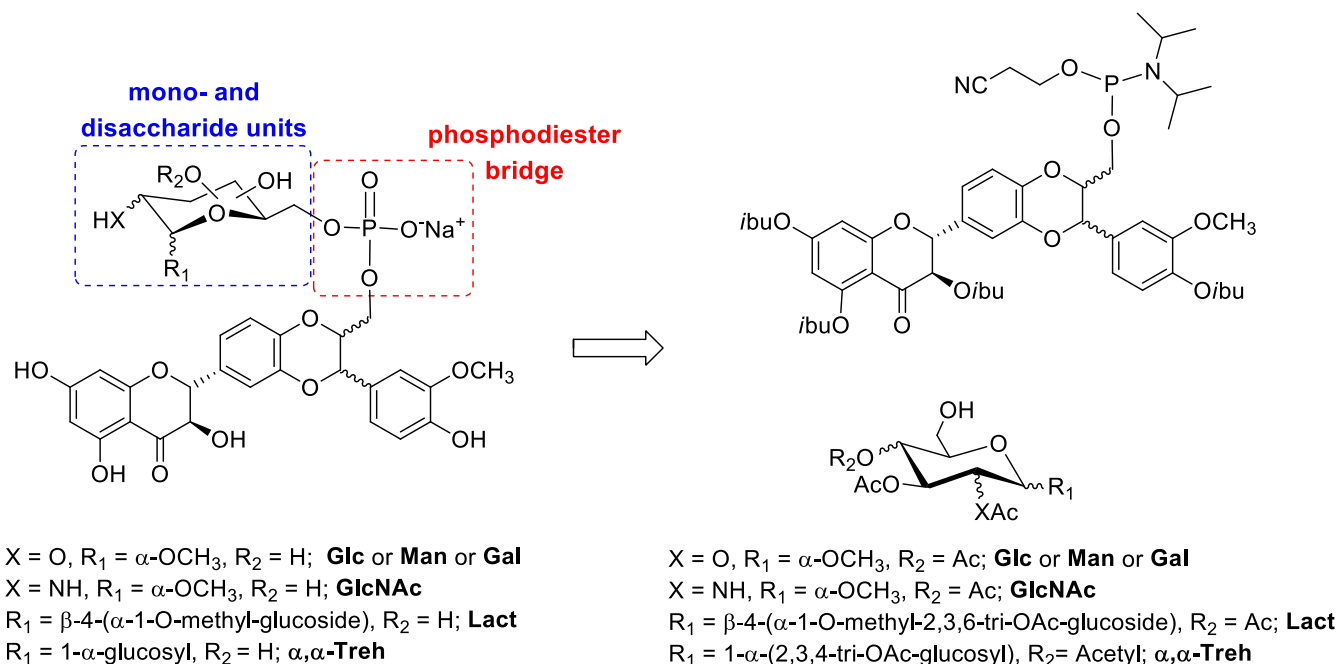
The DPPH test is a non-enzymatic method currently used to provide basic information on the scavenging potential of stable free radicals in vitro. On the other hand XO is considered to be the most important biological source of free radicals. Many references reveal cerebral microvascular injury resulting from XO production of superoxide free radicals.¹⁹ XO inhibition is thereby implicated as useful approach in treating cerebrovascular pathological changes or Central Nervous System (CNS) diseases.^{20,21} From the DPPH assay shown in Table 2.1, we observed that introducing any sugar moieties in position 9" did not led to a significant reduction of quenching properties. For comparison purposes, the antioxidant activities of 2,3-dihydrosilybin and quercetin were evaluated as controls. In particular the new silibinin derivatives (**6–9**) showed DPPH radical scavenging activities similar to that of the silibinin, confirming that the introduction in 9" of the sugar moiety did not influence the radical scavenging activity of this metabolite.^{22,23} In basal conditions, the pre-incubation of MKN28 cells with **6–9** and silibinin lead to two different results (Table 2.1). In fact, **6–9** analogues don't affect cell viability, while silibinin induced a cell death of about 50%, also at the lower dose used. The evaluation of cell viability in MKN28 cultured cells after incubation with **6–9** and silibinin, and the subsequent induction of oxidative stress, have shown that these molecules protect from cell death under oxidative stress, at least as the silibinin.

In conclusion, new silibinin glyco-conjugates were synthesized and their antioxidant properties were evaluated. Exploiting our solution phase strategy, a variety of structurally diverse silibinin glyco-conjugates were successfully realized in a short time and in very good yields. New derivatives were subjected to DPPH free radical scavenging assay and the Xanthine Oxidase inhibition models assay in preliminary study to evaluate their antioxidant activities. Irrespective of the sugar moiety examined, all compounds exhibited a radical scavenging activities slightly higher than to silibinin and XO inhibition at least as silibinin. In the other hand the new derivatives showed water solubility well above that of silibinin, in fact it was possible to prepare solutions of about 70 mg/mL of analogues in water. These two data encouraged our studies aimed to improve this synthetic strategy to realize libraries of optically pure glyco-conjugated silibinin.

2.3 Synthesis, biological and electrochemical investigation of new silibinin glyco-phosphodiester conjugates

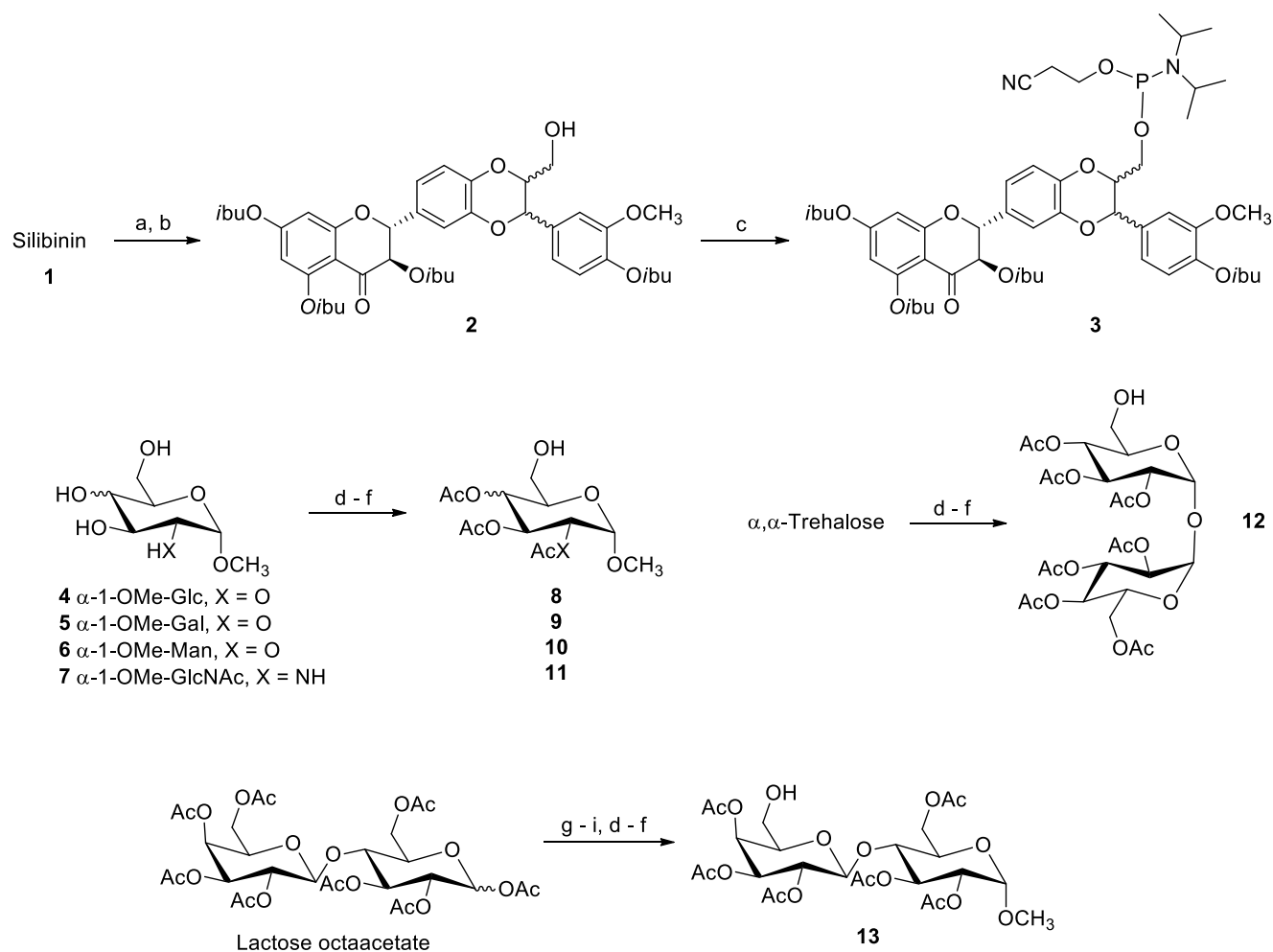
In our work, of which the details are explained in the previous paragraph, we have combined two important aspects of the design and synthesis of soluble *prodrugs*: the first is the possibility to create phosphate ester useful for phenolic and other metabolites containing poorly water soluble drugs to enhance their aqueous solubility. Moreover, phosphate *prodrugs* display excellent chemical stability and rapid bioconversion *in vivo* to the parent drug by phosphatases present in the intestinal brush border or in the liver. On the other hand, the second essential aspect for the *prodrug* strategy was that of using a carbohydrate-based conjugates that may be useful as carrier and a target of a certain class of cell membrane receptors, which recognize a specific carbohydrate motif and internalize in the cell their ligands by endocytosis. In this frame we reported the synthesis of a new class of phosphodiester silibinin glyco-conjugates with a good water solubility as well as an attractive antioxidant properties.²⁴ Unfortunately, NMR analysis has proved very complex, in fact ¹H and ³¹P NMR spectra of all compound showed a dramatic complexity, due to the presence of a complex mixture of diastereoisomers. This drawback has not allowed a complete and detailed NMR characterization of the new derivatives. This aspect, combined with the fact that the new conjugates are much more soluble in water and exhibit interesting biological properties compared to the starting molecule, have mainly encouraged us on the improving this synthetic strategy in order to realize libraries of optically pure glyco-conjugated silibinin derivatives. Infact, as a part of our continuing research effort towards the synthesis of silibinin conjugates, our project aimed at developing an efficient method for the synthesis of a new silibinin glyco-conjugates in which the structural elements necessary for the antioxidant activity are maintained and the saccharide units are linked by a phosphodiester bridge, which is hydrolytically labile in the biological fluid. Results of a recent study on the antiradical activity of silibinin have elucidated the functional groups responsible for this activity and suggest that the C-9'' position could be a site for useful modifications aimed to improve the bioavailability.

Key point of our strategy was the synthesis of 3,5,7,4''-O-tetra-isobutyryl-silibinin-9''-phosphoramidite, useful building block to obtain a wide range of silibinin 9''-conjugates with a many type of labels, following a reliable and well known phosphoramidite chemistry. Exploiting a combinatorial solution phase strategy, structurally diverse silibinin glyco-conjugates (gluco, manno, galacto, lacto, trehalo, and N-acetylgluco) were successfully obtained in very good yields (60-72% as sodium salts) and in short time (Scheme 2.2).



Scheme 2.2 Synthesis and general structures of new silibinin glycoconjugates.

New 9"-phosphoramidite (**3**, Scheme 2.3) have been used as silibinin substrate and primary OH full protected mono- and di-saccharide derivatives (**8–13**, Scheme 2.3) chosen as sugar starting materials. Using an a selectively and orthogonal approach we started from silibinin and from selected mono- and di-saccharides, to synthesized both **3** and **8–13** building blocks in a good yields. We initially converted silibinin **1** into 9-ODMT ether by a reaction with DMT-chloride (DMTCl) in pyridine and after an exhaustive acylation treatment with an excess of isobutyryl anhydride, the successive treatment using 5% formic acid in CH₂Cl₂ allowed the removal of the DMT protecting group to led **2** in a 61% yield. The choice of isobutyryl group was necessary for obtaining a more stable and easily manipulated intermediate. Indeed in the detrytilation step it was observed a partial lability of the acetyl group, used in previous works, which did not ensure a good efficiency in the purification procedures. The intermediate **2** was reacted with 2-cyanoethyl-N,N-diisopropylamino-chlorophosphoramidite and DIEA in anhydrous DCM. After purification the identity of compounds **3**, obtained in good yields (65%), was confirmed by NMR (¹H, ¹³C and ³¹P) and HRMS-ESI data.

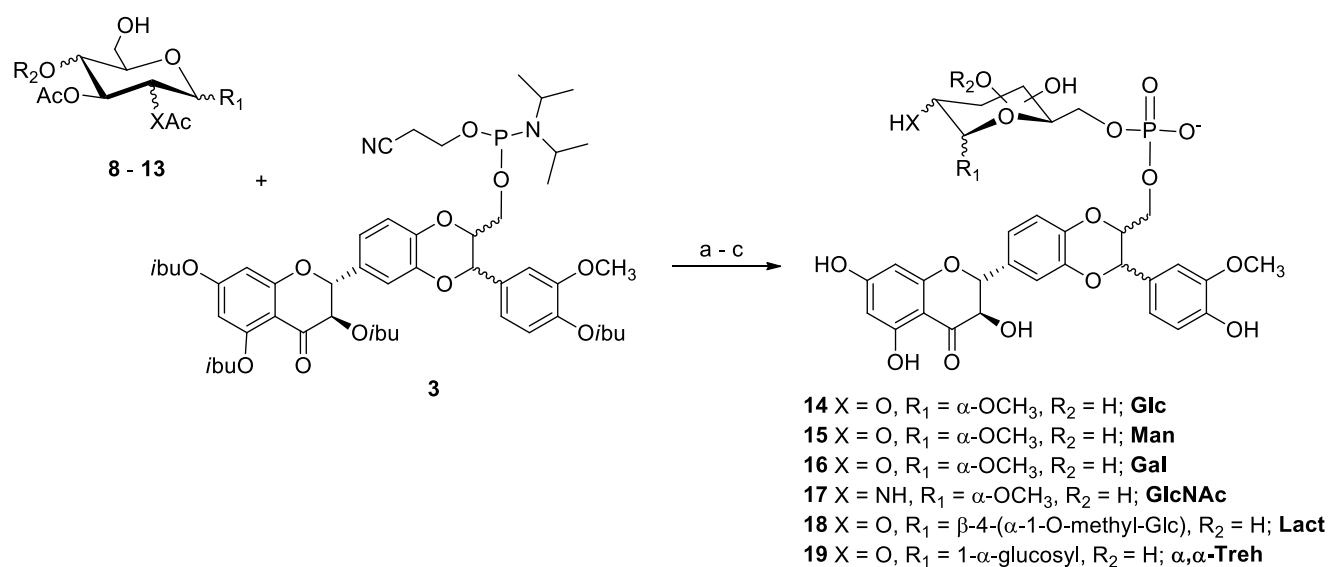


Scheme 2.3 a. DMTCl, pyridine, 50 °C; b. isobutyl anhydride, pyridine; c. 5% formic acid in CH₂Cl₂; d. DMTCl, pyridine; e. acetyl anhydride, pyridine; f. 5% formic acid in CH₂Cl₂; g. I₂/Et₃SiH, DMC; h. dry CH₃OH, Ag₂CO₃, 3 Å MS; i. 28% NH₄OH acq./CH₃OH (1:1, v/v), r.t.

For the synthesis of saccharide units (**8–13**, Scheme 2.3) were followed different routes according to the sugar units chosen. Starting from α -1-OMe-monosaccharides (**4–7**) we initially converted into 6-ODMT ether and then fully acylated by treatment with an excess of acetyl anhydride in pyridine. The treatment using 5% formic acid in CH₂Cl₂ allowed the removal of the DMT protecting group to led **8–11** in a good yields (50–70%). The same procedure was used starting from the commercially available α, α -trehalose to synthesized the eptaacetyl-trehalose (**12**, 60%). Regarding the lactose, we started from the precursor octaacetyl lactose, converting it into the corresponding glycosyl iodide intermediate by reaction with iodine (I₂) and triethylsilane (Et₃SiH) in CH₂Cl₂ at reflux, for 40 minutes. The intermediate obtained was subjected to glycosidation in situ for reaction with MeOH and silver carbonate (Ag₂CO₃) at room temperature for 6 hours, keeping the reaction mixture in low light

conditions. Later, also in this case were protected in a orthogonal way the primary and secondary hydroxyls with DMT-Cl and acetic anhydride, respectively.

Building blocks **8–13** (Scheme 2.4) were coupled with silibinin phosphoramidite **3** using 0.45 M 4,5-dicyanoimidazole (DCI). The treatment with 5.5 M tert-butyl hydroperoxide solution in decane (1 h, r.t.) and the subsequent treatment with conc. aq. ammonia and MeOH (1:1, v:v) at 50 °C, allowing full deprotection from isobutyryl and cyanoethyl groups.



Scheme 2.4 Coupling reaction between Silibinin phosphoramidite **3** and sugar building blocks **8–13**, giving conjugates **14–19**.

The crude materials were then subjected to reverse phase analysis and purification (RP-C18 HPLC) leading to the desired phosphodiester derivatives **14–19** (Scheme 2.4). All compounds were then converted into the corresponding sodium salts by cation exchange on a DOWEX (Na^+ form) resin to have crystalline samples. New compounds obtained in good yields (50–60%) were thus fully characterized by NMR (^1H , ^{13}C , ^{31}P) and MALDI-MS. The spectra of all analogues look like that of a single compound and the structures of new silibinin conjugates, were determined by NMR spectroscopy and the complete assignment was proved by COSY, HMBC and HSQC NMR experiments.

Table 2.2 ^{13}C NMR of **14–19** in CD_3OD at 400 MHz (*flavonolignan portion*).

sample carbon	14	15	16	17	18	19
2	85.46 85.00	85.03 84.97	85.05 84.97	85.04 84.97	83.33 83.26	83.36 83.28
3	74.29	73.96	73.98	73.96	72.25	71.37
4	198.54	198.51	198.67	198.62	198.70 198.68	198.43
4a	102.02	103.02 102.98	102.13	102.09	102.14	101.94
5	165.64 164.70	164.68	165.59	165.59	165.58	165.62
6	97.80	97.84	97.71	97.74	97.73	97.90
7	169.51 169.46	169.54	169.10	169.25	169.14	169.88 169.82
8	96.77	96.79	96.67	96.72	96.71	96.90
8a	164.70	164.68	164.70	164.70	164.70	164.69
1'	131.68	131.80	131.81	131.75	131.87 131.83	131.83 131.79
2'	117.98 117.85	117.96 117.86	117.95* 117.87*	117.98 117.85	117.97	118.00
3'	145.34	145.35 145.33	145.33	145.37 145.32	145.37 145.33	117.90
4'	145.65	145.61	145.62 145.61	145.67 145.62	145.59 145.57	145.68 145.61
5'	118.25 118.17	118.27 118.19	118.23* 118.17*	118.25 118.17	118.37 118.29	145.33
6'	122.55 122.37	122.57 122.46	122.59 122.49	122.59 122.43	122.71 122.68	168.30 118.23
1''	129.55	129.54	129.50	129.57	129.49	129.57 129.55
2''	112.64 112.60	112.59 112.56	112.58 112.49	112.59	112.60	112.64 112.60
3''	149.47	149.47	149.49 149.48	149.45	149.48	149.50 149.44
4''	148.53	148.54 148.51	148.55 148.54	148.53 148.47	148.54	148.53 148.49
5''	116.65 116.60	116.66 116.62	116.63	116.69 116.64	116.70 116.68	116.68
6''	122.08 122.05	122.09 122.02	122.00 121.96	122.06	122.13 122.08	122.07
7''	77.89 77.82	77.82 77.78	77.76	77.83	77.79 77.75	77.92 77.81
8''	78.72 d (4.3)§ 78.63 d (3.7)§	78.74 m 78.67 m	78.80 m 78.72 m	78.74 d (8.0)§ 78.69 d (7.4)§	78.83 d (7.5)§ 78.79 d (7.1)§	78.76 m 78.64 m
9''	66.11 m	66.36 d (5.3)	65.81 m	65.81 m 68.79 m	65.75	66.08 m 65.80 m
C-4''-OCH ₃	56.87 56.85	56.89 56.87	56.86	56.88 57.85	57.65	56.90

* Overlapped signals; § In parenthesis shows J_{CP} in Hz.

Table 2.3 ¹³C NMR of **14–19** in CD₃OD at 400 MHz (saccharide portion).

sample carbon	14	15	16	17	18	19
1	101.40	102.01	101.73	100.01	105.62	95.47 95.46
2	73.95 73.80	72.30	72.30	55.59	75.90 75.87	73.53 73.51
3	75.13 75.07	72.68	71.47	72.12 73.08	76.47 76.44	74.66 74.57
4	71.54	68.47	70.68 70.59	72.14 72.13	82.01	72.19 71.75
5	72.79 d (7.4) [§] 72.71 d (6.8) [§]	73.69 d (7.2) [§] 73.60 d (7.1) [§]	71.15 d (6.6) [§] 71.08 d (6.0) [§]	72.86 d (7.9) [§] 72.79 d (7.7) [§]	72.69 m	74.07 m 73.98 m
6	65.83 d (5.1) [§] 65.81 d (3.8) [§]	65.85 m 65.78 m	65.63 d (3.4) [§] 65.63 d (3.7) [§]	66.17 d (5.2) [§] 66.10 d (5.1) [§]	65.87 m	66.08 m 65.80 m
1'	-	-	-	-	105.31	95.39 95.33
2'	-	-	-	-	74.89 74.84	73.53 73.51
3'	-	-	-	-	75.80 75.70	74.74
4'	-	-	-	-	70.03 69.99	72.19 71.75
5'	-	-	-	-	76.67	74.07 73.98
6'	-	-	-	-	62.29	62.95
1-OCH ₃	55.86 55.84	55.59	56.07 56.03	55.85 55.83	56.92	-
NHCOCH ₃	-	-	-	173.99	-	-
NHCOCH ₃	-	-	-	22.91	-	-

[§] In parenthesis shows *J*_{CP} in Hz.**Table 2.4** ¹H NMR of **14–19** in CD₃OD at 400 MHz (saccharide portion).

proton sample	1	2	3	4	5	6	1	2	3	4	5	6	OCH ₃
14	4.55 d (4.5)	3.36 m	3.62 m	3.39 m	3.59 m	4.01 m	-	-	-	-	-	-	3.34 s
15	4.60 *	3.78 m	3.67 m	3.71 m	3.56 m	4.11 m 4.14 m	-	-	-	-	-	-	3.30 s
16	4.67 d (3.7)	3.76 m	3.94 m	4.06 m	3.89 m	4.00 m	-	-	-	-	-	-	3.33 s
17	4.55	3.92 m	3.65 m	3.45 m	3.60 m	4.03 m	-	-	-	-	-	-	3.37 s
18	4.36 d (1.3)	3.55 m	3.78 m	3.53 m	3.58 m	4.10 *	4.23	3.27 m	3.52 m	3.86 m	3.43 m	3.91 m 3.86 m 3.72 m	3.53 s
19	5.06 m	3.48 m	3.82 m	3.36 m	3.84 m	4.11 m 4.02 m	5.09	3.50 m	3.77 m	3.32 m	3.82 m	3.82 m	-

*Overlapped signals

Table 2.5. ¹H NMR of **14** **19** in CD₃OD 400 MHz (*flavonolignan portion*)

proton sample	2	3	6	8	2'	5'	6'	2''	5''	6''	7''	8''	9''	4''- OCH ₃	1- OCH ₃
14	4.99 d (11.2)	4.57 d (11.2) 4.54 d (11.2)	5.93 d (1.9)	5.91 d (1.9)	7.12 d (1.8) 7.11 d (1.9)	7.02 d (8.2) 7.01 d (8.2)	7.06 m	7.09 d (1.8)	6.87 d (8.1) 6.86 d (8.1)	6.98 dd (1.8;8.1)	5.01 d (7.5) 5.00 d (7.5)	4.35 m 4.33 m	4.11 m 3.85 m	3.91 s	3.34 s
15	4.98 d (11.5)	4.56 d (11.4)	5.94* d (2.0)	5.91* d (2.0)	7.12 d (1.8) 7.11 d (1.7)	7.03 d (7.5)	7.05 m	7.09 m	6.85 d (8.2)	6.97 dd (1.7;8.2)	5.03 d (7.8) 5.01 d (7.8)	4.31 m	4.12 m 3.85 m	3.91 s	3.30 s
16	5.03 d (11.5)	4.57 d (11.5)	5.95 d (1.8)	5.93 d (1.8)	7.12 s	7.03 d (8.3) 7.02 d (8.3)	7.06 m	7.08 m	6.86 d (8.1)	6.97 dd (1.8;8.1)	5.02 d (7.5)	4.27 m	4.10 m 3.85 m	3.91 s	3.33 s
17	4.99 d (11.8)	4.55 m 4.53 m	5.95 d (2.0)	5.93 d (2.0)	7.12 d (1.8) 7.11 d (1.7)	7.02 d (8.3) 7.00 d (8.3)	7.04 m	7.09 m	6.87 d (8.1) 6.86 d (8.3)	6.97 dd (2.0;8.3)	5.01 d (7.7) 5.00 d (7.7)	4.32 m	4.10 m 3.85 m	3.91 s 3.90 s	3.37 s
18	4.99 m	4.57* 4.55 m	5.95 d (2.1)	5.93 d (2.1)	7.12 s	7.05 d (8.5)	7.07 m	7.08 m	6.88 d (8.2) 6.87 d (8.2)	6.98 m	5.02 m 5.01 m	4.27 m 4.12 m	4.00 m 3.83 m	3.91 s	3.53 s
19	5.01 d (11.7)	4.56 d (11.5)	5.93 d (2.2)	5.92 d (2.2)	7.12 d (1.9) 7.11 d (1.9)	7.03 d (8.2) 7.02 d (8.2)	7.06 m	7.08 d (1.8)	6.88 d (8.0) 6.87 d (8.0)	6.97 dd (1.8;8.2)	5.02 d (8.0) 5.00 d (8.0)	4.33 m 4.28 m	4.13 m 4.11 m	3.91 s	

*Overlapped signals

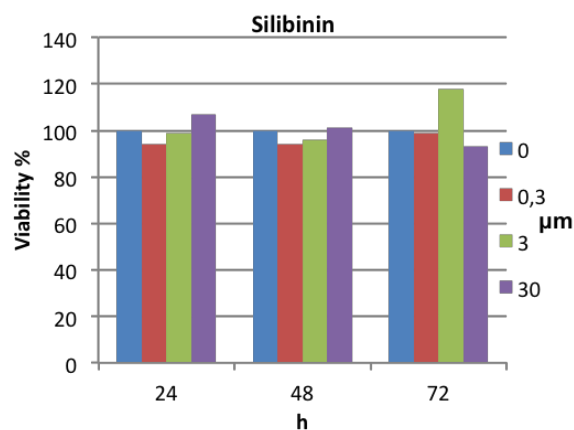
New derivatives were subjected to 2,2-diphenyl-1-picrylhydrazyl free radical scavenging assay (Table 2.6) and their activities results to be better than that of silibinin **1**, which confirmed that the introduction in 9" of the sugar moiety had little effect on radical scavenging activity. As expected the new glycosylated were very soluble in water and they exhibited a DPPH radical scavenging activities slightly higher than to silibinin.

Also, since the presence in blood and extracellular fluid of a great number of hydrolytic enzymes, among which phosphatase that can break a phosphodiester bond before the active complex can reach its molecular target, the stability of the new synthesized conjugates was evaluated in human serum and monitored over time by of HPLC analysis. New silibinins glyco-conjugates (**14–19**) were incubated at 37 °C with human serum from human male AB plasma in Tris•HCl buffer (pH = 8.0 containing 10 mM MgCl₂). Small aliquots of the mixture were taken at defined time intervals, kept at 85 °C for 5 min, equilibrated at room temperature, and analyzed by HPLC on an RP column. As shown in Table 2.6 the conjugates (**14–19**) are degraded with a time half-lives of 40 to 68 hours.

Table 2.6 Summary table of MALDI analysis, HPLC retention times, IC₅₀ and serum half lives for compound **14–19**.

	MALDI	t_R (min)	DPPH IC ₅₀ (mM)	$t_{1/2}$ (hours)
14	<i>calcd</i> for C ₃₂ H ₃₅ O ₁₈ P = 738.16 [M-H] ⁻ = 737.68; [MNa-H] ⁻ = 759.80; [MK-H] ⁻ = 775.20	14.04	1.23 ± 0.075	40.2
15	<i>calcd</i> for C ₃₂ H ₃₅ O ₁₈ P = 738.16 [M-H] ⁻ = 737.22; [MNa-H] ⁻ = 759.53 [MK-H] ⁻ = 775.28	14.07	1.00 ± 0.068	47.6
16	<i>calcd</i> for C ₃₂ H ₃₅ O ₁₈ P = 738.16 [M-H] ⁻ = 737.49; [MNa-H] ⁻ = 759.19 [MK-H] ⁻ = 775.68	14.03	0.89 ± 0.078	42.7
17	<i>calcd</i> for C ₃₄ H ₃₈ NO ₁₈ P = 779.18 [M-H] ⁻ = 778.58; [MNa-H] ⁻ = 800.37 [MK-H] ⁻ = 816.74	14.01	0.98 ± 0.069	45.6
18	<i>calcd</i> for C ₃₈ H ₄₅ O ₂₃ P = 900.21 [M-H] ⁻ = 900.35; [MNa-H] ⁻ = 921.43 [MK-H] ⁻ = 937.94	13.45	0.93 ± 0.084	59.0
19	<i>calcd</i> for C ₃₇ H ₄₃ O ₂₃ P = 886.19 [M-H] ⁻ = 886.65; [MNa-H] ⁻ = 907.34 [MK-H] ⁻ = 923.55	13.32	0.94 ± 0.071	68.1
Silibinin (1)			1.4 ± 0.06	
Quercetin			0.18 ± 0.01	

In order to verify the potential biological properties of these derivatives, a biological assay was utilized to evaluate the cytotoxicity of new glyco-conjugates, compared with the silibinin, on human liver cancer cell line (Hep G2). In this field, no significant changes in the viability of treated cells were observed when they were subjected to the action of glyco-conjugates, also considering the longest incubation (72 hours) and the maximum dose taken (30 µM).



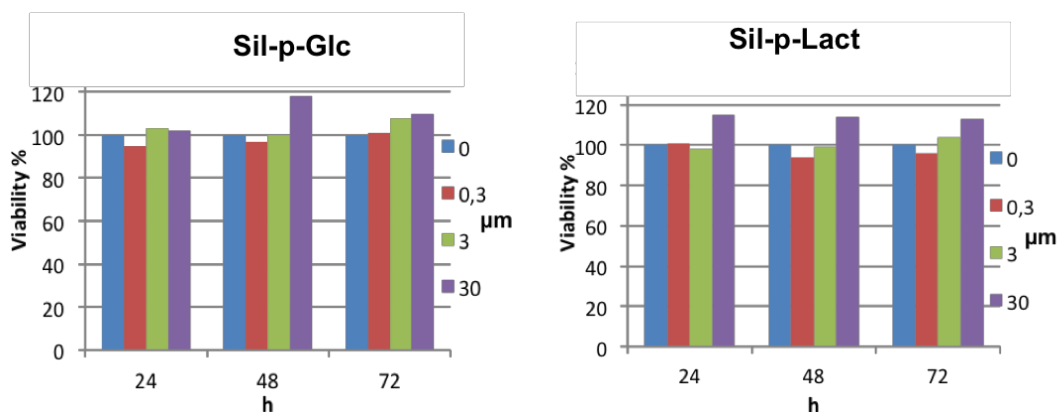


Figure 2.1 Diagrams of the cytotoxicity results for the silibinin and two of its glyco-conjugates (Sil-p-Glc and Sil-p-Lact).

In order to investigate the role of the different OH groups in the antioxidant activity of new glyco-conjugates in comparison with silibinin, their acidity and redox capacity have been evaluated. These results are obtained in collaboration with the group of Prof. Iuliano and Dr De Tommaso, of the Department of Chemical Sciences of University of Naples “Federico II”.

The determination of the acidity constants was conducted by potentiometric titrations measuring the hydrogenionic concentration with a glass electrode. The determination of acid-base silibinin and glyco-conjugates constants is made by evaluation of Z_H functions, that represents the average number of protons released per molecule. Bringing Z_H as function of pH the experimental data are in agreement with the solid line obtained with three equilibria. An example of the experimental curve (Z_H , $-\log[H^+]$) is shown in Figure 2.2.

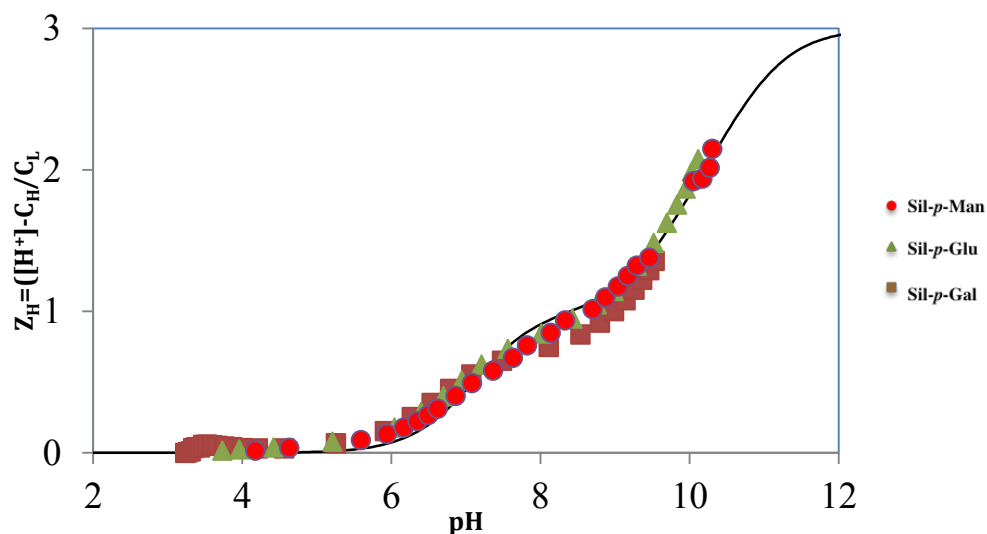


Figure 2.2 Z_H (pH) function, for Sil-p-Glc, Sil-p-Gal and Sil-p-Man systems. The solid line is calculated using the constants of Table 2.7.

By processing data, it was possible to define three equilibrium constants, both for silibinin and its conjugates. The obtained values for silibinin derivatives are similar to silibinin acidity constants. New glycosylated show a redox behavior similar to the silibinin and the acidic constants of the phenolic groups, very often implicated in the complexation with metals such as (Cu, Zn, Fe) remain unchanged.

Table 2.7. Protolysis constant of Silibinin and some conjugates in 0.1 M NaClO₄.

Compound	Silibinin ^a	Sil-p-Glc	Sil-p-Man	Sil-p-Gal	Sil-p-Lact
pKa₁	7.00 ± 0.1	7.20 ± 0.06	7.32 ± 0.08	7.28 ± 0.08	7.27 ± 0.07
pKa₂	10.25 ± 0.1	9.45 ± 0.09	9.56 ± 0.09	9.39 ± 0.09	9.40 ± 0.09
pKa₃	11.8 ± 0.1	10.9 ± 0.1	11.0 ± 0.1	11.0 ± 0.1	11.1 ± 0.1

a. Protolysis constant measured in 0.1 M NaClO₄/50% CH₃OH.

By analogy with the acid-base behavior of known molecules, the values of the acid constants can be assigned according to Figure 2.3.

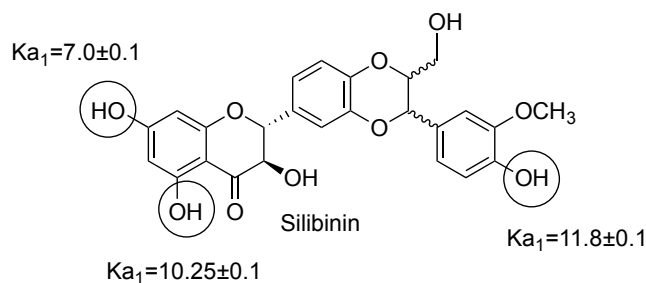


Figure 2.3 Molecular structure of silibinin where the OH groups relating to protolytic constants are shown.

Acidity constants obtained by spectrophotometric measurements (by Hyperquad program) were in good agreements with constants determined by potentiometric method.

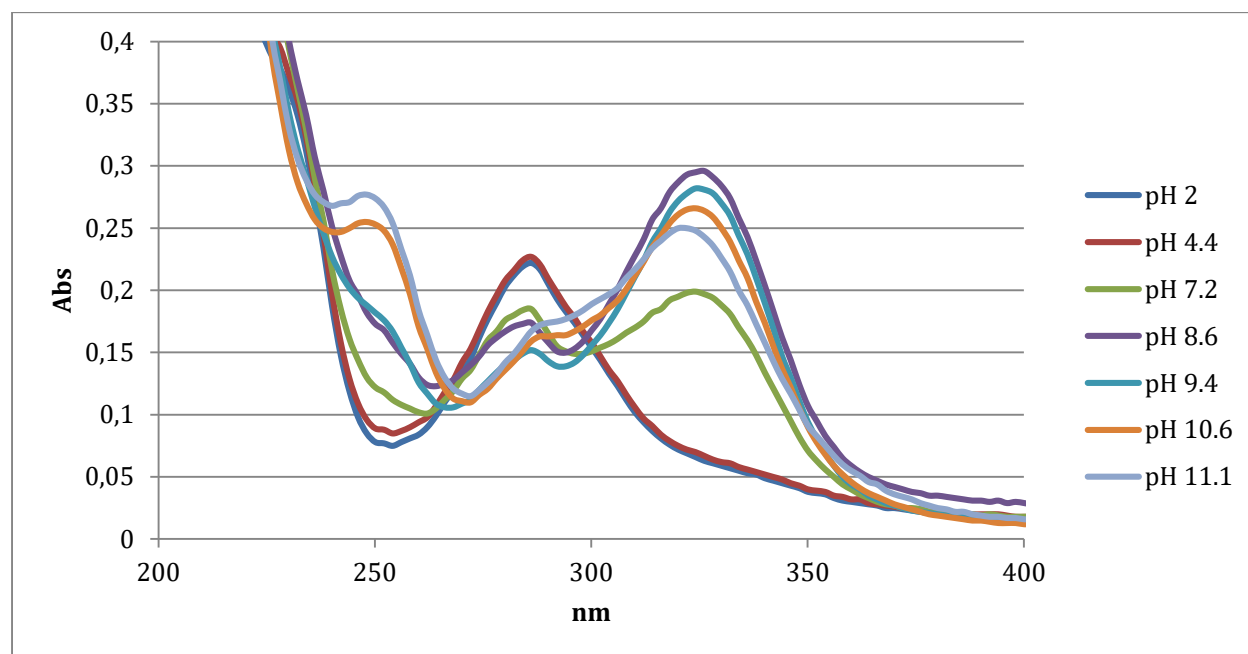


Figure 2.4 UV-Vis spectra of a Sil-p-Man solution of 5.0×10^{-5} M to varying pH (from 2 to 11.1).

CD-UV measurements in solution, at different pH, show variations in the CD spectrum that follow the same changes registered in UV-Vis spectra.

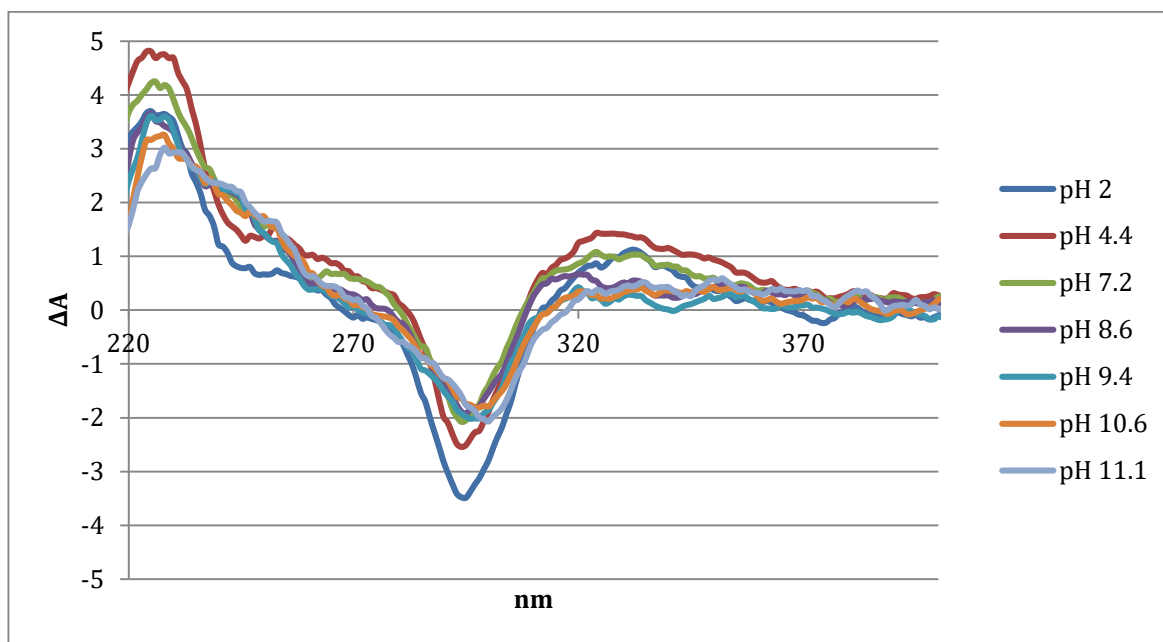


Figure 2.5 CD-Vis spectra of the glyco-conjugate Sil-p-Man, concentration of 5.0×10^{-5} M to vary pH from 2 to 11.1.

The electrochemical oxidation of flavonoids has been investigated using cyclic voltammetry (CV). All the above studies were based on in situ analyses using glassy carbon electrodes. Silibinin (**1**) and glyco-conjugates (**14–19**) are subjected to a single oxidation. This peak around 0.7 V (v.s. a saturated silver chloride reference electrode) appears in all the compounds under study and its anodic currents are probably associated with oxidation involving the OH group on C-4" (as Gyorgy et al. reported for silibinin)³¹. Cyclic voltammograms for silibinin gluco-conjugate Sil-p-Man, recorded at different pH, are reported in Figure 2.6.

Peak oxidation potential (E_P) and half-peak oxidation potential ($E_{P/2}$) are deducing from voltammograms. The number of electrons, n_e , involved in the process are obtained by equation:

$$E_P - E_{P/2} = 56.5 \text{ mV}/n_e,$$

The value of $E_P - E_{P/2}$ in the range pH 3.0–7.0, is $(37 \pm 8) \text{ mV}$, which corresponds to $n_e \approx 1.6$ (probably two electrons involved).

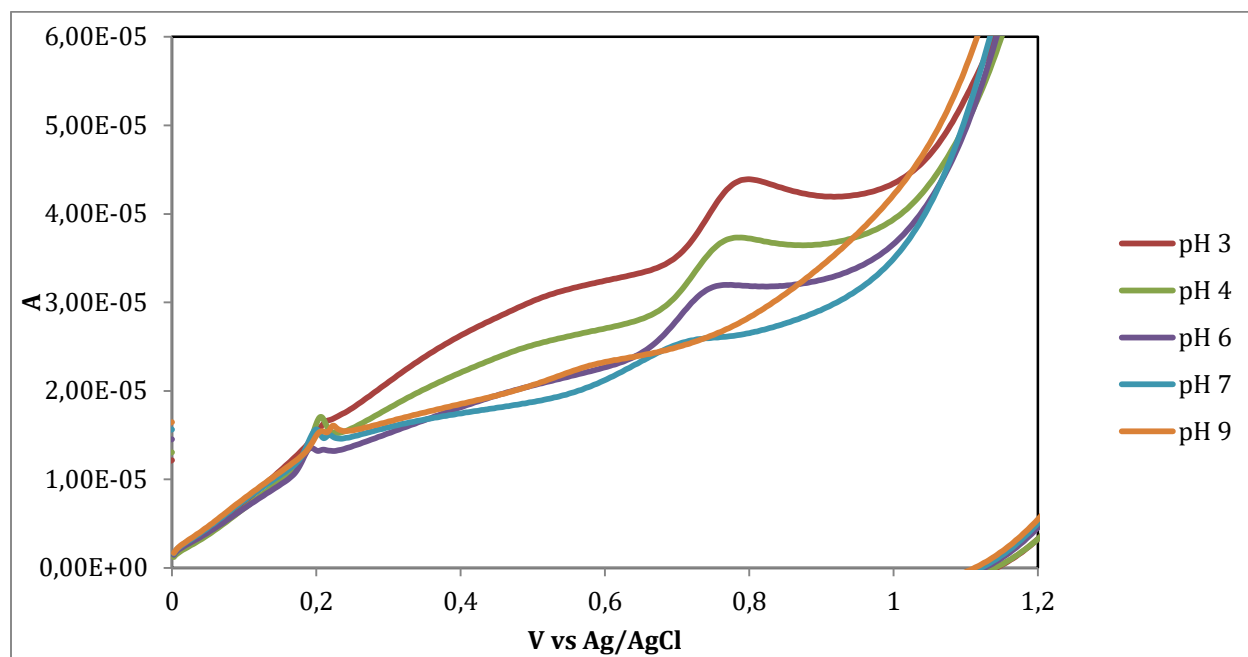


Figure 2.6. Cyclic voltammograms for Sil-p-Man recorded at different pH (from 3 to 9) (scan rate 50mV/s)

The reducing properties of compounds are very often characterized by formal redox potentials E° , determined at pH = 7.0 (v.s. saturated silver chloride reference electrode). This parameter can be obtained by half-wave potential ($E_{1/2}$), which is related to $E_{p/2}$ from equation:

$$E_{1/2} = E_{p/2} - 28 \text{ mV}/n_e$$

The results are presented in Table 2.8.

Table 2.8 Formal redox potentials at pH = 7.0 (v.s. saturated silver chloride reference electrode), for some silibinin glyco-conjugates.

Compound	Silibinin	Sil-p-Glc	Sil-p-Man	Sil-p-Gal	Sil-p-Lact
E° , Volt	0.65 ± 0.1	0.67 ± 0.02	0.64 ± 0.02	0.69 ± 0.02	0.68 ± 0.02

The electrochemical behavior is dependent on pH (Figure 2.7). The slope of $E_{p/2}$ vs pH is (33 ± 8) mV, corresponding at two protons involved in the oxidation.

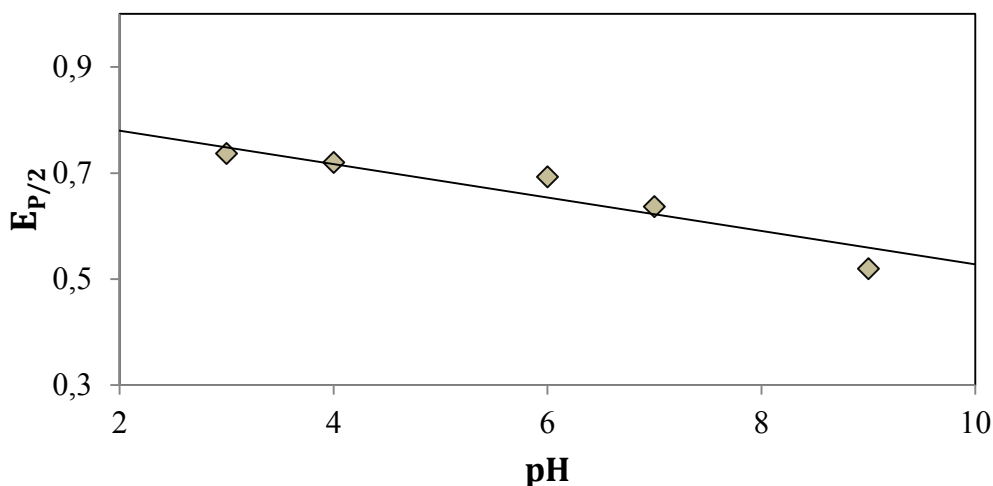


Fig. 2.7 $E_{p/2}$ values (mV, v.s. saturated silver chloride reference electrode) for Sil-p-Man determined over the pH range 3.0–9.0.

New glycosylated show a redox behavior similar to the silibinin and the acidic constants of the phenolic groups, very often implicated in the complexation with metals such as (Cu, Zn, Fe).

In conclusion, new silibinin glyco-phosphodiester conjugates were synthesized and their biological and electrochemical investigation were evaluated. Their synthesis was successfully realized in a short time and in very good yields. The serum stability of the new conjugates was evaluated, as well as their redox behavior was studied with different approaches. Infact, new derivatives were subjected to DPPH free radical scavenging assay in preliminary study to evaluate their antioxidant activities. Irrespective of the sugar moiety examined, all compounds exhibited a radical scavenging activities slightly higher than to silibinin. In the other hand, the new derivatives showed a water solubility well above that of silibinin, in fact it was possible to prepare solutions of about 70 mg/mL of analogues in water. Moreover, their acidity and redox capacity have been evaluated by potentiometric and voltammetric studies. The glyco-conjugation by phosphodiester bond has been to improve the water solubility of silibinin core without making any changes to the behavior redox. In collaboration with Prof. Pannecouque of Rega Institute of Leuven (Belgium) and prof. Agarwal of Department of Pharmaceutical Sciences, University of Colorado, future studies are in progress to evaluate the antiviral and anticancer activity, respectively.

2.4 Dimeric phosphate-linked silibinins: synthesis, spectroscopic properties, solubility and radical scavenger ability

Oxidative stress is a state present in all aerobic organisms. It occurs when the production of reactive oxygen species (ROS) exceeds the limited capacity of the cellular antioxidant system. ROS are small molecules, commonly produced in radical reactions, with the capacity to quickly interact with cellular structures. They are represented by various chemical entities: oxygen radicals (such as hydroxyl radical HO^\bullet and singlet oxygen $^1\text{O}_2$) or nonradical substances (such as hydrogen peroxide H_2O_2). ROS are usually very reactive; they do not have long lifetimes and cannot be transported for long distances in an organism. They therefore damage the cell structures that are closest to the site of their formation. They readily attack nucleic acids, proteins, and lipids. The molecule that is attacked is either damaged, destroyed, or has its function altered. Oxidative stress plays a key role in the origin and development of many diseases connected with inflammation, such as neurodegeneration, cardiovascular diseases, and cancer.

Free radicals are molecules containing unpaired electrons, which makes them very reactive. This reactivity leads to donating or accepting electrons from surrounding molecules to achieve a more stable state. The reaction of such a radical with a nonradical causes a radical chain reaction and creates new radicals, which, in turn, react with other molecules. This process is called propagation. The reaction is terminated when two radicals react with each other to form a nonradical. Hydroxyl radical is the most potent oxidant and one of the most reactive natural free radicals known. It has a very short half-life and thus reacts with molecules at the site of its formation. The reaction rate with other molecules is high, approximately 10^9 to $10^{10} \text{ M}^{-1} \times \text{s}^{-1}$.

As can be expected from the enormous reactivity of HO^\bullet , the exposure of DNA (or RNA) to it yields a large number of products. To prevent or overcome the damage to biomolecules and prevent the development of the diseases previously mentioned, the cells take advantage of various ways and enzymatic systems. Sometimes, the capacity of the endogenous enzymes and antioxidants is overwhelmed. For these cases human cells may use exogenous antioxidants supplied in the diet, such as vitamins, carotenoids, and flavonoids, such as polyphenols. Polyphenols are widely distributed in nature and are, in many cases, the active compounds of the medicinal plants from which they can be isolated. Despite the few and common biosynthetic origins, polyphenols encompass many subclasses of structurally diverse entities and from the different pharmacological properties. Many studies suggests

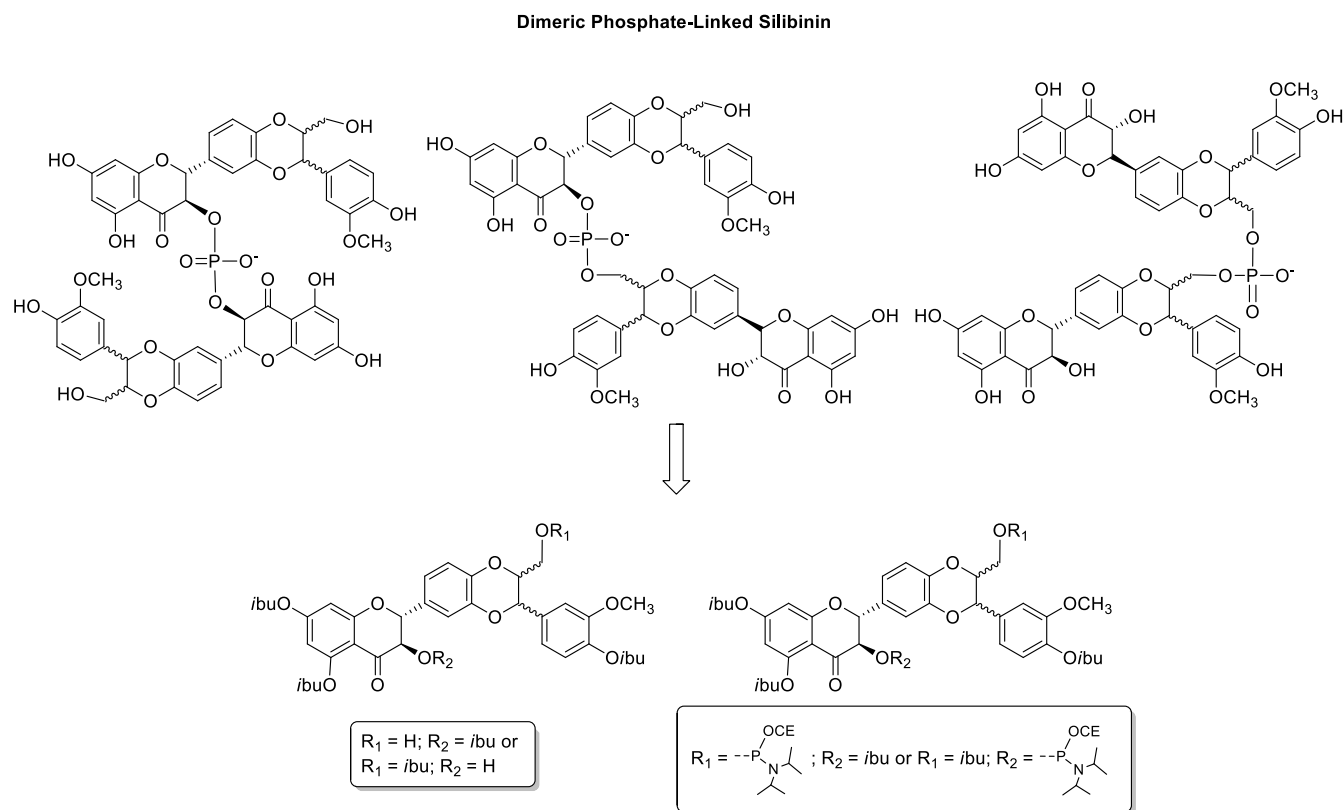
that the mechanisms by which plant polyphenols exert their protective actions against cardiovascular and neurodegenerative diseases, as well as cancer and diabetes, are not simply due to their redox properties, but rather to their ability to directly bind to bio-targets (peptides, proteins, nucleic acids).

It is the task of scientists to develop suitable methods for determining the antioxidant activity and to search for new antioxidants in natural sources. There are various ways of measuring the scavenging activity of HO[•] and ¹O₂. The choice of method substantially influences and determines the results. Obviously, we cannot expect the same results for all of the different methods used to measure ROS scavenging in antioxidant activity assays of flavonoids. Basically, there are 2 main steps in the procedure of ROS-scavenging methods: (1) production of ROS: this includes, in the case of HO[•], the employment of various physical processes, such as pulse radiolysis, γ -irradiation, or UV photolysis of H₂O₂, and also different chemical reactions, among which the most widely used is the Fenton reaction; (2) reaction of ROS with a suitable probe and the consequent detection of a reaction product or corresponding change in some physical parameter. Because HO[•] is a very reactive substance, it is not possible to measure its presence and concentration directly; but the first reaction of HO[•] with another molecule, a probe or trap, followed by the detection of a reaction product is crucial.³²

Recently we reported the synthesis of new silibinin conjugated by phosphodiester bond with a mono- and di-saccharides in the position 9". The presence of carbohydrate moiety and phosphate group increase the water solubility of the aglycon and brings the possibility of *prodrug* approach making possible the improvement of the its pharmaceutical, pharmacokinetic and/or pharmacodynamic properties. New derivatives showed a better solubility in water while maintaining a good antioxidant activity compared with that of the silibinin.

As a part of our continuing research effort towards the synthesis of new natural product analogues exploiting the phosphoramidite chemistry, we present here the efficient synthesis of new dimeric linked-phosphate silibinins and the related study of redox behavior. In order to achieve a more complete picture of spectroscopic properties, solubility and radical scavenger ability, we have studied the silibinin dimers in collaboration with Marcello Brigante, Maitre de Conférences at Université Clermont Auvergne, Institut de Chimie de Clermont-Ferrand (ICCF), Equipe Photochimie (France). Notably, during the period that I spent in the Brigante's group, I performed analysis to characterize spectroscopically the dimers in comparison with silibinin and assess their reactivity against some oxygen free radicals.

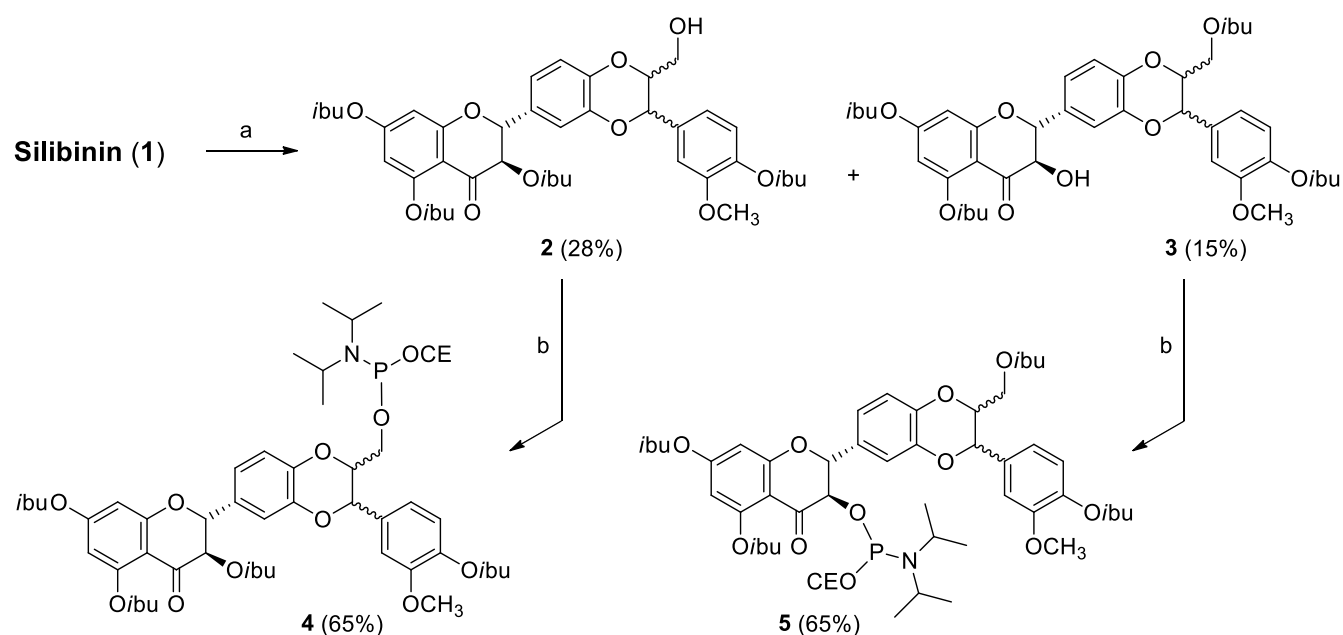
Exploiting the selective protection to hydroxyl groups of the silibinin we have developed an efficient strategy for the synthesis of new 3-9'', 3-3 and 9''-9'' dimers of silibinin (Scheme 2.5) in good yields.



Scheme 2.5 Synthetic strategy for the new 3-9'', 3-3 and 9''-9'' dimers of silibinin.

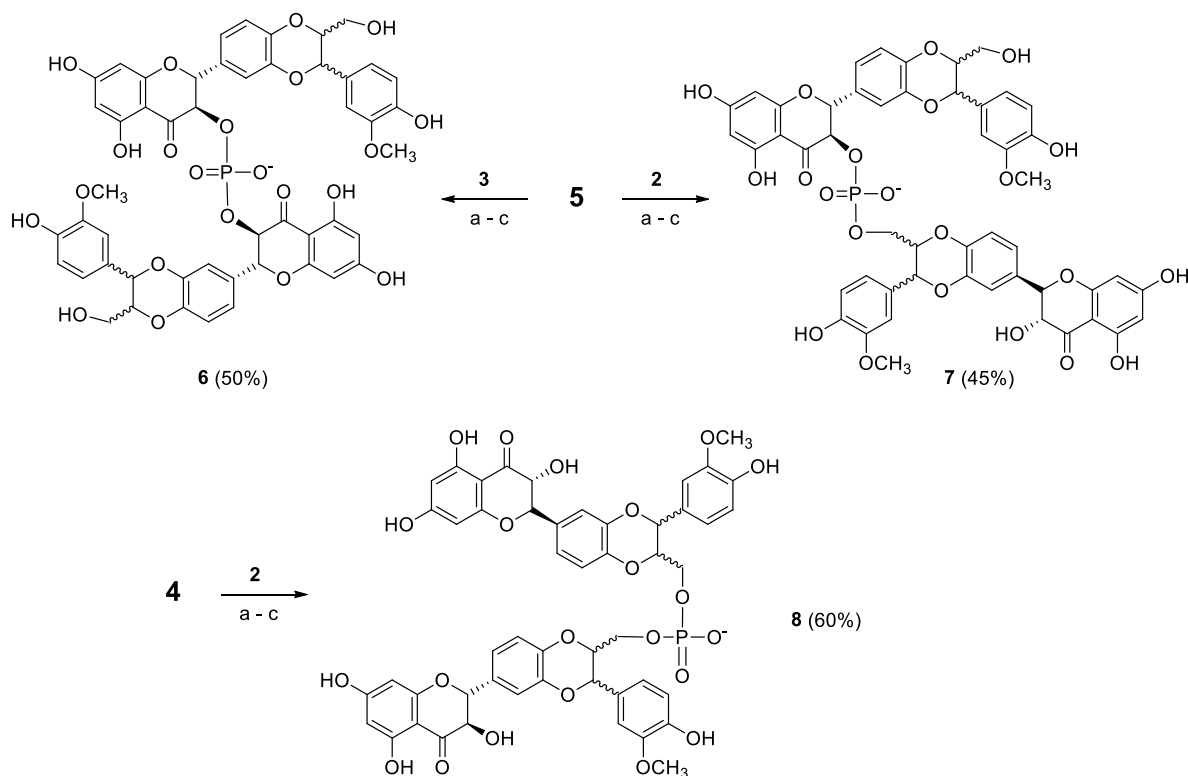
Solubility in water of silibinin and dimers as well as their ability to react with reactive oxygen species (ROS) were determined. Reactivity with ROS was quantified by estimating their second order rate constant with singlet oxygen and hydroxyl radical in solution. For this purpose, 545 nm centered excitation of Rose Bengal (RB) and laser flash photolysis (LFP) experiments were coupled with a kinetic competition approach. Laser Flash Photolysis is used for a kinetic study of short lived species, and was developed by George Porter and Ronald Norrish (Nobel Prize 1967). This technique provides direct transient measurement of reactions involving species such as radicals or excited states. In LFP the sample is firstly excited by a strong pulse of monochromatic light from a laser of a nanosecond pulse width. This first pulse starts a chemical reaction or leads to an increased population for energy levels other than the ground state within a sample of atoms or molecules. Differences in absorption compared to the ground state are measured with time resolved spectroscopy.

Silibinin is a flavonolignan with five hydroxy groups (three phenolic, one secondary and one primary) with a different reactivity. To obtain the suitable building blocks **2** and **3** (Scheme 2.6) we have developed a regioselective protection of the different hydroxyl functions with isobutyryl chloride in THF dry at low temperature. The desired compounds **2** and **3** were obtained with a 15 and 28% of yields, respectively.



Scheme 2.6 Silibinin building blocks and their corresponding phosphoramidites. (a) isobutyryl chloride, TEA, THF dry (b) 2-cyanoethyl-N,N-diisopropylamino-chlorophosphoramidite, DIEA, DCM dry, r.t.

In order to prepare the corresponding phosphoramidites, we converted **2** and **3** (Scheme 2.6) into the phosphoramidites **4** and **5** by a classic reaction with 2-cyanoethyl-N,N-diisopropylamino-chlorophosphoramidite. After work-up the silica gel chromatography afforded desired compounds **4** and **5** in a 65% yields. The identity of compounds **2–5** was confirmed by NMR (^1H , ^{13}C and ^{31}P) and ESI-MS data. Compounds **2** and **3** were coupled, in the three different provisions (Scheme 2.7), with phosphoramidites **4** and **5** using 0.45 M 4,5-dicyanoimidazole (DCI) in ACN. After oxidation treatment with 5.5 M tert-butyl hydroperoxide solution in decane, the crude material was purified by gel chromatography. Several complex fractions were recovered by gel chromatography, but only those with the signal ^{31}P NMR active, were treated with conc. aq. ammonia and MeOH (1:1, v:v) at 50 °C, allowing full deprotection from isobutyryl and cyanoethyl groups. After RP-18 HPLC purification the products were converted into the corresponding sodium salts by cation exchange on a DOWEX (Na^+ form) resin, leading to the desired phosphodiester derivatives **6–8** in good yields (50, 45 and 60 % respectively, Scheme 2.7).



Scheme 2.7 Phosphodiester silibinin dimers derivatives.

Given the very complex mixture (there are at least three diastereoisomers for each dimer) the NMR analysis was not possible. The products obtained by cation exchange were suspended in DMSO- d_6 , but the goodness of NMR spectra did not allow a detailed assignment of different spin systems. The structures of **6–8** were confirmed by ^{31}P NMR and MALDI-MS analyses.

In order to evaluate their antioxidant activity, all silibinin dimers were subjected to 2,2-diphenyl-1-picrylhydrazyl free radical scavenging assay.¹⁸ The DPPH test is the non-enzymatic method currently used to provide basic information on the scavenging potential of stable free radicals *in vitro*. From the DPPH assay shown in Table 2.8, we observed that conjugating two moieties of silibinin did not led to a significant reduction of quenching properties. For comparison purposes, the antioxidant activities of quercetin were evaluated as controls. In particular the new silibinin dimers **6–8** showed DPPH radical scavenging activities higher to that of the silibinin, confirming that the introduction in 9" of the moiety did not influence the radical scavenging activity of this metabolite.^{22, 23}

Blood and extracellular fluid contain a large number of hydrolytic enzymes, such as cholinesterase, aldolase, lipase, dehydropeptidase, alkaline and phosphatase. Several functional groups, such as ester, amide, carbamate, lactam, lactone, sulphonamide and, of course, phosphate, are susceptible to plasma

degradation. If the new compound has affinity for one of these enzymes and it has a hydrolyzable group in the right position, it can be decomposed in the plasma.

Hydrolysis in plasma can be a major cause of compound clearance, and pharmacologically efficacious concentrations may not be achievable in vivo pre-clinical study. Since the presence of a junction of phosphodiester type, which could be susceptible to the action of endogenous phosphatase before the active complex can reach its molecular target, the stability of the new synthesized conjugates was evaluated in human serum, and monitored over time by of HPLC analysis. New dimeric phosphate-linked silibinins (**6–8**) were incubated at 37 °C with human serum from human male AB plasma in Tris•HCl buffer (pH = 8.0 containing 10 mM MgCl₂). Small aliquots of the mixture were taken at defined time intervals, kept at 85 °C for 5 min, equilibrated at rt, and analyzed by HPLC on an RP column. As shown in Table 2.8 the dimers **6–8** are degraded with a time half-lives of 81 to 87 hours.

Table 2.8 Summary table of MALDI analysis, HPLC retention times, IC₅₀ and serum half lives for compound **6–8**.

	MALDI	<i>t_R</i> (min)	DPPH IC ₅₀ (mM)	<i>t</i> _{1/2} (hours)
6	<i>calcd</i> for C ₅₀ H ₄₃ O ₂₂ P = 1026.20 found [M-H] ⁺ = 1025.79; [MNa-H] ⁺ = 1047.25	17.32	0.99 ± 0.04	86.7
7	<i>calcd</i> for C ₅₀ H ₄₃ O ₂₂ P = 1026.20 found [M-H] ⁺ = 1025.11; [MNa-H] ⁺ = 1047.03	17.24	0.63 ± 0.05	85.6
8	<i>calcd</i> for C ₅₀ H ₄₃ O ₂₂ P = 1026.20 found [M-H] ⁺ = 1025.66; [MNa-H] ⁺ = 1047.05	17.17	0.99 ± 0.07	81.2
Silibinin (1)			1.4 ± 0.06	
Quercetin			0.18 ± 0.01	

As reported the water solubility of silibinin change according to the experimental conditions such as pH and temperature.^{25,26} In this work we determined the solubility of the new dimers (**6–8**) and then compared to the water solubility of the silibinin (**1**).

Solubility experiments were performed by dissolving selected compound in milli-Q water leaving that 10 min under magnetic stirring in the dark. Molar absorption coefficients (ε) for each compound in water were determined from calibration curves (absorbance vs concentration) with successive dilutions of a stock solution using the Beer-Lamberts law. For silibinin, stock solution was prepared in CH₃CN (1.18 mg in 1 mL) and absorption spectra were recorded by diluting silibinin solution in H₂O (2% of

CH₃CN used to increase the solubility and, as consequence, quantification limit) UV-Vis spectra were presented in Figure 2.7 revealing the presence of absorption band centered around 288 corresponding to the π - π^* transition and around 334 nm that should be also due to the transition n - π^* on the oxygen of ketones groups.

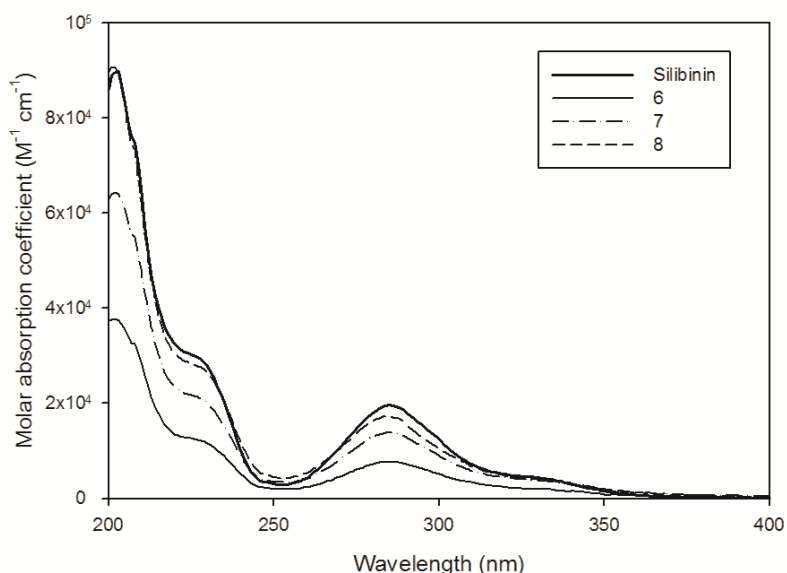


Figure 2.7. UV-vis spectra of Silibinin in H₂O:CH₃CN 98:2 solution and dimers in water at pH 6.5.

Silibinin results only slightly dissolved when 0.5 mg were suspended in 10 mL of water and, after filtration, the absorption spectrum reveals a maximum at 288 nm with an absorption of ~ 0.018 . The saturated concentration of silibinin in water was estimated to be $0.9 \mu\text{M}$ corresponding to $\sim 0.44 \text{ mg L}^{-1}$. The solution was also keep in the dark and stirred during 12 hours without any significant solubility increase.

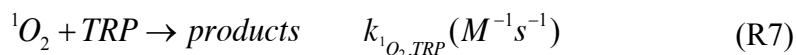
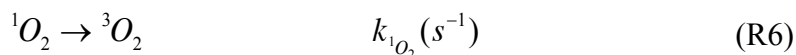
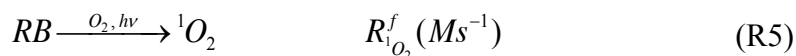
In order to investigate the possible increase of solubility as function of pH, a phosphate buffer solution at pH 8.0 was prepared (buffer concentration of 1 mM) and 0.35 mg of silibinin is suspended in 15 mL. After 10 min of magnetic stirring followed by $0.45 \mu\text{m}$ filtration, the UV-vis spectrum reveals the presence of two absorption maxima: the first at 288 attributed to the silibinin with an absorption of ~ 0.02 (in agreement with solubility determination) and a second centered around 330 nm indicating the formation of degradation products of silibinin in alkaline solutions.

Solubility experiments that are summarized in Table 2.9 indicate that dimers were completely dissolved (0.1 mg in 10 mL) in water and solubility can be estimated to be $\geq 19.5 \mu\text{M}$ corresponding to $> 20 \text{ mg L}^{-1}$.

Table 2.9. Spectroscopic characteristics and solubility of silibinin and dimers in solution. *solution is saturated and suspension is filtered before analysis.

Compound	ϵ_{288nm} (M ¹ cm ¹)	Suspended in 10 mL	Theoretical concentration	Abs _{288 nm} after filtration	Solubility in water
1	22158 (CH ₃ CN) 19469 (H ₂ O:CH ₃ CN 9.8:0.2)	0.44 mg	91.2 μM*	0.018	≤ 0.9 μM
6	9100 (H ₂ O)	0.10 mg	19.5 μM	0.18	≥ 19.5 μM
7	14435 (H ₂ O)	0.10 mg	19.5 μM	0.30	≥ 19.5 μM
8	18536 (H ₂ O)	0.10 mg	19.5 μM	0.35	≥ 19.5 μM

Reactivity between silibinin and dimers with ¹O₂ and HO• was determined by use of Rose Bengal and hydrogen peroxide as respective ROS sources. For singlet oxygen, the steady-state concentration in solution ([¹O₂]_{ss}) in water is determined following tryptophan disappearance at different initial concentrations. The kinetic approach is summarized in reaction R5-R7



With $R_{1O_2}^f$ the formation rate of ¹O₂ upon irradiation of RB, k_{1O_2} the pseudo-first decay of singlet oxygen in water (2.4×10^5 s⁻¹) and $k_{1O_2,TRP}$ the second order rate constant between ¹O₂ and TRP (3.2×10^7 M⁻¹s⁻¹).²⁷ Application of the steady-state approximation to [¹O₂] leads to the following equation:

$$\frac{1}{R_{TRP}^d} = \frac{1}{R_{1O_2}^f} \left(1 + \frac{k_{1O_2}}{k_{1O_2,TRP}[TRP]} \right) \quad (EQ1)$$

$\frac{1}{R_{TRP}^d}$ data vs. different TRP concentrations can be fitted with a linear equation $y = ax + b$ leading to the quantification of singlet oxygen steady state concentration ($[^1O_2]_{ss}$) that is equal to the ration $R_{1O_2}^f / k_{1O_2}$.

Dimers reactivity toward singlet oxygen results to be closed to the value determined for silibinin (see dimer **6**) or about 35% lower in the case of dimer **8** (Table 2.10). Second order rate constant fare in the same order of magnitude reported in literature for molecules with similar structure. Morales and co-workers²⁸ reported a reactivity ranging from 2.4 to $13.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for flavonoid derivative such as quercetin and morin.

Table 2.10 Singlet oxygen steady state concentrations $[^1O_2]_{ss}$ and second order rate constant ($k_{1O_2,X}''$) between singlet oxygen and silibinin or dimers in THF and H₂O solutions.

Compound	THF		H ₂ O	
	$[^1O_2]_{ss} \text{ (M)}$	$k_{1O_2,X}'' \text{ (M}^{-1}\text{s}^{-1})$	$[^1O_2]_{ss} \text{ (M)}$	$k_{1O_2,X}'' \text{ (M}^{-1}\text{s}^{-1})$
1	3.79×10^{13}	$6.1 \pm 0.4 \times 10^7$	-	-
6	-	-	4.07×10^{13}	$6.8 \pm 1.2 \times 10^7$
7	-	-	4.07×10^{13}	$5.4 \pm 0.3 \times 10^7$
8	-	-	4.07×10^{13}	$4.0 \pm 0.2 \times 10^7$

Experimental data like those reported in Figure 2.8 for dimers **7** and **8** (Abs/Abs₀ followed at 475 nm vs dimer concentration) are fitted with a linear equation (E1) leading to the estimation of second order rate constant between hydroxyl radical and selected molecule.

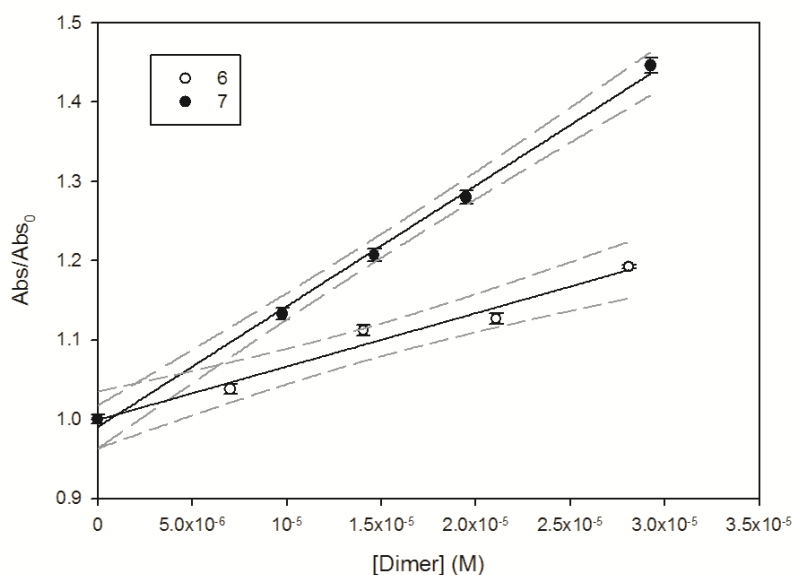


Figure 2.8. Abs/Abs₀ measured at 475 nm as function of dimers **6** and **7**. The solid line represents the linear fit of the experimental data and dashed lines denote the 95% confidence interval of this fit.

Estimation of second order rate constant indicates that the most reactive species are dimer **7** and **8** with a second order rate constant $\geq 1.5 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ (see Table 2.11 summarizing experimental results). Wang and co-workers²⁹ investigated the reactivity of hydroxyl radical with phenolic compounds in order to estimate their antioxidative ability using aqueous pulse radiolysis. The value of $1.5 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ was found for quercetin that is closed to those estimates for green tea polyphenols. Interestingly, Husain et al.³⁰ reported that reactivity of flavonoids toward photogenerated hydroxyl radical increases with the number of hydroxyl groups in the aromatic ring.

Table 2.11: Second order rate constant ($k_{HO^\bullet, X}^{II}$) between hydroxyl radical and silibinin or dimers in H₂O/CH₃CN (9/1) solutions.

Compound	$k_{HO^\bullet, X}^{II} (\text{M}^{-1}\text{s}^{-1})$
1	$8.26 \pm 1.64 \times 10^9$
6	$6.73 \pm 0.32 \times 10^9$
7	$1.52 \pm 0.08 \times 10^{10}$
8	$\geq 2.0 \times 10^{10}$

In conclusion, new dimeric linked-phosphodiester silibinins were synthesized and their biological and radical scavenger behaviour were evaluated. Exploiting our solution phase strategy, a variety of structurally diverse silibinin dimers were successfully realized in a short time and in very good yields. New derivatives were subjected to DPPH free radical scavenging assay in preliminary study to evaluate their antioxidant activities. Thanks to the conjugation through a phosphodiester junction of two units of molecule, all compounds exhibited radical scavenging activities quite higher than to silibinin. In the other hand, the new derivatives showed water solubility well above that of silibinin. Since the presence of a junction of phosphodiester type, which could be susceptible to the action of endogenous phosphatase before the active complex can reach its molecular target, the stability of the new synthesized conjugates was evaluated in human serum, and as shown in Table 2.8 the dimers (**6–8**) are degraded with a time half-lives of 81 to 87 hours and this makes them ideal candidates for further clinical trials. Moreover, for all the new analogues, their reactivity against some oxygen free radicals ($^1\text{O}_2$ and $\text{HO}\cdot$) was investigated. Dimers reactivity toward singlet oxygen results to be closed to the value determined for silibinin. Second order rate constant fare in the same order of magnitude reported in literature for molecules with similar structure. These data encouraged our future studies aimed to improve the synthetic strategy to realize libraries of optically pure linked-phosphate silibinin dimers.

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Chapter 3: Experimental Session

3.1 New Silibinin glyco-conjugates: Synthesis and evaluation of antioxidant properties.

3.1.1 General procedure for the synthesis of conjugates (6–9):

Phosphoramidite **1** (150 mg, 0.18 mmol) and the select saccharide compound **2–5** (0.19 mmol, previously dried and kept under reduced pressure, were reacted with a 0.45 M tetrazole solution in anhydrous CH₃CN (1.0 mL, 0.45 mmol). The reaction was left under stirring at room temperature and monitored by TLC in the eluent system *n*-hexane:AcOEt, (1:2, v:v). After 1 hour, a 5.5 M tert-butyl hydroperoxide (tert-BuOOH) solution in decane (70 µL, 0.40 mmol) was added to the mixture and left under stirring at room temperature. After 30 minutes the reaction mixture was diluted with CHCl₃, transferred into a separatory funnel, washed three times with water and dried under reduced pressure. The next treatment with TEA/pyridine (1:1, v/v) to 1 hour at 50 °C and then with aq. ammonia (28%)/CH₃OH (1:1, v:v) for 1 hour at room temperature, allowed full deprotection from acetyl and 2-cyanoethyl groups. The dried mixtures were purified by a Sep-Pak C18 Cartridge and then converted into the corresponding sodium salts by cation exchange on a DOWEX (Na⁺ form) resin to have a homogeneous samples in good yield (60-72%).

Compound **6** (brown powder, 88.1 mg, 68%): ³¹P NMR (161.98 MHz, D₂O, room temperature, mixture of diastereoisomers) δ 2.9, 1.4, 0.8. HRMS (MALDI-TOF, negative ions) m/z calcd for C₃₁H₃₂O₁₈P = 723.1332, found 723.1332 [M - H]⁻.

Compound **7** (brown powder, 94.0 mg, 72%): ³¹P NMR (161.98 MHz, D₂O, room temperature, mixture of diastereoisomers) δ 1.3, 0.9, 0.7. HRMS (MALDI-TOF, negative ions) m/z calcd for C₃₁H₃₂O₁₈P = 723.1332, found 723.1333 [M - H]⁻.

Compound **8** (brown powder, 82.3 mg, 63%): ³¹P NMR (161.98 MHz, D₂O, room temperature, mixture of diastereoisomers) δ 2.4, 1.6, 1.1, 0.8. HRMS (MALDI-TOF, negative ions) m/z calcd for C₃₁H₃₂O₁₈P = 723.1332, found 723.1333 [M - H]⁻.

Compound **9** (brown powder, 104.8 mg, 60%): ³¹P NMR (161.98 MHz, D₂O, room temperature, mixture of diastereoisomers) δ 1.1, 0.8. HRMS (MALDI-TOF, negative ions) m/z calcd for C₃₇H₄₂O₂₃P = 885.1860, found 885.1859 [M - H]⁻.

3.1.2 DPPH radical scavenging activity assay

The antioxidant activities of the compounds was assessed by examining their abilities to scavenge the 1,1-diphenyl-2-picrylhydrazyl free radical. DPPH solution (20 mg/mL) was prepared in methanol. The compounds was dissolved in methanol to prepare the stock solution (1 mM). Freshly prepared DPPH solution was taken in test tubes and analogue solutions (1 mM–1 μ M) were added to every test tube so that the final volume was 2.25 mL and after 10 min, the absorbance was read at 515 nm. Quercetin and 2,3-dehydrosilybin were used as a reference standards and dissolved in methanol to make the stock solution with the same concentration (1 mM).

3.1.3 Xanthine Oxidase Inhibition Assay.

Oxidative stress was induced by incubating MKN28 cells with XO (10-100 mU/mL) in the presence of its substrate xanthine (X) (1 mM) for periods of up to 3 hours. We examined the effect of silibinin and of our analogues **6–9** on X/XO induced cell damage. In particular, cells were incubated with serum free medium (control) for 1 - 48 hours; with serum free medium for 148 hours and then with X (1 mM) XO (50 mM) for 2 hours (X/XO control); with **6–9** and silibinin (10–200 μ M) for 148 hours and then, after washing, with X (1 mM) XO (50 mM) for 2 hours. Subsequently, we determined cell viability by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide] MTT assay in MKN28 cultured cells.

3.2 Synthesis, biological and electrochemical investigation of new silibinin glyco-phosphodiester conjugates

3.2.1 Synthesis of silibinin phosphoramidite building block 3

Silibinin (1.6 g, 3.3 mmol) previously dried by repeated coevaporate with anhydrous THF, was dissolved in 8 mL of anhydrous pyridine and DMTCl (1.48 g, 4.37 mmol) was added. The mixture was kept at 50 °C for 3 hours. After monitoring by TLC, the reaction was stopped by addition of CH₃OH and the mixture dried under reduced pressure. The crude material was purified by column chromatography with a gradient of *n*-hexane in CHCl₃, from 10% to 0%, and then increasing the polarity eluting with CHCl₃ and increasing percentages of CH₃OH from 0% to 5%. In all of the eluants, it was used 0.5% of Et₃N. The derivative 9"-ODMT was obtained in good yield. (2.5 g, 3.18 mmol, 96%).

9"-ODMT silibinin (2.5 g, 3.18 mmol) was dissolved in 8 mL of pyridine and isobutyric anhydride was added (6.5 mL, 60 mmol). The reaction mixture was kept under stirring for 2 hours at room temperature. After monitoring by TLC, the reaction was stopped by addition of CH₃OH and then kept at 0 °C for 1 hour. The mixture was extracted with a saturated aqueous solution of NaHCO₃ and CHCl₃ and the organic layer was dried over anhydrous Na₂SO₄ and concentrated under reduced pressure to lead 3,5,7,4"-tetra-O-isobutyryl-9"-O-DMT silibinin (2.70 g, 2.53 mmol, 79%).

3,5,7,4"-tetra-O-isobutyryl-9"-ODMT silibinin (2.70 g, 2.53 mmol) was selectively deprotected from DMT group with a solution of HCOOH 5% in CH₂Cl₂ (150 mL). The reaction mixture was kept under stirring for 1 hour at room temperature. After monitoring by TLC, the mixture was washed with a saturated aqueous solution of NaHCO₃ and the organic phase was dried over anhydrous Na₂SO₄ and evaporated under reduced pressure. The crude material was purified by column chromatography eluting with a gradient of ethyl acetate in hexane from 50% to 70% to obtain the compound **2** in good yield (1.5 g, 2.05 mmol, 81%).

To 3,5,7,4"-tetra-O-isobutyryl silibinin (240.0 mg, 0.33 mmol) dissolved in anhydrous CH₂Cl₂ (5 mL), DIEA (390 µL, 2.22 mmol), and 2-cyanoethyl-N,N-diisopropylamino-chlorophosphoramidite (107 µL, 0.48 mmol) were mixed. After 30 min the solution was diluted with EtOAc, and the organic phase was washed twice with brine and then concentrated. Silica gel chromatography of the residue (eluent *n*-hexane:EtOAc, 3:7, v/v, in the presence of 1% of Et₃N), afforded desired compound **3** (205.0 mg, 0.24 mmol) in a 65% yield.

3.2.2 General procedure for the synthesis of monosaccharide building blocks 8–12

Different 1-O-methyl-mono- and di-saccharides (Glc, Man, Gal, GlnAc, Thre, 2.07 mmol), previously coevaporated with anhydrous pyridine, were dissolved in anhydrous pyridine (8 mL) and reacted with DMTCl (1.86 mmol). The reaction mixture was stirred at room temperature for 2 hours (checked by TLC analysis) then quenched with MeOH and evaporated to dryness. The residue was extracted with CHCl₃, the organic phase was dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The crude material was purified by column chromatography (98:2 chloroform/methanol with 0.5% of pyridine), leading a good yield of the 6-O-DMT glycosides (55-85%). Then, the 6-O-DMT-1-O-methyl-glycoside (1.45 mmol) was solubilized in anhydrous pyridine (2 mL), and to the resulting mixture was added acetic anhydride (1.3 mL, 14 mmol). The mixture was stirring at room temperature for 12 hours (checked by TLC analysis). The reaction was quenched with MeOH at 0 °C and washed with CHCl₃, the organic phase was dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. Subsequently, we proceeded to remove the DMT group of full protected 1-O-methyl-glycoside (1.45 mmol) with a solution of HCOOH 5% in CH₂Cl₂ and triisopropylsilane (1.45 mmol) under stirring at 0 °C.

On completion of reaction, the resulting mixture was diluted with CH₂Cl₂ and washed with a saturated aqueous solution of NaHCO₃. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The crude material was purified by column chromatography (chloroform/methanol, 98:2, v:v), leading the compounds **8–12** in good yields (55–65%).

3.2.3 Synthesis of lactose building blocks 13

Lactose octaacetate (1 g, 1.47 mmol) and I₂ (523 mg, 2.06 mmol) were dissolved in anhydrous dichloromethane (10 mL). After completed dissolution, the reaction mixture was cooled to 0 °C and the Et₃SiH (329 µL, 2.06 mmol) was added. After 5 minutes the reaction was refluxed until TLC analysis displayed complete consumption of the peracetylated sugar (the glycosyl iodides are partially unstable on TLC). The mixture was cooled to RT and then diluted with CH₂Cl₂. The organic phase was washed with a 5% solution of NaHCO₃ containing Na₂S₂O₃ (to remove residual amounts of iodine). The aqueous phase was re-extracted with CH₂Cl₂, and the combined organic layers were dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The glycosyl iodide intermediate was obtained in good yield (1.01 g, 1.3 mmol, 90%).

A mixture of appropriate glycosyl iodide (1.01 g, 1.3 mmol) and Ag_2CO_3 (69 mg, 0.65 mmol) was stirred at room temperature in anhydrous MeOH (20 mL) in presence of 3-Å molecular sieves for one day. On completion of the reaction (checked by TLC analysis), the mixture was filtered with CHCl_3 , concentrated under reduced pressure and directly deprotected with a solution of MeOH/ $\text{NH}_4\text{OH}_{\text{aq}}$ (1:1, v:v) for 30 minutes. The mixture was purified by column chromatography (chloroform/methanol, 9:1) to give the pure product β -1-O-methyl-lactoside (454 mg, 1.27 mmol, 98%).

The methyl lactoside (454 mg, 1.27 mmol) was subsequently dissolved in anhydrous pyridine (8 mL) and reacted with DMTCl (386 mg, 1.14 mmol). The reaction mixture was stirred at room temperature for 2 hours, then quenched with CH_3OH and evaporated to dryness. The residue was extracted with CHCl_3 , the organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure.

The 6-O-DMT- β -1-O-methyl-lactoside was purified by column chromatography (9:1 chloroform/methanol with 0.5% of pyridine) (343 mg, 0.52 mmol, 41%).

Subsequently, 6-O-DMT- β -1-O-methyl-lactoside (343 mg, 0.52 mmol) was fully acetylated with acetic anhydride (4 mL, 42.3 mmol) in anhydrous pyridine (8 mL) for 12 hours. The reaction mixture was quenched with MeOH and then washed with CHCl_3 , the organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Finally, the fully acetylated 6-O-DMT- β -1-O-methyl-lactoside was regioselectively deprotected from DMT group at the position 6 of the galactosyl residue with a solution of 5% HCOOH in CH_2Cl_2 and triisopropylsilane (1 mmol) under stirring at 0 °C for 1 hour. The reaction mixture was diluted with CH_2Cl_2 and washed with 5% of NaHCO_3 aqueous solution and finally with H_2O . The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The product **13** was obtained in pure form and in good yield (196 mg, 0.32 mmol, 61%) by column chromatography (chloroform/methanol, 9:1).

3.2.4 General procedure for the synthesis of silibinin phosphodiester glyco-conjugates (14–19)

The coupling reaction between the phosphoramidite and the mono and disaccharides appropriately protected was carried out by suspending the derivatives **7–12** (0.6 mmol) in a solution of DCI in acetonitrile (0.45 M, Activator 42®) and by adding 3-Å molecular sieves. After few minutes, the mixture was added to the silibinin phosphoramidite (0.54 mmol) and was kept under stirring for 1 hour.

On completion of reaction monitoring by TLC analysis, tert-butyl hydroperoxide solution in decane (5.5 M) was added and after 30 minutes, the solvent was removed under vacuum and the crude material

was purified by column chromatography (chloroform/methanol, 99:1, 95:5). The products obtained were subsequently subjected to treatment with ammonia aqueous solution and methanol (1:1, v/v) at 25 °C for 3 hours for the full deprotection of protecting groups.

After the deprotection treatment, the crude material was purified by reverse phase HPLC using a Phenomenex Luna® C18 column eluted with ammonium acetate 0.1 M with a linear gradient 20-100% ACN in 50 minutes (flow = 7 mL/min). Then, the material was desalted on Chromafix® C18 column (deionized H₂O/CH₃OH, 8:2, 1:1, CH₃OH) and converted into the corresponding sodium salt by cation exchange on DOWEX resin (Na⁺) leading the compounds **14–19** in good yields (45–60 %).

3.2.5 Analysis to serum stability of silibinin glyco-conjugates

Reactions (500 µL reaction volume) were performed in 50 mM Tris•HCl pH 8.0 containing MgCl₂ (10 mM), silibinin conjugates (50 µL vol of a 10 µM solution), and human serum from human male AB plasma (400 µL). All reactions were incubated at 37 °C. From this solutions were taken samples of constant volume at different times, and the HPLC profile was analyzed in comparison with that of the intact compound.

3.2.6 DPPH radical scavenging activity assay

The antioxidant activities of the compounds was assessed by examining their abilities to scavenge the 1,1-diphenyl-2-picrylhydrazyl free radical. DPPH solution (20 mg/mL) was prepared in methanol. The compounds was dissolved in methanol to prepare the stock solution 2 mM). Freshly prepared DPPH solution was taken in test tubes and analogue solutions (2 mM–5 µM) were added to every test tube so that the final volume was 2.25 mL and after 10 min, the absorbance was read at 515 nm. Quercetin and Trolox were used as a reference standards and dissolved in methanol to make the stock solution with the same concentration (2 mM).

3.2.7 Citotoxicity on on human liver cancer cell (Hep G2)

Silibinin and its new glyco-conjugates were solubilised in DMSO at a final concentration of 13.5 mM and then diluted in water to obtain the operational concentrations to be used for the tests on cells. Cytotoxic activity of different amounts of silibinin or its derivatives (0.3, 3 and 30 µM) was measured

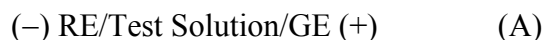
at different incubation times (24, 48 and 72 hours) by a colorimetric assay. As a control, cell viability was assessed in the absence of the effectors.

3.2.7 Determination of the acidity constants by potentiometric titrations, UV-Vis spectrophotometric measures and Circular Dichroism (CD-UV) of silibinin solutions. Redox behaviour carried out by cyclic voltammetry

In according to the chemical structure of the molecule, silibinin shows many OH groups, that are ionizable. The acid-base behavior can be represented by general equilibria:



The study is conducted by potentiometric methods at 25 °C in 0.1 M NaClO₄ as ionic medium. The measurements are carried out as potentiometric titrations where the hydrogenionic concentration is determined by the e.m.f. of cell (A):



in which GE symbolizes glass electrode and RE is the reference half-cell:



The test solution has the general composition:



C_{HnL} is chosen to be from $2.0 \times 10^{-3} \text{ M}$ to $8.0 \times 10^{-3} \text{ M}$ for silibinin glyco-conjugates (for silibinin the range is $2.0 \leq C_L \leq 6.0 \cdot 10^{-3} \text{ M}$, with 50% oh CH₃OH), whereas $(C_A - C_B) = C_H$ is varied into $-1.0 \times 10^{-3} \text{ M} - 9.0 \cdot 10^{-3} \text{ M}$. The e.m.f. of cell (A) at 25 °C may be written as:

$$E = E_0 - 0.05916 \times \log([H^+]_{y_H}) + E_J \quad (3.3)$$

where E_0 is a constant in each titration, y_H is the activity coefficient of the H⁺ ion and E_J represents the liquid junction potential at the test solution 0.1 M NaClO₄ boundary.¹

It depends on the hydrogen ions concentration in the equation:

$$E_J = \square 0.35 \times h \quad (3.4)$$

Activity coefficients tend to 1 and E_J tend to 0 as the composition of test solution approaches 0.1 M NaClO₄.

Each titration consists of two parts. In first, E is measured in the absence of silibinin, or its conjugates, and $(C_A - C_B) = [H^+]$ is varied between 2×10^{-3} M and 5×10^{-5} M. From these data E_0 values constant to within ± 0.05 V is calculated, assuming $E_J = -0.51 \times [H^+] + 0.24 \times K_w[H^+]^{-1}$. In the second part, in the presence of ligand, acid-base behavior is followed by alcalification and/or acidification.

Placing:

$$E_G^0 = E_G^{0'} + 0.05916 \times \log y_H$$

The equation (3.3) takes the form:

$$E_G = E_G^0 + 0.05916 \times \log h + E_J \quad (3.5).$$

From experimental data (C_L , C_{HnL} , $[H^+]$) the average number of protons (Z_H) released per L molecule is evaluated by:

$$Z_H = ([H^+] - C_H - K_w/[H^+])/C_{HnL} \quad (3.6)$$

where a value $K_w = 10^{-13.78}$ was utilized.

Bringing Z_H as function of pH the experimental data are in agreement with the solid line obtained with three equilibria.

A speciation model was obtained by processing the experimental data using *Hyperquad* program.²

The criterion used is to determine the values of the constants that make K_{An} minimize the sum U of squared weighted residuals:

$$U = \square (E_{G,i}^{\text{exp}} - E_{G,i}^{\text{calc}})^2 \quad (3.7)$$

where, for the i -th measurement, $E_{G,i}^{\text{exp}}$ and $E_{G,i}^{\text{calc}}$ represent, respectively, the experimental potential and that calculated for a set of constant value K_{An} .

The minimum of the function U is found by the method of Newton-Raphson. Once the minimum of U , U_{\min} , the agreement between the experimental and calculated data is given by the variance σ^2 defined as:

$$\sigma^2 = U_{min}/(n - m) \quad (3.8)$$

where n represents the number of experimental data and m is the number of K_{Am} constants.

The constants standard deviations are obtained by the diagonal elements of the variance-covariance matrix, calculated according to the equation:

$$M_v = \sigma^2 H^{-1} \quad (3.9)$$

where H is the matrix of the normal equations and it is a square matrix of order m.

The acid-base properties of the silibinin and its glyco derivatives are studied also by UV-VIS spectrophotometric procedures, that confirm the results obtained by potentiometric measurements. The experimental data collected are processed by the numerical minimization program Hyspec, Hyperquad package, which minimizes the standard deviation between the measured absorbance and those calculated by the proposed model constants. The results obtained are in agreement with those obtained from the potentiometric. Also, circular dichroism (CD-UV) of silibinin solutions were made. Measurements in solution, at different pH, show variations in the CD spectrum that follow the same changes registered in the UV-VIS spectra. For the voltammetric measurement, a polarograph Varian 767 METROM it was utilized, with a measurement electrode in glassy carbon, auxiliary electrode in Pt and a reference electrode of Ag/AgCl_(s).

3.3 Dimeric phosphate-linked silibinins: synthesis, spectroscopic properties, solubility and radical scavenger ability

3.3.1 Chemicals

Silibinin is purchased from Sigma-Aldrich. HPLC grade MeCN, MeOH are purchased from Carlo Erba Reagents and Sigma-Aldrich. Hydrogen peroxide (30 %), from Fluka. Unless otherwise indicated, other chemicals are obtained from Sigma Aldrich and purity is > 98 %.

Fresh aqueous solutions of H₂O₂, Rose Bengal and tryptophan are prepared before each experiment. The concentration of the stock solution of H₂O₂ in milli-Q water (Laser flash photolysis experiments) is determined using a molar absorption coefficient of $38.1 \pm 1.4 \text{ M}^{-1} \text{ cm}^{-1}$ at 240 nm.⁹

3.3.2 General methods

Reactions for dimers' synthesis are monitored by TLC (precoated silica gel plate F254, Merck) and Column chromatography: Merck Kieselgel 60 (70-230 mesh). The analysis is performed with a Shimadzu LC-8A PLC system equipped with a Shimadzu SCL-10A VP System control and Shimadzu SPD-10A VP UV-VIS Detector. HPLC purifications are carried out on Phenomenex RP18 column (5- μm particle size, 10.0 mm \times 250 mm i.d.) using a linear gradient of ACN in 0.1 M TEAA in H₂O, pH 7.0 from 5% to 100% over 30 minutes at a flow rate of 1.5 mL/min with detection at 260 nm.

For the ESI MS analyses, a Waters Micromass ZQ Instrument equipped with an Electrospray source, is used. MALDI TOF mass spectrometric analyses are performed on a PerSeptive Biosystems Voyager-De Pro MALDI mass spectrometer.

Absorption spectra are measured with a Varian Cary 100 Scan UV-vis spectrophotometer using 1 cm pathlength quartz cell.

3.3.3 Synthesis of silibinin building block 2

Silibinin **1** (1.5 g, 3.1 mmol) previously dried by repeated coevaporate with anhydrous THF and then was dissolved in 8 mL of anhydrous THF and TEA (2.1 mL, 15.4 mmol). Isobutyryl chloride (0.980 mL, 9.3 mmol) was added and the mixture was kept at 0 °C for 30 minutes. After monitoring by TLC, the reaction was stopped by addition of MeOH and the mixture was extracted with CH₂Cl₂. The organic phase was dried over anhydrous Na₂SO₄ and concentrated under reduced pressure, the crude material was purified by column chromatography (CHCl₃/acetone, 96:4, v/v). The derivative 3,5,7,4"-tetra-O-isobutyryl silibinin was obtained as pale amorphous solid (0.365 g, 0.47 mmol, 15%).

2 $R_f = 0.6$ (benzene/AcOEt, 80:20, v/v). ^1H NMR (CDCl_3 , 400 MHz, room temperature, mixture of two diastereoisomers): $\delta = 7.15\text{--}6.96$ (complex signals, 6H, H-2', H-5', H-6', H-2'', H-5'', H-6''), 6.77 (complex signal, 1H, H-8), 6.58 (*s*, 1H, H-6), 5.71 and 5.69 (*d*, 1H, $J = 12.2/12.3$ Hz, H-3), 5.38 (*m*, 1H, H-2), 5.08 and 5.06 (*d*, 1H, $J = 8.3/8.7$ Hz, H-7''), 4.04 (*m*, 1H, H-8''), 3.88–3.83 (overlapped signals, 4H, OCH_3 , H-9''a), 3.58 (*dd*, 1H, $J = 3.2$ Hz, $J = 12.0$ Hz, H-9''b), 2.98–2.74 (complex signals, 3H, CH of isobutyryl groups), 2.62–2.52 (*m*, 1H, CH of isobutyryl group), 1.41–1.25 (*m*, 18H, CH_3 of isobutyryl groups), 1.15–1.10 (*m*, 6H, CH_3 of isobutyryl group) ppm. ^{13}C NMR (CDCl_3 , 100 MHz, room temperature, mixture of diastereoisomers): $\delta = 185.0, 175.2, 175.1, 175.0, 174.1, 162.5, 162.4, 156.5, 151.7, 151.6, 144.0, 144.1, 143.7, 140.6, 134.5, 128.7, 123.1, 123.0, 121.0, 120.8, 119.8, 119.7, 117.1, 117.0, 116.6, 116.4, 111.2, 111.1, 108.7, 81.1, 81.0, 78.3, 78.4, 76.0, 75.9, 72.9, 72.8, 61.5, 56.1, 56.0, 34.3, 34.0, 33.7, 19.0, 18.8, 18.7, 18.6$ ppm. ESI-MS (positive ions): m/z calculated for $\text{C}_{41}\text{H}_{46}\text{O}_{14} = 762.29$; found: 763.84 $[\text{M-H}]^+$.

3.3.4 Synthesis of silibinin building block 3

Silibinin **1** (1.0 g, 2.7 mmol) previously dried by repeated coevaporate with anhydrous THF, was dissolved in 8 mL of anhydrous THF and TEA (2.1 mL, 15.4 mmol) and isobutyryl chloride (1.1 mL, 10.4 mmol) were added. The mixture was kept at 0°C for 30 minutes. After monitoring by TLC, the reaction was stopped by addition of MeOH and the mixture was extracted with CH_2Cl_2 , the organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude material was purified by column chromatography (eluent $\text{CHCl}_3/\text{acetone}$, 96:4, v/v). The derivative 5,7,4'',9''-tetra-O-isobutyryl silibinin was obtained as pale amorphous solid (0.447 g, 0.58 mmol, 28%).

3 $R_f = 0.9$ (benzene/AcOEt, 80:20, v/v). ^1H NMR (CDCl_3 , 400 MHz, room temperature, mixture of two diastereoisomers): $\delta = 7.21\text{--}6.98$ (complex signals, 6H, H-2', H-5', H-6', H-2'', H-5'', H-6''), 6.76 (complex signal, 1H, H-8), 6.59 (*s*, 1H, H-6), 5.07 (*d*, 1H, $J = 12.8$ Hz, H-2), 5.00 (*d*, 1H, $J = 8.3$ Hz, H-7''), 4.56 and 4.55 (*d*, 1H, $J = 12.3/12.4$ Hz, H-3), 4.44 (*dd*, 1H, $J = 2.3$ Hz, $J = 12.9$ Hz, H-9''a), 4.26 (*m*, 1H, H-8''), 3.98 (*dd*, 1H, $J = 2.3$ Hz, $J = 12.8$ Hz, H-9''b), 3.86 (*s*, 3H, OCH_3), 2.98–2.76 (complex signals, 3H, CH of isobutyryl groups), 2.61 (*ept*, 1H, $J = 6.8$ Hz, CH of isobutyryl group), 1.36 (complex signals, 18H, CH_3 of isobutyryl groups), 1.21 (*m*, 6H, CH_3 of isobutyryl group) ppm. ^{13}C NMR (CDCl_3 , 100 MHz, room temperature, mixture of diastereoisomers): $\delta = 191.2, 176.4, 175.0, 174.9, 174.1, 163.1, 157.0, 151.7, 151.5, 144.0, 143.5, 140.8, 134.3, 129.3, 123.2, 121.4, 121.2, 119.8,$

117.5, 117.4, 116.6, 116.5, 111.1, 110.9, 109.7, 108.9, 83.2, 76.2, 75.9, 75.8, 73.1, 73.0, 62.4, 56.0, 34.3, 34.1, 34.0, 33.9, 19.0, 18.9, 18.7, 18.6 ppm.

ESI-MS (positive ions): m/z calculated for $C_{41}H_{46}O_{14} = 762.29$; found: 763.66 $[M - H]^+$.

3.3.5 Synthesis of silibinin building block 4

To 3,5,7,4"-tetra-O-isobutyryl silibinin **2** (240.0 mg, 0.33 mmol) dissolved in anhydrous CH_2Cl_2 (5 mL), DIEA (390 μ L, 2.22 mmol), and 2-cyanoethyl-N,N-diisopropylamino-chlorophosphoramidite (107 μ L, 0.48 mmol) were mixed. After 30 min the solution was diluted with EtOAc, and the organic phase was washed twice with brine and then concentrated. Silica gel chromatography of the residue (eluent *n*-hexane/EtOAc, 30:70, v/v, in the presence of 1% of Et_3N), afforded desired compound **4** (205.0 mg, 0.24 mmol) in a 65% yield.

4 $R_f = 0.8$ (*n*-hexan/AcOEt, 60:40, v/v). 1H NMR ($CDCl_3$, 500 MHz, room temperature, mixture of four diastereoisomers): $\delta = 7.11$ – 6.94 (overlapped signals, 6H, H-2', H-3', H-6', H-2'', H-5'', H-6''), 6.76 (complex signals, 1H, H-8), 6.56 (*s*, 1H, H-6), 5.69 (*x*2), 5.68 and 5.67 (*d*, 1H, $J = 12.2$ Hz, H-3), 5.38, 5.37 (*x*2) and 5.36 (*d*, 1H, $J = 12.2$ Hz, H-2); 5.03 (*x*2) and 5.01 (*x*2) (*d*, 1H, $J = 7.2/7.5$ Hz, H-7''), 4.00–3.51 (complex signals, 12H, OCH_3 , H-8'', H-9''a, H-9''b, OCH_2CH_2CN , $N[CH(CH_3)_2]_2$ OCH_2CH_2CN), 3.01–2.68 (*m*, 3H, CH of isobutyryl groups), 2.56 (*m*, 1H, CH of isobutyryl group) 1.36–0.95 (complex signals, 36H, CH_3 of isobutyryl groups, $N[CH(CH_3)_2]_2$) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz, room temperature, mixture of diastereoisomers): $\delta = 185.0$, 175.2, 175.1, 175.0, 174.1, 162.5, 162.4, 156.5, 151.7, 151.5, 144.3, 143.5, 143.4, 140.5, 134.7, 128.3, 128.2, 123.0, 122.9, 121.0, 120.9, 120.8, 119.9, 119.8, 119.7, 117.7, 117.5, 117.1, 116.4, 116.3, 111.5, 111.4, 111.3, 111.1, 111.0, 110.8, 108.7, 81.2, 81.1, 76.2, 75.9, 75.8, 72.9, 72.8, 62.7, 62.5, 62.2, 62.0, 58.8, 58.6, 58.5, 58.3, 56.0, 55.9, 53.4, 43.3, 43.2, 34.2, 34.0, 33.9, 33.6, 24.6, 24.5, 19.0, 18.8, 18.7, 18.6 ppm. ^{31}P NMR ($CDCl_3$, 161.98 MHz): $\delta = 150.2$, 149.8, 149.7 ppm. ESI-MS (positive ions): m/z calculated for $C_{50}H_{63}N_2O_{15}P = 962.40$; found: 963.92 $[MH]^+$.

3.3.6 Synthesis of silibinin building block 5

To 5,7,4'',9"-tetra-O-isobutyryl silibinin **3** (240.0 mg, 0.33 mmol) dissolved in anhydrous CH_2Cl_2 (5 mL), DIEA (390 μ L, 2.22 mmol), and 2-cyanoethyl-N,N-diisopropylamino-chlorophosphoramidite (107 μ L, 0.48 mmol) were mixed. After 1 hour the solution was diluted with EtOAc, and the organic phase was washed twice with brine and then concentrated. Silica gel chromatography of the residue

(eluent *n*-hexane/EtOAc, 3:7, v/v, in the presence of 1% of Et₃N), afforded desired compound **5** (205.0 mg, 0.24 mmol) in a 65% yield.

5 $R_f = 0.8$ (*n*-hexan/AcOEt, 60:40, v/v). ¹H NMR (CDCl₃, 500 MHz, room temperature, mixture of four diastereoisomers): $\delta = 7.30\text{--}6.97$ (overlapped signals, 6H, H-2', H-3', H-6', H-2'', H-5'', H-6''), 6.73 (complex signals, 1H, H-8), 6.54 (complex signal, 1H, H-6), 5.02 (x2) and 5.01 (x2) (*d*, 1H, $J = 7.8/8.0$ Hz, H-7''), 4.94 (*d*, 1H, $J = 9.3$ Hz, H-2), 4.43 (complex signal, 1H, H-3), 4.34 (complex signal, 1H, H-9''a), 4.20 (complex signal, 1H, H-8''), 4.10–3.94 (complex signals, 1H, H-9''a), 3.86 (complex signal, 3H, OCH₃), 3.54–3.34 (complex signals, 9H, H-9''b, OCH₂CH₂CN, N[CH(CH₃)₂]₂ OCH₂CH₂CN) 2.96–2.74 (complex signals, 3H, CH of isobutyryl groups), 2.65 (complex signal, 1H, CH of isobutyryl group), 1.40–1.10 (complex signals, 36H, CH₃ of isobutyryl groups, N[CH(CH₃)₂]₂) ppm. ¹³C NMR (CDCl₃, 100 MHz, room temperature, mixture of diastereoisomers): $\delta = 189.0, 188.3, 176.4, 176.3, 174.9, 174.2, 174.1, 162.3, 156.3, 156.1, 149.9, 143.9, 143.8, 143.4, 143.3, 140.8, 135.9, 134.2, 129.5, 129.4, 129.3, 123.2, 121.7, 121.6, 119.7, 119.6, 117.8, 117.5, 117.4, 117.3, 116.8, 116.6, 111.3, 111.1, 111.0, 108.6, 83.1, 82.9, 76.2, 75.8, 75.6, 75.2, 62.4, 62.3, 59.7, 59.5, 58.5, 58.3, 56.0, 43.5, 43.4, 34.2, 34.1, 33.9, 24.8, 24.7, 24.6, 24.2, 24.1, 19.0, 18.9, 18.8, 18.7, 18.6$ ppm. ³¹P NMR (CDCl₃, 161.98 MHz): $\delta = 153.2, 153.0, 152.3, 152.2$ ppm. ESI-MS (positive ions): m/z calculated for C₅₀H₆₃N₂O₁₅P = 962.40; found: 963.43 [MH]⁺.

3.3.7 Synthesis of dimeric phosphate-linked silibinins 6–8

The coupling reaction between the phosphoramidites **4** or **5** and the suitable silibinin building blocks **2** or **3** was carried out by suspending 240 mg (0.32 mmol) the derivatives **2** or **3** in a solution of DCI in acetonitrile (0.45M, Activator 42[®]) and by adding 3-Å molecular sieves. After few minutes, the mixture was added to 270 mg (0.28 mmol) of silibinin phosphoramidites **4** or **5** and was kept under stirring for 1 hour. On completion of reaction monitoring by TLC analysis, 150 μ L of *tert*-butyl hydroperoxide solution in decane (5.5 M) was added and after 30 minutes, the solvent was removed under vacuum and the crude material was purified by column chromatography (eluent CHCl₃/CH₃OH, 99:1 to 95:5, v/v). The material obtained were subsequently subjected to treatment with ammonia aqueous solution (28%) and methanol (1:1, v/v) at 50 °C for 3 hours for the full deprotection. After the deprotection treatment, the crude material was analyzed by RP-HPLC using a Phenomenex Luna C18 (5 μ m particle size, 10.0 mm \times 250 mm i.d.) eluted with 0.1 M ammonium acetate with a linear gradient 20–100% ACN in 30 minutes (flow = 3.0 mL/min) and then purified by RP-HPLC using a

Gemini C18-110A preparative column (10 μ m particle size, 250 mm \times 21.2 mm i.d.) eluted with 0.1 M ammonium acetate with a linear gradient 20–100% ACN in 50 minutes (flow = 7 mL/min). The recovered material was desalted on Chromafix® C18 column eluted with H₂O/CH₃OH 8:2 (v/v) and converted into the corresponding sodium salt by cation exchange on DOWEX resin (Na⁺) leading the compounds **6** - **8** in good yields (50, 45 and 60 % respectively).

3.3.8 HPLC purification of **6**–**8**

The sample solutions were prepared by dissolving and sonicating the accurately weighed compound (**6** - **8**) in H₂O/CH₃OH (1:1, v/v). The obtained solution (ca 10 mg/mL) was then applied to Nylon filters (pore size = 0.45 μ m) and then purified.

The HPLC analysis was performed with a Shimadzu LC-9A system equipped with a Shimadzu SPD-6A Detector using a RP18 column Phenomenex LUNA (5- μ m particle size, 10.0 mm \times 250 mm i.d.) and eluted with ammonium acetate 0.1 M with a linear gradient 20–100% ACN in 30 minutes (flow = 1.5 mL/min). The chromatograms were monitored at 288 nm.

The preparative HPLC method was performed with a Shimadzu LC-8A PLC system equipped with a Shimadzu SCL-10A VP System control and Shimadzu SPD-10A VP UV-VIS Detector. A Phenomenex Gemini C18-110A preparative column (10- μ m particle size, 250 mm \times 21.2 mm i.d.) was used and eluted with ammonium acetate 0.1 M with a linear gradient 20–100% ACN in 30 minutes (flow = 7 mL/min). The chromatograms were monitored at 288 and 254 nm. The HPLC system was controlled by LC Real Time Analysis software (Shimadzu Corporation).

3.3.9 DPPH radical scavenging activity assay.

The antioxidant activities of the compounds was assessed by examining their abilities to scavenge the 1,1-diphenyl-2-picrylhydrazyl free radical. DPPH solution (20 mg/mL) was prepared in methanol. The compounds was dissolved in methanol to prepare the stock solution (2 mM). Freshly prepared DPPH solution was taken in test tubes and analogue solutions (2 mM–5 μ M) were added to every test tube so that the final volume was 2.25 mL and after 10 min, the absorbance was read at 515 nm. Quercetin and Trolox were used as a reference standards and dissolved in methanol to make the stock solution with the same concentration (2 mM).

3.3.10 Serum stability

Tests were performed in 500 μL of 50 mM Tris•HCl (pH 8.0 containing 10 mM MgCl_2), 50 μL of dimeric phosphate-linked silibinin (10 μM solution), and human serum from human male AB plasma (400 μL). All reactions were incubated at 37 $^\circ\text{C}$. From this solutions were taken samples of constant volume at different times, and the HPLC profile was analyzed in comparison with that of the intact compound.

3.3.11 Singlet oxygen reactivity

In order to estimate the reactivity of silibinin and dimers toward singlet oxygen, solutions are irradiated in a quartz cell of 1cm pathlength using 545 nm centered irradiation (1000 W Xenon lamp equipped with a monochromator) in the presence of 100 μM of RB as photosensitizer for singlet oxygen generation.³ An aliquot of solution was withdrawn at fixed interval time and analyzed by liquid chromatography

Figure 3.1 shows absorption spectra of RB and overlap with emission spectrum reaching the solution recorded using a fiber optics coupled with a CCD spectrophotometer (Ocean Optics USD 2000+UV-VIS). A reference lamp (DH-2000-CAL, Ocean Optics) is used for calibration.

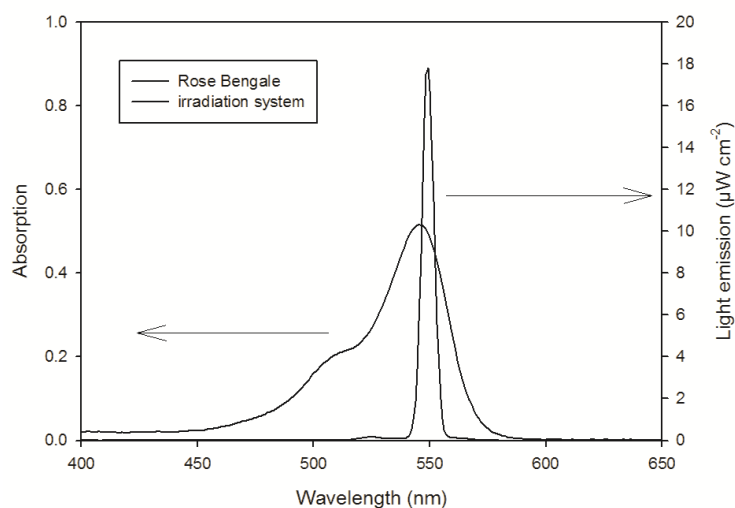


Figure 3.1 Emission spectrum reaching the solution (1000 W Xenon lamp equipped with a monochromator) and absorptions spectrum of 10 μM RB solution.

Silibinin is solubilized in THF, while dimers in H₂O/CH₃CN (9:1, v/v). THF and CH₃CN are used as solvent to increase the silibinin and dimer solubility and due to their similar reactivity with singlet oxygen compared to the water (*i.e.* only limited singlet oxygen scavenging is accounted for by the solvent).⁴ Silibinin and dimers degradations are determined as following: an aliquot (200 µL) was taken at fixed interval times during irradiation, stored in the dark and monitored by ultra-high performance liquid chromatography, using a Waters Acquity UPLC instrument equipped with a diode array detector and a Waters 2695 separation module.

The gradient is a linear increasing from 1% CH₃CN and 99% water acidified with 0.1% of formic acid to 50% acetonitrile within 5 min. Then the CH₃CN is increased linearly to 80% for 2 min and maintained constant for 2 min. The column is a Water Acquity UPLC C18 (100 mm × 2.1 mm × 1.7 µm) and flow rate of 0.3 mL min⁻¹. Retention times of silibinin, 3-3, 3-9" and 9"-9" dimers are 5.4, 6.3, 7.0 and 7.3 min respectively and detection wavelength set at 288 nm.

The time evolution of silibinin and dimers in the presence of singlet oxygen could be fitted with a pseudo-first order equation $C_t = C_0 \exp^{-k_{1O_2} t}$ where C_0 is the initial concentration of compound, C_t the concentration at time t and k_{1O_2} the pseudo-first order degradation rate constant of target compound (X). The degradation rate (R_X^d) is determined as $R_X^d = k_{1O_2} C_0$.

The transformation rate of silibinin or dimers in the presence of singlet oxygen is given by the following relation: $R_X^d = k_{1O_2, X}^{II} [^1O_2]_{ss} [X]$ where $[X]$ is the concentration of silibinin or dimers in solution. The second order rate constant between target molecule and singlet oxygen ($k_{1O_2, X}^{II}$ (M¹ s¹))

can be determined as: $k_{1O_2, X}^{II} = \frac{R_X^d}{[^1O_2]_{ss} [X]}$

Singlet oxygen steady-state concentration [¹O₂]_{ss} is quantified with tryptophan as probe using experimental conditions adopted for silibinin and dimers considering a second order rate constant between TRo and [¹O₂] of 3.2 × 10⁷ M¹ s¹.¹⁰

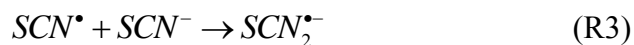
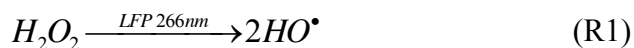
3.3.12 Hydroxyl radical generation and reactivity estimation

Due to the high reactivity of THF with hydroxyl radical ($\sim 4.0 \times 10^9$ M⁻¹ s⁻¹),⁵ stock solutions of H₂O₂, SCN⁻, silibinin and dimers are prepared in Milli-Q water or CH₃CN (for silibinin) and an appropriate volume is mixed just before each experiment, to obtain the desired concentration of all the species. Experiments in pure water are performed for dimers while for silibinin, CH₃CN/water (9:1,

v/v) solution is used. All experiments are performed at ambient temperature (295 ± 2 K) and in aerated solution.

Hydroxyl radical are generated from 266 nm excitation of H_2O_2 solution, corresponding to the fourth harmonic of a Quanta Ray GCR 130-01 Nd:YAG laser system instrument, used in a right-angle geometry with respect to the monitoring light beam. The single pulse is of 9 ns in duration, and energy of 40 mJ. Individual cuvette samples (~ 3 mL volume) are used for a maximum of two consecutive laser shots to avoid degradation products formation and interferences. The transient absorbance at the pre-selected wavelength is followed by a detection system consisting of a pulsed xenon lamp (150 W), monochromator and a photomultiplier (1P28). A spectrometer control unit is used for synchronising the pulsed light source and programmable shutters with the laser output. The signal from the photomultiplier is digitised by a programmable digital oscilloscope (HP54522A) and analysed using a 32 bits RISC-processor kinetic spectrometer workstation.

The UV-vis spectrum of HO^\bullet presents a maximum centered at 240 nm and direct quantification is difficult due to the low molar absorption coefficient (ϵ_{240nm}) of $\sim 600 \text{ M}^{-1} \text{ cm}^{-1}$.⁶ For this reason a kinetic approach based on the thiocyanate (SCN^-) competition reactivity is generally adopted to estimate the second order rate constant of target compounds with HO^\bullet in solution based on the following equations:



The first step (R1) is the hydrogen peroxide photolysis using a laser pulse at 266 nm followed by the competition reactivity of generated HO^\bullet with SCN^- (R2) and silibinin (or dimers) (R4). In the presence of high concentration of SCN^- , thiocyanate radical (SCN^\bullet) reacts with another equivalent of thiocyanate to form di-thiocyanate radical anion ($SCN_2^{\bullet-}$) (R3), which has a strong absorption molar coefficient at 475 nm ($\epsilon_{475nm} = 7600 \text{ M}^{-1} \text{ cm}^{-1}$).⁷

The absorption of $SCN_2^{\bullet-}$ in the presence of different concentration of HO^\bullet quenchers (X) allows the determination of the second order rate constant between hydroxyl radical and silibinin or dimers from the following equation:

$$\frac{Abs_0}{Abs} = 1 + \frac{k_{HO^\bullet, X}^{II} [X]}{k_{HO^\bullet, SCN^-}^{II} [SCN^-]} \quad (E1)$$

in which Abs_0 and Abs are the absorption of $SCN_2^{\bullet-}$ transient followed at 475 nm without and with quencher respectively; $k_{HO^\bullet, SCN^-}^{II}$ is the second order rate constant between HO^\bullet and thiocyanate that is $1.2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$,⁸ $[SCN^-]$ is the initial concentration of thiocyanate (0.1 or 0.5 mM in our study); $[X]$ is the concentration of target compound and $k_{HO^\bullet, X}^{II}$ the second order rate constant with hydroxyl radical.

$\frac{Abs_0}{Abs}$ values vs concentration of quencher ($[SY]$) can be fitted using a linear equation $y = ax + b$ in

which $y = \frac{Abs_0}{Abs}$, $b = 1$, $x = [X]$ and $a = \frac{k_{HO^\bullet, X}^{II}}{k_{HO^\bullet, SCN^-}^{II} [SCN^-]}$. $k_{HO^\bullet, X}^{II}$ value can be determined to be equal to

$$a \times k_{HO^\bullet, SCN^-}^{II} [SCN^-].$$

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PhD Course Activity Summary

Candidate: Dr. Raffaele Gravante

Supervisor: Prof. Giovanni Di Fabio

1) Attended Courses:

- Tecniche di estrazione solido-liquido, 8 hours, June 2014 (Prof. Naviglio);
- Spettrometria di Massa, 10 hours, July 2014 (Prof. Pucci);
- Sintesi, struttura ed applicazioni di oligonucleotide naturali e modificati, 8 hours, February 2015 (Prof. Montesarchio);
- Glicoscienza, 8 hours, July 2015 (Prof. Parrilli and Dr. Bedini);
- Produzione ricombinante di protein naturali e mutanti, 8 hours, July 2015 (Prof. Duilio);
- Neutron scattering, 8 hours, October 2015 (Prof. Zorn);
- Chimica Fisica degli Acidi Nucleici, 8 hours, July 2016 (Prof. Petraccone).

2) Attended Seminars:

Title	Speaker	Date	Place
Impact factor, citations, journal reputation, editorial policy: how to choose the right journal.	Dott. Thierry Tron; Dott.ssa Jalila Siman; Prof. Danilo Porro.	26/06/2014	Università degli Studi di Napoli "Federico II"
Evidenze genetiche sulle prime espansioni umane nell'Africa.	Prof. Guido Barbujani	23/10/2014	CEINGE BIOTECNOLOGIE AVANZATE
Pharmaceutical Companies: external manufacturing and quality assurance.	Prof. Domenico De Masi	25/05/2015	Università degli Studi di Napoli "Federico II"
Alessandro Ballio e la chimica a Napoli negli anni '60	Dr. Andrea Carpentieri	18/06/2015	Università degli Studi di Napoli "Federico II"
Le transglutaminasi..dalle poliammine alle bioplastiche	Prof. Raffaele Porta	30/06/2015	Università degli Studi di Napoli

			“Federico II”
Synthetic and biofunctional studies of microbial and animal glycans.	Dr. Fukase	29/09/2015	Università degli Studi di Napoli “Federico II”
Scientific calculator.	Prof. Vacatello	11/11/2015	
Introduction to bioinformatics	Dr. Remo Sanges	18/12/2015	Università degli Studi di Napoli “Federico II”
Biopesticides which target voltage-gated ion channels: efficacy and biosafety	Prof. Angharad M.R.Gatehouse	14/01/2016	Università degli Studi di Napoli “Federico II”
1,1,2,2-tetraethoxybutyne-Exciting to make and work with	Prof. Leiv K. Sydnes	14/01/2016	Università degli Studi di Napoli “Federico II”
Functionalized and artificial enzymes:newbio-derived catalysts	Prof. Therrytron	14/01/2016	Università degli Studi di Napoli “Federico II”
Secondary metabolites from higher land and sea plants in Chemical ecology and Chemosystematics	Prof. Christian Zidorn	27/01/2016	Università degli Studi di Napoli “Federico II”
Modified nucleotides and oligonucleotides for biomedical applications.	Prof. Daniela Montesarchio	18/01/2017	Università degli Studi di Napoli “Federico II”

3) Attended Integration Exams (for candidates not graduated in Chemical Science):

Title	Professor	Date
Chimica Fisica I	Castronuovo	11/02/2015
Chimica Inorganica	Vitagliano	29/02/2016

4) Visiting periods in Institutions different from University of Naples “Federico II”:

Host Institution	Country	Start Date	End Date
University Blaise Pascal, Institute of Chemistry of Clermont-Ferrand.	France	02/10/2016	02/01/2017

5) Publications (include submitted and in preparation):

- **New Silibinin glyco-conjugates: Synthesis and evaluation of antioxidant properties**
Armando Zarrelli, Valeria Romanucci, Concetta Tuccillo, Alessandro Federico, Carmela Loguercio, **Raffaele Gravante** and Giovanni Di Fabio; Bioorg Med Chem Lett. 2014; 24(22): 5147-9.
- **Isolation, structural determination, and biological activities of gymnemic acid, a molecule that does not exist**
Armando Zarrelli, Valeria Romanucci, **Raffaele Gravante**, Cinzia Di Marino and Giovanni Di Fabio; Nat Prod Commun. 2014; 9(10): 1429-32.
- **Scavenging Effect of Various Solvent Extracts of *Gymnema sylvestre* R. Br. and in vivo Antioxidant Activity of Isolated Triterpenes**
Armando Zarrelli, Valeria Romanucci, Giovanni Di Fabio, Daniele D'Alonzo, Cinzia Di Marino, **Raffaele Gravante**, Achille Cittadini and Alessandro Sgambato (forthcoming).
- **Synthesis, biological and electrochemical investigation of new Silibinin glyco-phosphodiester conjugates**
Valeria Romanucci, **Raffaele Gravante**, Cinzia Di Marino, Mauro Iuliano, Gaetano de Tommaso, Tonino Caruso, Armando Zarrelli and Giovanni Di Fabio (forthcoming).
- **Dimeric phosphate-linked Silibinins: Synthesis, spectroscopic properties, solubility and radical scavenger ability**
Raffaele Gravante, Valeria Romanucci, Martina Cimafronte, Cinzia Di Marino, Gilles Mailhot, Marcello Brigante, Armando Zarrelli and Giovanni Di Fabio (forthcoming).

6) Attended congresses/workshops/summer schools/contribution:

- **New Silibinin glyco conjugates: synthesis, characterization and preliminary evaluation of antioxidant properties**
Raffaele Gravante¹, Valeria Romanucci¹, Valeria Sgambati², Elio Pizzo², Daniele D'Alonzo¹, Annalisa Guaragna¹, Armando Zarrelli¹ and Giovanni Di Fabio¹
¹Department of Chemical Sciences; ²Department of Biology, University of Naples Federico II, Via Cintia 4, I-80126, Napoli, Italy
Poster session at “XXXVI CONVEGNO DELLA DIVISIONE DI CHIMICA ORGANICA - CDCO 2015”.
- **GEM-DCA: synthesis of a novel codrug**
Raffaele Gravante¹, Daniele D'Alonzo¹, Valeria Romanucci¹, Armando Zarrelli¹, Giovanni Di Fabio¹, Giovanni Palumbo¹ and Annalisa Guaragna¹
¹Department of Chemical Sciences, Università di Napoli "Federico II", Via Cintia 21, I-80126, Napoli, Italy.
Poster session at “XXXVI CONVEGNO DELLA DIVISIONE DI CHIMICA ORGANICA - CDCO 2015”.