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New silibinin glyco-conjugates: Synthesis and evaluation of antioxidant properties

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ABSTRACT

New silibinin glyco-conjugates have been synthesized by efficient method and in short time. Exploiting our solution phase strategy, several structurally diverse silibinin glyco-conjugates (gluco, manno, galacto, and lacto-) were successfully realized in very good yields and in short time. In preliminary study to evaluate their antioxidant and neuroprotective activities new derivatives were subjected to DPPH free radical scavenging assay and the Xanthine oxidase (XO) inhibition models assay. Irrespective of the sugar moiety examined, new glyco-conjugates are more than 50 times water-soluble of silibinin. In the other hand they exhibit a radical scavenging activities slightly higher than to silibinin and XO inhibition at least as silibinin.

Silibinin is the major biologically active component of an extract from the seeds of the milk thistle (*Silybum marianum*) known as silymarin.[1] Structurally natural silibinin is a diastereoisomeric mixture of two flavonolignans, namely silybin **A** and silybin **B** in a ratio of approximately 1:1.[2] Silibinin is a metabolite with multiple biological activities operating at various cell levels, most of them related to its radical scavenging activity and it is already used successfully in therapy of liver damage of various aetiology and as a liver-protecting drug.[3–7] Its therapeutic efficiency is rather limited by its low bioavailability and thus limited affectivity. Silibinin is typically administered orally, which limits the efficacy of the natural product because of its poor absorption and short half-life in the body.[8]

In order to reach their target tissues, cells, and organelles for their desired therapeutic effects a number of physical and chemical approaches by which to accomplish these difficult challenges have been proposed.[9] Only a few modifications have been introduced and most simply alter the chemical and physicochemical properties of the natural metabolite and enhance the biological efficacy of the derivatives by increasing their *in vivo* stability, binding affinity, and overall uptake. Generally tissue- and cell-specific drug targeting can only be achieved by employing carrier-drug complexes or conjugates that contain a ligand recognized by a receptor on the target cell. Carbohydrate-based conjugates allow targeting of a certain class of cell membrane receptors that are referred to as lectins which 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 the its pharmaceutical, pharmacokinetic and/or pharmacodynamic properties.[10]

In this frame it has been reported that the absorption of quercetin glycoside is more efficient than that of quercetin aglycon, indicating that the hydrophilic character of saccharide moieties increased the water solubility of the aglycon, and enhanced its bioavailability.[11–14]

We present here the preliminary results of a efficient synthetic procedure to obtain new 9″–phosphodiester silibinin conjugates with different mono- and di-saccharide labels through the anomeric hydroxyl group (Fig. 1). The introduction of the phosphate group is generally used to bring great pharmaceutical and pharmacokinetic benefits.

In our approach 9″-phosphoramidite **1**[15] (Scheme 1) have been used as silibinin substrate and 1-OH full protected mono- and di-saccharide derivatives (**2**–**5**, Scheme 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 1) were coupled with derivative **1** using 0.45 M tetrazole

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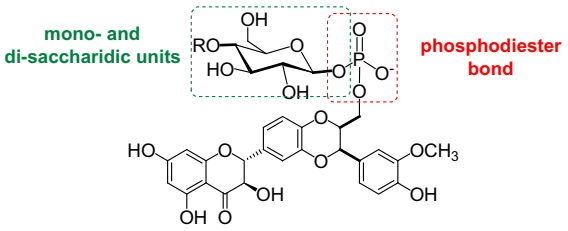
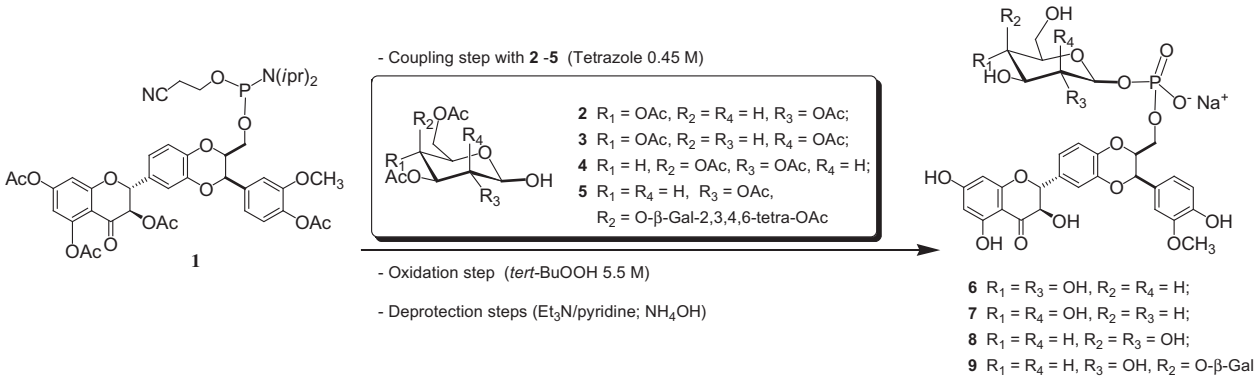


Figure 1. General structures of new silibinin glyco-conjugates.

in ACN/DCM (1:1, v:v). The treatment with 5.5 M *tert*-butyl hydroperoxide solution in decane and the subsequent treatment with conc. aq. ammonia and MeOH (1:1, v:v) at room temperature, allowing full deprotection from acetyl and cyanoethyl groups and leading to the desired phosphodiester derivatives **6–9** in good yields.[18] All compounds were then converted into the corresponding sodium salts by cation exchange on a DOWEX (Na^+ form) resin to have crystalline samples. The crude materials were then subjected to reverse phase analysis (RP-18 HPLC), with various columns and elution conditions, but the conditions for a separation of different diastereoisomers were not found. 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.[18] The NMR analysis has proved very complex, in fact ^{1}H and ^{31}P NMR spectra of all compound showed dramatic line broadening, already at 5.0 mg/mL (ca. 7.0 mM), diagnostic of a slow equilibrium on the NMR time scale, which could suggest a strong propensity toward aggregation in H_2O. 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.[19]

All silibinin derivatives were subjected to 2,2-diphenyl-1-picrylhydrazyl (DPPH)[20] free radical scavenging assay (Table 1) and the Xanthine oxidase (XO)[21,22] inhibition models assay were utilized to evaluate their antioxidative properties (Table 1). 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 important biological source of free radicals. Many references reveal cerebral microvascular injury resulting from XO production of superoxide free radicals.[23] XO inhibition is thereby implicated as useful approach in treating cerebrovascular pathological changes or central nervous system (CNS) diseases.[24,25] From the DPPH assay shown in Table 1, we observed that introducing an sugar moieties in 9″ position did not led to a general 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 that were similar to that of the silibinin **1**, which confirmed that the introduction in 9″ of the sugar moiety had little effect on radical scavenging activity.[26,27] In basal conditions, the pre-incubation of MKN28 cells with **6–9** and silibinin lead to two different results (Table 1). In fact, **6–9** analogues do not 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 subsequently the induction of oxidative stress shows that these molecules protect, from cell death after induction of oxidative stress, at least as 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 (XO) inhibition models assay in preliminary study to evaluate their antioxidant activities. Irrespective of the sugar moiety examined, all



Scheme 1. Synthesis of new 9″-phosphodiester glyco-conjugated silibinin analogues.

Table 1
Free radical scavenging capacity (DPPH) and Xanthine oxidase inhibition (X–XO)

Samples	IC_{50} (μM)[a]	Cell viability[b] (%)	
	DPPH scavenging		After induction of oxidative stress
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] IC_{50} values 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).

compounds exhibited a radical scavenging activities slightly higher than to silibinin and Xanthine oxidase (XO) inhibition at least as 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. These two data encourage our future studies that are aimed to improvement this synthetic strategy to realize libraries of optically pure glyco-conjugated silibinin and 2,3-dehydrosylibin derivatives.

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18. *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 CH_3CN (1.0 mL, 0.45 mmol). The reaction was left under stirring at room temperature and monitored by TLC in the eluent system 1:2 *n*-hexane/AcOEt, (v:v). After 1 h, 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 min the reaction mixture was diluted with $CHCl_3$, transferred into a separatory funnel, washed three times with water and dried under reduced pressure. The next treatment with Et_3N/pyridine (1:1, v/v) to 1 h at 50 °C and then with aq ammonia (28%)/CH_3OH (1:1, v:v) for 1 h 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%).
19. *Compound **6** (brown powder, 88.1 mg, 68%):* ^{31}P NMR (161.98 MHz, D_2O, room temperature, mixture of diastereoisomers) δ 2.9, 1.4, 0.8. HRMS (MALDI-TOF, negative ions) *m*/*z* calcd for $C_{31}H_{32}O_{18}P$ = 723.1332, found 723.1332 $[M-H]^-$. Compound **7** (brown powder, 94.0 mg, 72%): ^{31}P NMR (161.98 MHz, D_2O, room temperature, mixture of diastereoisomers) δ 1.3, 0.9, 0.7. HRMS (MALDI-TOF, negative ions) *m*/*z* calcd for $C_{31}H_{32}O_{18}P$ = 723.1332, found 723.1333 $[M-H]^-$. Compound **8** (brown powder, 82.3 mg, 63%): ^{31}P NMR (161.98 MHz, D_2O, room temperature, mixture of diastereoisomers) δ 2.4, 1.6, 1.1, 0.8. HRMS (MALDI-TOF, negative ions) *m*/*z* calcd for $C_{31}H_{32}O_{18}P$ = 723.1332, found 723.1333 $[M-H]^-$. Compound **9** (brown powder, 104.8 mg, 60%): ^{31}P NMR (161.98 MHz, D_2O, room temperature, mixture of diastereoisomers) δ 1.1, 0.8. HRMS (MALDI-TOF, negative ions) *m*/*z* calcd for $C_{37}H_{42}O_{23}P$ = 885.1860, found 885.1859 $[M-H]^-$.
20. *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 (DPPH) 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 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 2,3-dehydrosilybin were used as a reference standards and dissolved in methanol to make the stock solution with the same concentration (1 mM).
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