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New C-23 modified of silybin and 2,3-dehydrosilybin: Synthesis and preliminary evaluation of antioxidant properties

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ABSTRACT

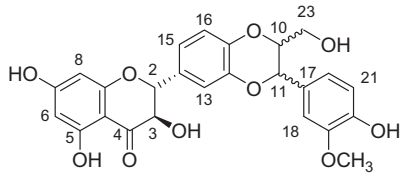
Silybin is the major flavonolignan of silymarin and it displays a plethora of biological effects, generally ascribed to its antioxidant properties. Herein we shall describe an efficient synthetic strategy to obtain a variety of new and more water-soluble silybin and 2,3-dehydrosilybin (DHS) derivatives in which the 23-hydroxyl group was converted to a sulfate, phosphodiester, or amine group, using a solution-phase approach. Furthermore a new and efficient method for the preparation of DHS from silybin was developed and optimised.

The antioxidant properties of the new compounds were evaluated in a cellular model *in vivo* and they displayed an antioxidant activity comparable to or higher than silybin and DHS, being able to prevent H_2O_2-induced generation of intracellular reactive oxygen species (ROS). Most of the derivatives also displayed a better hydrophilicity while retaining the biological activities of silybin and they might broaden the *in vivo* applications of this class of natural compounds.

Silybin is the major flavonolignan found in silymarin, isolated from the fruits of the milk thistle *Silybum marianum*, which is widely used as a natural remedy for the treatment of cirrhosis, chronic hepatitis, and liver diseases associated with alcohol consumption and exposure to environmental toxins.[1] Structurally silybin is a diastereoisomeric mixture of two flavonolignans, namely silybin **A** and silybin **B** (Fig. 1) in a ratio of approximately 1:1.

Silybin is a natural compound with multiple biological activities operating at various cell levels most of them related to its radical scavenging activity. In order to prevent an accumulation of reactive oxygen species (ROS), cells have developed protective mechanisms which either scavenge or detoxify ROS. Moreover, given the widespread involvement of ROS in several human diseases, there is a great interest for the developing of valid antioxidant therapies. Silybin is already used successfully in clinic but its therapeutic efficiency is rather limited by its low water-solubility. Recently silybin has received attention for its alternative beneficial activities that are not directly related to its hepatoprotective and/or antioxidant (radical scavenging) effects. These include mostly anticancer and chemopreventive actions, as well as hypocholesterolemic, cardioprotective and neuroprotective activities.[2–5]

Unfortunately the bioavailability and the therapeutic efficiency of silybin are rather limited by its low water-solubility, therefore



A (2*R*, 3*R*, 10*R*, 11*R*) **B** (2*R*, 3*R*, 10*S*, 11*S*)

Figure 1. Chemical structures of silybin **A** and silybin **B**.

new synthetic approaches for selectively modifying silybin are of high interest. In the last decade, a series of novel silybin analogues have been synthesized with the aim to improve the water-solubility while preserving its biological activity.[6–9] The synthesis of the 3,23-*O*-bis-hemisuccinate silybin, was the first example of a water-soluble analogue that enabled an intravenous application of silybin (Legalon-SIL, Madaus) for the treatment of acute liver intoxication by mycotoxins. Carboxylic acids derived from silybin and DHS with improved water solubility and good antioxidant properties were prepared by selective oxidation of the C-23 hydroxymethyl moiety.[10]

Different studies recently made on the antiradical activity of silybin and DHS have elucidated the functional groups responsible for this activity.[11] The results suggest that the C-23 position could

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be a site for modifications aimed to improve the bioactivity of silybin and/or DHS analogues since it is not involved in the antioxidant activity.

As part of our ongoing efforts towards the exploitation of a readily available natural product as a scaffold for chemical diversification, we recently considered silybin as a versatile starting material. We chose to modify the 23-position in the silybin to generate a series of new and more hydrophilic analogues, some of which could be useful templates for more chemical diversifications. Here we describe the results of a successful strategy of synthesis for a variety of silybin and DHS derivatives, bearing different substituents, some of which with a charge, at position C-23 and whose antioxidant activity has been evaluated *in vivo*.

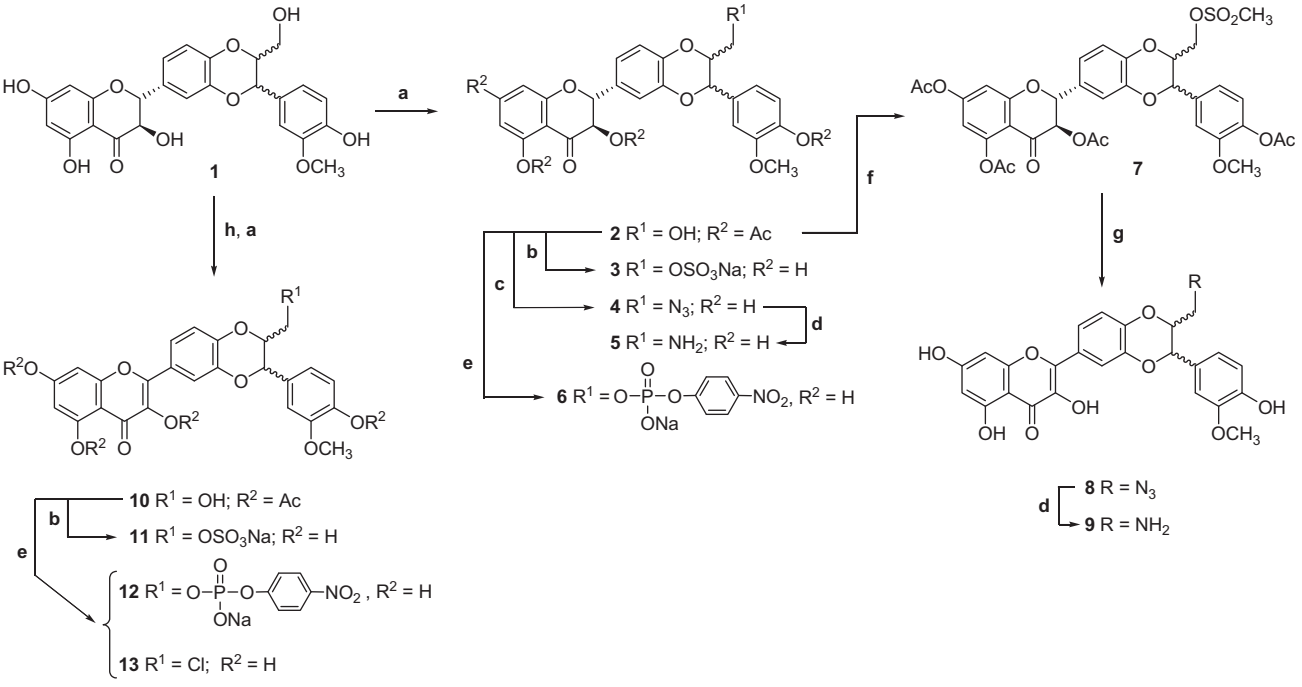
The proposed synthetic strategy provides an orthogonal protecting group approach, to obtain the key silybin and DHS intermediates with the 3,5,7,20-OH functions being protected and the 23-OH function free for suitable modifications. Our attempts were carried out using the 4,4′-dimethoxytriphenylmethyl (DMT) group for the transient protection of the 23-OH moiety while all other OH groups were protected with acetyl (Ac). The modifications introduced at the C-23 position included the sulfate, the phosphodiester and an amino function. The phosphodiester and amino functions were chosen with the objective of preparing the useful scaffold of silybin and DHS to generate a libraries of 23-modified as phosphodiesters, amides, urethanes and sulfonamides, through standard and well known chemistry. The preliminary results here described always concern a mixture of diastereoisomers and enantiomers, for silybin and DHS analogues, respectively.

Commercially available silybin **1** (Scheme) was first converted into 23-ODMT ether by a reaction with 4,4′-dimethoxytriphenylmethylchloride (DMTCl) in pyridine at 50 °C to give the desired product in a 90% yield.[12] After an exhaustive acetylation treatment with an excess of acetyl anhydride in pyridine, the successive treatment using 5% formic acid in CH_2Cl_2 allowed the removal of the DMT protecting group to led **2**[13] in a 73% yield. Starting from **2**, silybin analogues **3**–**6** were obtained in few steps in good overall yields. To introduce the sulfate group the compound **2** was reacted with $SO_3{\cdot}Et_3N$ and then treated with conc. aq. ammonia in CH_3OH at r.t., allowing full deprotection from acetyl groups, leading to the desired **3** in a 55% yield. Silybin analogue **4** was prepared starting from key intermediate **2**, which was first converted (in a 68% yield) into the corresponding 23-azido derivative by the Mitsunobu reaction and, after the removal of the acetyl groups, obtained by treatment with 0.1 M HCl/EtOH at 50 °C, the 23-azido function was reduced by catalytic hydrogenolysis, yielding derivative **5** in a 44% yield. The reaction of **2** with 4-nitrophenyl dichlorophosphate in pyridine at 0 °C, and subsequent deacetylation (conc. aq. ammonia in CH_3OH at r.t.), led to compound **6** in a 56% overall yield. In all cases, the intermediate compounds and final derivatives were purified by silica gel chromatography and then fully characterized by NMR (^1H, ^{13}C and also ^{31}P in the case of **6**) and MALDI-MS.[12]

Since the starting material was a mixture of specific diastereoisomers, all the intermediates and final derivatives were obtained as a mixture of two diastereoisomers and the ^1H and ^{13}C NMR spectra of many of them appeared like those of a single compound, and the assignment derived by concerted application of 2D NMR techniques (COSY, HMBC, HSQC and NOESY). However, for some nuclei different signals were observed, but these were not assigned to the individual silybin.

The synthesis of analogue **4** was originally designed to react with the corresponding 23-methansulfonyl derivative **7** (Scheme) with NaN_3. This reaction did not lead to the desired product, but it lead to the formation of 3,7,20-three-*O*-acetyl-23-azido-2,3-dehydrosilybin. In addition to replacement at the C-23 position, the oxidation of the silybin to 2,3-dehydrosilybin and a partial deacetylation were also observed.[12] After removal of the acetyl groups, obtained by treatment with conc. aq. ammonia in CH_3OH at r.t., to lead **8**, the next reduction of the 23-azido function was obtained by catalytic hydrogenolysis, yielding derivative **9** in a 35% yield.

Based on this result some experiments were designed to estimate the mean to oxidize the silybin to DHS in a similar reaction.



Scheme. *Reagents and conditions*: (a) DMTCl, pyridine, 50 °C, overnight; Ac_2O, pyridine, r.t., overnight; 5% formic acid in CH_2Cl_2, r.t., 30 min. (b) $SO_3{\cdot}Et_3N$, DMF, r.t., 30 min.; NH_4OH/CH_3OH, r.t., 30 min. (c) TPP, DPPA, THF, r.t., 5 h; 0.1 M HCl/EtOH (1:4, v/v), 50 °C, 20 min. (d) H_2, C/Pd, CH_3OH, r.t., overnight. (e) 4-Nitrophenyl dichlorophosphate, pyridine, 0 °C, 40 min; NH_4OH/CH_3OH, r.t., 30 min. (f) CH_3SO_2Cl, pyridine, 0 °C to r.t., 1 h. (g) NaN_3, DMF, 70 °C, 2 h; NH_4OH/CH_3OH, r.t., 30 min. (h) AcOK, DMF, 50 °C, 30 min.

Initially, a solution of silybin (**1**) in DMF was heated to 70 °C and TLC control and NMR analysis showed that silybin was still stable under these conditions after 5 h. Successively, the silybin (**1**) was treated with NaN_3 in DMF at 70 °C for 1 h, and we observed a complete conversion to DHS, as shown by ^1H NMR and MS data of the purified compound and by comparison with data of Refs.[10,13] The same product was obtained by treating the silybin with anhydrous potassium acetate (AcOK), in DMF at 50 °C in less than 30 min. Although it is a very convenient procedure, compared to previously reported methods,[10,14] we have not here investigated yet the mechanism of this reaction.

The synthetic route to key intermediate **10** was essentially similar to one previously adopted for the preparation of silybin derivative **2**, as summarized in Scheme. Thus commercially available silybin **1** was first converted into DHS by treatment with anhydrous AcOK in DMF at 50 °C (78% yield). Next DHS was converted into 23-ODMT ether by a reaction with DMTCl in pyridine at 50 °C and then treated with an excess of acetyl anhydride in pyridine. Successive treatment with 5% formic acid in CH_2Cl_2 to led the key intermediate **10** in 80% yield. The 3,5,7,20-tetra-*O*-acetyl-DHS **10** was reacted with $SO_3{\cdot}Et_3N$ and then treated with conc. aq. ammonia in CH_3OH at r.t., allowing full deprotection from acetyl groups, leading to the desired **11** in a 65% yield. The reaction of **10** with 4-nitrophenyl dichlorophosphate in pyridine at 0 °C, allows a mixture difficult to purify. After deacetylation, by treatment with conc. aq. ammonia in CH_3OH, two derivatives **12** and **13** were obtained, in a 76% and 7% yield, respectively. In all cases, the intermediate compounds and final derivatives were purified by silica gel chromatography and then fully characterized by NMR (^1H, ^{13}C and also ^{31}P in the case of **12**) and MALDI-MS.[12] As expected for a pair of enantiomers, the ^1H and ^{13}C NMR spectra of DHS analogues look like those of a single compound. The assignment was derived by a concerted application of 2D NMR techniques (COSY, HMBC, HSQC and NOESY).

The antioxidant activity of the new silybin analogues was evaluated *in vivo* using immortalized rat fibroblasts as cell model and the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) test.[15] In this assay, intracellular reactive oxygen species (ROS) levels are measured by the oxidative conversion of stable, non-fluorescent DCFH-DA to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) occurring in the presence of ROS.[16] As previously mentioned, most of the beneficial effects of silybin are attributed to its antioxidant properties and there is a great interest for the developing of more effective antioxidant therapies.

Different methods *in vitro*, chemical or enzymatic, and *in vivo*, intact cells, have been developed to evaluate the antioxidant activity of natural and synthetic chemicals, including polyphenols and it is likely to obtain different levels of activity in each system since each chemical can act at various levels (i.e., scavenging radicals and/or inhibiting their production) which are differentially detected by the various systems.[17] *In vitro* systems have been more frequently used to analyse antioxidant activity of silybin derivatives.[10,18]

We preferred to use this type of test, rather than classical *in vitro* assays, since it keeps into account the solubility and the cellular uptake of tested compounds which are important variables in view of a potential use of the compounds in humans. Moreover, it gives indications on different types of ROS, such as superoxide radical, hydroxyl radical and hydrogen peroxide, which are all relevant in an *in vivo* setting. As shown in Figure 2, all the derivatives retained the antioxidant activity of silybin when cells were pre-treated for 48 h with them. Indeed, all the derivatives were able to reduce the basal endogenous levels of ROS but, most importantly, they were also able to prevent H_2O_2-induced generation of intracellular ROS and, in most of the cases, more efficiently than silybin. Comparable results were observed with 16 h pre-incubation.

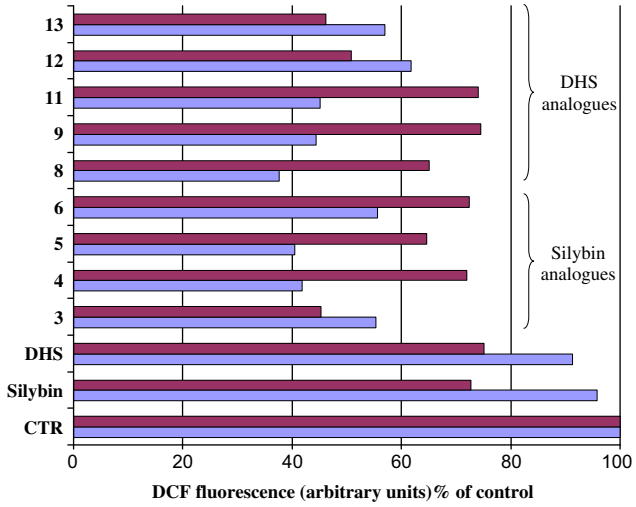


Figure 2. DCF fluorescence was measured in basal conditions (blue bars) or following cells exposure to 100 μM H_2O_2 for 15 min (dark bars). The values are expressed as% compared to the fluorescence value observed in control untreated cells which was set at 100.

In both series of assays (basal conditions and H_2O_2-exposed) all the analogues appeared more active than silybin and DHS in the following descending order: **8** > **5** > **4** > **9** > **11** > **3** > **6** > **13** > **12** > **DHS** > **Sy** (basal conditions) and **3** > **13** > **12** > **8** > **5** > **4** > **6** > **Sy** ≈ **11** ≈ **9** ≈ **DHS** (H_2O_2-exposed). These results confirm the assumption made whereby the simple modifications in position C-23 do not affect the radical scavenging activity of these analogues, but the data analysis did not point to a relationship between chemical modification (anionic, cationic and not charged group) and antioxidant activity.

It is noteworthy that, as previously mentioned, the antioxidant activity evaluated by *in vivo* assays, is affected not only by the intrinsic chemical modifications of the analogues, but also to differences in cell penetration ability. In order to better explain the results obtained, the partition coefficient (log *P*) for each new derivative was obtained using the shake-flask method (Table). The log *P* is an estimate of lipophilicity which has been found to be essential in predicting the transport and activity of many drugs.[19] Recently Křen and co-workers have recently correlated the log *P* values to the antioxidant activity of some more water-soluble analogues of silybin and DHS.[10]

The results obtained (Table) suggest that most of the analogues are more hydrophilic than silybin and DHS while, as expected, for some analogues (**4**, **8** and **13**) the log *P* values are not very different than those of silybin and DHS. The observed order (descending) of

Table
Partition coefficients (Log *P*) and IC_{50} values for new silybin and DHS derivatives.

Entry	log $P_{7.4}$[a]	IC_{50} (μM)[b]
3	−2.12	173 ± 27
4	3.98	166 ± 20
5	1.56	147 ± 22
6	1.80	143 ± 24
8	4.15	124 ± 21
9	3.31	165 ± 23
11	−1.32	178 ± 23
12	3.00	167 ± 25
13	4.52	142 ± 24
Silybin (**1**)	2.07	162 ± 31
DHS	3.75	155 ± 28

[a] For measurement procedure see the Supplementary data file.
[b] Concentration inhibiting cell growth by 50%.

lipophilicity is the following: **13** > **8** > **4** ≈ **DHS** > **9** > **12** > **Sy** > **6** > **5** > **11** > **3**. From a comparative analysis of the data, it appears that the observed variations in hydrophilicity do not affect the antioxidant activity of the analogues since derivatives at both ends of the lipophilicity order (i.e., **3**, **5**, **13** and **8**) were more active than silybin.

It is noteworthy that, as previously mentioned, in these preliminary assays here described, mixtures of diastereoisomers and enantiomers, of silybin and DHS, respectively, have been used and it cannot be excluded that they might display different activities. Thus, although considering the difficulties associated to the separation of the diastereoisomeric or enantiomeric mixture,[20] a future and more detailed study, carried out on separate diastereoisomers (or enantiomers), is warranted to complete the characterization of the newly synthesized analogues.

Using an *in vivo* cell-based test, we were also able to evaluate the concentration inhibiting cell growth by 50% (IC_{50}) for each tested compound. Cytotoxicity assays were carried out by use of the MTT test, as previously described.[14] Exponentially growing cultures of rat fibroblasts were exposed to increasing concentrations of each compound (0–800 μM) and cell viability was assessed after 48 h. A dose-dependent decrease in viable cells was observed with all tested compounds with an IC_{50} ranging between 124 and 178 μM, with silybin IC_{50} being 162 μM. Indeed, an evident toxic effect was not observed with any of the derivatives at the dose (30 μM) used to assess their antioxidant activity.

In conclusion, we have synthesized, exploiting a efficient orthogonal protecting groups approach, new silybin and 2,3-dehydrosilybin (DHS) analogues, bearing a sulfate, amino or phosphate group at C-23 substituents. We have also developed an efficient and faster procedure for the oxidation of the silybin to DHS, in the presence of potassium acetate in DMF at 50 °C in few minutes. The antioxidant properties of the new compounds were evaluated in a cellular model *in vivo* and most of them displayed an antioxidant activity comparable to or higher than silybin and DHS. These results confirm the assumption that modifications in position C-23 do not affect the radical scavenging activity of these analogues. Moreover, the results obtained clearly indicate that the new analogues do not display an increased toxicity compared to silybin thus further supporting their suitability for use in humans especially for the derivatives displaying an increased hydrophilicity. In the light of these preliminary results, these compounds have the potentiality to broaden their potential applications in humans for the prevention and/or treatment of several diseases associated with ROS-induced cellular damage. Future synthetic elaboration of the scaffolds **5**, **6**, **9** and **12**, through standard and reliable chemistry, are in progress to realize libraries of 23-conjugated silybin and DHS analogues.

Acknowledgments

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Supplementary data

Supplementary data (experimental procedures, NMR and MS characterization) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.06.049.

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