Enhanced Bioactivity of silybin B methylation Products

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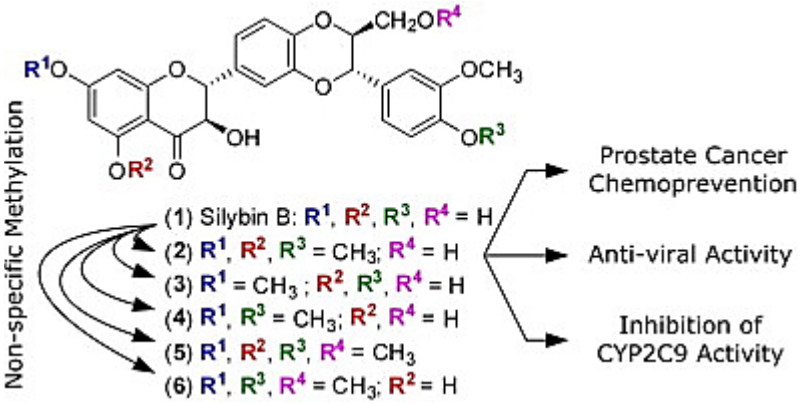
Abstract:

Flavonolignans from milk thistle (Silybum marianum) have been investigated for their cellular modulatory properties, including cancer chemoprevention and hepatoprotection, as an extract (silymarin), as partially purified mixtures (silibinin and isosilibinin), and as pure compounds (a series of seven isomers). One challenge with the use of these compounds in vivo is their relatively short half-life due to conjugation, particularly glucuronidation. In an attempt to generate analogues with improved in vivo properties, particularly reduced metabolic liability, a semi-synthetic series was prepared in which the hydroxy groups of silybin B were alkylated. A total of five methylated analogues of silybin B were synthesized using standard alkylation conditions (dimethyl sulfate and potassium carbonate in acetone), purified using preparative HPLC, and elucidated via spectroscopy and spectrometry. Of the five, one was monomethylated (3), one was dimethylated (4), two were trimethylated (2 and 6), and one was tetramethylated (5). The relative potency of all compounds was determined in a 72 h growth-inhibition assay against a panel of three prostate cancer cell lines (DU-145, PC-3, and LNCaP) and a human hepatoma cell line (Huh7.5.1) and compared to natural silybin B. Compounds also were evaluated for inhibition of both cytochrome P450 2C9 (CYP2C9) activity in human liver microsomes and hepatitis C virus infection in Huh7.5.1 cells. The monomethyl and dimethyl analogues were shown to have enhanced activity in terms of cytotoxicity, CYP2C9 inhibitory potency, and antiviral activity (up to 6-fold increased potency) compared to the parent compound, silybin B. In total, these data suggested that methylation of flavonolignans can increase bioactivity.

Graphical Abstract:



Keywords: Silybin B | Milk thistle | Silybum marianum | Flavonolignans | Methylation

Article:

1. Introduction

Our research team has been studying the chemopreventive properties of a series of flavonolignans isolated from an extract (termed 'silymarin')[1] of milk thistle [*Silybum marianum* (L.) Gaertn. (Asteraceae)]. [2, 3, 4, 5, 6,7 and 8] In vitro and human tumor xenograft anticancer activity of the commercial extracts, silymarin and silibinin, has been demonstrated in models of prostate, colon, and breast cancer. The extracts, and a subset of pure compounds derived therefrom, have been studied most extensively in prostate carcinoma cell lines, where they exhibit antiproliferative and pro-apoptotic activity via inhibition of expression of cyclins and cyclin-dependent kinases, induction of p21 and p27, degradation of androgen receptor, and activation of caspase-3 and caspase-[9. 2, 3, 4, 5, 6, 7 and 8] Studies on the natural products chemistry of these compounds led to the gram-scale isolation of the flavonolignans, [9] which consist of a series of seven diastereo or constitutional isomers, and the isolation and identification of two new minor analogs. [10] Besides chemoprevention, some of these materials have been evaluated as inhibitors of cytochrome P450 2C9 (CYP2C9) [11] activity and activities associated with hepatoprotection. [12, 13, 14 and 15]

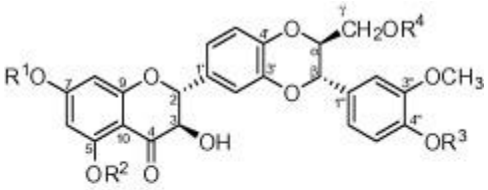
A challenge of working with milk thistle products has been translating the results from in vitro studies to the clinic, likely due to suboptimal pharmacokinetic characteristics of the compounds.[16] The pharmacokinetics of unconjugated and conjugated (sulfated and glucuronidated) flavonolignans from milk thistle were examined after oral administration of up to 700 mg/day silymarin, and 28% and 55% of the total silymarin compounds in plasma were found as sulfate and glucuronide conjugates, respectively, after 1–3 h.[17] Even with doses of 13 g/day with a silibinin derived product in clinical studies by Flaig et al.,[18] a major challenge to in vivo activity was extensive conjugation at phenolic positions. Conversely, these same phenolic moieties may present opportunities for the semi-synthetic modification of milk thistle compounds, if those that are or are not key for biological activity could be identified.

Other research groups have probed the semisynthesis of analogues of silibinin[19 and 20] (a 1:1 mixture of silybin A and silybin B)[1] and/or 2,3-dehydrosilybin.[21 and 22] The present semisynthetic studies were designed with two central goals. The primary chemistry goal was to determine which methylated analogues could be generated readily using a straightforward alkylation procedure. For this, selectivity was not critical, as it was desirable to examine the various permutations of single-, double-, triple-, and tetra-methylated silybin B analogues. The primary pharmacology goal was to determine how these structural variations affected the antiproliferative activity of silybin B (**1**). A secondary pharmacology goal was to examine the compounds for inhibition of CYP2C9 activity and of antiviral activity against hepatitis C virus (HCV). For the synthetic experiments, **1** was utilized as a representative flavonolignan, since it is a major component of both silibinin and silymarin,[2] there was an ample supply of >98% pure material on the gram scale available, and it was the easiest of the seven flavonolignans to purify.[9] Compounds that were monomethylated or dimethylated were more potent than **1**, with the most potent being the dimethylated analogue (compound **4**; approximately 6 times more active). These data suggested that alkylation of the phenolic moieties of the flavonolignans presents a strategy for their continued exploration.

2. Results and discussion

2.1. Synthesis and isolation

Silybin B (**1**) starting material was isolated in >98% purity from milk thistle extract (silymarin) as described in detail previously.[9] Methylated analogues of **1** were prepared in a manner similar to that of Dzubak and colleagues;[22] however, those researchers used a mixture of silybin A and silybin B (**1**) (often termed silibinin),[1] and thus their analogues were generated as diastereomeric mixtures.[22] Briefly, dimethyl sulfate was added to a solution of **1** and potassium carbonate in anhydrous acetone. The mixture was heated at reflux for 30 min, cooled to room temperature, acidified with dilute hydrochloric acid, and extracted with ethyl acetate. The organic extracts were combined and concentrated to dryness under reduced pressure. The resulting oil was purified using RP-HPLC to yield five major products (compounds **2**–**6**; Fig. 1) in greater than 95% purity as measured by analytical RP-HPLC (Fig. S1; the numbering of the compounds corresponds to their elution order).



(**1**) R^1, R^2, R^3, R^4 = H
(**2**) R^1, R^2, R^3 = CH_3; R^4 = H
(**3**) R^1 = CH_3 ; R^2, R^3, R^4 = H
(**4**) R^1, R^3 = CH_3; R^2, R^4 = H
(**5**) R^1, R^2, R^3, R^4 = CH_3
(**6**) R^1, R^3, R^4 = CH_3; R^2 = H

Figure 1. Structures of silybin B (**1**) and the methylated analogues **2**–**6**.

2.2. Structure elucidation

The structures of the five methylated analogues of silybin B (**1**) were elucidated using a suite of NMR experiments in conjunction with HRMS data. Table 1 and Table 2 summarize the ^{1}H and ^{13}C NMR data, respectively, for compounds **2**–**6** in comparison to the well established data for **1**. The supplement includes the ^{1}H and ^{13}C NMR data as stack plots (Figs. S2 and S3, respectively) and illustrates the ECD data (Fig. S4), which verified that all analogues retained the configuration of **1**.

Table 1.^{1}H NMR data for silybin B (**1**) and methylated analogues **2**–**6** acquired in DMSO-d_6

Position	**1**δ_H (*J* in Hz)	**2**δ_H (*J* in Hz)	**3**δ_H (*J* in Hz)	**4**δ_H (*J* in Hz)	**5**δ_H (*J* in Hz)	**6**δ_H (*J* in Hz)
2	5.08, d (12)	5.05, d (11)	5.13, d (12)	5.13, d (12)	5.04, d (12)	5.13, d (12)
3	4.61, d (12)	4.38, d (11)	4.66, br. d.	4.66, dd (12, 7)	4.66, dd (12, 7)	4.65, d (12)
6	5.92, s	6.22, d (2)	6.11, s	6.11, d (2)	6.22, d (2)	6.10, s
8	5.88, s	6.18, d (2)	6.09, s	6.09, d (2)	6.18, d (2)	6.08, s
2′	7.08, d (2)	7.08, d (2)	7.09, d (2)	7.10, d (2)	7.07, d (2)	7.11, d (2)

Position	**1**δ_H (*J* in Hz)	**2**δ_H (*J* in Hz)	**3**δ_H (*J* in Hz)	**4**δ_H (*J* in Hz)	**5**δ_H (*J* in Hz)	**6**δ_H (*J* in Hz)
5′	6.98, d (9)	6.97, d (9)	6.98, d (9)	6.97, d (9)	6.99, d (9)	7.01, d (9)
6′	7.01, dd (9, 2)	7.02, dd (9, 2)	7.03, dd (9, 2)	7.03, dd (9, 2)	7.09, dd (9, 2)	7.04, dd (9, 2)
α	4.17, ddd (8, 5, 2)	4.19, ddd (8, 4, 2)	4.17, ddd (8, 5, 2)	4.19, ddd (8, 4, 2)	4.36, m	4.38, ddd (8, 5, 2)
β	4.92, d (8)	4.97, d (8)	4.91, d (8)	4.97, d (8)	4.94, d (8)	4.95, d (8)
γ	3.54, ddd (11, 5, 2); 3.35, ddd (11, 5, 5)	3.82, m; 3.74, m	3.54, m; 3.35, m	3.55, m; 3.35, m	3.82, m; 3.74, m	3.81, m; 3.76, m
2″	7.02, d (2)	7.01, d (2)	7.02, d (2)	7.04, d (2)	7.05, d (2)	7.06, d (2)
5″	6.81 d, (8)	6.98, d (9)	6.80, d (8)	6.98, d (8)	6.97, d (8)	6.99, d (8)
6″	6.87, dd (8, 2)	6.99, dd (9, 2)	6.86, dd (8, 2)	6.99, dd (8, 2)	6.98, dd (8, 2)	7.00, dd (8, 2)
3-OH	—	—	—	5.88, d (7)	5.88, d (7)	—
5-OH	11.90, s	—	—	—	—	—
5-OCH_3	—	3.80, s	—	—	3.80, s	—
7-OCH_3	—	3.80, s	3.78, s	3.78, s	3.80, s	3.78, s
3″-OCH_3	3.78, s	3.77, s	3.77, s	3.77, s	3.78, s	3.78, s
4″-OCH_3	—	3.76, s	—	3.76, s	3.77, s	3.77, s

Position	**1**δ_H (*J* in Hz)	**2**δ_H (*J* in Hz)	**3**δ_H (*J* in Hz)	**4**δ_H (*J* in Hz)	**5**δ_H (*J* in Hz)	**6**δ_H (*J* in Hz)
γ-OCH_3	—	—	—	—	3.22, s	3.22, s

Table 2.^{13}C NMR data for silybin B (**1**) and methylated analogues **2**–**6** acquired in DMSO-d_6

Position	**1**δ_C, mult.	**2**δ_C, mult.	**3**δ_C, mult.	**4**δ_C, mult.	**5**δ_C, mult.	**6**δ_C, mult.
2	82.5, CH	82.1, CH	82.6, CH	82.9, CH	82.1, CH	82.6, CH
3	71.5, CH	72.5, CH	71.5, CH	71.6, CH	72.5, CH	71.5, CH
4	197.8, C	190.2, C	198.4, C	198.4, C	190.2, C	193.3, C
5	163.3, C	161.6, C	163.0, C	163.0, C	161.6, C	162.4, C
6	96.1, CH	92.9, CH	94.9, CH	94.9, CH	92.9, CH	93.8, CH
7	166.8, C	165.7, C	167.6, C	167.6, C	165.7, C	167.6, C
8	95.0, CH	93.6, CH	93.8, CH	93.8, CH	93.6, CH	95.0, CH
9	162.5, C	163.7, C	162.4, C	162.4, C	163.7, C	163.6, C
10	100.5, C	103.5, C	101.4, C	101.4, C	103.5, C	101.4, C
1′	130.1, C	130.3, C	129.9, C	130.0, C	130.4, C	130.2, C
2′	116.7, CH	116.5, CH	116.6, CH	116.6, CH	116.6, CH	116.8, CH
3′	143.2, C	143.1, C	143.2, C	143.2, C	143.1, C	143.1, C
4′	143.6, C	143.5, C	143.7, C	143.6, C	143.2, C	143.4, C
5′	116.4, CH	116.4, CH	116.4, CH	116.4, CH	116.4, CH	116.4, CH
6′	121.2, CH	121.0, CH	121.2, CH	121.0, CH	121.1, CH	121.3, CH
α	78.1, CH	78.0, CH	78.1, CH	78.1, CH	75.7, CH	75.8, CH

Position	**1**δ_C, mult.	**2**δ_C, mult.	**3**δ_C, mult.	**4**δ_C, mult.	**5**δ_C, mult.	**6**δ_C, mult.
β	75.9, CH	75.7, CH	75.8, CH	76.0, CH	76.4, CH	76.4, CH
γ	60.2, CH_2	60.1, CH_2	60.2, CH_2	60.1, CH_2	70.9, CH_2	70.9, CH_2
1″	127.5, C	129.0, C	127.5, C	129.0, C	128.7, C	128.7, C
2″	111.6, CH	111.1, CH	111.6, CH	111.7, CH	111.0, CH	111.0, CH
3″	147.6, C	149.1, C	147.6, C	149.1, C	149.1, C	149.2, C
4″	147.0, C	148.8, C	147.0, C	148.8, C	148.8, C	148.8, C
5″	115.3, CH	111.5, CH	115.3, CH	116.2, CH	111.5, CH	111.5, CH
6″	120.5, CH	120.2, CH	120.5, CH	120.5, CH	120.2, CH	120.2, CH
5-OCH_3	—	55.9, CH_3	—	—	56.0, CH_3	—
7-OCH_3	—	55.9, CH_3	56.0, CH_3	56.0, CH_3	55.8, CH_3	56.0, CH_3
3″-OCH_3	55.7, CH_3	55.6, CH_3	55.7, CH_3	55.6, CH_3	55.6, CH_3	55.6, CH_3
4″-OCH_3	—	55.5, CH_3	—	55.4, CH_3	55.5, CH_3	55.5, CH_3
γ-OCH_3	—	—	—	—	58.6, CH_3	58.6, CH_3

Of the five compounds, one was monomethylated (**3**), one was dimethylated (**4**), two were trimethylated (**2**and **6**), and one was tetramethylated (**5**). The signals for methoxy groups in the ^1H and ^{13}C NMR spectra (i.e. δ_H 3.2–3.8 and δ_C 55–59, respectively) helped identify the number of methyl groups incorporated in the structure of **1**; the HRMS data served to verify the number of additional methyl groups. For example, compound **2**, which was the major product of the reaction, was 5,7,4″-tri-*O*-methylsilybin B (**2**). Key HMBC signals that established the positions of the methyl moieties included correlations from OCH_3-5 to C-5, from OCH_3-7 to C-7, and from OCH_3-4″ to C-4″ (Fig. 2). HRESIMS data confirmed the addition of three methyl groups in the structure (*m/z* 547.1579 $[M+Na]^+$, calcd for $C_{28}H_{28}O_{10}Na$, 547.1580). The other analogues displayed similar HMBC correlations, thereby establishing the structures of 7-*O*-methylsilybin B (**3**), 7,4″-di-*O*-methylsilybin B (**4**), 5,7,4″, γ-tetra-*O*-methylsilybin B (**5**), and 7,4″, γ-tri-*O*-methylsilybin B (**6**). As with compound **2**, these structures were supported by HRMS data (see Section 4).

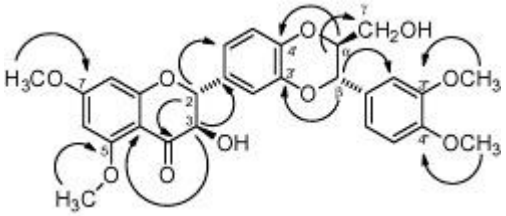


Figure 2. Key HMBC correlations for the major product of the reaction, 5,7,4″ -tri-*O*-methylsilybin B (**2**). The other analogues displayed similar data, depending on the number of methyl groups incorporated.

2.3. Bioassay results

2.3.1. Antiproliferative assays

The potency of the analogues (**2**–**6**) was determined in a 72 h growth-inhibition assay relative to silybin B (**1**) and against a panel of three prostate cancer cell lines (DU-145, PC-3, and LNCaP) and a human hepatoma cell line (Huh7.5.1; Table 3). For inhibition of prostate cancer cells, the most potent analogue (~6-fold increased potency) was compound **4**, corresponding to methylation of the phenolic moieties at positions 7 and 4″ , yielding IC_{50} values that were ⩽10 μ M against two of the cell lines (DU-145 and PC-3). The next most potent analogue, having a single methyl group at the 7 position (**3**), was approximately a factor of two less active than **4**. However, all of the compounds were equal to or slightly more potent (up to 6-fold increased potency) than the parent (**1**; mean IC_{50} = 61.8 μ M in DU-145 cells). Similarly, for inhibition of hepatoma cell growth, all of the methylated compounds were more potent than the parent. In particular, compounds **3** and **4**were over 5-fold more potent than the parent (**1**). In sum total, these cytotoxicity data, regardless of cell type, suggested that modifications at any of the phenolic moieties could generate compounds that enhance the antiproliferative potency of silybin B (**1**).

Table 3. Antiproliferative activity of silybin B (**1**) and analogues **2**–**6** against a panel of cell lines in culture

Compound	Mean IC_{50} values and standard deviations (μM)			
	DU-145	PC-3	LNCaP	Huh7.5.1
1	61.8 ± 3.3	60.5 ± 4.3	69.6 ± 3.8	56.2 ± 2.8[a]
2	46.9 ± 3.7	36.3 ± 3.7	40.7 ± 3.9	25.5 ± 1.8

Compound	Mean IC_{50} values and standard deviations (μM)			
	DU-145	PC-3	LNCaP	Huh7.5.1
3	19.3 ± 3.4	16.9 ± 3.6	20.1 ± 3.7	13.3 ± 1.8
4	10.0 ± 3.7	8.1 ± 3.5	11.3 ± 4.8	13.5 ± 1.8
5	24.0 ± 4.3[a]	14.4 ± 4.4	30.1 ± 3.7	15.6 ± 1.8
6	16.8 ± 3.9	21.9 ± 4.1	19.8 ± 4.8	nt[b]

a The IC_{50} values and standard deviations for these points were estimated based on visual inspection of sigmoidal dose‐response curves.

b Not tested.

2.3.2. Inhibition of CYP2C9 activity

The inhibitory effects of four of the major compounds found in silymarin (silybin A, silybin B (**1**), isosilybin A, and isosilybin B) on the CYP2C9-mediated metabolism of (*S*)-warfarin were reported recently. [11] Silybin B (**1**) was the most potent of the four, with an IC_{50} value of 8.2 μM. [11] Accordingly, the CYP2C9 inhibitory potencies of the methylated analogues (**2**‐**5**) were compared (Fig. 3); compound **6** was not evaluated due to paucity of sample. With the exception of **5**, all compounds inhibited CYP2C9 activity in a concentration-dependent manner (1 vs 10 μM). Compound **2** was less potent than **1** at 100 μM, whereas compounds **3** and**4**, representing monomethylation at the 7-OH and dimethylation at the 7- and 4″ -OH moieties, respectively, were more potent than **1**, with activities below the limit of quantification when tested at 100 μM. These results suggested that despite increased cytotoxic potency, methylation of silybin B may modulate the clearance of drugs metabolized by CYP2C9.

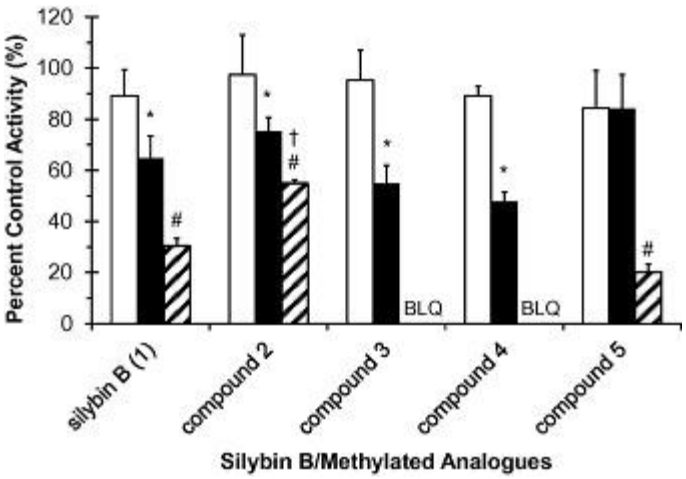


Figure 3. Effects of silybin B (**1**) and methylated analogues (**2**–**5**) on CYP2C9-mediated (*S*)-warfarin 7-hydroxylation in human liver microsomes (HLM). Incubation mixtures consisted of HLM (0.1 mg/mL), (*S*)-warfarin (4 μM), silybin B or methylated analogue (1, 10, or 100 μM; open, solid, and hatched bars, respectively) and potassium phosphate buffer (100 mM, pH 7.4). Reactions were initiated by the addition of NADPH (1 mM) and were terminated after 30 min with ice-cold MeOH (2 volumes). Activity in the presence of vehicle control (0.75% MeOH, v/v) was 4.3 $\pm$ 0.26 pmol/min/mg microsomal protein. Bars and error bars denote means and SDs, respectively, of triplicate incubations. *BLQ*, below limit of quantification. $^{*}p<0.05$, 1 versus 10 μM; $^{\#}p<0.05$, 10 versus 100 μM; $^{\dagger}p<0.05$ versus silybin B at 100 μM (two-way ANOVA followed by Tukey's test).

2.3.3. Inhibition of hepatitis C virus (HCV) infection

Figure 4 illustrates the antiviral profile of compounds **2**–**5** against HCV infection of human hepatoma Huh7.5.1 cells; compound **6** was not evaluated due to paucity of sample. Previous studies indicated that the IC_{50} value for inhibition of HCV infection by silybin B (**1**) was approximately 40 to 80 μM.[13] The tested analogues of **1** inhibited HCV infection at lower concentrations, and the dimethyl analogue **4** was the most potent. These data indicated that methylated silybin B analogues were more active than **1** as antivirals in the following order: dimethyl (**4**) > monomethyl (**3**) > tetramethyl (**5**) > trimethyl (**2**) > **1**. It was intriguing that both the cytotoxicity and antiviral activities were enhanced by methylation, even though the antiviral testing was conducted at non-toxic concentrations of the analogues.

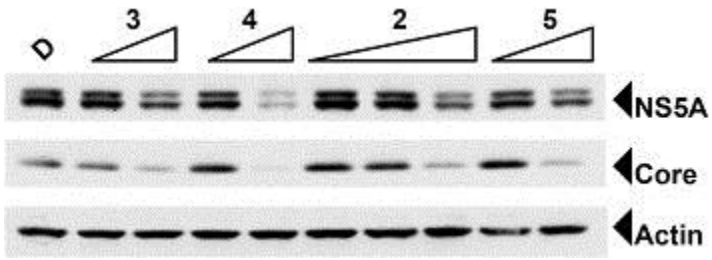


Figure 4. Methylated analogues (**2**–**5**) were more potent than silybin B (**1**) in blocking HCV infection. Human hepatoma Huh7.5.1 cells were infected with HCV in the presence of increasing concentrations of analogues for 72 h before cytoplasmic proteins were extracted and HCV NS5A and core proteins detected by Western blot. Detection of cellular actin protein served as a loading control. The concentrations selected for testing were nontoxic: compound **2**: 1.9, 9.4, 18.9 μM; compound **3**: 2.0, 10.1 μM; compound **4**: 1.9, 9.7 μM; compound **5**: 1.8, 9.2 μM. D refers to the DMSO solvent control.

3. Conclusion

In summary, a series of methylated analogues of silybin B (**1**) were synthesized; some of these analogues have been reported previously, albeit in the context of diastereomeric mixtures with silybin A analogues.[22] In the case of the dimethyl (**4**) and monomethyl (**3**) analogues, and irrespective of the biological assay, the bioactivity increased relative to **1** in terms of growth inhibition of prostate and liver cancer cells, inhibition of CYP2C9 activity, and antiviral activity against HCV. These data suggest the feasibility of retaining and even enhancing the bioactivity of silymarin-derived compounds, and provides a rationale for methylating other isomers to test in anticancer, antiviral, metabolic, and pharmacologic assays. Ultimately, such studies could provide valuable structure–activity relationships for delineating the mechanisms of action of flavonolignans, which could aid in understanding how these compounds act in many inflammatory disease states.

4. Experimental

4.1. General experimental methods

Optical rotation, UV, and IR data were acquired on a Rudolph Research Autopol® III polarimeter, a Varian Cary 3 UV–Vis spectrophotometer, and a Nicolet Avatar 360 FT-IR, respectively. Circular dichroism spectra were obtained from an Aviv Stopped Flow Model 202 circular dichroism spectrometer. All NMR experiments were acquired on a Varian Unity INOVA-500 instrument using a 5 mm broad-band inverse probe with z-gradient using DMSO-d_6. LRESIMS data were acquired using an API 150EX mass spectrometer. HRMS data were acquired on an Applied Biosystems 4700 TOF/TOF instrument operating in reflectron mode using 2,5 dihydroxybenzoic acid as the matrix. For those data, spectra were the average of 500–1000 laser shots, and the mass values were the mean of five independent measurements. Some HRMS data were also acquired on a Thermo LTQ Orbitrap XL mass spectrometer equipped with an ESI source. Preparative HPLC was carried out on a Varian Prostar HPLC system equipped with Prostar 210 pumps and a 335 photodiode array detector (PDA), with data collected and analyzed using Galaxie Chromatography Data System software (version 1.9), using an ODS-A column (250 $\times$ 20 mm, i.d., 5 μm; YMC, Wilmington, NC) at a flow rate of 7 mL/min. This

same system and software were used for analytical HPLC via an ODS-A column (150 × 4.6 mm, i.d., 5 μm) at a flow rate of 1 mL/min.

4.2. Synthesis and purification

Silybin B (**1**) was isolated in >98% purity from milk thistle extract (silymarin) as described in detail previously.[9] Methylated analogs of **1** were prepared in a manner analogous to that of Dzubak and colleagues.[22] Dimethyl sulfate (0.20 g, 1.55 mmol) was added to a solution of **1** (0.15 g, 0.31 mmol) and K_2CO_3 (0.35 g, 2.48 mmol) in anhydrous acetone (5 mL). The suspension was allowed to stir at reflux for 30 min and was then cooled to room temperature. The suspension was diluted with 1 N HCl (5 mL) and extracted with EtOAc (3 × 5 mL). The organic extracts were combined, dried ($MgSO_4$), and concentrated under reduced pressure to provide a brown oil (150 mg) as a mixture of methylated analogs.

4.2.1. Purification

The reaction product (150 mg) was dissolved in DMSO (500 μL) and purified via two separate rounds (~75 mg/round) of RP-HPLC using a gradient that initiated with 60:40 MeOH:H_2O and increased linearly to 70:30 MeOH:H_2O over 60 min, where it was held isocratic for another 40 min, to yield compounds **2**(25.1 mg; 16.7%), **3** (10.6 mg; 7.1%), **4** (14.5 mg; 9.7%), **5** (12.0 mg; 8.0%), and **6** (2.7 mg; 1.8%).

4.2.1.1. Silybin B (**1**)

The physical properties of **1** were described in detail previously.[23 and 24]

4.2.1.2. 5,7,4″ -Tri-*O*-methylsilybin B (**2**)

White solid (25.1 mg, yield 16.7% w/w); t_R 7.65 min in 60:40 → 100:0 MeOH–H_2O over 15 min with the ODS A column; $[\alpha]_D^{25}$ −10.67 (*c* 0.6, MeCN); UV (MeOH) λ_{max} (log ε) 283 (5.25), 205 (5.88) nm; CD (MeOH) λ_{ext}($\Delta\varepsilon$) 333 (+3.3), 293 (−19.7), 233 (+21.6) nm; IR (KBr) ν_{max} 3438, 2934, 1672, 1606, 1571, 1508, 1422, 1257, 1214, 1106, 1021, 985, 812 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1 and Table 2; HMBC data, H-2 → C-3, 4, 9, 1′, 2′, 6′; H-3 → C-2, 4, 10, 1′; H-6 → C-5, 7, 8, 10; H-8 → C-6, 7, 9, 10; H-2′ → C-2, 1′, 3′, 4′; H-5′ → C-1′, 3′, 4′, 6′; H-6′ → C-2, 1′, 2′, 4′, 5′; H-α → C-β, γ, 4′, 1″; H-β → C-α, γ, 3′, 1″, 2″, 6″; H_2-γ → C-α, β; H-2″ → C-1″, 3″, 4″, 6″; H-5″ → C-1″, 3″, 4″, 6″; H-6″ → C-β, 1″, 2″, 4″, 5″; OCH_3-3″ → C-3″; OCH_3-5 → C-5; OCH_3-7 → C-7; OCH_3-4″ → C-4″; HRESIMS *m*/*z* 547.1579 $[M+Na]^+$ (calcd for $C_{28}H_{28}O_{10}Na$, 547.1580).

4.2.1.3. 7-*O*-Methylsilybin B (**3**)

White solid (10.6 mg, yield 7.1% w/w); t_R 7.88 min in 60:40 → 100:0 MeOH–H_2O over 15 min with the ODS A column; $[\alpha]_D^{25}$ +2.67 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 287 (5.28), 204 (5.93) nm; CD (MeOH) λ_{ext} ($\Delta\varepsilon$) 333 (+3.7), 295 (−15.5), 234 (+12.4) nm; IR (KBr) ν_{max} 3400, 2936, 1668, 1606, 1571, 1508, 1422, 1258, 1214, 1105, 1021, 986, 813 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1 and Table 2; HMBC data, H-2 → C-3, 4, 9, 1′, 2′, 6′; H-3 → C-2, 4, 10, 1′; H-6 → C-5, 7, 8, 10; H-8 → C-6, 7, 9, 10; H-2′ → C-2, 1′, 3′, 4′; H-5′ → C-1′, 3′, 4′, 6′; H-6′ → C-2, 1′, 2′, 4′, 5′; H-α → C-β, γ, 4′, 1″; H-β → C-α, γ, 3′, 1″, 2″, 6″; H_2-γ → C-α, β; H-2″ → C-1″, 3″, 4″, 6″; H-5″ → C-1″, 3″, 4″, 6″; H-6″ → C-β, 1″, 2″, 4″, 5″; OCH_3-3″ → C-3″; OCH_3-7 → C-7; HRESIMS *m*/*z* 519.1271 $[M+Na]^+$ (calcd for $C_{26}H_{24}O_{10}Na$, 519.1267).

4.2.1.4. 7,4″-Di-*O*-methylsilybin B (**4**)

White solid (14.5 mg, yield 9.7% w/w); t_R 9.17 min in 60:40 → 100:0 MeOH–H_2O over 15 min with the ODS A column; $[\alpha]_D^{25}$ +8.77 (*c* 0.7, MeOH); UV (MeOH) λ_{max} (log ε) 286 (5.26), 205 (5.93) nm; CD (MeOH) λ_{ext} ($\Delta\varepsilon$) 335 (+5.1), 294 (-21.8), 233 (+17.7) nm; IR (KBr) ν_{max} 3432, 2937, 1670, 1606, 1572, 1508, 1422, 1258, 1214, 1105, 1021, 986, 811 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1 and Table 2; HMBC data, H-2 → C-3, 4, 9, 1′, 2′, 6′; H-3 → C-2, 4, 10, 1′; H-6 → C-5, 7, 8, 10; H-8 → C-6, 7, 9, 10; H-2′ → C-2, 1′, 3′, 4′; H-5′ → C-1′, 3′, 4′, 6′; H-6′ → C-2, 1′, 2′, 4′, 5′; H-α → C-β, γ, 4′, 1″; H-β → C-α, γ, 3′, 1″, 2″, 6″; H_2-γ → C-α, β; H-2″ → C-1″, 3″, 4″, 6″; H-5″ → C-1″, 3″, 4″, 6″; H-6″ → C-β, 1″, 2″, 4″, 5″; OCH_3-3″ → C-3″; OCH_3-7 → C-7; OCH_3-4″ → C-4″; HRESIMS*m*/*z* 533.1429 $[M+Na]^+$ (calcd for $C_{27}H_{26}O_{10}Na$, 533.1424).

4.2.1.5. 5,7,4″,γ-Tetra-*O*-methylsilybin B (**5**)

White solid (12.0 mg, yield 8.0% w/w); t_R 9.91 min in 60:40 → 100:0 MeOH–H_2O over 15 min with the ODS A column; $[\alpha]_D^{25}$ −6.18 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 285 (5.26), 205 (5.91) nm; CD (MeOH) λ_{ext} ($\Delta\varepsilon$) 334 (+3.1), 294 (−16.2), 233 (+15.5) nm; IR (KBr) ν_{max} 3424, 2936, 1670, 1606, 1571, 1508, 1422, 1257, 1214, 1106, 986, 812 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1 and Table 2; HMBC data, H-2 → C-3, 4, 9, 1′, 2′, 6′; H-3 → C-2, 4, 10, 1′; H-6 → C-5, 7, 8, 10; H-8 → C-6, 7, 9, 10; H-2′ → C-2, 1′, 3′, 4′; H-5′ → C-1′, 3′, 4′, 6′; H-6′ → C-2, 1′, 2′, 4′, 5′; H-α → C-β, γ, 4′, 1″; H-β → C-α, γ, 3′, 1″, 2″, 6″; H_2-γ → C-α, β; H-2″ → C-1″, 3″, 4″, 6″; H-5″ → C-1″, 3″, 4″, 6″; H-6″ → C-β, 1″, 2″, 4″, 5″; OCH_3-3″ → C-3″; OCH_3-5 → C-5; OCH_3-

7 → C-7; OCH_3-4″ → C-4″ ; OCH_3-γ → C-γ ; HRESIMS *m*/*z* 561.1746 $[M+Na]^+$ (calcd for $C_{29}H_{30}O_{10}Na$, 561.1737).

4.2.1.6. 7,4″ ,γ -Tri-*O*-methylsilybin B (**6**)

White solid (2.7 mg, yield 1.8% w/w); t_R 11.03 min in 60:40 → 100:0 MeOH–H_2O over 15 min with the ODS A column; $[\alpha]_D^{31}$ +9.0 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 286 (5.13), 205 (5.86) nm; CD (MeOH) λ_{ext} ($\Delta\varepsilon$) 335 (+4.0), 294 (−19.7), 232 (+17.4) nm; IR (KBr) ν_{max} 3400, 2935, 1670, 1606, 1572, 1508, 1423, 1258, 1215, 1107, 1023, 951, 815 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1 and Table 2; HMBC data, H-2 → C-3, 4, 9, 1′ , 2′ , 6′ ; H-3 → C-2, 4, 10, 1′ ; H-6 → C-5, 7, 8, 10; H-8 → C-6, 7, 9, 10; H-2′ → C-2, 1′ , 3′ , 4′ ; H-5′ → C-1′ , 3′ , 4′ , 6′ ; H-6′ → C-2, 1′ , 2′ , 4′ , 5′ ; H-α → C-β , γ , 4′ , 1″ ; H-β → C-α , γ , 3′ , 1″ , 2″ , 6″ ; H2-γ → C-α , β ; H-2″ → C-1″ , 3″ , 4″ , 6″ ; H-5″ → C-1″ , 3″ , 4″ , 6″ ; H-6″ → C-β , 1″ , 2″ , 4″ , 5″ ; OCH_3-3″ → C-3″ ; OCH_3-7 → C-7; OCH_3-4″ → C-4″ ; OCH_3-γ → C-γ ; HRESIMS *m*/*z* 525.1740 $[M+H]^+$ (calcd for $C_{28}H_{29}O_{10}$, 525.1755).

4.3. Biological assays

4.3.1. Antiproliferative assay

All compounds were tested for antiproliferative/cytotoxic activity against a panel of prostate cancer cell lines in culture using modifications of a published procedure[2] that have been described in detail.[10] The prostate cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA): DU145 (HTB-81, an androgen-independent line derived from a central nervous system metastasis of prostate adenocarcinoma), PC-3 (CRL-1435, an androgen-independent line derived from a bone metastasis of prostate adenocarcinoma), and LNCaP (CRL-1740, an androgen-dependent line derived from a lymph node metastasis of prostate adenocarcinoma). All cell lines were cultured and maintained as described previously,[2] with the recently noted modification for LNCaP.[10] We also tested the antiproliferative/cytotoxic activity of compounds against a liver cancer cell line, Huh7.5.1.[25] Briefly, 10,000 cells per well were plated in 96-well plates, and following overnight incubation, were incubated with increasing concentrations of compounds. Seventy-two hours later, cell viability was measured using the ATPlite kit, as described previously,[13] and an IC_{50} was calculated by linear regression using GraphPad Prism.

4.3.2. Inhibition of CYP2C9 activity

Silybin B (**1**) and methylated analogues (**2**–**5**) were evaluated as inhibitors of CYP2C9 activity using (*S*)-warfarin and pooled human liver microsomes (HLM) as described previously. [11] Incubation mixtures consisted of HLM (0.1 mg/mL microsomal protein), (*S*)-

warfarin (4 μM), silybin B or methylated analogue (1, 10, or 100 μM), and potassium phosphate buffer (100 mM, pH 7.4). The CYP2C9 inhibitor sulfaphenazole (1 μM) was used as a positive control. Control incubation mixtures contained 0.75% MeOH (v/v) in place of silybin B/methylated analogue or sulfaphenazole. Incubation mixtures were analyzed for 7-hydroxywarfarin by HPLC/MS-MS as described previously. [11]

All statistical analyses for this assay were conducted using SigmaStat (version 3.5; Systat Software, Inc., San Jose, CA). Data are presented as means ± SDs of triplicate incubations unless indicated otherwise. Concentration-dependent inhibition of silybin B and methylated analogs and comparisons between silybin B and methylated analogs were evaluated by two-way analysis of variance (ANOVA); post hoc comparisons were made using Tukey's test when an overall difference resulted (p <0.05).

4.3.3. Antiviral assays

The antiviral activity of **1**-**5** was evaluated by choosing two concentrations that were non-toxic to cells. For this, 150,000 cells were plated in 12-well plates, and the next day, cells were infected with JFH-1, a HCV that grows well in Huh7.5.1 cells, at a multiplicity of infection of 0.05 focus forming units per cell. Five hours post-infection, virus inocula were removed and replaced with fresh medium containing compounds. Protein lysates were harvested 72 h later, and HCV proteins were detected by Western blot analyses as described previously.[13]

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Supplementary data

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