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3-*O*-Alkyl-2,3-Dehydrosilibinins: Two Synthetic Approaches and *in vitro* Effects toward Prostate Cancer Cells

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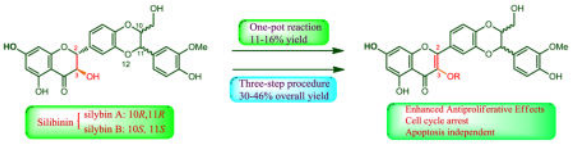
Abstract

Eight 3-*O*-alkyl-2,3-dehydrosilibinins have been synthesized from commercially available silibinin through two synthetic approaches. A one-pot reaction, starting with aerobic oxidation of silibinin followed by direct alkylation of the phenolic hydroxyl group in the subsequent 2,3-dehydrosilibinin, furnishes the desired derivatives in 11–16% yields. The three-step procedure employing benzyl ether to protect 7-OH in silibinin generates the desired derivatives in 30–46% overall yields. The antiproliferative activity of the 2,3-dehydrosilibinin derivatives against both androgen-sensitive and androgen-insensitive prostate cancer cells have been assessed using a WST-1 cell proliferation assay. All derivatives exhibited greater antiproliferative potency than silibinin, with 2,3-dehydrosilibinins each possessing a three- to five-carbon linear alkyl group to 3-OH (IC_{50} values in a range of 1.71 to 3.06 μM against PC-3 and LNCaP cells) as the optimal derivatives. The optimal potency was reached with three- to five-carbon alkyl groups. Our findings suggest that 3-*O*-propyl-2,3-dehydrosilibinin effectively inhibits the growth of PC-3 prostate cancer cells by arresting cell cycle in the G_0/G_1 phase, but not by activating PC-3 cell apoptosis.

Graphical abstract



Supplementary data

Supplementary data (synthetic procedures and structural characterization) associated with this article can be found, in the online version, at http://dx.doi.org/

Silibinin (**1**), isolated from milk thistle (*Silybum marianum* L. *Gaertner*, Asteraceae), represents the first identified and well-investigated flavonolignan. Milk thistle is a well-known traditional European medicine that has long been used for treating liver disorders and protecting the liver against a variety of xenobiotics and hepatotoxins. Its medicinal merits in this field were first recorded in Hieronymus Bock's book published in 1539. 2,3-Dehydrosilibinin (**2**), as the most important oxidized derivative of silibinin, was first synthesized from silibinin (**1**) and employed to revise the structure of silibinin by Pelter and Haensel in 1968. Several studies have so far confirmed that silibinin can be readily converted to 2,3-dehydrosilibinin through oxidation of the secondary aliphatic hydroxyl group to a ketone followed by enolization. So far, only two full reports have been published on the isolation of 2,3-dehydrosilibinin from natural sources including seeds of *S. marianum* subsp. *anatolicum* and the fruits of spotted milkweed (*S. marianum* L. *Gaertn.*) cultivated in Russia and CIS countries. Without publishing the detailed data, Gazak and co-workers pointed out that 2,3-dehydrosilibinin exists as a minor constituent in almost all crude extracts of milk thistle (silymarin) and is responsible for the yellow color of silymarin. It remains unclear whether 2,3-dehydrosilibinin is a naturally occurring or an artefact flavonolignan.

Recently, 2,3-dehydrosilibinin has been reported to display significant improvements over silibinin in numerous biological activities. As compared with silibinin, 2,3-dehydrosilibinin is superior by one order of magnitude in antioxidative properties; it is a 25 times more potent radical scavenger; it inhibits lipid peroxidation 10 times more efficiently;[,] it possesses more potent cytotoxicity against human prostate cancer cells; it exhibits better apoptotic activity in HTB cell model; and it exhibits a higher cytoprotective potential in hepatoma HepG2 cells. Additionally, *C*-isoprenylated or geranylated derivatives of 2,3-dehydrosilibinin were demonstrated to be effective P-glycoprotein modulators. Our previous studies showed that 7-*O*-alkyl-2,3-dehydrosilibinins with a C2–C3 double bond have better antiproliferative potency than 7-*O*-alkylsilibinins with a C2–C3 single bond against androgen-resistant human prostate cancer cell lines (DU145 and PC-3).

The ultimate goal of our program on 2,3-dehydrosilibinin is to engineer new derivatives with enhanced potency and bioavailability through appropriate structure manipulations for the treatment of castration-resistant prostate cancer. At the starting point of this long standing program, our ongoing studies aim to systematically explore the appropriate structure moieties of 2,3-dehydrosilibinin for further modifications. Recently, we reported that *in vitro* antiproliferative potency of 2,3-dehydrosilibinin against three prostate cancer cell lines can be significantly improved through appropriate chemical modifications on 7-OH. This encouraged us to investigate the effects of 3-OH modifications on prostate cancer cell proliferation. However, 3-*O*-alkyl-2,3-dehydrosilibinins cannot be achieved by the synthetic methods employed in our previous study, which can only yield 7-*O*-alkyl-2,3-dehydrosilibinins and 3,7-*O*-dialkyl-2,3-dehydrosilibinins. Consequently, the present study focuses on the exploration of general methods for the synthesis of 3-*O*-alkyl-2,3-dehydrosilibinins and *in vitro* evaluation of these derivatives as anti-prostate cancer agents.

3-*O*-Methyl-2,3-dehydrosilibinin was reported by Dzubak and co-workers to be capable of improving *in vitro* antiproliferative potency against K562 human myeloid leukemia cancer cells and of blocking functional activity of P-glycoprotein. No other 3-*O*-alkyl-2,3-

dehydrosilibinins have been reported. The challenge for the synthesis of 3-*O*-alkyl-2,3-dehydrosilibinins lies in the competitive reactivity of the four phenolic hydroxyl groups at C-3, C-5, C-7, and C-20 in 2,3-dehydrosilibinin. The relative reactivity of the phenolic hydroxyl groups in silibinin toward the etherification reaction is approximately 7-OH > 20-OH ≫ 5-OH. The only known 3-*O*-alkyl-2,3-dehydrosilibinin reported in the literature is the methyl derivative (**3**). It was synthesized in 45% yield by direct alkylation of 2,3-dehydrosilibinin, prepared by oxidation of silibinin in 13–90%, using sodium hydride as base and DMF as solvent.[9] This indicated that the 3-OH in 2,3-dehydrosilibinin is more reactive than 7-OH toward the etherification reaction.

Two synthetic approaches to a group of 3-*O*-alkyl-2,3-dehydrosilibinns have been developed in this paper. Our first synthetic approach to the 3-*O*-alkyl-2,3-dehydrosilibins is illustrated in Scheme 1. Specifically, the one-pot reaction starts from potassium acetate-mediated aerobic oxidation of silibinin followed by selective alkylation of 3-OH of the subsequent 2,3-dehydrosilibinin. In our hands, oxidation of silibinin to 2,3-dehydrosilibinin can be achieved under aerobic conditions using either potassium carbonate or potassium acetate as base and DMF as solvent. Using potassium carbonate to mediate the oxidation in the one-pot reaction led to decreased yields. This is probably due to the simultaneous deprotonation of 7-OH during oxidation, resulting in low selectivity of alkylation on 3-OH of 2,3-dehydrosilibinin. Prolonging the reaction time led to no significant change in yields. The one-pot reaction under the optimal conditions furnishes the desired derivatives in 11–16% yields (Table 1). Through this method, we could eliminate two steps required for the temporary protection/deprotection of other phenolic hydroxyl groups. However, it is challenging to further improve the yield due to the competitive reactivity of two phenolic hydroxyl groups at C-3 and C-7. The products from this reaction as determined by TLC analysis include the corresponding 7-*O*-alkyl-2,3-dehydrosilibinins and 3,7-*O*-dialkyl-2,3-dehydrosilibinins in addition to the desired 3-*O*-alkyl-2,3-dehydrosilibinins (**3**–**10**).

As shown in Scheme 2, the three-step procedure includes benzyl ether protection of 7-OH in silibinin to yield derivative **11**, oxidation of **11** followed by selective alkylation on 3-OH generates derivatives **12**–**18**, and debenzylation of **12**–**18** in the presence of ammonium formate catalyzed by palladium carbon provides the desired derivatives **3**–**9** in 30–46% overall yields for three steps (Table 1). The two- to three-fold improvement in overall yields is primarily attributed to higher efficiency of oxidation of 7-*O*-benzylsilibinin to 7-*O*-benzyl-2,3-dehydrosilibinin and higher selectivity of alkylation on 3-OH.

The structures of the eight 3-*O*-alkyl-2,3-dehydrosilibinins were characterized by interpreting their NMR, HRMS, and FTIR data. The ^{1}H and ^{13}C NMR data for compound **5** (Table 2) were fully assigned based on the interpretation of their COSY, HMQC, and HMBC data. The propyl group in compound **5** was assigned to 3-OH based on the key HMBC correlations from the triplet signal at δ_H 4.02 (CH_2 in propyl) to the signal at δ_C 138.8 (C-3, Fig. 1). This assignment is also supported by the absence of a broad singlet signal at around δ_H 6.5 for the proton of 3-OH in 2,3-dehydrosilibinin (**2**).

The *in vitro* anti-proliferative activities of the dehydrosilibinin derivatives were evaluated using a WST-1 cell proliferation assay in both androgen-sensitive (LNCaP) and androgen-

insensitive (PC-3 and DU145) human prostate cancer cell lines. The detailed procedure is described in the Experimental Section in Supplementary Data. Silibinin was used as a positive control for comparison in the parallel experiments and the IC_{50} values are listed in Table 3. The cytotoxicity of 2,3-dehydrosilibinin at 30 μM and 60 μM against PC-3 human prostate cancer cells has been reported, but without an IC_{50} value in the literature. Here, the antiproliferative activity of 2,3-dehydrosilibinin (**2**) is first reported with IC_{50} values against three human prostate cancer cell lines. All eight 3-*O*-alkyl-2,3-dehydrosilibinins (**3**–**10**) as well as 2,3-dehydrosilibinin exhibit significantly greater anti-proliferative potency by comparing their IC_{50} values with that of silibinin (Table 3). The potency is slightly enhanced with increasing length of the alkyl group, reaching the maximum with three- to five-carbon alkyl groups. Consequently, 2,3-dehydrosilibinins **5**–**7** each with a three- to five-carbon linear alkyl group attached to 3-OH were identified as the optimal derivatives with IC_{50} values in a range of 1.71–3.06 μM and 1.99–2.07 μM against PC-3 and LNCaP cells, respectively. All synthesized 3-*O*-alkyl-2,3-dehydrosilibinins (**3**–**10**) as well as 2,3-dehydrosilibinin (**2**) are more effective in inhibiting proliferation of LNCaP and PC-3 cells than of DU145 cells. Specifically, they are 5–42 times more potent toward LNCaP and PC-3 cell lines, but only 4–8 folds more potent against the DU145 cell line, as compared with silibinin.

Our data further corroborate that 2,3-dehydrosilibinin has greater anti-proliferative potency than silibinin against prostate cancer cells. Additionally, we found for the first time that 3-*O*-alkyl-2,3-dehydrosilibinins possess greater anti-proliferative potency than silibinin toward both androgen-sensitive and androgen-resistant human prostate cancer cell lines (LNCaP, DU145 and PC-3). Three- to five-carbon alkyl groups attached to 3-OH of 2,3-dehydrosilibinin maximize the *in vitro* antiproliferative potency. However, 7-*O*-methyl-2,3-dehydrosilibinin and 7-*O*-ethyl-2,3-dehydrosilibinin represent the most potent derivatives among the series of 7-*O*-alkyl-2,3-dehydrosilibinins.

Silibinin has been demonstrated to arrest cell cycle at G_1 phase in various prostate cancer cell models. [–] The effect of 3-*O*-propyl-2,3-dehydrosilibinin (**5**) on the PC-3 cell cycle was evaluated using flow cytometric analysis with propidium iodide DNA staining. Derivative **5** increased the population of PC-3 cells in a G_0/G_1 phase (Fig. 2), while fewer cells were observed in the G_2 phase. Specifically, the G_0/G_1 PC-3 cells were increased from 48% and 60% in control cells at 16 hours and 24 hours, respectively, to 68% in derivative **5**-treated cells at both time points (Table 4). The cell population in G_2 phase slightly decreased from 31% in control cells to 18% at 16 hours, and from 21% in control cells to 18% at 24 hours. Similarly, 2,3-dehydrosilibinin (**2**) also induces the PC-3 cell cycle arrest at the G_0/G_1 phase (Fig. 2). It increased the population of PC-3 cells in the G_0/G_1 phase from 48% and 60% (control cells) to 65% and 63% at 16 hours and 24 hours, respectively (Table 4).

Agarwal and co-workers have reported that silibinin can activate cell apoptosis in PC-3 tumor xenografts. F2N12S and CYTOX AADvanced double staining flow cytometry-based assay was chosen to discriminate PC-3 cells dying from apoptosis from those dying from necrosis in response to increasing concentrations of 2,3-dehydrosilibinin (**2**) and 3-*O*-propyl-2,3-dehydrosilibinin (**5**). PC-3 cells were incubated with **2** or **5** for 16 h. Staurosporine was used as a specific apoptotic inducer and positive apoptotic control in

these experiments (not shown). As illustrated in Fig. 3 and Fig. 5, derivative **5** with a propyl group at 3-OH in 2,3-dehydrosilibinin did not induce significant levels of apoptotic cell death in the androgen-insensitive PC-3 prostate cancer cell line at a dose of up to 100 μM after a 16-hour treatment. In contrast, 2,3-dehydrosilibinin (**2**) induced significant levels of PC-3 apoptotic cell death after a 16-hour treatment, as illustrated in Fig. 4–5. Specifically, 60 μM of **2** could induce detectable early phase of apoptosis in PC-3 cells as compared with control cells; treatment with 100 μM of **2** led to 46% early apoptotic cells and 16% late apoptotic/necrotic cells. Both apoptotic and necrotic cell populations increased in response to increasing concentration of **2** (0–100 μM final concentration range). Interestingly, 2,3-dehydrosilibinin (**2**), 3-*O*-propyl-2,3-dehydrosilibinin (**5**), and 7-*O*-ethyl-2,3-dehydrosilibinin show similar inhibitory effect on PC-3 cell proliferation but different inductive effect on PC-3 cell apoptosis, indicating that incorporation of an alkyl group to 7-OH in 2,3-dehydrosilibinin promotes the apoptotic activation and that introduction of an alkyl group to 3-OH in 2,3-dehydrosilibinin reverses the apoptotic response. Recently, the inhibitory effect of silibinin on PC-3 and other cancer cell proliferation was demonstrated to be associated with both cell apoptotic and autophagic induction.[–] Regulation of autophagy could be an important mechanism contributing to the significant anti-proliferative effect of 3-*O*-alkyl-2,3-dehydrosilibinins.

In summary, eight 3-*O*-alkyl-2,3-dehydrosilibinins have been successfully synthesized through one-pot reaction procedure. Seven of them have also been obtained by a three-step procedure in significantly improved yields. Their antiproliferative potency against three prostate cancer cell lines, as evaluated by WST-1 cell proliferation assay, is significantly greater than silibinin. 2,3-Dehydrosilibinins **5**–**7** each with a three- to five-carbon linear alkyl group attached to 3-OH were identified as the optimal derivatives with IC_{50} values in the range of 1.71 – 3.06 μM toward PC-3 and LNCaP prostate cancer cell lines, a 24- to 42-fold improvement in potency as compared with silibinin. Importantly, the antiproliferative potency of 3-*O*-propyl-2,3-dehydrosilibinin against PC-3 prostate cancer cells is not primarily associated with its capability to induce PC-3 cell apoptosis. However, 3-*O*-propyl-2,3-dehydrosilibinin appears to inhibit prostate cancer cell growth by confining more cells in the G_0/G_1 phase. Accordingly, this scaffold is worth further exploration to define the mechanism of action and to optimize the lead compounds *via* chemical modifications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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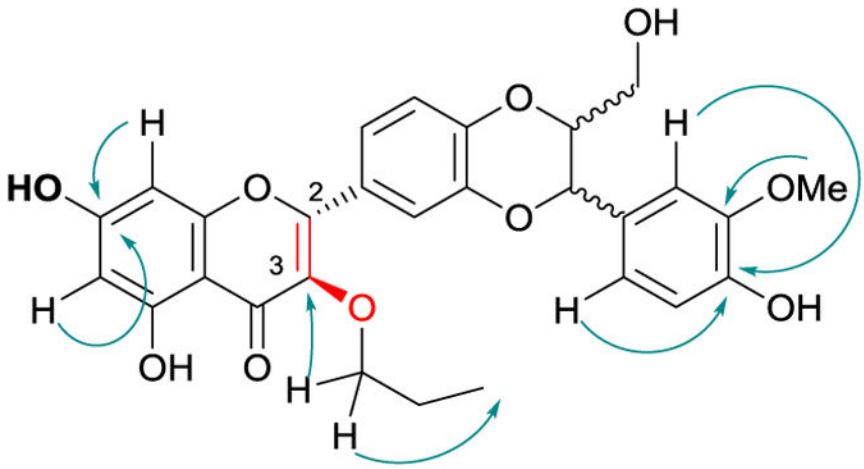
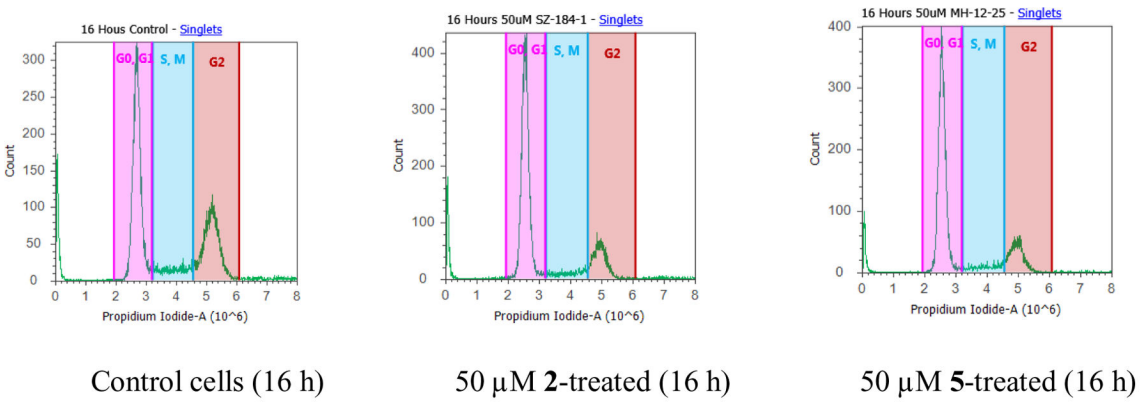


Figure 1.
Diagnostic HMBC correlations in 3-*O*-propyl-2,3-dehydrosilibinin (**5**)



Control cells (16 h) 50 μM **2**-treated (16 h) 50 μM **5**-treated (16 h)

Fig. 2.

Cell cycle analysis of PC-3 cells. PC-3 cancer cells were untreated or treated with **2** and **5**. Cells were harvested after 16 and 24 hours, fixed, stained, and analyzed for DNA content.

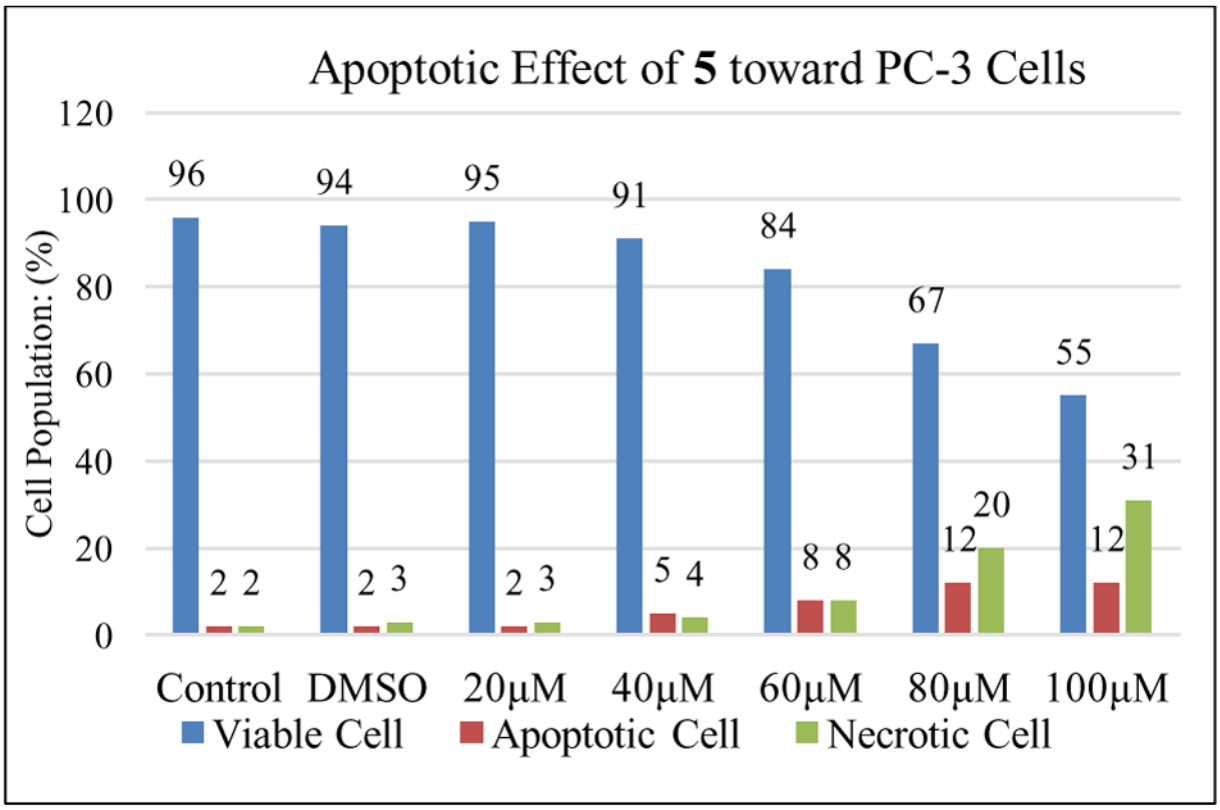


Fig. 3.

Evolution of viable, apoptotic, and necrotic PC-3 cells populations in response to increasing dosages of derivative **5**

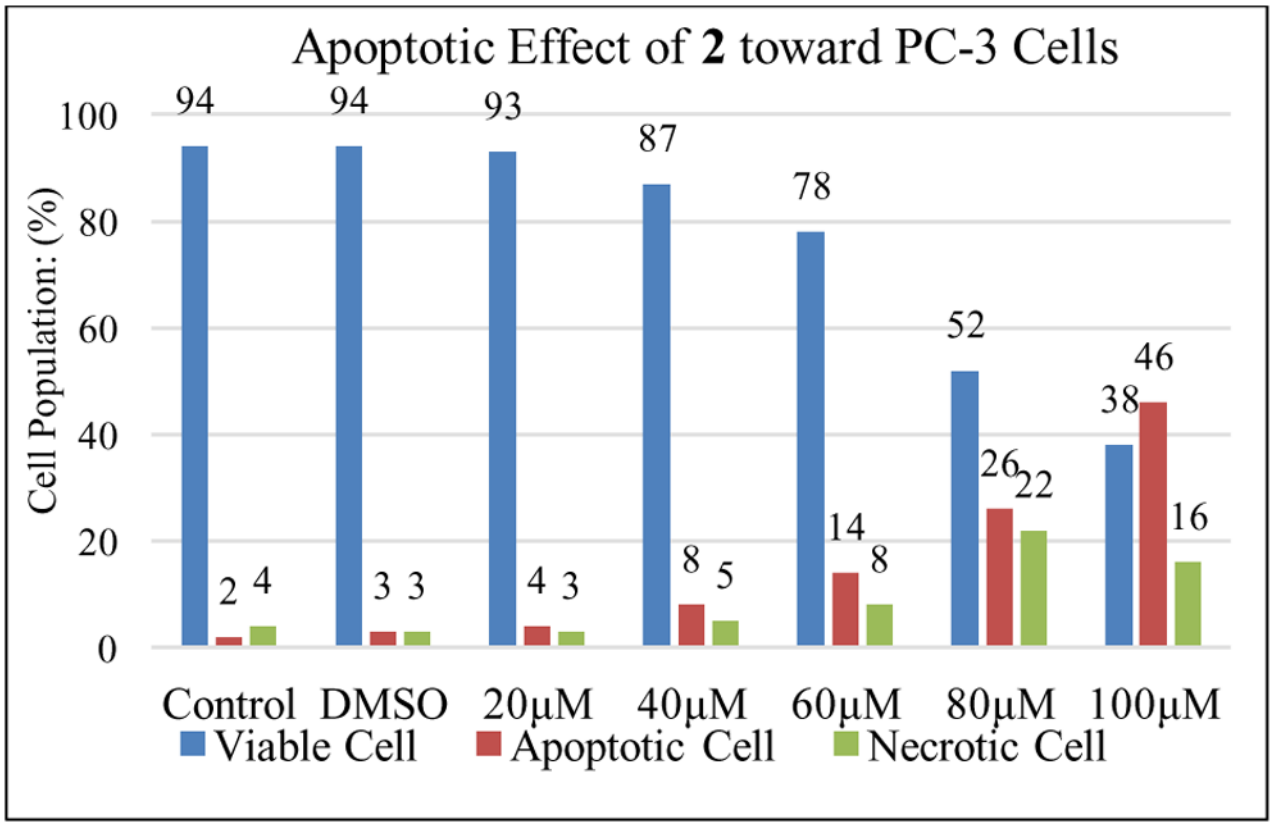


Fig. 4.

Evolution of viable, apoptotic, and necrotic PC-3 cells populations in response to increasing dosages of derivative **2**

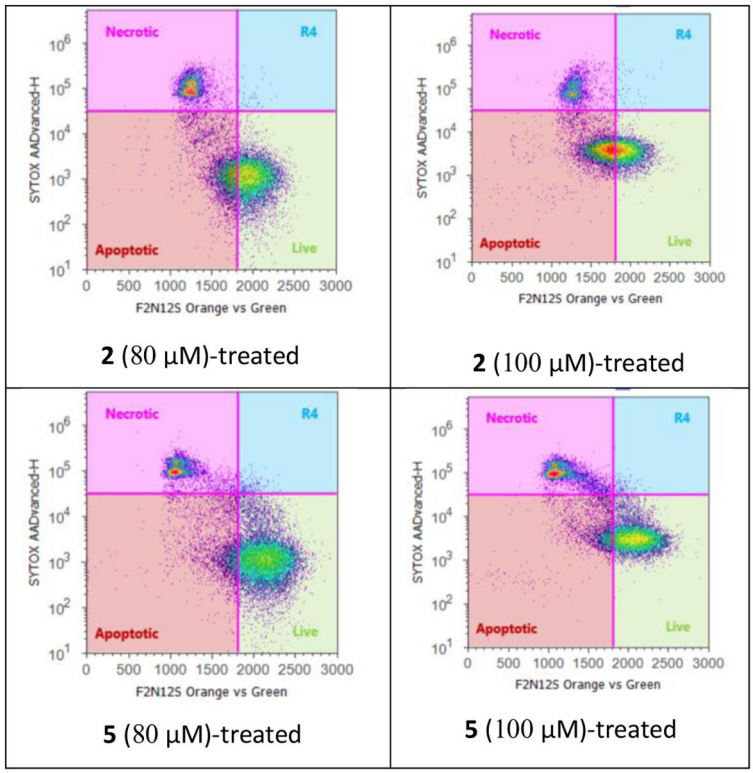
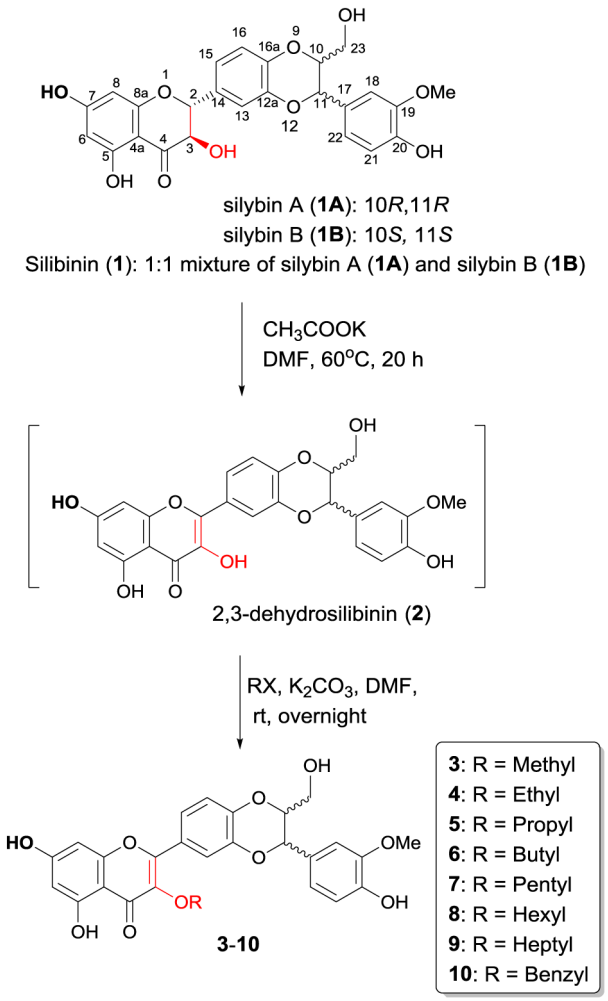


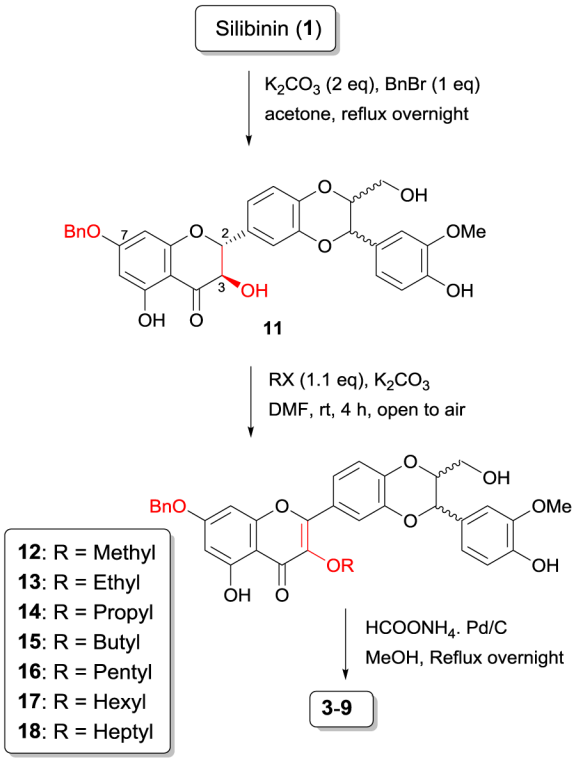
Fig. 5.

Apoptosis in PC-3 cells treated with derivatives **2** and **5** at 80 and 100 μM (by F2N12S and CYTOX AADvanced double staining)



Scheme 1.

One-pot synthesis of 3-*O*-alkyl-2,3-dehydrosilibinins (**3**–**10**)



Scheme 2.

Three-step synthetic procedure for 3-*O*-alkyl-2,3-dehydrosilibinins (**2**–**9**)

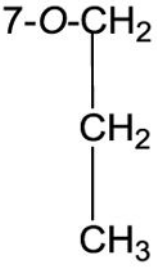
Table 1

Yields for the two alternative syntheses of 3-*O*-alkyl-2,3-dehydrosilibinins (**3**–**9**)

Derivative	One-pot method	Three-step method
3 (methyl**)**	13%	35%
4 (ethyl**)**	14%	30%
5 (propyl**)**	12%	38%
6 (butyl**)**	15%	46%
7 (pentyl**)**	11%	37%
8 (hexyl**)**	16%	32%
9 (heptyl**)**	11%	30%

Table 2

NMR Data for 3-*O*-propyl-2,3-dehydrosilibinin (**5**) (^{1}H NMR: 300 MHz; ^{13}C NMR: 75 MHz).

Position	3-*O*-propyl-2,3-dehydrosilibinin (acetone-d_6)	
	δ_C, type	δ_H, (*J* in Hz)
2	147.1, C	-
3	138.8, C	-
4	175.4, C	-
4a	105.9, C	-
5	163.2, C	-
6	99.4, CH	6.25, s
7	165.0, C	-
8	94.5, CH	6.52, s
8a	157.8, C	-
10	80.0, CH	4.25–4.23, m
11	77.2, CH	5.05, d (7.8)
12a	144.8, C	-
13	118.0, CH	7.70, s
14	124.4, C	-
15	123.2, CH	7.74, d (8.1)
16	117.7, CH	7.06, d (8.1)
16a	156.2, C	-
17	128.9, C	-
18	111.9, CH	7.16, s
19	148.6, C	-
20	148.1, C	-
21	115.8, CH	6.90, d (8.1)
22	121.7, CH	7.00, d (8.1)
23	61.7, CH_2	3.80, br.d (12.3) 3.55, br.d (12.3)
	74.8, CH_2	4.02, t (6.6)
	24.0, CH_2	1.73, Hex (7.2)
	10.8, CH_3	0.96, t (7.5)
19-OMe	56.3, CH_3	3.95, s
5-OH	-	11.67, s
20-OH	-	5.79, s

Position	3-*O*-propyl-2,3-dehydrosilibinin (acetone-d_6)	
	δ_C, type	δ_H, (*J* in Hz)
23-OH	-	

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Table 3

In vitro anti-proliferative activity (IC_{50}, μM)[a] of the compounds against prostate cancer cell lines

Comp. No	IC_{50} (μM)			IC_{50} (silibinin)/IC_{50} (derivitive)		
	LNCaP [b]	DU145 [c]	PC-3[d]	LNCaP	DU145	PC-3
Silibinin (1)	43.03 ± 7.84	93.34 ± 13.76	72.65 ± 3.15	1	1	1
2	3.09 ± 1.30	11.48 ± 1.42	9.45 ± 0.56	14	8	8
3	8.14 ± 2.35	21.64 ± 0.53	12.58 ± 1.28	5	4	6
4	3.22 ± 0.59	16.44 ± 0.49	7.52 ± 0.22	14	6	10
5	2.07 ± 0.18	11.04 ± 0.68	1.71 ± 0.45	21	8	42
6	1.99 ± 0.10	14.36 ± 0.40	2.29 ± 0.12	22	7	32
7	2.07 ± 0.35	14.03 ± 0.66	3.06 ± 0.48	21	7	24
8	3.50 ± 0.21	21.11 ± 0.76	6.04 ± 0.80	12	4	12
9	3.96 ± 0.38	19.24 ± 0.88	10.66 ± 1.62	11	5	7
10	3.77 ± 0.40	17.76 ± 1.98	4.46 ± 2.24	11	6	16

[a] IC_{50} is the drug concentration effective in inhibiting 50% of the cell viability measured by the WST-1 cell proliferation Assay after 3 days exposure.

[b] Human androgen-sensitive prostate cancer cell line

[c] Human androgen-independent prostate cancer cell line

[d] Human androgen-independent prostate cancer cell line

Table 4

The distribution and percentage of PC-3 cells in G_1/G_0 and G_2 phase of the cell cycle

PC-3 cells	**16 hours**		**24 hours**	
	G_0/G_1	**G_2**	**G_0/G_1**	**G_2**
Control cells	48%	31%	60%	21%
2-treated (50 μM)	65%	18%	63%	18%
5-treated (50 μM)	68%	18%	68%	18%