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Metal Coordination Protocol for the Synthesis 2,3-Dehydrosilybin and 19-O-Demethyl-2,3-dehydrosilybin from Silybin and Their Antitumor Activities

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ABSTRACT

An efficient and practical method to access bioactive 2,3-dehydrosilybin and 19-O-demethyl-2,3-dehydrosilybin using naturally abundant flavonolignan silybin in the presence of metal salt as a chelating agent is described. The procedure presented here has several advantages including one-pot, synthetic ease, and products in high yields with no side reactions, and large-scale feasibility. The dehydrogenation and demethylation proceed smoothly via a one-pot process using the $AlCl_3$/Pyridine system and I_2 as the additive. Furthermore, 2,3-dehydrosilybin and 19-O-demethyl-2,3-dehydrosilybin can inhibit the expression of intracellular mature miRNA-21 with IC_{50} values of 4.46 μM and 8.25 μM, respectively, and show moderate anticancer activities against HeLa cell lines.

Introduction

Silymarin applied in the treatment of cirrhosis, hepatitis, and alcohol-induced liver disease is a crude extract of milk thistle (Silybum marianum L. Gaertn., Asteraceae), which contains more than 10 structurally closely related flavonolignans such as silybin, silychristin, isosilybin, silydianin, taxifolin, quercetin, and 2,3-dehydrosilybin *etc.*[1-3] Among the flavonolignans, silybin (SIL, Figure 1) is the most abundant flavonolignan and the easiest to isolate from silymarin, but it exhibits quite poor biological activities. The structure-activity relationship analysis of silymarin flavonolignans reveals that 2,3 double bond or phenolic hydroxyl group enhance biological activities. For example, 2,3-dehydrosilybin (DHS, Figure 1) characterized by the presence of the 2,3 double bond possesses higher biological activities than silybin, such as anticancer,[4-6] antioxidant,[7-12] and modulation of P-glycoprotein.[13] Besides, 19-O-demethyl-2,3-dehydrosilybin (DHDMS, Figure 1) replaced methoxy group by the hydroxyl group at C-19 exhibits a much higher antioxidant activity compared to SIL or DHS, and better inhibitory activity against lipid peroxidation than quercetin.[14] Unfortunately, DHS and DHDMS are minor constituents in silymarin, therefore the synthesis of the 2,3-dehydro derivative and minor silymarin constituent DHS and DHDMS from the abundant silybin is of high interest due to their biological activities.

The first synthesis of 2,3-dehydrosilybin (DHS) was via the oxidation of silybin with iodine in K-acetate buffer.[15, 16] However, these methods have certain drawbacks, including no more than 50% yield, side products, the use of harmful solvents, hydrolysis of the formation of acetates prior to final purification, and low solubilities of silybin and iodine in acetic acid to scale-up difficult. In another method, 2,3-dehydrosilybin have been synthesized by the aerial or anaerobic oxidation of silybin with base as catalyst, such as *N*-methylglucamine, pyridine and alkaline milieu.[17] The disadvantage of the base-catalyzed method was the occurred side-reaction due to the unstable silybin in base condition, including the free radical polymerization and rearrangement reaction, to produce the insoluble substances and decomposition products.[7,18-20] 19-O-demethyl-2,3-dehydrosilybin (DHDMS) was prepared by the radical-coupling reaction of quercetin with (*E*)-3-O-benzyl-3,4-dihydroxy-cinnamyl alcohol,[21] only giving 9.6% yield which greatly limits it's biological applications. To the best of our knowledge, no reports existed to date for the synthesis of DHDMS from SIL or DHS due to the selective demethylation of 19-OMe was rather difficult.

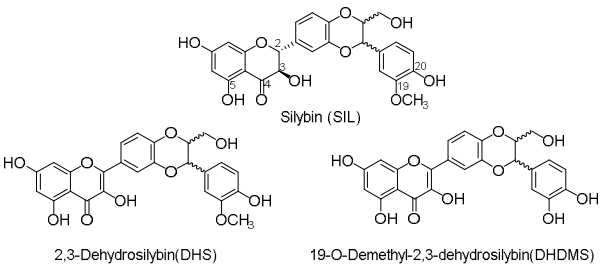

Figure 1. Structures of SIL, DHS and DHDMS

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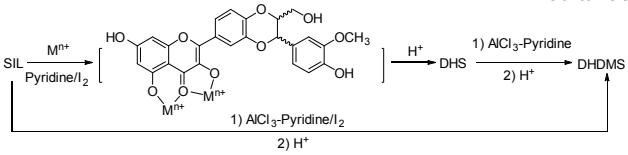


Scheme 1. Synthesis of DHS and DHDMS

Structurally, silybin is characterized by a flavonoid moiety associated with a phenylpropanoid unit. The flavonoid part consists of carbonyl and hydroxyl groups, they can coordinate metal ions and form complexes. In fact, a large number of experimental and theoretical studies demonstrate that metal ions such as Mg^{2+}, Zn^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+} and Al^{3+} ions can chelate with silybin at 3-OH, 5-OH, and 4-carbonyl group.[22-24] Besides, a survey of literature show that methoxy group of ortho-hydroxyphenyl alkyl ethers can be cleaved to provide catechol in a direct route with aluminum trichloride-pyridine.[25] Considering the unique ability of flavonoids to coordinate metal ions, it is envisaged that introduction of the metal ions to coordinate with the carbonyl and hydroxyl groups to stabilize the structure of silybin in base condition to avoid being oxidized or rearranged. On the other hand, due to the oxophilicity of aluminum ions, the ether cleavage reaction of 19-methoxy in phenylpropanoid unit of SIL possibly occurred during $AlCl_3$-pyridine induced the dehydrogenation of silybin. Hence, in the present work, we focus on the synthesis of DHS and DHDMS from silybin by dehydrogenation or(and) demethylation via metal coordination (Scheme.1)， along with the determination of their antitumor activities.

Results and Discussion

According to the O_2-induced oxidation protocols and the condition reported by Křen and co-workers[17], the reaction of silybin in the presence of various metals in the air using various solvents at reflux temperature for 24 h was initially conducted. Unfortunately, the desired products were obtained in only low isolated yields and most of original materials were observed (Table 1, entries 1-7), when the metal salts such as $MgCl_2$, $CaCl_2$, $ZnCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$ and $AlCl_3$ were used. The effect of the solvents, including MeOH, EtOH, DMF, and DMSO, did not show any affect in the transformation yields (Table 1, entries 8-11). Encouragingly, excellent yields were achieved when the I_2 was used as oxidizing agent with the addition of metal salts for 1 h (Table 2, entries 1-7). The reaction was found to give high conversions for all metal salts. Among the common metal salts studied for this reaction, $MgCl_2$ was found to be the most effective additive for this transformation since it resulted in the highest conversion to the desired product (Table 2, entry 1). Further optimization of the temperature revealed that without improving effect on the transformation of DHS when the temperature was decreased to 100℃ (Table 2, entry 8), but further decrease in temperature to 90 and 80 °C gave the DHS in 86% and 75% yield, respectively (Table 2, entries 9 and 10)。 We observed the yield was slight increased 92% when the reaction time was increased from 1 h to 2 h (Table 2, entry 11). Next, the amount of the $MgCl_2$ additive was examined. No substantial improvement in the yield of DHS was observed when the amount of $MgCl_2$ was increased from 2.0 to 2.2 eq (Table 2, entry 14), while for the decreasing the amount of $MgCl_2$ to 1.8 and 1.6 eq only gave DSH in 86% and 79% yield, respectively (Table 2, entries 12 and 13). Interestingly, use of $AlCl_3$, DHS and DHDMS were isolated in 75% and 19% yields, respectively (Table 2, entry 7), while no or trace DHDMS product was detected for the other metal salts (Table 2, entries 1-6).

In this regard, silybin was found to provide the 19-O-demethyl-2,3-dehydrosilybin (DHDMS) in 19% yield using 2.0 equiv $AlCl_3$, indicating a demethylation process. To explore if such a side reaction could be developed into a useful new method for the preparation of DHDMS, we directly treated SIL with 2.3 equiv, 2.5 equiv and 3.5 equiv $AlCl_3$ in pyridine. It is noteworthy that increasing the $AlCl_3$ amount from 2.0 to 3.5 equiv caused a substantial decrease in the yield of DHS and increase in the yield of DHDMS. (Table 1, entries 15-18). In contrast, only trace desired product of DHDMS were detected after 2 h using synthesized DHS as substrate in $AlCl_3$-pyridine at 110 °C with or without I_2 (Table 2, entries 20 and 21). Notably, the yield of DHDMS increased to be 85% when the KI was added (Table 2, entry 22). Like KI, similar effect was observed using AlI_3 instead of $AlCl_3$ (Table 2, entry 23). Since the reaction conditions of dehydrogenation and demethylation were quite similar, the *in site* generated iodide ion during the dehydrogenation by I_2 played a key role in promoting the demethylation.

Table 1. Survey of metal salts and solvents for the oxidation of SIL to DHS[a]

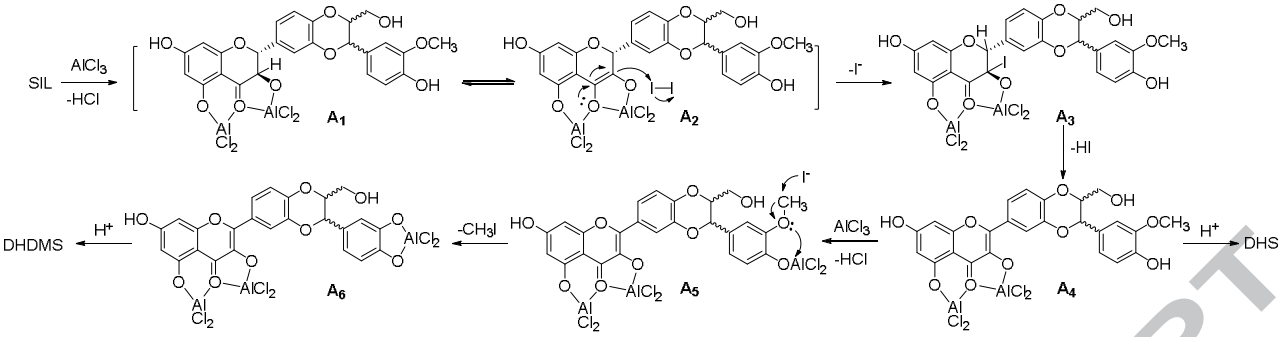
Entry	Metal	Solvent	Temp (℃)	Yield (%)[b]
1	$MgCl_2$	Pyridine	110	53
2	$CaCl_2$	Pyridine	110	37
3	$ZnCl_2$	Pyridine	110	55
4	$CuCl_2$	Pyridine	110	43
5	$FeCl_2$	Pyridine	110	38
6	$FeCl_3$	Pyridine	110	44
7	$AlCl_3$	Pyridine	110	56
8	$AlCl_3$	MeOH	Reflux	Trace
9	$AlCl_3$	EtOH	Reflux	Trace
10	$AlCl_3$	DMF	110	Trace
11	$AlCl_3$	DMSO	110	Trace

[a] Unless stated otherwise, reactions were performed on 0.2 mmol scale with silybin /metal salts as 1:2 ratio in 4.0 mL of solvent in air for 24 h.
[b] Isolated yield.

Table 2. Optimization of the reaction conditions[a]

Entry	Metal	Temp	Time	Yield (%)[b]	
	Salt (equiv)	(°C)	(h)	DHS	DHDMS
1	$MgCl_2$ (2.0 eq)	110	1	91	Trace
2	$ZnCl_2$ (2.0 eq)	110	1	89	Trace
3	$CaCl_2$ (2.0 eq)	110	1	84	N.r.[c]
4	$CuCl_2$ (2.0 eq)	110	1	60	N.r.[c]
5	$FeCl_2$ (2.0 eq)	110	1	87	Trace
6	$FeCl_3$ (2.0 eq)	110	1	85	Trace
7	$AlCl_3$ (2.0 eq)	110	1	75	19
8	$MgCl_2$ (2.0 eq)	100	1	91	N.r.[c]
9	$MgCl_2$ (2.0 eq)	90	1	86	N.r.[c]
10	$MgCl_2$ (2.0 eq)	80	1	75	N.r.[c]
11	$MgCl_2$ (2.0 eq)	100	2	92	N.r.[c]
12	$MgCl_2$ (1.8 eq)	100	1	86	N.r.[c]
13	$MgCl_2$ (1.6 eq)	100	1	79	N.r.[c]
14	$MgCl_2$ (2.2 eq)	100	1	91	N.r.[c]
15	$AlCl_3$ (2.3 eq)	110	2	62	32
16	$AlCl_3$ (2.5 eq)	110	2	50	43
17	$AlCl_3$ (3.0 eq)	110	2	Trace	92
18	$AlCl_3$ (3.5 eq)	110	2	Trace	91
19	$AlCl_3$ (3.0 eq)	110	2.5	Trace	90
20[d]	$AlCl_3$ (3.0 eq)	110	2		Trace
21[e]	$AlCl_3$ (3.0 eq)	110	2		Trace
22[f]	$AlCl_3$ (3.2 eq)	110	2		85
23[h]	AlI_3 (3.2 eq)	110	2		92

[a] Unless stated otherwise, reactions were performed on 0.2 mmol scale with silybin in 1 equiv. I_2 in 4.0 mL of pyridine in air.
[b] Isolated yield.
[c] Not reaction
[d] 0.2 mmol of DHS instead of silybin as substrate without I_2.
[e] 0.2 mmol of DHS instead of silybin as substrate in 1 equiv. I_2.
[f] 0.2 mmol of DHS instead of silybin as substrate in 2 equiv. KI.
[h] 0.2 mmol of DHS instead of silybin as substrate without I_2.



Scheme 2. Proposed mechanism of the Al-promoted dehydrogenation and demethylation.

Based on the above experimental results and literature reports,[25-28] a plausible dehydrogenation and demethylation mechanism using the $AlCl_3$/Pyridine system and I_2 as the additive is depicted in Scheme 2. Reaction occurred in the flavonoid and carbonyl and 5-hydroxyl group afford silybin aluminum complexes **A_1**, which subsequently undergoes keto-enol tautomerization with the assistance of Al^{3+}. Iodine then encounters the nucleophilic attack of the C=C bond of enol **A_2** to give intermediate **A_3**, which subsequently undergoes elimination of HI to furnish product 2,3-dehydrosilybin aluminum complexes **A_4**. On the other hand, according to the Lange's mechanism for $AlCl_3$-pyridine-catalyzed cleavage of alkyl o-hydroxyphenyl ethers,[25] the deprotonation of the 18-hydroxyl group of phenylpropanoid moiety by aluminum trichloride affords aluminum phenolate **A_5**. Coordination of the 19-methoxyl group to the Lewis acidic center of aluminum phenolate **A_5** gives a five-membered cyclic intermediate **A_6** after releasing methyl iodine. Only a trace mount o product DHDMS was observed when DHS was as substrate without iodine ion, whereas high yields of DHDMS was afforded with the iodine ion. Therefore, it seems possible that this demethylation reaction proceeds though releasing methyl iodine. Finally, hydrolysis of aluminum complexes **A_4** and **A_6** furnishes DHS and DHDMS, respectively. The dehydrogenation product DHS is major when the aluminum trichloride amount is not more than 2.0 equiv, whereas increasing the aluminum trichloride amount from 2.0 to 3.0 equiv caused the DHDMS as the major product, which indicates that the stepwise coordination of Al^{3+} to the chelation site and the demethylation proceeds slower than the dehydrogenation.[25]

HeLa-luciferase-miR-21 cells were constructed by transfected PCIneo-luciferase-miR-21 vector which quantitatively evaluate microRNA-21 (miR-21) activity by the insertion of miR-21 target sites downstream and screening with G418 (300 μg/mL). Luciferase is the primary reporter gene and reduced firefly luciferase expression indicates the binding of endogenous miRNAs to the cloned miR-21 target sequence. The cells were seeded at a density of 10^5 cells/well in 96-well plates and maintained at 37 ℃ under a humidified atmosphere of 5% CO_2 and 95% air in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and G418 (300 μg/mL). The cells were treated with vehicle (0.1% DMSO) alone, 10 μM SIL, DHS, DHDMS for 24 h, and then tested luminescence with D-luciferin sodium salt. All of them showed some effect, DHS and DHDMS inhibited production of miR-21 and proliferation with low IC_{50} values of 4.64 μM and 8.25 μM, respectively (Figure 2). Thus, DHS and DHDMS can be an alternative drug targeting miR-21 which is relevant to the tumorigenesis and progression.

In summary, we have developed an efficient method for the preparation of biologically relevant flavonolignans DHS and DHDMS via dehydrogenation or(and) demethylation of silybin in high yields. Metal coordination played a key role to avoid oxidizing and rearranging of silybin in alkaline aqueous solutions, which resulted in satisfactory yields of DHS and DHDMS, giving 91% and 92% yeild, respectively. Development of this method will greatly facilitate the biological studies of DHS and DHDMS that has demonstrated great potentials in pharmaceutical applications.

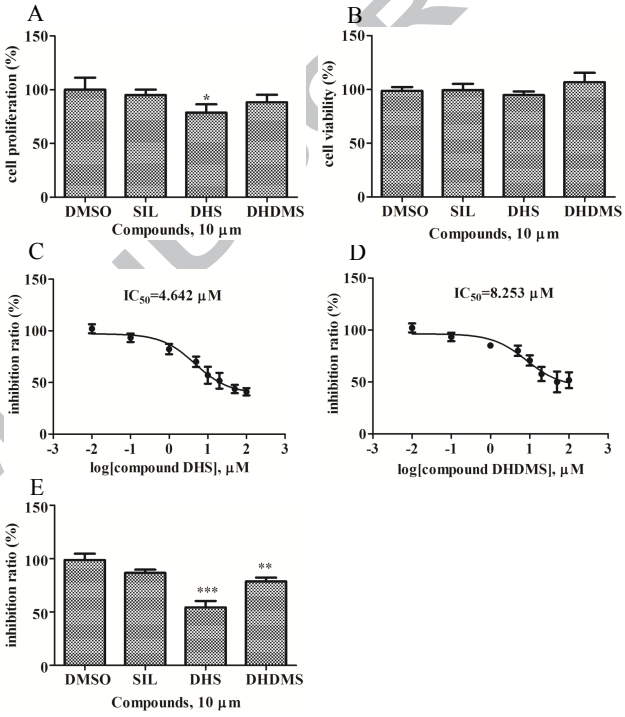


Figure 2. Effect of SIL, DHS and DHDMS on miR-21 production and cell viability. (A) HeLa-luciferase-miR-21 cells were treated with SIL, DHS and DHDMS at 10 μM for 24 h. (B) Cell viability and (E) cell proliferation were measured by Alamar-Blue Regent. (C-D) Hela-luciferase-miR-21 cells were treated with DHS and DHDMS at various concentrations for 24 h. Luciferase was measured by coenzyme A sodium hydrate and D-luciferin sodium salt and reduced firefly luciferase expression indicates the binding of endogenous miRNAs to the cloned miR-21 target sequence. All the data points represent means ± SD of triplicate times, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Acknowledgments

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Supplementary Material

Supplementary material that may be helpful in the review process should be prepared and provided as a separate electronic file. That file can then be transformed into PDF format and submitted along with the manuscript and graphic files to the appropriate editorial office.

The synthetic experimental procedures and of 2,3-dehydrosilybin (DHS) and 19-O-demethyl-2,3-dehydrosilybin (DHDMS), and their structure characterization data, 1H and 13C NMR spectra, HR-MS.

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Graphical Abstract

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Synthesis of 2,3-dehydrosilybin and 19-O-demethyl-2,3-dehydrosilybin is reported starting from naturally abundant flavonolignan silybin using metal-pyridine/I_2 with high yields of 91% and 92%, respectively. Synthesized compounds are tested for their anticancer activities against HeLa cell lines with IC_{50} values of 4.46 and 8.25 μM.

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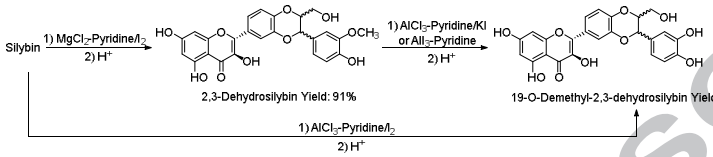

Metal Coordination Protocol for the Synthesis 2,3-Dehydrosilybin and 19-O-Demethyl-2,3-dehydrosilybin from Silybin and Their Antitumor Activities

1. Metal coordination protocol provided direct access to silybin derivatives.
2. The dehydrogenation and demethylation proceed via $AlCl_3$/pyridine/I_2 system.
3. Silybin derivatives exhibited the better anticancer activities than the silybin.