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Research Article

Preparation of Tritium-Labeled Silybin—A protectant for common liver diseases

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Summary
Silymarin, the seed extract of milk thistle plant, *Silybum marianum*, has been used traditionally for the treatment of liver diseases and bile duct infection. Silybin **1** is the main bioactive components of silymarin, consisting a pair of diastereomers: Silybin A and Silybin B. In this article, we report the preparation of tritium-labeled Silybin, which was accomplished by protection of Silybin as tritylated compound **2** and followed by oxidation of the primary alcohol to its corresponding aldehyde **3**. Subsequent reduction with $NaB[^3H]_4$ and deprotection of the trityl group provided the tritium-labeled Silybin **4**. Copyright © 2006 John Wiley & Sons, Ltd.

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Introduction
Common liver diseases such as cirrhosis, hepatitis, necroses, and liver damages, can be treated effectively with interferon, colchicines, penicillamine or corticosteroids, however, the severe side-effects[1] resulted from chronic administration of these medications greatly encouraged the development of an alternative therapeutic agent with a low incidence of side-effects. Traditional herbal remedies have been miraculous in fighting various liver diseases. Among hundreds of plants studied, *Silybum marianum* (milk thistle) is the most widely used medicinal plants in the treatment of chronic or acute liver disease without major side-effect.[2] Clinical trials have shown that silymarin, is effective in treating various forms of liver disease, including cirrhosis, hepatitis, necroses, and liver damage induced by drug and alcohol abuse.[1–3] It also

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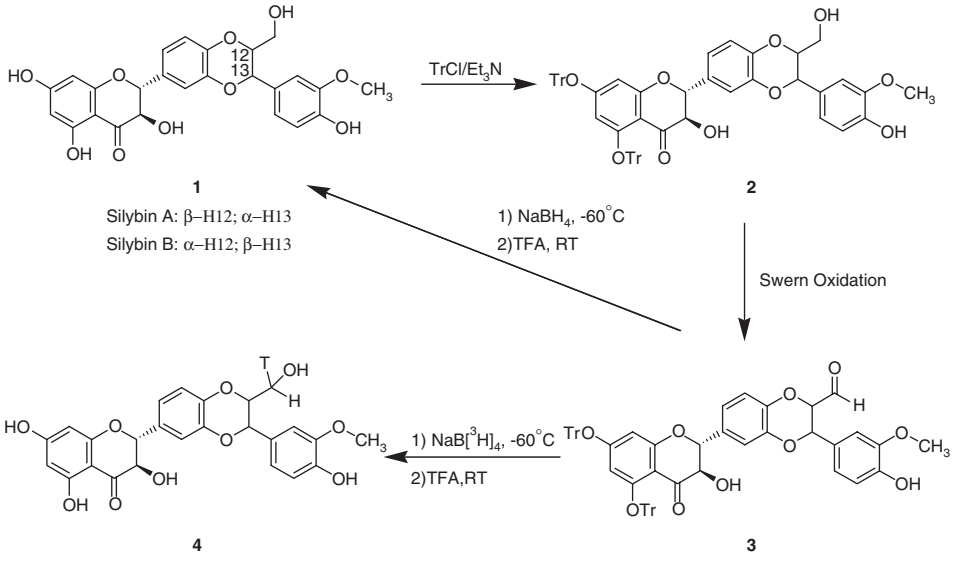

Scheme 1. Synthesis of the tritium labeled Silybin

shows liver protection from toxic chemicals, including acetaminophen, ethanol, carbon tetrachloride and D-galactosamine,[4] and from ischemic injury,[5] radiation,[6] iron toxicity, [7] viral hepatitis,[8] and shows promise as a clinical antidote to acute *Amanita* (deathcap mushroom) poisoning.[9] The hepatoprotective effect of silymarin appears to be related to different biochemical functions: antioxidation,[4d,7,10] anti-lipid peroxidation,[4b,10,11] cell-regeneration, and cytoprotective,[12] antifibrotic,[13] anti-inflammatory[14] and antiproliferative effects.[15]

Silybin **1** (3,5,7-trihydroxy-2-[8-(4-hydroxy-3-methoxy-phenyl)-9-(hydroxyl-methyl)-7,10-dioxabicyclo[4.4.0]deca-2,4,11-trien-4-yl]-chroman-4-one), the principal biologically active component in *Silybum marianum,* can be isolated from the crude extract by preparative HPLC. Silybin consists a pair of diasteroisomers: Silybin A and Silybin B. The difference between the two isomers as shown in Scheme 1 is the configuration at C12 and C13. Since both isomers are active in hepatoprotective activities, there is no need to separate individual isomers for tritium labeling. The tritium labeled Silybin will be used for bioavailability and pharmacokinetic study in rats.

Results and discussion

There are three types of hydroxyl groups in Silybin molecule. Direct oxidation of the primary hydroxyl moiety to its corresponding aldehyde by either Swern oxidation or Parikh-Doering oxidation was unsuccessful. The phenolic OH groups were selectively tritylated with 2.1 equivalents of triphenlymethyl chloride (TrCl) in triethylamine to give compound **2**, in which two phenolic OH

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groups were protected.[16] Parikh-Doering oxidation of compound **2** gave the corresponding aldehyde **3** in low yield (9%).[17] Swern oxidation of compound **2** afforded **3** in 35% yield.[18] Our initial attempts to reduce the aldehyde **3** at room temperature failed. When the reduction temperature was gradually lowered to $-60^{\circ}C$, the aldehyde **3** was reduced to the desired alcohol **2**. Subsequent removal of the trityl groups with chlorotrimethylsilane in the presence of NaI in acetonitrile[19] gave the target Silybin **1**. The synthetic Silybin **1** was identical to the natural Silybin as evidenced by ^{1}H, ^{13}C NMR analyses.

Following the synthetic procedure shown in Scheme **1**, tritium labeled Silybin **4** was obtained by reduction of the aldehyde **3** with $NaB[^{3}H]_4$ (5 mCi, 450 mCi/mmol) and removal of the trityl group with trifluoroacetic acid in tetrahedrofuran. After purification by preparative thin layer chromatography, tritium labeled Silybin **4** was obtained in 49% yield (2.08 mCi, 100 mCi/mmol) as a white solid. A small amount of tritium labeled Silybin **4** was further purified by HPLC with radiodetector. The HPLC retention time and the specific activity measurement by scintillation counter confirmed that the final product was tritium labeled Silybin.

Experimental

General

Tritium labeled sodium borohydride was obtained from American Radiolabeled Chemical Inc. All other chemicals were commercial reagents. TLC analysis with different solvent system was carried out using precoated-TLC silica gel plates (Merck, 60F254). Preparative purification of the labeled Silybin was carried out using EM science PLC (M13895-7). Column chromatography was run on a silica gel 60 Å (Merck 230–400 mesh). The HPLC was performed with waters HPLC system (Waters 1525 binary HPLC pump and Waters dual absorbance detector) at room temperature and the wavelength for UV-detection was 254 nm. YMC HPLC column (YMC-Pack, ODS-A, 150×4.6 mm I.D., S-5 μm, 12 nm) was used. ^{1}H NMR spectra were recorded on Varian NMR-300 spectrometers using the hydrogenated residue of the deuterated solvents ($CDCl_3$, DMSO-d_6, acetone-d_6) and/or TMS as internal standards. Radioactivity was measured by Multi-purpose Scintilation Counter (Beckman) and counter efficiency is 50% with negligible background radiation. The total radioactivity (mCi) is calculated by the formula (mCi = DPM × dilution factor/2.22×10^{9}) and specific activity is calculated as (mCi/mmol) = mCi × molecular weight (mg/mmol)/sample weight (mg).

*3-hydroxy-2-[3-(4-hydroxy-3-methoxy-phenyl)-2-hydroxymentyl-2,3-dihydrobenzo-1,4-dioxin-6-yl]-5,7-bis-trityloxy-chroman-4-one (**2**)*

To a slurry solution of Silybin (2.8 g, 5.8 mmol) in CH_2Cl_2, trityl chloride (3.23 g, 11.6 mmol) and triethylamine (1.76 g, 17.4 mmol) were added. The

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resulting solution was stirred at room temperature for 3 h. Ethyl acetate (300 ml) was added. The organic solution was washed with saturated NH_4Cl aqueous solution, dried ($MgSO_4$) and concentrated. The residue was purified by flash chromatography (ethyl acetate:hexane = 2:3) to give compound **2** as a white solid (3.5 g, 83%). ^{1}H NMR (acetone-d6) δ 3.36 (m, 1 H), 3.57 (s, 3 H), 3.63 (m, 1 H), 4.00 (m, 1 H), [4.62 (dd, J = 1.5, 3.9 Hz), 4.57 (dd, J = 1.5, 3.9 Hz), 1 H], 4.87 (d, J = 7.8 Hz, 1 H), 5.00 (d, J = 11.4 Hz, 1 H), 5.90 (m, 2 H), 6.69 (dd, J = 8.4 Hz, 1 H), 6.74 (d, J = 8.1 Hz), 6.90 (m, 1 H), 6.99 (m, 1 H), 7.30 (m, 18 H), 7.47 (m, 12 H).

*3-(4-Hydroxy-3-methoxy-phenyl)-6-(3-hydroxy-4-oxo-5,7-bis-trityloxy-chroman-2-yl)-2,3-dihydro-benzo[1,4]dioxine-2-carbaldehyde (**3**)*

To a stirred solution of DMSO (0.08 g, 1.0 mmol) in CH_2Cl_2 (10 mL) at $-78°C$, oxalyl chloride (0.5 mmol, 2 M in CH_2Cl_2) was added dropwise. The resulting solution was stirred at $-78°C$ for 10 min. Compound **2** (1.00 g, 1.03 mmol) in CH_2Cl_2 (10 ml) was added drop by drop and the mixture was stirred at $-78°C$ for 45 min. Triethylamine (0.52 g, 5.14 mmol) was introduced to the solution, The mixture was stirred at $-78°C$ for 15 min and allowed to warm up to room temperature. H_2O was added and the product was extracted with ethyl acetate (3 $\times$ 100 ml). The combined organic phase was washed with brine and dried over sodium sulfate. After removal of the solvent, the residue was purified by flash column chromatography (ethyl acetate : hexane = 4:6) to afford compound **(3)** (0.35 g, 35%) as a white solid. ^{1}H NMR ($CDCl_3$) δ 3.56 (s, 3 H), 4.40 (m, 1 H), 4.55 (m, 1 H), 4.87 (m, 2 H), 5.91 (dd, J = 2.1, 6.0 Hz, 1 H), 5.96 (t, J = 2.4 Hz, 1 H), 6.53 (dd, J = 2.1, 8.1 Hz, 1 H), 6.69 (m, 2 H), 7.05 (m, 3 H), 7.26 (m, 18 H), 7.43 (m, 12 H), 9.27 (s, 1 H), 10.88 (s, 1 H).

*Tritium labeled Silybin (**4**)*

$NaB[^3H]_4$ (5 mCi, 450 mCi/mmol) was added into ethanol (1 ml). The slurry solution was cooled down to $-60°C$. Compound **3** (41.2 mg, 4.3×10^{-2} mmol) in THF (2 ml) was added drop by drop. The resulting solution was stirred at 0°C for 10 min. Thin layer chromatography indicated that the reaction was completed. Ethyl acetate (3 ml) and water (2 ml) was added. The organic layer was separated and the aqueous solution was extracted with ethyl acetate (2×3 ml). The organic layers were combined and dried (Na_2SO_4). Part of the organic solvent was evaporated and the rest of the organic solution was quickly passed a very short column (silica gel, ethyl acetate). Solvent was evaporated. Tetrahydrofuran (1 ml) and 10 µL of trifluroacetic acid were added. The resulting solution was stirred at room temperature for 3 h. Purification by preparative thin layer chromatography ($CHCl_3$: MeOH = 9:1) afforded tritium labeled Silybin **4** (10.0 mg, 49%, 2.08 mCi, 100 mCi/mmol) as a white solid. HPLC analysis (MeOH : H_2O : HOAc = 50:49.75:0.25) revealed

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that the retention time (10.65 and 12.19 min) of the prepared tritium labeled Silybin **4** was the same as the retention time of the cold Silybin. Radioactivity measurement confirmed that the tritium labeled Silybin fraction contained the correct amount of radioactivity.

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