Synthesis of [5,6-$^3H_2$]CP-88,818 (β-[5,6-$^3H_2$]Tigogenin Cellobioside)

Peter A. McCarthy

Department of Medicinal Chemistry
Central Research, Pfizer Inc.,
Eastern Point Road, Groton, Connecticut 06340

SUMMARY

CP-88,818 (β-tigogenin cellobioside) is a novel hypocholesterolemic and anti-atherosclerosis agent which acts by inhibiting the absorption of dietary and biliary cholesterol. While the exact mechanism of this inhibition is unclear, CP-88,818 is believed to block cholesterol absorption without entering the body. In order to prove this hypothesis, radiolabelled CP-88,818 was needed. Herein, we report the stereoselective synthesis of [5,6-$^3H_2$]CP-88,818 in four steps from diosgenin acetate.

Key words: CP-88,818, β-tigogenin cellobioside, cholesterol absorption inhibition, tritium, stereoselective reduction.

INTRODUCTION

Reduction of serum cholesterol is a proven therapeutic means of halting and reversing atherosclerosis and reducing the incidence of myocardial infarctions - the number one cause of death in Western industrialized countries.[1] Although a small percentage of the population are severe hypercholesterolemics, the vast majority of patients at risk are mild to moderate hypercholesterolemics. It has been suggested that these patients could return their serum cholesterol levels to a more acceptable range through proper dietary control of lipid intake.[2]

Should dietary controls prove unsuccessful, an approach to reducing cholesterol intake and thus atherosclerosis is to pharmacologically inhibit cholesterol absorption. Not only would such an approach inhibit the absorption of dietary cholesterol, but it would also block the re-absorption of the cholesterol contained in normal bile secretions. Such an approach might also avoid the risks associated with therapies that affect key lipid metabolism or biosynthesis pathways.[3]

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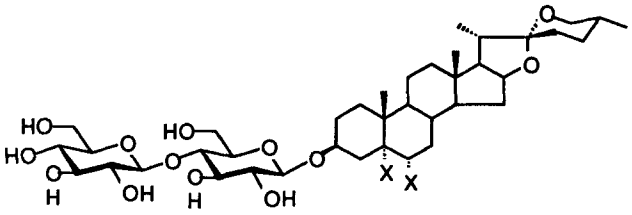


CP-88,818 X = H
[5,6-$^{3}H_2$]CP-88,818 X = ^{3}H

CP-88,818 (β-tigogenin cellobioside) is a novel inhibitor of cholesterol absorption. As has been detailed elsewhere,[4] CP-88,818 inhibits cholesterol absorption and lowers serum cholesterol levels in hamsters, rats, dogs and monkeys. Serum cholesterol reductions have been observed in both cholesterol- and chow-fed animals, suggesting inhibition of both dietary and biliary cholesterol absorption.

The exact mechanism of action whereby CP-88,818 inhibits cholesterol absorption is not known. *In vitro* binding studies[4b] suggest that CP-88,818 forms a complex with cholesterol in the intestinal lumen, thereby rendering the cholesterol unavailable for absorption. As such, CP-88,818 may lower serum cholesterol levels without being absorbed itself. Such a mechanism of action would appear to have an intrinsic safety advantage over systemic agents. Thus, CP-88,818 could be an ideal agent for the vast majority of patients at risk from atherosclerosis due to mild hypercholesterolemia.

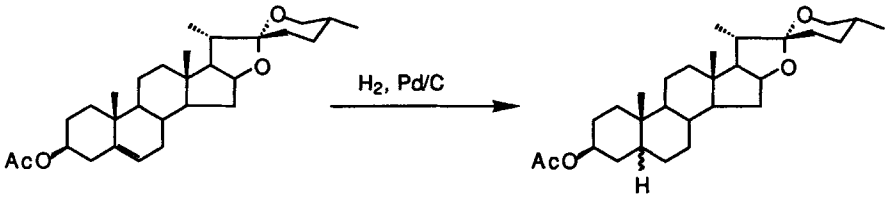
In order to clearly establish the degree of absorption, if any, of CP-88,818, a radioactive tracer needed to be synthesized. Consideration of the study's requirements, potential metabolism, and synthetic accessibility led us to choose [5,6-$^{3}H_2$]CP-88,818 as our target. The most direct way to incorporate tritium into the 5,6-positions of CP-88,818 would be to reduce a CP-88,818 analog containing an 5,6-olefin (e.g. β-diosgenin cellobioside) with tritium gas. Because we desired a sample of [5,6-$^{3}H_2$]tigogenin as a control for absorption studies, we opted to incorporate tritium into the sterol nucleus through reduction of a diosgenin derivative with tritium gas.

DISCUSSION

Catalytic reduction of diosgenin could provide two diastereomers, smilagenin and tigogenin. Literature[5] in this area suggested reducing a diosgenin derivative with the 3-hydroxyl group protected in order to avoid this functional group directing reduction to the β-face. With direct literature precedence lacking, however, we decided to investigate the stereochemical course of this reduction

(Scheme 1). Hydrogenation of diosgenin acetate (1) at atmospheric pressure using palladium on carbon as catalyst cleanly provides tigogenin acetate (3) as the sole product in quantitative yield. Tigogenin acetate (3) could be distinguished from smilagenin acetate (2) by proton and carbon NMR (see Table 1).

Scheme 1



1

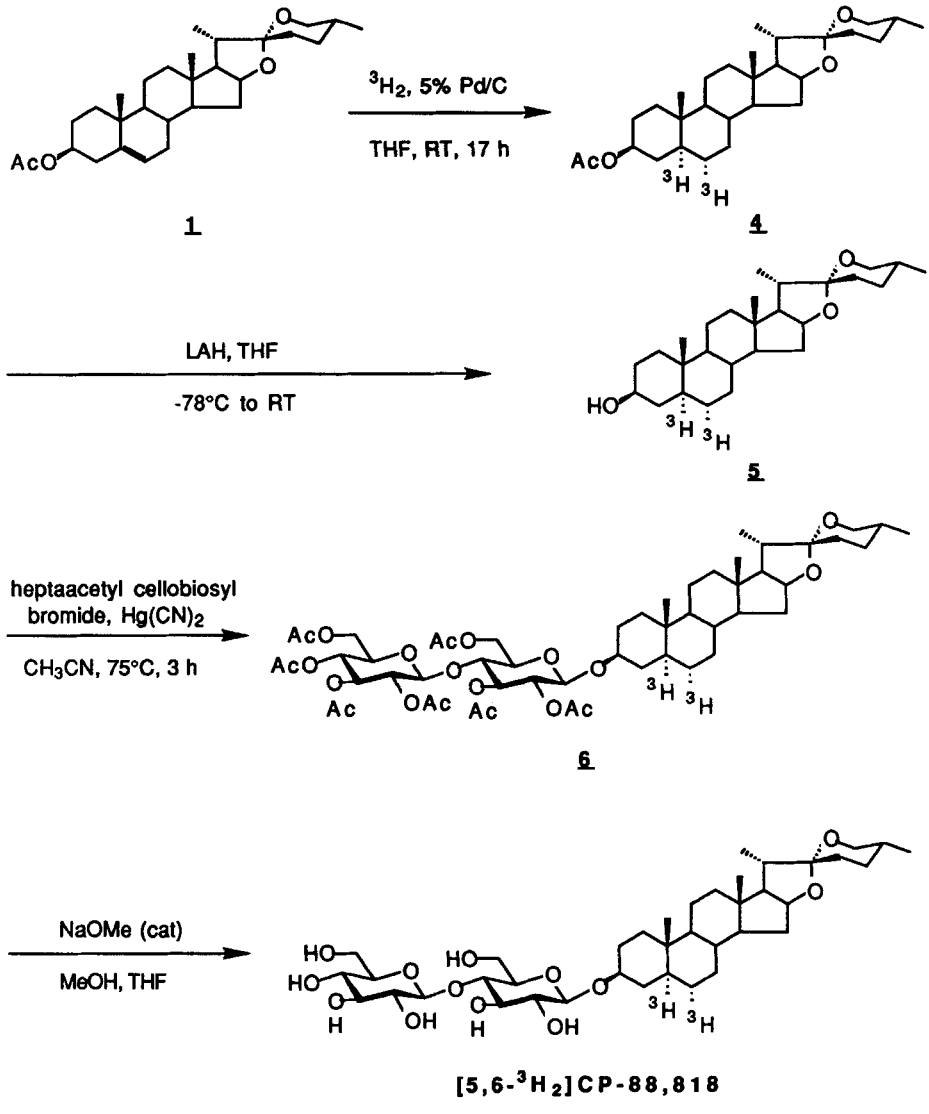
2 5β (smilagenin acetate)
3 5α (tigogenin acetate)

Table 1: ^1H and ^{13}C NMR Resonances Distinguishing Smilagenin Acetate from Tigogenin Acetate

assigned nuclei	chemical shift (δ, ppm) smilagenin acetate (2)	tigogenin acetate (3)
C_3-H	5.04	4.65
C_{18}-H	0.96	0.82
CH_3CO_2	23.90	12.29
C_1	25.02	32.19
C_2	30.66	34.05
C_4	30.80	36.61
C_5	40.05	54.25
C_9	37.35	44.67
C_{22}	70.77	73.68

This same reduction could be carried out using deuterium gas, so a sample of diosgenin acetate was reduced with 4 Ci of tritium gas to give a crude product containing 3.88 Ci of radioactivity.[6] TLC analysis of this crude product showed that nearly 70% of the radioactivity was contained in a higher R_f spot.[7] Purification twice by PTLC gave a material which was chromatographically homogeneous with regard to both chemical and radiochemical impurities. NMR analysis of this material indicated that tigogenin acetate and diosgenin acetate were present in a 53:47 ratio. No peaks from smilagenin could be detected. Analysis of this purified mixture showed that all radioactivity co-migrated with this mixture. A quantitative mass balance was obtained, but given that the material was a mixture, only a 53% yield of tigogenin acetate was realized.

Scheme 2


AcO
$^3H_2$, 5% Pd/C
THF, RT, 17 h
1
4
LAH, THF
-78°C to RT
HO
5
heptaacetyl cellobiosyl bromide, $Hg(CN)_2$
CH_3CN, 75°C, 3 h
Ac
OAc
6
NaOMe (cat)
MeOH, THF
OH
H
[5,6-$^3H_2$]CP-88,818

Using cold material, several procedures (TLC, HPLC, selective chemical modifications)[8] failed to purify tigogenin acetate from diosgenin acetate. Presuming that diosgenin acetate was unlabelled and would represent a minor impurity after cutting the final product, we decided to carry this material on without further purification. HPLC analyses of subsequent intermediates and final product (*vide infra*) showed a radiolabelled impurity (possibly diosgenin-derived products)[9] to the extent of 20% of the total radioactivity. Thus, preparative HPLC was needed to purify these products.

Elaboration to [5,6-$^3H_2$]CP-88,818 was accomplished in the following manner (Scheme 2). Removal of the acetate was accomplished in 96% yield using lithium aluminum hydride in tetrahydrofuran. More typical acetate hydrolysis procedures were unworkable due to the insolubility of the spirostane in the necessary solvents. TLC analysis showed that the [5,6-$^3H_2$]tigogenin (5) obtained co-migrated with an authentic sample of tigogenin. No separation of diosgenin was observed in any TLC system examined.

At this point, the material was cut with 500 mg of recrystallized tigogenin and separated into two lots. One lot of 500 mg was carried on in the synthesis while another lot of 42 mg was purified by PTLC to give 40 mg of [5,6-$^3H_2$]tigogenin. NMR and TLC analyses showed this material to be >95% chemically pure. Although initial analyses indicated that this material was radiochemically pure, a subsequently identified HPLC system showed that 20% of the radioactivity had been incorporated in a minor chemical impurity (possibly diosgenin).[9] Using preparative HPLC, pure [5,6-$^3H_2$]tigogenin could be prepared on a lot by lot basis with 60% yield.

The next step in the synthesis involved the coupling with the cellobioside moiety. Treatment of unpurified [5,6-$^3H_2$]tigogenin (5) with excess heptaacetylcellobiosyl bromide and mercuric cyanide in acetonitrile at 75°C for three hours gave complete conversion to heptaacetyl β-[5,6-$^3H_2$]tigogenin cellobioside (6). Washing with potassium iodide to remove traces of mercuric ions followed by flash chromatography gave this product in quantitative yield and free of chemical impurities as judged by TLC.

The final synthetic step involved removal of seven acetates from the cellobioside moiety. Here, we used a procedure involving catalytic sodium methoxide in methanol and tetrahydrofuran. After workup, a 49% yield was obtained of [5,6-$^3H_2$]CP-88,818 which was identical with authentic material by TLC and NMR. Examination of the proton NMR (C_1-H, 4.29 ppm, J = 7.7 Hz) showed that the desired β-linkage was obtained.[4c] HPLC analysis showed that [5,6-$^3H_2$]CP-88,818 also contained a

radiochemical impurity (possibly β-diosgenin cellobioside)[9] to an extent of 20%. Radiochemically-pure [5,6-$^3H_2$]CP-88,818 (>98%) has been obtained on a lot by lot basis in 60% yield using preparative HPLC.

CONCLUSION

We have completed the synthesis of [5,6-$^3H_2$]CP-88,818 (β-[5,6-$^3H_2$]tigogenin cellobioside), a novel hypocholesterolemic and anti-atherosclerosis agent which acts by inhibiting the absorption of dietary and biliary cholesterol. The stereoselective synthesis of [5,6-$^3H_2$]CP-88,818 was accomplished in four steps from diosgenin acetate. [5,6-$^3H_2$]Tigogenin was also isolated from during this synthesis. In the first step of the synthesis, nearly 70% of the tritium radioactivity was incorporated into a higher R_f impurity[7]. Crude [5,6-$^3H_2$]tigogenin acetate, produced in 53% chemical yield, contained 25% of the tritium radioactivity. From that point on, pure [5,6-$^3H_2$]tigogenin was obtained in 58% chemical yield and 29% radiochemical yield. From [5,6-$^3H_2$]tigogenin acetate, pure [5,6-$^3H_2$]CP-88,818 was obtained in 28% chemical yield and 18% radiochemical yield. The specific activities of [5,6-$^3H_2$]tigogenin and [5,6-$^3H_2$]CP-88,818 were 400 mCi/mmole and 420 mCi/mmole, respectively.[10] Absorption studies in rats and dogs using this material are now in progress and will be reported in due course.[11]

EXPERIMENTAL

The synthesis of [5,6-$^3H_2$]CP-88,818 was fully explored with unlabelled material prior to the radiolabelled synthesis. All radiolabelled products were compared with authentic unlabelled material prepared according to literature procedures.[4b-d] Tetrahydrofuran was distilled from sodium-benzophenone ketal immediately prior to use. Anhydrous acetonitrile was purchased from Aldrich and used as received. Radiochemical purities were determined using either a Berthold L2842 TLC Linear Analyzer or a HPLC apparatus outfitted with a Berthold radioactivity monitor. HPLC eluate from the monitor was collected for quantification by liquid scintillation counting. Specific activities were determined by making serial dilutions with DMSO, dissolving an aliquot in Packard Ultima Gold liquid scintillation fluid and counting in a Packard Tri-Carb 2000 CA scintillation counter. ^1H and ^{13}C NMR spectra were recorded on either a Varian XL300 (300 MHz) or a Bruker AM300 (300 MHz) FT-NMR spectrometer. NMR data are reported in parts per million (δ) and are referenced to the deuterium lock

signal from the NMR solvent. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected.

Diosgenin Acetate (1)

Diosgenin (10 g, 24.1 mmol) was dissolved in pyridine (100 ml) and acetic anhydride (3.00 ml, 31.8 mmol) was added dropwise. The mixture was allowed to stir overnight, then the volatiles were removed. The crude product was partitioned between ether and aqueous ammonium chloride. A flocculant solid that formed at the interface was filtered off and dissolved in tetrahydrofuran. The combined organic phases were washed twice with aqueous ammonium chloride and concentrated to give a solid. This crude product was contaminated with some starting material which was removed by flash chromatography (20:1 hexane:ethyl acetate eluent). The resulting chromatographically-homogeneous product was recrystallized from ethyl acetate to give 6.044 g (55%) of diosgenin acetate, mp 200-201.5°C (lit.[12] mp 198°C).

Smilagenin Acetate (2)

By use of the procedure described for diosgenin acetate excluding recrystallization, smilagenin was converted to smilagenin acetate, mp 150-150.5°C (lit.[13] mp 152-154°C).

Tigogenin Acetate (3) via Acetylation of Tigogenin

By use of the procedure described for diosgenin acetate, tigogenin was converted to tigogenin acetate, mp 209.5-211°C (lit.[12] mp 206°C).

Tigogenin Acetate (3) via Reduction of Diosgenin Acetate

Diosgenin acetate (1, 46 mg, 0.10 mmol) was dissolved in tetrahydrofuran (5 ml) and 5% Pd/C (30 mg) was added. The reduction was stirred under atmospheric pressure of hydrogen for 17 h, then the catalyst was removed by filtration through celite. The filtrate was concentrated to give 56 mg (quantitative yield) of tigogenin acetate as a white amorphous solid. NMR analysis showed this material to be identical in all respects with tigogenin acetate prepared via acetylation of tigogenin. To the limits of detection, no diosgenin acetate or smilagenin acetate was observed.

[5,6-$^3H_2$]Tigogenin Acetate (4)

Using the procedure described for reducing diosgenin acetate with hydrogen gas, diosgenin acetate (1, 46 mg, 0.10 mmol) was reduced with tritium gas (4 Ci) by Chemsyn Science Laboratories in Lenexa, Kansas. The crude product contained 3.88 Ci of radioactivity.

Upon receipt of this material at Pfizer, TLC analysis of this crude product showed that nearly 70% of the radioactivity was contained in a higher R_f spot.[7] Purification twice by PTLC (two 1000 um plates developed in 1.5:1 hexane: ether, followed by two 1000 um plates developed in 3:1 hexane:ether) gave a material which was chromatographically homogeneous with regard to both chemical and radiochemical impurities. NMR analysis of this material indicated that tigogenin acetate and diosgenin acetate were present in a 53:47 ratio. No peaks from smilagenin could be detected. Analysis of this purified mixture showed that all radioactivity co-migrated with this mixture. A quantitative mass balance was obtained, but given that the material was a mixture, only a 53% yield of tigogenin acetate was realized.

Using cold material, several procedures (TLC, HPLC, selective chemical modifications)[8] failed to purify tigogenin acetate from diosgenin acetate. Presuming that diosgenin acetate was unlabelled and would represent a minor impurity after cutting the final product, we decided to carry this material on without further purification. HPLC analyses of subsequent intermediates and final product (*vide infra*) showed a radiolabelled impurity (possibly diosgenin-derived products)[9] to the extent of 20% of the total radioactivity. Thus, preparative HPLC was used to purify these products.

[5,6-$^3H_2$]Tigogenin (5)

[5,6-$^3H_2$]Tigogenin acetate (4, 46 mg, 0.1 mmol) was dissolved in tetrahydrofuran (5 ml) and cooled to -78°C. A solution of lithium aluminum hydride in tetrahydrofuran (1.0 ml of 1.0 M soln, 1.0 mmol) was added dropwise via syringe. The cooling bath was then removed and the reaction mixture was allowed to warm to RT. After 2 h, the reaction was quenched by cautious addition of aqueous ammonium chloride. The organic layer was removed via pipet, dried with magnesium sulfate, filtered and concentrated to give a white solid (40 mg, 96% yield). TLC analysis showed that this material co-migrated with an authentic unlabelled sample of tigogenin.

The [5,6-$^3H_2$]tigogenin obtained above was dissolved in tetrahydrofuran and recrystallized tigogenin (500 mg, 1.19 mmol, recrystallized from methanol, mp 206-208°C, lit.[12] mp 203°C) was added to give a solution. Concentration to dryness gave 542 mg of a white solid. Of this lot, 500 mg was carried on to the next synthetic step, while 42 mg was purified by PTLC (two 1000 um plates developed in 2:1 chloroform:ethyl acetate) to give 40 mg of [5,6-^{3}H]tigogenin. NMR and TLC analyses showed this material to be >95% chemically pure. Although initial analyses indicated that this material was radiochemically pure, a subsequently identified HPLC system showed that 20% of the radioactivity had been incorporated in a minor chemical impurity (possibly diosgenin).[9] Using preparative HPLC, pure [5,6-$^3H_2$]tigogenin could be prepared on a lot by lot basis in 60% yield.

Heptaacetyl β-[5,6-$^3H_2$]Tigogenin Cellobioside (6)

[5,6-$^3H_2$]Tigogenin (5, 500 mg, 1.19 mmol), heptaacetylcellobiosyl bromide (3.50 g, 4.99 mmol) and mercuric cyanide (2.40 g, 9.52 mmol) were combined in a flask which was sealed with a septum, degassed and charged with anhydrous acetonitrile (100 ml). The septum was quickly replaced with a reflux condenser and the suspension was warmed to 75°C at which point it became a solution. This solution was stirred at 75°C for 3 h, then allowed to cool to RT. The volatiles were removed and the resulting yellow residue was partitioned between aqueous sodium bicarbonate and dichloromethane. The organic phase was separated, washed sequentially with aqueous sodium bicarbonate, aqueous potassium iodide and water, dried with sodium sulfate, filtered and concentrated to give a white solid. This material was purified by flash chromatography (2:1 chloroform:ethyl acetate as eluent) to give 1.35 g (quantitative yield) of the title compound. By TLC analysis (1:1 chloroform:ethyl acetate), this material co-migrated with an authentic sample of heptaacetyl β-tigogenin cellobioside and was free of chemical impurities.

[5,6-$^3H_2$]CP-88,818 (β-[5,6-$^3H_2$]Tigogenin Cellobioside)

Heptaacetyl β-[5,6-$^3H_2$]tigogenin cellobioside (6, 1.35 g, 1.31 mmole) was dissolved in methanol (10 ml) and tetrahydrofuran (10 ml). The mixture was warmed to reflux and a catalytic amount of sodium methoxide in methanol (87 µl of 4.35 M soln, 0.37 mmol) was added. After stirring at reflux for 5 min, the solution changed to a white suspension. After stirring at reflux overnight, TLC analysis (4:1:1 butanol:acetic acid:water) showed that reaction was complete. The reflux condenser was

replaced with a short path still head and the mixture was concentrated to approximately 10 ml total volume. Another 10 ml of methanol was added and the mixture was again concentrated to approximately 10 ml total volume. Water (0.1 ml) was added and the flocculant white precipitate quickly changed to a sandy material. The mixture was heated to near reflux for 3 h, then allowed to stir at RT overnight. The mixture was filtered and the resulting white powder was dried in a vacuum oven at 45°C overnight to give 480 mg (49% yield) of the title compound. By NMR and TLC analyses, this material was identical with authentic unlabelled CP-88,818 and appeared to be free (>95%) of chemical impurities. HPLC analysis (Waters Novapak C-18 reverse phase HPLC column using a 60:15:25 methanol:acetonitrile:water mobile phase) showed that [5,6-$^3H_2$]CP-88,818 contained a radiolabelled impurity (possibly β-diosgenin cellobioside)[9] to an extent of 20%. Using this HPLC solvent system with a preparative column (Waters Bondapak Semi-prep C-18 reverse phase column), radiochemically-pure (>98%) [5,6-$^3H_2$]CP-88,818 was obtained in 60% yield.

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6. The reduction with tritium gas was carried out by Chemsyn Science Laboratories, Lenexa, Kansas.

7. This impurity was not identified, but given its R_f and the fact that a quantitative mass balance for spirostanes was realized, this impurity may be BHT from the tetrahydrofuran used in the reduction. Diosgenin acetate demonstrated poor solubility in more typical hydrogenation solvents (i.e. ethanol, ethyl acetate), thereby necessitating the use of tetrahydrofuran.

8. Neither bromination nor hydroboration converted diosgenin acetate into products which were separable from tigogenin acetate by PTLC.

9. It is known that under catalytic reduction conditions, exchange of a proton for tritium can take place without reduction of the olefinic system (see March, J. - Advanced Organic Chemistry (Third Edition), John Wiley and Sons, New York, 1985, pg 696; also see reference 5b).

10. Both products show poor solubility characteristics in most solvents. For this reason, the calculated specific activities are not significantly different from one another. In order to study absorption, solutions can be made, filtered and assayed radioactivity per unit volume.

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