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Synthesis and stability of a carbon-14-labeled 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitor

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Inhibition of 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMGR) is an effective method of lowering plasma low-density lipoprotein cholesterol levels. Hemi-calcium (3R,5S,E)-7-(4-(4-fluorophenyl)-6-isopropyl-2-(methyl(1-methyl-1H-1,2,4-triazol-5-yl)amino)pyrimidin-5-yl)-3,5-dihydroxyhept-6-enoate (1) is a cholesterol-lowering statin drug that effectively inhibits HMGR. An important step in the development of this compound was the synthesis of a carbon-14-labeled analog for use in preclinical absorption, distribution, metabolism and excretion studies.

The synthesis of a carbon-14-labeled analog of the cholesterol-lowering statin drug 1 is described. The carbon-14-labeled compound [^{14}C]-1 was prepared in 11 steps from [^{14}C]-labeled urea. The overall radiochemical yield for the synthesis was 22% and the radiochemical purity of [^{14}C]-1 was 99.9% immediately after synthesis. It was found that [^{14}C]-1 with a specific activity of 43.2 μCi/mg decomposed at a rate of about 1.9%/month when stored at −78°C under argon. Three samples of [^{14}C]-1 were prepared to study the chemical stability of the molecule. One sample had a specific activity of 3.8 μCi/mg and the other two contained radical inhibitors, L-ascorbic acid (1% by weight, specific activity of 10.5 μCi/mg) or BHT (1% by weight, specific activity of 9.8 μCi/mg). For these samples the decomposition rates were decreased to 0.5%/month, 0.2%/month and 0.1%/month, respectively.

Keywords: carbon-14 labeling; radical inhibitors; 3-hydroxy-3-methylglutaryl coenzyme-A reductase; statin drugs; radiochemical decomposition

Introduction

Coronary heart disease is the leading cause of death in the United States.[1] Studies have shown that elevated plasma levels of low-density lipoprotein (LDL) cholesterol increases ones risk of experiencing a coronary event.[1,2] 3-Hydroxy-3-methylglutaryl coenzyme-A reductase (HMGR) is the rate-limiting enzyme in the biosynthesis of LDL cholesterol. Inhibition of HMGR has been clinically proven to be one of the most effective methods of lowering plasma LDL cholesterol levels and reducing event rates associated with cardiovascular disease.[2] Statins are a class of drugs that effectively lower plasma LDL cholesterol levels by inhibiting HMGR. These drugs are considered the gold-standard in treating hypercholesterolemia.

Bristol-Myers Squibb has investigated hemi-calcium (3R,5S,E)-7-(4-(4-fluorophenyl)-6-isopropyl-2-(methyl(1-methyl-1H-1,2,4-triazol-5-yl)amino)pyrimidin-5-yl)-3,5-dihydroxyhept-6-enoate (**1**, Figure 1) in an effort to develop a new statin drug that was more effective at lowering plasma LDL cholesterol levels than the statin drugs currently on the market.[3] This new compound was designed to have minimal statin-induced side effects, namely myotoxicity and rhabdomyolysis.[3] An important step in the early development of this compound was the initiation of preclinical absorption, distribution, metabolism and excretion (ADME) studies to determine the metabolic fate of the molecule using *in vivo* animal models. These preclinical ADME studies typically require a [^{14}C]-labeled analog of the parent compound. To support the development of **1**, a [^{14}C]-labeled analog needed to be prepared. The labeling strategy for this molecule not only took into consideration the availability of labeled starting materials and degree of synthetic complexity but also the known biotransformation. A conscious effort was made to make sure the [^{14}C]-label was not in a position known or suspected to be metabolically labile. After collaboration with our Biotransformation colleagues, it was decided that the [^{14}C]-label should be incorporated into the pyrimidine core of the molecule. This paper describes the synthesis of [^{14}C]-labeled **1** and its chemical stability.

Experimental procedure

Materials and methods

All experimental procedures were optimized using unlabeled material. Reactions were run under an inert atmosphere of

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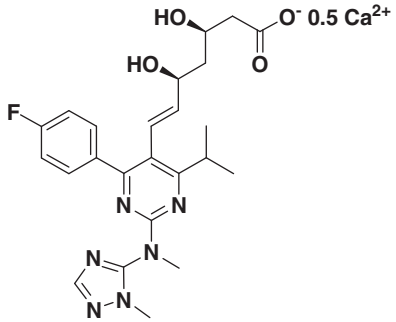

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Figure 1. Structure of the HMGR Inhibitor (1).

argon and stirred magnetically unless otherwise noted. Reactions were monitored by HPLC, LC/MS, TLC and NMR. When possible, comparisons were made to authentic materials. All reagents and solvents were ACS grade or better and used without further purification. Carbon-14-labeled urea (400 mCi, radiochemical purity 99.8%, specific activity 950 μCi/mg, 59 mCi/mmol) was obtained from GE Healthcare (formerly Amersham Biosciences). The intermediates **8** and **13** and all authentic samples were obtained from Bristol-Myers Squibb's Process Research & Development department.

Instruments

Solvent removal under vacuum was accomplished using a Buchi R-124 rotary evaporator. Column chromatography was performed using a Biotage® Flash Chromatography system. Proton NMR spectra were recorded on a 300 MHz Bruker Avance spectrometer or a 400 MHz Bruker Avance spectrometer. Mass spectra were recorded on a Finnigan LCQ system. TLC analyses (EMD 60 F_{254} silica gel-coated plates) were performed as described and using UV (254 nm) and radiochemical detection (QC-Scan, Bioscan Model B-QC). Specific activities were determined by gravimetric analysis using liquid scintillation counting (Wallac Model 1409).

HPLC

HPLC analyses were performed on a Varian instrument equipped with two PrepStar pumps (Model SD-218, 25 mL pump heads), a ProStar PDA detector (model 330, for analytical UV detection), a ProStar UV-1 detector (model 320, for semi-preparative UV detection) and an IN/US β-ram detector (model 3B, for radiochemical purity measurements). The following analytical method (method A) was used for all in process and final product purity analyses.

HPLC Method A:
Column: YMC-Pack Pro C18, 3.0 μm (4.6 × 150 mm).
Mobile Phase A: Water with 0.05% trifluoroacetic acid.
Mobile Phase B: Acetonitrile with 0.05% trifluoroacetic acid.
Conditions: 0% B, 0–5 min; 0–100% B, 5–20 min; 100% B, 20–25 min; 100–0% B 25–30 min.
Flow rate: 1 mL/min, Injection size: 20 μL.
Detection: UV at 254 nm and radiochemical (β-ram).
All Semi-preparative HPLC purifications were conducted on the Varian instrument described above using the methods described in the experimental section.

[^{14}C]-Methyl 4-(4-fluorophenyl)-6-isopropyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate ([14C]-5)[4]

4-Fluorobenzaldehyde (**2**, 1.89 g, 15.24 mmol), methylisobutyryl acetate (**3**, 2.20 g, 15.24 mmol), [^{14}C]-labeled urea (**[^{14}C]-4**, 0.42 g, 6.78 mmol, 400 mCi, 59 mCi/mmol), unlabeled urea (0.55 g, 9.22 mmol) (total urea used (0.97 g, 16.0 mmol, 1.05 eq)), copper (I) chloride (15.1 mg, 0.15 mmol), concentrated H_2SO_4 (0.145 mL) and anhydrous methanol (15 mL) were added to a heat-dried flask.[4] The mixture was heated to 64°C and stirred for 47 h under argon. Afterwards, the reaction mixture was cooled to room temperature. After 30 min at room temperature a white solid formed, which was vacuum filtered and washed with methanol (2 × 10 mL). To maximize yield, the mother liquor was heated to 65°C and stirred for an additional 66 h. The mixture was cooled to room temperature and after 30 min a white solid formed. The solid was isolated as described above and washed with methanol (3 × 5 mL). The white solids were combined and vacuum dried for 17 h to give 3.95 g (84% chemical yield based on urea) of **[^{14}C]-5**.[4] The product was analyzed by: HPLC, method A, RT 16.6 min, UV detector 254 nm 94.5% pure, β-Ram detector 100% pure; LC/MS *m/z* $[^{14}C\text{-}M+H]^+$ 295, $[^{12}C\text{-}M+H]^+$ 293; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.18 (d, J = 6.97 Hz, 3 H), 1.19 (d, J = 6.97 Hz, 3 H), 3.62 (s, 3 H), 4.16 (sept, J = 6.97 Hz, 1 H), 5.29 (brs, 1 H), 5.39 (d, J = 2.07 Hz, 1 H), 6.52 (brs, 1 H), 6.96–7.06 (m, 2 H), 7.21–7.31 (m, 2 H).

[^{14}C]-Methyl 4-(4-fluorophenyl)-2-hydroxy-6-isopropyl-pyrimidine-5-carboxylate ([14C]-6)[4,5]

Dihydropyrimidinone **[^{14}C]-5** (3.95 g, 13.50 mmol) was added to a round-bottomed flask under a nitrogen atmosphere. The flask was cooled to 0°C and concentrated HNO_3 (8.93 g, 135.0 mmol) was added dropwise with stirring over 10 min.[4,5] The mixture was warmed to room temperature and then stirred for 1 h. At that time the solution was cooled to 0°C and 2 N NaOH was cautiously added dropwise until the pH was adjusted to 6. The solution was diluted with water (20 mL) and extracted with CH_2Cl_2 (3 × 20 mL). The organic solutions were combined and concentrated under reduced pressure to give a slightly yellow solid. The solid was vacuum dried for 16 h to give 3.97 g of crude **[^{14}C]-6**.[4–6] The product was analyzed by: HPLC, method A, RT 15.7 min, UV detector 254 nm 99.2% pure, β-Ram detector 100% pure; LC/MS *m/z* $[^{14}C\text{-}M+H]^+$ 293, $[^{12}C\text{-}M+H]^+$ 291; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.43 (d, J = 6.78 Hz, 6 H), 3.25 (sept, J = 6.78 Hz, 1 H), 3.62 (s, 3 H), 7.09–7.19 (m, 2 H), 7.57–7.67 (m, 2 H).

[^{14}C]-Methyl 2-chloro-4-(4-fluorophenyl)-6-isopropylpyrimidine-5-carboxylate ([14C]-7)[4,5]

Crude **[^{14}C]-6** (3.97 g, 13.66 mmol) and $POCl_3$ (14.01 mL, 23.05 g, 150.3 mmol) were added to a round-bottomed flask.[4] The solution was heated to 100°C and stirred for 3 h under nitrogen. At that time the mixture was cooled to 0°C and slowly quenched with a dropwise addition of water (100 mL). When the addition was complete the mixture was warmed to room temperature and stirred for 5 min. The aqueous solution was extracted with CH_2Cl_2 (4 × 25 mL). The organic solutions were combined and concentrated under reduced pressure. The resulting solid residue was dissolved in tert-butyl methyl ether (TBME, 40 mL) and concentrated. The concentrate was vacuum dried for 15 h to give 3.90 g of yellow/brown solid. The crude material was purified by column chromatography (silica, 90:10 hexanes/ethyl

acetate) to give 3.40 g (82% for the two steps) of **[^{14}C]-7** as a white solid.[4] The product was analyzed by: TLC R_f = 0.54 (UV) using 70:30 hexanes/ethyl acetate; HPLC, method A, RT 21.3 min, UV detector 254 nm 93.0% pure, β-Ram detector 96.4% pure; LC/MS *m/z* [^{14}C-M+H]$^+$ 311, [^{12}C-M+H]$^+$ 309; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.33 (d, *J* = 6.78 Hz, 6 H), 3.13 (sept, *J* = 6.78 Hz, 1 H), 3.75 (s, 3 H), 7.11–7.21 (m, 2 H), 7.62–7.72 (m, 2 H).

[^{14}C]-Methyl 4-(4-fluorophenyl)-6-isopropyl-2-(1-methyl-1H-1,2,4-triazol-5-ylamino)pyrimidine-5-carboxylate ([^{14}C]-9)[5,7]

The chloride **[^{14}C]-7** (3.40 g, 11.03 mmol), amino triazole **8**[5,7,8] (1.30 g, 13.25 mmol) and DMF (44 mL) were added to a round-bottomed flask under argon.[5,7] The solution was cooled to 0°C and sodium tert-butoxide (2.12 g, 22.06 mmol) was added portion-wise over 20 min. After the addition was complete, the dark yellow solution was warmed to room temperature and stirred for 1 h 45 min. At that time the solution was quenched with water (250 mL) and the mixture was extracted with CH_2Cl_2 (4 × 50 mL). The combined organic solutions were concentrated under reduced pressure. The oil that remained was diluted with toluene (50 mL) and concentrated. This process was repeated two more times. The concentrate was vacuum dried for 15 h to give 3.69 g of **[^{14}C]-9** as a yellow solid.[5,7] The crude product was analyzed by: HPLC, method A, RT 16.8 min, UV detector 254 nm 94.1% pure, β-Ram detector 97.7% pure; LC/MS *m/z* [^{14}C-M+H]$^+$ 373, [^{12}C-M+H]$^+$ 371; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.24 (d, *J* = 6.78 Hz, 6 H), 3.15 (sept, *J* = 6.78 Hz, 1 H), 3.68 (s, 3 H), 3.83 (s, 3 H), 7.07–7.18 (m, 2 H), 7.52–7.63 (m, 2 H), 7.84 (s, 1 H).

[^{14}C]-Methyl 4-(4-fluorophenyl)-6-isopropyl-2-(methyl(1-methyl-1H-1,2,4-triazol-5-yl)amino)pyrimidine-5-carboxylate ([^{14}C]-10)[5]

Crude **[^{14}C]-9** (3.69 g, assume 9.97 mmol), dimethylcarbonate (18.5 mL, 5 mL/g of **[^{14}C]-9**) and 1,4-diazabicyclo[2.2.2]octane (1.12 g, 9.97 mmol) were added to a round-bottomed flask.[5] The mixture was heated to 90°C and stirred for 6 h under argon. At that time the solution was cooled to room temperature and diluted with water (75 mL). The aqueous solution was extracted with CH_2Cl_2 (3 × 25 mL). The organic solutions were combined, washed with 10% H_3PO_4 (35 mL), brine (25 mL) and then concentrated under reduced pressure. The material that remained was diluted with toluene (50 mL) and concentrated to remove residual water. The concentrate was vacuum dried for 12 h to give 3.67 g of **[^{14}C]-10** as a dark yellow viscous oil. The crude product was analyzed by: HPLC, method A, RT 19.3 min, UV detector 254 nm 81.9% pure, β-Ram detector 89.3% pure; LC/MS *m/z* [^{14}C-M+H]$^+$ 387, [^{12}C-M+H]$^+$ 385; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.19 (d, *J* = 6.59 Hz, 6 H), 3.14 (sept, *J* = 6.59 Hz, 1 H), 3.61 (s, 3 H), 3.67 (s, 3 H), 3.68 (s, 3 H), 7.06–7.15 (m, 2 H), 7.52–7.63 (m, 2 H), 7.88 (s, 1 H).

[^{14}C]-4-(4-Fluorophenyl)-6-isopropyl-2-(methyl(1-methyl-1H-1,2,4-triazol-5-yl)amino)pyrimidin-5-yl)methanol ([^{14}C]-11)[5,7]

Crude **[^{14}C]-10** (3.67 g, assume 9.55 mmol) and anhydrous toluene (30 mL) were added to a round-bottomed flask. The mixture was cooled to −78°C and DIBAL (1 M in toluene, 28.6 mL, 28.6 mmol) was added dropwise over 20 min.[5,7] The internal temperature was kept below −60°C during the addition. When the addition was complete the solution was warmed to 0°C and stirred for 1 h. At that time the reaction was slowly quenched with the careful addition of methanol (5 mL) and then 2 N NaOH (50 mL). The mixture was warmed to room temperature and stirred for 30 min. The solution was diluted with water (200 mL) and extracted with CH_2Cl_2 (4 × 50 mL). The organic solutions were combined and concentrated under reduced pressure. Toluene (50 mL) was added and then removed under reduced pressure. The concentrate was vacuum dried for 14 h to give 3.18 g of **[^{14}C]-11** as a yellow solid. The crude product was analyzed by: HPLC, method A, RT 17.4 min, UV detector 254 nm 84.7% pure, β-Ram detector 87.0% pure; LC/MS *m/z* [^{14}C-M+H]$^+$ 359, [^{12}C-M+H]$^+$ 357; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.22 (d, *J* = 6.78 Hz, 6 H), 3.41 (sept, *J* = 6.78 Hz, 1 H), 3.59 (s, 3 H), 3.67 (s, 3 H), 4.60 (s, 2 H), 7.08–7.15 (m, 2 H), 7.67–7.75 (m, 2 H), 7.87 (s, 1 H).

General procedure for the preparation and titration of the bleach solution

A commercial bleach solution that was advertized to contain 5% aqueous NaOCl was added to a round-bottomed flask. The pH of the solution was adjusted to 9.1–9.2 (actual pH was 9.15) by the addition of solid $NaHCO_3$. The buffered bleach solution (approximately 1.5 g, the exact amount was recorded) was added to an Erlenmeyer flask along with water (50 mL), KI (2.0 g) and 6 N acetic acid (10 mL). The black solution was titrated to a near end point with 0.1004 N sodium thiosulfate. A 1% starch solution (3 mL) was added and the titration was continued until a clear end point was reached. The exact amount of sodium thiosulfate added was recorded. This data was used to calculate the percentage of NaOCl in the stock solution. This process was repeated a second time and the results were averaged (the average concentration was 5.06%). This stock solution was used in the next step.

[^{14}C]-4-(4-Fluorophenyl)-6-isopropyl-2-(-(methyl(1-methyl-1H-1,2,4-triazol-5-yl)amino)pyrimidine-5-carboxaldehyde ([^{14}C]-12)[5,7]

Crude **[^{14}C]-11** (3.18 g, assume 8.92 mmol), TEMPO (69.7 mg, 0.45 mmol), KBr (106 mg, 0.89 mmol) and CH_2Cl_2 (25 mL) were added to a round-bottomed flask. The solution was cooled to 0°C and the aqueous bleach solution (pH 9.15, 5.06% NaOCl, 14.43 g, 9.81 mmol) was added dropwise over 30 min.[5,7] The internal temperature was kept below 5°C during the addition. The mixture was stirred for 1 h under argon at 0°C. At that time the reaction was quenched with 1 N sodium thiosulfate (15 mL) and then warmed to room temperature. Next, 0.5 N NaOH (20 mL) was added and the solution was stirred for 10 min. Finally, water (100 mL) was added and the mixture was extracted with CH_2Cl_2 (3 × 40 mL). The organic solutions were combined and concentrated under reduced pressure. The concentrate was diluted with TBME (40 mL) and concentrated under reduced pressure. The material that remained was vacuum dried for 68 h to give 2.98 g of a dark yellow solid. The crude material was purified by column chromatography (silica, 1:1 hexanes/ethyl acetate) to give 2.56 g (65% for the four steps) of **[^{14}C]-12** as a slightly yellow solid. The product was analyzed by: TLC R_f = 0.22 (UV) using 1:1 hexanes/ethyl acetate; HPLC, method A, RT 19.2 min, UV detector 254 nm 90.3% pure, β-Ram detector 97.5% pure; LC/MS *m/z* [^{14}C-M+H]$^+$ 357, [^{12}C-M+H]$^+$ 355; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.18 (d, *J* = 6.59 Hz, 6 H), 3.66 (s, 3 H), 3.71 (s, 3 H), 3.96 (sept, *J* = 6.59 Hz, 1 H), 7.13–7.22 (m, 2 H), 7.47–7.57 (m, 2 H), 7.92 (s, 1 H), 9.90 (s, 1 H).

[^{14}C]-tert-Butyl 2-((4R,6S)-6-((E)-2-(4-(4-fluorophenyl)-6-isopropyl-2-(methyl(1-methyl-1H-1,2,4-triazol-5-yl)amino)pyrimidin-5-yl)vinyl)-2,2-dimethyl-1,3-dioxan-4-yl)acetate ([^{14}C]-14)[3,5,7]

The aldehyde **[^{14}C]-12** (2.56 g, 7.22 mmol), sulfone **13**[7,9] (3.59 g, 7.94 mmol) and THF (30 mL) were added to a round-bottomed flask. The solution was cooled to −78°C and lithium bis (trimethylsilyl)amide (1 M in THF, 9.39 mL, 9.39 mmol) was added dropwise over 15 min.[5,7] The internal temperature was kept below −70°C during the addition. The reaction was stirred for 30 min at −78°C under argon. At that time the solution was quenched with 7.5% aqueous NH_4Cl (20 mL). The mixture was warmed to room temperature and diluted with water (200 mL). The aqueous solution was extracted with CH_2Cl_2 (4 × 50 mL). The organic solutions were combined, washed with 5% $NaHCO_3$ (25 mL) and concentrated under reduced pressure. TBME (40 mL) was added and the solution was concentrated to give a yellow oil. Analysis by HPLC showed the E/Z ratio to be greater than 95:5 in favor of the desired E isomer. The crude material was purified by column chromatography (silica, 1:1 hexanes/ethyl acetate) to give a white solid. The white solid was vacuum dried for 21 h to yield 3.50 g (84%) of **[^{14}C]-14** with a specific activity of 39.8 μCi/mg (23.1 mCi/mmol, total activity 139.2 mCi, 35% radiochemical yield from urea).[5,7] The product was analyzed by: TLC R_f = 0.22 (UV) using 1:1 hexanes/ethyl acetate; HPLC, method A, RT 23.0 min, UV detector 254 nm 90.9% pure, β-Ram detector 93.4% pure; LC/MS *m/z* [^{14}C-M+H]$^+$ 583, [^{12}C-M+H]$^+$ 581; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.07–1.19 (m, 7 H), 1.38 (s, 3 H), 1.42–1.53 (m, 13 H), 2.22–2.34 (m, 1 H), 2.36–2.48 (m, 1 H), 3.22–3.37 (m, 1 H), 3.60 (s, 3 H), 3.68 (s, 3 H), 4.20–4.32 (m, 1 H), 4.32–4.44 (m, 1 H), 5.40 (dd, *J* = 16.20, 5.65 Hz, 1 H), 6.47 (dd, *J* = 16.20, 1.13 Hz, 1 H), 6.99–7.09 (m, 2 H), 7.50–7.61 (m, 2 H), 7.89 (s, 1 H).

[^{14}C]-Hemi-calcium (3R,5S,E)-7-(4-(4-fluorophenyl)-6-isopropyl-2-(methyl(1-methyl-1H-1,2,4-triazol-5-yl)amino)pyrimidin-5-yl)-3,5-dihydroxyhept-6-enoate ([^{14}C]-1)[5]

The acetonide **[^{14}C]-14** (0.20 g, 0.34 mmol, 8.0 mCi, 39.8 μCi/mg), acetonitrile (2 mL) and 0.02 N HCl (1 mL, 0.02 mmol) were added to a round-bottomed flask.[3,5,7] The solution was heated to 40°C and stirred for 6 h. Analysis by HPLC showed complete conversion to the free diol (method A, β-Ram detector 88.5% pure). Care was taken to stop the reaction as quickly as possible after completion to minimize impurity formation.[10] At that time the solution was diluted with methanol (10 mL) and concentrated under reduced pressure. The off-white solid that remained was vacuum dried for 16 h. The solid was dissolved in acetonitrile (5 mL) and the desired product was purified by semi-preparative HPLC (0.75 mL injections on a YMC Pack Pro C18 column s-5 μm, 250 × 20 mm. Solvent: A = water with 0.05% TFA; B = acetonitrile with 0.05% TFA. Conditions: 75% B, 0–10 min; 75–100% B, 10–12 min; 100% B, 12–19 min; 100–75% B, 19–20 min. Flow 10 mL/min. Wavelength: 230 nm). The fractions that contained the desired product were combined and concentrated to give 0.148 g of a white solid. The diol product was analyzed by: HPLC, method A, RT 19.4 min, UV detector 254 nm 99.3% pure, β-Ram detector 99.4% pure.

The diol (0.148 g, 0.27 mmol), acetonitrile (2 mL) and 1 N NaOH (0.5 mL, 0.5 mmol) were added to a round-bottomed flask.[3,5,7] The solution was stirred for 4 h at room temperature. Analysis by HPLC showed complete hydrolysis of the ester (method A, β-Ram detector 99.2% pure). Care was taken to stop the reaction as quickly as possible after the reaction was complete to minimize impurity formation.[10] At that time the solution was diluted with methanol (10 mL) and concentrated under reduced pressure. The material that remained was dissolved in 65:35 water/acetonitrile with 17.5 mmol/L of ammonium acetate (5 mL) and the desired product was purified by semi-preparative HPLC (0.5 mL injections on a YMC Pack Pro C18 column s-5 μm, 250 × 20 mm. Solvent: A = 65:35 water/acetonitrile with 17.5 mmol/L of ammonium acetate; B = 90:10 acetonitrile/water with 17.5 mmol/L of ammonium acetate. Conditions: 0% B, 0–13 min, 6 mL/min; 0–100% B, 13–15 min, 6–15 mL/min; 100% B, 15–23 min, 15 mL/min; 100–0% B, 23–25 min, 15 mL/min; 0% B, 25–30 min, 15 mL/min; 0% B, 30–31 min, 15–6 mL/min, 0% B, 31–33 min, 6 mL/min. Wavelength: 230 nm). The fractions that contained the desired product were combined and partially concentrated. The pH of the aqueous solution was lowered to 5.2 by the addition of saturated aqueous NH_4Cl (approximately 200 mL were added, the pH of the saturated NH_4Cl solution was adjusted to 3.3 with 1 N HCl prior to the addition). Care was taken to insure the pH of the solution containing the product did not go below 5. When the pH was lowered below 5 large amounts of the lactone (**[^{14}C]-17**) impurity were formed.[5,7] The aqueous solution was extracted with CH_2Cl_2 (8 × 50 mL). The organic solutions were combined and concentrated to a volume of about 10 mL. The CH_2Cl_2 solution was filtered to remove traces of solid ammonium chloride. The filter was washed with CH_2Cl_2 (3 × 5 mL). The organic solutions containing the acid product were combined (total CH_2Cl_2 volume 25 mL) and treated with NH_3 (2 M solution in MeOH, 2.5 mL).[5] The reaction flask was capped and allowed to stand at room temperature for 1.5 h. At that time the suspension was concentrated using a stream of nitrogen to give a white solid. The solid was vacuum dried for 5 h to yield 0.104 g (61% for the two steps and salt formation) of **[^{14}C]-15** with a specific activity of 48.6 μCi/mg (24.4 mCi/mmol, total activity 5.1 mCi, 64% radiochemical yield). The ammonium salt was analyzed by: HPLC, method A, RT 16.1 min, UV detector 254 nm 99.7% pure, β-Ram detector 99.9% pure; the material co-eluted with an authentic sample of **15**; the ^{1}H NMR was also identical to an authentic sample.

The ammonium salt **[^{14}C]-15** (48.6 mg, 0.097 mmol, 48.6 μCi/mg, 2.4 mCi) and water (1 mL) were added to a round-bottomed flask. An aqueous solution of $CaCl_2$-$2H_2O$ (7.4 mg, 0.0504 mmol, 0.52 eq, in 0.3 mL of water) was added to the reaction mixture.[5] The suspension was stirred for 16 h at room temperature. At that time the reaction mixture was diluted with MeOH (2 × 10 mL) and concentrated to give a white solid. The solid was vacuum dried for 17 h to yield 55.3 mg (100%) of **[^{14}C]-1** with a specific activity of 43.2 μCi/mg (21.8 mCi/mmol, total activity 2.4 mCi, 100% radiochemical yield).[11] The calcium salt was analyzed by: HPLC, method A, RT 16.1 min, UV detector 254 nm 99.1% pure, β-Ram detector 99.9% pure; the material coeluted with an authentic sample of **1**; ^{1}H NMR (400 MHz, DMSO-D_6) δ ppm 1.09 (d, *J* = 6.80 Hz, 3 H), 1.10 (d, *J* = 6.80 Hz, 3 H), 1.27–1.36 (m, 1 H), 1.46–1.54 (m, 1 H), 2.02–2.10 (m, 1 H), 2.15–2.22 (m, 1 H), 3.27–3.37 (m, 1 H), 3.45 (s, 3 H), 3.61 (s, 3 H), 3.70–3.79 (m, 1 H), 4.12–4.20 (m, 1 H), 5.44 (dd, *J* = 15.99, 5.67 Hz, 1 H), 6.43 (d, *J* = 15.99 Hz, 1 H), 7.21–7.28 (m, 2 H), 7.59–7.65 (m, 2 H), 7.90 (s, 1 H); the ^{1}H NMR spectrum was identical to an authentic sample.

Stability studies

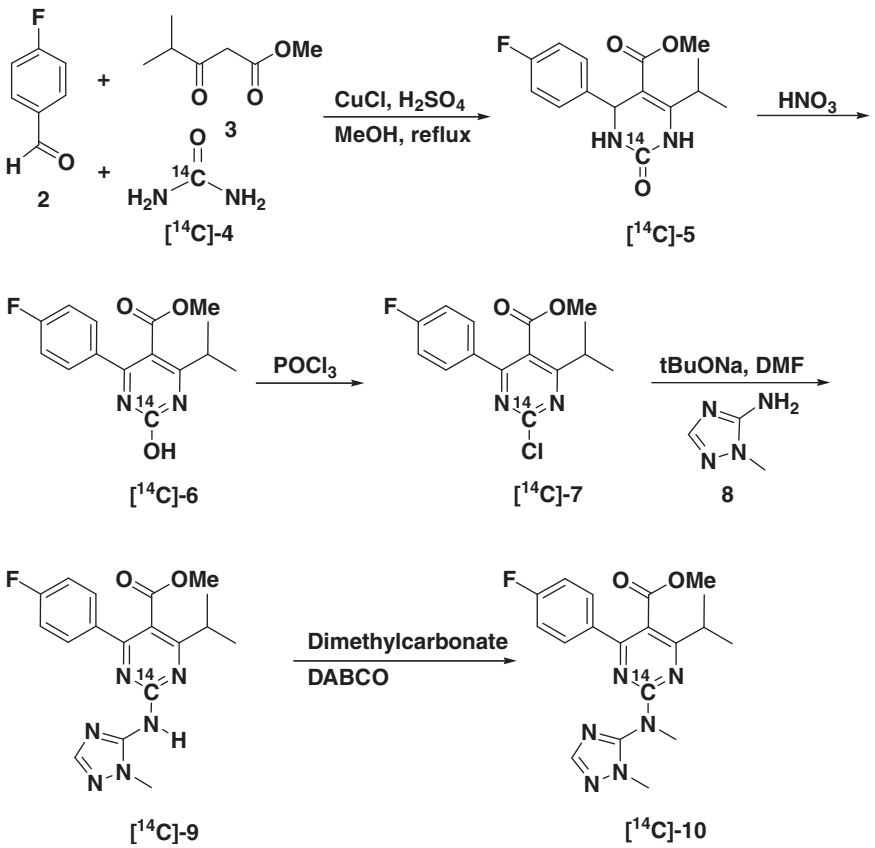
Small samples of **[^{14}C]-15** (48.6 μCi/mg) and **[^{14}C]-1** (43.2 μCi/mg) were saved to study the chemical stability of high specific

activity material. A sample of **[^{14}C]-1** with lower specific activity was prepared by dissolving **[^{14}C]-1** (34.0 μCi/mg, 10.1 mg) and unlabeled **1** (91.4 mg) in EtOH (190 proof, 10 mL). The clear solution was concentrated and vacuum dried for 65 h to give a white solid. The specific activity of this material was determined to be 3.8 μCi/mg. A sample with lower specific activity containing L-ascorbic acid (1% by weight) was prepared by dissolving **[^{14}C]-1** (43.2 μCi/mg, 16.4 mg), unlabeled **1** (50.4 mg) and L-ascorbic acid (1 mg/mL solution in EtOH (190 proof), 0.65 mL, 0.65 mg) in EtOH (190 proof, 5 mL). The clear solution was concentrated and vacuum dried for 15 h to give a white solid. The specific activity of this material was determined to be 10.5 μCi/mg. This material was analyzed by: HPLC, method A, RT 16.1 min, UV detector 254 nm 99.5% pure, β-Ram detector 99.9% pure. Finally, a sample with lower specific activity containing BHT (1% by weight) was prepared by dissolving **[^{14}C]-1** (43.2 μCi/mg, 14.8 mg), unlabeled **1** (50.6 mg) and BHT (1 mg/mL solution in EtOH (190 proof), 0.65 mL, 0.65 mg) in EtOH (190 proof, 5 mL). The clear solution was concentrated and vacuum dried for 5 h to give a white solid. The specific activity of this material was determined to be 9.8 μCi/mg. This material was analyzed by: HPLC, method A, RT 16.1 min, UV detector 254 nm 99.5% pure, β-Ram detector 99.9% pure.

All of the samples prepared above were stored at −78°C under argon and analyzed by HPLC (method A) about once a month over the next 14 weeks. The samples of **[^{14}C]-15** (48.6 μCi/mg) and **[^{14}C]-1** (43.2 μCi/mg) with high specific activities showed decomposition rates of about 1.0%/month and 1.9%/month, respectively. The sample of **[^{14}C]-1** (3.8 μCi/mg) with low specific activity showed a decomposition rate of about 0.5%/month. The samples of **[^{14}C]-1** containing the radical inhibitors L-ascorbic acid and BHT showed decomposition rates of about 0.2%/month and 0.1%/month, respectively. As many as five decomposition products were observed. The major decomposition product (4.8% for the sample of **[^{14}C]-1** with a specific activity of 43.2 μCi/mg) was isolated by semi-preparative HPLC (YMC Pack Pro C18 column s-5 μm, 250 × 20 mm. Solvent: A = 65:35 water/acetonitrile with 17.5 mmol/L of ammonium acetate. Conditions: 10% A, 0–15 min, 6 mL/min. Wavelength: 230 nm). This material was determined to be the allylic ketone **[^{14}C]-16** (method A, RT 16.8 min) after comparison of its NMR spectrum with an authentic sample. The next largest impurity (0.7% for the sample of **[^{14}C]-1** with a specific activity of 43.2 μCi/mg) was determined to be the lactone **[^{14}C]-17** (method A, RT 17.3 min) by coinjection with an authentic sample. The structures of the other minor impurities were not determined.

Results and discussion

Our labeling strategy for compound **1** was to incorporate the [^{14}C]-label into the pyrimidine core. The synthetic plan that was developed took advantage of some existing synthetic methodology that was being used by the Bristol-Myers Squibb PR&D department.[5] The key step in the construction of the pyrimidine core utilized a Biginelli three-component coupling reaction.[12] This provided the option of using any one of the three starting materials to incorporate the [^{14}C]-label (Scheme 1). Carbon-14-labeled urea was chosen as the label source because it was the cheapest and most readily available of the three potential starting materials.

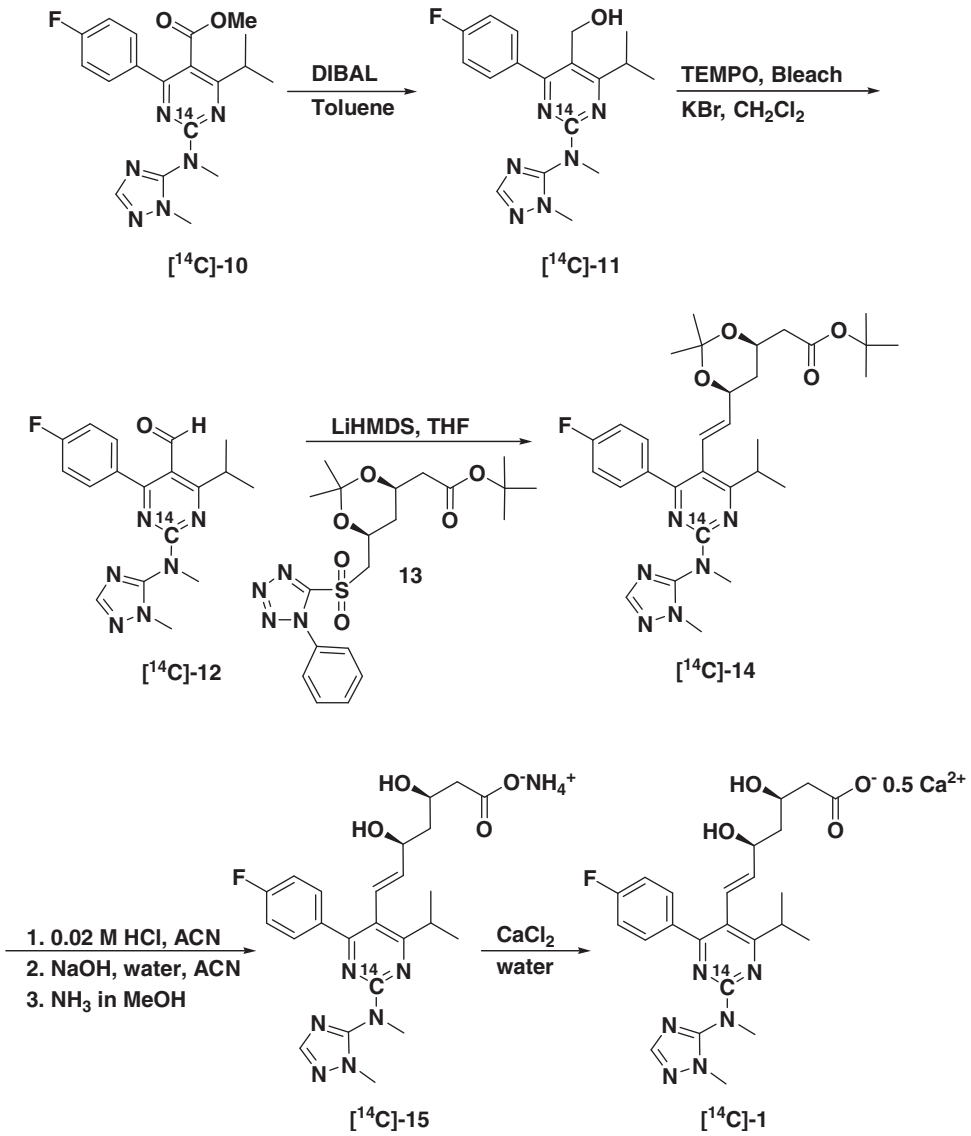


Scheme 1.

The Biginelli reaction was conducted using 4-fluorobenzaldehyde (**2**), methylisobutyryl acetate (**3**) and [^{14}C]-labeled urea ([^{14}C]-**4**, 400 mCi) as shown in Scheme 1. This gave the desired dihydropyrimidinone ([^{14}C]-**5**) in an 84% yield.[4] Most importantly this intermediate had all of the functionality around the core in the appropriate positions to complete the synthesis. The dihydropyrimidinone ([^{14}C]-**5**) was oxidized with nitric acid to produce the pyrimidinol [^{14}C]-**6**.[4–6] Treatment of this intermediate with $POCl_3$ produced the corresponding chloropyrimidine ([^{14}C]-**7**) in an 82% yield for the two steps.[4] The chloropyrimidine ([^{14}C]-**7**) was coupled with amino triazole **8**[5,7,8] to give the secondary amine [^{14}C]-**9**.[5,7] The secondary amine [^{14}C]-**9** was methylated with dimethylcarbonate to yield the tertiary amine [^{14}C]-**10**.[5]

Next, the olefinic top portion of the molecule was installed and the synthesis was completed as shown in Scheme 2. The methyl ester [^{14}C]-**10** was reduced with DIBAL to produce the primary alcohol [^{14}C]-**11**.[5,7] A TEMPO oxidation of [^{14}C]-**11** gave the corresponding aldehyde [^{14}C]-**12** in a 65% overall yield for the four steps.[5,7] The aldehyde [^{14}C]-**12** and sulfone **13**[7,9] were coupled via a Julia olefination to give [^{14}C]-**14** in an 84% yield.[3,5,7] Deprotection of the acetonide ([^{14}C]-**14**) with HCl, hydrolysis of the tert-butyl ester with NaOH and then formation of the ammonium salt of the carboxylic acid gave [^{14}C]-**15** in a 61% yield.[3,5,7] The ammonium salt [^{14}C]-**15** was treated with $CaCl_2$ to produce the desired calcium salt [^{14}C]-**1** in 100% yield.[5] This material had a radiochemical purity of 99.9% and a specific activity of 43.2 μCi/mg.

Radiolabeled [^{14}C]-**1** was used successfully in preclinical ADME studies to determine the metabolic fate of the molecule. In the course of this work it was discovered that the ammonium salt [^{14}C]-**15** (48.6 μCi/mg) and the calcium salt [^{14}C]-**1**, (specific activity above 30 μCi/mg) had poor chemical stability. These materials decomposed at rates of about 1.0%/month and 1.9%/month respectively to two major components and several minor components when stored at −78°C under argon (Table 1). A semi-preparative HPLC method was developed to purify impure [^{14}C]-**1**. However this purification was difficult in practice since the impurities eluted close to the desired product. Fortunately the bulk supply of the material was stored as the



Scheme 2.

Table 1. Summary of stability data (14 weeks)

	[^{14}C]-15	[^{14}C]-1	[^{14}C]-1	[^{14}C]-1	[^{14}C]-1
	48.6 μCi/mg	43.2 μCi/mg	3.8 μCi/mg	10.5 μCi/mg Ascorbic acid	9.8 μCi/mg BHT
Rate of decomposition (%/month)	1.0	1.9	0.5	0.2	0.1
[^{14}C]-**16** formed (%)	2.1	4.8	0.7	0.3	0.2
[^{14}C]-**17** formed (%)	0.4	0.7	0.4	0.2	0.1

fully protected intermediate **[^{14}C]-14**, which was chemically stable. The requests for highly pure **[^{14}C]-1** were met by preparing fresh batches of material as needed from **[^{14}C]-14** and using them as quickly as possible after preparation. This strategy was fine for the preclinical ADME studies, but would not work for the preparation of clinical supplies needed to fund the human ADME study. For these studies the radiolabeled material needed to be relatively stable for several weeks to several months to allow for drug product manufacturing and dosing.

Confirming the identity of the two major decomposition products was the first step taken to understand how and why **[^{14}C]-1** was decomposing. The larger of the two impurities as measured by HPLC peak integration was isolated by semi-preparative HPLC. This compound was determined to be allylic ketone **[^{14}C]-16** by comparison of its ^{1}H NMR spectrum to an authentic sample (Figure 2). The smaller impurity was determined to be lactone **[^{14}C]-17** by coinjection with an authentic sample. Based on the structure of the largest impurity (**[^{14}C]-16**), the decomposition pathway is believed to be a radical promoted, oxidative decomposition process that is initiated by β particle emission from carbon-14. This phenomenon has been documented for other [^{14}C]-labeled compounds.[13–15] It is worth noting that under similar conditions, this decomposition was not observed with unlabeled **1**.

Three samples were prepared to study the stability of **[^{14}C]-1**. Sample one, with a specific activity of 3.8 μCi/mg, was prepared by diluting **[^{14}C]-1** (34.0 μCi/mg) with unlabeled **1**. This sample was prepared because **[^{14}C]-1** with lower specific activity should have better stability. Samples two and three were prepared to study the effects of radical inhibitors on sample stability.[14,15] Sample two (10.5 μCi/mg) was prepared by diluting **[^{14}C]-1** (43.2 μCi/mg) with unlabeled **1** and L-ascorbic acid (1% by weight).[15,16] The hope was that radical inhibitors would reduce or even inhibit the decomposition of **[^{14}C]-1**. Sample three (9.8 μCi/mg) was prepared by diluting **[^{14}C]-1** (43.2 μCi/mg) with unlabeled **1** and 2,6-di-tert-butyl-4-methylphenol (BHT, 1% by weight).[15,16] This sample was prepared to compare the effectiveness of the different radical inhibitors. The radical inhibitors L-ascorbic acid and BHT were specifically chosen because they are commonly found in foods, supplements and consumer products, making them acceptable additives to a drug substance administered to human subjects.

All samples were stored at −78°C under argon and analyzed periodically by HPLC over the next 14 weeks. The sample of **[^{14}C]-1** with a specific activity of 3.8 μCi/mg decomposed at a rate of about 0.5%/month. This was better than the high specific activity material but the stability still did not meet the requirements necessary for a human ADME study. The samples of **[^{14}C]-1** with L-ascorbic acid (1% by weight, specific activity of 10.5 μCi/mg) and BHT (1% by weight, specific activity of 9.8 μCi/mg) decomposed at rates of about 0.2%/month and 0.1%/month, respectively. These decomposition rates were

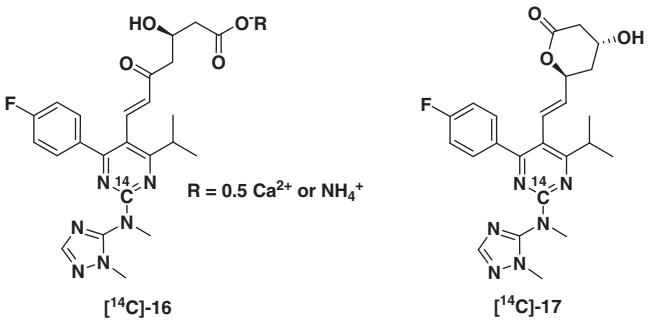


Figure 2. Structures of the two major decomposition products.

significantly lower than the sample with specific activity 3.8 μCi/mg with no radical inhibitor. These results show that there is an advantage to adding radical inhibitors to **[^{14}C]-1**, a compounds susceptible to radical promoted decomposition. These results also show that BHT is slightly better than L-ascorbic acid at stabilizing this [^{14}C]-labeled compound. However, both provided potential solutions to the stability issue that needed to be resolved before the human ADME study could be conducted.

Conclusion

The synthesis of **[^{14}C]-1** was completed in 11 steps from [^{14}C]-labeled urea in a 22% overall yield. Unfortunately **[^{14}C]-1** with a specific activity of 43.2 μCi/mg decomposed at a rate of 1.9%/month when stored at −78°C. As the decomposition pathway was believed to be radical initiated, samples were prepared that contained the radical inhibitors L-ascorbic acid or BHT. By adding L-ascorbic acid the decomposition rate was reduced to about 0.2%/month and by adding BHT the decomposition rate was reduced to about 0.1%/month. The use of radical inhibitors to stabilize compounds labeled with radioactive isotopes has been shown to be effective numerous times in the literature. Most of these examples focus on the use of radical inhibitors in the solution phase. It is not always practical or feasible to store radiolabeled compounds as solutions, especially when the radiolabeled compounds are intended for use in human clinical studies. The radical inhibitors L-ascorbic acid and BHT have not yet been shown to be effective at stabilizing a large number of [^{14}C]-labeled compounds being stored as solids. However, it is believed that this approach could be applicable to other [^{14}C]-labeled compounds that undergo rapid degradation due to radical-promoted decomposition.

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